# VISIOPHARM®

midt regionmidtjylland | Aarhus University Hospital DENMARK

# Importance of automated invasive tumor detection and hot spot identification in Ki-67 assessment in breast carcinomas

Henrik Klingberg[1], Henrik Holten-Rossing[1], Gitte Tindbæk[2,3], Torben Steiniche[3], Michael Lippert[1]

[1] Visiopharm A/S, Hørsholm, Denmark. [2] Lillebælt Hospital, Denmark. [3] Department of Pathology, Aarhus University Hospital, Aarhus, Denmark

## INTRODUCTION

Accurate assessment of the Ki-67 proliferation index (PI) is an important tool in breast carcinoma classification. According to the Danish national guidelines by the Danish Breast Cancer Cooperative Group (DBCG) the Ki-67 assessment should only be assessed in the invasive front and hot spot of invasive tumors. Non-invasive breast tumor regions (DCIS) are identified by the presence of myoepithelial cells (p63 positive nuclei). Ki-67 assessments in hot spots within invasive tumor regions, have been shown to be superior in performance compared to manual assessments giving rise to more accurate diagnosis[1,2]. Our aim was to evaluate the PI values in hot spots for either all tumor regions or limited to invasive tumor regions only using digital image analysis (DIA) and compare this to manual Ki-67 PI assessment.
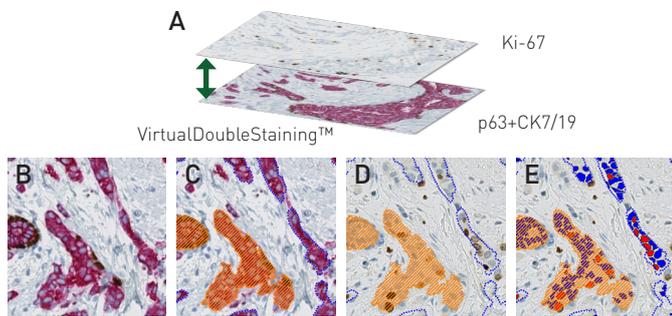


**Figure 1** Image analysis workflow. A) Slide-scans of serial slides stained against Ki-67 (top) and slides stained against p63 (myoepithelial) and cytokeratin 7 and 19 (CK7/19) (cancer tumor) are aligned using the VirtualDoubleStaining™ method. B) Tumor cells (red) and p63 positive nuclei (brown) are detected, and C) invasive (blue outline) and non-invasive (DCIS) (orange hashed) tumor regions are identified using the CE IVD Invasive Tumor Detection APP (20101). D) The defined tumor regions are transferred to the aligned Ki-67 image with Ki-67 positive nuclei (brown) and Ki-67 negative nuclei (light blue). E) The Ki-67 proliferation index (PI) is estimated by detecting Ki-67 positive nuclei (red overlay) and negative nuclei (blue overlay) in the invasive tumor regions using the CE IVD Ki-67 APP (90004), we also estimated the PI in the DCIS tumor regions for further analysis and comparisons.

## MATERIALS AND METHODS

72 breast cancer tissue samples (all with invasive cancer and DCIS present) were included in this study. Slides were stained using the BenchMark ULTRA Ventana platform and digitization by a Hamamatsu NanoZoomer 2.0HT slide-scanner.

Visiopharm® image analysis software was utilized for the DIA and tumor/stroma separation by the VirtualDoubleStaining™ method. This was followed by automatic invasive and non-invasive tumor detection, Ki-67 PI assessment and hot spot detection using the newly developed and clinically approved (CE IVD) Invasive Tumor Detection APP (20101), Hot Spot APP (20114) and Ki-67 APP (90004) (see Figure 1). For the Hot Spot APP a circular hot spot with a diameter of 550 µm was used. Manual Ki-67 assessment scores was performed by a pathologist and was estimated in the invasive front (according to DBCG guidelines of 2016).

## RESULTS

When performing digital image analysis (DIA) Ki-67 PI assessment of hot spots in all tumor regions (DIA HS A) ≈ 20% of the hot spots were located in or with pronounced overlap of DCIS regions (an example is shown in Figure 2).

We then investigated the absolute % PI discrepancies between DIA HS A and DIA Ki-67 PI assessment of hot spots in invasive tumor regions (DIA HS IV) by Bland Altman and plotting against DIA HS IV values (Figure 3 left). The bias was 0.01% PI the limits of agreement (LOA) was ±8% PI. The LOA defines within which 95% of the differences between one method and the other are included.

The treatment category changed for 5.6% of samples (20% cut-off) and 3 were considered "false negatives" (red dot) and 1 considered "false positive" (blue dot) when using DIA HS IV as the reference. For comparison we also compared DIA HS IV with DIA Ki-67 assessment by average of all invasive tumor regions (DIA IV) (see Figure 3 right).

