Graduate thesis

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Faculty of Medicine
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PHH3 in breast cancer

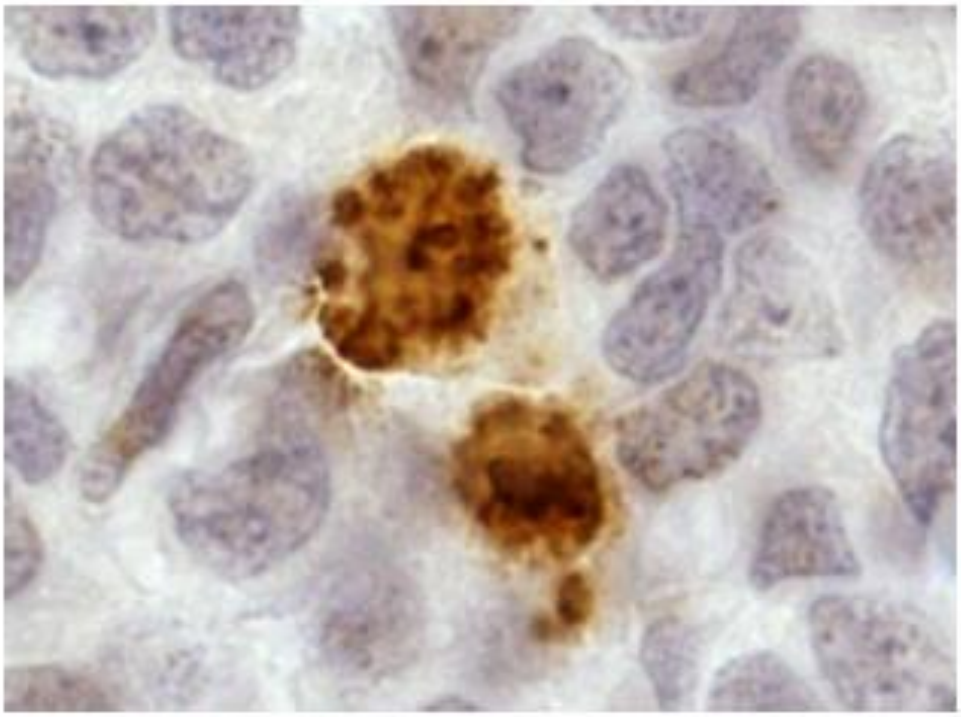
Using immunohistochemistry to visualize mitoses

Graduate thesis in Medicine

Trondheim, January 2016

Main supervisor: Professor Anna M. Bofin, Department of Laboratory Medicine, Children's and Women's Health, NTNU

Co-supervisor: MD PhD Monica J. Engstrøm, Department of public Health and General Practice, NTNU and Department of Breast and Endocrine Surgery, St. Olavs Hospital



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Abstract

Mitotic activity is an independent prognostic indicator in breast cancer and is one of the three morphological characteristics assessed when determining histopathological grade. However, mitotic counts carried out on routinely stained sections are time-consuming and may be subject to inter- and intra-observer variation.

In this study we compared mitotic counts performed on routine, haematoxylin-erythrosine-saffron (HES) stained sections with mitotic counts carried out on sections stained by immunohistochemistry for the mitotic marker Anti-Phospho-Histone H3 (Ser10)(PHH3), a marker of cells in late G2 to M phase. The study population comprised tissue samples from 250 cases of symptomatic breast cancer assembled in tissue microarrays (TMA). All cases were stained with HES and PHH3 and mitoses were counted. Mitotic counts carried out on HES-stained whole sections from the same cases were also available.

We found that mitotic counts in PHH3-stained slides were consistently higher compared to HES-stained slides. However, the results showed good correlation between the methods (0.72 by Pearson correlation test). PHH3 counts correlated well with histopathological grade and molecular subtypes. No clear association with breast cancer specific survival was observed. The number of cases is relatively low and TMA is an additional limitation. However, the results of this study indicate that further studies of PHH3 as an alternative method of visualizing mitoses are warranted.

Introduction

Breast cancer is the most common cancer among women worldwide comprising 25 % of all cancers with an estimated 1.67 million new cases diagnosed in 2012. It is also the second most frequent cause of cancer death after lung cancer among women in more developed regions of the world, while remaining the main cause in less developed areas (1). When diagnosing breast cancer, tumors are classified into histopathological type (2) and grade (3) according to current guidelines. In recent years, molecular subtyping based on gene expression analysis (4) has become more common but is not yet included in standard procedures. Histopathological grade is a well-established, independent prognostic factor, and is determined by microscopic assessment of tubule formation, nuclear pleomorphism, and number of mitoses (Table 1). However, the mitotic count has been shown to be the most reliable constituent of the histopathological grading system (5) and is the only one of the three components with a significant prognostic value when analyzed separately (2).

Table 1: Method for assessing histological grade in breast carcinomas (3)

Feature	Score
Tubule formation	
Majority of tumour (>75%)	1
Moderate degree (10–75%)	2
Little or none (<10%)	3
Nuclear pleomorphism	
Small, regular uniform cells	1
Moderate increase in size and variability	2
Marked variation	3
Mitotic counts	
Dependent on microscope field area	1–3

A number of factors may influence the ease with which mitotic figures are recognized in HES-stained sections. Preanalytical conditions, such as suboptimal fixation, that may distort or perturb the appearance of mitotic figures in the section may lead to over- or under-estimation of mitotic activity. Also, it is difficult to distinguish mitotic cells from apoptotic cells and necrotic cells with hyperchromatic nuclei using HES-staining, making the method vulnerable to inter- and intra-observer variation (Figure 1) (6, 7).

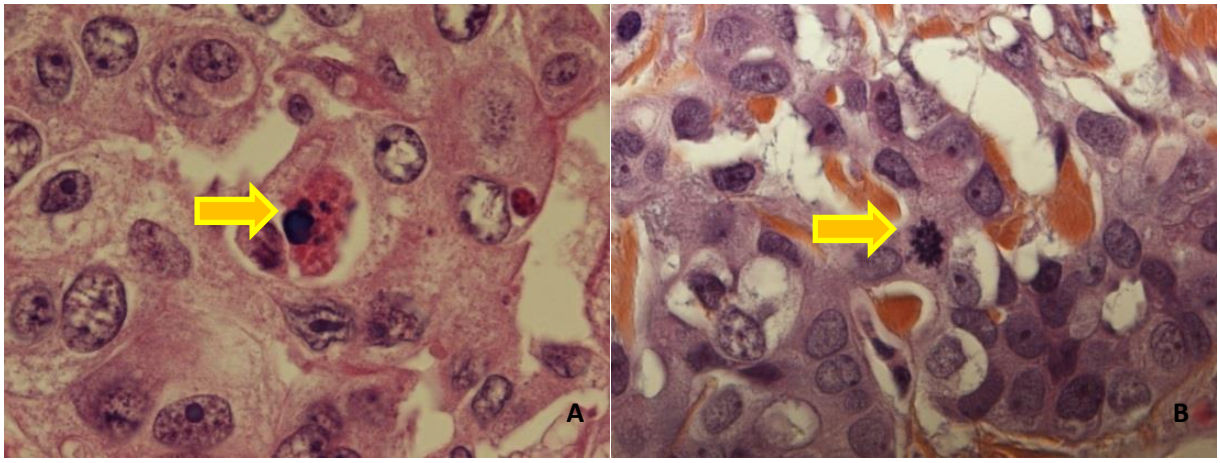

Figure 1: A Apoptosis and B Mitosis (HES x1000) (Photo: A. M. Bofin, NTNU)

Immunohistochemical staining with an antibody that reliably identifies mitosis may result in more precise estimation of mitotic activity. PHH3 is a protein demonstrable from the start of mitosis until the spindle dissolves. By using an antibody that specifically stains for PHH3 when determining mitotic count it may be possible to more accurately identify which cells are undergoing mitosis, and avoid counting apoptotic and damaged cells.

