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Tissue Selection for PD-L1 Testing in Triple Negative Breast Cancer (TNBC)

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Abstract: Atezolizumab in combination with nab-paclitaxel has been introduced for the treatment of locally advanced or recurrent triple negative breast cancer (TNBC). Patient selection relies on the use of immunohistochemistry using a specific monoclonal PD-L1 antibody (clone SP142) in a tightly controlled companion diagnostic test (CDx) with a defined interpretative algorithm. Currently there are no standardized recommendations for selecting the optimal tissue to be tested and there is limited data to support decision making, raising the possibility that tissue selection may bias test results. We compared PD-L1 SP142 assessment in a collection of 73 TNBC cases with matched core biopsies and excision samples. There was good correlation between PD-L1-positive core biopsy and subsequent excision, but we found considerable discrepancy between PD-L1 negative core biopsy and matched excision, with a third of cases found negative on core biopsies converting to positive upon examination of the excision tissue. In view of these findings, we developed a workflow for the clinical testing of TNBC for PD-L1 and implemented it in a central referral laboratory. We present audit data from the clinical PD-L1 testing relating to 2 years of activities, indicating that implementation of this workflow results in positivity rates in our population of TNBC similar to those of IMpassion130 clinical trial. We also developed an online atlas with a precise numerical annotation to aid pathologists in the interpretation of PD-L1 scoring in TNBC.

Key Words: triple negative breast cancer (TNBC), PD-L1 (SP142) immunohistochemistry, atezolizumab companion diagnostic, clinical workflow, immune oncology

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Programmed cell death ligand-1 (PD-L1, also known as CD274 and B7-H1), is a 40 kD transmembrane glycoprotein that plays a role in acquired cell-mediated immunity and is part of a family of T-cell regulatory molecules known as immune checkpoints.[1] Immune checkpoints are a complex network of membrane-based molecules that require cell-to-cell proximity to convey inhibitory or stimulating signals. PD-L1 is expressed on a variety of immune cells, parenchymal cells and tumor cells.[1] Programmed cell death protein-1 (PD-1 or CD 279), is a cell surface receptor with inhibitory function that belongs to the family of immune checkpoints and is present on subpopulations of T- and B-cells.[2] It has 2 cognate ligands: PD-L1 and PD-L2 (also known as CD273 and B7-DC). PD-L2 is also a transmembrane protein that competes with PD-L1 for PD-1 with higher affinity but lower expression levels than PD-L1. It is also the ligand for another exclusive receptor, RGM-b (Repulsive Guidance Molecule-b), also known as DRAGON or DRG-11, a molecule involved in bone morphogenesis and modulation of the immune response.[3,4] Immune checkpoint interactions are complex and the crosstalk can result in different outcomes. For instance, the binding

between PD-L1 (and PD-L2) and PD-1 occurs on the surface of 2 neighboring cells but PD-L1 can also bind CD80 (B7-1) on the surface of the same cell. CD80 is another transmembrane protein that functions as a ligand for CTLA-4 and B28, which are 2 more receptors of the immune checkpoint system. PD-L1-CD80 dimerization prevents PD-L1 interaction with PD-1 while CD80 retains its ability to bind CTLA-4 or CD28.[3]

In the last decade there has been renewed interest in immune oncology (IO), thanks to a number of biological compounds that modulate the immune checkpoint inhibition as a means to boost the host immune response against cancer. Clinical trials have demonstrated superiority of several of these treatment options for a number of cancer subtypes. These are monoclonal antibody-based drugs that recognize either a ligand or a receptor of the immune checkpoint system. The use of checkpoint-targeted therapy was initially successful in the treatment of melanoma, then in non-small cell lung cancer (NSCLC), followed by a large number of other tumor types.[5] In 2018, atezolizumab (an anti-PD-L1 drug) in combination with nab-paclitaxel was approved for the first-line treatment of locally advanced or metastatic triple negative breast cancer (TNBC) in both the US and Europe.[6,7] Clinical trials indicated that this treatment almost doubled the overall survival (OS) at 2 years (42% vs. 26%) and the progression-free survival (PFS) at 1 year (22% vs. 11%) in a subpopulation of TNBC patients with expression of PD-L1 on immune cells in the tumor tissue.[6,8] As patients who have PD-L1 negative tumors do not benefit from this treatment, a companion diagnostic test (CDx) is necessary for patient selection to ensure safe and effective use of the corresponding therapeutic drug.

