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Mitotic index matter: how to improve the assessment of mitosis in order to better classify G2 breast cancer and luminal A category

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Summary. G2 ductal infiltrating carcinomas are a heterogeneous group of tumours with ambiguous clinical significance. This is because G2 carcinomas are almost always the largest category and poorly reproducible. Mitotic count (MC) is one of the causes of poor histological grading reproducibility. The phosphoistone H3 (PPH3) antibody improves identification of mitotic figures. The aim of our study is to demonstrate whether using a new histological grading system based on PPH3 immunostaining to assess MC can re-stratify G2 category. We selected 100 cases of G2 invasive carcinoma. The mitotic score was accurately re-evaluated performing MC on PPH3 immunostained sections. 21/100 G2 cases (21%) showed the same mitotic score both with hematoxilin and eosin (H&E) and PPH3 while 79 cases (79%) with PPH3 shifted to a higher mitotic score. After re-grading the 100 G2 cases based on the assessment of mitotic score with PPH3 only 53 cases (53%) were confirmed as G2, while 47 cases (47%) had shifted to G3. Finally we reclassified early tumours in the surrogate molecular subtype according to the 2013 St. Gallen Conference criteria and found that 13/40 cases (33%) classified as luminal A were G3 with the PPH3 mitotic score and could benefit from chemotherapy.

In conclusion, PPH3 improving MC gives a better categorization by halving the G2 group. In particular, applied to the surrogate subtype luminal A breast cancer it identified cases that could benefit from adjuvant cytotoxic chemotherapy.

Key words: Nottingham Grading System, Mitotic count, G2 category, PPH3, Luminal A breast cancer

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Introduction

Despite the advent of molecular tests to predict survival and treatment response of patients with breast cancer, the Nottingham (Elston-Ellis) modification of the Scarff-Bloom-Richardson grading system, also known as the Nottingham Grading System (NGS) has been confirmed as one of the most accurate methods to assess tumor biological characteristics and patient prognosis (Rakha et al., 2008, 2010; Schwartz et al., 2014) .

However, several authors have shown that NGS has some limits. NGS is a combined histological grade and is based on three morphological criteria: degree of tubule or gland formation, nuclear pleomorphism and mitotic count (MC). Although all these parameters are subjective, MC reproducibility is commonly responsible for differences in assessment of NGS. In fact MC can be influenced by several factors such as quality of tissue fixation and processing, thickness of the section, size of ocular lens, failure to locate the mitotically most active area of the tumour, criteria for identifying mitotic figures (hyperchromatic, karyorrhectic or apoptotic nuclei must be excluded). One of the main consequences is that the grade 2 (G2) category is poorly reproducible. In fact in several published series G2 carcinomas constitute 30-60% of all cases (Meyer et al., 2005; Longacre et al.,

2006). Moreover, the G2 category is almost always the largest (Le Doussal et al., 1989). Consequently, G2 is a category with ambiguous clinical significance and its prognosis often overlaps with prognosis of grade 1 (G1) or grade 3 (G3). It has been demonstrated that G2 includes a mixed population with heterogeneous gene expression profiles that range from those for G1 tumours to those for G3 tumours (Ivshina et al., 2006; Viale, 2011).

To improve the reproducibility of NGS and MC, consensus criteria and guidelines for standardization of pre-analytic and analytic parameters (van Diest et al., 1992; Robbins et al., 1995; Paradiso et al., 2009; Pathology Reporting of Breast Disease, 2005; Ellis et al., 2006) have been provided. Nevertheless, the problem remains unresolved.

The phosphoistone H3 antibody (PPH3) labeling has been proposed to improve the MC in breast cancer (Bossard et al., 2006).

Specifically, it targets cells in mitosis and allows, compared to hematoxylin and eosin (H&E) staining, a clear and non-ambiguous identification of mitotic figures, especially prophase, and distinguishes mitosis from nuclei of cells in apoptosis or necrosis allowing a quicker detection of the area of highest mitotic activity. The antibody PPH3 recognizes histone H3 after it becomes phosphorylated on serine 10 when the chromosomes condense during prophase. Histone 3 remains phosphorylated until telophase, when it becomes dephosphorylated by specific phosphatases.

