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Original Research

Intra-patient and inter-metastasis heterogeneity of HER2-low status in metastatic breast cancer

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Abstract ***Introduction:*** Anti-HER2 antibody-drug conjugates (ADCs) have shown important efficacy in HER2-low metastatic breast cancer (mBC). Criteria for receiving ADCs are based on a single assay on the primary tumour or a small metastatic biopsy. We assessed the intra-patient inter-metastasis heterogeneity of HER2-low status in HER2-negative mBC.

Patients and Methods: We included samples of 10 patients (7 ER-positive and 3 ER-negative) donated in the context of our post-mortem tissue donation program UPTIDER. Excisional post-mortem biopsies of 257 metastases and 8 breast tumours underwent central HER2 immunohistochemistry (IHC), alongside 41 pre-mortem primary or metastatic samples. They were classified as HER2-zero, HER2-low (HER2-1+ or HER2-2+, in situ hybridisation [ISH] negative) or HER2-positive (HER2-3+ or HER2-2+, ISH-

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positive) following ASCO/CAP guidelines 2018. HER2-zero was further subdivided into HER2-undetected (no staining) and HER2-ultralow (faint staining in ≤10% of tumour cells).

Results: Median post-mortem interval was 2.5 h. In 8/10 patients, HER2-low and HER2-zero metastases co-existed, with the proportion of HER2-low lesions ranging from 5% to 89%. A total of 32% of metastases currently classified as HER2-zero were HER2-ultralow. Intra-organ inter-metastasis heterogeneity of HER2-scores was observed in the liver in 3/6 patients. Patients with primary ER-positive disease had a higher proportion of HER2-low metastases as compared to ER-negative disease (46% versus 8%, respectively). At the metastasis level, higher percentages of ER-expressing cells were observed in HER2-low or -ultralow as compared to HER2-undetected metastases.

Conclusions: Important intra-patient inter-metastasis heterogeneity of HER2-low status exists. This questions the validity of HER2-low in its current form as a theranostic marker.

1. Introduction

Antibody-drug conjugates (ADCs) have reshaped the concept of targeted treatments. While HER2-overexpression/amplification was historically indispensable to benefit from HER2-targeted treatments, the DESTINY-Breast04 trial recently showed major survival benefits for the ADC trastuzumab deruxtecan (T-DXd) in comparison to chemotherapy in patients with HER2-negative metastatic breast cancer with low levels of expression of the HER2 protein (HER2-low) [1]. HER2-low disease was defined in this trial as centrally assessed immunohistochemical (IHC) HER2-scores of 1+, or 2+ with negative reflex in situ hybridisation (ISH) testing on any given tumour biopsy of the patient, leading to T-DXd now being clinically implemented in that same setting. While subgroup analyses of the DESTINY-Breast04 trial showed benefit of T-DXd irrespective of the site of HER2-low status assessment (primary versus metastatic tumour tissue) [2], discordance in HER2-low status of different tumour biopsies of a patient can complicate treatment decision making. These discordances have been reported on different levels: between matched pre- and post-neoadjuvant treatment samples [3], primary and metastatic samples [4–8], and two metastatic samples in the same patient [5,6], with a status switch between HER2-zero and HER2-low being observed both in oestrogen receptor (ER) positive as well as ER-negative disease. Up to date, no reports have evaluated the discordance in HER2-low status between more than two metastases per patient, or between multiple metastases of a patient at a single point in time. Here, we assessed the intra-patient inter-metastasis heterogeneity of HER2-low status in patients participating in our institutional rapid post-mortem tissue donation program.

2. Methods

2.1. Patients and samples

UPTIDER (UZ/KU Leuven Program for Post-mortem Tissue Donation to Enhance Research) is a monocentric tissue donation program enroling patients with any type of metastatic breast cancer in their last line(s) of treatment (NCT04531696). Upon death, a rapid research autopsy is performed, acquiring formalin-fixed paraffin embedded (FFPE) and other samples from all primary or metastatic tumour lesions identified during the procedure. For the purpose of this study, ten patients who had undergone tissue donation since start of the program in November 2020, were reportedly HER2-negative as per ASCO/CAP 2018 guidelines at time of initial diagnosis [9], and had adequate FFPE fixation times were considered.

