Defining the area of mitoses counting in invasive breast cancer using whole slide image

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Although counting mitoses is part of breast cancer grading, concordance studies showed low agreement. Refining the criteria for mitotic counting can improve concordance, particularly when using whole slide images (WSIs). This study aims to refine the methodology for optimal mitoses counting on WSI. Digital images of 595 hematoxylin and eosin stained sections were evaluated. Several morphological criteria were investigated and applied to define mitotic hotspots. Reproducibility, representativeness, time, and association with outcome were the criteria used to evaluate the best area size for mitoses counting. Three approaches for scoring mitoses on WSIs (single and multiple annotated rectangles and multiple digital high-power (×40) screen fields (HPSFs)) were evaluated. The relative increase in tumor cell density was the most significant and easiest parameter for identifying hotspots. Counting mitoses in 3 mm² area was the most representative regarding saturation and concordance levels. Counting in area <2 mm² resulted in a significant reduction in mitotic count (P = 0.02), whereas counting in area ≥4 mm² was time-consuming and did not add a significant rise in overall mitotic count (P = 0.08). Using multiple HPSF, following calibration, provided the most reliable, timesaving, and practical method for mitoses counting on WSI. This study provides evidence-based methodology for defining the area and methodology of visual mitoses counting using WSI. Visual mitoses scoring on WSI can be performed reliably by adjusting the number of monitor screens.

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INTRODUCTION

Histological grade of breast cancer (BC) is a strong prognostic and predictive factor for disease behavior and outcome. Mitotic count as a component of grade reflects the rate of tumor proliferation and aggressiveness. However, the level of concordance of mitotic count among pathologists remains low. Such discrepancy is not only attributed to the variability in pathologists’ performance but also reflects the subjectivity and variation in methodologies used for the assessment of mitotic scores.

Mitotic scores often represent the highest proliferative activity of the tumor, and they are obtained by counting mitotic figures within hotspots (areas showing the highest number of mitoses within the tumor). When using light microscope, it is recommended that mitotic figures are counted in ten high-power fields (HPFs) in hotspots. Different microscopes have different field areas, which vary widely (they range from 1.26 to 3.74 mm² per 10 HPFs). To achieve consistency of scoring when using different microscopes, tables detailing mitotic cut-offs per each field area are published. Changes of mitotic cut-offs rather than standardizing the area by adjusting the number of the HPFs accordingly are currently recommended, despite evidence indicating that using a fixed area size is more reliable for mitotic counts in BC. The approach toward standardizing the area for counting mitoses rather than the cut-offs per microscope field diameters has already been adopted in several other tumors such as melanoma and gastrointestinal stromal tumors.

Although the recent introduction of whole slide image (WSI) technology in primary histopathology reporting has several advantages, it can make visual assessment of mitoses more challenging. Identification of mitotic figures on WSI is difficult due to loss of some of the fine details of mitoses resulting from the lack of fine-tuning ability. Although this could be overcome by using higher quality scanners with focus stacking (Z stacking) functionality, the time, cost, and the image storage capacity make the availability of such options challenging in routine practice. Moreover, the methodology for defining the area for measurement on the screen is still ambiguous. One HPF on the conventional light microscope is not equivalent to a digital HPF, while the microscopic resolution is dependent on the objective and ocular lens only, the digital resolution is more complicated with additional influencing factors including the scanner objective lens magnification, the resolution of the digital camera sensor, viewing software, and the display monitor characteristics. Although most the available imaging viewing software can generate absolute area size on the monitor screens, the practical application still face challenges with standardization of the methodology. In addition, the use of different combinations of scanners, imaging viewing software, and monitor screens of variable features in routine practice makes
Fig. 1  Case selection flowchart. Overview of the studied cases selection.

This task more challenging. Even though several studies have shown high overall diagnostic concordance between both platforms27,30–32 more standardization of mitotic count methodology using WSI is needed to improve the consistency of its assessment and to provide guidelines when applying artificial intelligence (AI) for automatic scoring of mitosis.

In this study, we aimed to provide evidence-based data to define the optimal area for counting mitoses in terms of the geographical distribution within the tumor and the best practical and accurate methodology for counting mitoses using WSI.

MATERIALS AND METHODS
Study cohort
This study was conducted on a cohort primary invasive BC (n = 595). A chart outlining an overview summary of the cases selected is provided in Fig. 1.

