PATHOLOGICA 2023;115:292-301;
DOI: 10.32074/1591-951X-942

Review

Think "HER2" different: integrative diagnostic approaches for HER2-low breast cancer

Caterina Marchiò[1,2], Carmen Criscitiello[3,4], Cristian Scatena[5,6], Alfredo Santinelli[7], Paolo Graziano[8], Umberto Malapelle[9], Giulia Cursano[10], Konstantinos Venetis[10], Giuseppe Nicolò Fanelli[5,6], Francesco Pepe[9], Enrico Berrino[1,2], Carmine De Angelis[11], Giuseppe Perrone[12,13], Giuseppe Curigliano[3,4], Nicola Fusco[4,10]

[1] Division of Pathology, Candiolo Cancer Institute, FPO-IRCCS, Candiolo, Italy; [2] Department of Medical Sciences, University of Turin, Turin, Italy; [3] Division of Early Drug Development for Innovative Therapy, IEO, European Institute of Oncology, IRCCS, Milan, Italy; [4] Department of Oncology and Hemato-Oncology, University of Milan, Milan, Italy; [5] Department of Laboratory Medicine, Pisa University Hospital, Anatomic Pathology 1 Universitaria, Pisa, Italy; [6] Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Italy; [7] Anatomic Pathology, Azienda Sanitaria Territoriale di Pesaro-Urbino, Pesaro, Italy; [8] Pathology Unit, Fondazione IRCCS Ospedale 'Casa Sollievo della Sofferenza', San Giovanni Rotondo (FG), Italy; [9] Department of Public Health, Federico II University of Naples, Naples, Italy; [10] Division of Pathology, IEO, European Institute of Oncology, IRCCS, Milan, Italy; [11] Department of Clinical Medicine and Surgery, Federico II University of Naples, Naples, Italy; [12] Department of Medicine and Surgery, Research Unit of Anatomical Pathology, Università Campus Bio-Medico di Roma, Roma, Italy; [13] Anatomical Pathology Operative Research Unit, Fondazione Policlinico Universitario Campus Bio-Medico, Roma, Italy

Received: November 2, 2023
Accepted: November 7, 2023

Correspondence
Nicola Fusco
Division of Pathology, IEO, European Institute of Oncology IRCCS, and Department of Oncology and Hemato-Oncology, University of Milan, via G. Ripamonti 435, Milano, Lombardy 20141, Italy
E-mail: nicola.fusco@ieo.it

How to cite this article: Marchiò C, Criscitiello C, Scatena C, et al. Think "HER2" different: integrative diagnostic approaches for HER2-low breast cancer. Pathologica 2023;115:292-301. https://doi.org/10.32074/1591-951X-942

Summary
This work explores the complex field of HER2 testing in the HER2-low breast cancer era, with a focus on methodological aspects. We aim to propose clear positions to scientific societies, institutions, pathologists, and oncologists to guide and shape the appropriate diagnostic strategies for HER2-low breast cancer. The fundamental question at hand is whether the necessary tools to effectively translate our knowledge about HER2 into practical diagnostic schemes for the lower spectrum of expression are available. Our investigation is centered on the significance of distinguishing between an immunohistochemistry (IHC) score 0 and score 1+ in light of the clinical implications now apparent, as patients with HER2-low breast cancer become eligible for trastuzumab-deruxtecan treatment. Furthermore, we discuss the definition of HER2-low beyond its conventional boundaries and assess the reliability of established diagnostic procedures designed at a time when therapeutic perspectives were non-existent for these cases. In this regard, we examine potential complementary technologies, such as gene expression analysis and liquid biopsy. Ultimately, we consider the potential role of artificial intelligence (AI) in the field of digital pathology and its integration into HER2 testing, with a particular emphasis on its application in the context of HER2-low breast cancer.

Key words: HER2-low, breast cancer, pathology, testing methods, liquid biopsy, Artificial Intelligence

Introduction

The field of breast pathology has experienced a profound transformation with the emergence of the DESTINY-Breast04 (DB-04) findings in 2022 [1]. This trial, further supported by the DAISY study [2], brought HER2 back into the spotlight as a predictive biomarker in breast cancer [3]. Patients with metastatic breast cancer classified as "HER2-low" (i.e., HER2 immunohistochemistry (IHC) score 1+ or score 2+ without

HER2 gene amplification by in situ hybridization (ISH) testing) were the focus of DB-04 [4]. Treating these patients with trastuzumab deruxtecan (T-DXd), a novel antibody-drug conjugate (ADC), instead of conventional chemotherapy led to remarkable improvements in survival [1,5].