For these patients, samples retrieved at autopsy that had microscopically confirmed tumoral invasion were considered. Additionally, archived FFPE blocks from primary tumours and metastases, when available, were requested (pre-mortem samples). In the rare event where no FFPE block could be retrieved, the historical HER2 IHC stained slide was requested.

2.2. Immunohistochemistry and fluorescence ISH

All retrieved FFPE samples underwent IHC staining for HER2 (HercepTest™, ready to use [RTU], ISO15189 accredited) in our institution. Consensus scoring on these and historically stained slides (Agilent, clone A0485, RTU, CE IVD) was performed between two breast pathologists according to ASCO/CAP 2018 guidelines [9]. The observers were blinded for patient ID. Fluorescence ISH testing was performed for

samples with IHC score of 2+ (Her2 IQFISH pharmDx™, ISO15189 accredited). HER2 status was then categorised as HER2-zero (HER2-0), HER2-low (HER2-1+ or HER2-2+ with negative ISH) or HER2-positive (HER2-3+ or HER2-2+ with positive ISH). HER2-zero was further classified into HER2-undetected (no IHC staining, corresponding with the HER2-0 category of the ASCO-CAP 2007 guidelines [10]) and HER2-ultralow (faint or barely perceptible IHC staining in ≤10% of tumour cells). IHC staining for ER (EP1, DAKO, RTU, ISO15189 accredited) was performed, and scored as the percentage of positive staining tumour cells. A cut-off of 1% was used to distinguish ER-positive from ER-negative, in accordance with ASCO/CAP 2020 guidelines [11].

2.3. Statistical analyses

The association between HER2 status and percentage of ER-staining cells was assessed by linear regression, with ER percentage as dependent variable, HER2 status as independent variable (HER2-undetected as reference and considering two definitions: (i) any HER2-detection [grouping HER2-ultralow and HER2-low], (ii) HER2-staining as HER2-ultralow, 1+, or 2+ with negative ISH) and accounting for the clustering of the data by patient using the generalised estimating equation method. Two nested models – one with constant and one with linear relationship – were compared using Analysis of Variance (ANOVA) testing strategy. Sample-specific post-mortem interval (ssPMI) for each sample was defined as the time between death of the patient and the fixation in formalin of the sample. Associations between HER2 category (HER2-low versus HER2-zero) and ssPMI were assessed by logistic linear regressions for longitudinal data, with HER2 category as dependent variable, ssPMI as independent variable, and accounting for the clustering of the data by patient using the generalised estimating equation method. We performed the Wald test on regression coefficients. All analyses were performed in R version 4.2.1.

3. Results

In total, 257 metastases or pathological axillary lymph nodes (median 25 per patient, range 9–41) and 8 breast tumour samples collected at autopsy, as well as 41 pre-mortem samples were collected from the 10 patients and analysed in this study (Fig. 1). The main clinicopathological characteristics of these patients are presented in Table A.1 and Table A.2. Seven had ER-positive and three had ER-negative disease at time of primary diagnosis. Median age at diagnosis of first metastasis was 52.5 years (range: 37–78), median time from first metastasis till death was 4.8 years (range: 0.9–10.3). Median post-mortem interval, defined as the time between the death of the patient and the start of the tissue donation procedure, was 2.5 h (range: 1.9–3.5).

Three main results emerged from our analyses. First, intra-patient inter-metastasis heterogeneity of HER2 status was observed in 8 out of 10 patients, meaning that in these patients HER2-zero and HER2-low lesions co-existed at end-stage disease (Fig. 2A and B, Table A.3). The percentage of HER2-low metastases in these 8