PHH3 is a marker of cells in late G2 to M phase. It is one of the five main histone proteins present in eukaryotic chromatin. Phosphorylation of the amino-terminus of histone H3 (Ser10), begins in late G2 interphase cells at the beginning of mitosis, peaks before metaphase, and is complete in most cells just prior to the formation of prophase chromosomes(8). As explained by Hendzel and co-workers, it is proposed that the main functions of the phosphorylation of histone H3 is regulating protein-protein interactions to promote bindings of trans-acting factors that “drive” chromatin condensation as cells enter the M-phase, and coordinating chromatin decondensation associated with the M-phase.

Dephosphorylation of H3 begins in anaphase, and is complete immediately prior to chromosome decondensation in telophase cells right before the spindle dissolves during the cytoplasm division at the end of mitosis(9). With the strong correlation between H3 phosphorylation and chromatin condensation, and also the fact that histone H3 phosphorylation is low in remaining interphase cells, PHH3 as a proliferation marker is specific exclusively for mitotic cells. In addition, PHH3 is not present in apoptotic cells (10). The antibodies used to detect PHH3 are highly site-specific for the phosphorylated form of histone H3, making them among the better “mitotic markers” available today (11).

The aims of this study were to compare mitotic counts estimated according to current guidelines on HES-stained sections with mitotic counts carried out on sections from the same tumors that have undergone immunohistochemical staining with an antibody for PHH3. The results of both measurements were correlated with histopathological grade and compared.

Materials and methods

Tissue samples

The study population comprises 250 consecutive cases of symptomatic breast cancer included in a cohort previously described by Engstrøm et al (12). The original cohort consists of 909 Norwegian women, born between 1886 and 1928, who developed breast cancer between 1961 and 2008 and for whom formalin-fixed, paraffin-embedded (FFPE) tumor tissue was available. Briefly, new 4µ thick whole sections were cut from representative FFPE blocks from each tumor and stained with HES. All cases were then reviewed independently by two pathologists, and classified according to histopathological type (2) and grade (3), and areas of the tissue material were selected for inclusion in TMA. The TMA method entails using a hollow needle to extract tissue punches from paraffin-embedded donor blocks and insert them into an empty recipient block. Three 1-mm-in-diameter tissue cores were extracted from the tumor edge, based on the assumption that this is the zone with highest cell proliferation. The tissue cores were then inserted into TMA recipient blocks using the Tissue Arrayer Mini-Core® 3 with TMA Designer2 software (Alphelys). This method enables processing and examination of several tissue samples at once by arraying them in a single paraffin block Figure 2.

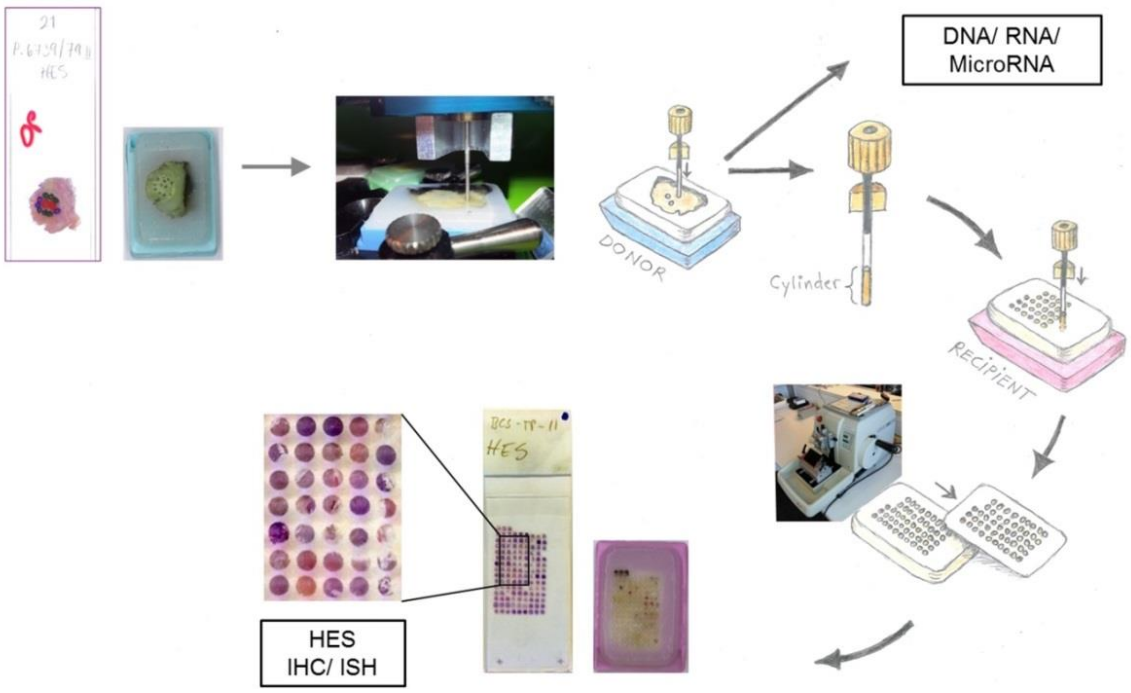

Figure 2 Tissue microarrray procedure, Illustration: LA Dyrnes (NTNU)

The cases in this study were selected by examining HES-stained TMA sections. Cases of insufficient quality or quantity (<500 tumor cells) were excluded. The same series of 250 cases has previously been included in a study of the proliferation marker Ki67 (9).

All cases had been reclassified into molecular subtypes using six different biomarkers as described in Engstrøm et al (12). Respectively Luminal A, Luminal B (HER2-), Luminal B (HER2+), HER2 subtype, five negative phenotype (5NP) and Basal-like phenotype (BP) (Figure 3).