We evaluated the agreement between DIA and manual PI assessment scores by Bland Altman analysis (see Figure 4). When comparing manual assessment with DIA HS IV (Figure 4 left panel) or DIA HS A (data not shown) we observed similar results with a bias ≈ 0.05% and LOA ≈ ±18%. When comparing manual assessment with DIA IV (Figure 4 right panel) a bias of +8.4% and LOA ≈ ±19% was observed.
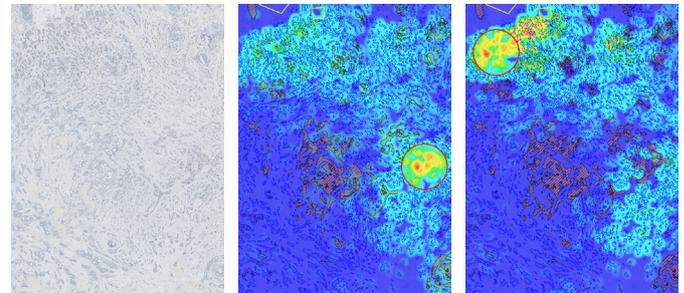


**Figure 2** Hot spot analysis of Ki-67 positive nuclei. Left panel) shows Ki-67 stained tumor slide. Center panel) shows the detected hot spot (red circle) when the analysis was performed on all tumor regions. Right panel) Shows the detected hot spot when the analysis was performed on invasive tumor regions. Invasive tumor regions (blue outlined) and non-invasive tumor region (orange hashed) are overlaid the heat map (blue to red) for increasing Ki-67 using the Hot Spot APP (20114).

We evaluated the theoretical change in treatment categories for manual assessment with DIA HS IV and we observed that the treatment categories were altered for 16.6% of the samples (see Figure 4 left). Of these 6 samples were considered "false negatives" (red dots) while 6 were considered "false positives" (blue dots).
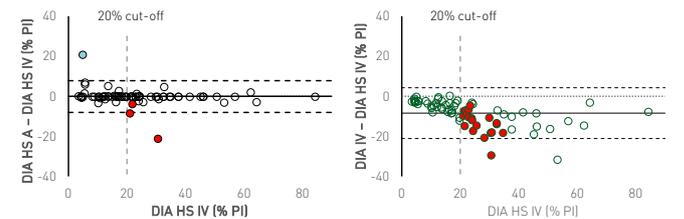


**Figure 3** Bland Altman method agreement analysis. Left panel) shows the agreement of digital image analysis (DIA) Ki-67 proliferation index (PI) assessment for hot spots in all tumor regions (DIA HS A) and DIA PI assessment of hot spots in invasive tumor regions (DIA HS IV). "False negatives" (red dots) and "false positives" (blue dots) relative to a treatment PI cut-off at 20%. Right panel) shows the agreement of DIA HS IV and DIA of the averaged PI in invasive tumor regions (DIA IV). The bias (black solid lines), limits of agreement (black dashed lines) and zero difference (grey dotted line) are included
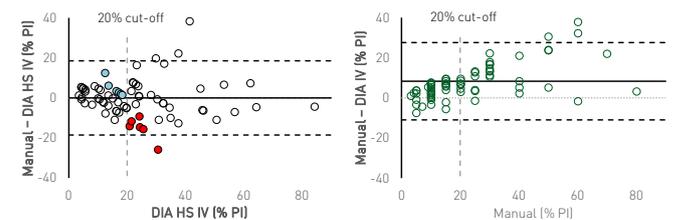


**Figure 3** Bland Altman method agreement analysis. Left panel) shows the agreement of manual Ki-67 proliferation index (PI) assessment (Manual) and DIA HS IV. "False negatives" (red dots) and "false positives" (blue dots) relative to DIA HS IV result and a cut-off at 20% PI. Right panel) shows the agreement of Manual and DIA IV relative to manual score.

## DISCUSSION AND CONCLUSION

Our results highlight the importance of excluding DCIS regions when using DIA for Ki-67 assessments in hot spots as these can lead to detection of hot spots within DCIS regions or the unintentional contributions of these regions to the final PI assessment score. This could potentially lead to false treatment exclusion or inclusion, which emphasize the importance of identifying invasive tumor regions before evaluating PI values by hot spot analysis.

At the same time the results demonstrate that the DIA hot spot analysis can produce unbiased results compared with manual PI assessment, further strengthening the value of this approach. When interpreting the LOA between all DIA methods and manual counting it is clear that the range is relatively large. An important factor here is the inherent variation in manual compared with DIA Ki-67 scoring[1-2]

1. Stålhammar G et al. *Mod Pathol.* 2016. doi:10.1038/modpathol.2016.34
2. Røge R et al. *Breast Cancer Res* Treat. 2016. doi:10.1007/s10549-016-3852-6