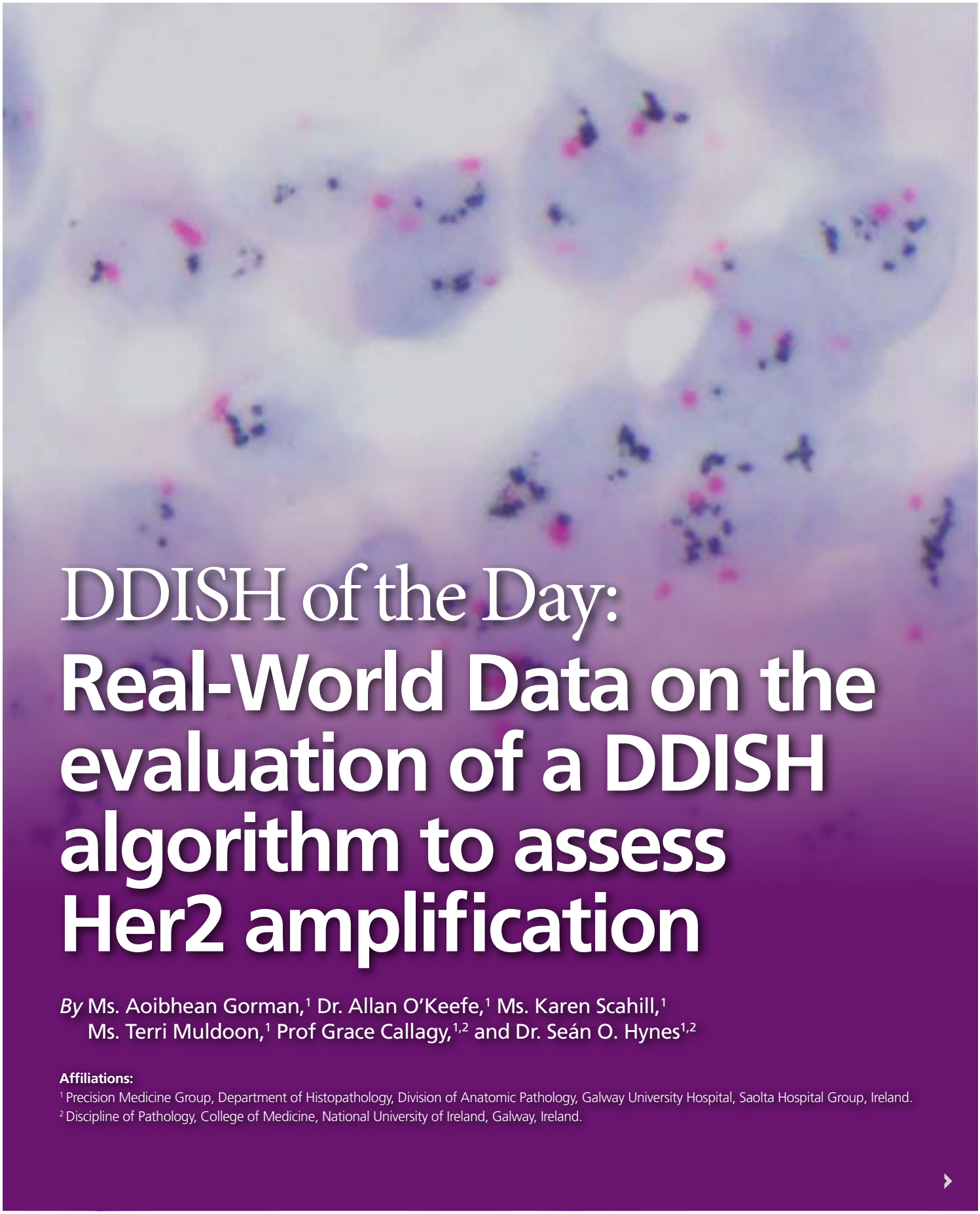

DDISH of the Day: Real-World Data on the evaluation of a DDISH algorithm to assess Her2 amplification

