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Paolo Giovanni Nuciforo

Esta tesis se desarrolló en el Departamento de Anatomía Patológica, el Departamento de Oncología del H.U. Vall d'Hebron y el Vall d'Hebron Institute of Oncology bajo la supervisión de los directores doctores:

Javier Cortes Castán

Joan Carles Ferreres Piñas

Tutor: Vicente Peg Cámara

A Valentino e Roberta: perche siete la mia vita, per sempre.

La presente tesis doctoral se ha estructurado siguiendo la Normativa interna de la Universidad Autónoma de Barcelona para la presentación de tesis doctorales como compendio de publicaciones, aprobada por Comisión de Doctorado de la Facultad de Medicina.

Los trabajos que forman parte de la memoria del proyecto de tesis doctoral pertenecen a la misma línea de investigación, enmarcada en la definición y valor del concepto de análisis de la familia de proteínas HER determinada mediante técnicas cuantitativa de espectrometría de masas en las pacientes con cáncer de mama HER2-positivo y su valor clínico. Como resultado de estos trabajos se han publicado dos artículos originales en la Literatura internacional.

«Se uno non corre qualche rischio per un'idea, o non vale niente l'idea o non vale niente lui»

Roberto Benigni

TABLE OF CONTENTS

ADCC	Antibody-dependent cell cytotoxicity
amol/µg	Attomol/microgram
ASCO	American Society of Clinical Oncologists
CAP	College of American Pathologists
DM	Double minutes
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
FFPE	Formalin fixed, paraffin embedded
FISH	Fluorescent in situ hybridization
HER	Human epidermal growth factor receptor
HER2+	HER2-positive
HER2-	HER2-negative
IGF1R	Insulin-like growth factor receptor 1
IHC	Immunohistochemistry
ISH	In situ hybridization
mRNA	Messenger ribonucleic acid
OS	Overall survival
pCR	Pathologic complete response
PFS	Progression-free survival
RTK	Tyrosine kinase receptor
SRM-MS	Selected reaction monitoring-Mass spectrometry
TKI	Tyrosine kinase inhibitor
TNBC	Triple-negative breast cancer

INTRODUCTION

The HER family (also called ErbB or epidermal growth factor receptor (EGFR) family) comprises four transmembrane receptor tyrosine kinases, EGFR (or HER1), HER2, HER3, and HER4 (Figure 1). These receptors signal through homo- and heterodimerization and promote cell proliferation, motility, and invasion (Holbro, 2003). Dysregulated expression and activity of HER family members is frequent in breast cancer (BC). Overexpression of EGFR1, HER2 and HER3 is generally associated with poor prognosis whereas high expression of HER4 is associated with a better outcome (Naidu 1998, Pawlowski 2000, Suo 2001 and 2002, Tovey 2004, Witton, 2003). Gene amplification or protein overexpression of the human epidermal growth factor receptor type 2 (HER2) has been reported in 25% to 30% of invasive BC and is usually associated with a worse prognosis (Slamon 1989, Dendukuri 2007, Slaghmon 2001, Yu 2000, Ross 2009).

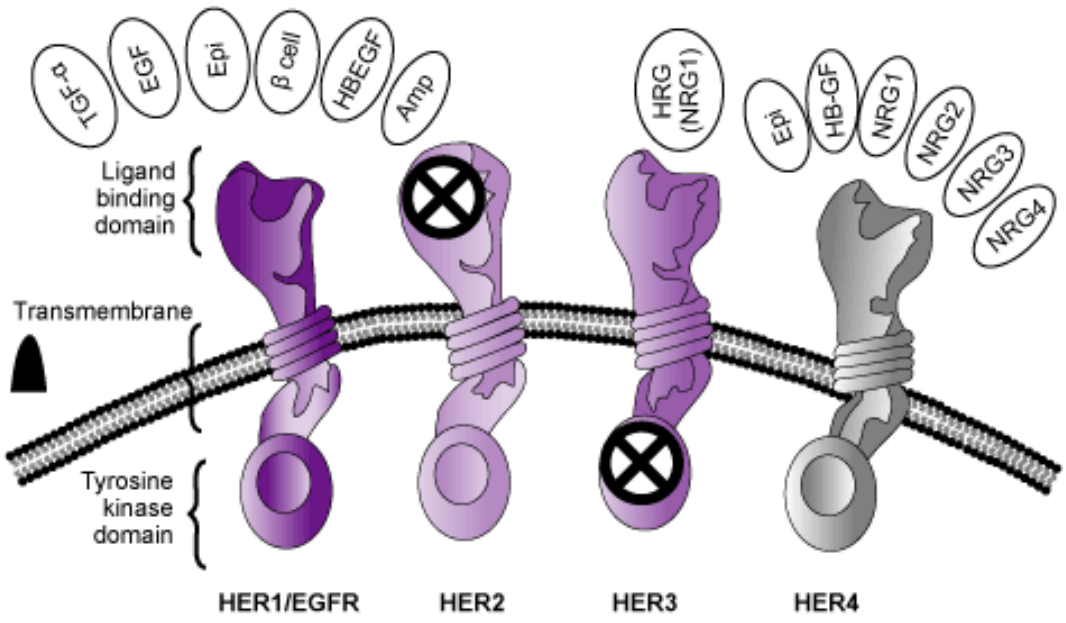

Figure 1. The epidermal growth factor receptor family of proteins.

Monoclonal antibodies (i.e. trastuzumab, pertuzumab, T-DM1) and small molecules kinase inhibitors (i.e. lapatinib, neratinib and afatinib) are the main strategies to target HER2 in BC (Figure 2). Trastuzumab (Herceptin®), a humanized monoclonal antibody, was the first drug developed to target HER2

amplified BC. The addition of trastuzumab to cytotoxic chemotherapy significantly improved disease-free and overall survival in metastatic HER2-positive (HER2+) BC, resulting in FDA first approval of the drug in 1998 (Cobleigh 1999). In 2006, approval was extended to use of the drug in combination with chemotherapy in the adjuvant setting in early stage HER2+ BC (Piccart-Gebhart 2005, Romond 2005). The addition of trastuzumab to chemotherapy in neoadjuvant setting has also been tested in several phase II studies, with pathologic complete response (pCR) rates ranging from 18% to 47% (Burstein 2003, Coudert 2006, Van Pelt 2003, Hurley 2006, Limentani 2007). Lapatinib, given in combination with capecitabine, has shown clinical activity in HER2+ BC patients that became refractory to trastuzumab-based therapy (Geyer 2006). Moreover, measurable clinical benefit is observed also when lapatinib is administered as single agent or in combination with paclitaxel as first-line treatment (Gomez 2008; Di Leo 2008). Recently, the antitumor activity of dual HER2 blockade (trastuzumab in combination with either lapatinib or pertuzumab) was proven to be significantly superior to single agents in a neoadjuvant setting (Baselga 2012; Gianni 2012).

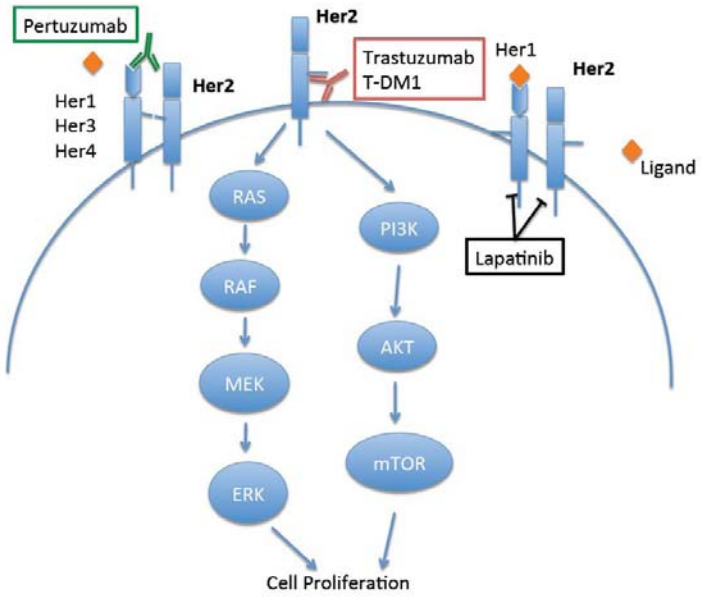

Figure 2. Approved monoclonal antibodies and small molecules kinase inhibitors targeting HER2 in breast cancer.

Despite the remarkable success of trastuzumab, however, HER2+ BC frequently display primary resistance and even in patients initially sensitive, acquired resistance almost inevitably emerges over time. In the preclinical setting, numerous potential mechanisms of resistance to anti-HER2 agents have been proposed (increased HER2-ectodomain shedding, epitope masking or inaccessibility, activation of compensatory signalling pathways, resistance to apoptosis, co-amplification of genes such as Cyclin E and Myc, dimerization with other tyrosine kinase receptors (RTKs) such as c-MET or IGF1R, and escape from antibody-dependent cell cytotoxicity (ADCC)).

EGFR has been showed to be highly expressed in triple-negative breast cancer (TNBC), both in cell lines and in patients (Nielsen 2004). Moreover, preclinical studies have demonstrated that the inhibition of EGFR affects growth in TNBC cells lines (Hoadley 2007). These findings provided the rationale to test the efficacy of anti-EGFR agents, such as the antibodies cetuximab and panitumumab, in TNBC patients. In metastatic setting, cetuximab in combination with chemotherapy showed some promising activity (Baselga 2013; Carey 2012; O'Shaughnessy 2007). Nevertheless, no substantial improvements in either PFS or OS were achieved in these patients. A better scenario seems to be the neoadjuvant setting, where a pathological complete response rate of 46.8% was observed in TNBC patients enrolled in a pilot clinical trial testing the efficacy of panitumumab in combination with standard chemotherapy (Nabholtz 2014).

There are several unanswered questions about which patients with BC are most likely to benefit from one or another form of anti-HER targeted therapy and which type of determination methodology is most appropriate.

A biomarker to identify those patients who are not likely to benefit from trastuzumab would be clinically useful- especially in the adjuvant setting where one cannot measure tumor response to assess the effectiveness of a therapy - allowing patients to move in other therapeutic directions. Similarly, a marker that pegged tumors as exquisitely sensitive to anti-HER2 drugs might spare the addition of chemotherapy or define patients most suited for novel targeted or immunological approaches centering on HER2 expression. Retrospective studies conducted in tumor samples from the major adjuvant

studies of trastuzumab have failed to demonstrate a single biomarker or biomarker signature predictive of resistance to trastuzumab (Perez 2010, Gianni 2011). Even the most obvious candidate biomarker for a predictor of trastuzumab benefit, HER2 itself, has proven to be surprisingly ambiguous. Neither levels of gene amplification nor soluble HER2 levels predicts benefit of adjuvant trastuzumab (Dowsett 2009, Perez 2010, Moreno-Aspitia 2013). Most surprisingly, NSABP B-31 and NCCTG N9831 finding of a group of patients turned out to be HER2- after central confirmation derived as much benefit from trastuzumab as those whose tumors confirmed to be HER2+ (Perez 2010, Paik 2008) raise the provocative notion that overexpression of HER2 might not be essential to realizing benefit from adjuvant trastuzumab. To date, exploitation of the overexpression of HER2 is part of the management of a BC patient whereas EGFR, HER3, and HER4 determinations are still exploratory and not used in clinical practice. As a matter of fact, to be eligible for an anti-HER therapy such as trastuzumab, specimens have to be HER2+ as determined by immunohistochemistry (IHC) analysis or harbor *HER2* gene amplification by DNA in situ hybridization (ISH) (Figure 3).

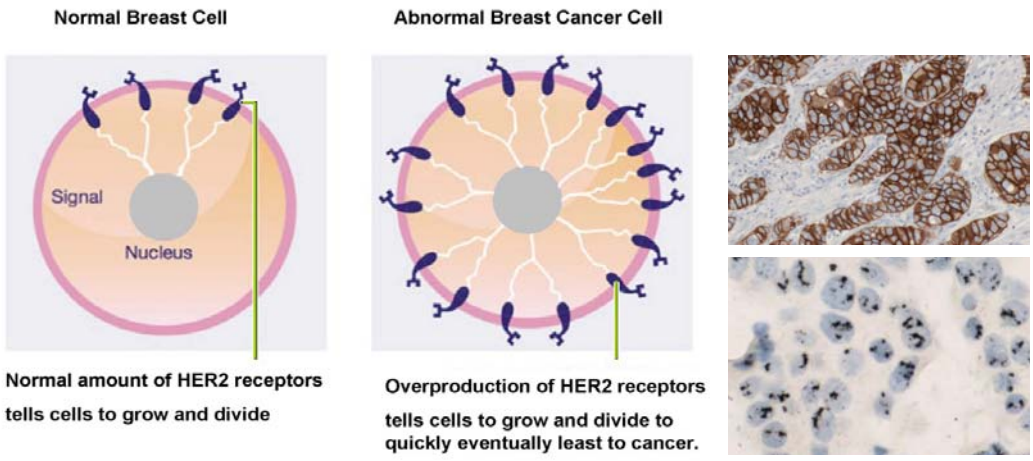

Figure 3. A cartoon illustrating HER2 abundance in a breast cancer cell as compared to normal cell (left). HER2 positive tumor by standard immunohistochemistry (top right) and in situ hybridization (bottom right).

Although these tests have become the benchmarks for defining tumors as HER2+, considerable controversy still exists regarding the accuracy, reliability, and inter-observer variability of these assay methods. It is estimated that approximately 20% of HER2 testing performed in the field may be inaccurate when validated against central or "expert" laboratories (Paik 2002, Roche 2002). In an effort to improve the accuracy and consistency of HER2 testing, a joint task force of the American Society of Clinical Oncologists (ASCO) and the College of American Pathologists (CAP) proposed guideline recommendations for HER2 testing using either an IHC or FISH (Wolff 2007). The assessment of HER2 expression by immunohistochemical analysis is inherently subjective and semiquantitative (scored as 0, 1+, 2+, and 3+), whereas the FISH test, in which the *HER2* gene copy number is counted, is considered to be more quantitative analytically. However, neither test is a perfect predictor of response to trastuzumab, and both tests are affected by interlaboratory variability.

Algorithms for evaluation of HER2 protein expression by IHC assay and gene amplification by ISH of the invasive component of a BC specimen are illustrated below (Wolff 2013) (Figure 4-6).

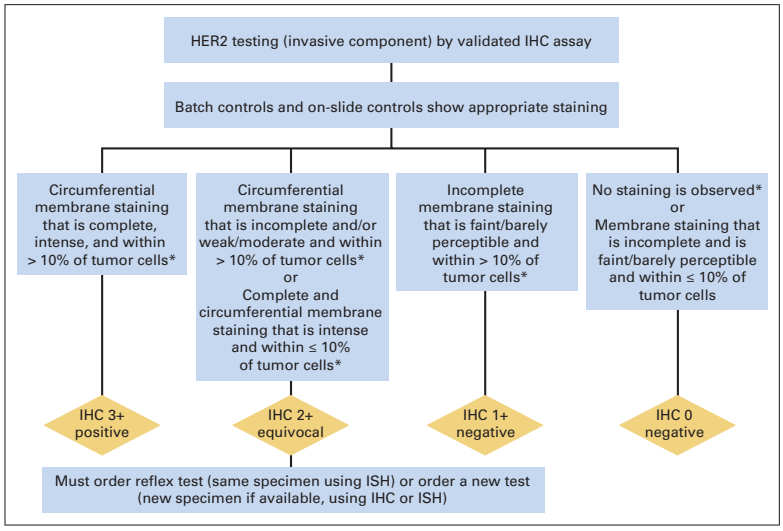

Figure 4. Algorithm for evaluation of human epidermal growth factor receptor 2 (HER2) protein expression by immunohistochemistry (IHC) assay.

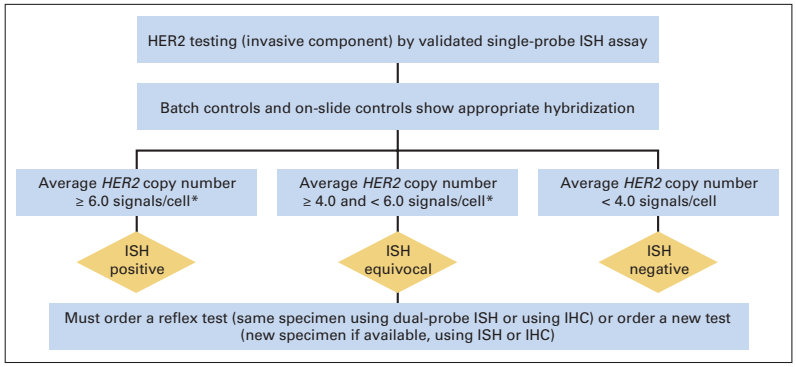

Figure 5. Algorithm for evaluation of human epidermal growth factor receptor 2 (HER2) gene amplification by in situ hybridization (ISH) using a single-signal (HER2 gene) assay (single-probe ISH).

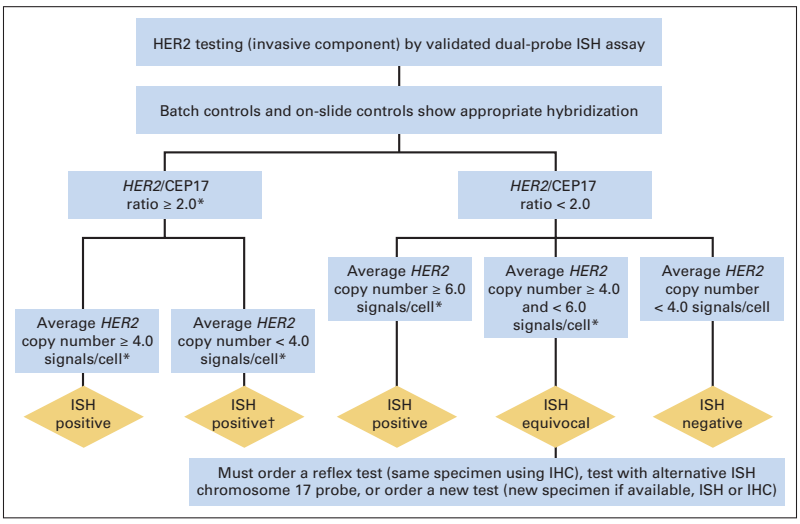

Figure 6. Algorithm for evaluation of human epidermal growth factor receptor 2 (HER2) gene amplification by in situ hybridization (ISH) assay using a dual-signal (HER2 gene) assay (dual-probe ISH).

Among HER2+ tumors (defined by consensus criteria), there is a wide range of variability in terms of *HER2* gene amplification and protein expression measured by conventional semi-quantitative methods such as the HercepTest®. The possibility that a quantitative analysis of HER-family protein expression could improve the prediction of HER-targeting drugs has led to the evaluation of alternative and more quantitative tests.

The VeraTag™ proximity-based assay (HERmark® Breast Cancer Assay; Monogram Biosciences, Inc., South San Francisco, CA) has been shown to enable precise quantitative measurements of total HER1 and HER2 proteins and their respective homodimers in formalin-fixed, paraffin-embedded tissue specimens (Shi 2009). Higher HER2 expression, as determined using this assay, was associated with a longer survival time after trastuzumab treatment in HER2+ advanced BC patients (Lipton 2010, Toi 2010). Most recently, measurement of HER2 protein using AQUA technology - a quantitative fluorescence immunohistochemistry-based method - was able to predict which HER2+ patients was more or less likely to achieve a pCR after trastuzumab containing neoadjuvant treatment regime (Cheng, 2014). These quantitative antibody-based protein assays may overcome some of the limitation of IHC (subjective, qualitative, lack of reproducibility…), but still suffers from numerous drawbacks including antibody specificity/sensitivity, lack of multiplexing capabilities, sensitivity to preanalytical factors and limited dynamic range. As such, a protein assay technology platform for application to formalin fixed patient tissue that overcomes these limitations is warranted. Advances in mass spectrometry have resulted in development of strategies for large-scale protein analysis of complex cellular protein lysates. In contrast to gene expression, mass spec protein analysis directly addresses the products of gene expression present in a given cell state by characterizing protein expression levels and post translational modifications. It is now possible to identify thousands of expressed proteins from a single sample (Washburn, 2001; Gavin, 2002; Ho, 2002). In addition, it is possible to precisely quantitate proteins and analyze post translation modifications of specific peptides directly in protein samples (Aebersold and Goodlett, 2001; Gerber et al, 2003). Selected reaction monitoring (SRM-MS) is a mass spectrometry-based method that can provide for protein assays with high sensitivity, absolute specificity, objective quantitation, and multiplex capabilities (Aebersold 2003, Ong 2005, Addona 2009, Nilsson 2010, Rudnick 2010) thus overcoming the many limitations of IHC. The reliability of this approach for analysis of proteins in any biological sample including FFPE patient tumor tissue has been demonstrated (Prieto 2005, Hood 2005, Hembrough 2012, Bateman 2011 Desouza 2010, Huang 2009, Jain 2008,

Cheung 2008, Patel 2008). However, the clinical utility of this assay for patient stratification, choice of therapy, and drug resistance prediction have not yet been shown.

Other groups have used mRNA measurements to either predict pCR or survival in HER2+ tumors treated with trastuzumab in both neoadjuvant and adjuvant setting. Denkert and colleagues found that quantitative assessment of mRNA for ESR1 and HER2 can predict pCR (Denkert 2013). Similarly, Pogue-Geile et al (Pogue-Geile 2013) developed a gene expression-based predictive model for degree of benefit from trastuzumab with a total of eight genes associated with expression of either the ER (ESR1, NAT1, GATA3, CA12, IGF1R) or the HER2 (ERBB2, c17orf37, GRB7) amplicon. The eight-gene model stratified tumors into 3 subsets with different clinical outcomes. A distinct subset (called Group 3 by the investigators) of approximately 45% of the patients with tumors characterized by high-level HER2 expression and low- or absent levels of ER which was associated with an extraordinary benefit from trastuzumab. By contrast, 10% of cases (classified as Group 1), showed no apparent benefit from trastuzumab. Interestingly, this subset had tumors characterized by intermediate-level but clear HER2 expression and very high-level ESR1 expression. In between were the Group 2 patients, which contained tumors with low/no HER2 expression and with variable degrees of ESR1 expression who still derived moderate benefit from trastuzumab, thus providing justification for testing trastuzumab in HER2- patients (NSABP B-47).

The development of these new quantitative diagnostic methods is a substantial technical achievement. Despite that, the 2013 ASCO/CAP Update Committee concluded that there was insufficient evidence to warrant inclusion of these new assays to determine HER2 status in unselected patients due to lack of a consistent body of evidence on their analytical validity, clinical validity, and clinical utility (Wolff 2013).

HYPOTHESIS

Despite the remarkable success of trastuzumab, HER2+ BC frequently display primary resistance and even in patients initially sensitive, acquired resistance almost inevitably emerges over time.

Biomarkers to assess the effectiveness of anti-HER therapies with identification of exquisitely sensitive or resistant patients would be clinically useful.

Expression levels of HER2 and related family proteins (HER1 and HER3) may dictate response to HER-targeting drugs.

Considerable controversy exists regarding the accuracy, reliability, and interobserver variability of current methods for the determination of HER2 status which are not quantitative and do not predict response to anti-HER treatments.

Based on the above, we hypothesize that multiplex quantitative expression analysis of HER family proteins using mass spectrometry could overcome current limitations and improve cancer patient treatment decision.

OBJECTIVES

1) To provide analytical validation of SRM-MS technology as applied to the clinical quantitative measurement of HER2 expression in BC FFPE samples.

2) To compare HER2 quantitative expression levels determined by SRM-MS with reference assays routinely used for HER2 testing such as IHC and FISH.

3) To demonstrate that quantitative analysis of HER2 (and related family proteins HER1 and HER3) may improve current prediction of response or resistance to HER-targeting agents.

PUBLICATIONS: METHODS AND RESULTS

1.- STUDY METHODOLOGY:

The study design, the study population, and the methodology used are described in the "Material and methods" section of the published articles that constitute this doctoral thesis.

These articles are included in the following pages as they have been published in the scientific literature.

2.- PUBLICATIONS:

2.1.- Study 1:

"Quantification of HER family receptors in breast cancer".