The CDx for atezolizumab in TNBC is based on the immunohistochemical (IHC) staining of formalin-fixed and paraffin-embedded (FFPE) TNBC tissue sections. It uses an anti-PD-L1 antibody (clone SP142), a specific set of reagents and a locked-down staining protocol. SP142 recognizes an epitope that is present on the intracellular portion of the PD-L1 molecule.[9] In numerous tumor types, it stains fewer tumor cells compared with other anti-PD-L1 antibodies.[10,11] Conversely, it stains immune cells (IC) more intensely.[12,13] The interpretation of SP142 IHC uses a prescribed algorithm which can be found in the publicly-available interpretation guide published by the manufacturer and the original paper.[7] Briefly, the stained tumor tissue is assessed for the presence of PD-L1-expressing IC that are associated with invasive carcinoma. The percentage of the tumor tissue area covered by PD-L1-expressing IC is then estimated with a threshold for positivity set at 1%. IC associated with normal breast tissue, in situ carcinoma or intravascular carcinoma are not included in the scoring. The IC population includes all types of immune cells, including granulocytes and plasma cells. Tumor cells staining for PD-L1 are not scored.

There are 2 major elements that can cause concern when it comes to quantitative predictive biomarkers with defined thresholds for a negative or positive clinical status and eligibility for treatment. The first is preanalytics; recent recommendations have been published to improve the quality of on-slide oncology biomarker testing [14] and these are fully aligned with ASCO-CAP guidelines.[15,16] Resection samples are more susceptible to poor preanalytics due predominantly to unpredictable cold ischemia times. In fact, several studies have shown the superiority of core biopsy over excision specimens for breast cancer biomarkers ER & PR.[17] The second element is sampling in the context of heterogeneity of PD-L1 staining. Due to the nature of the interpretive algorithm, the staining in samples with low PD-L1 expression is likely to be distributed heterogeneously, especially if organized in focal aggregates rather than diffuse single cell infiltrates. On one hand therefore, it would seem better to choose core biopsies because of better preanalytics. Conversely, excision biopsies may overcome sampling problems.

To address these two elements, we set up a study to provide guidance on the selection of the best sample type to use for PD-L1 status in TNBC. The study compares PD-L1 expression between a small tissue sample (the initial core biopsy) and the corresponding whole tumor tissue (the excision biopsy). This initial work enabled us to set up a workflow for determination of PD-L1 status in TNBC and develop an atlas to aid interpretation. Thereafter, we conducted an audit of the clinical service in one of the participating laboratories to validate the guidance and assess its impact.

MATERIALS AND METHODS

Case selection: this study falls under the banner of performance assessment/quality assurance and, as such, did not require approval by a UK Research Ethics Committee.

A retrospective selection of FFPE tissue blocks from 73 TNBC cases with matched diagnostic core biopsies and excision biopsies were collected from the participating hospitals. Inclusion criteria were TNBC diagnosis on pathology records and availability of FFPE blocks from both core biopsy and excision. All cases included in this study were treated by primary surgery (no neoadjuvant chemotherapy) and therefore the time elapsed from core biopsy to excision was minimal. For each case, we stained the core biopsy and all the available tumor tissue from the excision biopsy (up to a maximum of 5 blocks) with H&E and PD-L1, using the SP142 clone on-label protocol.

Immunohistochemistry: Paraffin sections were cut at 3 to 5 μm and mounted on TOMO slides (TOM-11, Matsunami Glass Ind Ltd., Japan) together with the appropriate on-slide tissue controls and stained with anti-PD-L1 (SP142) rabbit monoclonal primary antibody (Roche Ventana) on a BenchMark Ultra platform (Roche Ventana) using the validated locked-down protocol. The staining protocol for the SP142 CDx was available in the package insert. Briefly, antigen retrieval was undertaken for 48 minutes, the primary antibody was applied for 16 minutes, amplification was done for 8 minutes/8 minutes multimer, and sections were counterstained for 4 minutes with haematoxylin II and post-counterstained

with bluing reagent for 4 minutes. All sections were stained within a week from being cut. All slides were mounted with glass coverslips.