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HER2: Where Precision Medicine Began

In 1992, fifteen women became pioneers of the era of precision medicine as they embarked on their treatment pathway for breast cancer. They had enrolled in a trial to test trastuzumab (a novel humanised monoclonal antibody) for its ability to block growth signals to breast cancer cells that over-expressed human epidermal growth factor receptor 2 (HER2). The bravery and efforts of these patients, as well as the foresight of the researchers and doctors from this era of medicine, resulted in the transformation of an aggressive subtype of breast cancer with a poor prognosis to one which could respond to a specific therapy.[1] Perhaps as important was the demonstration of the proof of principle of the first precision drug to fight cancer.

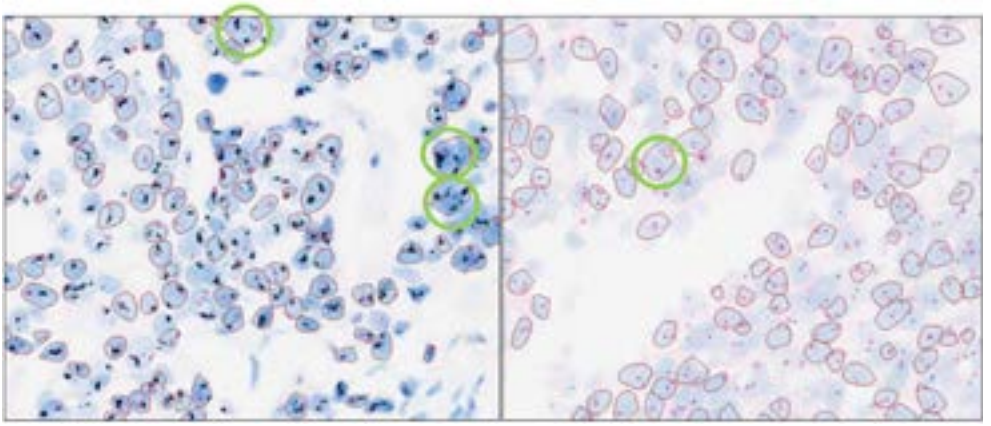
The over-expression of the HER2 protein, via amplification of the *HER2* gene, results in an alteration that enables the cancer cell to outcompete its non-amplified normal tissue neighbours for resources and result in uncontrolled growth. The process represents Darwinian selection at the cellular level; antibody therapy that targets the receptor introduces a selective pressure against these cells. Fortunately, the *HER2* oncogene is a trunk mutation that is also a driver alteration, i.e., one that the cancer cell becomes addicted to and limits its escape pathways. Unfortunately, heterogeneity can be an issue, causing other sub-clones to come to the fore once their HER2 over-expressing "kin" have been eliminated.[2]

HER2 And Other Cancers

HER2 is a receptor that is expressed by many different epithelial cell types and, as such, this makes HER2 attractive for targeting therapies to cancer cells in other sites. HER2 amplification and mutations have been described in many different carcinomas including gastric, colorectal, non-small cell lung cancer and many others.[3] Thanks to the work in the ToGA trial, the use of anti-HER2 based therapies has also been shown to be useful and effective in gastric cancer.[4] Many different reports also show the promise of this approach in colorectal cancer although not yet at the level of any first line therapies.[5]

Primum Non Nocere

Given the potential advantages in eliminating (at least) a clone of cancer cells – be they breast, gastric or other cancer – one could ask why we cannot give all cancer patients a trial of anti-HER2 therapy on diagnosis (assuming the approach is affordable). One of the most oft-quoted parts of the Hippocratic oath is "primum non nocere" ("first do no harm") and although humanised monoclonal antibody anti-HER2 therapies are well tolerated overall, the toxicities of anti-HER2 therapies are not inconsequential. These toxicities include increased risk of infection, anaemia, thrombocytopenia, pain, muscle spasms along with the much less common cardiotoxicity and pulmonary fibrosis.[6] Therefore, it is vitally important that only those patients who have the best chance of response for their cancer be exposed to the risks associated with this particular therapy – the very raison d'etre for precision medicine.