Paolo Nuciforo, Nina Radosevic-Robin, Tony Ng, and Maurizio Scaltriti.

Breast Cancer Research 2015 Apr 9, 17:53: 1-12.

In this review article, we evaluated the current methodologies used for HER family status determination and discussed the clinical implications of HER family quantification on response to anti-HER treatment.

Nuciforo et al. Breast Cancer Research (2015) 17:53
DOI 10.1186/s13058-015-0561-8

Breast Cancer RESEARCH

REVIEW Open Access

Quantification of HER family receptors in breast cancer

Paolo Nuciforo[1,2], Nina Radosevic-Robin[3,4], Tony Ng[5,6,7] and Maurizio Scaltriti[8*]

Abstract

The clinical success of trastuzumab in breast cancer taught us that appropriate tumor evaluation is mandatory for the correct identification of patients eligible for targeted therapies. Although HER2 protein expression by immunohistochemistry (IHC) and gene amplification by fluorescence *in situ* hybridization (FISH) assays are routinely used to select patients to receive trastuzumab, both assays only partially predict response to the drug. In the case of epidermal growth factor receptor (EGFR), the link between the presence of the receptor or its amplification and response to anti-EGFR therapies could not be demonstrated. Even less is known for HER3 and HER4, mainly due to lack of robust and validated assays detecting these proteins. It is becoming evident that, besides FISH and IHC, we need better assays to quantify HER receptors and categorize the patients for individualized treatments. Here, we present the current available methodologies to measure HER family receptors and discuss the clinical implications of target quantification.

Introduction

The HER family (also called ErbB or epidermal growth factor receptor (EGFR) family) comprises four transmembrane receptor tyrosine kinases, EGFR (or HER1), HER2, HER3, and HER4. These receptors signal through homo- and heterodimerization and promote cell proliferation, motility, and invasion [1]. Dysregulated expression and activity of HER family members is frequent in breast cancer. Overexpression of EGFR1, HER2 and HER3 is generally associated with poor prognosis whereas high expression of HER4 is associated with a better outcome [2-7]. Up to 25% of breast carcinomas overexpress HER2. High levels of this oncogene, almost invariably as a consequence of genomic amplification of a region of chromosome 17 (17q21) including the HER2 locus, drives aggressive disease and is an important therapeutic target.

Monoclonal antibodies (that is, trastuzumab, pertuzumab, T-DM1) and small molecule kinase inhibitors (that is, lapatinib, neratinib and afatinib) are the main strategies to target HER2 in breast cancer. Trastuzumab, in combination with chemotherapy, has significantly increased both progression-free survival (PFS) and overall survival in patients with advanced disease [8,9] as well as in the early (adjuvant) setting [10,11]. Lapatinib, given in combination with capecitabine, has shown clinical activity in HER2-positive breast cancer patients that became refractory to trastuzumab-based therapy [12]. Moreover, measurable clinical benefit is observed also when lapatinib is administered as a single agent or in combination with paclitaxel as first-line treatment [13,14]. Recently, the antitumor activity of dual HER2 blockade (trastuzumab in combination with either lapatinib or pertuzumab) was proven to be significantly superior to single agents in a neoadjuvant setting [15-17].

EGFR has been shown to be highly expressed in triple-negative breast cancer (TNBC), both in cell lines and in patients [18]. Moreover, preclinical studies have demonstrated that the inhibition of EGFR affects growth in TNBC cell lines [19]. These findings provided the rationale to test the efficacy of anti-EGFR agents, such as the antibodies cetuximab and panitumumab, in TNBC patients. In the metastatic setting, cetuximab in combination with chemotherapy showed some promising activity [20-22]. Nevertheless, no substantial improvements in either PFS or overall survival were achieved in these patients. A small pilot study testing the efficacy of panitumumab in combination with standard chemotherapy in TNBC patients in the neoadjuvant setting showed a pathological complete response rate of 46.8% [23]. However, the relevance of these findings will be assessed only when PFS and/or overall survival data are available.

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There are several unanswered questions about which patients with breast cancer are most likely to benefit from one or another form of anti-HER targeted therapy and which type of determination methodology is most appropriate.

DNA-based, RNA-based, and protein-based assays have been developed to determine the HER status of breast tumors. To date, exploitation of the overexpression of HER2 is part of the management of a breast cancer patient whereas EGFR, HER3, and HER4 determinations are still exploratory and not used in clinical practice. As a matter of fact, to be eligible for anti-HER therapy such as trastuzumab, specimens have to be HER2-positive as determined by immunohistochemistry (IHC) analysis or harbor HER2/neu gene amplification by fluorescence *in situ* hybridization (FISH). Although these tests have become the benchmarks for defining tumors as HER2-positive, considerable controversy still exists regarding the accuracy, reliability, and inter-observer variability of these assay methods. It is estimated that up to 20% of HER2 testing performed in the field may be inaccurate when validated against central or 'expert' laboratories [24,25]. A recent round-robin study conducted to evaluate current HER2 testing methods and their potential impact on clinical outcomes showed that interpretation issues (especially when dealing with IHC or FISH equivocal results as defined by the American Society of Clinical Oncologists (ASCO)/College of American Pathologists (CAP) guidelines) and/or HER2 tumor heterogeneity may play a significant role in discordant results [26].

In an effort to improve the accuracy and consistency of HER2 testing, a joint task force of ASCO and CAP proposed guideline recommendations for HER2 testing using either IHC or FISH [27]. Among 'HER2-positive' tumors (defined by consensus criteria), there is a wide range of variability in terms of HER2-gene amplification and protein expression measured by conventional semi-quantitative methods such as the HercepTest®. The possibility that a quantitative analysis of HER family protein expression could improve the prediction of HER-targeting drugs has led to the evaluation of alternative and more quantitative tests. Despite that, the 2013 ASCO/CAP Update Committee concluded that there was insufficient evidence to warrant inclusion of these new assays to determine HER2 status in unselected patients due to lack of a consistent body of evidence on their analytical validity, clinical validity, and clinical utility [27].

In this review we address these issues by evaluating the current methodologies used for HER family status determination and discussing the clinical implications of HER family quantification on response to anti-HER treatment. In Additional file 1 we list the Food and Drug Administration (FDA) approved/Clinical Laboratory Improvement Amendments certified diagnostic tests available to measure HER receptors in the clinic.

Methodologies

HER status assessment at the protein level

Immunohistochemistry

IHC is the primary technique used to determine protein expression status in a patient sample. It is a simple, fast, easy to implement and relatively inexpensive method for protein detection. Slides are incubated with an antibody directed against the HER receptor protein, labeled, and finally made visible with a chromogen, resulting in a staining localized in the cellular compartment where the protein target is expressed (membrane, cytosol, nucleus). The more the protein is present, the stronger the staining will be. Traditionally, assessment of protein expression is done by visual estimation of staining intensity and is reported as binary (positive versus negative), four-tiered (0, 1+, 2+, and 3+), or semiquantitative continuous variable as for the H score ((% at 0) × 0 + (% at 1+) × 1 + (% at 2+) × 2 + (% at 3+) × 3; range = 0 to 300) results [28].

For companion diagnostic tests, guidelines are generally issued to guide pathologists in the interpretation and scoring of the staining. The HER2 scoring guidelines recommended by ASCO/CAP classified HER2 expression as 0 (no staining or faint incomplete membrane staining observed in ≤10% of tumor cells), 1+ (faint/barely perceptible incomplete membrane staining in >10% of tumor cells), 2+ (circumferential membrane staining that is incomplete and/or weak/moderate and within >10% of tumor cells or complete and circumferential membrane staining that is intense and within ≤10% of tumor cells) or 3+ (circumferential membrane staining that is complete, intense, and within >10% of tumor cells). Tumors with scores 0 and 1+ were considered negative; 2+ was considered equivocal and required FISH reflex testing; 3+ was considered positive and eligible for trastuzumab [29].

Despite the effort to standardize HER2 status determination, current guidelines do not restrict the type and characteristics of IHC assay to be used for HER2 protein expression. The use of FDA approved tests such as HercepTest® (DAKO, Carpinteria, CA, USA), PATHWAY anti-HER-2/neu (Ventana Medical systems, Roche, Tuscon, AZ, USA), InSite™ Her-2/neu (Biogenex, Freemont, CA, USA) as well as fully automated staining systems such as Ventana Benchmark (Ventana Medical systems, Roche, Tuscon, AZ, USA) and Leica Microsystems Bondmax (Leica, Newcastle, UK) may certainly minimize process variability and improve assay repeatability and reproducibility. Nevertheless, many laboratories developed tests with different antibodies directed against other HER2 epitopes (intracellular versus

extracellular), which may show non-overlapping specificities and be differently influenced by preanalytical factors [30-32].

Interpretation of membrane staining can be optimized using quantitative image analysis such as the automated quantitative analysis (AQUA) system [33]. AQUA is a fluorescence IHC-based method that provides objective and continuous protein expression scores for tissues by using automated fluorescence microscopy and advanced image analysis algorithms. It is important to note, however, that there are as yet no clinical data related to AQUA's predictive ability.

Other software applications include Aperio Scanscope (Aperio Technologies; Vista, CA, USA), Definiens (Carlsbad, CA, USA) and Vysis AutoVysion (Abbott Molecular, Des Plaines, IL, USA) among others. These applications can reduce the subjectivity of a traditional scoring system and provide a more reproducible protein expression score [34,35].

Enzyme-linked immunosorbent assay

HER2 receptor protein extracellular domain (ECD, p105) is released into the circulation after cleavage by matrix metalloproteinases and its levels can be measured in the serum using an enzyme-linked immunosorbent assay approved by the FDA (Siemens Healthcare, Erlangen, Germany). Elevated levels of serum HER2 ECD have been shown to be both prognostic and predictive of response to trastuzumab in HER2-positive tumors [36,37]. Serum ECD values have been suggested, therefore, as an alternative technique for determining HER2 status, although available results are controversial. First, not all patients with HER2-positive tumors appear to have elevated serum ECD values and patients with HER2-negative tumors can also have elevated ECD values. Second, the reported data come from studies including a limited number of patients, thus making current evidences still insufficient to consider basing treatment decisions on ECD levels in routine clinical practice. A large meta-analysis study [38] has combined the data of four trastuzumab trials in metastatic breast cancer and showed that, from the combined dataset (N = 322 patients), there was no correlation between baseline ECD value and tumor response. ECD values decreased upon initiation of combination therapy irrespective of treatment and tumor response. Furthermore, disease progression was not reliably predicted by an increase in ECD levels. Therefore, the use of ECD values in treatment decision making was not recommended.

VeraTag™ proximity-based assay

The VeraTag™ proximity-based assay (HERmark® Breast Cancer Assay; Monogram Biosciences, Inc., South San Francisco, CA, USA) enables precise quantitative measurements of total HER-2 expression and HER2 homodimers in formalin-fixed, paraffin-embedded (FFPE) tissue specimens [39,40]. The HERmark assay was developed based on a proprietary proximity-based technology platform that enables accurate quantification of proteins and protein-protein complexes through the release of a fluorescent tag (VeraTag reporter, Monogram Biosciences) conjugated to a pair of monoclonal antibodies directed to unique epitopes on the HER2 receptor in molecular proximity [40]. The continuous total HER-2 expression results are grouped as HERmark negative, HERmark equivocal, and HERmark positive. The threshold for a positive HERmark test is based on the comparison with HER2 tests performed in 1,090 breast tumor reference samples (central IHC and central *in situ* hybridization) from three different study cohorts. The HERmark assay can detect HER2 at amounts of 2,500 up to more than 1 million receptors per cell, and is thus said to be 7 to 10 times more sensitive than IHC. The assay has been validated according to the specifications prescribed by the Clinical Laboratory Improvement Amendments and is performed only in the CAP-certified clinical reference laboratory at Monogram Biosciences (US). VeraTag™ proximity-based assays have been developed also to measure total EGFR, EGFR-EGFR homodimers and EGFR-HER2 heterodimers [40-42], p95 [43], total HER3, HER3-HER3 homodimers and HER3-phosphoinositide 3-kinase (PI3K) complex heterodimers [44] and the phosphorylated forms of EGFR, HER2, and HER3.

Protein interaction measurements

Clinical application of protein-protein interactions has uncovered many potential targets for novel drug development or drug resistance mechanisms [45], with the MDM2-p53 interaction [46,47] and B-Raf inhibition being examples of recent successes [48]. More recently, incorporation of protein interaction data was shown to also improve the predictive performance of prognostic gene expression signatures [49,50]. Despite the importance of adjunct information supplied by the protein interactome configuration to improve the existing prognostic signatures for predicting patient outcome [50], this protein interaction information has rarely been incorporated in diagnostic/prognostic assays.

Fluorescence lifetime imaging microscopy (FLIM) is based on quantifying the non-radiative transfer of energy between the donor and acceptor fluorophores and can only occur when the two molecules are no further apart than 10 nm, consistent with being in molecular contact [51-53]. Various automated imaging platforms, including ours, measure Förster resonance energy transfer (FRET) - the decrease in donor lifetime, the gold standard for FRET measurements (reviewed recently in [54]) - to directly monitor validated protein-protein interactions [55-61] and post-translational modifications, including conformational

changes, in cultured cells [58,62-66]. A two antibody FRET/FLIM approach was originally applied, by ourselves and others, to human cancer tissues to detect the nano-proximity between a donor fluorophore-conjugated anti-protein kinase C or anti-EGFR antibody, and an acceptor fluorophore-labeled phospho-specific antibody, providing highly specific quantification of phosphorylation [67,68]. Detailed methodology for sample preparation and instrumentation can be found elsewhere [69,70]. We have now extended this method to measure endogenous protein-protein interactions in archived pathological material [71]. The presence of autofluorescence in stromal and epithelial components may cause difficulties in accurately determining the fluorescence lifetime of fluorophores in FFPE tissue samples [72]. By circumventing the autofluorescence issue using a new analysis algorithm [73], we have recently described the first clinical utilization of this refined FLIM assay (using Alexa546 and Cy5 as donor and acceptor fluorophores, respectively) to quantify the level of HER1-HER3 dimer formation in FFPE tissues from basal-like breast cancer patients who were treated with a neoadjuvant anti-EGFR treatment (cetuximab or panitumumab) [74]. Moreover, we have demonstrated the existence of EGFR-HER4 dimers in breast cancer cells and how these dimers are important for cell motility [75].

Liquid chromatography-tandem mass spectrometry-based proteomics has emerged as the most effective method to study complex proteomes. In this approach, the proteins representing a proteome are analyzed after enzymatic digestion by liquid chromatography coupled to mass spectrometry (MS). Although this approach is a powerful tool to identify proteins in complex biological samples [76,77], it is not optimal for systematic quantification of these proteins because of the stochastic nature and the limited sensitivity of the approach. During the past few years, targeted proteomics has been shown to be complementary to the more widely used discovery proteomic methods. In targeted proteomics, only predetermined peptide ions are selected for detection and quantification in a sample. The main MS approach supporting targeted proteomics is selected reaction monitoring (SRM), where specific MS assays are generated *a priori* and used to selectively detect and quantify proteins of interest in a sample. This approach can provide objective quantification and multiplex capabilities with high sensitivity and in an antibody-free setting [78-80]. SRM methods have long been used to quantify low-abundance protein targets in plasma [81] but application of these techniques to FFPE tissue samples has, until recently, been hindered by incomplete solubilization of samples [82,83]. The Liquid Tissue-(SRM) diagnostic technology platform is a newly developed proteomic method that overcomes this limitation, allowing for precise protein quantification in FFPE tissues. Microdissected FFPE tumor tissues are subjected to Liquid Tissue processing to reverse formalin crosslinks. This is followed by trypsinization to completely solubilize all of the protein in the sample. This tryptic peptide mixture is then subjected to SRM analysis using stable isotope-labeled control peptides for accurate quantification [83-85]. Multiple reports have demonstrated that comparable results may be obtained between formalin fixed and matching frozen tissue [84,86]. The reliability of this approach for analysis of proteins in any biological sample including FFPE patient tumor tissue has been demonstrated [87-91], thus widening the application of MS to patient-derived tissue with a consequent profound impact on patient stratification and targeted cancer therapeutics.

Reverse phase protein array (RPPA) and collaborative enzyme enhanced reactive-immunoassay (CEER) are nano-scaled dot blot platforms allowing the detection of multiple proteins (both total and phosphorylated) in many samples simultaneously. They do not require large amounts of sample but are not suitable for FFPE tissue. For RPPA protein lysates are immobilized onto microarrays and then probed with the primary antibodies of choice. Detection is performed by quantification of the labels (fluorescent, colorimetric or other kinds) bound to either the primary or, more often, the secondary antibody added to amplify the signal. RPPA allows testing hundreds of samples at the same time and multiplexing is performed by analyzing multiple arrays spotted with the same protein lysates with different antibodies [92].

CEER takes advantage of the immunocomplexes formed between antibodies printed on a nitrocellulose microarray surface with the target molecules in cell lysates. Once the complexes are formed, two detector antibodies (one conjugated to glucose oxidase and another conjugated to horse radish peroxidase (HRP)) are added. Target detection (expressed as computational units (CU)) requires the presence of both detector antibodies, and the enzyme channeling event between glucose oxidase and HRP will not occur unless both antibodies are in close proximity [93]. The main difference with RPPA is that, instead of protein lysate, antibodies are immobilized in cellulose arrays. This means that, contrary to RPPA, CEER is capable of measuring the expression of dozens of targets simultaneously in the same sample.

Further studies are needed to prove the clinical relevance of the above described methods.

HER status assessment at the DNA level

In situ hybridization

FISH is considered the gold standard method for gene amplification status determination. FISH uses fluorescently labeled probes (usually red) that are complementary to a part of the target gene. After hybridization to

the complementary DNA on the slide, the probes can be visualized with a fluorescence microscope. A second probe labeled with a different fluorochrome (usually green) directed against the centromeric region of the chromosome containing the target gene is generally used as control for polysomy. The number of copies of the target gene and centromere probe can be estimated and the ratio determined.

CISH (chromogenic *in situ* hybridization) is an alternative for FISH. It uses an immunoperoxidase reaction to visualize the target gene probe, which allows scoring with a conventional light microscope. CISH has several advantages over FISH: signal does not fade and the slides can be kept permanently and allows better preservation of morphology. One of the main limitations of CISH is that most of the available assays are still monoprobe assays, meaning that there is no correction with a centromere control probe and only the absolute gene copy number is scored.

Similar to CISH, silver *in situ* hybridization (SISH) technology uses a non-fluorescent method where the HRP bound to the probe catalyses the reduction of silver acetate to produce a black signal. Several studies showed a good correlation between FISH, CISH, SISH, and IHC for HER2 status determination [94-100].

Recently released ASCO/CAP guidelines recommended that HER2 must be considered *in situ* hybridization (ISH)-positive based on a single-probe average HER2 copy number ≥6.0 signals/cell or dual-probe HER2/CEP17 ratio ≥2.0 or dual-probe HER2/CEP17 ratio <2.0 with an average HER2 copy number ≥6.0 signals/cell [27]. Whether the centromere control probes for polysomy 17 are really necessary is a matter of debate given that it has been proven by several studies that true polysomy 17 is very rare in breast carcinomas [101]. Concurrent evaluation of several chromosome 17 genes using multiple-probe FISH or multiplex ligation-dependent probe amplification showed that focal amplifications encompassing the centromere - and not true polysomy - are the most common explanation for increases in CEP17 signals [102,103]. These results suggest that CEP17 copy number assessment by standard ISH is not a useful surrogate for polysomy 17. Compared with IHC, ISH assays, in which the target gene copy number is counted, are considered to be more quantitative analytically. However, ISH is not a direct measurement of the protein and just because a change in gene copy number is observed does not necessarily mean that it is expressed. In addition, the procedure is time consuming and new 'fast' FISH assays are under development to reduce the turnaround time [104].

PCR-based techniques

PCR-based techniques such as multiplex ligation-dependent probe amplification [105] have several advantages over ISH-based assays. First, they are more quantitative and results are easier to interpret. Second, they require only small amounts of DNA and are not affected by DNA degradation, thus performing well with FFPE samples. Third, they can be multiplexed, allowing simultaneous interrogation of multiple genes or different parts of genes, representing an ideal and low cost prescreening tool. Head-to-head comparisons between IHC, FISH, and CISH have shown good correlation among technologies [106-109]. The main weaknesses of PCR-based assays are that they do not preserve tissue morphology, may require sample macro- or microdissection to enrich for tumor content, heterogeneity can be missed and contamination with normal or ductal carcinoma *in situ* may lead to both false-negative and false-positive results.

HER status assessment at the RNA level

Due to multiplexing capability, RNA-based tests are usually used to generate global gene expression signatures rather than single gene measurements. All these signatures work using proprietary algorithms that generate a score based on the expression levels of the genes measured that can determine risk factors, incidence, prognoses and responses to systemic therapies. Clinically validated gene expression tests that include one or more HER family members in their gene lists are discussed below.

The Onco*type* DX assay (Genomic Health, Redwood City, CA, USA) uses RT-PCR as a primary technique and work on RNA extracted from FFPE samples. The assay measures the expression of a panel of 21 genes (only *HER2* is included among the HER family genes) and the results are provided as a recurrence score. Although the assay was approved as a prognostic test predictive of breast cancer recurrence in women with newly diagnosed, early stage breast cancer, it also assesses the benefit from certain types of chemotherapy [110]. Recently, Genomic Health started reporting estrogen receptor (ER), progesterone receptor (PR), and HER2 results separately in addition to the recurrence score. Although high overall concordance (greater than 91%) between HER2 by IHC or FISH assay and quantitative RT-PCR using the Onco*type* DX test has been reported [111,112], an independent study showed a false-negative rate for Onco*type* DX RT-PCR for HER2 of >50% [113].

TargetPrint™ (Agendia, Irvine, CA, USA/Amsterdam, The Netherlands) is a microarray-based gene expression test that allows quantitative assessment of ER, PR and HER2 at the RNA level in breast cancer. Compared with IHC results, HER2 gene expression levels provided by TargetPrint™ have been shown to be more reproducible and truly quantitative. Results were validated against

IHC and showed an overall concordance greater that 95% [114-116]. Its use is currently proposed in case of equivocal or unreliable IHC results, discordance between two separate tests, discordance of test results and clinicopathologic features or technical failure of IHC/FISH/CISH.

The NanoString Prosigna™ (NanoString Technologies, Seattle, WA, USA) assay measures the expression levels of 50 target genes (including HER2) plus eight constitutively expressed normalization genes (PAM50) to classify a tumor as one of four intrinsic subtypes (luminal A, luminal B, HER2-enriched, and basal-like), which have been shown to be prognostic [117,118]. In addition to identifying a tumor's intrinsic subtype, the PAM50 signature generates an individualized score estimating a patient's probability of disease recurrence by weighting the molecular subtype correlations, a subset of proliferation genes, and pathologic tumor size [118,119]. Based on these data, the FDA-cleared and CE-marked Prosigna™ assay, based on the PAM50 gene expression signature, has recently been shown to predict the risk of distant recurrence in women with hormone receptor-positive early stage breast cancer treated with 5 years of endocrine therapy [120-122]. The Nanostring nCounter system uses color-coded probes that bind directly to the RNA transcript without reverse transcription and PCR amplification [123] and work in frozen or FFPE tissues with equivalent ease and efficiency [124]. Assay controls are included to ensure that test samples and the test process meet pre-defined quality thresholds.

The PAM50 gene signature may be run also by classic quantitative PCR and can also provide quantitative and qualitative gene expression scores for the standard biomarkers usually measured semi-quantitatively by IHC - ER, PR and HER2. Using the quantitative PCR cutoff for ERBB2 expression, a study found high specificity (609/624 samples that were low ERBB2 were also HER2-negative by IHC/CISH), while 53% (109/190) of tumors with intermediate-high ERBB2 expression were HER2-positive [125]. This same study and the MA.5 trial [126] found that only about two-thirds of clinically HER2-positive tumors are classified as HER2-enriched. Thus, only a subset of the IHC-defined groups overlap with PAM50 subtype classification.