It has been demonstrated that PPH3 in breast cancer has a strong prognostic value in patients with lymph node negative breast cancer (Skaland et al., 2007, 2009a,b)

Recently, a surrogate molecular classification of early breast cancer based on immunohistochemistry that provides information to determine individual treatment and predict treatment efficacy has been proposed (Goldhirsh et al., 2013). The aim of our paper is to demonstrate that using a new histological grading system based on immunohistochemistry can re-stratify the heterogeneous G2 category and provide more information regarding treatment strategies compared to conventional NGS using clinicopathological subtyping (St. Gallen 2013). This system uses PPH3 to assess MC. It has been demonstrated that NGS may play a critical role in treatment strategies for breast cancer based on this current molecular subtype classification method (Maisonneuve et al., 2014).

To achieve this we selected from our files 100 consecutive cases of invasive ductal carcinoma, originally diagnosed as G2 tumours and re-graduated them according to NGS evaluating MC on PPH3 immuno-stained sections. Finally, we reclassified all early breast cancers in surrogate intrinsic subtypes according to the 2013 St. Gallen Conference (Goldhirsh et al., 2013).

Materials and methods

We selected 100 cases of invasive ductal carcinoma G2 from a cohort observed consecutively at our Institution between May 2008-April 2011. Cases with insufficient or non-representative material remaining in the paraffin block or cases with a suboptimal level of tissue fixation were excluded. The study was approved by the Institution's ethics committee. All cases were surgical specimens. Patient age, surgical treatment, primary tumour size, growth pattern, lymph node stage, main prognostic and predictive parameters, estrogen receptor (ER), progesterone receptor (PgR), HER-2 and ki-67 proliferative index, were obtained from our database. At diagnosis, no patient had systemic metastases. Staging was according to the 7th edition of the American Joint Committee on Cancer (AJCC) TNM. All tumours were graded according NGS (Elston and Ellis, 1991). In the surgical pathology report of each case in addition to the grade, individual NGS score components were reported. In order to compare MC on PPH3 staining with MC on H&E we have revalued it on the H&E section.

For revaluation of MC with H&E stain and immunohistochemistry for PPH3 new consecutive sections were prepared from the representative paraffin blocks. Immunohistochemistry was performed on 4 μm sections using the polyclonal antibody phospho-histone H3 (Cell Signaling, diluition 1:200). The primary antibody was detected using a biotin -free polymeric-horseradish peroxidase (HRP)-linker antibody conjugate system (Bond Polymer Refine Detection, Leica BioSystems, Newcastle UK) with heat-induced epitope retrieval, using the Bond III automated immunostainer (Leica BioSystems, Melbourne).

H&E and immunostaining sections were evaluated jointly by two pathologists under a multi-head microscope, Nikon Optiphot-2 diagnostic microscope 0,50 mm diameter, 40x magnification field, corresponding to score cut-off for mitotic counts of ≤ 7 mitosis (score 1), 8-14 mitosis (score 2) and ≥15 mitosis (score 3).

Mitotic count with H&E staining (CMC)

The van Diest et al. guidelines (van Diest et al., 1992) were applied for correct identification of mitotic figures. They were counted in 10 consecutive high power fields starting from the area with highest mitotic density. We selected the area with subjectively the highest density of mitotic figures in the tumour periphery in the invasive growing zone avoiding fields with necrosis, inflammation or tissue folds. Two parallel separate chromosome clots were counted as separate mitosis. Only those mitotic figures for which consensus was obtained by both observers were counted.

Mitotic count with PPH3 immunostaining (PPH3MC)

The cells showing nuclear immunostaining for PPH3 were counted in 10 consecutive fields in a similar manner to MC determined on H&E slides.