Amplified Nuclei Detected and Outlined Aberrant Conglomerate Cells Highlighted

Non-amplified Nuclei Detected and Outlined Aberrant Conglomerate Cells Highlighted

Figure 1

"Given the potential advantages in eliminating (at least) a clone of cancer cells – be they breast, gastric or other cancer – one could ask why we cannot give all cancer patients a trial of anti-HER2 therapy on diagnosis (assuming the approach is affordable)."

Current testing regimens to identify these patients may need to employ a two-step approach: an immunohistochemistry assay and, if needed, *in situ* hybridisation to check for amplification of the *HER2* gene. A colorimetric immunohistochemistry assay with a HER2 monoclonal antibody on tissue slides from breast cancer biopsies allows the pathologist to score the slide by sight (see **Table 1** and **Figure 1** (for an example)).

- Those samples with cells that show intense circumferential staining in >10% of cells are scored as HER2 3+. These samples are positive for HER2 and suitable for treatment with a high likelihood of response.
- If there is no staining, or only weak partial staining, the slide is scored as 0 or 1+ by the consultant/attending pathologist. These cases are deemed negative and not suitable for treatment.
- For slides with cells with weak circumferential staining in >10% of cells, the final score is 2+ and is called as equivocal, which require the next step in testing. This next step harks back