The significance of this clinical achievement challenges the established binary HER2 classification system, which categorizes breast carcinomas as either positive (i.e., HER2 ISH score 3+ or score 2+ ISH-positive) or negative (all other cases), in line with the ASCO/CAP 2018 guideline [6] reaffirmed by ASCO/CAP 2023 guideline update and 2023 ESMO consensus statements on HER2-low breast cancer [7,8]. The HER2-low category does not delineate a specific subtype of breast cancer but rather it encompasses a heterogeneous group of tumors, accounting for approximately half of all cases of breast cancer [9-11]. Nonetheless, HER2-low signifies a distinctive biomarker status that corresponds to a favorable prognosis after treatment with T-DXd [12]. At present, unlike with conventional HER2 targeting, differentiating between a HER2 IHC score of 0 and score 1+ holds clinical significance [13]. To ensure precise identification of these tumors, it is essential to implement standardized procedures, guidelines, and specific training for pathologists in interpreting HER2-low [14,15]. The prospective integration of artificial intelligence (AI) in this context holds promise for supporting these efforts, although its implementation in the present appears less likely [16]. In addition to the aforementioned approaches, the use of liquid biopsy (LB), may represent a possible future opportunity for the assessment of HER2-low status using circulating tumor cells (CTCs) [17].

In this collective effort, we critically examine the current state of assessing HER2-low status in breast cancer, addressing unresolved issues, and providing recommendations for conducting high-quality testing in line with the 2023 ASCO/CAP updates [7] and the 2023 ESMO consensus statements [18] on HER2-low breast cancer.

Clinical rationale for HER2-low identification

By definition, HER2-negative breast cancer includes a significant proportion of cases with low HER2 expression levels (i.e., IHC score 2+, score 1+, and a subset of score 0) that are not underpinned by HER2-amplification as investigated by ISH (Fig. 1) [9]. Previous studies showed limited efficacy of trastuzumab and ado-trastuzumab emtansine (T-DM1) in this subgroup, hence they were not approved for use in tumors other than HER2-positive [19,20]. However, recent advancements in therapy, particularly with second-generation HER2 ADCs such as T-DXd, have brought to the forefront an interest in the whole spectrum of HER2-expressing carcinomas [21]. ADCs stud-

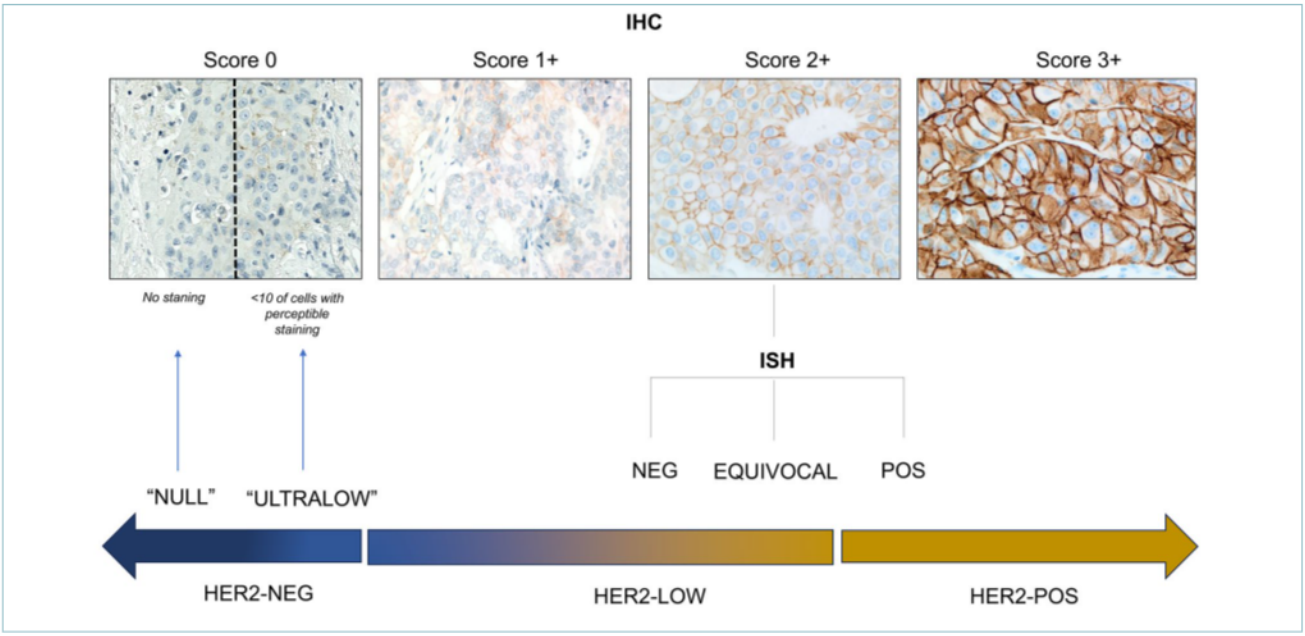

Figure 1. Schematic depiction of HER2 expression range, according to ASCO/CAP guidelines.