Slide assessment: slides were digitized using Panoramic 250 Flash-3 scanners (3D-Histech) using x20 lens. Digital slides were stored on a CaseCenter v2.9 (3D-Histech) and viewed using CaseViewer v2.2.1 (3D-Histech). Glass slides were viewed with transmitted light microscopes (Olympus BX series) fitted with planapo lenses and multi-head facility. All pathologists (FD, AB, AC, KB, M-PC, RV, ML-T, EA, MC, PT and CD) had been trained in PD-L1 assessment for TNBC according to the SP142 algorithm. Pathologists scored either glass slides, digital slides or both. Three PD-L1 scores were given as described in Figure 1: (A) one score for each individual slide, (B) a cumulative score of each individual excision sample by averaging the PD-L1 expression over the entire tumor area present in all slides from the excision, and (C) a cumulative score of each individual patient by averaging the PD-L1 expression over the entire tumor area present in all slides from both core biopsy and excision. All cases showing discrepancy between pathologists were re-assessed by two pathologists (P.T. and C.D.) and a consensus diagnosis reached using a multi-head microscope. A slide, case or patient status was considered positive if the PD-L1 score was $\geq 1\%$.

Approach to scoring and Atlas: we realize that pathologists use different approaches for assessing PD-L1, especially in complex cases with heterogeneous staining. An example of a systematic approach to scoring is described in Figure 2.

In the course of the work, we realized that it would be useful to have a reference atlas, demonstrating different PD-L1 staining patterns to help achieve better concordance. This atlas was prepared by taking microphotographs from digital slides all taken at the same magnification (digital ×20) from the cases used in this study. Each image was overlaid with a grid of 100 equal tiles (each tile equaling 1% of the total area). The microphotographs were then scored by 3 pathologists independently for PD-L1 IC expression (% of area occupied by PD-L1 positive IC in relation to the total area) according to the interpretation guide provided by the manufacturer and described in the original paper.[7]

Once the individual scores were collected, we realized that there was discrepancy in the assessment of some images. We therefore decided to employ the encircling technique, as described by Vennapusa et al (7, Supplemental Digital Content 3). To do this, we remeasured PD-L1 scores using a manual geometrical scoring approach: each image was reviewed and re-scored in consensus by 2 pathologists. Briefly, the positive immune cells (singly or in clusters) on the digital slides were each encircled manually with an overlaid smooth perimeter; the individual perimeters were then manually clustered together and a PD-L1 score was obtained by adding the number of tiles occupied by the clustered perimeters (see Fig. 3).

Figure 4 is an extract from the atlas, the full collection is available as an online atlas (see Supplemental Digital Content 1 & 2, http://links.lww.com/AIMM/A357 & http://links.lww.com/AIMM/A358, for images representing the