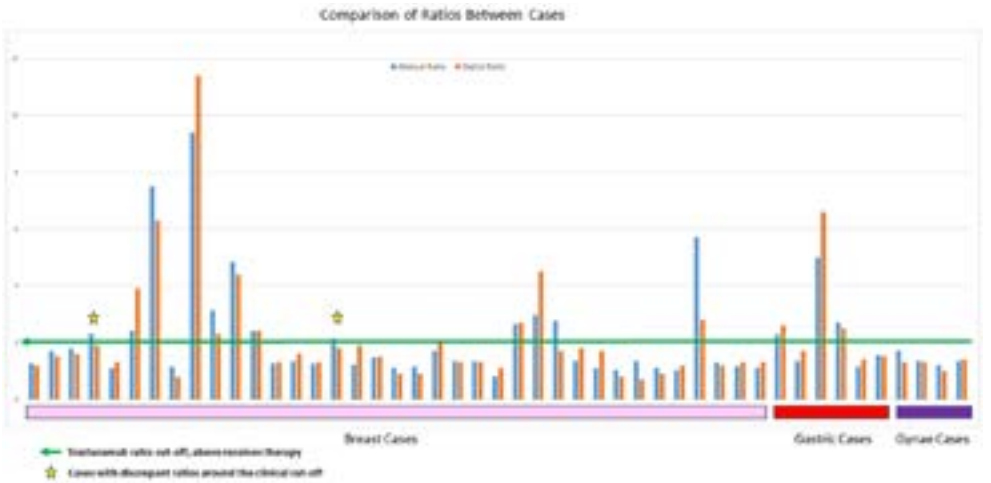


Figure 2

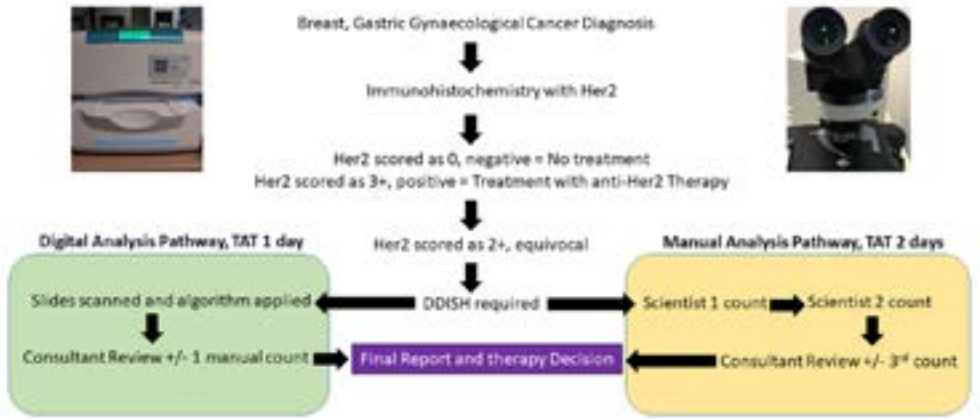


Figure 3

to the central dogma of molecular biology, DNA to RNA to protein [classic Franklin, Crick, Watson *et al.*]. That is, slides with equivocal protein-based results are further assessed using DNA for any sign of potential amplification.[7]

Changing From FISH To D-DISH

The presence of excess copies of the *HER2* gene is assessed by utilising complementary strands of DNA or probes that bind to the *HER2* gene for slides that carry signals that can be differentiated visually by colorimetric *in situ* hybridisation (see above). This can be done with fluorescent probes as in Fluorescent In-situ Hybridisation (FISH) or with brightfield probes as in D-DISH.[8] In D-DISH or dual-hapten, dual-colour *in situ* hybridisation, a black signal is used for *HER2* copy number and a red signal is used for a housekeeping portion of the chromosome 17 on which *HER2* resides, called *Cep17*. For this assay, if the *HER2* gene alone is amplified by a ratio with the housekeeping probe greater than 2:1, then the patient is likely to respond to therapy. The amplified gene may also be detected based on copy number alone as in some polysomic cases that put the patient into different ASCO groups.[7]

> *"Digital pathology is an obvious next step in the improvement of histopathology laboratory workflow in general and biomarker assessment in particular."*

The assessment of the staining – using either FISH or DDISH – includes the copy number count by two scientific staff within at least 20 cells for each count with confirmation and sign off by a consultant pathologist. A copy number per cell is then calculated for *HER2* and the housekeeping *Cep17*. The use of DDISH on an automated VENTANA system rather than manual fluorescent methodology represents a step forward in efficient usage of resources, however, there is still a significant dedication of resources to the assessment of these equivocal cases.[8]

Digital Pathology To Improve Workflow

Digital pathology is an obvious next step in the improvement of histopathology laboratory workflow in general and biomarker assessment in particular.[9,10] To improve workflow on the assessment of equivocal HER2 cases, whole slide images can be enumerated by computer algorithms. There are two steps required (refer to **Figure 1**): first, the computer must count the cells correctly by outlining the nuclei accurately and precisely. The second step is then somewhat simpler, to count the number of distinct black and red signals within the outlined nucleus. Calculation of the ratio is then automatically carried out.

To this end we have carried out a laboratory evaluation of a commercially available algorithm (uPath, Roche) to enumerate the signals and cells in a DDISH context. We examined a validation set of 48 previously scored HER2 DDISH slides, involving a mix of breast cancer, gastric cancer and gynaecological cancer slides scored predominantly as 2+ on immunohistochemistry. The slides were scanned on a DP200 scanner and cases built on the uPath image management system. A DDISH algorithm from Roche was then applied to the cases and compared to the current gold standard of manually scored results enumerated by two scientists and a consultant pathologist. The algorithmic approach was carried out by a biomedical student with some training in distinguishing in situ and invasive disease.[11] Given the expanded use of trastuzumab and other anti-Her2 therapies in the realms of gastric and gynaecological cancers, we also examined the applicability of this algorithm to other tumour types.