Although some literature shows an overall high concordance between standard techniques such as IHC and FISH assays and quantitative RT-PCR [111,127-129], there are several practical issues that should be considered when conducting RNA-based analyses. First, the presence of normal tissue within the tumor sample is a major source of subtype misclassification [130]. Therefore, identification of the region of viable invasive breast carcinoma by a pathologist is critical before any RNA extraction is performed. Second, RNA shows greater instability compared with DNA and proteins and thus the selection of technologies that may prevent/overcome RNA degradation is important.

Clinical implications

The fundamental principle of targeted therapy is to specifically harm tumor cells that depend on a definite target for proliferation and survival, sparing non-tumor cells from damage. In many cases, the target is a protein with activating mutations that is present only in tumor cells, facilitating the specificity of the therapy (for example, Braf-mutant melanomas, EGFR-mutant lung cancer), allowing profound inhibition of the target before the emergence of side effects. In the case of HER receptors in breast cancer the target is a protein that, although not carrying any activating alterations, is present in much higher amounts in tumor cells compared with normal cells. In these cases one would guess that the higher is the difference in target expression between normal and tumor cells, the wider is the therapeutic window. However, only the presence of the target or its semiquantitative expression (and not the absolute levels) is currently taken into consideration in clinical practice.

There is an increasing body of evidence indicating that the levels of HER2 in HER2-positive tumors can influence the response to HER2-targeted therapy, converging to the common conclusion that 'more HER2, more response' [131-137]. Quantitative HER2 expression or homodimer levels determined by the HERmark assay correlated with clinical outcome of trastuzumab therapy better than IHC or central FISH studies in patients with metastatic breast cancer. Interestingly, patients with *HER2* gene amplification by FISH but low HER2 protein expression or homodimer levels as measured by HERmark responded poorly to trastuzumab-containing therapy, suggesting that not all gene-amplified tumors overexpress the target of trastuzumab [135]. Similarly, absolute HER2 quantification in an homogeneous group of HER2-positive breast cancer (IHC 3+) using triple quadrupole MS was predictive of a better response to trastuzumab in both adjuvant and metastatic settings [136].

But perhaps this is valid until a certain limit. First, the link between the level of HER2 amplification and outcome in patients treated with trastuzumab has been proven only in the neoadjuvant setting [138], whereas other studies failed to demonstrate this association [139,140]. Second, although the clinical benefit from HER2 blockade increases with the level of the target, there may be tumors with extraordinarily high levels of HER2 that are actually more resistant to the therapeutic pressure [141-144]. It is unclear whether this is due to insufficient engagement of the receptor by the targeted agents. In any case, validation of these findings in a larger cohort of patients is necessary. Third, the intriguing

observation from B-31 and N9831 studies that tumors that failed to be confirmed as HER2-positive after central laboratory testing may still derive benefit from trastuzumab [145,146] and the complex relationship between HER2, ER, and trastuzumab sensitivity outlined by the study suggest that quantitative HER2 measure alone may not be sufficient, and combination with other markers may be more predictive of trastuzumab response [147].

Since dual HER2 blockade (trastuzumab combined with either pertuzumab or lapatinib) is proving to be more effective than single agent treatment, it will be interesting to investigate whether HER2 absolute levels predict response in this setting as well. In the neoadjuvant setting, this seems to be the case. HER2 levels were measured by HERmark in the primary tumors of patients enrolled in the NeoALTTO trial, testing the activity of trastuzumab in combination with lapatinib compared with single agent treatments, and a positive correlation was found between constitutive HER2 expression and benefit from dual blockade [148].

One of the mechanisms proposed for the synergy observed when combining lapatinib and trastuzumab (at least in preclinical models) is the stabilization and membrane accumulation of HER2 as a consequence of receptor kinase inhibition [149]. One may wonder, therefore, whether lapatinib could sensitize tumors with relatively low levels of HER2 to the antitumor activity of trastuzumab. Testing this possibility, however, is not as easy as it sounds. First, a threshold above which tumors benefit from anti-HER2 therapy (but are still considered 'low expressing tumors') needs to be defined by quantitative methodology. Then, other therapeutic options should be considered to exclude the possibility that these patients can achieve better response from other agents. Genomic analysis of the tumors would be very helpful in these cases as the identification of actionable genetic alterations may guide the choice of therapy. Finally, HER-targeted therapeutic agents such as lapatinib have been shown to stabilize/enhance the HER2-HER3 dimer in preclinical cell models [149]. The quantification of this dimer (as described above), which is believed to be the most potent of all HER dimers with regard to driving cellular proliferation [150,151], will provide important and non-redundant information to that provided by HER protein expression to help clinicians to understand and/or predict the heterogeneity in clinical response.

The quantification of HER3 in response to lapatinib-containing therapies may also be of relevance. In fact, compensatory upregulation of HER3 upon lapatinib treatment has been described both in preclinical models and in patients with HER2-positive breast cancer [152]. The addition of compounds blocking HER3 or the downstream PI3K/AKT pathway significantly potentiates the antitumor effects of lapatinib, underscoring the importance of this occurrence. Because of the mechanistic relationship between EGFR and HER2, EGFR measurement may provide a method for personalizing treatment in breast cancer, beyond the single assay for HER2. Patients with high EGFR using the EGFR antibody D38B1 did not appear to benefit from concurrent trastuzumab in the N9831 trial using the fluorescence-based AQUA quantitative platform [153]. Based on these results, it may be hypothesized that the subset of tumors with high EGFR expression may better respond to lapatinib or dual HER blockade compared with trastuzumab alone.

The absolute levels of EGFR may be predictive for response to anti-EGFR therapy in TNBC patients. We recently showed that patients with tumors expressing high levels of EGFR were more likely to achieve pathological complete response following panitumumab-based therapy [74]. Furthermore, we found that EGFR levels tended to decrease in the residual tumors collected at surgery compared with the primary tumor before the commencement of therapy, indicating that the levels of EGFR may be influenced by the therapeutic pressure. It remains to be defined whether this is a global downregulation of EGFR in all tumor cells or is a positive selection of cells with lower EGFR expression.

As a matter of fact, the acquired loss of expression of HER receptors may be an obvious mechanism of resistance to targeted therapy according to the simple paradigm 'no target, no response'. This has also been described in HER2-positive breast cancer patients upon treatment with trastuzumab-based therapy [154]. Therefore, measuring the levels of HER receptors at the time of progression to targeted therapy should be encouraged to avoid persevering with similar targeted approaches.

Conclusion and perspectives

It is becoming evident that the 'simple detection' of the HER receptors in breast cancer is not sufficient to predict the benefit that patients will achieve from anti-HER therapy. The example of HER2 is archetypal. We know that HER2-positive patients benefit from anti-HER2 therapy, but now we also know that 15 to 20% of these patients express levels of the receptors that are almost comparable with HER2-negative tumors. And, more importantly, these patients do not achieve the same benefit from anti-HER2 therapy as do patients with high HER2 expression. This is especially true in the neoadjuvant setting in patients undergoing dual HER2 blockade [148].

Let's make an example of how relevant these findings can be. The disease-free survival data from the ALTTO adjuvant trial (comparing patients that received lapatinib, trastuzumab or the combination of the two agents) were recently released [155]. The take home message was that the combination was not significantly superior

to trastuzumab single agent in preventing relapses to therapy. These findings were somehow surprising since the NeoALTTO trial clearly demonstrated that dual HER2 blockade is more effective than monotherapy in the neoadjuvant setting. But if we dissect the data we realize that many variables could have influenced this outcome. First, the number of PFS events taken into consideration was lower than the one needed for the planned statistical analysis. Second, a significant percentage of patients enrolled in the combination arm were not treated with a full dose of lapatinib (for toxicity reasons). In a study where the 'control arm' (trastuzumab-based therapy) is known to cure more than 80% of patients, these factors may have diluted the possible improvement in PFS. Thus, it is not so surprising that the difference observed in the ALTTO trial was not significant. It would be interesting to quantify the levels of HER2 in these samples and correlate them with clinical response. Perhaps we will identify a subset of patients with high HER2 expression that is more sensitive to dual HER2 blockade and shows significant clinical benefit in the long term. Fortunately, these samples are available for future biomarker analyses, including HER2 quantification.

For EGFR things are far behind. The basis for testing anti-EGFR therapy in TNBC was the knowledge that overexpression of EGFR occurs in up to 50% of cases [156]. But a real stratification based on how much EGFR these tumors express has never been made. Now we have evidence that, the higher the levels of EGFR, the higher the probability to achieve pathological complete response from cetuximab- or panitumumab-based therapy in the neoadjuvant setting [74]. Again, one would wonder whether the reported activity of anti-EGFR therapy in TNBC (or even in head and neck and colon cancers) would be different if stratification based on the EGFR levels had been done in these clinical trials.

Conclusion

In conclusion, HER receptor quantification may be more tedious than FISH or IHC but it can help in stratifying and selecting patients for anti-HER therapy. Measuring the levels of the targets in patients undergoing 'targeted' therapy sounds like a good idea.

Additional file

Additional file 1: A table listing laboratory diagnostic tests cleared by the Food and Drug Administration or offered by central laboratories under Clinical Laboratory Improvement Amendments measuring HER receptors in the clinic. *Epidermal growth factor (EGFR), HER2 and HER3. #Approved for colorectal cancer. LDT, laboratory developed test; Q, quantitative; QL, qualitative; SQ, semiquantitative.

Abbreviations

AQUA: Automated quantitative analysis; ASCO: American Society of Clinical Oncologists; CAP: College of American Pathologists; CEER: Collaborative enzyme enhanced reactive-immunoassay; CISH: Chromogenic *in situ* hybridization; ECD: Extracellular domain; EGFR: Epidermal growth factor receptor; ER: Estrogen receptor; FDA: Food and Drug Administration; FFPE: Formalin-fixed, paraffin-embedded; FISH: Fluorescence *in situ* hybridization; FLIM: Fluorescence lifetime imaging microscopy; FRET: Förster resonance energy transfer; HRP: Horse radish peroxidase; IHC: Immunohistochemistry; ISH: *In situ* hybridization; MS: Mass spectrometry; PFS: Progression-free survival; PI3K: Phosphoinositide 3-kinase; PR: Progesterone receptor; RPPA: Reverse phase protein array; SISH: Silver *in situ* hybridization; SRM: Selected reaction monitoring; TNBC: Triple-negative breast cancer.

Competing interests

The authors declare that they have no competing interests.

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References

1. Holbro T, Beerli RR, Maurer F, Koziczak M, Barbas 3rd CF, Hynes NE. The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. Proc Natl Acad Sci U S A. 2003;100:8933–8.
2. Naidu R, Yadav M, Nair S, Kutty MK. Expression of c-erbB3 protein in primary breast carcinomas. Br J Cancer. 1998;78:1385–90.
3. Pawlowski V, Revillion F, Hebbar M, Hornez L, Peyrat JP. Prognostic value of the type I growth factor receptors in a large series of human primary breast cancers quantified with a real-time reverse transcription-polymerase chain reaction assay. Clin Cancer Res. 2000;6:4217–25.
4. Suo Z, Berner HS, Risberg B, Karlsson MG, Nesland JM. Estrogen receptor-alpha and C-ERBB-4 expression in breast carcinomas. Virchows Arch. 2001;439:62–9.
5. Suo Z, Risberg B, Kalsson MG, Willman K, Tierens A, Skovlund E, et al. EGFR family expression in breast carcinomas. c-erbB-2 and c-erbB-4 receptors have different effects on survival. J Pathol. 2002;196:17–25.
6. Tovey SM, Witton CJ, Bartlett JM, Stanton PD, Reeves JR, Cooke TG. Outcome and human epidermal growth factor receptor (HER) 1–4 status in invasive breast carcinomas with proliferation indices evaluated by bromodeoxyuridine labelling. Breast Cancer Res. 2004;6:R246–51.
7. Witton CJ, Reeves JR, Going JJ, Cooke TG, Bartlett JM. Expression of the HER1-4 family of receptor tyrosine kinases in breast cancer. J Pathol. 2003;200:290–7.
8. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med. 2001;344:783–92.
9. Hudis CA. Trastuzumab - mechanism of action and use in clinical practice. N Engl J Med. 2007;357:39–51.

10. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med. 2005;353:1659–72.
11. Smith I, Procter M, Gelber RD, Guillaume S, Feyereislova A, Dowsett M, et al. 2-year follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer: a randomised controlled trial. Lancet. 2007;369:29–36.
12. Geyer CE, Forster J, Lindquist D, Chan S, Romieu CG, Pienkowski T, et al. Lapatinib plus capecitabine for HER2-positive advanced breast cancer. N Engl J Med. 2006;355:2733–43.
13. Gomez HL, Doval DC, Chavez MA, Ang PC, Aziz Z, Nag S, et al. Efficacy and safety of lapatinib as first-line therapy for ErbB2-amplified locally advanced or metastatic breast cancer. J Clin Oncol. 2008;26:2999–3005.
14. Di Leo A, Gomez HL, Aziz Z, Zvirbule Z, Bines J, Arbushites MC, et al. Phase III, double-blind, randomized study comparing lapatinib plus paclitaxel with placebo plus paclitaxel as first-line treatment for metastatic breast cancer. J Clin Oncol. 2008;26:5544–52.
15. Baselga J, Bradbury I, Eidtmann H, Di Cosimo S, de Azambuja E, Aura C, et al. Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): a randomised, open-label, multicentre, phase 3 trial. Lancet. 2012;379:633–40.
16. Baselga J, Cortes J, Kim SB, Im SA, Hegg R, Im YH, et al. Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer. N Engl J Med. 2012;366:109–19.
17. Gianni L, Pienkowski T, Im YH, Roman L, Tseng LM, Liu MC, et al. Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial. Lancet Oncol. 2012;13:25–32.
18. Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, et al. Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin Cancer Res. 2004;10:5367–74.
19. Hoadley KA, Weigman VJ, Fan C, Sawyer LR, He X, Troester MA, et al. EGFR associated expression profiles vary with breast tumor subtype. BMC Genomics. 2007;8:258.
20. Baselga J, Gomez P, Greil R, Braga S, Climent MA, Wardley AM, et al. Randomized phase II study of the anti-epidermal growth factor receptor monoclonal antibody cetuximab with cisplatin versus cisplatin alone in patients with metastatic triple-negative breast cancer. J Clin Oncol. 2013;31:2586–92.
21. Carey LA, Rugo HS, Marcom PK, Mayer EL, Esteva FJ, Ma CX, et al. TBCRC 001: randomized phase II study of cetuximab in combination with carboplatin in stage IV triple-negative breast cancer. J Clin Oncol. 2012;30:2615–23.
22. O'Shaughnessy J, Weckstein DJ, Vukelja SJ, McIntyre K, Krekow L, Holmes FA, et al. Preliminary results of a randomized phase II study of weekly irinotecan/carboplatin with or without cetuximab in patients with metastatic breast cancer. Breast Cancer Res Treat. 2007;106:S32. Abstract 308.
23. Nabholtz JM, Abrial C, Mouret-Reynier MA, Dauplat MM, Weber B, Gligorov J, et al. Multicentric neoadjuvant phase II study of panitumumab combined with an anthracycline/taxane-based chemotherapy in operable triple-negative breast cancer: identification of biologically defined signatures predicting treatment impact. Ann Oncol. 2014;25:1570–7.
24. Perez EA, Suman VJ, Davidson NE, Martino S, Kaufman PA, Lingle WL, et al. HER2 testing by local, central, and reference laboratories in specimens from the North Central Cancer Treatment Group N9831 intergroup adjuvant trial. J Clin Oncol. 2006;24:3032–8.
25. McCullough AE, Dell'orto P, Reinholz MM, Gelber RD, Dueck AC, Russo L, et al. Central pathology laboratory review of HER2 and ER in early breast cancer: an ALTTO trial [BIG 2-06/NCCTG N063D (Alliance)] ring study. Breast Cancer Res Treat. 2014;143:485–92.
26. Perez EA, Press MF, Dueck AC, Jenkins RB, Kim C, Chen B, et al. Immunohistochemistry and fluorescence in situ hybridization assessment of HER2 in clinical trials of adjuvant therapy for breast cancer (NCCTG N9831, BCIRG 006, and BCIRG 005). Breast Cancer Res Treat. 2013;138:99–108.
27. Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. Arch Pathol Lab Med. 2014;138:241–56.
28. Taylor CR, Levenson RM. Quantification of immunohistochemistry - issues concerning methods, utility and semiquantitative assessment II. Histopathology. 2006;49:411–24.
29. Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol. 2007;25:118–45.
30. Engel KB, Moore HM. Effects of preanalytical variables on the detection of proteins by immunohistochemistry in formalin-fixed, paraffin-embedded tissue. Arch Pathol Lab Med. 2011;135:537–43.
31. Khoury T. Delay to formalin fixation alters morphology and immunohistochemistry for breast carcinoma. Appl Immunohistochem Mol Morphol. 2012;20:531–42.
32. Portier BP, Wang Z, Downs-Kelly E, Rowe JJ, Patil D, Lanigan C, et al. Delay to formalin fixation 'cold ischemia time': effect on ERBB2 detection by in-situ hybridization and immunohistochemistry. Mod Pathol. 2013;26:1–9.
33. Camp RL, Chung GG, Rimm DL. Automated subcellular localization and quantification of protein expression in tissue microarrays. Nat Med. 2002;8:1323–7.
34. Gustavson MD, Bourke-Martin B, Reilly D, Cregger M, Williams C, Mayotte J, et al. Standardization of HER2 immunohistochemistry in breast cancer by automated quantitative analysis. Arch Pathol Laboratory Med. 2009;133:1413–9.
35. McCabe A, Dolled-Filhart M, Camp RL, Rimm DL. Automated quantitative analysis (AQUA) of in situ protein expression, antibody concentration, and prognosis. J Natl Cancer Inst. 2005;97:1808–15.
36. Colomer R, Llombart-Cussac A, Lluch A, Barnadas A, Ojeda B, Caranana V, et al. Biweekly paclitaxel plus gemcitabine in advanced breast cancer: phase II trial and predictive value of HER2 extracellular domain. Ann Oncol. 2004;15:201–6.
37. Kostler WJ, Steger GG, Soleiman A, Schwab B, Singer CF, Tomek S, et al. Monitoring of serum Her-2/neu predicts histopathological response to neoadjuvant trastuzumab-based therapy for breast cancer. Anticancer Res. 2004;24(2C):1127–30.
38. Lennon S, Barton C, Banken L, Gianni L, Marty M, Baselga J, et al. Utility of serum HER2 extracellular domain assessment in clinical decision making: pooled analysis of four trials of trastuzumab in metastatic breast cancer. J Clin Oncol. 2009;27:1685–93.
39. Huang W, Reinholz M, Weidler J, Yolanda L, Paquet A, Whitcomb J, et al. Comparison of central HER2 testing with quantitative total HER2 expression and HER2 homodimer measurements using a novel proximity-based assay. Am J Clin Pathol. 2010;134:303–11.
40. Shi Y, Huang W, Tan Y, Jin X, Dua R, Penuel E, et al. A novel proximity assay for the detection of proteins and protein complexes: quantitation of HER1 and HER2 total protein expression and homodimerization in formalin-fixed, paraffin-embedded cell lines and breast cancer tissue. Diagn Mol Pathol. 2009;18:11–21.
41. DeFazio-Eli L, Strommen K, Dao-Pick T, Parry G, Goodman L, Winslow J. Quantitative assays for the measurement of HER1-HER2 heterodimerization and phosphorylation in cell lines and breast tumors: applications for diagnostics and targeted drug mechanism of action. Breast Cancer Res. 2011;13:R44.
42. Dua R, Zhang J, Nhonthachit P, Penuel E, Petropoulos C, Parry G. EGFR over-expression and activation in high HER2, ER negative breast cancer cell line induces trastuzumab resistance. Breast Cancer Res Treat. 2010;122:685–97.
43. Sperinde J, Jin X, Banerjee J, Penuel E, Saha A, Diedrich G, et al. Quantitation of p95HER2 in paraffin sections by using a p95-specific antibody and correlation with outcome in a cohort of trastuzumab-treated breast cancer patients. Clin Cancer Res. 2010;16:4226–35.
44. Mukherjee A, Badal Y, Nguyen XT, Miller J, Chenna A, Tahir H, et al. Profiling the HER3/PI3K pathway in breast tumors using proximity-directed assays identifies correlations between protein complexes and phosphoproteins. PLoS One. 2011;6:e16443.
45. Arkin MR, Wells JA. Small-molecule inhibitors of protein-protein interactions: progressing towards the dream. Nat Rev Drug Discov. 2004;3:301–17.
46. Tovar C, Rosinski J, Filipovic Z, Higgins B, Kolinsky K, Hilton H, et al. From the Cover: Small-molecule MDM2 antagonists reveal aberrant p53 signaling in cancer: implications for therapy. Proc Natl Acad Sci U S A. 2006;103:1888–93.

47. Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z, et al. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science. 2004;303:844–8.
48. Solit DB, Rosen N. Resistance to BRAF inhibition in melanomas. N Engl J Med. 2011;364:772–4.
49. Chuang HY, Lee E, Liu YT, Lee D, Ideker T. Network-based classification of breast cancer metastasis. Mol Syst Biol. 2007;3:140.
50. Taylor IW, Linding R, Warde-Farley D, Liu Y, Pesquita C, Faria D, et al. Dynamic modularity in protein interaction networks predicts breast cancer outcome. Nat Biotechnol. 2009;27:199–204.
51. Peter M, Ameer-Beg SM, Hughes MK, Keppler MD, Prag S, Marsh M, et al. Multiphoton-FLIM quantification of the EGFP-mRFP1 FRET pair for localization of membrane receptor-kinase interactions. Biophys J. 2005;88:1224–37.
52. Barber PR, Ameer-Beg SM, Gilbey J, Carlin LM, Keppler M, Ng TC, et al. Multiphoton time-domain fluorescence lifetime imaging microscopy: practical application to protein-protein interactions using global analysis. J R Soc Interface. 2008;6:S93–S105.
53. Kelleher MT, Fruhwirth G, Patel G, Ofo E, Festy F, Barber PR, et al. The potential of optical proteomic technologies to individualize prognosis and guide rational treatment for cancer patients. Target Oncol. 2009;4:235–52.
54. Fruhwirth GO, Fernandes LP, Weitsman G, Patel G, Kelleher M, Lawler K, et al. How Forster resonance energy transfer imaging improves the understanding of protein interaction networks in cancer biology. Chemphyschem. 2011;12:442–61.
55. Anilkumar N, Parsons M, Monk R, Ng T, Adams JC. Interaction of fascin and protein kinase Calpha: a novel intersection in cell adhesion and motility. EMBO J. 2003;22:5390–402.
56. Legg JW, Lewis CA, Parsons M, Ng T, Isacke CM. A novel PKC-regulated mechanism controls CD44 ezrin association and directional cell motility. Nat Cell Biol. 2002;4:399–407.
57. Ng T, Parsons M, Hughes WE, Monypenny J, Zicha D, Gautreau A, et al. Ezrin is a downstream effector of trafficking PKC-integrin complexes involved in the control of cell motility. EMBO J. 2001;20:2723–41.
58. Ng T, Squire A, Hansra G, Bornancin F, Prevostel C, Hanby A, et al. Imaging protein kinase Calpha activation in cells. Science. 1999;283:2085–9.
59. Parsons M, Keppler MD, Kline A, Messent A, Humphries MJ, Gilchrist R, et al. Site-directed perturbation of protein kinase C-integrin interaction blocks carcinoma cell chemotaxis. Mol Cell Biol. 2002;22:5897–911.
60. Parsons M, Monypenny J, Ameer-Beg SM, Millard TH, Machesky LM, Peter M, et al. Spatially distinct binding of Cdc42 to PAK1 and N-WASP in breast carcinoma cells. Mol Cell Biol. 2005;25:1680–95.
61. Prag S, Parsons M, Keppler MD, Ameer-Beg SM, Barber P, Hunt J, et al. Activated ezrin promotes cell migration through recruitment of the GEF Dbl to lipid rafts and preferential downstream activation of Cdc42. Mol Biol Cell. 2007;18:2935–48.
62. Ganesan S, Ameer-Beg SM, Ng TT, Vojnovic B, Wouters FS. A dark yellow fluorescent protein (YFP)-based Resonance Energy-Accepting Chromoprotein (REACh) for Forster resonance energy transfer with GFP. Proc Natl Acad Sci U S A. 2006;103:4089–94.
63. Dadke S, Cotteret S, Yip SC, Jaffer ZM, Haj F, Ivanov A, et al. Regulation of protein tyrosine phosphatase 1B by sumoylation. Nat Cell Biol. 2007;9:80–5.
64. Makrogianneli K, Carlin LM, Keppler MD, Matthews DR, Ofo E, Coolen A, et al. Integrating receptor signal inputs that influence small Rho GTPase activation dynamics at the immunological synapse. Mol Cell Biol. 2009;29:2997–3006.
65. Morris JR, Boutell C, Keppler M, Densham R, Weekes D, Alamshah A, et al. The SUMO modification pathway is involved in the BRCA1 response to genotoxic stress. Nature. 2009;462:886–90.
66. Carlin LM, Evans R, Milewicz H, Fernandes L, Matthews DR, Perani M, et al. A targeted siRNA screen identifies regulators of Cdc42 activity at the natural killer cell immunological synapse. Sci Signal. 2011;4:ra81.
67. Keese M, Magdeburg RJ, Herzog T, Hasenberg T, Offterdinger M, Pepperkok R, et al. Imaging epidermal growth factor receptor phosphorylation in human colorectal cancer cells and human tissues. J Biol Chem. 2005;280:27826–31.
68. Kong A, Leboucher P, Leek R, Calleja V, Winter S, Harris A, et al. Prognostic value of an activation state marker for epidermal growth factor receptor in tissue microarrays of head and neck cancer. Cancer Res. 2006;66:2834–43.
69. Parsons M, Ng T. Intracellular coupling of adhesion receptors: molecular proximity measurements. Methods Cell Biol. 2002;69:261–78.
70. Barber PR, Tullis ID, Pierce GP, Newman RG, Prentice J, Rowley MI, et al. The Gray Institute 'open' high-content, fluorescence lifetime microscopes. J Microsc. 2013;251:154–67.
71. Weitsman G, Lawler K, Kelleher M, Barrett J, Barber PR, Shamil E, et al. Imaging tumour heterogeneity of the consequence of a PKC-substrate interaction in breast cancer patients. Biochem Soc Trans. 2014;in press.
72. Berezin MY, Achilefu S. Fluorescence lifetime measurements and biological imaging. Chem Rev. 2010;110:2641–84.
73. Barber PR, Tullis IDC, Rowley MI, Martins CD, Weitsman G, Lawler K, et al. The Gray Institute open microscopes applied to radiobiology and protein interaction studies. In: Brown TG, Cogswell CJ, Wilson T, editors. SPIE Proceedings. Volume 8949. Three-Dimensional and Multidimensional Microscopy: Image Acquisition and Processing XXI. 2014;in press.
74. Tao J, Castel P, Radosevic-Robin N, Elkabets M, Auricchio N, Aceto N, et al. Blockade of EGFR and HER3 enhances PI3K/Akt anti-tumor activity in triple negative breast cancer. Sci Signal. 2014;7:ra29.
75. Kiuchi T, Ortiz-Zapater E, Monypenny J, Matthews DR, Nguyen LK, Barbeau J, et al. The ErbB4 CYT2 variant protects EGFR from ligand-induced degradation to enhance cancer cell motility. Sci Signal. 2014;7:ra78.
76. Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature. 2003;422:198–207.
77. Domon B, Aebersold R. Mass spectrometry and protein analysis. Science. 2006;312:212–7.
78. Addona TA, Abbatiello SE, Schilling B, Skates SJ, Mani DR, Bunk DM, et al. Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. Nat Biotechnol. 2009;27:633–41.
79. Nilsson T, Mann M, Aebersold R, Yates 3rd JR, Bairoch A, Bergeron JJ. Mass spectrometry in high-throughput proteomics: ready for the big time. Nat Methods. 2010;7:681–5.
80. Rudnick PA, Clauser KR, Kilpatrick LE, Tchekhovskoi DV, Neta P, Blonder N, et al. Performance metrics for liquid chromatography-tandem mass spectrometry systems in proteomics analyses. Mol Cell Proteomics. 2010;9:225–41.
81. Keshishian H, Addona T, Burgess M, Kuhn E, Carr SA. Quantitative, multiplexed assays for low abundance proteins in plasma by targeted mass spectrometry and stable isotope dilution. Mol Cell Proteomics. 2007;6:2212–29.
82. Bateman NW, Sun M, Bhargava R, Hood BL, Darfler MM, Kovatich AJ, et al. Differential proteomic analysis of late-stage and recurrent breast cancer from formalin-fixed paraffin-embedded tissues. J Proteome Res. 2011;10:1323–32.
83. Prieto DA, Hood BL, Darfler MM, Guiel TG, Lucas DA, Conrads TP, et al. Liquid Tissue: proteomic profiling of formalin-fixed tissues. Biotechniques. 2005;Suppl:32–5.
84. Hood BL, Darfler MM, Guiel TG, Furusato B, Lucas DA, Ringeisen BR, et al. Proteomic analysis of formalin-fixed prostate cancer tissue. Mol Cell Proteomics. 2005;4:1741–53.
85. Hembrough T, Thyparambil S, Liao WL, Darfler MM, Abdo J, Bengali KM, et al. Application of selected reaction monitoring for multiplex quantification of clinically validated biomarkers in formalin-fixed, paraffin-embedded tumor tissue. J Mol Diagn. 2013;15:454–65.
86. Huang SK, Darfler MM, Nicholl MB, You J, Bemis KG, Tegeler TJ, et al. LC/MS-based quantitative proteomic analysis of paraffin-embedded archival melanomas reveals potential proteomic biomarkers associated with metastasis. PLoS One. 2009;4:e4430.
87. Bateman NW, Sun M, Hood BL, Flint MS, Conrads TP. Defining central themes in breast cancer biology by differential proteomics: conserved regulation of cell spreading and focal adhesion kinase. J Proteome Res. 2010;9:5311–24.
88. Cheung W, Darfler MM, Alvarez H, Hood BL, Conrads TP, Habbe N, et al. Application of a global proteomic approach to archival precursor lesions: deleted in malignant brain tumors 1 and tissue transglutaminase 2 are upregulated in pancreatic cancer precursors. Pancreatology. 2008;8:608–16.
89. DeSouza LV, Krakovska O, Darfler MM, Krizman DB, Romaschin AD, Colgan TJ, et al. mTRAQ-based quantification of potential endometrial carcinoma biomarkers from archived formalin-fixed paraffin-embedded tissues. Proteomics. 2010;10:3108–16.
90. Hembrough T, Thyparambil S, Liao WL, Darfler MM, Abdo J, Bengali KM, et al. Selected reaction monitoring (SRM) analysis of epidermal growth factor receptor (EGFR) in formalin fixed tumor tissue. Clin Proteomics. 2012;9:5.

91. Patel BN, Sharma N, Sanyal M, Shrivastav PS. High throughput and sensitive determination of trazodone and its primary metabolite, m-chlorophenylpiperazine, in human plasma by liquid chromatography-tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2008;871:44–54.
92. Spurrier B, Ramalingam S, Nishizuka S. Reverse-phase protein lysate microarrays for cell signaling analysis. Nat Protocols. 2008;3:1796–808.
93. Kim P, Liu X, Lee T, Liu L, Barham R, Kirkland R, et al. Highly sensitive proximity mediated immunoassay reveals HER2 status conversion in the circulating tumor cells of metastatic breast cancer patients. Proc Natl Acad Sci U S A. 2011;9:75.
94. Arnould L, Denoux Y, MacGrogan G, Penault-Llorca F, Fiche M, Treilleux I, et al. Agreement between chromogenic in situ hybridisation (CISH) and FISH in the determination of HER2 status in breast cancer. Br J Cancer. 2003;88:1587–91.
95. Bartlett JM, Campbell FM, Ibrahim M, Wencyk P, Ellis I, Kay E, et al. Chromogenic in situ hybridization: a multicenter study comparing silver in situ hybridization with FISH. Am J Clin Pathol. 2009;132:514–20.
96. Francis GD, Jones MA, Beadle GF, Stein SR. Bright-field in situ hybridization for HER2 gene amplification in breast cancer using tissue microarrays: correlation between chromogenic (CISH) and automated silver-enhanced (SISH) methods with patient outcome. Diagn Mol Pathol. 2009;18:88–95.
97. Hanna WM, Kwok K. Chromogenic in-situ hybridization: a viable alternative to fluorescence in-situ hybridization in the HER2 testing algorithm. Modern Pathol. 2006;19:481–7.
98. Lebeau A, Deimling D, Kaltz C, Sendelhofert A, Iff A, Luthardt B, et al. Her-2/neu analysis in archival tissue samples of human breast cancer: comparison of immunohistochemistry and fluorescence in situ hybridization. J Clin Oncol. 2001;19:354–63.
99. Pauletti G, Dandekar S, Rong H, Ramos L, Peng H, Seshadri R, et al. Assessment of methods for tissue-based detection of the HER-2/neu alteration in human breast cancer: a direct comparison of fluorescence in situ hybridization and immunohistochemistry. J Clin Oncol. 2000;18:3651–64.
100. Brugmann A, Lelkaitis G, Nielsen S, Jensen KG, Jensen V. Testing HER2 in breast cancer: a comparative study on BRISH, FISH, and IHC. Appl Immunohistochem Mol Morphol. 2011;19:203–11.
101. Hanna WM, Ruschoff J, Bilous M, Coudry RA, Dowsett M, Osamura RY, et al. HER2 in situ hybridization in breast cancer: clinical implications of polysomy 17 and genetic heterogeneity. Mod Pathol. 2014;27:4–18.
102. Moelans CB, de Weger RA, van Diest PJ. Absence of chromosome 17 polysomy in breast cancer: analysis by CEP17 chromogenic in situ hybridization and multiplex ligation-dependent probe amplification. Breast Cancer Res Treat. 2010;120:1–7.
103. Yeh IT, Martin MA, Robetorye RS, Bolla AR, McCaskill C, Shah RK, et al. Clinical validation of an array CGH test for HER2 status in breast cancer reveals that polysomy 17 is a rare event. Modern Pathol. 2009;22:1169–75.
104. Franchet C, Filleron T, Cayre A, Mounie E, Penault-Llorca F, Jacquemier J, et al. Instant-quality fluorescence in-situ hybridization as a new tool for HER2 testing in breast cancer: a comparative study. Histopathology. 2014;64:274–83.
105. Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res. 2002;30:e57.
106. Moerland E, van Hezik RL, van der Aa TC, van Beek MW, van den Brule AJ. Detection of HER2 amplification in breast carcinomas: comparison of multiplex ligation-dependent probe amplification (MLPA) and fluorescence in situ hybridization (FISH) combined with automated spot counting. Cell Oncol. 2006;28:151–9.
107. Moelans CB, de Weger RA, Ezendam C, van Diest PJ. HER-2/neu amplification testing in breast cancer by multiplex ligation-dependent probe amplification: influence of manual- and laser microdissection. BMC Cancer. 2009;9:4.
108. Moelans CB, de Weger RA, van Blokland MT, van der Wall E, van Diest PJ. Simultaneous detection of TOP2A and HER2 gene amplification by multiplex ligation-dependent probe amplification in breast cancer. Modern Pathol. 2010;23:62–70.
109. Kuijpers CC, Moelans CB, van Slooten HJ, Horstman A, Hinrichs JW, Al-Janabi S, et al. Added value of HER-2 amplification testing by multiplex ligation-dependent probe amplification in invasive breast cancer. PLoS One. 2013;8:e82018.
110. Jackisch C, Untch M. Systemic therapy for women with ErbB2-positive breast cancer: new options, new challenges. Breast Care (Basel). 2010;5(s1):1–2.
111. Baehner FL, Achacoso N, Maddala T, Shak S, Quesenberry Jr CP, Goldstein LC, et al. Human epidermal growth factor receptor 2 assessment in a case–control study: comparison of fluorescence in situ hybridization and quantitative reverse transcription polymerase chain reaction performed by central laboratories. J Clin Oncol. 2010;28:4300–6.
112. Perez E, Butler S, Dueck A, Baehner F, Cherbavaz D, Thompson E, et al. The relationship between quantitative HER2 gene expression by the 21-gene RT-PCR assay and adjuvant trastuzumab (H) benefit in NCCTG (Alliance) N9831. J Clin Oncol. 2013;31:520.
113. Dabbs DJ, Klein ME, Mohsin SK, Tubbs RR, Shuai Y, Bhargava R. High false-negative rate of HER2 quantitative reverse transcription polymerase chain reaction of the Oncotype DX test: an independent quality assurance study. J Clin Oncol. 2011;29:4279–85.
114. Ach RA, Floore A, Curry B, Lazar V, Glas AM, Pover R, et al. Robust interlaboratory reproducibility of a gene expression signature measurement consistent with the needs of a new generation of diagnostic tools. BMC Genomics. 2007;8:148.
115. Glas AM, Floore A, Delahaye LJ, Witteveen AT, Pover RC, Bakx N, et al. Converting a breast cancer microarray signature into a high-throughput diagnostic test. BMC Genomics. 2006;7:278.
116. Roepman P, Horlings HM, Krijgsman O, Kok M, Bueno-de-Mesquita JM, Bender R, et al. Microarray-based determination of estrogen receptor, progesterone receptor, and HER2 receptor status in breast cancer. Clin Cancer Res. 2009;15:7003–11.
117. Majidzadeh AK, Esmaeili R, Abdoli N. TFRC and ACTB as the best reference genes to quantify Urokinase Plasminogen Activator in breast cancer. BMC Res Notes. 2011;4:215.
118. Parker JS, Mullins M, Cheang MC, Leung S, Voduc D, Vickery T, et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. J Clin Oncol. 2009;27:1160–7.
119. Nielsen TO, Parker JS, Leung S, Voduc D, Ebbert M, Vickery T, et al. A comparison of PAM50 intrinsic subtyping with immunohistochemistry and clinical prognostic factors in tamoxifen-treated estrogen receptor-positive breast cancer. Clin Cancer Res. 2010;16:5222–32.
120. Dowsett M, Sestak I, Lopez-Knowles E, Sidhu K, Dunbier AK, Cowens JW, et al. Comparison of PAM50 risk of recurrence score with oncotype DX and IHC4 for predicting risk of distant recurrence after endocrine therapy. J Clin Oncol. 2013;31:2783–90.
121. Filipits M, Nielsen TO, Rudas M, Greil R, Stoger H, Jakesz R, et al. The PAM50 risk-of-recurrence score predicts risk for late distant recurrence after endocrine therapy in postmenopausal women with endocrine-responsive early breast cancer. Clin Cancer Res. 2014;20:1298–305.
122. Gnant M, Filipits M, Greil R, Stoeger H, Rudas M, Bago-Horvath Z, et al. Predicting distant recurrence in receptor-positive breast cancer patients with limited clinicopathological risk: using the PAM50 Risk of Recurrence score in 1478 postmenopausal patients of the ABCSG-8 trial treated with adjuvant endocrine therapy alone. Ann Oncol. 2014;25:339–45.
123. Geiss GK, Bumgarner RE, Birditt B, Dahl T, Dowidar N, Dunaway DL, et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. Nat Biotechnol. 2008;26:317–25.
124. Reis PP, Waldron L, Goswami RS, Xu W, Xuan Y, Perez-Ordonez B, et al. mRNA transcript quantification in archival samples using multiplexed, color-coded probes. BMC Biotechnol. 2011;11:46.
125. Bastien RR, Rodriguez-Lescure A, Ebbert MT, Prat A, Munarriz B, Rowe L, et al. PAM50 breast cancer subtyping by RT-qPCR and concordance with standard clinical molecular markers. BMC Med Genomics. 2012;5:44.
126. Cheang MC, Voduc KD, Tu D, Jiang S, Leung S, Chia SK, et al. Responsiveness of intrinsic subtypes to adjuvant anthracycline substitution in the NCIC.CTG MA.5 randomized trial. Clin Cancer Res. 2012;18:2402–12.
127. Benohr P, Henkel V, Speer R, Vogel U, Sotlar K, Aydeniz B, et al. Her-2/neu expression in breast cancer - a comparison of different diagnostic methods. Anticancer Res. 2005;25(3B):1895–900.
128. Gjerdrum LM, Sorensen BS, Kjeldsen E, Sorensen FB, Nexo E, Hamilton-Dutoit S. Real-time quantitative PCR of microdissected paraffin-embedded breast carcinoma: an alternative method for HER-2/neu analysis. J Mol Diagn. 2004;6:42–51.
129. Christopherson C, Chang M, Eberhard DA, Sninsky JJ, Anderson SM, Wang AM, et al. Comparison of immunohistochemistry (IHC) and quantitative RT-PCR: ER, PR, and HER2 receptor status. J Clin Oncol. 2012;30:abstr 47.
130. Elloumi F, Hu Z, Li Y, Parker JS, Gulley ML, Amos KD, et al. Systematic bias in genomic classification due to contaminating non-neoplastic tissue in breast tumor samples. BMC Med Genomics. 2011;4:54.

131. Toi M, Sperinde J, Huang W, Saji S, Winslow J, Jin X, et al. Differential survival following trastuzumab treatment based on quantitative HER2 expression and HER2 homodimers in a clinic-based cohort of patients with metastatic breast cancer. BMC Cancer. 2010;10:56.
132. Cheng H, Bai YL, Sikov W, Sinclair N, Bossuyt V, Abu-Khalaf MM, et al. Quantitative measurements of HER2 and phospho-HER2 expression: correlation with pathologic response to neoadjuvant chemotherapy and trastuzumab. BMC Cancer. 2014;14:326.
133. Montemurro F, Prat A, Rossi V, Valabrega G, Sperinde J, Peraldo-Neia C, et al. Potential biomarkers of long-term benefit from single-agent trastuzumab or lapatinib in HER2-positive metastatic breast cancer. Mol Oncol. 2014;8:20–6.
134. Duchnowska R, Biernat W, Szostakiewicz B, Sperinde J, Piette F, Haddad M, et al. Correlation between quantitative HER-2 protein expression and risk for brain metastases in HER-2+ advanced breast cancer patients receiving trastuzumab-containing therapy. Oncologist. 2012;17:26–35.
135. Lipton A, Kostler WJ, Leitzel K, Ali SM, Sperinde J, Weidler J, et al. Quantitative HER2 protein levels predict outcome in fluorescence in situ hybridization-positive patients with metastatic breast cancer treated with trastuzumab. Cancer. 2010;116:5168–78.
136. Nuciforo P, Thyparambil S, Garrido-Castro A, Peg V, Prudkin L, Jimenez J, et al. Correlation of high levels of HER2 measured by multiplex mass spectrometry with increased overall survival in patients treated with anti-HER2-based therapy. J Clin Oncol. 2014;32:649.
137. Christiansen J, Barakat N, Murphy D, Rimm D, Dabbas B, Nerenberg M, et al. Her2 expression measured by AQUA analysis on BCIRG-005 and BCIRG-006 predicts the benefit of Herceptin therapy. Cancer Res. 2012;72:PD02-01.
138. Guiu S, Gauthier M, Coudert B, Bonnetain F, Favier L, Ladoire S, et al. Pathological complete response and survival according to the level of HER-2 amplification after trastuzumab-based neoadjuvant therapy for breast cancer. Br J Cancer. 2010;103:1335–42.
139. Perez EA, Reinholz MM, Hillman DW, Tenner KS, Schroeder MJ, Davidson NE, et al. HER2 and chromosome 17 effect on patient outcome in the N9831 adjuvant trastuzumab trial. J Clin Oncol. 2010;28:4307–15.
140. Press MF, Finn RS, Cameron D, Di Leo A, Geyer CE, Villalobos IE, et al. HER-2 gene amplification, HER-2 and epidermal growth factor receptor mRNA and protein expression, and lapatinib efficacy in women with metastatic breast cancer. Clin Cancer Res. 2008;14:7861–70.
141. Bates M, Sperinde J, Kostler WJ, Ali SM, Leitzel K, Fuchs EM, et al. Identification of a subpopulation of metastatic breast cancer patients with very high HER2 expression levels and possible resistance to trastuzumab. Ann Oncol. 2011;22:2014–20.
142. Joensuu H, Sperinde J, Leinonen M, Huang W, Weidler J, Bono P, et al. Very high quantitative tumor HER2 content and outcome in early breast cancer. Ann Oncol. 2011;22:2007–13.
143. Gullo G, Bettio D, Torri V, Masci G, Salvini P, Santoro A. Level of HER2/neu gene amplification as a predictive factor of response to trastuzumab-based therapy in patients with HER2-positive metastatic breast cancer. Invest New Drugs. 2009;27:179–83.
144. Dowsett M, Procter M, McCaskill-Stevens W, de Azambuja E, Dafni U, Rueschoff J, et al. Disease-free survival according to degree of HER2 amplification for patients treated with adjuvant chemotherapy with or without 1 year of trastuzumab: the HERA Trial. J Clin Oncol. 2009;27:2962–9.
145. Paik S, Kim C, Wolmark N. HER2 status and benefit from adjuvant trastuzumab in breast cancer. N Engl J Med. 2008;358:1409–11.
146. Romond EH, Perez EA, Bryant J, Suman VJ, Geyer Jr CE, Davidson NE, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. N Engl J Med. 2005;353:1673–84.
147. Pogue-Geile KL, Kim C, Jeong JH, Tanaka N, Bandos H, Gavin PG, et al. Predicting Degree of Benefit From Adjuvant Trastuzumab in NSABP Trial B-31. J Natl Cancer Inst. 2013;105:1782–8.
148. Scaltriti M, Nuciforo P, Bradbury I, Sperinde J, Agbor-Tarh D, Campbell C, et al. High HER2 expression correlates with response to the combination of lapatinib and trastuzumab. Clin Cancer Res. 2015;21:569–76.
149. Scaltriti M, Verma C, Guzman M, Jimenez J, Parra JL, Pedersen K, et al. Lapatinib, a HER2 tyrosine kinase inhibitor, induces stabilization and accumulation of HER2 and potentiates trastuzumab-dependent cell cytotoxicity. Oncogene. 2009;28:803–14.
150. Jura N, Shan Y, Cao X, Shaw DE, Kuriyan J. Structural analysis of the catalytically inactive kinase domain of the human EGF receptor 3. Proc Natl Acad Sci U S A. 2009;106:21608–13.
151. Aertgeerts K, Skene R, Yano J, Sang BC, Zou H, Snell G, et al. Structural analysis of the mechanism of inhibition and allosteric activation of the kinase domain of HER2 protein. J Biol Chem. 2011;286:18756–65.
152. Garrett JT, Olivares MG, Rinehart C, Granja-Ingram ND, Sanchez V, Chakrabarty A, et al. Transcriptional and posttranslational up-regulation of HER3 (ErbB3) compensates for inhibition of the HER2 tyrosine kinase. Proc Natl Acad Sci U S A. 2011;108:5021–6.
153. Cheng H, Ballman K, Vassilakopoulou M, Dueck AC, Reinholz MM, Tenner K, et al. EGFR expression is associated with decreased benefit from trastuzumab in the NCCTG N9831 (Alliance) trial. Br J Cancer. 2014;111:1065–71.
154. Mittendorf EA, Wu Y, Scaltriti M, Meric-Bernstam F, Hunt KK, Dawood S, et al. Loss of HER2 amplification following trastuzumab-based neoadjuvant systemic therapy and survival outcomes. Clin Cancer Res. 2009;15:7381–8.
155. Piccart-Gebhart M, Holmes A, Baselga J, De Azambuja E, Dueck A, Viale G, et al. First results from the phase III ALTTO trial (BIG 2–06; NCCTG [Alliance] N063D) comparing one year of anti-HER2 therapy with lapatinib alone (L), trastuzumab alone (T), their sequence (T→L), or their combination (T+L) in the adjuvant treatment of HER2-positive early breast cancer (EBC). J Clin Oncol. 2014;32:LBA2.
156. Carey L, Winer E, Viale G, Cameron D, Gianni L. Triple-negative breast cancer: disease entity or title of convenience? Nat Rev Clin Oncol. 2010;7:683–92.