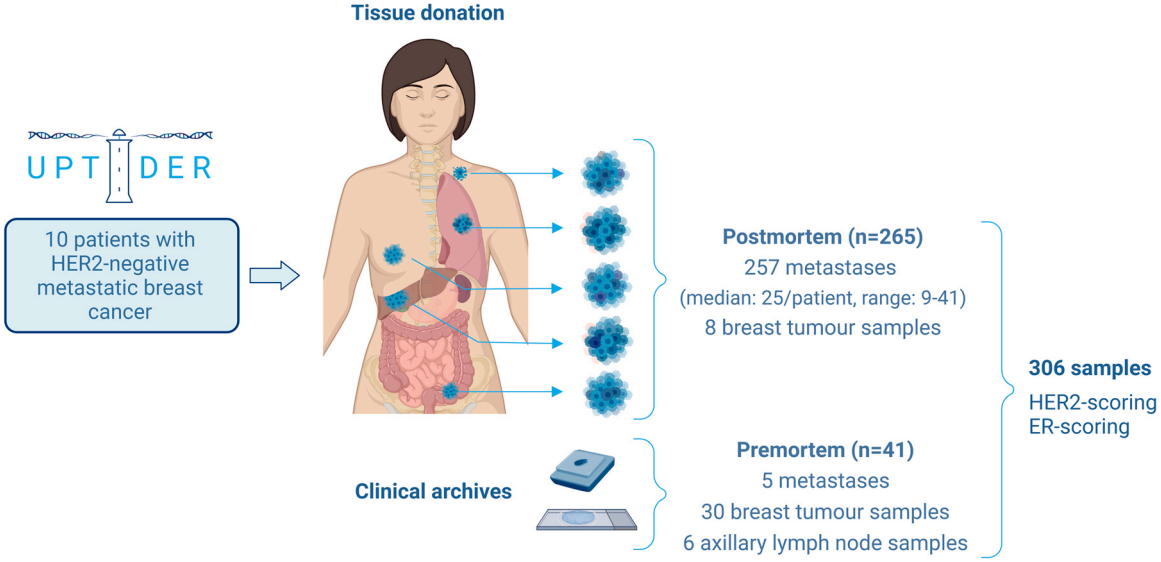

Fig. 1. Sample collection and analysis. Samples from multiple metastases (including pathological axillary lymph nodes) were collected rapidly after death for 10 patients with HER2-negative metastatic breast cancer included in our UPTIDER post-mortem tissue donation program (NCT04531696). Additionally, pre-mortem samples were collected from clinical archives. In total, we scored 306 samples for HER2 and ER as described in the methods section of this manuscript. Created with BioRender.com.