ies have shown convincing efficacy, thus challenging conventional treatment approaches [22]. Around 50% of patients with breast cancer are affected by HER2-low disease, with about 60% expressing hormone receptors (HR), while 40% are triple-negative breast cancers (TNBC) with HER2-low expression [5,11,23]. The DB-04 (NCT03734029) study demonstrated the superiority of T-DXd versus chemotherapy in treating patients with HER2-low breast cancer [21]. In this trial, metastatic HER2-low breast cancer patients had been previously treated with one or two lines of chemotherapy and were refractory to endocrine therapies in case of HR-positive disease. T-DXd significantly improved progression-free survival (PFS) and overall survival (OS) compared to chemotherapy in the intention-to-treat (ITT) population and in the HR-positive subgroup. Moreover, an exploratory analysis of 58 patients with HR-negative disease demonstrated that T-DXd was also effective in this subgroup of patients [21]. Subanalyses showed consistent activity of T-DXd in both IHC score 1+ and score 2+/ISH-negative patients without significant differences [3,21]. The efficacy of T-DXd in HER2-low breast cancer was further confirmed by the DAISY trial, an open-label phase II study evaluating T-DXd in three cohorts of patients with HER2-positive (score 3+ on IHC or score 2+/ISH+; Cohort 1), HER2 low (score 1+ or score 2+/ISH-negative; Cohort 2), and HER2-null (score 0+, Cohort 3) advanced breast cancer. Patients in Cohort 2 achieved an objective response of 33.5% with a median duration of response of 7.6 months and a median PFS of 6.7 months. Notably, T-DXd showed anti-tumor activity in Cohort 3, albeit the magnitude of the treatment effect was smaller compared with Cohorts 1 and 2 [2]. At present, the ongoing Destiny Breast-06 (DB-06) (NCT04494425) study includes patients with HR-positive tumors, HER2-low, and the so-called "HER2 ultra-low", namely IHC score 0 greater than zero, including IHC values > 0 and $\leq 10\%$. This study aims to determine the effectiveness of T-DXd in both HER2 ultra-low and HER2-low conditions, as well as compare it to traditional chemotherapy in less pre-treated patients [24]. This study will provide data on the efficacy of trastuzumab deruxtecan after CDK4/6 inhibition.

Critical factors of HER2 assessment in breast cancer

The foundations of a diagnosis of HER2-low disease stem from the traditional scoring system historically adopted to identify HER2-addicted breast carcinomas. This means that IHC and ISH are the standard techniques to identify HER2-low disease without the need for additional assay, at least up to now. Pathologists should equally curate all the diagnostic phases (i.e. pre-analytical, analytical, and post-analytical phases) in collaboration with radiologists and surgeons, as also highlighted by the ASCO/CAP guidelines over the years (Fig. 2) [25, 26]. This concept has been reinforced by the ESMO expert consensus statements [18], as well as by national societies of pathologists [27]. An essential factor is represented by the assay employed for HER2 testing, as different IHC assays, although clinically validated and/or CE-IVD, can give slightly different results [28]. In a recent work comparing the performance of the two most widely used assays in clinical practice, the monoclonal Dako HercepTest™ GE001 and the VENTANA anti-HER2/neu (4B5), a high concordance (98.2%) was observed in assigning a positive versus negative result [29]. Nevertheless, in the lower spectrum of expression, a higher sensitivity was demonstrated for the GE001 assay compared to the 4B5. While all cases scored as 0 using the GE001 remained consistent with a score of 0 when assessed with 4B5, it is noteworthy that 37.5% of cases initially classified as score 0 by 4B5 were subsequently re-classified as score 1+ or 2+ when the same slides were stained using the GE001 [29]. Of note, the assay used in the DB-04 as well as in all of the other DB studies is the 4B5. At present, there is a lack of specific data regarding the performance of alternative assays (e.g., CB11 and A0485) in distinguishing the lower spectrum of HER2 expression, specifically in differentiating between scores 0 and 1+. In the absence of alternative validated options to conduct the test, in-house validations are imperative, utilizing both internal and external controls that encompass the full range of HER2 scoring intensities and patterns [14].