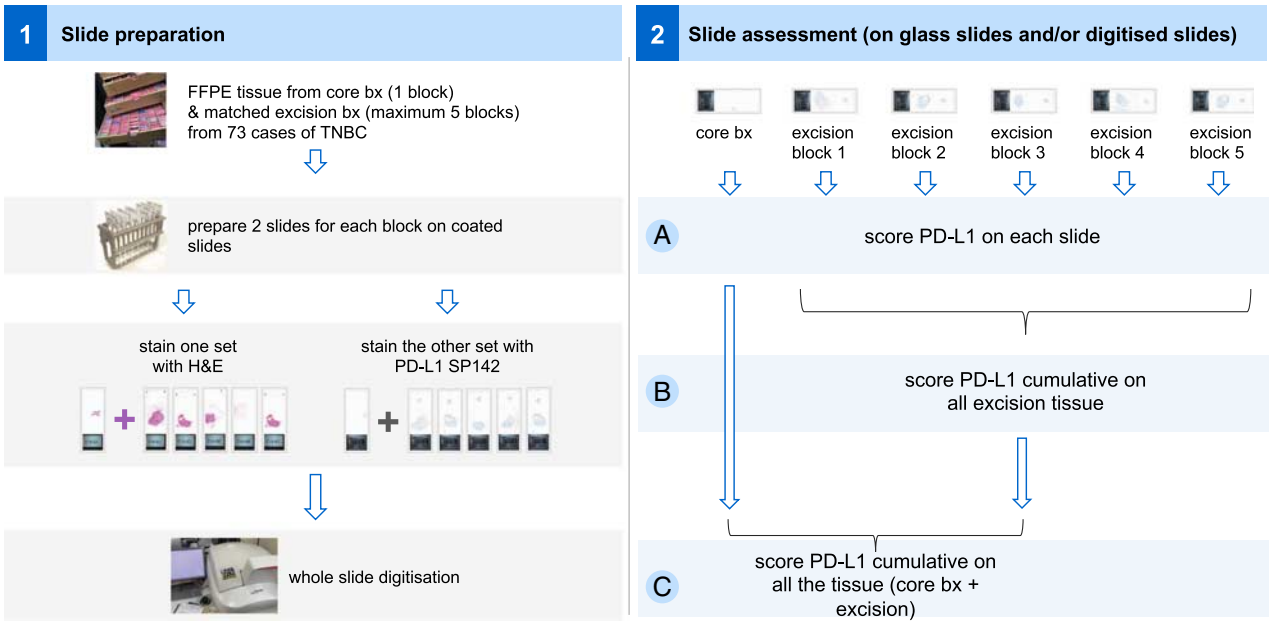

FIGURE 1. Overview of the slide preparation and assessment. (1). For each case, H&E and PD-L1 stained slides were produced from the blocks representing the core biopsy and a maximum of five blocks from the corresponding excision. All slides were digitized. (2). The slides were assessed as follows: (A) Individual scores per slide were first recorded. (B) A cumulative score for the excision tissue was then obtained by considering the total PD-L1 expression over the entire tumor area present in all slides. (C) Lastly, for each case, the cumulative score was reassessed including all available blocks (core biopsy and excision specimen).

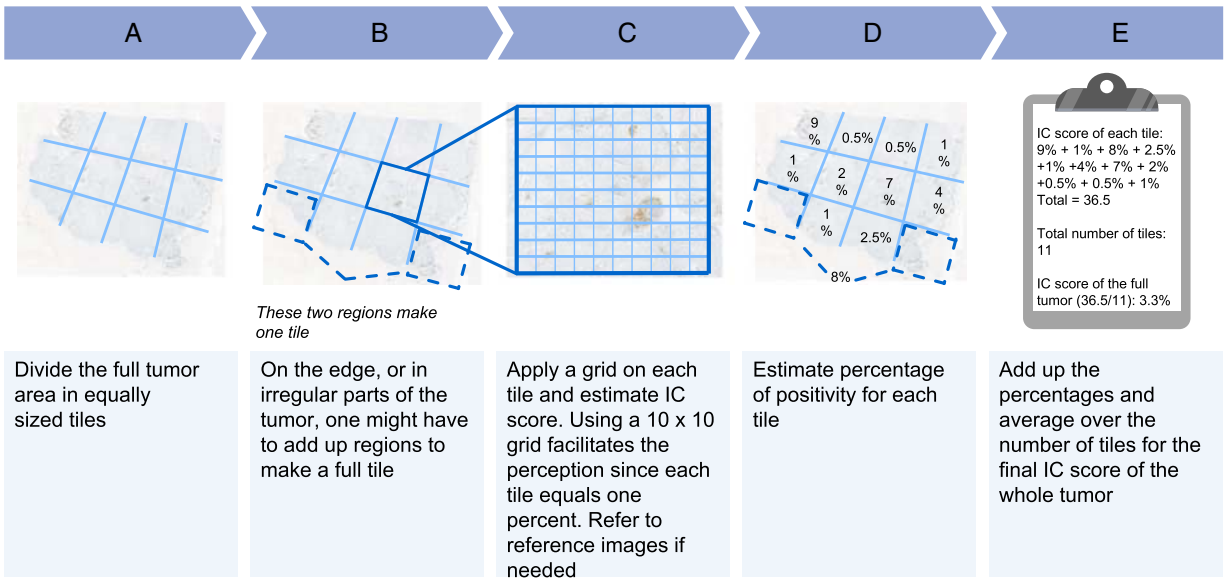

FIGURE 2. Systematic scoring method. Our proposed systematic approach to assess a large tumor area with heterogeneous distribution of PD-L1 stained immune cells.

full range of PD-L1 scoring). Our hope is that now this atlas can support diagnostic pathologists to find solutions to their interpretation problems in daily practice.