The clinicopathological data including the molecular subtypes of the cases included in the study were available as previously described33. Tumors were classified into molecular subtypes based on the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 (HER2) as follows: (i) ER+ and/or PR+ and HER2–, (ii) HER2 enriched (ER– and/or PR– and HER2+), and (iii) triple negative (TNBC).

Four-µm-thick, full-face tumor sections stained with hematoxylin and eosin (H&E) were used. The slides were scanned at ×40 magnification using a high throughput slide scanner (Pannoramic 250 Flash II; 3DHISTECH, Budapest, Hungary), and the images were viewed using Case Viewer software (version 2.2.0.85; 3DHISTECH) on a full-screen panel (27 inches with resolution 2560 × 1440 pixels).

Defining the criteria for hotspot identification
To define the criteria for selecting the representative area for mitotic counting on WSIs, the mitotic figures in 113 cases were annotated manually in the entire WSI to create heatmaps. One observer screened each WSI twice for mitotic figures. Slides were examined in a systematic manner at ×40 magnification. The whole tumor area within the WSIs was examined starting from the upper left corner, moving in a Z-shaped manner to the lower right corner. All figures demonstrating the morphological criteria of true mitoses were annotated. To ensure the specificity of annotation by the first observer, all the cases were reviewed by a second observer to confirm the nature of the annotated structures (i.e., mitotic or nonmitotic cell). Only evident mitotic figures were considered, and the agreement was achieved by consensus. 56,169 mitotic figures were agreed between the two observers as true mitotic figures.

Areas with the highest numbers of mitoses (hotspots) in each slide were selected and zoomed out at ×0.5 magnification to attain a general overview of their characteristics regarding distribution (location), the relative increase in the tumor cell density, pattern of tumor growth, mitotic counts, relation between hotspots with areas of tumoral necrosis, DCIS, presence of central necrosis and fibrosis, tumor infiltrating lymphocytes (TILs), and tumor border either pushing or infiltrative.

The distribution of hotspots in each case was determined using heatmaps generated by the annotated true mitotic figures. The hotspot distribution was classified as either peripheral (at or close to the invasive tumor fronts), central, scattered/dispersed (hotspots evenly distributed all over the tumor including both peripheral and central areas), or no hotspot could be detected where only a few separated mitotic figures present (Fig. 2). Stromal TILs were evaluated based on the International Immunohistochemistry and Immunomarker Working Group guidelines for invasive breast carcinoma34. Central fibrosis refers to central acellular zones occupied by fibrotic tissues, whereas central necrosis refers to central acellular zones occupied by necrotic tissues, based on the following criterion: necrosis/fibrosis extends for 1 mm or more over the cross-section of tumors, with abrupt transition between necrosis/fibrosis and viable tumor cells, no evidence of squamous, osseous, or cartilaginous metaplasia and of matrix-producing features.

The tumor cell density was evaluated subjectively within the areas of interest (hotspots) compared to the cellularity in other tumor areas on the same slide taking in consideration tumor cell overlapping, cellular cohesion, spacing, morphological pattern, and intervening stroma. The pattern of tumor growth was visually assessed by one observer and according to the predominant pattern (>90%), it was classified into one of the following: sheets, nests and trabeculae, tubular, cribriform, (single or papillary patterns (Supplementary Fig. S1). Mitotic counts were recorded in hotspots with the average mitotic count in the WSI and after applying the criteria using 40 cases. Two pathologists used a copy of the same WSI, and each of them separately annotated mitotic figures present (Fig. 2). The degree of agreement was assessed statistically using Cohen’s Kappa test.

To validate the defined criteria for hotspot identification (Tables 2 and 3), we assessed the effect of the studied characteristics on identifying mitotically active areas using low digital power.

We measured the level of agreement between two pathologists, before and after applying the criteria using 40 cases. Two pathologists used a copy of the same WSI, and each of them separately annotated mitotic hotspot as region of interest using the circle annotation tool of Case Viewer software. Each observer was blinded to the results of the other observer. The agreement between them was considered when these two circles overlapped or intersected (Supplementary Fig. S2). The degree of agreement was assessed statistically using Cohen’s Kappa test.