During the post-analytical phase of the test (i.e., interpretation of the staining), caution should be paid to possible artifactual staining caused by pre-analytical and/or analytical issues, resulting -for example- in non-linear or cytoplasmic dot-like staining [30]. Internal and external controls are recommended in each slide run [31]. The final step of HER2 testing is represented by the pathology report of the biomarker, which should be optimized for HER2-low breast cancer in compliance with 2023 ASCO/CAP updates and 2023 ESMO consensus statements, as summarized in Figure 3 [30]. By synergistically combining IHC and ISH techniques, it is essential to provide a clear, precise, and comprehensive report of HER2 status to enable improved treatment decisions and personalized patient management.

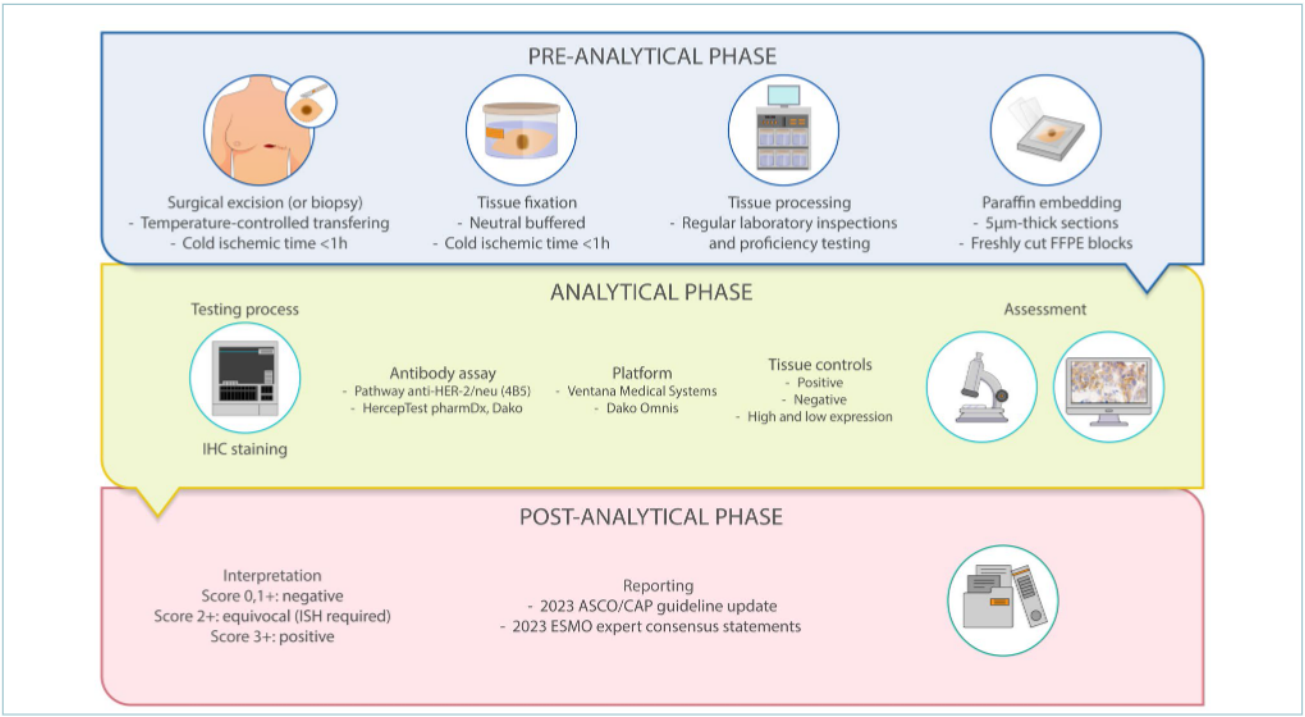

Figure 2. Laboratory procedures for accurate assessment of low HER2 expression. Following excision (biopsy or surgery) the specimen should be transferred to the pathology lab using a temperature-controlled system. The cold ischemia time should not exceed 1 hour. Sample preservation during transport can be achieved either by vacuum sealing or submerging in 4% neutral buffered formalin. The time interval before sampling should fall within a range of 6 to 72 hours. Upon tissue processing, the pathologist should select the most representative sample, subjecting it to immunohistochemical analysis, with the possible option to employ validated digital pathology tools. The HER2 report must include details on the percentage of positive neoplastic cells, staining intensity, and pattern of membrane staining.