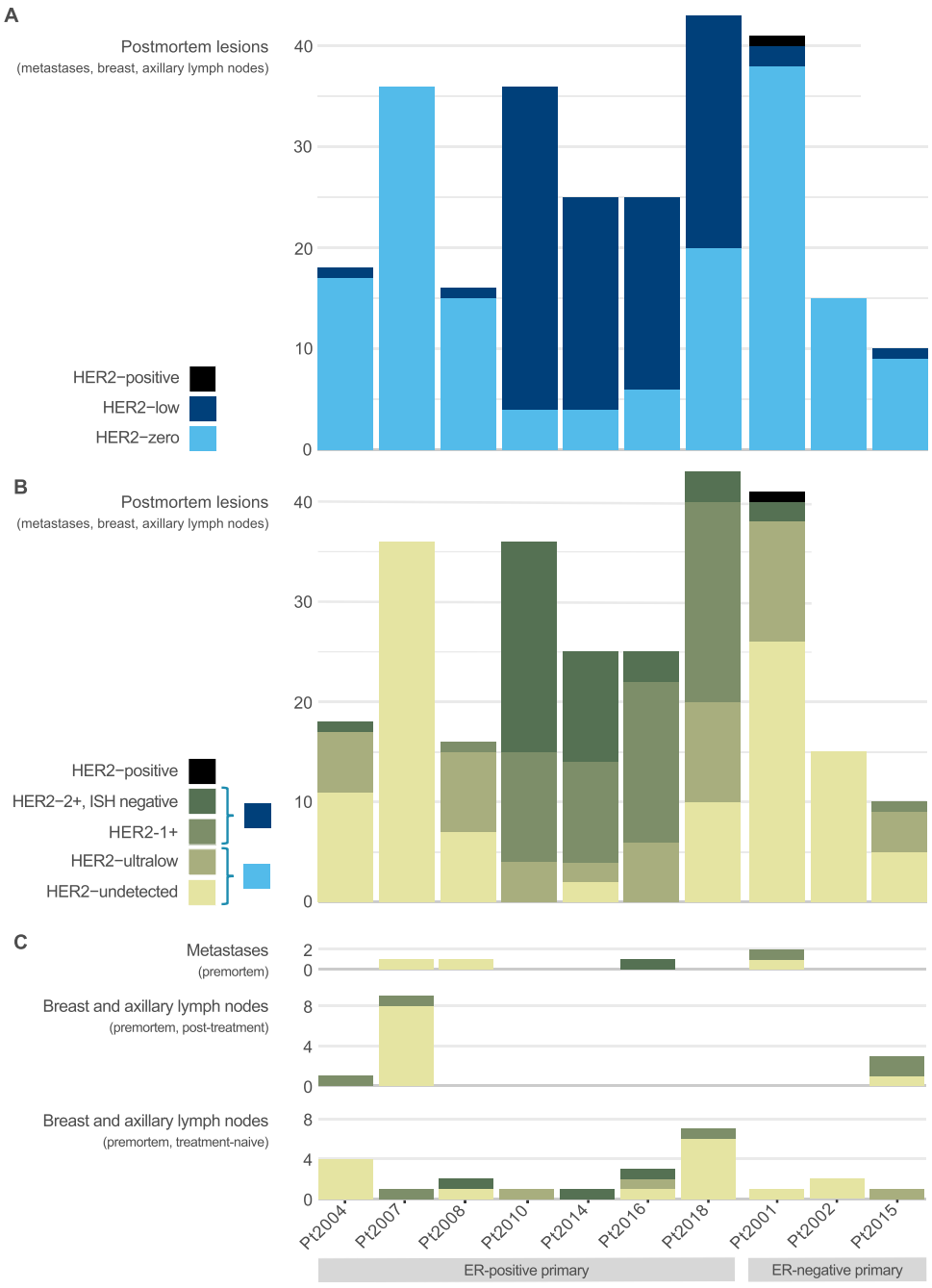

Fig. 2. Intra-patient inter-lesion heterogeneity in HER2 status. (A) HER2 statuses per patient of the different metastatic, axillary lymph node and breast tumour samples taken at autopsy, categorised as HER2-zero (HER2-0 as per ASCO-CAP 2018 guidelines), HER2-low (HER2-1+ or HER2-2+ with negative in situ hybridisation (ISH)), or HER2-positive (HER2-2+ with positive ISH or HER2-3+). The one HER2-positive lesion in patient Pt2001 was a HER2-2+ ISH-positive peritoneal lesion. (B) HER2 statuses per patient of the different metastatic, axillary lymph node and breast tumour samples taken at autopsy, categorised as HER2-undetected (no staining), HER2-ultralow (faint/barely perceptible incomplete membranous staining in ≤10% of tumour cells), HER2-1+, HER2-2+ with negative ISH, or HER2-positive (HER2-2+ with positive ISH or HER2-3+). (C) HER2 statuses per patient of pre-mortem samples.

patients at time of death ranged between 5% and 89%, with a median of 33%. When looking in detail at the pre-mortem primary and metastatic samples of the 8 patients with co-existing HER2-zero and HER2-low metastases, 7 of them had had at least one HER2-low sample during life (Fig. 2C, Table A.2). Two patients (Pt2002 and Pt2007) presented with HER2-zero, and even HER2-undetected, disease at time of death in all evaluated metastatic samples. One of these patients (Pt2007) had had a diagnostic core needle biopsy and a post-neoadjuvant chemotherapy breast resection sample with HER2-low status during life. Across all patients, at