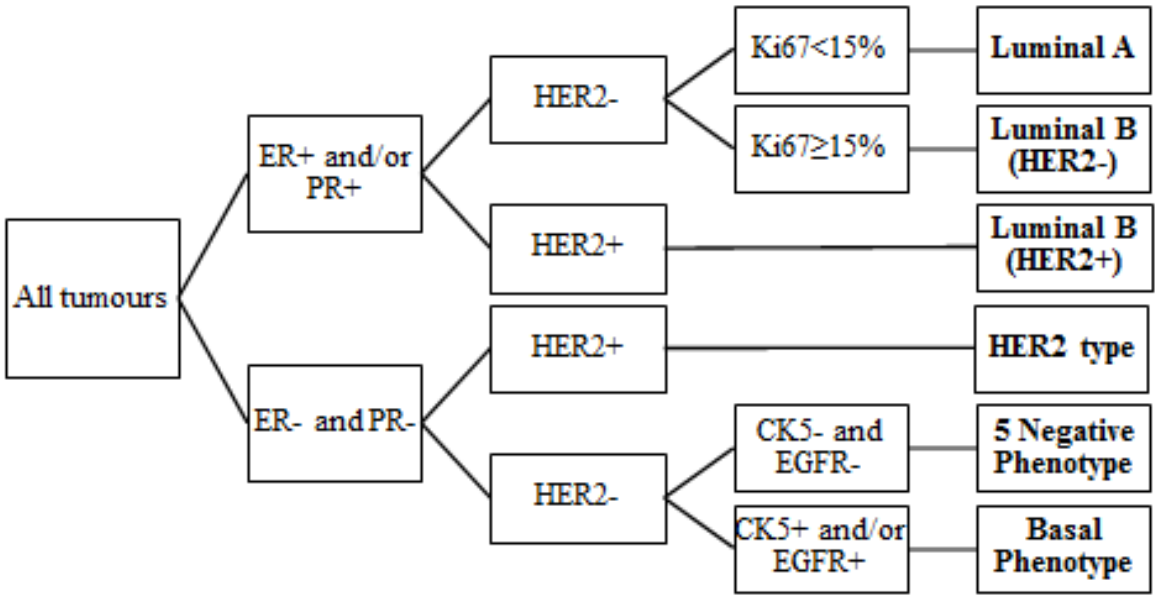

Figure 3: Classification algorithm for molecular subtypes (12)

Immunohistochemistry

From the TMA blocks, 4-µm sections were cut for HES-staining and immunohistochemistry (IHC). For IHC, paraffin sections were mounted on Superfrost + slides (Menzel, Braunschweig, Germany) and dried overnight at 37 °C, followed by 1 hour at 60 °C. Sections were deparaffinized 3 x 5 min in TissueClear (Sakura FineTek, Europe) and rehydrated in decreasing concentrations of alcohol (2 x 3 min in respectively 100 %, 96 % and 80 % alcohol), and 5 min in tap water. Antigen retrieval was performed with Heat Induced Epitope Retrieval (HIER) in DAKO PT-Link (DAKO Denmark A/S, Produktionsvej 42 DK-2600 Glostrup, Denmark) using EnVision FLEX Target Retrieval Solution High pH (pH 9), DM 828, dilution 1:50, as the retrieval buffer. Sections were heated for 20 min at 97 °C and cooled for 30 min to 65 °C. Sections were immunostained using the rabbit polyclonal Anti-Phospho-Histone H3 (Ser10) Mitosis Marker, from MerckMillipore (MerckKGaA, Darmstadt, Germany), at a dilution of 1:2000 for 40 min at 22 °C. Dako REAL Antibody Diluent (S2022) was used. Endogenous peroxidase activity was blocked with Dako REALPeroxidase Blocking Solution (S2023) for 10 min. The immune complex was visualized with the DakoREAL™ EnVision™ Detection System Peroxidase/DAB+ Rabbit/Mouse (K5007).

Sections were incubated with EnVision/HRP Rabbit/Mouse for 30 min and then diaminobenzidine DAB+ chromogen for 10 min. All steps were performed using Dako Autostainer PLUS with Dako Wash buffer (S3006), 1:10. The sections were counterstained with haematoxylin for 30 sec, followed by dehydration for 2 x 3 min in respectively 80 %, 96 % and 100 % alcohol, and then 3 x 3 min TissueClear. Sections were mounted with TissueMount (Sakura FineTek, Europe). Normal liver tissue was used as a control.

Mitotic counts and quantification of PHH3-positive nuclei

HES- and IHC-stained sections from TMA blocks were used to estimate mitotic activity. Mitotic activity was evaluated using a light microscope (Nikon Labophot-2) with an ocular grid in 3 fields of vision (FOV) per case, with a 40x objective (radius 0,235 mm), making the total area examined per case 3,14*0,235*0,235*3=0,52 mm².

In both HES- and PHH3-stained TMA sections visual fields with the highest density of mitoses were targeted. Mitotic counts were assessed on HES- sections by counting only nuclei in which clear morphological features of metaphase, anaphase and telophase were present. Hyperchromatic and apoptotic nuclei were ignored(3). PHH3 assessment was performed according to the guidelines of Skaland et al. (7). PHH3-positive figures in the invasive tumor in a TMA core were counted. Nuclei with fine granular staining were not counted as these are not in the G2-phase (13).

Others studies have shown a cut off value for long-term disease-specific survival of >13 for mitotic counts assessed on 10 FOV on PHH3-stained whole sections (7, 14, 15) . The total area of 10 FOV would be 1.73 mm², while on 3 FOV on TMA sections the total area is 0,52 mm². In the present study the number of positively stained nuclei per case (3 FOVs) was multiplied by 1.73 and dividing by the total area of 3 FOV, 0.52 mm², as described in Klintman et al. (16), to simulate mitotic count in 10 FOV, when comparing to studies on whole sections.

Statistical analysis

The program Stata version 13.1 (Stata Corp. TX) was used for all statistical analyses. Mitotic counts performed on PHH3- and HES-stained sections were compared and visualized by scatter plots. Pearson Correlation was used to test the linear relationship between mitotic counts on HES- and PHH3-stained TMA sections. Breast cancer specific survival for various cut offs of mitotic counts was estimated by Kaplan-Meier methods.

Ethical statement

The Breast Cancer Subtypes project has been approved by the Regional Committee for Medical Health Sciences Research Ethics (REK Midt-Norge, ref. nr: 836/2009) and has been granted dispensation from the usual requirement of informed consent from participants.

Results

Description of the population

Of the 250 cases, 18 (7.2 %) were histopathological grade 1, 141 (56.4 %) were grade 2 and 91 (36.4 %) were grade 3. A total of 178 (72.2 %) were histopathological type ductal and 30 (12.0 %) were lobular carcinomas (Table 2). The characteristics of this population are representative for the original cohort described by Engstrøm et al. (12).

Examples of HES-stained sections and corresponding PH3-stained sections are shown in Figure 4.

Figure 5 shows that mitotic counts on PHH3-stained TMA sections are consistently higher than mitotic counts on equivalent HES-stained sections. However, Pearson Correlation between HES and PHH3 shows a high correlation (0.72). The mean PHH3-stained mitotic count was 14.4, while the mean HES-stained mitotic count was 4.2.

Figure 6 shows mitotic counts on PHH3-stained TMA sections and equivalent HES-stained whole sections (Pearson correlation 0.61). Mitotic counts performed on HES-stained TMA sections and equivalent HES-stained whole sections have a Pearson correlation of 0.63, shown in Figure 7, meaning that PHH3 and HES on TMA give quite similar information.