Results Of A Real-World Evaluation

We considered two questions for the real-world evaluation. The first question to be answered was whether the correct cells could be identified by a commercial algorithm. There was good concordance with visual on-screen assessment by a pathologist with a 1% error rate where conglomerates of cells were identified as single cells. This was noted more often in specimens with crowded cellular architectures, e.g., in a gastric carcinoma environment (**Figure 1**). This may also be the case in ductal carcinoma *in situ* in breast cancer, but this is not evaluable for HER2

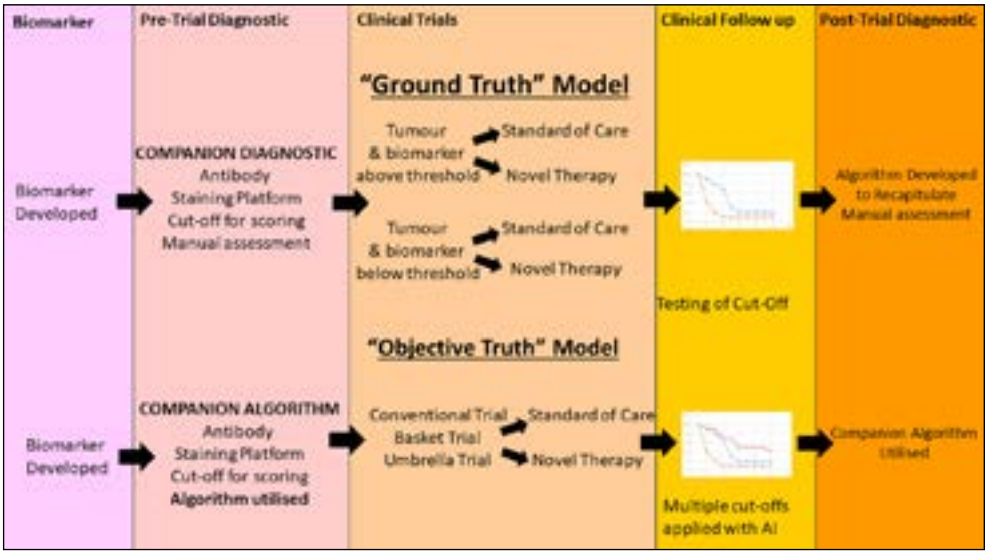


Figure 4

> *"The subjectivity of some PDL1 scores such as combined proportion score (CPS) makes the use of algorithmic analysis of biomarkers very attractive for reproducibility and creating considerable savings in a real-world laboratory environment."*

in any case as *in situ* carcinoma is not treated with anti-HER2 therapies.

The second question to be addressed was whether the signals per cell could be counted correctly. In 46 of the 48 cases examined there was concordance between amplified and non-amplified. Overall, 4/48 cases had discrepant ratios or copy numbers. In a further 2/48 cases, the results for amplified versus non-amplified were concordant but the value of the ratio differed significantly (**Figure 2**).

The discordant/discrepant cases could be broken down into three common areas:

1) Clusters of Her2 signal not counted in the same manner by manual and digital assessment.

When there are clusters of HER2 signals, the pathologist needs to do a mental overlay of single signals onto the nucleus to try to enumerate the

Table 1: Changes in the ASCO/CAP guidelines: interpretation of HER2 immunohistochemistry

HER2 IHC status	2007 ASCO/CAP guidelines	2013 ASCO/CAP guidelines	2018 ASCO/CAP guidelines
Positive (3+)	Uniform intense membrane staining of >30% of invasive tumor cells	Circumferential membrane staining that is complete, intense, and in >10% of tumor cells	Circumferential membrane staining that is complete, intense, and in >10% of tumor cells
Equivocal (2+)	Complete membrane staining that is either non-uniform or weak in intensity but with obvious circumferential distribution in at least 10% of cells	Circumferential membrane staining that is incomplete and/or weak to moderate and within >10% of the invasive tumor cells	Weak to moderate complete membrane staining observed in >10% of tumor cells[a]
Negative (1+)	Weak incomplete membrane staining in any proportion of tumor cells Weak, complete membrane staining in <10% of tumor cells No staining	Complete and circumferential membrane staining that is intense and within ≤10% of the invasive tumor cells Incomplete membrane staining that is faint or barely perceptible and within >10% of the invasive tumor cells	Incomplete membrane staining that is faint or barely perceptible and within >10% of the invasive tumor cells
Negative (0)		No staining observed Incomplete membrane staining that is faint or barely perceptible and within ≤10% of the invasive tumor cells	No staining observed Incomplete membrane staining that is faint or barely perceptible and within ≤10% of the invasive tumor cells