2.2.- Study 2:

"High HER2 protein levels correlate with increased survival in breast cancer patients treated with anti-HER2 therapy".

Paolo Nuciforo, Sheeno Thyparambil, Claudia Aura, Ana Garrido-Castro, Marta Vilaro, Vicente Peg, José Jimenez, Rocio Vicario, Fabiola Cecchi, William Hoos, Jon Burrows, Todd Hembrough, Juan Carles Ferreres, José Perez-Garcia, Joaquin Arribas, Javier Cortes, Maurizio Scaltriti

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Using selected reaction monitoring mass spectrometry (SRM-MS), we quantified the levels of HER2 protein in FFPE tissue samples that had been previosly classified as HER2- or HER2+ by standard IHC and FISH. We demonstrated that accurate HER2 status determination may be achieved in FFPE diagnostic samples by SRM-MS. Cases which could not be properly classified (SRM-MS-negative/ISH-positive) showed a characteristic amplification pattern known as double minutes. More importantly, HER2 levels >2200 amol/µg were significantly associated with better survival in patients treated with anti-HER2 therapies in both adjuvant metastatic settings. This study demonstrated, for the first time, the successful application of a mass spectrometry-based method to precision oncology.

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High HER2 protein levels correlate with increased survival in breast cancer patients treated with anti-HER2 therapy

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ABSTRACT

Introduction: Current methods to determine HER2 (human epidermal growth factor receptor 2) status are affected by reproducibility issues and do not reliably predict benefit from anti-HER2 therapy. Quantitative measurement of HER2 may more accurately identify breast cancer (BC) patients who will respond to anti-HER2 treatments.
Methods: Using selected reaction monitoring mass spectrometry (SRM-MS), we quantified HER2 protein levels in formalin-fixed, paraffin-embedded (FFPE) tissue samples that had been classified as HER2 0, 1+, 2+ or 3+ by immunohistochemistry (IHC). Receiver operator curve (ROC) analysis was conducted to obtain optimal HER2 protein expression thresholds

Abbreviations: HER2, human epidermal growth factor receptor 2; SRM-MS, selected reaction monitoring mass spectrometry; ROC, receiver operating characteristic; MS, mass spectrometry; CEP17, centromere 17; DM, double minutes; HSR, homogeneously staining regions; MIX, mixed; GCN, gene copy number; HR, hormone receptor; ADCC, antibody-dependent cell cytotoxicity.

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predictive of HER2 status (by standard IHC or in situ hybridization [ISH]) and of survival benefit after anti-HER2 therapy.
Results: Absolute HER2 amol/μg levels were significantly correlated with both HER2 IHC and amplification status by ISH ($p < 0.0001$). A HER2 threshold of 740 amol/μg showed an agreement rate of 94% with IHC and ISH standard HER2 testing ($p < 0.0001$). Discordant cases (SRM-MS-negative/ISH-positive) showed a characteristic amplification pattern known as double minutes. HER2 levels >2200 amol/μg were significantly associated with longer disease-free survival (DFS) and overall survival (OS) in an adjuvant setting and with longer OS in a metastatic setting.
Conclusion: Quantitative HER2 measurement by SRM-MS is superior to IHC and ISH in predicting outcome after treatment with anti-HER2 therapy.

1. Introduction

Gene amplification or protein overexpression of the human epidermal growth factor receptor type 2 (HER2) has been reported in ~20% of invasive breast cancer (BC) and is usually associated with worse prognosis (Slamon et al., 1987, 1989). The monoclonal antibody trastuzumab has dramatically increased survival in patients with HER2-overexpressing metastatic disease (Hudis, 2007; Slamon et al., 2001) and has often proved curative when used in combination with chemotherapy in the adjuvant setting (Joensuu et al., 2006; Piccart-Gebhart et al., 2005; Romond et al., 2005; Smith et al., 2007).

The benchmarks for defining tumors as HER2-positive (HER2+) is the presence of protein overexpression (3+) by immunohistochemistry (IHC) or gene amplification by in situ hybridization (ISH), according to current clinical guidelines (Wolff et al., 2013). However, considerable controversy still exists regarding the accuracy, reliability, and inter-observer variability of these methods. Studies in patients treated with trastuzumab indicate that neither test is a perfect predictor of response to trastuzumab (Dowsett et al., 2009; Perez et al., 2010). It is estimated that up to 20% of tumors initially classified as HER2+ by IHC are actually false-positives (Paik et al., 2002; Perez et al., 2006; Roche et al., 2002), and an estimated 1.1%–11.5% of HER2-negative (HER2-) patients by IHC that never received anti-HER2 therapy harbor HER2 gene amplification by ISH (Hanna et al., 2014). False positives HER2 results increase treatment costs (trastuzumab costs $50,000/person/year in the US) and expose patients to a likely ineffective therapy; false negative results deny patients the potential benefits of anti-HER2 therapy.

It is widely accepted that the levels of HER2 are not homogeneous among the HER2+ population defined by conventional semi-quantitative methods such as IHC. Tests capable of absolute quantitation of HER-family protein expression have demonstrated that HER2 protein expression can vary up to 100 fold and that tumors with high HER2 expression are more likely to benefit from anti-HER2 therapy in the neoadjuvant (Cheng et al., 2014; Denkert et al., 2013), adjuvant (Pogue-Geile et al., 2013), and metastatic (Montemurro et al., 2014) settings.

We have recently developed a mass spectrometry (MS)-based proteomic BC panel to measure the absolute abundance of targeted proteins in patient-derived formalin fixed, paraffin embedded (FFPE) tissue for use in clinical decision-making. The reliability of this assay for protein analysis has been demonstrated (Hembrough et al., 2012), however, its clinical utility for patient stratification, choice of therapy, and drug resistance prediction is still being evaluated. In this work, we tested this methodology's ability to predict HER2 status as determined by standard IHC/ISH in a panel of breast tumors. We also assessed the value HER2 quantitation by MS for predicting disease-free survival (DFS) or overall survival (OS) of patients with HER2-positive BC after treatment with anti-HER2 therapy.

2. Material and methods

2.1. Patients and tissue samples

Samples of histologically confirmed invasive BC diagnosed at Vall d'Hebron University Hospital (Barcelona, Spain) were retrospectively identified by one study pathologist (CA) between 1997 and 2013. Samples were selected to ensure a representative number of HER2- and HER2+ samples and to include cases treated with trastuzumab to enable survival analyses in a subset of patients. Sample selection criteria were: known HER2 status tested in the setting of the routine surgical pathology laboratory and available FFPE tumor sample for SRM-MS analysis. For survival analyses, samples with available data on type of anti-HER2 treatment and outcome were included. The study was approved by the hospital ethical committee, including a waiver of consent for the use of archival material for research.

2.2. HER2 standard testing (combined IHC/ISH)

HER2 status was retrieved from hospital Vall d'Hebron pathology laboratory reports (HER2 local). The diagnostic algorithm for HER2 testing used was IHC on all cases and ISH assays done on all IHC2+ equivocal cases (per ASCO/CAP guidelines). Protein expression was determined in paraffin-embedded sections using the 4B5 (Ventana Medical Systems, Tucson, AZ) antibody. *HER2* amplification was determined using silver-enhanced ISH (SISH) and carried out with an INFORM HER2

Dual ISH DNA Probe Assay (Ventana). Testing was performed and scored according to both the 2007 ASCO/CAP guidelines and the 2013 update of these guidelines.

For the purpose of this study, and to exclude any possibility of heterogeneity in the tumor with respect to HER2 expression, IHC and ISH were repeated on all HER2 3+ patients with available tissue samples and on discordant cases on the same tissue block of the resection specimen sent for SRM-MS testing (HER2 central). Central testing was performed using IHC (HercepTest) and FISH (HER2 FISH pharmDx™ Kit) or SISH (INFORM HER2 Dual ISH DNA Probe Assay, Ventana) according to ISO15189 standards and interpreted following the most recent ASCO/CAP guidelines. *HER2* gene status was assessed by two pathologists (CA and VP) blindly scoring 30 nuclei for the number of *HER2* and *centromere 17* (*CEP17*) signals in each cell. The *HER2/CEP17* probe signal ratio was determined and the patterns of *HER2* amplification were analyzed in those cases with *HER2/CEP17* ratio $\geq$2. Samples with >70% of the cells with double minutes (DM, small dispersed dots distributed through the nucleus) or homogeneously staining regions (HSR, tightly clustered dots in discrete regions of the nucleus) patterns were classified accordingly. Cases with both HSR and DM patterns in the same sample were classified as mixed (MIX).

2.3. *HER2 quantification by SRM-MS*

HER2 protein was quantitated by SRM-MS as previously described (Hembrough et al., 2013). Briefly, tissue sections (10 μM) were cut from FFPE blocks, placed onto DIRECTOR® microdissection slides, deparaffinized and stained with hematoxylin. Tumor areas were marked by a board-certified pathologist and a cumulative area of a 12 mm^2 (from multiple sections of a single tumor if necessary) containing approximately 45,000 malignant cells was microdissected from each tumor and then solubilized to tryptic peptides using Liquid Tissue® technology. This tryptic peptide mixture was then subjected to SRM-MS analysis using stable isotope-labeled internal standard for accurate quantitation of analytical targets. The peptide that was chosen for HER2 was ELVSEFSR (located in the intracellular region of HER2, aa 971–978). This peptide is unique to HER2 and has been reported to be the best sequence for SRM in FFPE tissue (Schoenherr et al., 2012; Steiner et al., 2015). On-column injection resulted in 1 μg (~4000 cells) of solubilized tissue and 5 fmol of internal standard measured by microBCA (ThermoFisher Scientific, San Jose, CA). Instrumental analyses were performed on TSQ series (Vantage or Quantiva) triple quadrupole mass spectrometer (Thermo Scientific, San Jose, CA). The MS and chromatography conditions have been previously described (Catenacci et al., 2014).

2.4. *Statistical methods*

To select a SRM-MS threshold for stratifying tumors into HER2+ and HER2-, receiver operating characteristic (ROC) curves were constructed by computing the sensitivity and specificity of increasing quantities of HER2 (by SRM-MS) in predicting HER2 positivity (by combined IHC/ISH). Differences

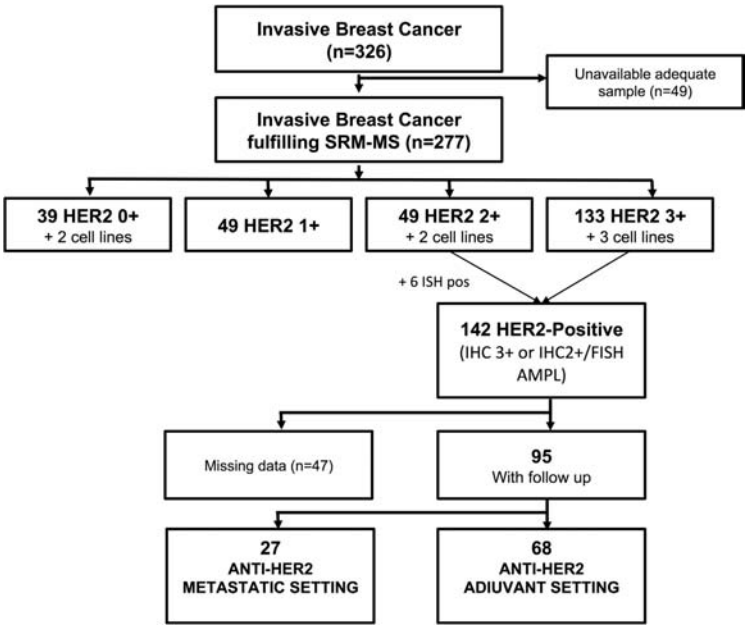

Figure 1 – Breast cancer tumor samples selected for analysis. SRM, selected reaction monitoring; IHC, immunohistochemistry; ISH, in situ hybridization; AMPL, amplified.

in continuous HER2 measurements among various IHC and ISH subgroups were analyzed by using Kruskal–Wallis tests. The Spearman rank correlation coefficient (Spearman ρ) was used to describe the relationship between the HER2 protein levels by SRM-MS, *HER2* gene copy number (GCN) and *HER2/CEP17* ratio by central ISH. Among patients who had received anti-HER2 therapy, ROC analysis was used to establish an optimal cutoff for HER2 levels (by SRM-MS, *HER2/CEP17* ratio and *HER2* GCN) that would predict disease-free, progression-free, and overall survival in the adjuvant and metastatic settings. Chi-square test and Fisher's exact test were used to determine the nature of the associations between optimal cut-off points and clinicopathological parameters. Survival was modeled using the Kaplan–Meier curves, and the significance of differences between these curves was determined using hazard ratio (HR) and its confidence interval of 95%, and the p-value obtained by the log-rank test. Multivariate survival analysis was performed using the Cox proportional hazards model adjusted for hormone receptor status, tumor stage, lymph node status and HER2 SRM levels. Results were considered significant when p-values (p) were less than 0.05. Statistical analyses were conducted using R software, version 3.0.3.

3. Results

3.1. Patients and tissue samples

We identified 326 samples, of which 277 (85%) were suitable for SRM-MS analysis. Forty-nine samples were discarded for lack of sufficient tumor tissue for SRM-MS. Of the 277 study samples, 270 were FFPE breast cancer samples and 7 were cell lines (Figure 1, Table 1 and Supplementary Table 1). Patient specimens were obtained mainly from surgical resection (n = 255), and a small part from diagnostic core biopsies (n = 6) or sampling of recurrent disease (n = 9). The study series included 41 HER2 0+, 49 HER2 1+, 51 HER2 2+, and 136 HER2 3+ assessed by IHC. Of the 142 samples classified as HER2+ by combined IHC/ISH approach, 95 were included in the survival analysis (Supplementary Table 2). Forty-seven were excluded due to the following: twenty-five were replicated samples from the same patients; seven were lost to follow up; five were still under treatment at the time of the analysis; three received trastuzumab after 12 months from diagnosis (atypical adjuvant); three were cell lines; two had a bilateral invasive breast carcinoma; and two had received trastuzumab as neoadjuvant treatment. Sixty-eight patients received adjuvant chemotherapy in combination with trastuzumab alone (76%, n = 52) or combined with another anti-HER2 agent (24%, lapatinib, n = 6 and pertuzumab, n = 10). Twenty-seven received anti-HER2 therapy in the metastatic setting. Trastuzumab alone was the preferred anti-HER2 treatment (70%, n = 19), followed by trastuzumab combined with another anti-HER2 (22%, pertuzumab, n = 5; lapatinib, n = 1), T-DM1 (4%, n = 1), and T-DM1 plus pertuzumab (4%, n = 1).

3.2. SRM-MS versus standard IHC/ISH

The average HER2 protein level in the analyzed dataset (n = 277) as measured by SRM-MS was 2217.9 amol/μg (median: 643.5; sd: 3299.4; range: 0 to 17,446.7). Absolute HER2 amol/μg levels increased with increasing IHC scores with averages values of 189.1, 259.9, 406.7 and 4214.1 in HER2 IHC 0+, 1+, 2+, and 3+, respectively ($p < 0.001$, Kruskal–Wallis rank sum test) (Supplementary Table 3). Samples scored as HER2 IHC3+ expressed the widest dynamic range of HER2 protein levels as quantified by SRM-MS (range: 163.7–17,446.7 amol/μg). When correlated with amplification status, HER2 protein levels were also substantially higher in ISH-amplified (mean: 4151.2 amol/μg; sd: 3682.1; range: 272.8–17,446.7) than non-amplified samples (mean: 383.8 amol/μg; sd: 339.1; range: 0–1748.0; $p < 0.001$, Wilcoxon test, Supplementary Table 4). In our study we analyzed samples collected from 1997 to 2013. Although the SRM-MS is an epitope-independent technology and its robustness has been proven (Catenacci et al., 2014), we addressed the stability of

Table 1 – Characteristics of 270 clinical samples used in the study.

Characteristics	N°	%
Patient specimen		
Surgical resection	255	95
Diagnostic core biopsy	6	2
Recurrent disease	9	3
Histological grade		
G1	16	6
G2	112	41
G3	134	50
Unknown	8	3
Pathological stage T		
Tx–T1	143	53
T2–T4	123	46
Unknown	4	1
Pathological stage N		
Nx–N0	147	54
N1–N3	119	44
Unknown	4	1
Hormone receptor status (HR)		
Negative	47	17
Positive	223	83
HER2 overexpression by IHC		
0	39	14
1	49	18
2	49	18
3	133	49

Table 2 – Concordance between SRM-MS and local and central combined IHC/ISH.

	HER2 Status (IHC/ISH)	n	SRM-MS Agreement, n (%)
Local	Negative	135	130 (96%)
	Positive	142	125 (88%)
Central	Negative	144	137 (95%)
	Positive	133	123 (92%)

Local, local HER2 testing result; Central, central HER2 re-testing results; SRM-MS, selected reaction monitoring mass spectrometry. IHC, Immunohistochemistry; ISH, in situ hybridization.

HER2 as detected by SRM-MS over time. The average SRM-MS values did not differ significantly with age of the tissue blocks thus supporting the validity of results generated using samples collected over a period of many years (Supplementary Table 5).

Per ROC analysis, the SRM-MS threshold that best correlated with HER2 status by combined local IHC/ISH was 740 amol/μg (area under the ROC curve: 0.963). When stratified according to this threshold, 130 samples (47%) were classified as overexpressors and 147 (53%) as non-overexpressors. The overall percent agreement between SRM-MS and combined local IHC/ISH was 92% (255 of 277). The percent positive agreement was 88% (125 of 142), and the percent negative agreement was 96% (130 of 135) (Table 2). HER2 status by SRM-MS for 277 breast cancer samples that had been previously scored by local IHC testing and subsequently evaluated by ISH reflex central testing are shown in Figure 2. In the HER2 IHC negative group (0+ and 1+, n = 90), 86 samples (96%) were correctly classified as negative and 4 (4%) as positive by SRM-MS. After central retest, none of these 4 positive samples showed *HER2* amplification. In the HER2 IHC equivocal group (2+, n = 51), 47 (92%) and 4 (8%) samples were classified as negative and positive by SRM-MS, respectively. Three of these 4 SRM-MS-positive samples were *HER2* amplified (the non-amplified discordant sample was the ZR75-1 cell line). Three out of 47 samples (6%) classified as negative by SRM-MS showed *HER2* gene amplification.

In the HER2 IHC positive group (3+, n = 136), 122 samples (90%) were correctly classified as positive whereas 14 samples (10%) as negative by SRM-MS. *HER2* gene amplification was centrally confirmed in 111 (98%) of the 113 evaluable IHC3+/SRM-MS-positive samples (not amplified, n = 2; data not available, n = 9). Among IHC3+/SRM-MS-negative (n = 14), seven harbored *HER2* gene amplification. Overall agreement after central retest was 94% (260 of 277), the percent positive agreement was 92% (123 of 133), and the percent negative agreement was 95% (137 of 144) (Table 2). Details of the discordant samples between SRM-MS and combined IHC/ISH are shown in Supplementary Table 6.

3.3. *Relationship between HER2 gene amplification pattern and HER2 protein levels*

After central retest, 6% (17/277) of samples remained discordant. The 7 SRM-MS-positive/ISH-negative samples showed absolute HER2 protein levels below the average dataset value (2217.9 amol/μg) and very close to the 740 amol/μg threshold distinguishing overexpressors from non-overexpressors. The remaining 10 samples showed low protein levels (<740 amol/μg) despite *HER2* gene amplification. When stratified by *HER2* amplification pattern, 8 of 10 samples had patterns involving extrachromosomal circles of DNA known as DM and the remaining 2 showed a mixed amplification pattern. No significant differences in *HER2/CEP17* ratios were evident (data not shown).

We therefore investigated whether, in the presence of *HER2* gene amplification, the levels of HER2 protein in the tumor tissue may be influenced by its amplification pattern rather than the levels of gene amplification itself. To test this hypothesis, we correlated HER2 expression by SRM-MS with *HER2* GCN, *HER2/CEP17* ratio and pattern of amplification (HSR, DM, MIX) in HER2 IHC 2+ (n = 6) and IHC3+ (n = 117) cases amplified by central ISH. The mean HER2 protein SRM-MS level was 4047.1 amol/μg (sd: 3508.9; range: 272.8–17,446.7). Mean *HER2* GCN was 14.0 (sd: 4.2; range: 5.2–22.2). Mean *HER2/CEP17* ratio was 7.2 (sd: 2.5; range: 2.1–15.0) (Supplementary Table 7). HER2 SRM-MS levels showed weak positive correlations with *HER2* GCN (Spearman $\rho = 0.44$; $p < 0.001$) and *HER2/CEP17* ratio (Spearman $\rho = 0.31$; $p < 0.001$) (Figure 3). Forty-one percent of samples (n = 50) had HSR patterns, 37% (n = 46) had DM patterns and 22% (n = 27) were mixed. Average HER2 protein levels were significantly higher in tumors amplified with HSR (mean: 5462.9; sd: 3368.4; range: 1099.3–17,446.7) compared to those with DM (mean: 2176.4; sd: 1908.1; range: 272.8–8070.0) (Supplementary Table 8).

DM amplification patterns were present in 80% (8/10) of samples with low HER2 protein levels; only 34% (38/113) of samples with high HER2 protein levels had DM patterns (Supplementary Table 9).

		IHC NEG (0+, 1+), n=90		IHC EQUIV (2+), n=51		IHC POS (3+), n=136	
		SRM-MS		SRM-MS		SRM-MS	
		(-)	(+)	(-)	(+)	(-)	(+)
		86 (96%)	4 (4%)	47 (92%)	4 (8%)	14 (10%)	122 (90%)
Central ISH	(-)	na	4 (100%)	44 (94%)	1 (25%)	7 (50%)	2 (2%)
	(+)	na	0	3 (6%)	3 (75%)	7 (50%)	111 (91%)
	(na)	na	0	0	0	0	9 (7%)

Figure 2 – HER2 selected reaction monitoring-mass spectrometry (SRM-MS) results for 277 breast cancers previously classified as IHC negative (0+, 1+), equivocal (2+) or positive (3+) with subsequent ISH central retesting results. SRM-MS-positive = HER2 protein >740 amol/μg. Discordant cases are highlighted. −, negative; +, positive; na, not assessed.

3.4. *Survival analyses*

ROC analysis of patients treated with anti-HER2 therapy (n = 95) resulted in cutoff values of 2200 amol/μg for HER2 SRM-MS, 6.4 for *HER2/CEP17* ratio and 12.5 for *HER2* GCN (Supplementary Table 10). The 2200 amol/μg HER2 SRM-MS cutoff outperformed the 740 cutoff in predicting DFS and OS (Supplementary Table 11) and was used for survival analyses.