Table 2: Descriptive statistics for the 250 breast cancer cases

		Total	Percentage
Number (%)		250	100
Mean age at diagnosis (SD[1])		73.9 (7.4)	
Median years of follow-up after diagnosis (IQR[2])		8 (10.2)	
Histopathological grade (%)			
1		18	7.2
2		141	56.4
3		91	36.4
Histopathological type (%)			
Ductal		178	71.2
Lobular		30	12.0
Other		42	16.8
Tumor size (histologically confirmed) (%)			
<2 cm		67	26.8
2-5 cm		116	46.4
>5 cm		18	7.2
Uncerain		49	19.6
Lymph node invasion (histologically confirmed)			
Yes		84	43.2
No		108	33.6
Uncertain		58	23.2
Marker status			
Oestrogen receptor (ER)	Negative	34	13.6
	Positive	216	86.4
Progesterone reseptor (PR)	Negative	101	40.4
	Positive	149	59.6
Human epidermal growth factor receptor 2 (HER2)	Negative	218	87.2
	Positive	32	12.8
Ki67	Low	148	59.2
	High	101	40.4
	Uncertain	1	0.4
Molecular subtype			
Basal phenotype (BP)		15	6
Five negative phenotype (5NP)		6	2.4
Human epidermal growth factor receptor 2 (HER2)		13	5.2
Luminal B (HER2-)		19	7.6
Luminal B (HER2+)		65	26
Luminal A		132	52.8

[1] Standard deviation
[2] Interquartile range

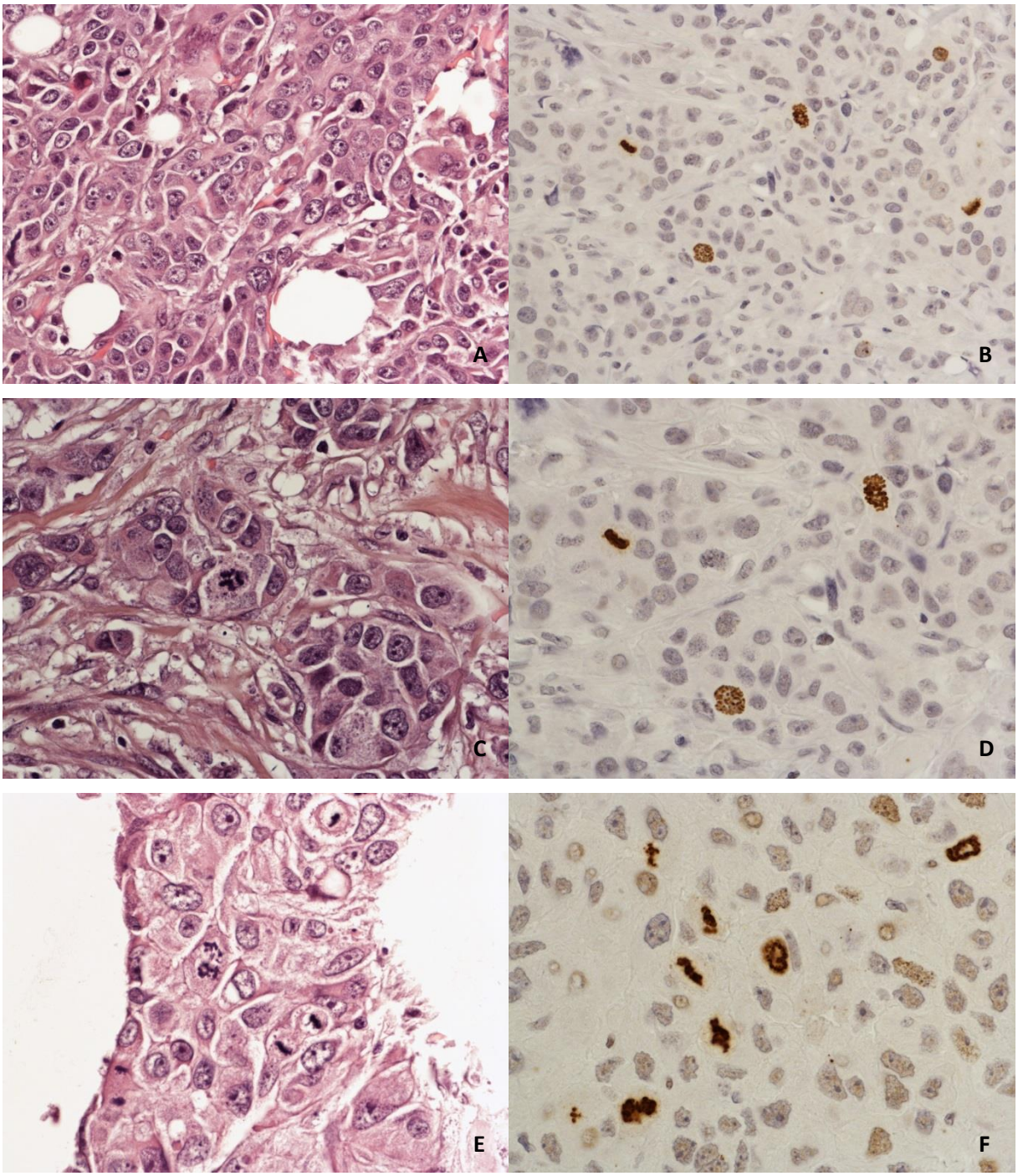

Figure 4: ID624: A HES, B PHH3 (x400). ID624: C HES, D PHH3 (x600). ID599: E HES, F PHH3 (x600). (Photo: Michelle Hansen, NTNU)

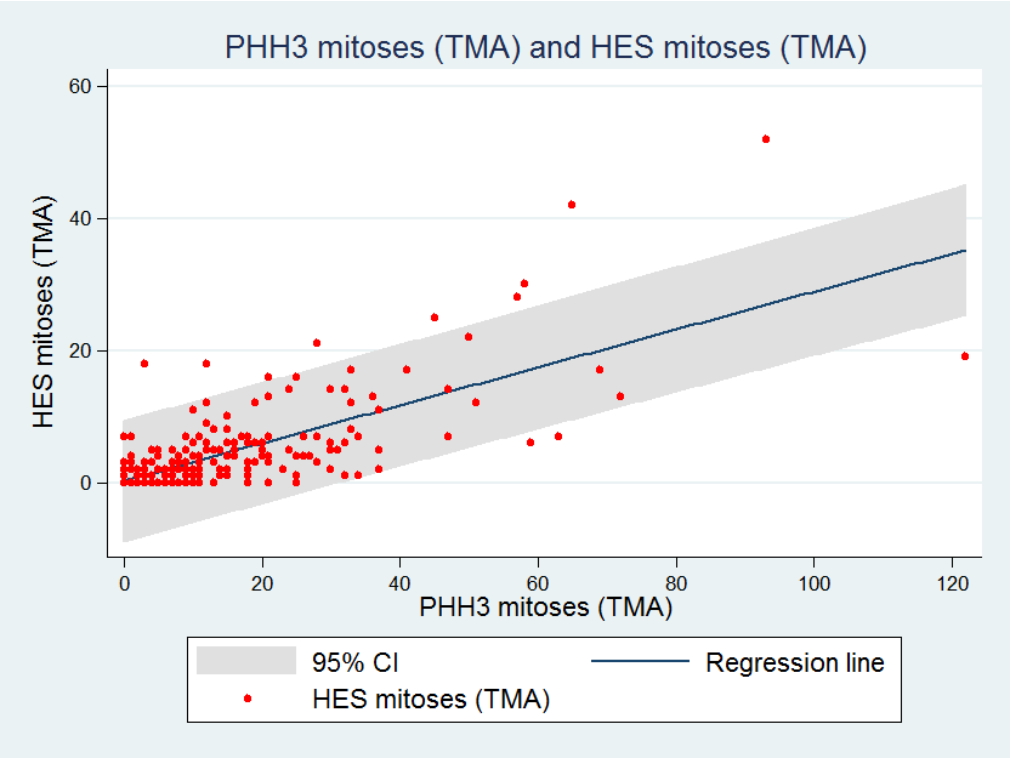

Figure 5: Mitotic counts on phosphohistone H3 and haematoxylin-erythrosine-saffron stained tissue microarray sections for all 250 cases

