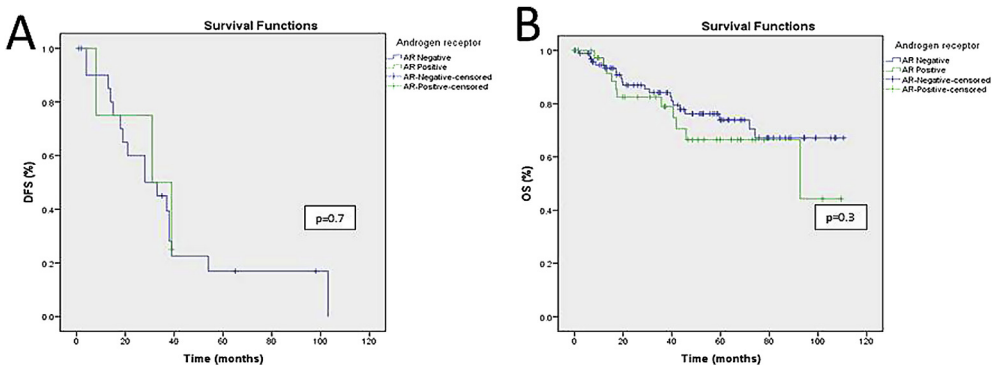

Fig. 2 Kaplan–Meier curves of estimated disease free survival (A) and overall survival (B) for TNBC patients grouped based on androgen receptor immunoexpression.

p=0.3, respectively). This finding remained valid after adjusting for confounders such as age, tumour size, grade and overall pathological stage using a Cox regression model (Fig. 2).

Expression of AR was more frequent in younger patients and in tumours with apocrine differentiation and less common in tumours with medullary-like features ($p<0.005$). Positive AR immunostaining was also shown to be correlated with mitotic rate ($p<0.05$) and overall tumour grade (G2>G3) ($p<0.001$).

We accept that this study was based on TMA sections rather than whole sections (WS) and heterogeneous expression may be a confounding factor. Indeed, we did find occasional cases where one core was positive for AR and the other negative which were resolved by staining whole sections (n=9). Søiland *et al.*[10] demonstrated that there was generally very good agreement between IHC-WS and IHC-TMA when the percent of AR positive nuclei using IHC was >80%, however discrepancies occurred when AR positive nuclei were <80%.

Another potential limitation is the lack of detailed treatment data for this cohort, including the fact that treatment algorithms are likely to have been inconsistent over the 10 year time frame of this study. Of note, AR expression was not routinely performed in our department during this time and therefore knowledge of AR expression status would not have altered treatment.

In conclusion, in our large unselected TMA-based cohort of 137 TNBCs, we found a rate of AR expression of 29.2% and no significant association between AR expression and disease free survival or overall survival. We hope that this information informs the use of AR immunohistochemistry in routine clinical practice.

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Digital evaluation of proliferative 'hotspots' of more than 16,000 cells negatively impacts Ki-67 assessment in breast carcinoma

Sir,

Ki-67 is a nuclear protein expressed during all phases of cell proliferation, which can be detected by a rapid and affordable immunohistochemical assay, widely available in pathology laboratories. Ki-67 proliferative index (PI) has strong prognostic and predictive value in invasive breast carcinoma (IBC). High Ki-67 levels are associated with poorer outcomes

in 'intermediate risk' cancers (i.e., grade 2, hormone receptor positive, HER2 negative tumours with no or low nodal burden) and better responses to (neo)adjuvant chemotherapy.[1–4] However its utility is hampered by suboptimal standardisation and accuracy. This is due to issues of measurement (e.g., visual vs digital assessment; semi-quantitative vs formal quantitative assessment), interpretation (e.g., unclear cut-off values for 'low' vs 'high' Ki-67 PI) and even the marker itself (e.g., different clones produce subtly different Ki-67 PI values).[5] Thereby, an International Ki-67 in Breast Cancer Working Group devised a set of recommendations addressing pre-analytical, analytical and interpretation factors in the hope of standardising practice and reducing variability.[6] These guidelines included a recommendation to evaluate Ki-67 PI incorporating highly proliferative 'hotspots' on a minimum of 500−1000 cells, but no upper limit on the number of cells to count was provided.[6,7] Digital image analysis allows rapid enumeration of large numbers of cells and is being increasingly applied to the evaluation of Ki-67 PI.[5] The effect of analysing hotspots in excess of 1000 cells on the assessment of Ki-67 PI is unknown.