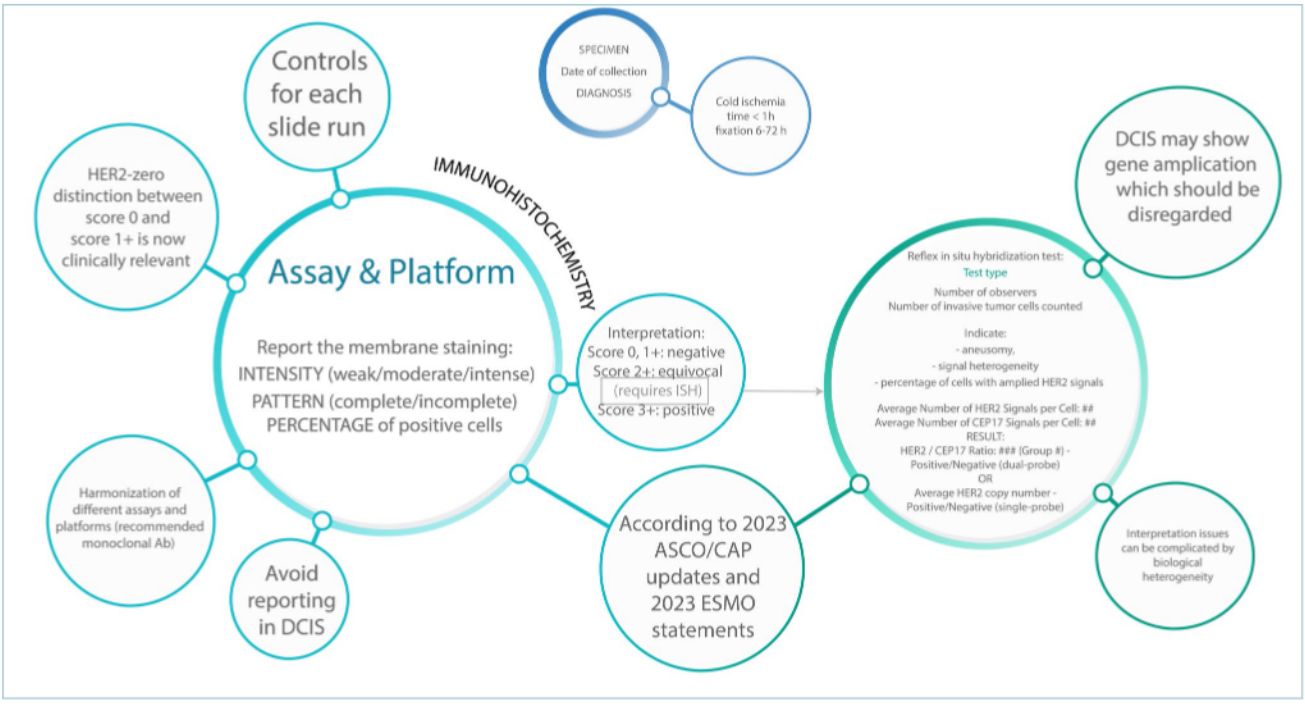

Figure 3. Overview of the existing barriers that may trouble HER2-low identification in breast cancer and practical solutions that could enhance the test.

What to test and when to (re)-assess HER2

Spatial variability and temporal evolution in HER2 expression is a well-known phenomenon [32]. By assessing matched primary and metastatic tumors recent studies have shown that HER2-low status can vary along the disease evolution [33,34]. This variation may be attributable to biological factors, but could also be inherent in the definition of low HER2 expression. There is a mutual shift from HER2 negative to HER2-low disease and *vice versa* with a higher conversion from score 0 to HER2-low in the metastatic setting. Nevertheless, the true status metastatic setting can be difficult to capture given the possible multiple metastatic deposits. In this respect, precious information stems from a recent work where multiple metastatic deposits were sampled and assessed within the context of a rapid autopsy program [35]. Out of the n = 10 patients analyzed for a total of n = 306 samples, only n = 2 cases had a homogeneous lack of HER2 expression. The remaining 8 cases showed a high variability of HER2 expression from score 0 to score 2+ (ISH-negative), even when analyzing a single organ (liver) with multiple metastatic deposits. It is noteworthy that the effectiveness of T-DXd compared to TPC remained consistent across various tumor sample types, as highlighted by the DB-04 trial [36]. These findings may suggest a hypothesis or speculation: whenever a 'HER2-low profile' is observed at any point in the disease history, patients should be considered as HER2-low candidates [37]. Importantly, a significant proportion of historical score 0 results, upon re-evaluation in the context of HER2-low, are now reclassified as score 1+ [38]. This highlights the importance of considering a retest, especially for cases with a historical score 0. This notion has been corroborated by a worldwide, multicenter, noninterventional, retrospective study (NCT04807595) of tissue samples and medical records from n = 789 patients with HER2-negative unresectable/metastatic breast carcinomas previously HER2-negative (i.e., IHC score 0, 1+, or 2+/ISH-negative) [39]. The study design included the re-evaluation of HER2 IHC (4B5 or other) slides by trained pathologists and revealed that > 30% of historical IHC score 0 results were rescored as HER2-low. Education and training are instrumental in this context to drive the attention to the nuances of HER2-low breast cancer, and the complexities involved in reporting HER2 scores 1+ or 0 [18,40,41].