The correlations between the optimal HER2 SRM-MS, *HER2/CEP17* ratio and *HER2* GCN cutoffs for survival and clinic–pathological parameters are shown in Supplementary Table 12 and 13. Patients showing HER2 levels by SRM-MS above the threshold of 2200 amol/μg (n = 58, 61%) were defined as super-expressors. In the adjuvant setting (n = 68), super-expressors had a statistically significantly better outcome than non-super-expressors (Figure 4). The number of

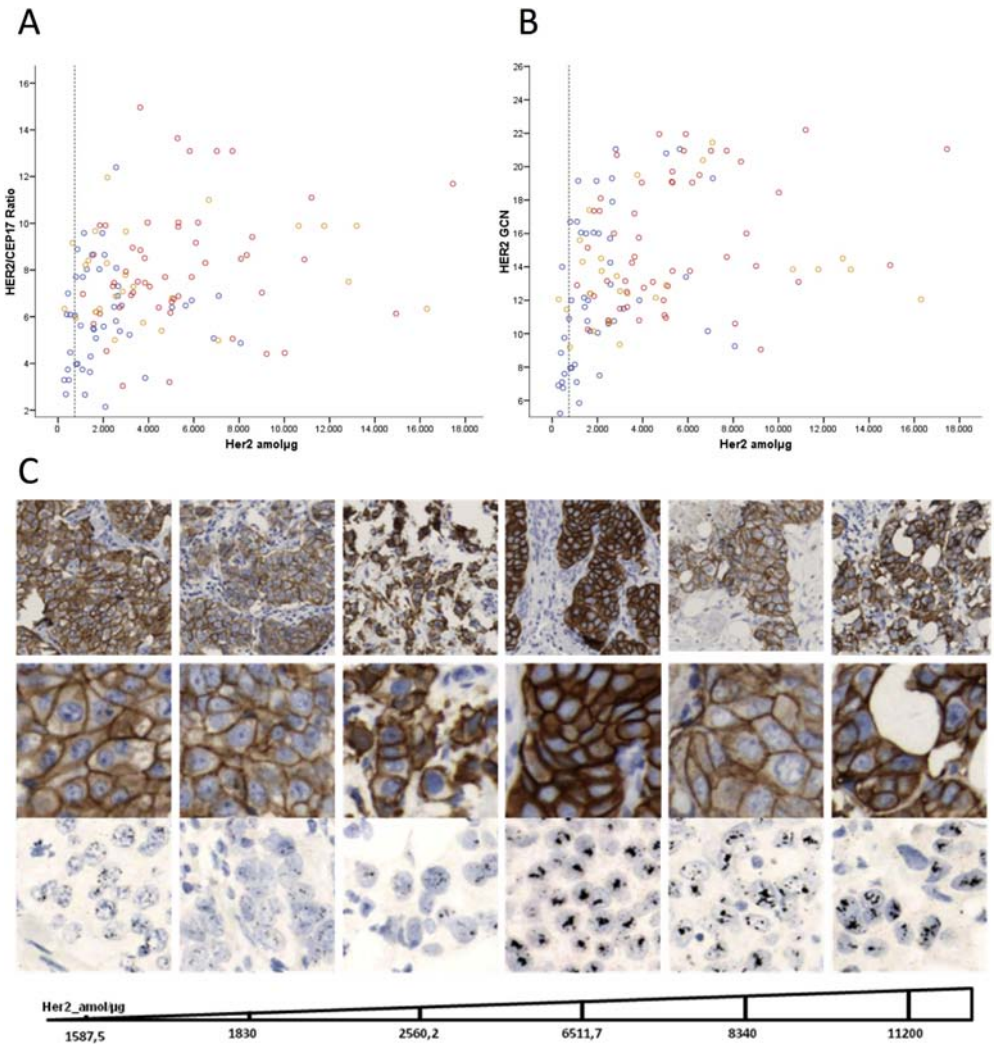

Figure 3 – Correlation between the HER2 protein expression by SRM-MS and HER2/CEP17 ratio (A), and HER2 GCN (B). Dotted gray line indicates HER2 SRM-MS 740 amol/μg threshold. Spearman rank correlation coefficient was used to describe the relationship between SRM-MS and HER2 GCN (ρ, 0.44; $p < 0.001$) and SRM-MS and HER2/CEP17 (ρ, 0.31; $p < 0.001$). Pattern of amplification by in situ hybridization is shown. Red circle, homogeneously staining regions (HSR); blue circle, double minutes (DM); orange circle, Mixed pattern. c, Representative images of protein expression by IHC and amplification patterns by ISH are shown together with SRM-MS protein levels.

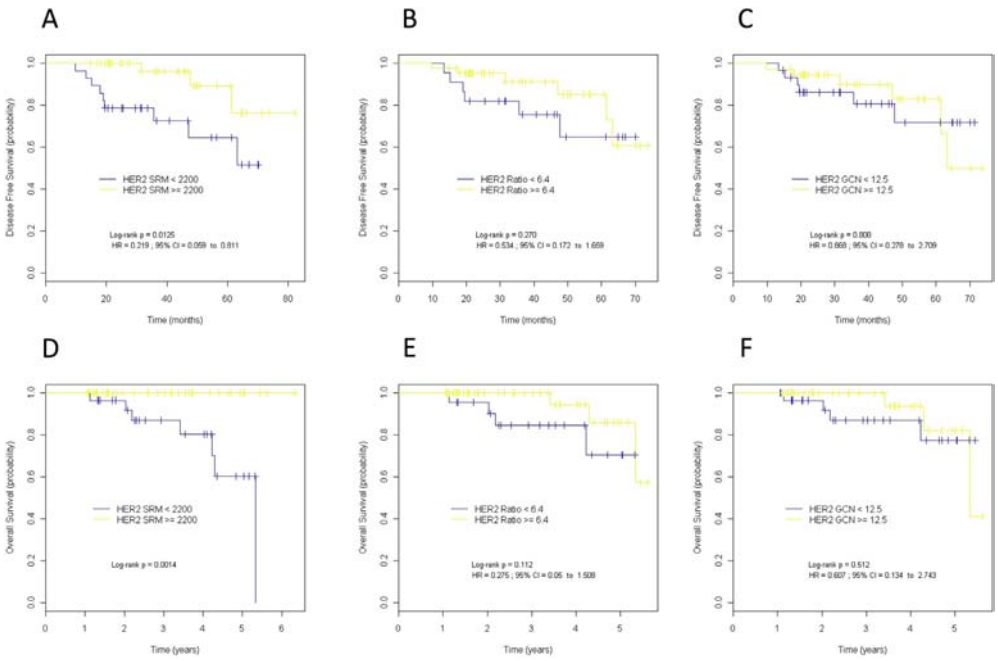

Figure 4 – Kaplan–Meier curves for disease-free survival (A–C) and overall survival (D–F) according to HER2 protein expression by SRM-MS (A,D), HER2/CEP17 ratio (B,E) and HER2 gene copy number (GCN) (C,F) in patients treated with anti-HER2 in the adjuvant setting. Optimal cutoff values were determined by receiver operating characteristic (ROC) analysis. Disease-free survival and overall survival were superior for the group of patients with high HER protein levels (>2200). HR, hazard ratio; HER2 Ratio, HER2/CEP17 ratio; CI, confidence interval.

observed DFS events were 3 in the super-expressors compared to 9 events observed in tumors with HER2 levels below 2200 amol/μg (HR = 0.22, 95% CI 0.06–0.81, log rank p = 0.013). Differences in DFS were even greater between refractory patients (relapse within 24 months) and patients without relapse or recurrent disease within 24 months (OR = 23, 95% CI, 1.26–434.86, p 0.003).

Similar results were observed for OS. None of the super-expressors died of the disease compared to 7 patients whose tumors were below 2200 amol/μg (HR = na, p 0.001). Neither *HER2/CEP17* ratio nor HER2 GCN was predictive of longer DFS or OS in the adjuvant setting (Figure 4).

In the first-line metastatic setting (n = 27), 18 (67%) patients were classified as super-expressors. Median OS was significantly longer in super-expressors (7.84; 95% CI: 5.23 to NA) as compared to non-super-expressors (2.91; 95% CI: 1.61 to NA), (HR = 0.20; 95% CI: 0.07 to 0.57; p < 0.001), (Figure 5). In this setting, HER2 GCN (HR = 0.15; p = 0.001) and, to a lesser extent, *HER2/CEP17* ratio (HR = 0.32; p = 0.050) were also predictive of a better OS. No significant correlations were found between HER2 protein levels or gene status and PFS, likely due to the fact that all but two patients relapsed during follow up. When looking at relapse within 24 months, nine of 18 (50%) super-expressors were refractory to anti-HER2 therapy compared to all (n = 9) patients with HER2 lower than 2200. No correlation was found between HER2 pattern of amplification and survival in both adjuvant and metastatic series (Supplemental Table 14).

In the multivariate model which includes hormone receptor status, tumor size (T) and presence of lymph-node metastases (N), HER2 levels by SRM-MS independently predicted DFS in the adjuvant setting (HR = 0.25; 95% CI: 0.06 to 0.96; p = 0.044) (Table 3). The model could not be run for OS due to lack of events in the HER2 super-expressor group.

4. Discussion

This report demonstrates the application of a MS-based method to objectively quantify HER2 protein in FFPE clinical tissue samples from BC patients. We showed that within IHC-positive (3+) ISH-amplified tumors, a wide dynamic range of HER2 protein expression is found and the subgroup of tumors with the highest levels benefitted most from HER2 inhibition. Our findings suggest that quantitative HER2 measurement is superior to gene amplification levels in

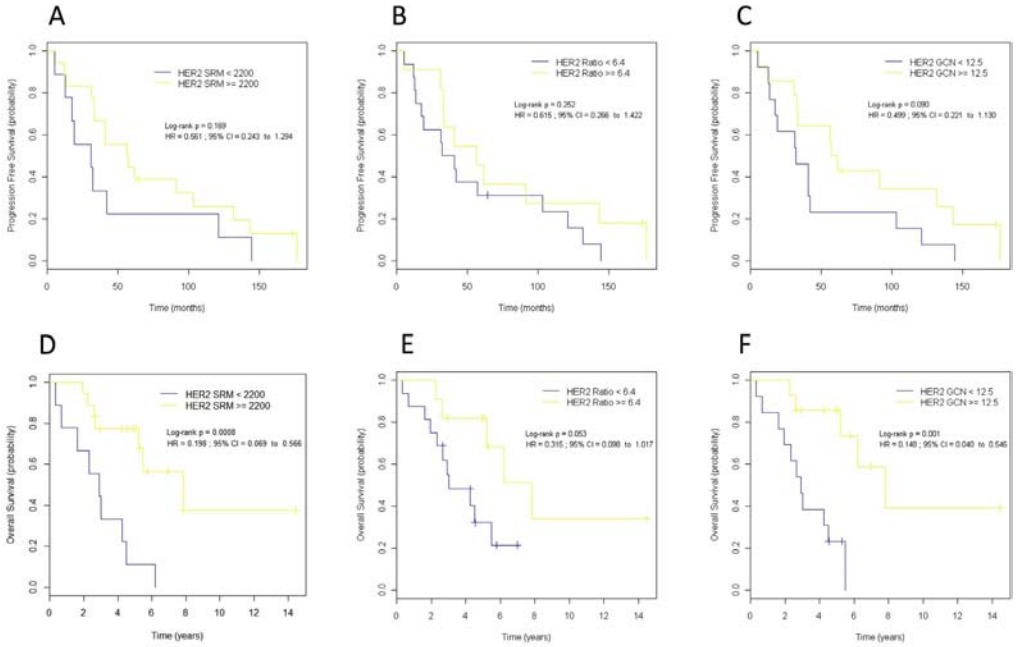

Figure 5 – Kaplan–Meier curves for progression-free survival (A–C) and overall survival (D–F) according to HER2 protein expression by SRM-MS (A,D), HER2/CEP17 ratio (B,E) and HER2 gene copy number (GCN) (C,F) in patients treated with anti-HER2 in the metastatic setting. HR, hazard ratio; *HER2* Ratio, *HER2/CEP17* ratio; CI, confidence interval.

determining which patient will benefit from trastuzumab treatment in both adjuvant and metastatic settings.

The ASCO/CAP guidelines (Wolff et al., 2013) recommend initial HER2 screening of all BC, followed by ISH for samples with equivocal staining; the results of these tests determine a patient's eligibility for trastuzumab. However, lack of concordance between IHC and ISH (IHC-negative/ISH-positive) occurs in up to 11.5% of cases (Hanna et al., 2014). Our findings suggest that these conflicting results may be only marginally due to pre-analytic (fixation affects antibody sensitivity), analytic (limited dynamic range of chromogenic IHC, different antibodies used), or post-analytic (subjectivity in interpretation of the results) factors (Camp et al., 2002; Rimm, 2006). In fact, despite the high correlation observed with IHC score or gene amplification detected by ISH, we found that approximately 10% of *HER2*-amplified breast tumors expressed very low amounts of HER2 protein; all of these discordant cases were associated with a gene amplification pattern known as DM.

Evidence indicates that the amplification of genes in DM may result in a dynamic regulation of gene expression and resistance to EGFR TKIs for EGFR*vIII*-positive glioblastomas (Nathanson et al., 2014). Conversely, data from our group did not find any significant correlation between amplification of HER2 in DM content and sensitivity to anti-HER2 therapy (Vicario et al., 2015). Quantitative HER2 protein analysis, however, may identify a subset of HER2 tumors amplified in DM with low HER2 expression that are less sensitive to anti-HER2 treatment.

Based on our analysis, patients expressing greater than 740 amol/μg of HER2 should receive anti-HER2 treatment, as this was the optimal threshold that correlated with standard IHC/ISH. However, the most meaningful endpoint of HER2 testing is not prediction of HER2 status by IHC or ISH, but outcome after HER2-targeted therapies. Using quantitative HER2 measurement, we found that patients whose tumors expressed HER2 protein level >2200 amol/μg) benefitted

Table 3 – Multivariate analyses of disease-free survival (DFS) in the 68 patients included in the adjuvant series.

DFS	HR	CI95%(HR)	*p-value*
$\frac{\text{Hazard(Hormone receptor positive)}}{\text{Hazard(Hormone receptor negative)}}$	0.19	0.05–0.70	0.012
$\frac{\text{Hazard(TX–T1)}}{\text{Hazard(T2–T4)}}$	0.20	0.05–0.73	0.015
$\frac{\text{Hazard(NX–N0)}}{\text{Hazard(N1–N3)}}$	1.19	0.36–3.94	0.777
$\frac{\text{Hazard(HER2}\geq 2200)}{\text{Hazard(HER2}< 2200)}$	0.24	0.06–0.96	0.044

more from anti-HER2 therapy than patients with lower HER2 expression levels. Strikingly, relapse within 24 months was observed in 21% of patients with HER2 expression levels below 2200 amol/μg and none of the super-expressors progressed to therapy in this period of time. One possible explanation is that tumors with high levels of HER2 are enriched with "true" HER2-dependent disease and therefore potentially more susceptible to HER2 blockade (Montemurro et al., 2014). Another explanation is that the more HER2 receptors are present in the membrane of tumor cells, the more molecules of trastuzumab (or other anti-HER2 antibodies) can bind and prime antibody-dependent cell cytotoxicity (ADCC). A direct correlation between HER2 levels and ADCC has been reported in preclinical models (Scaltriti et al., 2009).

Our findings should be considered in light of certain limitations. The number of patients included in this proof-of-concept study is small and the cutoff point of 2200 amol/μg was based on the survival outcomes in patients whose tissues were selected for the analysis. This cutoff needs to be validated in a larger, independent set of patients. Also, survival analyses included only individuals who had received anti-HER2 treatment. Prospective trials will be needed to address the question of whether varying levels of HER2 positivity are truly predictive of response in all BC patients. Studies are underway to validate the cut-off in an expanded BC cohort.

5. Conclusions

HER2 protein quantitation by SRM-MS in FFPE tissues is predictive of response to anti-HER2 therapy and survival in HER2-positive (by standard IHC/FISH) BC patients. Moreover, this methodology may allow the identification of FISH positive cases that express low amounts of HER2 and respond poorly to anti-HER2 therapy.

Disclosure statement

ST, WH, TH, FC, and JB are/were paid employees and stock owners at OncoPlexDx, which developed the assay approach described in this report. The remaining authors have no conflict of interests to declare.

Author agreement

The corresponding author certifies that all authors of this manuscript have seen and approved the version being submitted. The manuscript is the authors' original work, has not received prior publication and is not under consideration for publication elsewhere.

Authors' contributions

PN, JC, MS, TH, and JB designed the study. ST, CA, AGC, VP, JJ, RV, JCF, JPG, FC, WH, participated in the data collection and generation of results. PN, MS, JC, and MV analyzed data. PN, ST, MS drafted the article. JC, TH, JA revised the article critically for important intellectual content.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2015.09.002.

REFERENCES

Camp, R.L., Chung, G.G., Rimm, D.L., 2002. Automated subcellular localization and quantification of protein expression in tissue microarrays. Nat. Med. 8, 1323–1327.

Catenacci, D.V., Liao, W.L., Thyparambil, S., Henderson, L., Xu, P., Zhao, L., Rambo, B., Hart, J., Xiao, S.Y., Bengali, K., Uzzell, J., Darfler, M., Krizman, D.B., Cecchi, F., Bottaro, D.P., Karrison, T., Veenstra, T.D., Hembrough, T., Burrows, J., 2014. Absolute quantitation of met using mass spectrometry for clinical application: assay precision, stability, and correlation with MET gene amplification in FFPE tumor tissue. PloS One 9, e100586.

Cheng, H., Bai, Y., Sikov, W., Sinclair, N., Bossuyt, V., Abu-Khalaf, M.M., Harris, L.N., Rimm, D.L., 2014. Quantitative measurements of HER2 and phospho-HER2 expression: correlation with pathologic response to neoadjuvant chemotherapy and trastuzumab. BMC Cancer 14, 326.

Denkert, C., Huober, J., Loibl, S., Prinzler, J., Kronenwett, R., Darb-Esfahani, S., Brase, J.C., Solbach, C., Mehta, K., Fasching, P.A., Sinn, B.V., Engels, K., Reinisch, M., Hansmann, M.L., Tesch, H., von Minckwitz, G., Untch, M., 2013. HER2 and ESR1 mRNA expression levels and response to neoadjuvant trastuzumab plus chemotherapy in patients with primary breast cancer. Breast Cancer Res.: BCR 15, R11.

Dowsett, M., Procter, M., McCaskill-Stevens, W., de Azambuja, E., Dafni, U., Rueschoff, J., Jordan, B., Dolci, S., Abramovitz, M., Stoss, O., Viale, G., Gelber, R.D., Piccart-Gebhart, M., Leyland-Jones, B., 2009. Disease-free survival according to degree of HER2 amplification for patients treated with adjuvant chemotherapy with or without 1 year of trastuzumab: the HERA Trial. J. Clin. Oncol.: Off. J. Am. Soc. Clin. Oncol. 27, 2962–2969.

Hanna, W.M., Barnes, P.J., Chang, M.C., Gilks, C.B., Magliocco, A.M., Rees, H., Quenneville, L., Robertson, S.J., SenGupta, S.K., Nofech-Mozes, S., 2014. Human epidermal growth factor receptor 2 testing in primary breast cancer in the era of standardized testing: a Canadian prospective study. J. Clin. Oncol.: Off. J. Am. Soc. Clin. Oncol. 32, 3967–3973.

Hembrough, T., Thyparambil, S., Liao, W.L., Darfler, M.M., Abdo, J., Bengali, K.M., Hewitt, S.M., Bender, R.A., Krizman, D.B., Burrows, J., 2013. Application of selected reaction monitoring for multiplex quantification of clinically validated biomarkers in formalin-fixed, paraffin-embedded tumor tissue. J. Mol. Diagn.: JMD 15, 454–465.

Hembrough, T., Thyparambil, S., Liao, W.L., Darfler, M.M., Abdo, J., Bengali, K.M., Taylor, P., Tong, J., Lara-Guerra, H.,

Waddell, T.K., Moran, M.F., Tsao, M.S., Krizman, D.B., Burrows, J., 2012. Selected reaction monitoring (SRM) analysis of epidermal growth factor receptor (EGFR) in formalin fixed tumor tissue. Clin. Proteom. 9, 5.

Hudis, C.A., 2007. Trastuzumab–mechanism of action and use in clinical practice. New Engl. J. Med. 357, 39–51.

Joensuu, H., Kellokumpu-Lehtinen, P.L., Bono, P., Alanko, T., Kataja, V., Asola, R., Utriainen, T., Kokko, R., Hemminki, A., Tarkkanen, M., Turpeenniemi-Hujanen, T., Jyrkkio, S., Flander, M., Helle, L., Ingalsuo, S., Johansson, K., Jaaskelainen, A.S., Pajunen, M., Rauhala, M., Kaleva-Kerola, J., Salminen, T., Leinonen, M., Elomaa, I., Isola, J., FinHer Study, I., 2006. Adjuvant docetaxel or vinorelbine with or without trastuzumab for breast cancer. New Engl. J. Med. 354, 809–820.

Montemurro, F., Prat, A., Rossi, V., Valabrega, G., Sperinde, J., Peraldo-Neia, C., Donadio, M., Galvan, P., Sapino, A., Aglietta, M., Baselga, J., Scaltriti, M., 2014. Potential biomarkers of long-term benefit from single-agent trastuzumab or lapatinib in HER2-positive metastatic breast cancer. Mol. Oncol. 8, 20–26.

Nathanson, D.A., Gini, B., Mottahedeh, J., Visnyei, K., Koga, T., Gomez, G., Eskin, A., Hwang, K., Wang, J., Masui, K., Paucar, A., Yang, H., Ohashi, M., Zhu, S., Wykosky, J., Reed, R., Nelson, S.F., Cloughesy, T.F., James, C.D., Rao, P.N., Kornblum, H.I., Heath, J.R., Cavenee, W.K., Furnari, F.B., Mischel, P.S., 2014. Targeted therapy resistance mediated by dynamic regulation of extrachromosomal mutant EGFR DNA. Science 343, 72–76.

Paik, S., Bryant, J., Tan-Chiu, E., Romond, E., Hiller, W., Park, K., Brown, A., Yothers, G., Anderson, S., Smith, R., Wickerham, D.L., Wolmark, N., 2002. Real-world performance of HER2 testing–national surgical adjuvant breast and bowel project experience. J. Natl. Cancer Inst. 94, 852–854.

Perez, E.A., Reinholz, M.M., Hillman, D.W., Tenner, K.S., Schroeder, M.J., Davidson, N.E., Martino, S., Sledge, G.W., Harris, L.N., Gralow, J.R., Dueck, A.C., Ketterling, R.P., Ingle, J.N., Lingle, W.L., Kaufman, P.A., Visscher, D.W., Jenkins, R.B., 2010. HER2 and chromosome 17 effect on patient outcome in the N9831 adjuvant trastuzumab trial. J. Clin. Oncol.: Off. J. Am. Soc. Clin. Oncol. 28, 4307–4315.

Perez, E.A., Suman, V.J., Davidson, N.E., Martino, S., Kaufman, P.A., Lingle, W.L., Flynn, P.J., Ingle, J.N., Visscher, D., Jenkins, R.B., 2006. HER2 testing by local, central, and reference laboratories in specimens from the North Central Cancer Treatment Group N9831 intergroup adjuvant trial. J. Clin. Oncol.: Off. J. Am. Soc. Clin. Oncol. 24, 3032–3038.

Piccart-Gebhart, M.J., Procter, M., Leyland-Jones, B., Goldhirsch, A., Untch, M., Smith, I., Gianni, L., Baselga, J., Bell, R., Jackisch, C., Cameron, D., Dowsett, M., Barrios, C.H., Steger, G., Huang, C.S., Andersson, M., Inbar, M., Lichinitser, M., Lang, I., Nitz, U., Iwata, H., Thomssen, C., Lohrisch, C., Suter, T.M., Ruschoff, J., Suto, T., Greatorex, V., Ward, C., Straehle, C., McFadden, E., Dolci, M.S., Gelber, R.D.Herceptin Adjuvant Trial Study, T, 2005. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. New Engl. J. Med. 353, 1659–1672.

Pogue-Geile, K.L., Kim, C., Jeong, J.H., Tanaka, N., Bandos, H., Gavin, P.G., Fumagalli, D., Goldstein, L.C., Sneige, N., Burandt, E., Taniyama, Y., Bohn, O.L., Lee, A., Kim, S.I., Reilly, M.L., Remillard, M.Y., Blackmon, N.L., Kim, S.R., Horne, Z.D., Rastogi, P., Fehrenbacher, L., Romond, E.H., Swain, S.M., Mamounas, E.P., Wickerham, D.L., Geyer Jr., C.E., Costantino, J.P., Wolmark, N., Paik, S., 2013. Predicting degree of benefit from adjuvant trastuzumab in NSABP trial B-31. J. Natl. Cancer Inst. 105, 1782–1788.

Rimm, D.L., 2006. What brown cannot do for you. Nat. Biotechnol. 24, 914–916.

Roche, P.C., Suman, V.J., Jenkins, R.B., Davidson, N.E., Martino, S., Kaufman, P.A., Addo, F.K., Murphy, B., Ingle, J.N., Perez, E.A., 2002. Concordance between local and central laboratory HER2 testing in the breast intergroup trial N9831. J. Natl. Cancer Inst. 94, 855–857.