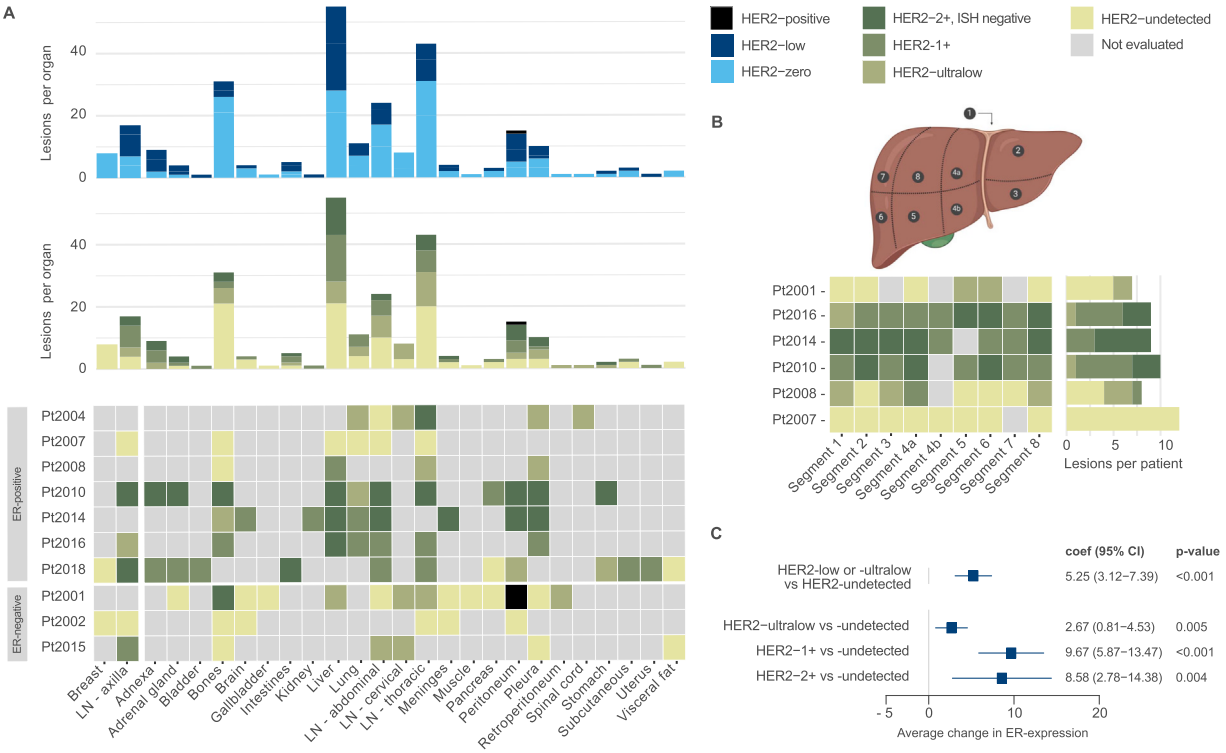

Fig. 3. (A) Distribution of HER2 statuses of lesions per organ for all patients (histograms) and per organ per patient (matrix). For the matrix, in case multiple samples were taken from the same organ in one patient, the highest score is shown. (B) Distribution of HER2 statuses of samples taken from metastases in different segments within the liver in 6 patients (matrix) and per patient for the liver (horizontal histogram). For the matrix, in case multiple samples were taken from the same organ in one patient, the highest score is shown. In 3 patients (Pt2008, Pt2010, Pt2016) both HER2-low and HER2-zero metastases were present. (C) Association between HER2 status and ER-expression of metastases in the 7 patients with ER-positive disease at diagnosis (Pt2001 is considered ER-negative, see also Table A.1). The association was assessed by linear regressions, with ER-expression as dependent variable, HER2-categories as independent variable (HER2-undetected as reference) and accounting for the clustering of the data by patient using the generalised estimating equation method. Two nested models – with constant and linear relationship – were compared using ANOVA testing strategy. LN = lymph nodes, ER = oestrogen receptor, ISH = in situ hybridisation, coef = coefficient, CI = confidence interval. HER2-zero = HER2-0 as per ASCO-CAP 2018 guidelines), HER2-low = HER2-2+ with negative ISH or HER2-1+, HER2-positive = HER2-2+ with positive ISH or HER2-3+.

least some membrane staining (HER2-ultralow) was observed in 32% (52/163) of metastases currently classified as HER2-zero according to ASCO-CAP 2018 guidelines.

Secondly, when grouping all patients together, a variety of HER2-scores was seen in the different organs and HER2-low lesions were found across almost all organ categories (Fig. 3A). Moreover, even within one and the same patient, intra-organ heterogeneity could be observed: out of the 6 patients where metastases were sampled from different segments of the liver (total number of samples=57), the copresence of HER2-low and HER2-zero lesions was seen in 3 of them (Fig. 3B). Two of the other patients were consistently HER2-low across the sampled liver metastases but did exhibit heterogeneity in terms of HER2 IHC scores. The last patient was HER2-undetected across all metastases, including the liver.

Thirdly, our results show an association between HER2- and ER-expression at the patient and metastatic level. Patients with ER-positive breast cancer at diagnosis on average had a higher percentage of HER2-low metastases as compared to ER-negative patients (46% (range: 0–89%) versus 8% (range: 0–18%)). We then focused on the metastases of the 7 patients with ER-positive breast cancer at primary diagnosis and observed the same trend on a lesion-level: HER2-low and HER2-ultralow lesions had slightly but significantly higher ER scores as compared to HER2-undetected lesions (coefficient 5.25, 95% confidence interval [CI] 3.12–7.39, p-value < 0.001) (Fig. 3C, Fig. A.1). The association was also seen within each HER2 category (HER2-ultralow, -1+ and -2+ with negative ISH versus HER2-undetected) (Fig. 3C).