Clinical audit: the Poundbury laboratory provided centralized testing for PD-L1 in TNBC as part of the UK EAMS (Early Access Medicines Scheme) from 2018 to 2021. It was also a referral laboratory for all patients with TNBC from Ireland from 2019 to 2022. From the onset, all cases that were negative for PD-L1 and had relatively small amounts of tumor tissue in the examined blocks were offered repeat testing of additional tissue. Following retesting of the requested tissue, a final PD-L1 diagnosis was issued based on an average score for PD-L1 on the total area of tumor tissue tested (first test plus second test). The following details were recorded for each patient: the type of tissue selected for testing, the PD-L1 status, whether additional tissue was requested for testing, whether the additional tissue was sent, the PD-L1 status of the additional tissue and the final PD-L1 clinical status. This work was audited to determine the proportion of cases identified as requiring retesting and whether retesting changed the final PD-L1 diagnosis.

RESULTS

PD-L1 status in core biopsies and resections: All 73 cases entered in this study were successfully assessed for PD-L1 status on both core biopsy and resection material. Of the initial core biopsies, 22 (30%) were PD-L1 positive and 51 (70%) negative. However, 16 of the negative cases converted to final positive PD-L1 status upon assessment of the matching resection tissue. This represents 22% of the total number of patients and almost a third of the PD-L1 negative cases on core biopsies. When both excision and biopsy samples were considered together, 38 patients (52%) had a positive PD-L1 status and 35 patients (48%) remained PD-L1 negative. None of the initial positive scores on core biopsies was converted to negative status upon assessment of the excision.

Clinical audit: In the period audited (2019-2021), the laboratory received a total of 1458 original requests relative to 1395 patients (some patients had more than one biopsy tested at different times). The audit data is summarized in Table 1A. In the initial testing, 490 samples (33.6%) were positive, but after retesting on additional tissues from the patients with initial negative status, a total of 558 (38.9%) were found positive. Of the initial requests, only 22 (1.5%) were deemed insufficient for analysis. Of the 946 negative cases, 392 were considered relatively small for reliable testing and retesting was requested, however only 288 of these had sufficient tissue material available. Table 1B extrapolates these figures to provide a theoretical positivity rate if all negative tissue were available for retesting.

DISCUSSION

With the approval of therapy targeting checkpoint inhibitors for the treatment of TNBC, pathologists are being asked to assess tumor tissue for PD-L1 status to guide patient selection. A number of questions have been raised in relation to the choice of tissue for testing, but very limited guidance exists. The most common questions are: is it preferable to use tissue from primary tumor or metastatic or local recurrence lesions? What tissue is the best predictor of patient response to anti-PD-L1 treatment – that taken before or after chemotherapy and/or radiotherapy? Is there a preference for using resection samples over core biopsies or vice versa?

Studies of other cancer types and TNBC [16] have highlighted differences in PD-L1 status between primary and metastasis, and there are some indications that particular metastatic sites, such as liver, may be