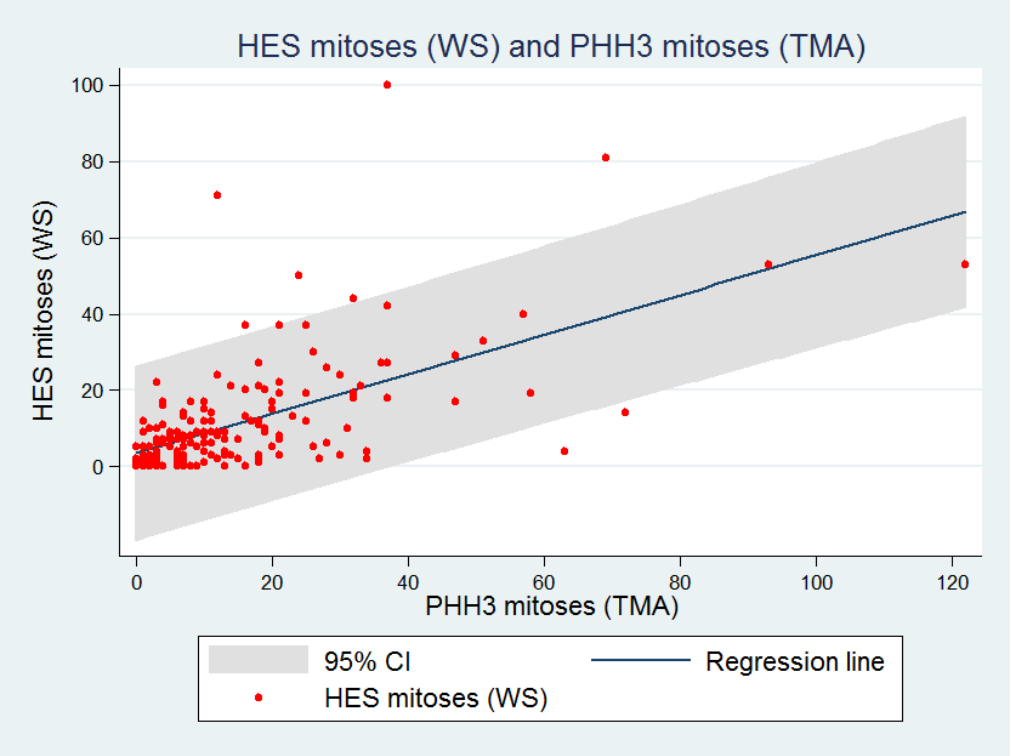

Figure 6: Phosphohistone H3 stained tissue microarray sections and equivalent haematoxylin-erythrosine-saffron stained whole sections for all 250 cases

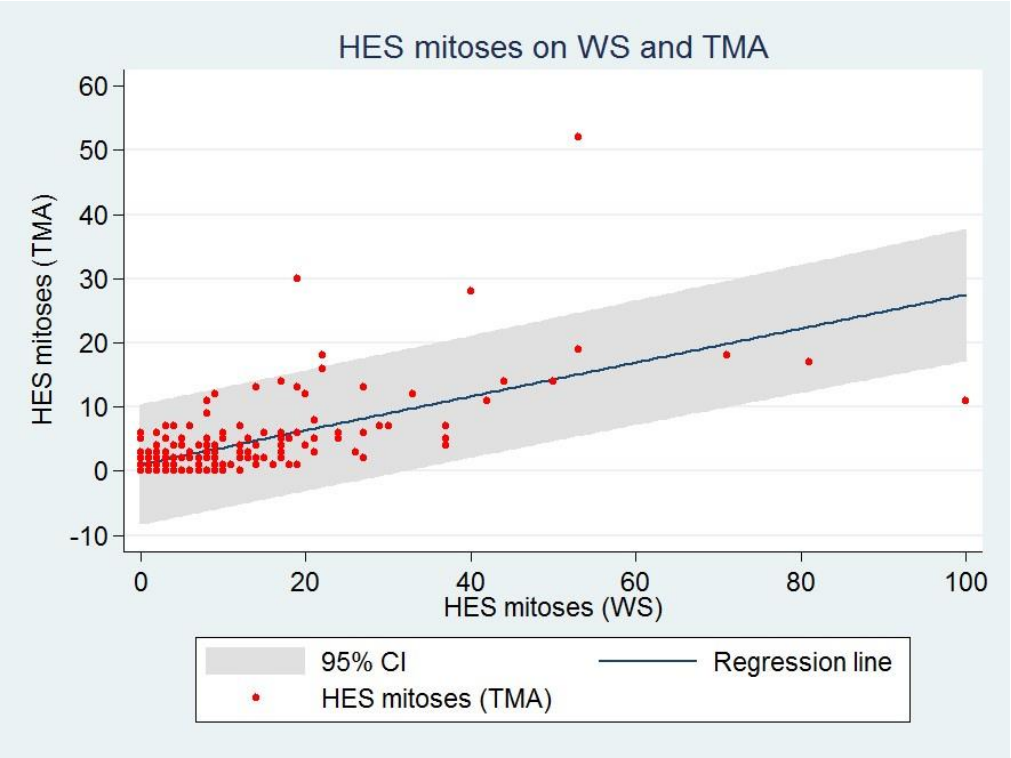

Figure 7: Haematoxylin-erythrosine-saffron stained mitotic counts on whole sections and equivalent tissue microarray sections for all 250 cases

HES	Freq.	Percent
0	67	26.80
1	37	14.80
2	29	11.60
3	20	8.00
4	22	8.80
5	19	7.60
6	10	4.00
7	12	4.80
8	3	1.20
9	1	0.40
10	1	0.40
11	3	1.20
12	4	1.60
13	3	1.20
14	4	1.60
16	2	0.80
17	3	1.20
18	2	0.80
19	1	0.40
21	1	0.40
22	1	0.40
25	1	0.40
28	1	0.40
30	1	0.40
42	1	0.40
52	1	0.40
Total	250	100.00

PHH3	Freq.	Percent
0	12	4.80
1	10	4.00
2	16	6.40
3	20	8.00
4	12	4.80
5	7	2.80
6	11	4.40
7	14	5.60
8	9	3.60
9	8	3.20
10	12	4.80
11	9	3.60
12	6	2.40
13	8	3.20
14	5	2.00
15	6	2.40
16	5	2.00
17	1	0.40
18	9	3.60
19	3	1.20
20	3	1.20
21	7	2.80
23	1	0.40
24	2	0.80
25	4	1.60
26	2	0.80
27	1	0.40
28	3	1.20
30	4	1.60
31	1	0.40
32	3	1.20
33	3	1.20
34	2	0.80
36	1	0.40
37	4	1.60
41	1	0.40
45	1	0.40
47	2	0.80
50	1	0.40
51	1	0.40
57	1	0.40
58	1	0.40
59	1	0.40
63	1	0.40
65	1	0.40
69	1	0.40
72	1	0.40
93	1	0.40
122	1	0.40
999	11	4.40
Total	250	100.00

Table 3: A Mitotic counts in haematoxylin-erythrosine-saffron stained tissue microarray sections B Mitotic counts in Phosphohistone H3 stained tissue microarray sections. 11 sections are registered as 999, due to inadequate quality of sections

Table 3 shows registered mitotic counts for A HES-stained sections and B PHH3-stained sections. Also here it is clear that mitotic counts assessed on PHH3-stained sections are consistently higher.