To test the hypothesis that counting in hotspots represents the highest proliferative activity within the tumor, we have compared the average mitotic count in hotspots with the average mitotic count in the WSI and with mitotic counts in randomly selected areas. To record mitotic counts in randomly selected areas, the tumor on WSIs (n = 60) was divided into four quadrants. A grid was drawn (with squares of 1 mm² area size) in each
quadrant and another one was drawn in the central part of the tumor. Each small square was assigned a number. Numbers were randomly ordered using free access random number generator (https://stattrek.com/statistics/random-number-generator.aspx) and the first 5 numbers in the generated list were chosen to count mitosis within them. This was designed to represent a non-selective manner of counting mitoses in areas of 5 mm² in total. Figure 3 shows the selection of these random areas.

Defining the optimal area size

The optimal area for counting mitoses within hotspots was evaluated by counting mitoses at ×40 digital magnification in multiple annotated areas including 1, 2, 3, 4, and 5 mm² areas. Five rectangles, each measuring 1 mm², were annotated separately in selected hotspots, avoiding areas of very low cellularity, necrosis, DCIS, tissue artifact, or areas out of focus on WSI. All cases were scored by three observers using the same protocol; the first observer scored all cases, and the other two scored 25% of the cases. The optimal size of the area for mitotic count evaluation was determined based on the level of reproducibility, scoring time, association with outcome, practicality (as determined subjectively by the observers), and level of saturation defined as the area beyond which the increase in mitotic count, if any, is not statistically significant. The latter was determined using the non-parametric, Mann–Whitney U test, to assess the two-level differential mitotic counts against the baseline (area size).

Method of navigating WSIs when counting mitoses

To select the most reliable approach for counting mitotic figures on WSI, we compared three methods including (1) multiple display screens at ×40 magnification (multiple digital high-power (×40) screen fields (HPSFs)) equivalent to of 3 mm² area (refer to Table 6 for how to calculate the number of HPSFs), (2) counting within a pre-annotated (3 mm²) as a single...
area. (3) counting within a pre-annotated (3 mm²) as multiple separate rectangles (non-adjacent small areas in different hotspots to avoid areas with low cellularity or artifacts that collectively equivalent to 3 mm². Measurement of accuracy is challenging as no ground truth is available to compare each method against. We have checked the accuracy by annotating and re-assessing some of the figures that were detected by one method and missed by the others, and they were agreed by the two observers to be true mitotic figures; high concordance was used as evidence of acceptance. One of the main aims for the choice of the method is the reproducibility of the technique, as well as the consistency and concordance of scoring. Other variables include time, pathologist’s preferences, matching with the existing guidelines and current practice.

The number of HPSFs was determined by the viewing area size only, excluding the toolbars, menu bars, and any other annotation windows. The size of the viewing areas was calculated, and the number of the screens (HPSFs) was determined to produce a 3 mm² area. The effect of different variables (display monitor size, screen resolution (measured in pixel density), different scanner types and viewing software) were tested on the size of the area on the WSI, by changing one variable while fixing the others and identifying which of them had the major impact. For this aim, cases were scanned by three scanners Philips IntelliSite Ultra-Fast Scanner, and Leica Aperio AT2 scanners were used in addition to Pannoramic 250 Flash III: 3DHISTECH with the relevant viewer software including IMS Philips, Aperio Image Scope and Case Viewer, respectively.

Statistical analysis
All statistical analyses were performed using SPSS ver.26. The correlations between categorical variables were analyzed by $\chi^2$ test. The differences between the two independent groups were compared by Mann–Whitney U test. The degree of interobserver agreement was assessed by use of the intraclass correlation coefficient (ICC) for continuous data. Cohen’s statistic was used to assess the concordance between two observers for categorical variables while Fleiss Kappa was used for more than two variables/scores.

Univariate Cox Regression model was used for outcome analysis against the BC-specific survival using the continuous mitotic score within different area size. For all tests, $P < 0.05$ (two-tailed) was considered statistically significant.

RESULTS
Defining hotspots
Table 1 summarizes the identified morphological characteristics of hotspots using WSI.

The most frequently seen hotspot distribution pattern was the scattered pattern, accounting for 48%. Cases showing regional variability of mitotically active areas represented 42% and these either had a peripheral pattern in 34% or central in 8%, while 10% of the tumors showed no hotspots with only few separated mitoses without any clustering, which was determined subjectively by visual assessment. One of the main aims for the choice of the method is the reproducibility of the technique, as well as the consistency and concordance of scoring. Other variables include time, pathologist’s preferences, matching with the existing guidelines and current practice.