Artificial intelligence to enhance HER2 test predictivity

The interest in digital pathology is boundless, particularly in the field of artificial intelligence (AI) and machine learning applications for predictive pathology [16,42,43]. Recent evidence on HER2-low assessment using AI methods revealed that digital and computational pathology can provide valuable insights into the number and distribution of different HER2-expressing cells in breast cancer [44]. The study demonstrated that reliable results can be obtained using either supervised or even unsupervised machine learning algorithms. To date, several platforms offer these types of analyses, albeit mostly in academic settings. Among these, Visiopharm HER2-CONNECT™, Ibex Galen™ Breast HER2 are for research use only, while Paige HER2Complete and Ventana uPath HER2 (4B5) are CE-IVD [45,46]. However, these algorithms continue to grapple with several significant challenges [47-49]. First, it is essential to recognize that even minor variations in preanalytical processes (e.g., slide preparation, staining procedures, handling of tissue samples) can introduce subtle yet critical discrepancies in color and image quality. These discrepancies, in turn, have the potential to exert a considerable influence on the overall quality of the digitalized tissue slides and, subsequently, on the accuracy of the ensuing analysis [50]. This underscores the need for meticulous attention to detail in the pre-analytical phase to ensure consistency and reliability in digital pathology. Moreover, the lack of standardization across various machine learning algorithms poses a notable obstacle. The field of digital pathology is marked by a proliferation of diverse algorithms, each with its distinct approach and architecture. The absence of a unified framework or standardized practices can result in interoperability challenges and hinder the seamless integration of these algorithms into clinical workflows. Consequently, achieving a consensus on standardized practices and interoperable solutions is pivotal for enhancing the effectiveness and efficiency of digital pathology applications. Finally, a critical concern lies in the absence of prospective clinical trials evaluating the performance of these software solutions in real-world scenarios for HER2-low breast cancer. While the potential of machine learning algorithms in pathology is promising, their true clinical utility and reliability can only be substantiated through rigorous randomized clinical trials.

Perspectives on molecular testing

Gene expression-based assays of HER2 mRNA have been proposed for a more direct measurement of HER2 expression, providing a quantitative assessment of *HER2* gene amplification [51-54]. Various commercial kits and platforms are available for performing

RT-PCR-based HER2 testing, offering standardized and reproducible results, albeit not validated in clinical trials [55,56]. Another mRNA-based assay is the NanoString nCounter® system, which utilizes digital barcode technology [57]. In this method, specific probes are designed to capture and count individual mRNA molecules. The nCounter® system provides highly sensitive and accurate quantification of HER2 expression levels and offers the advantage of multiplexing, enabling simultaneous assessment of other relevant genes. Next-generation sequencing (NGS) techniques have also emerged as powerful tools for HER2 status assessment [58]. NGS allows for the sequencing of thousands of RNA molecules simultaneously, providing comprehensive information about *HER2* and other relevant genes. This approach enables the identification of novel HER2 alterations and can help in subclassifying HER2-positive tumors based on their genomic profiles [59]. mRNA/gene expression-based assays have the potential to overcome some limitations of IHC and FISH, such as inter-observer variability and tissue heterogeneity [60], hence these assays may have the potential to improve the assessment of HER2 status in BC patients. By offering quantitative and comprehensive measurements of HER2 expression, one could argue that these techniques could aid in complementing the treatment selection and improving patient outcomes, although specific studies applied to clinically relevant cohorts have yet to be performed. The critical point of this quite vast literature is that it does not consistently show a sufficient level of agreement between molecular and tissue-based tests in defining HER2 status when evaluated according to ASCO/CAP criteria. Consequently, the use of molecular tests in place of conventional in situ methods is still not recommended [61]. Of note, it has been previously demonstrated that *HER2* mRNA levels [10,11,62] and *HER2* copy number [11] show proportionally increased values across HER2 IHC scores. Nevertheless, whether these features may have an impact on response to specific therapies is yet to be demonstrated. If on one hand there are data supporting the association between *HER2* mRNA levels and higher levels of response to standard anti-HER2 blockade [63-66], on the other data on HER2-low and HER2-ultralow carcinomas are missing. In an exploratory analysis performed in the phase 2 DAISY trial, no efficacy difference based on *HER2* gene expression within the group of patients with HER2 IHC score 0 carcinomas was observed, further suggesting potential drug activity in patients with very low, if any, HER2 expression [2].