PPH3-positive nuclei with morphological features of prophase, metaphase, anaphase and telophase were counted. Two parallel separate chromosome clots were counted as separate mitosis.

Molecular biomarkers

ER (Leica, clone 6F11, ready to use) was scored positive when nuclear staining was present in ≥1 % of tumour cells. PgR (Leica, clone 16, ready to use) and ki-67 (Dako, clone MIB-1, diluition 1:100) were considered high when nuclear staining was present in ≥20% of tumour cells. For ki-67 assessment, we used a hot-spot method: at 10x a hot spot was identified and 100 tumour cells were counted at 40x. HER-2 protein was detected and scored according to the Dako Herceptest (for automated Link Platforms, Copenhagen, Denmark). All 2+ cases were tested for gene amplification by HER-2 FISH (fluorescent in situ hybridization) (Path Vysion HER-2 DNA Probe kit II Abbott). All cases with amplified HER-2 gene, and all with Herceptest 3+ were considered HER-2 positive.

Tumours were classified by immunohistochemical staining according to 2013 St Gallen subtypes (Goldhirsh et al., 2013) as follows: luminal A (ER+, PgR high, HER-2-, Ki-67 low), luminal B HER- (ER+, HER-2-, and Ki-67 high or PgR - or low), luminal B HER-2+ (ER+ and HER-2+), HER-2+ (non luminal) (HER-2+, ER- and PgR-), triple negative (HER-2-, ER-, PgR-).

Statistical analysis

Continuous variables were compared using t test. To asses the relationship between MC on H&E and on PPH3, Spearman's correlation was used. χ^2 tests were used to compare category variables. Values of $p < 0.05$