Figure 8 shows the correlation between mitotic counts on A PHH3-stained and B HES-stained TMA sections and histopathological grade. The number of mitoses is higher in the PHH3-stained sections. However, both for PHH3- and HES-stained sections the plots show an increasing number of mitoses with increasing histopathological grade.

Figure 9 shows the correlation between mitotic counts on PHH3-stained and HES-stained TMA sections and molecular subtypes. These results show that the subtype Luminal A, which has the best prognosis, also has a consistently low mitotic count.

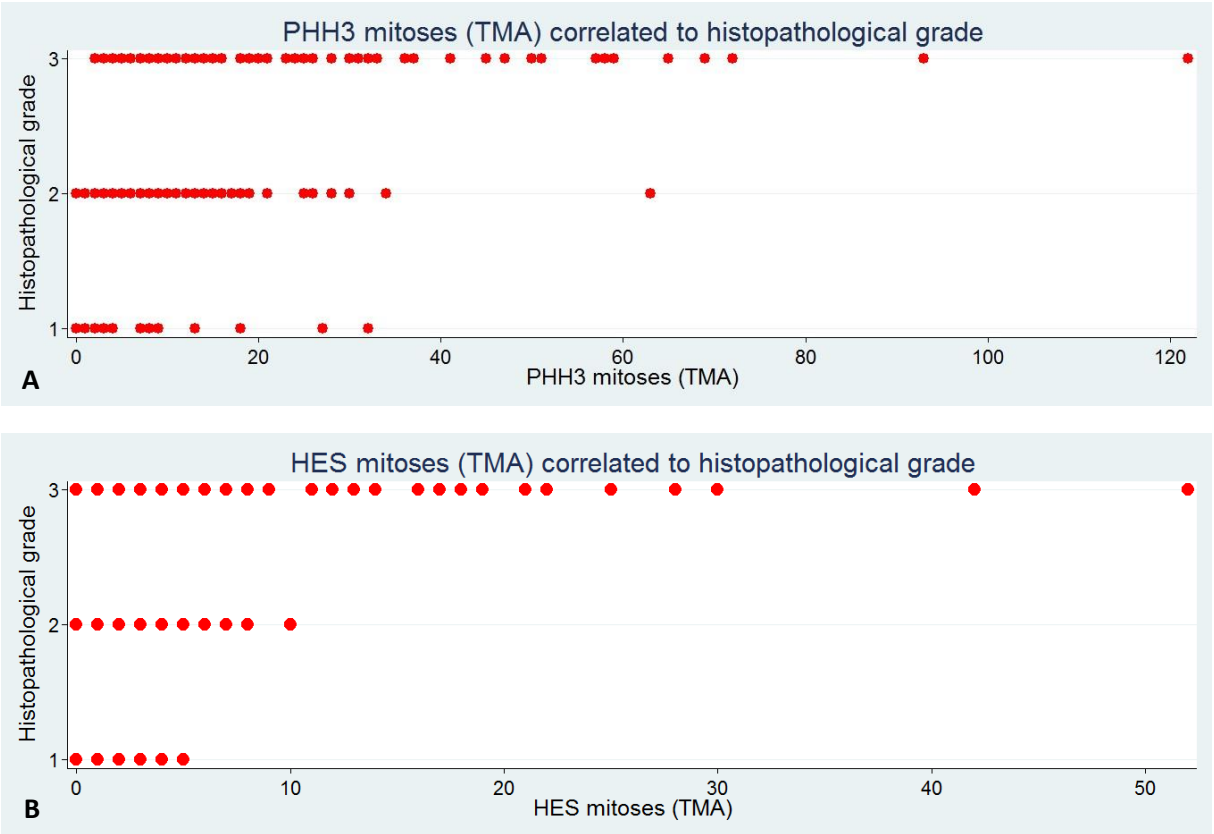

Figure 8: A Phosphohistone H3 stained tissue microarray mitotic counts correlated to histopathological grade B Haematoxylin-erythrosine-saffron stained tissue microarray mitotic counts correlated to histopathological grade

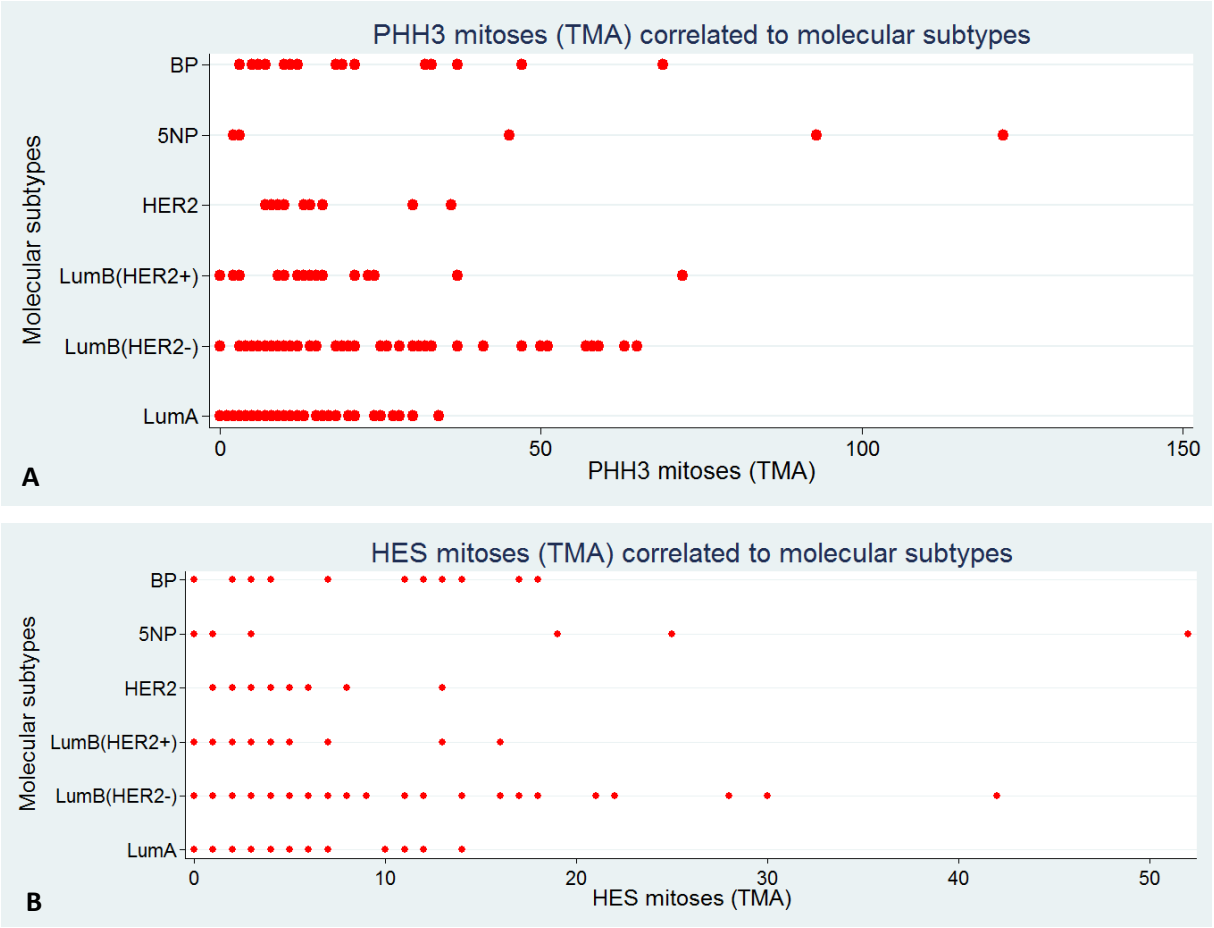

Figure 9: A Phosphohistone H3 stained tissue microarray mitotic counts correlated to subtypes B Haematoxylin-erythrosine-saffron stained tissue microarray mitotic counts correlated to subtypes

The scatterplots in Figures 10, 11 and 12 show the correlation between PHH3 and HES mitotic counts for grade 1, 2 and 3 separately. These highlight that the counts are more specific for grade 1 and 3 than for grade 2, where the results are widely spread.