HER2 assessment on circulating tumor cells

Circulating tumor cells (CTCs) comprise a heterogeneous group of a cancer-derived cell population relapsed from a primary or metastatic tumor into the bloodstream [67-69]. Detecting and analyzing CTCs has significant clinical implications in different types of solid tumors, including breast cancer. So far, several groups within the International Society of Liquid Biopsy (ISLB) have proposed clinical applications for circulating tumor cells (CTCs), specifically emphasizing the enumeration of CTCs in breast cancer patients [70]. To date, CellSearch® (Menarini, Italy) is considered the gold standard approach to isolate and count CTCs from peripheral blood. The CTC enumeration procedure is carried out by categorizing BC patients based on 5 cells as positive cutoff values [71]. On the other hand, a more complex and technically valuable CTC isolating strategy includes antibody-based platforms, which can combine physical parameters (size) and immune-specific selection (antibody) for the identification of antigens on the surface of CTCs. The characterization of surface antigens on CTCs enables the comprehensive three-dimensional analysis of biological components shed in the bloodstream by tumor tissue [72]. This approach expands the landscape of predictive biomarkers beyond DNA and RNA, encompassing specific proteins carried by CTCs. HER2 expression in CTCs may represent a key weapon for the stratification of patients according to HER2 expression level. Interestingly, shorter OS [9.7 (7.1-12.3)] in strong HER2 staining breast cancer patients exhibiting ≥ 1 CTC has been observed compared to patients with negative-to-moderate HER2 staining breast cancer patients without any signal for CTC detection [16.5 (14.9-18.1) months, P = 0.013] [73]. In particular, this dynamic stratification of HER2 expression fills a gap in the understanding of positivity levels within the population and complements the static tissue assessment. This precision in gradation is achieved by positively identifying the antigen contact, verifying the nucleated and epithelial nature of the component using contrast with DAPI and cytokeratins, respectively. Evaluation of HER2 expression on CTCs aims to complement tissue assessment by providing a dynamic perspective in addition to the static information obtained from tissue analysis. With these principles in mind, it becomes evident that HER2 expression levels on CTCs or HER2 mutations in patients with HER2-low/unamplified breast cancer patients could potentially serve as a valuable source of information [74]. However, it is important to note that, as of now, there is a lack of substantial data in this regard.

Conclusions and future perspectives

The emergence of HER2-low as an umbrella term for a subset of patients with breast cancer that can be responsive to T-DXd has shaken the pathology community to re-think the way biomarkers can be used. Although data explaining more in-depth predictors of response/resistance in patients treated with ADCs are eagerly awaited, at present the *escamotage* of identification of low levels of HER2 expression in tissue samples represents a novel approach for the selection of candidate patients.

Hence, pathologists are now faced with the task of identifying the subtle nuances of HER2 expression dynamics across a wider range, which was previously considered clinically insignificant. To meet this challenge, rigorous quality control and clear assessment guidelines are essential. Although the techniques behind this testing (IHC/ISH) are well established and largely available at pathology laboratories worldwide, education is needed across laboratories because of the historical set-up of the assay. Novel HER2 molecular testing methods, machine learning technologies, and CTC show promise in addressing methodological and biological variations.

By merging pathology and oncology standpoints we were able to define five pillars in the assessment of HER2-low disease and possible response to ADC:

1 the concept of low levels of HER2 expression in breast cancer encompasses a possible diagnostic strategy for advanced-stage tumors, rather than a biological subgroup of patients;
2 if feasible, it is important to re-assess advanced disease, to confirm the diagnosis and biological profile of the metastatic tumor, although, primary tumor samples can be a source of the HER2 testing or (re)evaluation;
3 it is recommended to provide exact scoring categories in the pathology report (essential parameter), possibly including the percentage of cells displaying HER2 expression even at low levels (desirable parameter); the use of the term "HER2-low" in pathology reports is discouraged;
4 pathologists are the curators of preanalytical features, analytical assays, and interpretation;
5 the lowest limit of clinically relevant HER2 levels is yet to be elucidated.