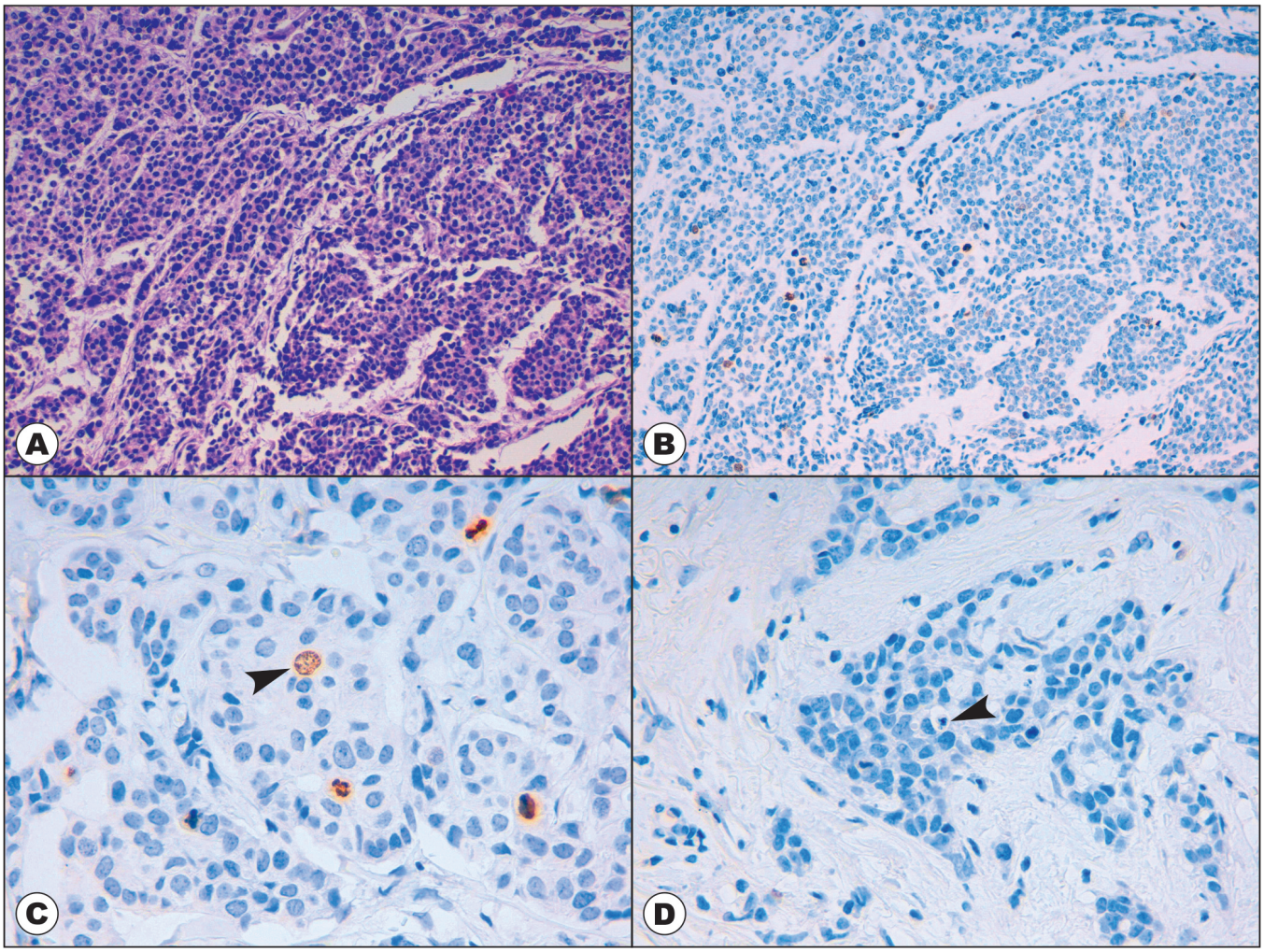

Fig. 1. PPH3 staining rather than H&E allows a quick identification of the areas of highest mitotic activity at low-power magnification **(A, B)**. PPH3 staining labels prophase nuclei (arrow head) **(C)** but not apoptotic nuclei (arrow head) **(D)**. A, B, x 200; C, D, x 400.

were considered statistically significant. Analyses were conducted using SPSS software.

Results

On PPH3 sections we quickly identified at the periphery of the tumour the areas of highest mitotic activity (hot spot) at low-power magnification (Fig. 1A,B) and the time spent to perform MC was significantly less. PPH3 staining strongly labeled all mitotic figures as metaphase, anaphase or telophase. In addition it also allowed us to identify prophase nuclei (Fig. 1C) that in H&E stain are not easily identifiable. They are characterised by intense, dense staining of chromatin clumps. The apoptoses were negative for PPH3 immunostaining (Fig. 1D). The interphasic nuclei (non-mitotic nuclei) were excluded from the counts. They were characterized by weak, granular, nuclear immunostaining and intact nuclear membranes (Fig. 2). The MC of 100 carcinomas, performed on H&E sections, showed a mean number of mitoses of 6.87 (median 5.5, range 1-37) while on PPH3 sections there were 16.86 mitoses (median 16, range 2-60). The difference between the means was statistically significant ($p<0.000$). As expected there was a positive correlation between CMC and PPH3MC ($r_s=0.539$, $p<0.000$). Finally, we correlated the other NGS parameters, tubule formation and nuclear pleomorphism, with MC. Comparing PPH3 with H&E an average of more mitoses in the tumours with morphological features, poorly differentiated as marked nuclear pleomorphism (score 3) (16.63 vs 5.47, $p<0.03$) or with little or no tubule formation (score 3) (15.86 vs 5.81, $p<0.000$) was observed.

Histological grading

Table 1 gives the distribution of mitotic score with the two stains in the 100 G2 cases. Twenty-one (21%) cases had the same mitotic score with both stains while 79 (79%) cases shifted with PPH3 stain to a higher mitotic score. Among the 63 cases with mitotic score 1 on H&E, 30 (48%) shifted to score 2 with PPH3 and 22 (35%) cases to score 3. Of the 35 cases with H&E score 2, 26 (76%) shifted to score 3 and only 1 case (3%) shifted to score 1.

Table 1. Distribution of mitotic scores between two stains in 100 G2 cases.