Romond, E.H., Perez, E.A., Bryant, J., Suman, V.J., Geyer Jr., C.E., Davidson, N.E., Tan-Chiu, E., Martino, S., Paik, S., Kaufman, P.A., Swain, S.M., Pisansky, T.M., Fehrenbacher, L., Kutteh, L.A., Vogel, V.G., Visscher, D.W., Yothers, G., Jenkins, R.B., Brown, A.M., Dakhil, S.R., Mamounas, E.P., Lingle, W.L., Klein, P.M., Ingle, J.N., Wolmark, N., 2005. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. New Engl. J. Med. 353, 1673–1684.

Scaltriti, M., Verma, C., Guzman, M., Jimenez, J., Parra, J.L., Pedersen, K., Smith, D.J., Landolfi, S., Ramon y Cajal, S., Arribas, J., Baselga, J., 2009. Lapatinib, a HER2 tyrosine kinase inhibitor, induces stabilization and accumulation of HER2 and potentiates trastuzumab-dependent cell cytotoxicity. Oncogene 28, 803–814.

Schoenherr, R.M., Whiteaker, J.R., Zhao, L., Ivey, R.G., Trute, M., Kennedy, J., Voytovich, U.J., Yan, P., Lin, C., Paulovich, A.G., 2012. Multiplexed quantification of estrogen receptor and HER2/Neu in tissue and cell lysates by peptide immunoaffinity enrichment mass spectrometry. Proteomics 12, 1253–1260.

Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A., McGuire, W.L., 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235, 177–182.

Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Keith, D.E., Levin, W.J., Stuart, S.G., Udove, J., Ullrich, A., et al., 1989. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 244, 707–712.

Slamon, D.J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., Baselga, J., Norton, L., 2001. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. New Engl. J. Med. 344, 783–792.

Smith, I., Procter, M., Gelber, R.D., Guillaume, S., Feyereislova, A., Dowsett, M., Goldhirsch, A., Untch, M., Mariani, G., Baselga, J., Kaufmann, M., Cameron, D., Bell, R., Bergh, J., Coleman, R., Wardley, A., Harbeck, N., Lopez, R.I., Mallmann, P., Gelmon, K., Wilcken, N., Wist, E., Sanchez Rovira, P., Piccart-Gebhart, M.J.team, H.s., 2007. 2-year follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer: a randomised controlled trial. Lancet 369, 29–36.

Steiner, C., Tille, J.C., Lamerz, J., Kux van Geijtenbeek, S., McKee, T.A., Venturi, M., Rubbia-Brandt, L., Hochstrasser, D., Cutler, P., Lescuyer, P., Ducret, A., 2015 Jul 6. Quantification of HER2 By Targeted mass spectrometry in formalin-fixed paraffin-embedded breast cancer tissues. Mol. Cell. Proteomics pii: mcp.O115.049049 [Epub ahead of print].

Vicario, R., Peg, V., Morancho, B., Zacarias-Fluck, M., Zhang, J., Martinez-Barriocanal, A., Navarro Jimenez, A., Aura, C., Burgues, O., Lluch, A., Cortes, J., Nuciforo, P., Rubio, I.T., Marangoni, E., Deeds, J., Boehm, M., Schlegel, R., Tabernero, J., Mosher, R., Arribas, J., 2015. Patterns of HER2 gene amplification and response to anti-HER2 therapies. PloS One 10, e0129876.

Wolff, A.C., Hammond, M.E., Hicks, D.G., Dowsett, M., McShane, L.M., Allison, K.H., Allred, D.C., Bartlett, J.M., Bilous, M., Fitzgibbons, P., Hanna, W., Jenkins, R.B., Mangu, P.B., Paik, S., Perez, E.A., Press, M.F., Spears, P.A., Vance, G.H., Viale, G., Hayes, D.F.American Society of Clinical, O., College of American, P, 2013. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. J. Clin. Oncol.: Off. J. Am. Soc. Clin. Oncol. 31, 3997–4013.

Supplementary Table 1. Breast cancer cell lines used in study.

Cell Line	Study Sample ID	HER2 IHC	HER2 FISH	Her2 amol/µg
MDA231	ctrl_1	+0	no ampl	0.0
MCF7	ctrl_2	+0	no ampl	0.0
T47D	ctrl_3	+2	no ampl	697.0
ZR75-1	ctrl_4	+2	no ampl	988.0
SKBR3	ctrl_5	+3	ampl	7717.0
HCC1954	ctrl_6	+3	ampl	10020.0
ZR 75-30	ctrl_7	+3	ampl	16964.0

Supplementary Table 2. Clinicopathological characteristics of patients included in the survival analysis.

Characteristics	Nº	%
Histological grade		
G1-G2	33	35
G3	62	65
Pathological stage T		
Tx-T1	50	53
T2-T4	42	44
Unknown	3	3
Pathological stage N		
Nx-N0	40	42
N1-N3	52	55
Unknown	3	3
Hormone receptor status (HR)		
Negative	27	28
Positive	68	72
HER2 amplification by central ISH		
No amplification	6	6
Amplification	87	92
Not determined	2	2
HER2/CEP17 ratio by central ISH		
<2	6	6
2-6.3	32	34
≥6.4	55	58
Not determined	2	2
Pattern of amplification		
Double Minutes	32	34
Homogeneous stained regions	37	39
Mixed	18	19
Not determined	8	8
Anti-HER2 treatment		
Trastuzumab (single agent)	71	75
Trastuzumab + lapatinib	7	7
Trastuzumab + pertuzumab	15	16
T-DM1	2	2
Anti-HER2 treatment setting		
Adjuvant	68	72
Metastatic	27	28
Disease/Progression-free survival, years		
Adjuvant, Mean (95% CI)	3.2 (2.8–3.6)	
Metastatic, Mean (95% CI)	5.2 (3.5-6.8)	
Overall survival, years		
Adjuvant, Mean (95% CI)	3.1 (2.7-3.4)	
Metastatic, Mean (95% CI)	4.4 (3.3-5.3)	

Supplementary Table 3. HER2 expression as measured by SRM-MS according to the different immunohistochemistry (IHC) scores.

SD, standard deviation. [1]Kruskal-Wallis rank sum test.

Her2 (amol/µg)							
		Mean	**SD**	**Min**	**Max**	**N total**	**P-value[1]**
HER2 IHC	**+0**	189.1	324.2	0.0	1348.7	41	**<0.001**
	+1	259.9	357.2	0.0	1748.0	49	
	+2	406.7	481.9	0.0	2731.5	51	
	+3	4214.1	3768.0	163.7	17446.7	136	

Supplementary Table 4. HER2 expression as measured by SRM-MS according to the HER2 gene amplification status by in situ hybridization (ISH).

No ampl, absence of amplification; Ampl, presence of amplification; SD, standard deviation; [1]Wilcoxon test.

		Her2 (amol/µg)					
		Mean	**SD**	**Min**	**Max**	**N total**	**P-value[1]**
HER2 ISH	**No ampl**	383.8	339.1	0.0	1748.0	60	**<0.001**
	Ampl	4151.2	3682.1	272.8	17446.7	124	

Supplementary Table 5. Average HER2 levels as detected by SRM-MS according to sample collection year by IHC category (cat)

SD, Standard deviation; *P* value calculated with ANOVA.

IHC cat	Sample year	n	Her2 (amol/µg) Average	SD	*P* value
+0	<2008	2	365,25	516,54	.22
	2008	5	232,76	323,61	
	2009	15	131,21	226,17	
	2010	11	353,70	457,82	
	2011	6	,00	,00	
+1	<2008	3	174,00	301,38	.17
	2008	3	424,68	391,54	
	2009	9	375,90	289,88	
	2010	23	310,03	425,25	
	2011	11	38,63	128,11	
+2	2008	4	534,75	392,33	.31
	2009	16	245,58	181,15	
	2010	18	555,78	713,87	
	2011	5	194,01	265,66	
	2012-13	6	336,28	177,04	
+3	<2008	25	3820,55	3366,02	.40
	2008	21	4570,58	3071,53	
	2009	25	5155,53	4751,08	
	2010	19	2879,73	2523,23	
	2011	15	3932,04	3646,72	
	2012-13	28	3726,41	3520,68	

Supplementary Table 6. Details of discordant cases between SRM-MS and IHC/ISH after central ISH retest.

SRM-MS cat: Negative, <740 amolμg; Positive, >740 amolμg. HER2 status local: Positive, IHC positive (3+) or ISH amplified (IHC equivocal). HER2 status central: Positive, ISH amplified; Negative, ISH non amplified. In **bold** discordant cases between SRM-MS and ISH after central re-test. DM, double minutes; MIX, mixed pattern. SR, surgical resection; RD, recurrent disease. IDC, invasive ductal carcinoma; HR, Hormone receptors. POS, positive; NEG, negative. [1]results based on literature data.

Oncoplex ID	HER2 amolμg	SRM-MS cat	HER2 IHC Local	HER2 status Local	HER2 status Central	Amplification pattern	pTN	Specimen	Diagnosis	Grade	HR
D0606	349.8	Negative	Equivocal	Positive	**Positive**	DM	T1N0	SR	IDC	3	POS
D0595	435.3	Negative	Equivocal	Positive	**Positive**	DM	T2N1	SR	IDC	2	POS
D0596	478.0	Negative	Equivocal	Positive	**Positive**	DM	T1N0	SR	IDC	3	NEG
C0593	163.8	Negative	Positive	Positive	Negative	-	T4NX	SR	IDC	2	NEG
D0188	272.8	Negative	Positive	Positive	**Positive**	DM	T2N1	SR	IDC	3	POS
C0568	283.3	Negative	Positive	Positive	Negative	-	T2N1	SR	IDC	3	POS
D0152	287.8	Negative	Positive	Positive	**Positive**	MIX	T1N0	SR	IDC	3	NEG
D0156	382.4	Negative	Positive	Positive	**Positive**	DM	T1N0	SR	IDC	1	POS
D0665	424.9	Negative	Positive	Positive	Negative	-	T1N1	SR	TC	1	POS
D0656	443.6	Negative	Positive	Positive	**Positive**	DM	T2N0	SR	IDC	2	POS
A0090	500.0	Negative	Positive	Positive	Negative	-	T1N1	SR	IDC	3	POS
C0594	522.8	Negative	Positive	Positive	Negative	-	TXN2	RD	IDC	3	NEG
D0165	535.3	Negative	Positive	Positive	**Positive**	DM	T3N3	RD	IDC	3	NEG
D0177	542.8	Negative	Positive	Positive	**Positive**	DM	T1N0	SR	IDC	2	POS
A0097	550.0	Negative	Positive	Positive	Negative	-	T2N1	SR	IDC	3	POS
A0091	580.0	Negative	Positive	Positive	Negative	-	T1N2	SR	IDC	2	POS
D0187	639.8	Negative	Positive	Positive	**Positive**	MIX	T2N0	SR	IDC	3	POS
D0574	1348.7	Positive	Negative	Negative	**Negative**	-	TXN0	SR	IDC	xx	POS
D0503	771.3	Positive	Negative	Negative	**Negative**	-	T1N0	SR	IDC	2	POS
D0547	834.5	Positive	Negative	Negative	**Negative**	-	T2N1	SR	IDC	3	POS
D0569	1748.0	Positive	Negative	Negative	**Negative**	-	T1N0	SR	PC	3	POS
Ctrl_4	988.0	Positive	Equivocal	Negative[1]	**Negative**[1]	-	xx	xx	xx	xx	xx
D0664	764.3	Positive	Positive	Positive	**Negative**	-	T4N2	SR	IDC	3	POS
D0173	959.8	Positive	Positive	Positive	**Negative**	-	T1N0	SR	IDC	2	POS

Supplementary Table 7. HER2 expression levels by SRM, HER2 gene copy number (GCN) and HER2/CEP17 ratio in ISH-positive breast cancer.

SD, standard deviation. ZR 75-30 cell line was not included in the analysis as central ISH re-test was not available.

	N	Mean	SD	Min	Max
Her2 amol/µg	123	4047.1	3508.9	272.8	17446.7
HER2 GCN	123	14.0	4.2	5.2	22.2
HER2/CEP17 Ratio	123	7.2	2.5	2.1	15.0

Supplementary Table 8. HER2 expression levels by SRM-MS according to amplification pattern.

DM, double minutes; HSR, homogeneously stained regions; MIX, mixed pattern. SD, standard deviation. [1]Kruskal-Wallis rank sum test.

		Her2 (amol/µg)					
		Mean	**SD**	**Min**	**Max**	**N total**	**P-value[1]**
Pattern of amplification	**DM**	2176.4	1908.1	272.8	8070.0	46	**<0.001**
	HSR	5462.9	3368.4	1099.3	17446.7	50	
	MIX	4612.1	4439.6	287.8	16303.3	27	

Supplementary Table 9. Distribution for pattern of HER2 amplification in SRM-MS negative (<740) and positive (>740) tumors.

DM, double minutes; HSR, homogeneously stained regions; MIX, mixed pattern.

		DM	MIX	HSR	
HER2 (amol/µg)	<740	8	2	0	
	>740	38	25	50	
	Total	46	27	50	123

Supplementary Table 10. ROC analysis.

The optimal cutoff for HER2 levels by SRM-MS, HER2/CEP17 ratio and HER2 GCN discriminating between a positive or negative result, in terms of response to treatment and prolonged survival, was determined using ROC analysis. Patients were dichotomized into good responders (absence of disease progression at last follow up) or poor responders (presence of disease progression at last follow up) and into good survival (alive at last follow up) versus poor survival (dead at last follow up) groups. The cutoff values of HER2 amol/µg of 2200, HER2/CEP17 ratio of 6.4 and HER2 GCN of 12.50 were chosen as the optimal cutoff values for survival analyses. The HER SRM of 2200 amol/µg threshold represents an intermediate value between optimal thresholds distinguishing good versus poor survival (2110.25 amol/µg) and good versus poor responders (2302.25 amol/µg) in the adjuvant population. The sensitivity and specificity of this threshold in predicting survival was 100% and 65.57% in the adjuvant setting, and 56.25% and 100% in the metastatic setting. AUC: Area under ROC curve.

			Threshold	Sensitivity	Specificity	AUC
HER2 by SRM	All cohort, n=95	Good vs poor survival	2110.25	69.6%	73.6%	0.698
	Anti HER2 adjuvant, n=68	Good vs poor survival	2110.25	100%	68.9%	0.690
		Good vs poor responder	2302.25	75%	66.1%	0.688
	Anti HER2, metastatic, n=27	Good vs poor survival	2758.75	62.5%	90.9%	0.727
		Good vs poor responder	6286.65	80%	50%	0.580
HER2 by GCN	All cohort, n=93	Good vs poor survival	11.80	56.5%	71.4%	0.658
	Anti HER2 adjuvant, n=66	Good vs poor survival	12.45	57.1%	56.9%	0.548
		Good vs poor responder	13.45	50%	63%	0.513
	Anti HER2, metastatic, n=27	Good vs poor survival	12.62	68.8%	81.8%	0.810
		Good vs poor responder	15.10	72%	100%	0.800
HER2/Chr17 Ratio	All cohort, n=93	Good vs poor survival	6.41	69.6%	65.7%	0.737
	Anti HER2 adjuvant, n=66	Good vs poor survival	6.27	57.1%	71.2%	0.630
		Good vs poor responder	6.27	50%	72.2%	0.514
	Anti HER2, metastatic, n=27	Good vs poor survival	5.31	56.2%	90.9%	0.741
		Good vs poor responder	10.20	100%	50%	0.740

Supplementary Table 11. DFS and OS HRs comparison using two different cut points (740 and 2200).

NA, Not assessable; HR, hazard ratio; CI, confidence interval.

Adjuvant (n=68)		HR	$CI_{95\%}$	*p-value*
DFS	**740**	0.705	0.09-5.514	0.738
	2200	0.219	0.059 - 0.811	0.0125
OS	**740**	0.274	0.031-2.375	0.209
	2200	NA	NA - NA	0.0014
Metastatic (n=27)		**HR**	**$CI_{95\%}$**	***p-value***
DFS	**740**	0.331	0.104-1.056	0.05
	2200	0.561	0.243-1.294	0.169
OS	**740**	0.183	0.054-0.623	0.002
	2200	0.198	0.069 - 0.566	0.0008

Supplementary Table 12. Correlation between HER2 status by SRM and ISH and clinicopathological characteristics in the adjuvant series.

[1]Chi square test; [2]Fisher exact test; [3]Wilcoxon test.

		HER2 (amol/ug)			HER2 Ratio			HER2 GCN		
		< 2200 (n=28, 41.2%)	**>= 2200 (n=40, 58.8%)**	***p-value***	**< 6.4 (n=22, 33.3%)**	**>= 6.4 (n=44, 66.7%)**	***p-value***	**< 12.5 (n=30, 45.4%)**	**>= 12.5 (n=36, 54.6%)**	***p-value***
HR	**Negative**	7 (10.3%)	10 (14.7%)	*0.968[1]*	7 (10.6%)	10 (15.2%)	*0.619[1]*	8 (12.1%))	9 (13.6%)	*0.898[1]*
	Positive	21 (30.89%)	30 (44.1%)		15 (22.7%)	34 (51.5%)		22 (33.3%)	27 (40.9%)	
Grade	**G1**	1 (1.5%)	0 (0%)	***0.023[2]***	1 (1.5%)	0 (0%)	*0.208[2]*	1 (1.5%)	0 (0%)	*0.695[2]*
	G2	15 (22.1%)	11 (16.2%)		10 (22.7%)	15 (16.7%)		12 (18.2%)	13 (19.7%)	
	G3-G4	12 (17.7%)	29 (42.7%)		11 (18.2%)	29 (43.9%)		17 (25.8%)	23 (34.9%)	
Pahological T	**TX - T1**	14 (20.6%)	28 (41.2%)	*0.157[1]*	14 (21.2)	26 (39.4)	*0.929[1]*	17 (25.8%)	*23 (34.9%)*	*0.730[1]*
	T2 - 4	14 (20.6%)	12 (17.7%)		8 (12.1%)	18 (27.3%)		*13 (19.7%)*	*13 (19.7%)*	
Pathological N	**NX – N0**	15 (22.1%)	20 (29.4%)	*0.965[1]*	*12 (18.2%)*	*22 (33.3%)*	*0.931[1]*	*20 (30.3%)*	*14 (21.2%)*	***0.045[1]***
	N1 – N3	13 (19.1%)	20 (29.4%)		*10 (15.2%)*	*22 (33.3%)*		10 (15.2%)	*22 (33.3%)*	
Follow up, Years Mean (sd)		3.5 (1.5)	3.4 (1.5)	*0.699[3]*	3.5 (1.4)	3.3 (1.5)	*0.414[3]*	3.3 (1.5)	3.4 (1.4)	*0.918[3]*
Disease progression	**No**	19 (27.9%)	37 (54.4%)	***0.021[2]***	16 (24.2%)	38 (57.6%)	*0.310[1]*	24 (36.4%)	30 (45.5%)	*0.977[1]*
	Yes	9 (13.2%)	3 (4.4%)		6 (9.1%)	6 (9.01%)		6 (9.1%)	6 (9.1%)	
Vital status	**Dead**	7 (10.3%)	0 (0%)	***0.001[2]***	4 (6.1%)	3 (4.6%)	*0.210[2]*	4 (6.1%)	3 (4.6%)	*0.693[2]*
	Alive	21 (30.9%)	40 (58.8%)		18 (27.3%)	41 (62.1%)		26 (39.4%)	33 (50%)	

Supplementary Table 13. Correlation between HER2 status by SRM and ISH and clinicopathological characteristics in metastatic series.

[1]Chi square test; [2]Fisher exact test; [3]Wilcoxon test.

		HER2 (amol/ug)			HER2 Ratio			HER2 GCN		
		< 2200 (n=9, 33.3%)	**>= 2200 (n=18, 66.7%)**	***p-value***	**< 6.4 (n=16, 59.3%)**	**>= 6.4 (n=11, 40.7%)**	***p-value***	**< 12.5 (n=13, 48.1%)**	**>= 12.5 (n=14, 51.9%)**	***p-value***
Follow up, Years	**Mean (sd)**	6.0 (4.7)	8.5 (5.5)	*0.232*[3]	6.2 (4.3)	9.8 (6.0)	*0.056*[3]	5.8 (4.2)	9.4 (5.7)	*0.105*[3]
Disease progression	**No**	0 (0%)	2 (7.4%)	*0.539*[2]	1 (3.7%)	1 (3.7%)	*0.987*[2]	0 (0%)	2 (7.4%)	*0.482*[2]
	Yes	9 (33.3%)	16 (59.3%)		15 (55.6%)	10 (31.0%)		13 (48.1%)	12 (44.4%)	
Vital status	**Dead**	9 (33.3%)	7 (25.9%)	***0.003***[2]	11 (40.7%)	5 (18.5%)	*0.264*[2]	11 (40.7%)	5 (18.5%)	***0.018***[2]
	Alive	0 (0%)	11 (40.7%)		5 (18.5%)	6 (22.2%)		2 (7.4%)	9 (33.3%)	

Supplementary Table 14. Disease free survival and overall survival hazard ratios according to HER2 pattern of amplification. DM, doble minutes, HSR, homogeneously stained regions; MIX, mixed; HR, hazard ratio; CI, confidence interval.

Adjuvant (n=63)		HR	CI_{95}	*p-value*
DFS	DM/HSR	1.59	0.35-7.14	0.546
	DM/MIX	0.83	0.18-3.70	0.804
	HSR/MIX	0.52	0.10-2.60	0.427
OS	DM/HSR	2.62	0.23-29.80	0.438
	DM/MIX	0.96	0.10-8.93	0.973
	HSR/MIX	0.37	0.03-4.44	0.431
Metastatic (n=24)		**HR**	**CI_{95}**	***p-value***
DFS	DM/HSR	0.50	0.13-1.85	0.297
	DM/MIX	1.13	0.21-6.01	0.883
	HSR/MIX	2.28	0.39-13.42	0.362
OS	DM/HSR	4.14	0.89-8.41	0.067
	DM/MIX	5.63	0.71-44.45	0.101
	HSR/MIX	1.36	0.12-15.56	0.804

DISCUSSION

The fundamental principle of targeted therapy is to specifically harm tumor cells that depend on a definite target for proliferation and survival, sparing non-tumor cells from damage. In many cases, the target is a protein with activating mutations that is present only in tumor cells, facilitating the specificity of the therapy (for example, *Braf*-mutant melanomas, *EGFR*-mutant lung cancer), allowing profound inhibition of the target before the emergence of side effects. In the case of HER receptors in BC the target is a protein that, although not carrying any activating alterations, is present in much higher amounts in tumor cells compared with normal cells. In these cases one would guess that the higher is the difference in target expression between normal and tumor cells, the wider is the therapeutic window. However, only the presence of the target or its semiquantitative expression (and not the absolute levels) is currently taken into consideration in clinical practice.

The ASCO/CAP guidelines (Wolff 2013) recommend initial HER2 screening of all BC, followed by ISH for samples with equivocal staining; the results of these tests determine a patient's eligibility for trastuzumab. However, lack of concordance between IHC and ISH (IHC-negative/ISH-positive) occurs in up to 11.5% of cases (Hanna 2014). Our findings suggest that these conflicting results may be only marginally due to pre-analytic (fixation affects antibody sensitivity), analytic (limited dynamic range of chromogenic IHC, different antibodies used), or post-analytic (subjectivity in interpretation of the results) factors (Camp 2002, Rimm 2006). In fact, despite the high correlation observed with IHC score or gene amplification detected by ISH, we found that approximately 10% of *HER2*-amplified breast tumors expressed very low amounts of HER2 protein; all of these discordant cases were associated with a gene amplification pattern known as double minutes (DM). Evidence indicates that the amplification of genes in DM may result in a dynamic regulation of gene expression and resistance to EGFR TKIs for EGFR*vIII*-positive glioblastomas (Nathanson 2014). Conversely, data from our group did not find any significant correlation between amplification of *HER2* in DM content and sensitivity to anti-HER2 therapy (Vicario 2015). Quantitative HER2 protein analysis, however, may identify a subset of HER2+ tumors

amplified in DM with low HER2 expression that are less sensitive to anti-HER2 treatment.