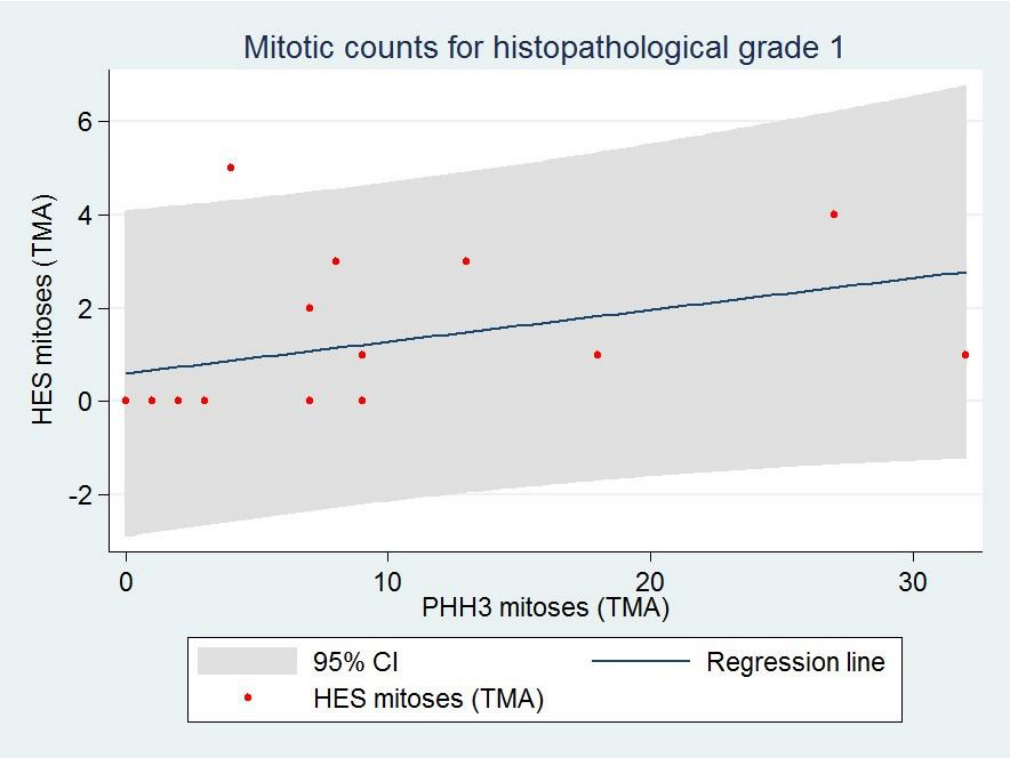

Figure 10: Mitotic counts on phosphohistone H3 and haematoxylin-erythrosine-saffron stained tissue microarray sections for cases of histopathological grade 1

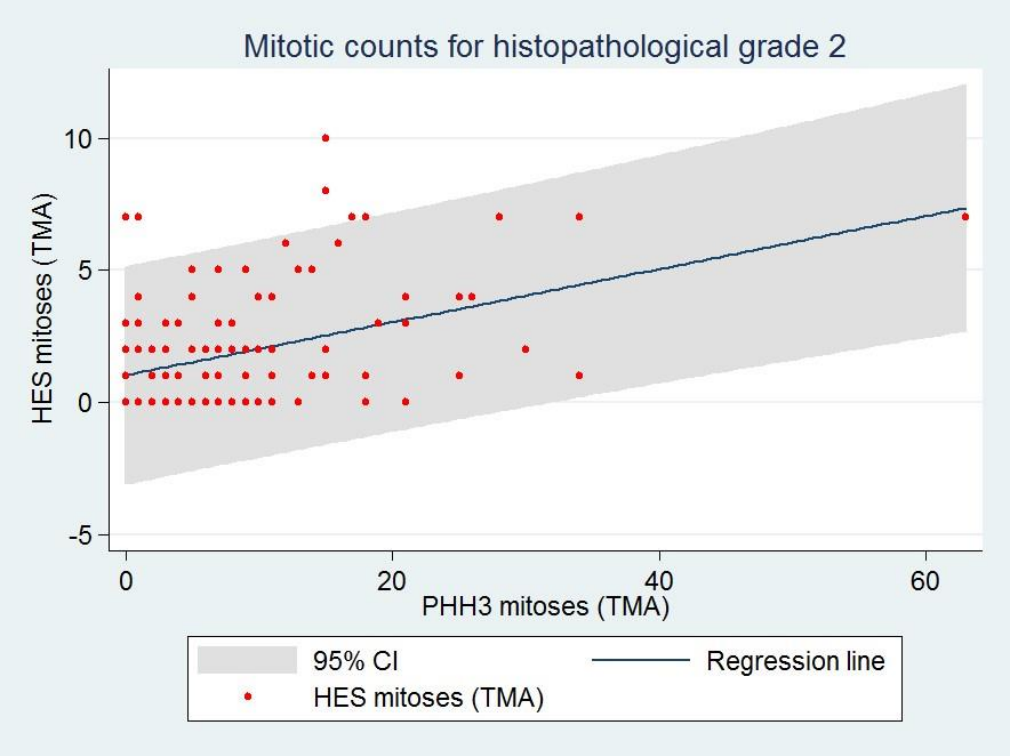

Figure 11: Mitotic counts on phosphohistone H3 and haematoxylin-erythrosine-saffron stained tissue microarray sections for cases of histopathological grade 2