ASCO: American Society of Clinical Oncology; CAP: College of American Pathologists; HER2: human epidermal growth factor receptor 2; IHC: immunohistochemistry.

[a] Unusual staining patterns of HER2 by IHC can be encountered that are not covered by these definitions. As one example, some specific subtypes of breast cancers can show IHC staining that is moderate to intense but incomplete (basolateral or lateral) and can be found to be *HER2* amplified. Another example is circumferential membrane staining that is intense but in ≤ 10% tumor cells. Such cases can be considered equivocal (2+).

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actual number of HER2 signals. Our group suggests that this is achieved more readily and accurately by digital means (**Figure 3**).

Other cases with concordant amplified status but discrepant ratios again appeared to be due to the difficulties in counting clusters visually. These cases are also likely more accurately assessed digitally whereas a pathologist will deem the cells as amplified well above threshold and suitable for therapy but perhaps not counted as precisely as would happen if they were close to the cut-off of 2 as a ratio.

2) Borderline cases

One discordant case was found to be manually scored as 2.15 vs 1.8 digitally; this is in a borderline territory around the ratio of 2. Therefore, it behoves us as pathologists that these borderline cases be recounted manually.

3) Region of Interest Limitations

A single discordant case resulted from a limitation of the region of interest whereby sufficient numbers of positively amplified cells could not be captured by the square shaped region of interest in one single field utilised by the algorithm.

Our final question around whether the algorithm could be applied to other tumour types showed that the system worked very well in that regard, having a high level of agreement (100%) for determining ratios for treatment application in the gastric and gynaecological tumours examined (Figure 2).

"The design of future clinical trials, be they traditional two-armed trials or the increasingly more common basket or umbrella trials, should recognise the use of digital algorithms in their trial assessment."

Potential Impact On Workflow

The application of such systems in a real-world setting of a busy tertiary referral centre's histopathology laboratory has potentially significant impacts on workflow and throughput. The study outlined in this paper shows that work requiring a double count from two separate scientists followed by a third assessment from a consultant pathologist could now be undertaken by a single consultant reviewing the case at the screen with technical input for scanning the slides (**Figure 3**). This potential saving in scientific labor and review time is crucial, given the many demands on biomedical scientist time in a histopathology service.

The expansion of such systems to include other biomarker analysis has potentially even further time and human resource savings (even in isolation as the first increment in developing digital pathology capabilities within a laboratory). The subjectivity of some PDL1 scores such as combined proportion score (CPS) makes the use of algorithmic analysis of biomarkers very attractive for reproducibility and creating considerable savings in a real-world laboratory environment.[10]

Ground Truth vs Objective Reproducible Truth

Overall, there was good concordance between two methods used to assess HER2 status in breast, gastric, and gynaecological cancer cases with a Cohen's kappa of 0.75.[11]

One test relied on two senior biomedical scientists trained in HER2 assessment and a consultant pathologist. The other relied on a commercial algorithm with a trainee scientist with some rudimentary tutorials around *in situ* and invasive carcinoma.

Given the worldwide shortage of trained pathologists, it is likely that future biomarker assessments will increasingly be carried out by technical staff using algorithms and overseen by a consultant pathologist.[12] However, it is our group's opinion that these algorithms should be linked to clinical trial evaluation at any earlier stage to allow for standardisation and reproducibility.[13] Particularly in an era of precision medicine where patients are stratified based on molecular and proteomic data from their tumour, it is important to have an objective assessment of their biomarker status (**Figure 3**).