A single slide from 32 consecutive IBCs from the PathWest archives of January 2017 was subjected to Ki-67 staining and then digital evaluation of six progressively enlarging, highly proliferative hotspots of 1000−2000, 2001−4000, 4001−8000, 8001−16000, 16001−32000 and >32001 cells corresponding to mean hotspot areas of 4.2, 8.3, 14.6, 33.3, 71.2 and 170.7 high power fields respectively (0.55 mm field diameter). The progressively larger hotspots each encompassed the smaller hotspot(s) within the new target area (Fig. 1).

Ki-67 immunostaining was performed on 4 µm sections on a Ventana Ultra automated platform (Ventana, USA). Antigen was heat retrieved in propriety Ventana CCI buffer at 95°C for 64 min, followed by incubation with the Dako M7240 clone of the Ki-67 antibody (Dako, Denmark) for 60 min at 1/400 dilution. Antibody binding was detected by DAB chromogen, visualised by the Ultraview DAB detection kit (Ventana, USA) and haematoxylin counterstain. Control lymph node tissue was included in each run. Ki-67 slides were scanned at ×200 using Aperio ScanScope AT and viewed on Aperio ImageScope software. The region for evaluation and the digital mark-up were always viewed onscreen by a pathologist before accepting Ki-67 PI results. The algorithm is designed to exclude populations of small cells (such as lymphocytes) and spindled cells, and to quantify positive nuclear staining as a percentage of all tumour nuclei.

Sensitivity and specificity were calculated by comparing the dichotomised Ki-67 'high'/'low' PI value of each hotspot at a cut-off of ≥14% (a consensus cut-off value suggested at the 2015 14th St Gallen International Breast Cancer Conference[3]) against the Ki-67 'high'/'low' PI value at the 1000−2000 cell hotspot (the minimum hotspot size as suggested by the Ki-67 Working Group;[6] i.e., the gold standard).

All hotspot sizes were evaluated in 29 cases. In one case there was insufficient cells for evaluation of hotspots above 16,001 cells and in two there were insufficient cells for evaluation of the >32,000 cell hotspot.

Regarding the 'gold standard' hotspot; the range for Ki-67 PI was 2−59%, mean 21% and median 17%. Twenty cases were classified as 'high' Ki-67 PI with a mean of 32%, median of 31% and a range from 14% to 59%, and 12 as 'low' Ki-67 PI with a mean of 7%, median of 6% and values ranging from 2% to 11%.

There was excellent sensitivity and specificity of digitally evaluated Ki-67 PI of hotspots up to 16,000 cells. While specificity remained at 100% for all hotspots (no false 'high' Ki-67 PI cases were observed), the sensitivity dropped from 95% to 85% once more than 16,000 cells were evaluated, then to 75% at hotspots above 32,000 cells (Table 1). Five cases changed from a 'high' Ki-67 PI value to a 'low' value at this largest hotspot. The largest Ki-67 PI drop of these five cases was from 31% to 11%.

As a readily available, rapid and affordable ancillary test, with demonstrable prognostic and predicative power, the potential for Ki-67 PI to influence management of IBC is exciting, but requires resolution of standardisation, reproducibility and accuracy issues.

We have demonstrated in this small pilot study that the sensitivity of digital Ki-67 PI evaluation is adversely affected when more than 16,000 cells are enumerated, when taking assessment of 1000 cells as the gold standard. It is intuitive that a drop in the Ki-67 PI (and thus sensitivity) will result from enlarging hotspots due to the most proliferative area being progressively 'diluted' by non-proliferative cells. However, this aspect of Ki-67 PI evaluation is previously unexplored and has potential to represent an additional factor