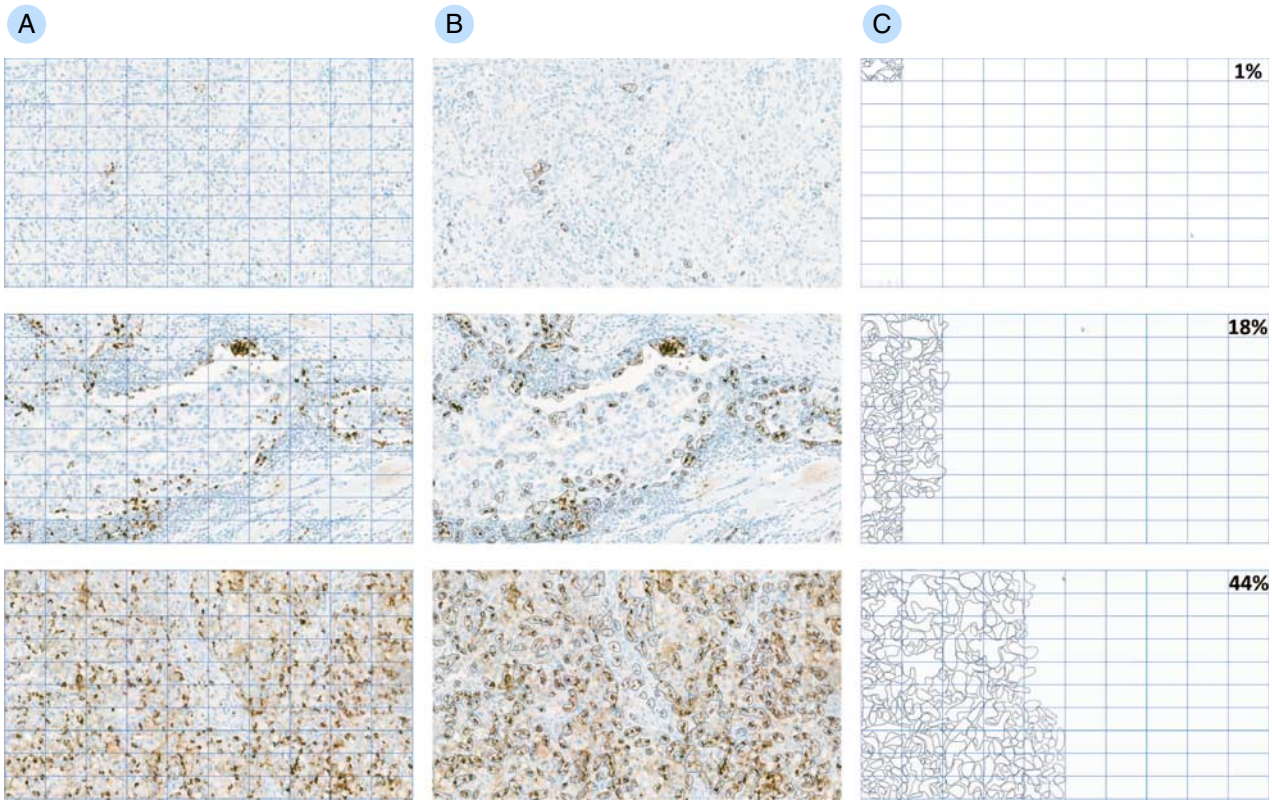

FIGURE 3. Grid and encircling method. Illustrative examples of the application of the grid and encircling technique used to determine the precise IC scores in selected areas from digitized PD-L1 (SP142) stained slides (all at the same magnification). This method was applied to all images provided in the atlas (see Fig. 4 and images within Supplemental Digital Content 1 & 2, http://links.lww.com/AIMM/A357 & http://links.lww.com/AIMM/A358). These are low resolution images to illustrate the encircling method, but the encircling of PD-L1 stained immune cells was performed on high resolution images. (A) A 10×10 grid was placed over the selected area in the IHC PD-L1 (SP142) digitized image. (B) Manual encircling of the immune cell area expressing PD-L1 was performed (the grid is removed in this figure, to better visualize the annotations). Stained tumor cells, if present, were not included, as per the scoring recommendation. (C) Finally, the slide image was removed and the encircling annotations were aggregated tightly to fill tiles in the grid, to determine the precise total area occupied by PD-L1 IC staining.

immunologically "protected". The clinical implications are not yet well understood. Although testing of an archival biopsy from the primary carcinoma at first clinical presentation may not be representative of the current PD-L1 status in the patient, the IMpassion130 study [9] used any tissue types available (except for cytology samples and bone biopsies, due to lack of stroma and use of decalcification respectively). Apart from a lower incidence of PD-L1 positivity in tissue of metastatic TNBC compared to primary, no trial data suggest that particular tissue sample types may be less effective at identifying responders.[18] At present, therefore, there is no recommendation to favor any tissue sample type over another, but there is a need to clarify this.

There are also studies reporting that PD-L1 expression is likely to change after (neo)-adjuvant therapy.[19] It remains unclear, however, if these changes are also reflected in the clinical response to anti-PD-L1 treatment. In the IMpassion130 study, patients were allowed to have received previous radiation and chemotherapy in a curative context, if treatment was completed more than 12 months before randomization. Still, the question remains to be further investigated and guidelines are needed.

In immune-excluded tumors, immune cells positive for PD-L1 accumulate along the invasive margin.[20] This has prompted the concern that tissue samples that do not contain the tumor margin may result in underestimation of PD-L1. In addition, we have observed that in a significant proportion of all clinical samples the distribution of the immune infiltrate is heterogeneous. Thus, sampling and size of biopsy might introduce bias in PD-L1 assessment.

In this study we aimed to address whether the size of the tissue tested had an influence on PD-L1 status. To do so, we compared core biopsies with excision samples. We did not attempt to measure the tumor area in each sample, but assumed that core biopsies in general are smaller than excision samples. Indirectly, our aim was also to look for indications of the impact of non-controlled preanalytical differences between biopsies and surgical specimens in daily practice.