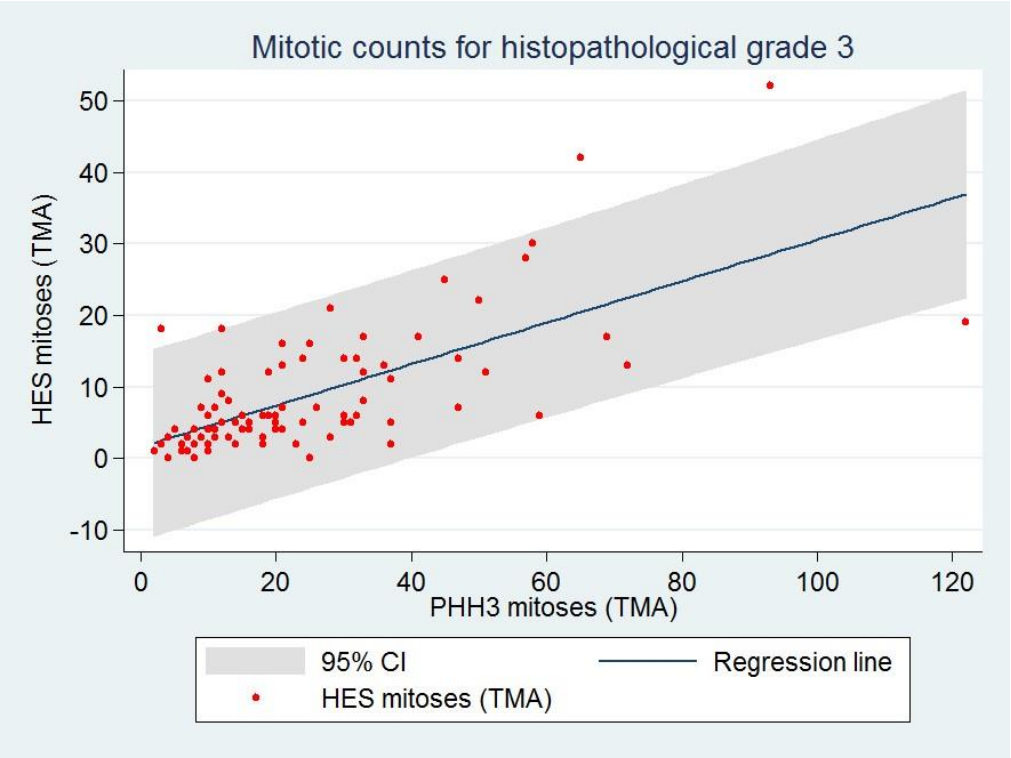

Figure 12: Mitotic counts on phosphohistone H3 and haematoxylin-erythrosine-saffron stained tissue microarray sections for cases of histopathological grade 3

Using Kaplan-Meier methods we tried to find a cut off value for PHH3 mitotic count converted to 10 FOV. Other studies have shown a cut off of mitotic counts >13 on 10 FOV assessed by PHH3. In the present study there were no significant differences in breast cancer specific survival (BCSS) when analyzed with cut offs of 2, 3, 4, 6, 7 or 13 (data not shown).

Discussion

The main findings in the study were that mitotic counts assessed on HES- and PHH3-stained sections show consistently higher numbers of mitoses in PHH3-stained sections (on average 3x higher), but the results still correlate to the HES counts. We were not able to confirm the prognostic cut off value of >13 mitoses/10 FOV for PHH3 shown by others (7, 14, 15).

We experienced that PHH3-staining made evaluation of mitoses easier and quicker, but that it gave the same information as mitotic counts on HES-stained sections.

The varied results of the mitotic counts in grade 2, Figure 11, support our current knowledge that grade 2 tumors present a heterogeneous range of characteristics. Grades 1 and grade 3 tumors are more homogenous in their characteristics and prognosis (5) (Figures 10 and 12). Both mitotic counts assessed on PHH3- and HES-stained sections support this.

PHH3 staining as a method

When assessing mitotic count on PHH3-stained sections, more mitoses are counted as it is possible to identify cells in earlier stages of mitosis which are not visible in HES-stained sections(11). The staining was contrast-rich, and differentiating fine granular staining from positively stained mitotic nuclei was difficult only in a few suboptimal stained sections. In addition, PHH3 does not stain apoptotic cells which can be mistaken for mitotic cells by routine assessment. There was no cytoplasmic staining.

Application of the PHH3-staining method facilitated identification of PHH3-positive nuclei, even in histologically suboptimal sections, and on low magnification. PHH3-staining was especially useful in samples that were difficult to assess on routine HES-stained sections, such as those with a lot of apoptotic cells.

PHH3-staining clearly was helpful to quickly determine the area of the tumor with most cells in proliferation. It also made assessment of mitotic count easier and faster, but the ability to individually evaluate whether each cell is undergoing mitosis or not was still a prerequisite for the observer.

Mitotic activity index (MAI) has been shown to be a robust prognostic marker in younger women (<55 years of age) with node-negative breast cancer. MAI is defined as the number of mitoses in 10 consecutive FOV in a preselected area of the tumor with the highest number of mitoses (10). PHH3-staining could contribute to an improved assessment of mitoses in these cases.

Another advantage with the PHH3 method, as opposed to other methods of assessing cell proliferation, such as Ki67, is that PHH3 assessment only requires counting of positively stained cells, which is less time consuming than current guidelines for assessing Ki67.

Assessment of mitotic count by HES-staining is time consuming and hard to reproduce, as it requires experienced histopathologists. The question remains if PHH3 does give more accurate information than HES-staining, or if it might be easier to use for less experienced pathologists. PHH3 can potentially make assessment of mitotic count faster, easier to assess and reproduce and could be suitable for counting by highly accurate automated digital image analysis (7, 13). It could also be useful as a method to determine histopathological grade. It has been proposed that mitotic counts could be replaced by a much faster but less reliable method called mitotic impression (15). This is a method for estimating an approximate mitotic count by taking a quick glance at a FOV, also known as eyeballing. PHH3-staining could be useful for such a technique, though the value of such an approach is highly questionable.

We found that the number of mitoses varied with molecular subtype and that a low mitotic count was seen in Luminal A tumors using both HES- and PHH3-stained sections. Skaland and co-workers found that PHH3 was a prognosticator of survival in Luminal, Triple negative and Basal-like breast cancers in a cohort of 240 cases of breast cancer among women <70 years of age (15). We failed to demonstrate association with survival. One reason may be the fact that the mean age of our population is higher (73.9 years).

Strengths and weaknesses

The main weakness of this study is the limited area of tumor tissue available for PHH3 – assessment. Furthermore, despite the fact that tissue for TMA was selected from the edge of the tumor, an area that presumptively has a high proliferative capacity, it is known that the density of mitoses varies also at the edge of a tumor. This study did not test inter-observer variation. The low number of mitoses observed particularly in HES-stained TMA-sections, would almost inevitably result in poor inter-observer reproducibility since a difference of 1-2 mitoses would represent a relatively large discrepancy.

The tissue material used in this study was a representative selection of cases, compared to the original cohort of 909 cases. However, the mean age is somewhat high, compared to the general population of breast cancer patients. Most cases were histopathological grade 2 and Luminal A subtype. There were few grade 1 tumors and the numbers of cases in the remaining molecular subtypes were also low. This implies that the results should be interpreted with caution.

Use of the TMA method leads to many different samples stained according to the same procedure, without individual adjustment. It was therefore expected that the PHH3-staining method would not be optimal for all cases in a given TMA section, and overstaining was observed in some samples.

The amount of tissue is considerably smaller in a TMA sample then in a whole sample, so we only assessed mitotic count in 3 FOV instead of 10 FOV. It could be discussed whether these small “biopsies” are representative for the entire tumor. We started out in an identified hot spot. When gradually moving the FOV away from the hot spot there might occur a dilution of mitoses, where the choice between 3 and 10 FOV can make a difference. IHC is relatively inexpensive, and especially when used on TMA slides.

Conclusion

In conclusion, the number of mitoses counted on PHH3-stained sections was higher than on the equivalent HES-stained sections. In the present study, PHH3-staining did not give any more information than HES-staining. The clear, contrast-rich PHH3-staining has an advantage of being easily assessed, and to help distinguish mitotic cells from apoptotic cells (7, 14).

Assessment of mitotic count was easier with PHH3-staining, as it was easier to recognize a cell in mitoses and exclude apoptosis. It was also quicker to identify the area of highest density of mitoses. Using the TMA method when applying PHH3-staining was suboptimal, as the staining is not adjusted to each case.

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