Finally, the observed results were unlikely to be importantly influenced by the cold ischaemia time, as no

s-

ignificant association between HER2 category (HER2-low versus HER2-zero) and the ssPMI was observed (odds ratio 0.93, CI 0.82–1.06, p-value = 0.29).

4. Discussion

Samples retrieved post-mortem, either through research autopsies (tissue donation programs) or clinical autopsies, represent invaluable sources for translational research. The unique and comprehensive sample repository of our tissue donation program UPTIDER allowed us to demonstrate, for the first time on a large number of metastases per patient and at one point in time, that a patient's metastases can be very heterogeneous with regard to their HER2 status. Even within the liver, often biopsied for biomarker testing, we show that intra-organ heterogeneity of HER2-scores was common. Our findings are in line with the previously reported discordance between the HER2 status of a maximum of two metastases evaluated per patient [5,6], but describe it to a much bigger extent on an individual patient-level. Secondly, no other series has thus far assessed the discordance in HER2-low status between synchronous metastases, and it has been shown in the preclinical setting that differences in treatment exposures can affect HER2 expression which can partially explain heterogeneity between metachronous metastases [12–14]. Of note, in our series, the HER2 status of lesions assessed at different points in time during the life of the patient did not accurately reflect the HER2 status at autopsy.

The observed intra-patient heterogeneity puts into question the assessment of HER2-low status on a single biopsy at any point in time, which was set as the inclusion criterion in DESTINY-Breast04 and was subsequently adopted as a predictive marker for benefit of T-DXd [1]. As pointed out before, patients included in this trial based on testing of a primary sample as well as those tested on a metastatic sample derived benefit of T-DXd over treatment of physician's choice [2], justifying the indication in both scenarios while the results of further prospective trials are awaited. Two trials are indeed currently evaluating whether treatment eligibility for T-DXd can be expanded to all patients with metastatic breast cancer, irrespective of HER2 status. The DAISY trial already presented preliminary evidence for efficacy in patients with HER2-zero disease [15] and Destiny-Breast06 is expected to report on the benefit in HER2-ultralow in earlier treatment lines (NCT04494425). The simultaneous presence of HER2-low and HER2-zero lesions observed in our study in a substantial proportion of patients could help to interpret the responses in different HER2-cohorts in these trials, as well as in real life.

With regard to the association between HER2 and ER status on a lesion-level, our results are consistent with the convincing data currently available in early breast cancer [4,16–18], and the more limited data available in metastatic disease [4]. While in our series the association between HER2-detection (HER2-ultralow or -low) and the percentage of cells staining positive for ER was modest in effect, it likely reflects the interplay between HER2- and ER-pathways observed since long [19]. When thus assessing the prognostic effect of HER2-low status, ER status inevitably needs to be considered, and further research will hopefully elucidate how both unique markers play interlinked roles in tumour progression.

Our study does come with limitations. Firstly, a low number of patients was included. However, high numbers of samples were analysed per patient. Moreover, large excisional biopsies were evaluated, reducing the known issues of reproducibility of smaller core needle biopsies, especially for HER2-low assessment [20,21]. Secondly, the post-mortem setting has peculiarities of its own. Post-mortem samples represent a heavily pretreated situation, possibly complicating the translation of our results into earlier treatment lines. Post-mortem samples also often come with long cold ischaemia times, which are known to sometimes affect IHC results [22,23]. In our series we did not observe an effect of the ssPMI on the HER2 status, which is reassuring in the interpretation of our results. Thirdly, IHC- and ISH-based assays represent the only assays of established clinical value for the assessment of HER2 but come with many limitations of their own. The ASCO/CAP guidelines were indeed designed to identify tumours with targetable HER2-overexpression and have shown to be less reliable in distinguishing between the categories with low levels of HER2 [24–26]. Up until recently, there was no clinical relevance of distinguishing HER2-zero from HER2-1+ cases, and thus pathologists may have been less stringent in applying the exact cut-offs defined by the ASCO/CAP guidelines. Furthermore, the interobserver agreement between pathologists for HER2-zero and HER2-low declines when HER2 is expressed in around 10% of the cells (cut-off value) [26]. Additionally, HER2-categories defined by the ASCO/CAP guidelines are heterogeneous by definition. The cut-off that is used reduces the entire group of tumours with any incomplete membranous HER2-staining in 10–100% of tumour cells into one single category, and it is currently unknown if this is relevant for predicting response to ADCs. More sensitive and quantitative assays exist, having the ability to detect HER2 protein expression in cases where the current HER2 assay does not [27–29]. Heterogeneity, however, remains a challenge as these techniques average the quantity of protein

by area. A clinically meaningful cut-off, validated on clinical trial cohorts, is also still lacking for these techniques.