We already know that the PD-L1 SP142 CDx is sensitive to variations in preanalytics, including cold ischemia time, prolonged or insufficient fixation and suboptimal processing.[21] There is more abundant tissue from excision

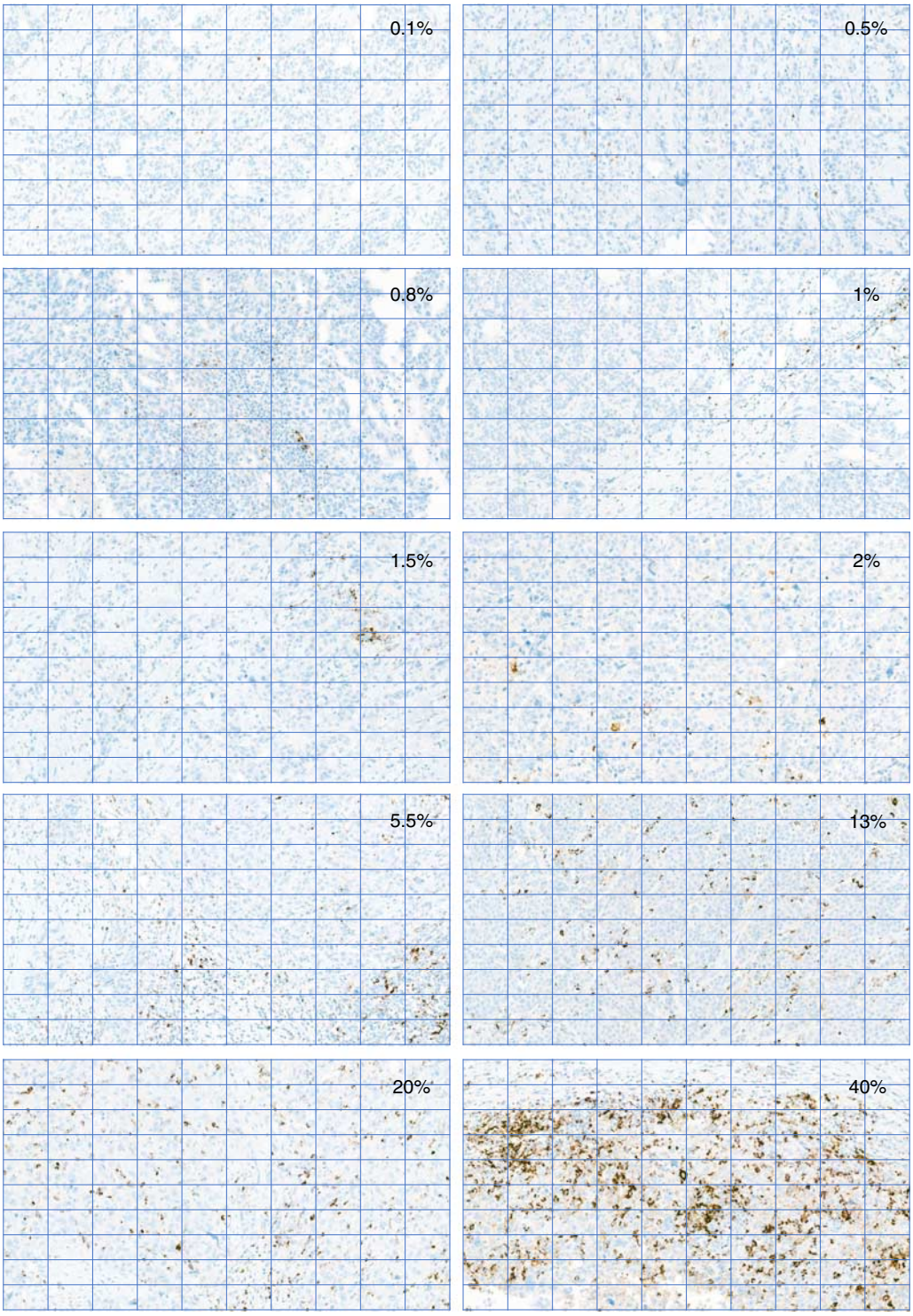

FIGURE 4. Example from online atlas. Extract from the atlas representing a range of PD-L1 IC scores obtained by the grid and encircling method described in Figure 3. The full collection is available as an online atlas (see Supplemental Digital Content 1 & 2 for high and low resolution versions, http://links.lww.com/AIMM/A357 & http://links.lww.com/AIMM/A358).

samples (either mastectomy or wide local excision) and, in general, they include the invasive margin, but they are more prone to preanalytical variations, while core biopsy tissue is usually small but has better preanalytics.