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cases with scattered or central patterns showed neither central necrosis nor fibrous scar (76% and 82% of cases, respectively). Hotspots were frequent next to areas of necrosis (81% of cases with necrosis) but not adjacent to DCIS (11% of the cases with DCIS). No significant correlation was found between the distribution of hotspots and the presence of TILs or type of tumor border. The relative increase in tumor cellularity within hotspots was one of the most significant parameters and was observed in 78% of cases. Relative increase in tumor cell density was associated with peripheral, scattered, and central hotspot distribution (90%, 83%, and 55%, respectively).

In most cases with peripheral and scattered hotspot distribution, tumor cells were arranged in sheets, nests, or trabeculae, whereas the majority of cases with a central hotspot distribution showed tubular and single file patterns.

Hotspots with a high mitotic count were associated with sheeted, nested, and trabeculated patterns, while hotspots with lower counts were associated with single file, papillary, cribriform, and tubular patterns ($\chi^2 = 60.45, P < 0.001$).

The mitotic count within the hotspot, the relative increase in tumor cell density, and the pattern of tumor growth, all showed a statistically significant correlation with hotspot distribution ($P < 0.001$) (Supplementary Table S2).

There is a statistically significant correlation between the distribution of hotspots and the presence of central necrosis with the molecular subtypes ($\chi^2 = 19.19, P = 0.024$) and ($\chi^2 = 10.285, P = 0.016$), respectively. Peripheral distribution and central necrosis were associated with triple-negative BC.

**Interobserver concordance in choosing the same hotspot**

Table 2 shows a summary of additional and existing information on mitotic hotspot identification. The criteria for defining hotspots are as follows: (1) hotspots are located at the most cellular areas of the tumor (more basophilic areas with nuclear overlapping and increased tumor cell density relative to other areas); (2) hotspots are frequently located at the peripheral invasive part of the tumor, but they can be central or scattered throughout the tumor; and (3) hotspots are more frequent in areas with solid growth pattern, sheets, and nested architecture than in areas with tubular or single cell infiltrative pattern.

These defining criteria were applied to test for improvement in interobserver concordance on hotspot identification (Table 3).

Interobserver agreement on choosing the same hotspot was tested before using these refined criteria for hotspot identification on WSI and it showed a moderate interobserver agreement (kappa = 0.53). An improvement of interobserver agreement on choosing the same hotspots was observed after applying these criteria (kappa = 0.75).

**Mitotic counts in hotspots versus non-selected areas of similar size**

The median mitotic counts in a defined area within selected hotspots were higher than the median mitotic count in the same size of the randomly selected areas of the same size and the difference was statistically significant ($P < 0.001$). Figure 4a shows the relationship (scatter plots) of mitotic count between hotspot and randomly selected areas. Moreover, the median mitotic counts in hotspots per mm$^2$ were significantly higher than the median mitotic count per mm$^2$ when mitotic figures were scored in the whole slide and the difference was statistically significant ($P < 0.001$) (Supplementary Fig. S4).

**The optimal area of counting mitoses**

Supplementary Table S3 shows the mean, median, 95% confidence interval for the mean, SD, and variance of mitotic count in different areas, while Supplementary Fig. S4 boxplot shows the median, minimum, and maximum range of average mitotic count (mitotic count/area) in different areas within the hotspots.

Counting mitoses in 1 and 2 mm$^2$ showed a statistically significant difference in median counts ($P < 0.05$). We found that counting in areas larger than 3 mm$^2$ does not have a significant statistical difference (level of saturation) in the median count when comparison was run between 3 and 4 mm$^2$ ($P = 0.234$), between 4 and 5 mm$^2$ ($P = 0.528$), or between 3 and 5 mm$^2$ ($P = 0.79$) (Fig. 5).

The highest degree of interobserver agreement among three pathologists was observed when counting mitoses in 3 mm$^2$ (ICC = 0.691), while counting in 1 mm$^2$ had the lowest interobserver agreement (ICC = 0.595) (Supplementary Table S4).

The average time (mean ± SD) for counting mitotic figures using WSI is illustrated in Fig. 4b, which shows a progressive increase in time from 1 mm$^2$ (50 ± 30 s) to 5 mm$^2$ (240 ± 50 s).