Despite these limitations, the heterogeneity in HER2 status we observed in this study using the only currently clinically validated assays likely reflects real differences in HER2 expression profiles between different metastases. Assessment of a patient's HER2 status on a single biopsy might thus not accurately represent their likelihood to respond to ADCs. We do acknowledge that sampling of a large number of metastases is not a feasible solution. In fact, the key might lie in targeted imaging or other *in vivo* techniques that could comprehensively capture HER2 status beyond IHC, or the omission of HER2 status as a biomarker for benefit of T-DXd in case benefit would be observed across all patients.

5. Conclusions

We observed important intra-patient, inter-metastases heterogeneity of HER2-low status in post-mortem samples using the currently clinically used HER2 assay. These results will impact our view on the validity of HER2 status assessment on a single tumour sample as a predictive marker for treatment with T-DXd and aid in the interpretation of results of the efficacy of HER2-targeted ADCs in presumed HER2-zero populations. Caution is thus advised for using the current HER2 assay as a theranostic marker in the metastatic setting.

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CRediT authorship contribution statement

Tatjana Geukens: Conceptualisation, Methodology, Investigation, Resources, Data Curation, Writing – Original Draft, Visualisation, Project Administration, Funding Acquisition. Maxim De Schepper: Conceptualisation, Methodology, Investigation, Resources, Data Curation, Writing – Original Draft, Visualisation, Project Administration. François Richard: Conceptualisation, Methodology, Software, Validation, Formal Analysis, Resources, Data Curation, Writing – Original Draft, Visualisation, Project Administration. Marion Maetens: Conceptualisation, Resources, Writing – Review & Editing, Project Administration, Funding Acquisition. Karen Van Baelen, Amena Mahdami, Ha-Linh Nguyen, Edoardo Isnaldi, Sophia Leduc, Anirudh Pabba, Gitte Zels, Kevin Punie, Patrick Neven, Wouter Van Den Bogaert: Resources, Writing – Review & Editing. Freya Mertens, Sara Vander Borght: Investigation, Writing – Review & Editing. Ann Smeets, Ines Nevelsteen: Writing – Review & Editing. Hans Wildiers: Resources, Writing – Review & Editing, Funding Acquisition. Giuseppe Floris: Conceptualisation, Methodology, Investigation, Resources, Writing – Review & Editing, Supervision, Project Administration, Funding Acquisition. Christine Desmedt: Conceptualisation, Methodology, Resources, Writing – Review & Editing, Supervision, Project Administration, Funding Acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: TG, MDS, FR, KVB, MM, AM, H-LN, EI, SL, AP, GZ, FM, SVB, AS, IN, PN, WVDB, GF, CD: no conflicts to declare. KP: research grants paid to institution: MSD and Sanofi, speaker fees and honoraria for consultancy and advisory board functions: Astra Zeneca, Eli Lilly, Exact Sciences, Focus Patient, Gilead, MSD, Novartis, Pfizer, Roche, Seagen, speaker fees and honoraria for consultancy and advisory board functions paid to institution: Astra Zeneca, Eli Lilly, Exact Sciences, Gilead, MSD, Novartis, Pfizer, Roche, Seagen, stock options: Need Inc, travel grants: Astra Zeneca, Novartis, Pfizer, PharmaMar, Roche. HW: his institution received financial compensation on his behalf for advisory boards, lecture fees and/or consultancy fees from Immutep Pty, MSD, Astrazenca, Daiichi, AbbVie, Lilly, Roche, EISAI, Pfizer, Sirtex, Gilead. He received travel support from Pfizer and Roche.

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Data sharing statement

Data related to this study is available in the Appendix (Table A.2 and Table A.3). Detailed code for the analysis is available upon request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejca.2023.04.026.

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