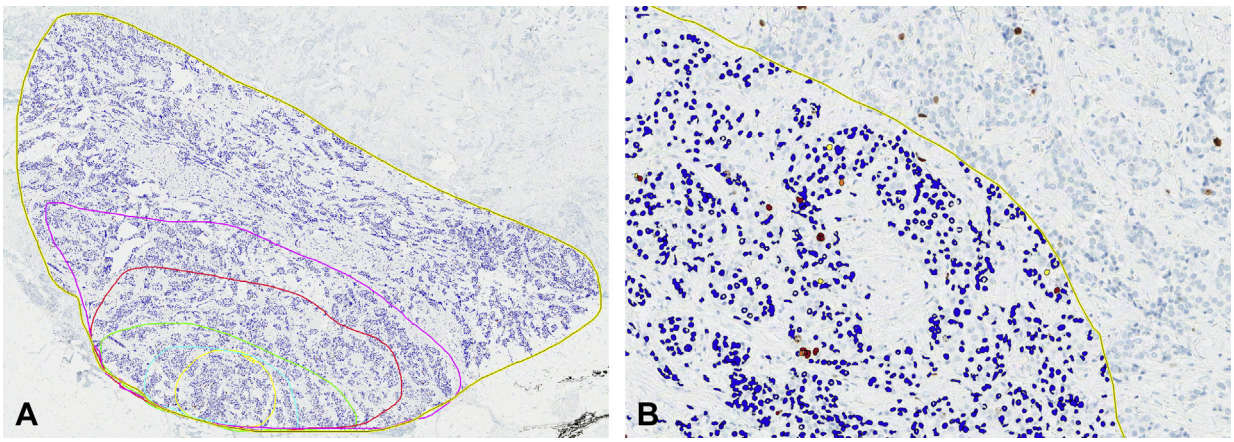

Fig. 1 (A) Low power magnification of a Ki-67 stained IBC demonstrating progressively enlarging hotspots for evaluation, delineated by different coloured lines. Each new hotspot incorporates the smaller hotspot(s). (B) High power magnification demonstrating the Aperio nuclear algorithm which marks negative tumour cells as blue and positive tumour cells as yellow, orange or brown, thus allowing enumeration of the percent positive cells.

Table 1 Sensitivity and specificity of Ki-67 proliferative index (PI) at progressively enlarging hotspots in comparison to the minimum recommended (gold standard) hotspot size of 1000 cells

Hotspot size	*n*	True high Ki-67 PI	True low Ki-67 PI	False high Ki-67 PI	False low Ki-67 PI	Sensitivity	Specificity
1000−2000	32	20	12	–	–	–	–
2001−4000	32	19	12	0	1	0.95	100
4001−8000	32	19	12	0	1	0.95	100
8001−16,000	32	19	12	0	1	0.95	100
16,001−32,000	31	17	11	0	3	0.85	100
>32,000	29	15	9	0	5	0.75	100

which may contribute to poor interobserver correlation, albeit one unique to groups/laboratories utilising digital systems for such evaluations.

This is a small study and requires verification in a larger cohort and ideally against outcome data in order to determine the optimal counting area with the strongest correlation to clinical outcomes. However, until such studies are available it seems prudent for those reporting Ki-67 PI using digital technology to limit their maximum hotspot size; PathWest will adopt a 16,000 cell hotspot size maximum in evaluations going forward. There is also a need to consider an upper cell enumeration limit in any future international guidelines for Ki-67 PI evaluation in IBC.

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Clear cell sarcoma of the soft palate mimicking unclassified melanoma

Sir,

Clear cell sarcoma (CCS) of soft tissue is a rare sarcoma that shares overlapping histogenic, morphologic and immunophenotypic features with melanoma. Soft tissue CCS typically arises in lower extremity tendons and aponeuroses of young adults, with a female predominance. It has very rarely been reported to involve the gastrointestinal tract or head and neck region. Herein, we describe CCS of the soft palate.

A 27-year-old man with no significant past medical history presented with a 4 cm lesion on the palate. Over the past three years, the patient had reportedly presented to the clinic for palatal ulcerations on multiple occasions. On examination, the lesion was ill-defined and erythematous, with a friable centre, superficial erosions, and irregular, raised edges (Fig. 1A). Two successive biopsies were performed, and pathology obtained at the outside institution described an unclassifiable melanocytic tumour in both cases. We received the second biopsy in consultation. Histological sections demonstrated a small sample of a larger tumour, comprised of nests of oval to epithelioid cells which appeared to abut the overlying epithelium (Fig. 1B). Mitotic figures were readily identifiable. Immunohistochemical staining showed diffuse MelanA positivity and clonal loss of p16 protein expression. A diagnosis of unclassified melanoma was rendered. Radiological staging was performed using magnetic resonance imaging (MRI) and positron emission tomography (PET) scanning. The lesion was not visible on MRI, and palatal osteolysis was not identified. Full body PET scan was positive for hypermetabolic activity in the draining jugular-carotid lymph nodes but was otherwise negative.

Given the unusual clinical and histopathological features of this case, as well as the advanced stage of disease at presentation, RNA-sequencing was performed to guide management. The results were negative for hotspot mutations in *BRAF, NRAS*, and *CKIT*, but did identify an *EWSR1-ATF1* fusion. The diagnosis was revised from unclassified melanoma to clear cell sarcoma of the palate. The