Survival analysis using the univariate cox regression model showed that mitotic counts in 2, 3, 4, and 5 mm$^2$ areas were associated with BC-specific survival ($HR = 1.025, P = 0.02$), ($HR = 1.018, P = 0.016$),
Table 4 shows a constellation of factors for each area size and justifying the choice of the optimal area size of 3 mm².

Methods of navigating WSIs when counting mitoses

To assess the best approach for counting mitoses on WSI, we compared three counting methods utilizing the same area (3 mm²): multiple HPSFs (×40), pre-annotated single area, and multiple areas. We found that the median mitotic count using multiple HPSFs that were calculated to produce 3 mm² (i.e., 15 full monitor screen at ×40, using 27-inch monitor, BenQ, 2560 × 1440 pixels) was higher than counting within multiple or single annotated rectangles of the same area size and the difference was statistically significant (P < 0.001) (Table 5).

When using multiple HPSFs, the interobserver agreement between two observers was higher compared to using multiple annotated and single annotated rectangles (ICC = 0.911, ICC = 0.877, and ICC = 0.840, respectively). In addition, we found that using the multiple HPSFs was more practical (timesaving) (Table 6) as it did not require annotation on the WSI or consideration for counting fields within the annotated areas to avoid overlaps with counting the same mitotic figures or missing some mitotic figures in between fields.

Factors affecting size displayed on digital platforms

As HPSF showed the best method for counting mitoses, it is important to standardize the number of screen fields required to measure a defined area (e.g., 3 mm²). Therefore, we tested the impact of several factors on the size of the screen field. We found that the monitor screen resolution is the most important factor.

Figure 6 shows the effect of different resolutions on the area size displayed on monitors of the same size (27 inches). We found that while fixing all the variables and changing only the display resolution, the size of the area on the monitor changed proportionately and significantly with a linear relationship (Fig. 7). When the monitor screen resolution is fixed, the monitor screen size resulted in an insignificant change in the size of the area. The monitor screen size had an almost negligible impact on the field size and subsequently, the number of HPSF used to cover the defined area.

When scanning slides by two different scanners using the same image viewing software, we found that the area size...
changed, confirming the effect of the scanner’s camera sensor and resolution on the overall viewing area size (Supplementary Fig. S6).

Accordingly, our results confirmed that the size of the viewing area displayed on the monitor at the same magnification (×40) varies with screen resolution, scanner type used, and viewing software, while the monitor size does not have any significant effect. With the available data, we were able to produce the equation below to determine the number of HPSFs (the area size effect. With the available data, we were able to produce the software, while the monitor size does not have any signi

\[ \text{Number of HPSFs} = a_1 \times \text{scanner camera resolution} + a_2 \times \text{monitor resolution} + a_3 \times \text{software viewing area} \]

The relationship between variables can be described using this equation, where \( a_1, a_2, \) and \( a_3 \) are constants that control how much these elements contribute to the viewer area.

Some viewing software features built-in algorithms for determining the area displayed on screen. Table 7 shows different monitor sizes with different resolutions and the number of HPSFs needed to account for area size of 2 and 3 mm², respectively.

**DISCUSSION**

The introduction and implementation of WSI in routine clinical settings for primary diagnosis have brought some potential benefits as well as some challenges, although WSI provides the advantage of an enhanced low-power overview of the slide and allows the integration of diagnostic AI algorithms.

Studies on mitoses detection using AI have been published. However, the consistency in the methodology and definitions of the mitotic index or score remain lacking, which makes it difficult to be integrated in the final Nottingham histological grade. The current study aimed to provide evidence of the methodology and a protocol that can improve concordance using WSIs and to guide future AI-based mitosis detection studies in refining performance and improving consistency of reporting.

The mitotic hotspots are most representative of the proliferative capacity of the tumor. Our results showed that there is a tendency to underestimate mitotic count in randomly selected areas.

In the present study, we revealed that mitotic figures in the majority of cases show regional variation either located at the periphery or situated at the center, suggesting that this variation in the distribution of mitotic figures can be considered as a major cause of interobserver variability. Using WSIs, we searched for clues to...
found that counting mitoses per mm² have better reproducibility.