As a final remark, we need to be prepared to embrace further developments, either stemming from simple IHC scores (ultra-low HER2 levels), AI-based solutions, or other techniques. Further studies may add valuable information to this scenario, especially for the identification of mechanisms of resistance or poor response to therapy.

Acknowledgements

The Authors extend their sincere gratitude to Medica Editoria s.r.l. for their invaluable support. This work received partial funding from the Italian Ministry of Health, specifically with "Ricerca Corrente" and "5 × 1000" initiatives. K. V. acknowledges support from the Fondazione IEO-MONZINO, while CM's research is backed by the FONDAZIONE AIRC (IG 2019 - ID. 22850 project), led by P.I. Caterina Marchiò. The final proofreading process for the grammar and syntax of the manuscript was performed using ChatGPT v.3.5 and Grammarly v.6.8.263.

Conflicts of interest

CM reports personal fees from Bayer, Roche, Daiichi Sankyo, AstraZeneca, Illumina, and Veracyte. CC reports personal fees for consulting, advisory role, and speakers' bureau from Lilly, Roche, Novartis, MSD, Seagen, Gilead, Daiichi Sankyo, AstraZeneca, and Pfizer. CS reports personal fees for consulting, advisory role, and speakers' bureau from Astra Zeneca, Bristol Myers Squibb, Daiichi-sankyo, Gilead, Novartis, and Veracyte. AS Roche-Ventana, Novartis, Astrazeneca, Amgen, Gilead PG Consulting/Honoraria from Eli-Lilly, Pfizer, Novartis, AstraZeneca, Boehringer-Ingelheim, Roche, MSD, Amgen. UM has received personal fees (as consultant and/or speaker bureau) from Boehringer Ingelheim, Roche, MSD, Amgen, Thermo Fisher Scientific, Eli Lilly, Diaceutics, GSK, Merck and AstraZeneca, Janssen, Diatech, Novartis and Hedera unrelated to the current work. GNF reports personal fees for advisory role from Astra Zeneca. CDA reports advisory role for Roche, Lilly, Novartis, AstraZeneca, Pfizer, Seagen, Daicii-Sankyo, Gilead, and GSK and speaker honoraria from Roche, Lilly, Novartis, Pfizer, Seagen, GSK, GILEAD, and Daiichi-Sankyo. Travel Grants from Gilead and research support (to the Institution) from Novartis, GILEAD, and Daiichi-Sankyo outside the submitted work. GP reports research funding (to Institution) from AstraZeneca, Exact Sciences, Diapath; personal honoraria as invited speaker from Amgen, AstraZeneca, Bio-Optica, Boehringer-Ingelheim; Diatech Pharmacogenetics, Exact Sciences, GlaxoSmithKline, Incyte, Janssen-Cilag; Lilly, Novartis, Roche, Merck Serono, Veracyte; participation in advisory board for Amgen, Astrazeneca, Novartis, Exact Sciences, Roche. GC reports funding from AstraZeneca, Daiichi Sankyo, and Merck; consulting fees from BMS, Roche, Pfizer, Novartis, Lilly, Astra Zeneca, Daiichi Sankyo, Merck, Seagen, and Ellipsis; honoraria from Pfizer, Lilly; support for attending meetings from Roche, Pfizer. NF has received honoraria for consulting, advisory role, speaker bureau, travel, and/or research grants from Merck Sharp & Dohme

(MSD), Merck, Novartis, AstraZeneca, Roche, Menarini, Daiichi Sankyo, GlaxoSmithKline (GSK), Gilead, Adicet Bio, Sermonix, Reply, Veracyte Inc., Leica Biosystems, Lilly. These companies had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and/or in the decision to publish the results. All other authors declare no potential conflicts of interest.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Authors' contributions

The preparation of this consensus article was a collaborative effort, with each author contributing their expertise in various aspects of the subject matter. The contributions of each author are outlined as follows. Conceptualization and Consensus Meeting Coordination: NF; Methodology: CM and NF; Data Collection, Literature Analysis, and Interpretation: All Authors. Writing and First Draft Preparation: GC and KV Review and Editing: All Authors. Supervision and Project Administration: CM and NF Iconography: CM, GC, KV, and NF.

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