H&E stain	PPH3 immunostain				
	score 1 n (%)	score 2 n (%)	score 3 n (%)	Total n (%)	p
score 1	11 (17)	30 (48)	22 (35)	63	0.002
score 2	1 (3)	8 (23)	26 (74)	35	
score 3	0	0	2 (100)	2	
Total	12 (12)	38 (38)	50 (50)	100	

score 1: ≤7 mitosis; score 2: 8-14 mitosis; score 3: ≥15 mitosis (according to Elston and Ellis 1991).

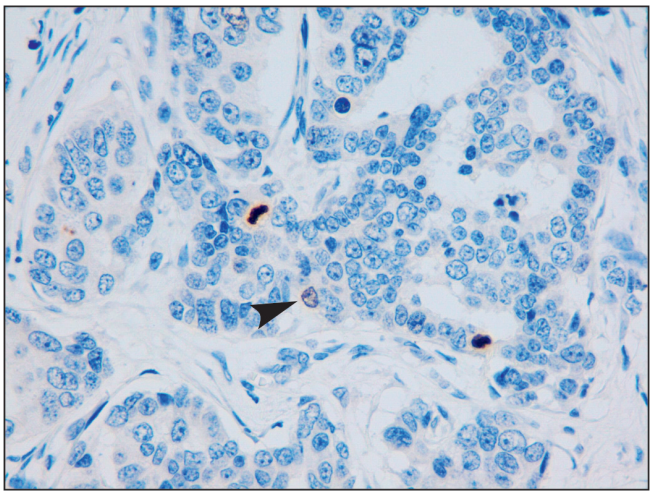

Fig. 2. All mitotic figures show strong positivity for PPH3. The interphasic nuclei (non-mitotic) are weakly positive (arrow head). x 400.

Table 2. Distribution of nuclear and tubule scores in relation to the mitotic score on H&E and PPH3 stains.

NGS score components		H&E stain			PPH3 stain		
Mitotic score	Pleomorphism Tubule	score 1	score 2 *n (%)*	score 3	score 1	score 2 *n (%)*	score 3
1	score 1	0	0	0	0	0	0
	score 2	0	0	7 (11)	0	0	0
	score 3	0	46 (73)	10 (16)	0	10 (83)	2 (17)
2	score 1	0	0	0	0	0	0
	score 2	0	9 (26)	2 (9)	0	3 (8)	3 (8)
	score 3	0	24 (68)	0	0	27 (71)	5 (13)
3	score 1	0	0	0	0	0	0
	score 2	0	2 (100)	0	0	8 (16)	6 (12)
	score 3	0	0	0	0	33 (66)	3 (6)

Finally we re-graded the 100 G2 cases according to the new NGS based on assessment of mitotic score with PPH3 (PPH3NGS) and found that only 53 cases (53%) were confirmed as G2, and 47 cases (47%) had shifted to G3. Table 2 shows the distribution of nuclear pleomorphism and tubule scores in relation to the mitotic score on H&E and PPH3 stains.

Histological grading system based on immunohistochemistry and histopathological features

Table 3 shows the clinicopahological features of 100 cases re-graded according to PPH3NGS. We found significantly positive correlation between grading and Ki-67 and HER-2 status. Due to the short follow-up period no correlations were observed with outcome. To verify if the re-grading of tumours according to PPH3NGS provides more precise information regarding therapeutic management compared to CNGS we reclassified the early tumours (stage I and II) in the surrogate subtype according to the 2013 St. Gallen Conference criteria (Goldhirsh et al., 2013). Seventy-seven (77%) of the 100 G2 carcinomas were early breast cancers.

Table 4 shows the stratification of 77 G2 early breast cancers among PPH3NGS. We found that 14/40 (35%) luminal A cases were G3 (Fig. 3) and of these 13 cases were pN0. The mean size was 15 mm (range 8-25), and mean age of patients was 67 years (range 42-83).