In a recent study, we showed that there is an average 20% reduction of mitotic counts on WSI compared to light microscopy. Although this can be explained partially by the scanner and/or image quality, and lack of familiarity with WSI, we noted this reduction with most of the commercially available clinical grade tools without significant improvement with training. Therefore, a new cut-off of mitotic count for scores 1, 2, and 3 as a component of the Nottingham grade is needed. This should

attain better agreement in choosing mitotic hotspots, to re
determine area (e.g., 2 or 3 mm²). In this

defined region of the tissue

It was recently suggested that a predefined region of the tissue
measured in mm² is favored over methods focusing on the
number of HPFs when using a microscope43, and some studies
found that counting mitoses per mm² have better reproducibility
than per HPFs16,21,22. However, the practice in counting mitoses in
BC remains dependent on the use of ten HPFs and estimating the
size of the area of these ten HPFs rather than using a variable
number of HPFs to achieve a defined area (e.g., 2 or 3 mm²). In this
study, we tested several area sizes to identify the optimal area;
however, we did not exceed 5 mm² as larger areas are believed to
be impractical50, time-consuming, and represent more than any
area covered by ten HPFs even with the widest ×40 fields on
conventional microscope lenses currently available in the clinical
setting. Our findings showed that 1 mm² is not reliable for
counting mitoses as it is associated with lower mitotic count and
low concordance; in addition, it did not show significant
association with the patient outcome. While 2 mm² was the
smallest area associated with patient outcome, mitoses counting
in 3 mm² appeared to be more representative when considering the
level of saturation, scoring time, and reproducibility; there was
no statistically significant impact on scoring when compared to a
wider area. It is worth mentioning that 3 mm² area is comparable
to ten HPF of a high field diameter microscope (0.62 mm), which
we currently use in our clinical practice. If we convert these
assessed factors into acquired points, we can see that 3 mm²
achieved the highest points. Consistent with our finding, Meyer
et al.20 showed that less sampling error is associated with larger
sample areas and this has a non-linear relationship with the size of
the area to be sampled, where a slight increase in the area has a
significant effect on reducing the misclassification rate. This fits
more in tumors with a low mitotic count, and the misclassification
rates nearly reduced to half when increasing the area from 1.26 to
3.74 mm²14. The need to count mitoses in ≥2 mm² to adjust
the number of microscopes HPFs has also been stated in many
previous studies1,14,17,20.

When testing several approaches for scoring mitoses on the
WSI, we have found that using screen fields was the most reliable,
timesaving, and practical compared with annotated areas. In this
study, we suggest using the term digital HPSFs to be used in
digital reporting instead of the conventional term HPF applied to
microscopes. In this situation, high power (×40) refers to the
scanner lens magnification and/or the magnification on the
screen. Although some scanners can use a ×20 magnification lens
but the ×40 is the standard. In this study, we used a ×40 lens and
×40 magnification power on the screen. Higher magnification can
be used to improve the visualization of mitotic figures but the
standard mitoses counting on the defined area was performed on
×40 magnification.

Several variables could affect the size of the displayed area at
×40 magnification on WSIs. These are mainly the scanning
resolution, the viewing monitor resolution, the image viewer
software, and the type of scanner used26. We investigated the
parameters influencing the size of the area displayed on the
screen at ×40 digital magnification and discovered that monitor
resolution has a substantial effect, whereas display size has an
insignificant effect. It is worth noting that when scanning the
same slide with different scanners at a fixed magnification while
keeping and monitor screen resolution unchanged, there was an
insignificant change in the size area of viewing. However, this is
unlikely to be a noticeable issue if the same scanner is routinely
used in a department. The viewing software can also have a
limited effect due to window and toolbar size. Kim et al.28
addressed this point and found that the size of the area
displayed on the screen at ×40 digital magnification is mainly
affected by display resolution, and WSI viewer, but not by
changing the scanner type or scanning resolution. Therefore, it is
important to adjust the area of viewing on the screen to calculate
the number of HPSFs that is needed to produce a defined area
on WSI. A toggle to HPSF could be added to the viewer software
may help in this regard. Table 6 shows the details of the area size
and number of HPSF to cover a defined area, which is either 3 or
2 mm² when counting mitosis using common monitor sizes and
resolution. From the table, it is clear that ten HPSF produced
variable areas and when using certain monitor screens, this area
can be <2 mm².