Rather than choosing random tissue samples from the excision, we elected to investigate a set of cases with matched core biopsy and excision, as these would resemble more closely the clinical settings, particularly for

TABLE 1. (a) Audit of Central Service PD-L1 SP142 Testing in TNBC for UK and Ireland. (b) Estimation of Theoretical Positivity Rate

(a)

Number of individual cases	1458
PD-L1 positive on 1st test	490 (33.6%)
Negative/NA on 1st test	968 (66.4%)
Positive cases on retesting	68 (4.7%)
Total positivity rate including retesting	558 (38.3%)

Retesting for cases negative/NA for PD-L1 on 1st test	
Cases requested for retesting	392
Cases received for retesting	288
Positive on retesting	68 (23.6% of received)

(b)

Negative cases on 1st testing not requested or not available for retesting	680
Theoretical number of positive cases in total nonretested population (assuming 0-23.6% positivity rate)	0-160
Theoretical number of total positive cases if all negative cases could be retested	558-718 (38.3%-49.2%)

(a) Summary of PD-L1 positivity rate in the central service PD-L1 SP142 testing in TNBC for UK and Ireland during 2019 to 2021. The initial positivity rate was 33.6%. When retesting was applied the total positivity rate rose to 38.3%.

(b) As not all initially negative cases had tissue available for retesting, a theoretical maximum of positive cases was estimated based on the audit finding of 23.6% positive rate in the retested cases. The theoretical maximum positivity rate would then be as high as 49.2%.

what is affected by bias related to anatomic access during the core biopsy procedure.

Our data do not support the idea that core biopsies should be preferred due to preanalytical reasons. However, it might be necessary to opt for biopsies for reasons of patient management workflow. We could not find any negative resection sample that had a positive antecedent biopsy in our study.

The Impassion130 positivity rate was 40.9% based on a cohort of 902 patients, with a mix of available FFPE tumor tissue (primary and metastatic, biopsy and resection). In our series, only 30% of the cohort had a positive PD-L1 score when core biopsies alone were evaluated. This is in contrast to 52% when resection samples were assessed. We do not know if the 22% of patients who "converted" from negative to positive PD-L1 status would have clinical benefit from anti-PD-L1 treatment. The published trial data did not provide any precise information regarding tissue selection and indicated that any available tissue can be used for testing. The failure to consider tissue selection is common in clinical trials but it is a limitation, and a better understanding of the impact of tissue selection is crucial for effective implementation into clinical routine.

Our results indicate that tissue selection should be considered as an important parameter to be assessed in clinical trials, especially for heterogeneous biomarkers, as has been indicated in other studies.[22] The primary objective of the pathologist, however, is to identify all patients eligible for treatment, so with the information we have at hand, we propose the implementation of a workflow which follows up patients with a negative PD-L1 status on core biopsies with a repeat test on the subsequent resection.

Our data (including our theoretical extrapolation from the audit data in Table 1B) indicate that this workflow will result in the capture of more patients eligible for treatment, and more closely represent the numbers seen in the initial clinical study. To date, we have no reason to believe that this approach would include patients unlikely to benefit from the treatment, but future studies are required to narrow down the best algorithm for identifying the right patients and ruling out the nonresponders.

Ever since the introduction of PD-L1 scoring in TNBC, pathologists have had a need for a visual guide to aid their daily practice. A limited number of examples are provided as a part of the interpretation guide, but they are without precise numerical scores. Our work resulted in a large number of digital cases, from which it was easy to produce an atlas providing examples of different amounts of IC staining with a precise numerical annotation. We decided to use the manual encircling technique to validate the pathologist consensus score since we would like our atlas to provide a precise, definitive and practical aid to support PD-L1 scoring in TNBC.

In conclusion, our study demonstrates that small tissue samples with PD-L1 status below the 1% threshold should be re-assessed using additional tissue (resection samples or additional biopsy tissue). Although we are still uncertain of the real-world prevalence of PD-L1-positive

TNBC, and await larger trials, we have obtained positivity rates closer to the published rates by repeat testing in our clinical practice, and we have developed an atlas to aid other pathologists.

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