Table 3. Clinicopathological features of the 100 cases studied subdivided in relation to PPH3 grading.

Features	G2	G3	Total	p
Mean age year (range)	62 (33-91)	67 (35-96)		
Surgical treatment				
Quadrantectomy	36 (53%)	32 (47%)	68	ns
Mastectomy	17 (53%)	15 (47%)	32	
T				
T1b	8 (57%)	6 (43%)	14	ns
T1c	32 (56%)	25 (44%)	57	
T2-3-4	13 (46%)	15 (54%)	28	
Vascular invasion				
No	42 (53%)	37 (47%)	79	ns
Yes	11 (52%)	10 (48%)	21	
Growth pattern				
Expansive	13 (43%)	17 (57%)	30	ns
Infiltrative	40 (57%)	30 (43%)	70	
Lymph-node status				
Negative	29 (53%)	26 (47%)	55	ns
Positive	18 (55%)	15 (45%)	33	
Nx	6 (50%)	6 (50%)	12	
Stage				
I	26 (53%)	23 (47%)	49	ns
II	16 (57%)	12 (43%)	28	
III	5 (45%)	6 (55%)	11	
ND	6 (50%)	6 (50%)	12	
ER				
≥1%	50 (55%)	40 (45%)	90	ns
<1%	3 (30%)	7 (70%)	10	
PgR				
≥20%	41 (57%)	31 (43%)	72	ns
<20%	12 (43%)	16 (57%)	28	
Ki-67				
<20%	44 (62%)	28 (38%)	72	0.01
≥20%	9 (32%)	19 (68%)	28	
HER-2				
Negative	47 (58%)	34 (42%)	81	0.03
Positive	5 (28%)	13 (72%)	18	

Discussion

G2 infiltrating ductal carcinoma is a heterogeneous group of tumours with an unpredictable course of disease, due to the fact that class G2 is the less reproducible category (Meyer et al., 2005; Longacre et al., 2006) and in several published series the proportion ranged between 30% and 60% cases. The inter and intra-

Table 4. Surrogate intrinsic subtypes in 77 early breast cancers subdivided in relation to PPH3 grading.

Subtypes	G2 n (%)	G3 n (%)	Total n (%)	p
Luminal A	26 (65)	14 (35)	40 (52)	ns
Luminal B (HER-2-)	10 (48)	11 (52)	21 (27)	
Luminal B (HER-2+)	4 (40)	6 (60)	10 (13)	
HER-2+	0	2 (100)	2 (3)	
Triple negative	2 (50)	2 (50)	4 (5)	

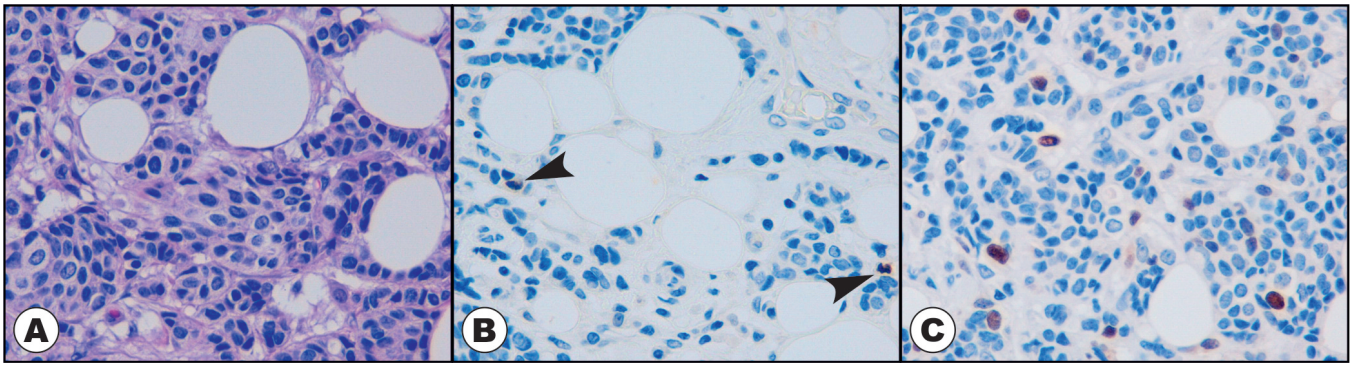

Fig. 3. Luminal A case (**A,** H&E) become G3 with PPH3 mitotic count (mitosis arrow head) **(B)**. Ki-67 proliferative index is 7% **(C)**. x 400.