In a recent study, we showed that there is an average 20% reduction of mitotic counts on WSI compared to light micro-
scopy26. Although this can be explained partially by the scanner
and/or image quality, and lack of familiarity with WSI, we noted
this reduction with most of the commercially available clinical
grade tools without significant improvement with training.
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**Fig. 7** The effect of changing the resolution on the area on WSI. Scatter plots and regressions describing the relation between the area on WSIs measured in mm², and Y-axis represents monitor resolution in megapixels. The size of the area on the WSI, we have found that using screen fields was the most reliable, timesaving, and practical compared with annotated areas. In this study, we suggest using the term digital HPSFs to be used in digital reporting instead of the conventional term HPF applied to microscopes. In this situation, high power (×40) refers to the scanner lens magnification and/or the magnification on the screen. Although some scanners can use a ×20 magnification lens but the ×40 is the standard. In this study, we used a ×40 lens and ×40 magnification power on the screen. Higher magnification can be used to improve the visualization of mitotic figures but the standard mitoses counting on the defined area was performed on ×40 magnification. Several variables could affect the size of the displayed area at ×40 magnification on WSIs. These are mainly the scanning resolution, the viewing monitor resolution, the image viewer software, and the type of scanner used26. We investigated the parameters influencing the size of the area displayed on the screen at ×40 digital magnification and discovered that monitor resolution has a substantial effect, whereas display size has an insignificant effect. It is worth noting that when scanning the same slide with different scanners at a fixed magnification while keeping and monitor screen resolution unchanged, there was an insignificant change in the size area of viewing. However, this is unlikely to be a noticeable issue if the same scanner is routinely used in a department. The viewing software can also have a limited effect due to window and toolbar size. Kim et al.28 addressed this point and found that the size of the area displayed on the screen at ×40 digital magnification is mainly affected by display resolution, and WSI viewer, but not by changing the scanner type or scanning resolution. Therefore, it is important to adjust the area of viewing on the screen to calculate the number of HPSFs that is needed to produce a defined area on WSI. A toggle to HPSF could be added to the viewer software may help in this regard. Table 6 shows the details of the area size and number of HPSF to cover a defined area, which is either 3 or 2 mm² when counting mitosis using common monitor sizes and resolution. From the table, it is clear that ten HPSF produced variable areas and when using certain monitor screens, this area can be <2 mm².
ideally be based on the level of reduction of the mitotic count, and the association with patient’s outcome utilizing a large well-established BC cohort with long term outcome data.

Finally, as we are approaching the approval for scanner use in primary diagnosis, the first objective would be to improve reproducibility and concordance in BC grading, as a result a proposed protocol for agreement on a standard area to count mitoses is a priority when using WSI. Standardizing the area using WSI, together with better recognition of mitotic figures will facilitate the adoption of WSI in routine practice. Counting mitoses in BC can reliably be done on WSI in 3 mm², thereby we recommend using a high-resolution display monitor in routine practice to save time and produce more reliable BC grading. We believe these steps will provide the base evident approach that will be the cornerstone template to develop and validate robust AI-based tools to count mitoses in a standardized manner. They also provide a reliable stopgap for eyeballing assessment of mitoses until these AI algorithms are well validated and implemented.