At present, the assessment/enumeration of biomarkers including ER, PR, HER2, Ki67, PDL1,

CASE STUDY:

Scanner and software use, a personal experience. (AG)

In terms of the use of both the Roche DP200 scanner and the uPath software, there were no significant negative issues. Both the hardware and associated applications were simple to understand and easy to run. The scanning process took approximately 2 minutes per core biopsy sample and the same time again to copy the file to the uPath enterprise software™. Once the files had been sent across, building the cases in order to run the image analysis algorithm was very easy to do.

The image analysis was similarly intuitive, especially with the heat map (**Figure 5**) to aid in the determination of where the analysis should be carried out. There were however some issues noted. The main issue was that the heatmap works by finding areas where there is a high concentration of black HER2 signals, as this is what it deems to be the tumour and therefore the place where the enumeration of the ratio would be best carried out. The software on occasion would pick up ink and folded artefacts on the slide in the heatmap. However, this was easily discounted on inspection.

When the software began analysing the digital images the timeframe for it to finish and be ready for analysis depended greatly on the speed of the connection available on the hospital network. This varied between taking only seconds to finish running the software to five or more minutes to analyse a single image. In most cases, the analysis was quick and no amendments to the cells chosen for analysis were needed. In some cases, cells had to be manually deleted and reselected as the algorithm may have chosen overlapping cells or cells that were too large in comparison to the surrounding tumour cells. This process was relatively easy to carry out and once the problematic cell analysis had been deleted a new cell could be chosen and its count simply added to the overall tally.

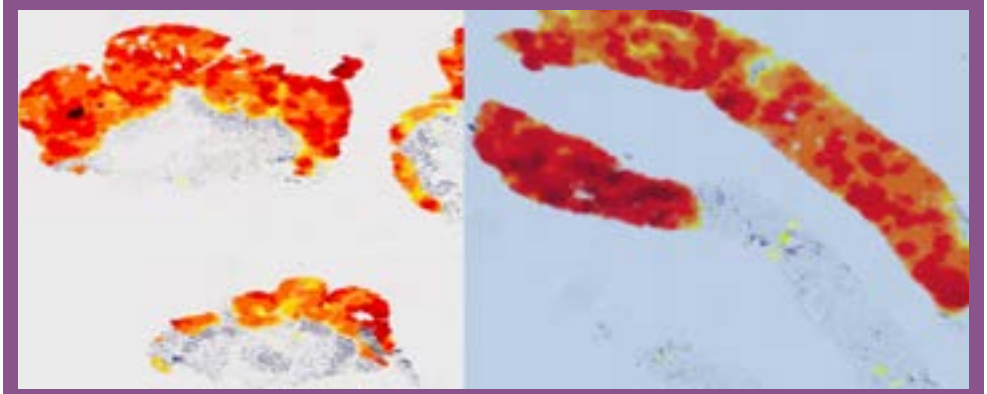

Figure 5: The "heatmap" function in the Upath system allows identification of areas of amplification more easily

ALK, and NTRK is by immunohistochemistry with crucial management decisions being made on these results.[14-19] The trials on which these are based have all been scored manually. Whilst it is arguable about whether digital or manual represents the ground truth of the biomarker expression in a tumour, it is undisputable that a reproducible and objective truth needs to be established for correct clinical trial assessment. In this group's opinion, such objective truths can be more readily obtained without unconscious bias toward a positive result by using digital means.[13]

The Future

The design of future clinical trials, be they traditional two-armed trials or the increasingly more common basket or umbrella trials, should recognise the use of digital algorithms in their trial assessment. Currently, algorithms attempt to recapitulate what has been assessed manually by an expert pathologist. The use of an algorithm to assess biomarker expression prior to treatment on a trial may lead to a more objective and reproducible value resulting in a more precise separation or overlap of the treatment curves. We will shortly be entering the era of companion algorithms to go with companion diagnostics (**Figure 4**). JoPM

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