There is an increasing body of evidence indicating that the levels of HER2 in HER2+ tumors can influence the response to HER2-targeted therapy, converging to the common conclusion that 'more HER2, more response' (Toi 2010, Cheng 2014, Montemurro 2014, Duchnowska 2012, Lipton 2010, Nuciforo 2014, Christiansen 2012). Quantitative HER2 expression or homodimer levels determined by the HERmark® assay correlated with clinical outcome of trastuzumab therapy better than IHC or central FISH studies in patients with metastatic BC. Interestingly, patients with *HER2* gene amplification by FISH but low HER2 protein expression or homodimer levels as measured by HERmark® responded poorly to trastuzumab-containing therapy, suggesting that not all gene-amplified tumors overexpress the target of trastuzumab (Lipton 2010).

Based on our analysis, patients expressing greater than 740 amol/µg of HER2 should receive anti-HER2 treatment, as this was the optimal threshold that correlated with standard IHC/ISH. However, the most meaningful endpoint of HER2 testing is not prediction of HER2 status by IHC or ISH, but outcome after HER2-targeted therapies. Using quantitative HER2 measurement, we found that patients whose tumors expressed HER2 protein level > 2200 amol/µg) benefitted more from anti-HER2 therapy than patients with lower HER2 expression levels. Strikingly, relapse within 24 months was observed in 21% of patients with HER expression levels below 2200 amol/µg and none of the super-expressors progressed to therapy in this period of time. One possible explanation is that tumors with high levels of HER2 are enriched with "true" HER2-dependent disease and therefore potentially more susceptible to HER2 blockade (Montemurro 2014). Another explanation is that the more HER2 receptors are present in the membrane of tumor cells, the more molecules of trastuzumab (or other anti-HER2 antibodies) can bind and prime ADCC. A direct correlation between HER2 levels and ADCC has been reported in preclinical models (Scaltriti 2009).

Our findings should be considered in light of certain limitations. The number of patients included in this proof-of-concept study is small and the cutoff point of 2200 amol/µg was based on the survival outcomes in patients whose tissues were selected for the analysis. This cutoff needs to be validated in a larger, independent set of patients. Also, survival analyses included only individuals who had received trastuzumab as single anti-HER2 treatment. Since dual HER2 blockade (trastuzumab combined with either pertuzumab or lapatinib) is proving to be more effective than single agent treatment, it will be interesting to investigate whether HER2 absolute levels predict response in this setting as well. In the neoadjuvant setting, this seems to be the case. HER2 levels were measured by HERmark® in the primary tumors of patients enrolled in the NeoALTTO trial, testing the activity of trastuzumab in combination with lapatinib compared with single agent treatments, and a positive correlation was found between constitutive HER2 expression and benefit from dual blockade (Scaltriti 2015) .

Because of the mechanistic relationship among HER family proteins, EGFR and HER3 measurements may provide a method for personalizing treatment in BC, beyond the single assay for HER2. HER-targeted therapeutic agents such as lapatinib have been shown to stabilize/enhance the HER2-HER3 dimer in preclinical cell models (Scaltriti 2009). The quantification of this dimer (as described above), which is believed to be the most potent of all HER dimers with regard to driving cellular proliferation, will provide important and non-redundant information to that provided by HER protein expression to help clinicians to understand and/or predict the heterogeneity in clinical response. Similarly, it may be hypothesized that the subset of tumors with high EGFR expression may better respond to lapatinib or dual HER blockade compared with trastuzumab alone. Also, the absolute levels of EGFR may be predictive for response to anti-EGFR therapy in TNBC patients. Patients with tumors expressing high levels of EGFR were more likely to achieve pCR following panitumumab-based therapy (Tao 2014). Furthermore, EGFR levels tended to decrease in the residual tumors collected at surgery compared with the primary tumor before the commencement of therapy, indicating that the levels of EGFR may be influenced by the therapeutic pressure. Overall, these

data, although still exploratory, suggest that quantitative measurement of EGFR and HER3, may provide additional important information.

Our results demonstrate the application of a mass spectrometry-based method to objectively quantify HER2 protein in FFPE clinical tissue samples from BC patients. We showed that within IHC-positive (3+) ISH-amplified tumors, a wide dynamic range of HER2 protein expression is found and the subgroup of tumors with the highest levels benefitted most from HER2 inhibition. Our findings suggest that quantitative HER2 measurement is superior to gene amplification levels in determining which patient will benefit from trastuzumab treatment in both adjuvant and metastatic settings. Moreover, this methodology may allow the identification of FISH positive cases that express low amount of HER2 and respond poorly to anti-HER2 therapy. Prospective trials will be needed to address the question of whether varying levels of HER2 positivity are truly predictive of response in all BC patients.

CONCLUSIONS

1) Unlike gene mutations, the simple presence/absence of HER2 protein may not be sufficient to predict the benefit that a patient will achieve from a targeted therapy.

2) In current clinical practice, only the presence of HER2 or its qualitative or semiquantitative expression (and not the absolute levels) is taken into consideration.

3) In our study, we demonstrated that accurate HER2 protein quantification can be achieved using SRM-MS and can be conducted in FFPE clinical tissue samples from BC patients.

4) Quantitative HER2 protein determination is objective and reproducible as compared to qualitative or semiquantitative methods such as IHC and it is not affected by the stability of the protein over time being an epitope-independent technology.

5) Within IHC-positive (3+), a wide dynamic range of HER2 protein expression (from 163.7 to 17,446.7 amol/µg) is present, thus highlighting the limited resolution of diagnostic IHC.

6) Not all ISH-positive tumors show high HER2 protein expression despite being IHC 3+.

7) The levels of HER2 proteins are influenced by the pattern of *HER2* gene amplification. Tumors amplified in DM show significantly lower HER2 protein levels compared to those with HSR pattern.

8) Mass spectrometry-based HER2 quantification may identify the subgroup of tumors with the highest benefit from HER2 inhibition.

9) Patients whose tumors express HER2 protein level >2200 amol/mg benefit more from anti-HER2 therapy than patients with lower HER2 expression levels.

10) Prospective trials will be needed to address the question of whether varying levels of HER2 positivity are truly predictive of response in all BC patients.

BIBLIOGRAPHY

Addona TA, Abbatiello SE, Schilling B, Skates SJ, Mani DR, Bunk DM, et al: Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. Nat Biotechnol 2009, 27(7):633-641.

Aebersold R, Goodlett DR. Mass spectrometry in proteomics. Chem Rev. 2001 Feb;101(2):269-95.

Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature 2003, 422:198 -207.

Baselga J, Bradbury I, Eidtmann H, Di Cosimo S, de Azambuja E, Aura C, et al. Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): a randomised, open-label, multicentre, phase 3 trial. Lancet 2012, 379(9816):633-640.

Baselga J, Cortes J, Kim SB, Im SA, Hegg R, Im YH, et al. Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer. The New England journal of medicine 2012, 366(2):109-119.

Baselga J, Gomez P, Greil R, Braga S, Climent MA, Wardley AM, et al. Randomized phase II study of the anti-epidermal growth factor receptor monoclonal antibody cetuximab with cisplatin versus cisplatin alone in patients with metastatic triple-negative breast cancer. J Clin Oncol 2013, 31(20):2586-2592.

Bateman NW, Sun M, Bhargava R, Hood BL, Darfler MM, Kovatich AJ, et al. Differential proteomic analysis of late-stage and recurrent breast cancer from formalin-fixed paraffin-embedded tissues. J Proteome Res 2011, 10:1323 -1332.

Burstein HJ, Harris LN, Gelman R, Lester SC, Nunes RA, Kaelin CM, et al. Preoperative therapy with trastuzumab and paclitaxel followed by sequential adjuvant doxorubicin/cyclophosphamide for HER2 overexpressing stage II or III breast cancer: a pilot study. J Clin Oncol 2003, 21(1):46 -53.

Camp RL, Chung GG, Rimm DL. Automated subcellular localization and quantification of protein expression in tissue microarrays. Nat Med 2002, 8: 1323-1327.

Carey LA, Rugo HS, Marcom PK, Mayer EL, Esteva FJ, Ma CX, et al. TBCRC 001: randomized phase II study of cetuximab in combination with carboplatin in stage IV triple-negative breast cancer. J Clin Oncol 2012, 30(21):2615-2623.

Cheng H, Bai Y, Sikov W, Sinclair N, Bossuyt V, Abu-Khalaf MM, et al. Quantitative measurements of HER2 and phospho-HER2 expression: correlation with pathologic response to neoadjuvant chemotherapy and trastuzumab. BMC Cancer 2014, 14:326

Cheung W, Darfler MM, Alvarez H, Hood BL, Conrads TP, Habbe N, et al. Application of a Global Proteomic Approach to Archival Precursor Lesions:

Deleted in Malignant Brain Tumors 1 (DMBT1) and Tissue Transglutaminase-2 (TG2) are upregulated in pancreatic cancer precursors. Pancreatology 8(6):608-616, 2008.

Christiansen J, Barakat N, Murphy D, Rimm D, Dabbas B, Nerenberg M, et al. Her2 expression measured by AQUA analysis on BCIRG-005 and BCIRG-006 predicts the benefit of Herceptin therapy. Cancer Res. 2012;72:PD02-01.

Cobleigh MA, Vogel CL, Tripathy D, Robert NJ, Scholl S, Fehrenbacher L, et al. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J Clin Oncol 1999, 17(9):2639 -2648.

Coudert BP1, Arnould L, Moreau L, Chollet P, Weber B, Vanlemmens L, et al: Pre-operative systemic (neo-adjuvant) therapy with trastuzumab and docetaxel for HER2-overexpressing stage II or III breast cancer: results of a multicenter phase II trial. Ann Oncol 2006, 17(3):409 -414.

Dendukuri N, Khetani K, McIsaac M, Brophy J. Testing for HER2-positive breast cancer: a systematic review and cost-effectiveness analysis. CMAJ 2007, 176:1429-1434.

Denkert C, Huober J, Loibl S, Prinzler J, Kronenwett R, Darb-Esfahani S, et al. HER2 and ESR1 mRNA expression levels and response to neoadjuvant trastuzumab plus chemotherapy in patients with primary breast cancer. Breast Cancer Res 2013, 15(1):R11.

DeSouza LV, Krakovska O, Darfler MM, Krizman DB, Romaschin AD, Colgan TJ, et al. mTRAQ-based quantification of potential endometrial carcinoma biomarkers from archived formalin-fixed paraffin-embedded tissues. Proteomics 2010, 10(17):3108-3116.

Di Leo A, Gomez HL, Aziz Z, Zvirbule Z, Bines J, Arbushites MC, et al. Phase III, double-blind, randomized study comparing lapatinib plus paclitaxel with placebo plus paclitaxel as first-line treatment for metastatic breast cancer. J Clin Oncol 2008, 26(34):5544-5552.

Dowsett M, Procter M, McCaskill-Stevens W, de Azambuja E, Dafni U, Rueschoff J, et al. Disease free survival according to degree of HER2 amplification for patients treated with adjuvant chemotherapy with or without 1 year of trastuzumab: the HERA Trial. J Clin Oncol 2009, 27:2962-2969.

Duchnowska R, Biernat W, Szostakiewicz B, Sperinde J, Piette F, Haddad M, et al. Correlation between quantitative HER-2 protein expression and risk for brain metastases in HER-2+ advanced breast cancer patients receiving trastuzumab-containing therapy. Oncologist 2012, 17:26–35.

Gavin AC, Bösche M, Krause R, Grandi P, Marzioch M, Bauer A, et al. Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature. 2002, 415(6868):141-147.

Geyer CE, Forster J, Lindquist D, Chan S, Romieu CG, Pienkowski T, et al. Lapatinib plus capecitabine for HER2-positive advanced breast cancer. N Engl J Med 2006, 355(26):2733-2743.

Gerber SA, Rush J, Stemman O, Kirschner MW, Gygi SP. Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. Proc Natl Acad Sci U S A 2003, 100(12):6940-5.

Gianni L, Dafni U, Gelber RD, Azambuja E, Muehlbauer S, Goldhirsch A, et al. Herceptin Adjuvant (HERA) Trial Study Team. Treatment with trastuzumab for 1 year after adjuvant chemotherapy in patients with HER2-positive early breast cancer: a 4-year follow-up of a randomised controlled trial. Lancet Oncol 2011, 12(3):236-44.

Gianni L, Pienkowski T, Im YH, Roman L, Tseng LM, Liu MC, et al. Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial. Lancet Oncol 2012, 13(1):25-32.

Gomez HL, Doval DC, Chavez MA, Ang PC, Aziz Z, Nag S, et al. Efficacy and safety of lapatinib as first-line therapy for ErbB2-amplified locally advanced or metastatic breast cancer. J Clin Oncol 2008, 26(18):2999-3005.

Hanna WM, Rüschoff J, Bilous M, Coudry RA, Dowsett M, Osamura RY, et al. HER2 in situ hybridization in breast cancer: clinical implications of polysomy 17 and genetic heterogeneity. Mod Pathol 2014, 27(1):4-18.

Hembrough T, Thyparambil S, Liao WL, Darfler MM, Abdo J, Bengali KM, et al. Selected Reaction Monitoring (SRM) Analysis of Epidermal Growth Factor Receptor (EGFR) in Formalin Fixed Tumor Tissue. Clin Proteomics 2012, 9(1):5.

Ho Y, Gruhler A, Heilbut A, Bader GD, Moore L, Adams SL, et al. Systematic identification of protein complexes in Saccharomyces cerevisiae by mass spectrometry. Nature 2002;415(6868):180-3.

Hoadley KA, Weigman VJ, Fan C, Sawyer LR, He X, Troester MA, et al. EGFR associated expression profiles vary with breast tumor subtype. BMC genomics 2007, 8:258.

Holbro T, Beerli RR, Maurer F, Koziczak M, Barbas CF 3rd, Hynes NE. The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. Proc Natl Acad Sci U S A 2003, 100(15):8933-8938.

Hood BL, Darfler MM, Guiel TG, Furusato B, Lucas DA, Ringeisen BR, et al. Proteomic analysis of formalin-fixed prostate cancer tissue. Mol Cell Proteomics 2005, 4:1741-1753.

Huang SK, Darfler MM, Nicholl MB, You J, Bemis KG, Tegeler TJ, et al. LC/MS-based quantitative proteomic analysis of paraffin-embedded archival melanomas reveals potential proteomic biomarkers associated with metastasis. PLoS One. 2009, 4(2):e4430.

Hurley J, Doliny P, Reis I, Silva O, Gomez-Fernandez C, Velez P, et al. Docetaxel, cisplatin, and trastuzumab as primary systemic therapy for human epidermal growth factor receptor 2-positive locally advanced breast cancer. J Clin Oncol 2006, 24(12):1831-1838.

Jain MR, Liu T, Hu J, Darfler M, Fitzhugh V, Rinaggio J, et al. Quantitative Proteomic Analysis of Formalin Fixed Paraffin Embedded Oral HPV Lesions from HIV Patients. Open Proteomics J 2008, 1:40-45.

Limentani SA, Brufsky AM, Erban JK, Jahanzeb M, Lewis D. Phase II study of neoadjuvant docetaxel, vinorelbine, and trastuzumab followed by surgery and adjuvant doxorubicin plus cyclophosphamide in women with human epidermal growth factor receptor 2-overexpressing locally advanced breast cancer. J Clin Oncol 2007, 25(10):1232-1238.

Lipton A, Köstler WJ, Leitzel K, Ali SM, Sperinde J, Weidler J, et al. Quantitative HER2 protein levels predict outcome in fluorescence in situ hybridization-positive patients with metastatic breast cancer treated with trastuzumab. Cancer 2010, 116:5168 -5178.

Moreno-Aspitia A, Hillman DW, Dyar SH, Tenner KS, Gralow J, Kaufman PA, et al. Soluble human epidermal growth factor receptor 2 (HER2) levels in patients with HER2-positive breast cancer receiving chemotherapy with or without trastuzumab: results from North Central Cancer Treatment Group adjuvant trial N9831. Cancer 2013, 119(15):2675-82.

Montemurro F, Prat A, Rossi V, Valabrega G, Sperinde J, Peraldo-Neia C, et al. Potential biomarkers of long-term benefit from single-agent trastuzumab or lapatinib in HER2-positive metastatic breast cancer. Mol Oncol 2014, 8:20–26.

Nabholtz JM, Abrial C, Mouret-Reynier MA, Dauplat MM, Weber B, Gligorov J, et al. Multicentric neoadjuvant phase II study of panitumumab combined with an anthracycline/taxane-based chemotherapy in operable triple-negative breast cancer: identification of biologically defined signatures predicting treatment impact. Ann Oncol 2014, 25(8):1570-1577.

Naidu R, Yadav M, Nair S, Kutty MK. Expression of c-erbB3 protein in primary breast carcinomas. Br J Cancer 1998, 78(10):1385-1390.

Nathanson DA, Gini B, Mottahedeh J, Visnyei K, Koga T, Gomez G, et al. Targeted therapy resistance mediated by dynamic regulation of extrachromosomal mutant EGFR DNA. Science 2014, 343:72-76.

Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, et al. Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin Cancer Res 2004, 10(16):5367-5374.

Nilsson T, Mann M, Aebersold R, Yates JR 3rd, Bairoch A, Bergeron JJ. Mass spectrometry in high-throughput proteomics: ready for the big time. Nat Methods 2010, 7:681-685.

Nuciforo P, Thyparambil S, Garrido-Castro A, Peg V, Prudkin L, Jimenez J, et al. Correlation of high levels of HER2 measured by multiplex mass spectrometry with increased overall survival in patients treated with anti-HER2-based therapy. J Clin Oncol 2014, 32:649

Ong SE, Mann M. Mass spectrometry-based proteomics turns quantitative. Nat Chem Biol 2005, 1:252-262.

O'Shaughnessy J, Weckstein D, Vukelja S, McIntyre K. Randomized phase II study of weekly irinotecan/carboplatin with or without cetuximab in patients with metastatic breast cancer. Presented at: Thirty Annual CTRC-AACR San Antonio Breast Cancer Symposium 2007; December 13-16, 2007; San Antonio, TX Abstract 308.

Paik S, Bryant J, Tan-Chiu E, Romond E, Hiller W, Park K, et al. Real-world performance of HER2 testing: National Surgical Adjuvant Breast and Bowel Project experience. J Natl Cancer Inst 2002, 94:852-854.

Paik S, Kim C, Wolmark N. HER2 status and benefit from adjuvant trastuzumab in breast cancer. N Engl J Med 2008, 358(13):1409-1411.

Patel V, Hood BL, Molinolo AA, Lee NH, Conrads TP, Braisted JC, et al. Proteomic Analysis of Laser-Captured Paraffin-Embedded Tissues: A Molecular Portrait of Head and Neck Cancer Progression. Clinical Cancer Research 2008, 14(4):1002-1014.

Pawlowski V, Révillion F, Hebbar M, Hornez L, Peyrat JP. Prognostic value of the type I growth factor receptors in a large series of human primary breast cancers quantified with a real-time reverse transcription-polymerase chain reaction assay. Clin Cancer Res 2000, 6(11):4217-4225.

Perez EA, Reinholz MM, Hillman DW, Tenner KS, Schroeder MJ, Davidson NE, et al. HER2 and chromosome 17 effect on patient outcome in the N9831 adjuvant trastuzumab trial. J Clin Oncol 2010, 28(28):4307-4315.

Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, et al: Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med 2005, 353(16):1659 -1672.

Pogue-Geile KL, Kim C, Jeong JH, Tanaka N, Bandos H, Gavin PG, et al. Predicting degree of benefit from adjuvant trastuzumab in NSABP trial B-31. J Natl Cancer Inst 2013, 105(23):1782-8.

Prieto DA, Hood BL, Darfler MM, Guiel TG, Lucas DA, Conrads TP, et al. Liquid Tissue: proteomic profiling of formalin-fixed tissues. Biotechniques 2005, Jun (Suppl):32-35.

Rimm, DL. What brown cannot do for you. Nat Biotechno 2006, 24:914-916.

Roche PC, Suman VJ, Jenkins RB, Davidson NE, Martino S, Kaufman PA, et al. Concordance between local and central laboratory HER2 testing in the Breast Intergroup Trial N9831. J Natl Cancer Inst 2002, 94:855-857.

Romond EH, Perez EA, Bryant J, Suman VJ, Geyer CE Jr, Davidson NE, et al: Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. N Engl J Med 2005, 353(16):1673-1684.

Ross JS, Slodkowska EA, Symmans WF, Pusztai L, Ravdin PM, Hortobagyi GN. The HER-2 receptor and breast cancer: ten years of targeted anti-HER-2 therapy and personalized medicine. Oncologist 2009, 14(4):320 -368.

Rudnick PA, Clauser KR, Kilpatrick LE, Tchekhovskoi DV, Neta P, Blonder N, et al. Performance metrics for liquid chromatography-tandem mass spectrometry systems in proteomics analyses. Mol Cell Proteomics 2010, 9:225-241.

Scaltriti M, Verma C, Guzman M, Jimenez J, Parra JL , Pedersen K, et al. Lapatinib, a HER2 tyrosine kinase inhibitor, induces stabilization and accumulation of HER2 and potentiates trastuzumab-dependent cell cytotoxicity. Oncogene 2009; 28: 803-814.

Scaltriti M, Nuciforo P, Bradbury I, Sperinde J, Agbor-Tarh D, Campbell C, et al. High HER2 expression correlates with response to the combination of lapatinib and trastuzumab. Clin Cancer Res 2015,21:569–576.

Shi Y, Huang W, Tan Y, Jin X, Dua R, Penuel E, et al. A novel proximity assay for the detection of proteins and protein complexes: quantitation of HER1 and HER2 total protein expression and homodimerization in formalin-fixed, paraffin-embedded cell lines and breast cancer tissue. Diagn Mol Pathol 2009, 18:11-21.

Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 1989, 244:707-712.

Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Eng J Med 2001, 344:783-792.

Suo Z, Berner HS, Risberg B, Karlsson MG, Nesland JM. Estrogen receptor-alpha and C-ERBB-4 expression in breast carcinomas. Virchows Arch 2001, 439(1):62-69.

Suo Z, Risberg B, Kalsson MG, Willman K, Tierens A, Skovlund E, et al. EGFR family expression in breast carcinomas. c-erbB-2 and c-erbB-4 receptors have different effects on survival. J Pathol 2002, 196(1):17-25.

Tao J, Castel P, Radosevic-Robin N, Elkabets M, Auricchio N, Aceto N, et al. Blockade of EGFR and HER3 enhances PI3K/Akt anti-tumor activity in triple negative breast cancer. Sci Signal 2014, 7:ra29.

Toi M, Sperinde J, Huang W, Saji S, Winslow J, Jin X, et al. Differential survival following trastuzumab treatment based on quantitative HER2 expression and HER2 homodimers in a clinic-based cohort of patients with metastatic breast cancer. BMC Cancer 2010, 10:56.

Tovey SM, Witton CJ, Bartlett JM, Stanton PD, Reeves JR, Cooke TG. Outcome and human epidermal growth factor receptor (HER) 1-4 status in invasive breast carcinomas with proliferation indices evaluated by bromodeoxyuridine labelling. Breast Cancer Res 2004, 6(3):R246-251.

Van Pelt AE, Mohsin S, Elledge RM, Hilsenbeck SG, Gutierrez MC, Lucci A Jr, et al: Neoadjuvant trastuzumab and docetaxel in breast cancer: preliminary results. Clin Breast Cancer 2003, 4(5):348-353.

Vicario R, Peg V, Morancho B, Zacarias-Fluck M, Zhang J, Martínez-Barriocanal Á, et al. Patterns of HER2 Gene Amplification and Response to Anti-HER2 Therapies. PloS one 2015, 10, e0129876.

Yu D, Hung MC. Overexpression of ErbB2 in cancer and ErbB2-targeting strategies. Oncogene 2000, 19(53):6115-6121.

Washburn MP, Wolters D, Yates JR 3rd. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat Biotechnol 2001, 19(3):242-247.

Witton CJ, Reeves JR, Going JJ, Cooke TG, Bartlett JM. Expression of the HER1-4 family of receptor tyrosine kinases in breast cancer. J Pathol 2003, 200(3):290-297.

Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol 2007, 25:118-145.

Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. J Clin Oncol 2013, 31:3997-4013.

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