**DATA AVAILABILITY**
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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| Monitor sizes (inches) | Aspect ratio | Resolution (pixels) | Resolution (megapixels) | WSI viewer area (mm²) | WSI Viewer software | ×40 digital HPSF nearly equivalent to 3 mm² | ×40 digital HPSF nearly equivalent to 2 mm² | Size area of 10 digital ×40 HPSF |
|------------------------|-------------|---------------------|------------------------|----------------------|-------------------|--------------------------------|--------------------------------|-------------------------------|
| 32” Samsung LU32J590UQXEN | 16:09 | 3840 × 2160 | 8.29 | 0.460 | Case Viewer (3DHISTECH) | 7 (3.2 mm²) | 5 (2.3 mm²) | 4.6 mm² |
|                        | 16:09 | 3840 × 2160 |               | 0.436 | IMS (Philips) | 7 (3.1 mm²) | 5 (2.2 mm²) | 4.4 mm² |
|                        | 16:09 | 3840 × 2160 |               | 0.517 | Aperio (Leica) | 6 (3.1 mm²) | 4 (2.1 mm²) | 5.2 mm² |
| 27” BenQ GW2765         | 16:9  | 2560 × 1440 | 3.68 | 0.209 | Case Viewer (3DHISTECH) | 15 (3.1 mm²) | 10 (2.1 mm²) | 2.1 mm² |
|                        | 16:09 | 2560 × 1440 |               | 0.193 | IMS (Philips) | 16 (3.1 mm²) | 11 (2.1 mm²) | 1.9 mm² |
|                        | 16:09 | 2560 × 1440 |               | 0.230 | Aperio (Leica) | 13 (3.0 mm²) | 9 (2.1 mm²) | 2.3 mm² |
| 27” Dell U2719D         | 16:09 | 1600 × 900  | 1.44 | 0.078 | Case Viewer (3DHISTECH) | 39 (3.0 mm²) | 26 (2.0 mm²) | 0.8 mm² |
|                        | 16:09 | 1600 × 900  |               | 0.067 | IMS (Philips) | 45 (3.0 mm²) | 30 (2.0 mm²) | 0.7 mm² |
|                        | 16:09 | 1600 × 900  |               | 0.088 | Aperio (Leica) | 35 (3.1 mm²) | 23 (2.0 mm²) | 0.9 mm² |
| 24” Samsung S24E450B    | 16:09 | 1920 × 1080 | 2.07 | 0.115 | Case Viewer (3DHISTECH) | 26 (3.0 mm²) | 18 (2.1 mm²) | 1.2 mm² |
|                        | 16:09 | 1920 × 1080 |               | 0.102 | IMS (Philips) | 20 (3.1 mm²) | 20 (2.0 mm²) | 1.0 mm² |
| 22” iiyama P2252HS-B1   | 16:09 | 1920 × 1080 | 2.07 | 0.115 | Case Viewer (3DHISTECH) | 26 (3.0 mm²) | 18 (2.1 mm²) | 1.2 mm² |
|                        | 16:09 | 1920 × 1080 |               | 0.102 | IMS (Philips) | 20 (3.1 mm²) | 20 (2.1 mm²) | 1.0 mm² |
|                        | 16:09 | 1920 × 1080 |               | 0.128 | Aperio (Leica) | 24 (3.1 mm²) | 16 (2.0 mm²) | 1.3 mm² |
| 20” HPLA2006            | 16:09 | 1600 × 900  | 1.44 | 0.078 | Case Viewer (3DHISTECH) | 39 (3.0 mm²) | 26 (2.0 mm²) | 0.8 mm² |
|                        | 16:09 | 1600 × 900  |               | 0.067 | IMS (Philips) | 45 (3.0 mm²) | 30 (2.0 mm²) | 0.7 mm² |
| 19” iiyama ProLite E19005D | 05:04 | 1280 × 1024 | 1.31 | 0.072 | Case Viewer (3DHISTECH) | 42 (3.0 mm²) | 28 (2.0 mm²) | 0.7 mm² |
|                        | 05:04 | 1280 × 1024 |               | 0.063 | IMS (Philips) | 48 (3.0 mm²) | 32 (2.0 mm²) | 0.6 mm² |
|                        | 05:04 | 1280 × 1024 |               | 0.081 | Aperio (Leica) | 38 (3.1 mm²) | 25 (2.0 mm²) | 0.8 mm² |
| 17” DELL 210-AEUR       | 04:03 | 1280 × 1024 | 1.31 | 0.072 | Case Viewer (3DHISTECH) | 42 (3.0 mm²) | 28 (2.0 mm²) | 0.7 mm² |
|                        | 04:03 | 1280 × 1024 |               | 0.063 | IMS (Philips) | 48 (3.0 mm²) | 32 (2.0 mm²) | 0.6 mm² |
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**AUTHOR CONTRIBUTIONS**

A. L. scored the cases and wrote the manuscript draft, data analysis, and interpretation. A. G. L. and A. K. helped with double scoring. G. B. and M. S. T. helped in data interpretation. A. G. L., M. S. T., R. M., and A. K. agreed with manuscript results and conclusions and critically reviewed the article. E. A. R. conceived and planned the study, contributed to data interpretation, made critical revisions, and approved the final version.

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**COMPETING INTERESTS**

The authors declare no competing interests.

**ETHICAL APPROVAL**

This study was approved by the Yorkshire & The Humber – Leeds East Research Ethics Committee (REC reference: 19/YH/0293) under the IRAS Project ID: 266925. Informed consent was obtained from all individuals prior to surgery to use their tissue materials in research. All samples were properly coded and anonymized. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The release of data was also pseudo-anonymized as per the UK Human Tissue Act regulations. This article does not contain any studies with animals performed by any of the authors. Informed consent was obtained from all individuals prior to surgery to use their tissue materials in research.

**ADDITIONAL INFORMATION**

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