observed studies (Longacre et al., 2006) have shown that the greatest cause of poor reproducibility of NGS is nuclear pleomorphism followed by MC and tubular formation. However, it has been shown that it is possible to improve reproducibility of NGS by carefully following guidelines and consensus criteria that provide recommendations on tissue handling, histological grading methods and promoting training courses (Robbins et al.,1995; Pathology Reporting of Breast Disease, 2005; Ellis et al., 2006, Paradiso et al., 2009).

Regarding this, we selected for NGS revaluation on H&E only those cases with an optimal fixation level and performed MC on H&E and PPH3 sections following the protocol suggested by van Diest (van Diest et al., 1992) . A recent paper has suggested that the automatic MC with a computerized system can accelerate the pathologist's work and improve reproducibility (Malon et al., 2012).

Proliferative activity is a well-established prognostic marker in breast carcinoma and MC is not only one of the components of NGS but, used independent of grade, can divide the G2 category patients into two different prognostic groups, particularly among patients with positive lymph node or ER+ (Lynch et al., 2002).

PPH3 immunohistochemistry has been used for mitotic cell counting, also using automated image analysis (Bossard et al., 2006; Zbytek et al., 2013) in different types of breast (Bossard et al., 2006) and female genital tract (Scott et al., 2004; Aune et al., 2011; Brunner et al., 2012), meninges (Ribalta et al., 2004; Fukushima et al., 2009) brain (Colman et al., 2006), larynx (Chatrath et al., 2006), urothelium (Gunia et al., 2012), lung (Tsuta et al., 2011), soft tissue (Idriss et al., 2013), esophagus (Nakashima et al., 2013), adrenal gland (Duregon et al., 2014), prostate (Goltz et al., 2015) and melanoma (Nasr et al., 2008) tumours .

The strong correlation between PHH3 count and mitotic index has been confirmed in multiple studies (Bossard et al., 2006) and detection of mitotic figures via PPH3 has been described as having greater sensivity due to enhanced detection of prophase cells and better specificity due to lack of staining in apoptotic cells.

In breast ductal carcinoma it has been shown that PPH3 count correlates strongly with CMC (Bossard et al., 2006) and is a strong prognostic variable in early invasive breast cancer (Skaland et al., 2007, 2009a,b).

Also in our study the average number of mitoses counted with PPH3 was significantly higher than that counted with H&E with a positive correlation between CMC and PPH3MC (r_s=0.539, p<0.000).

PPH3 has been correlated with other markers of cellular proliferation such as ki-67, cyclin B1, cyclin A and MAI (proliferation factor mitotic activity index) (Gudlaugsson et al., 2013; Lee et al., 2014) and the literature reports a considerable variability in correlation, especially between ki-67 and PPH3 count. These discrepancies (Klintman et al., 2013) were explained in part by the heterogeneity of tumours and in part by cell cycle abnormalities found in malignant tumours. In fact, in high-grade tumours that are likely aneuploid, there is an increase in the duration of the interphase (G1-S-G2 phase), especially S phase, compared to the mitotic phase. In addition, Ki-67 unlike PPH3, is expressed not only in M phase of the cell cycle but also in G1, S, and G2. Moreover, some cells labelled by ki-67 will not complete the cell cycle but undergo apoptosis.

We found, as expected, more mitosis in tumours with poorly differentiated features (marked nuclear pleomorphism and little or no tubule formation). This observation was amplified when the mitotic score was obtained with PPH3 staining, since a significant increase in score 3 cases and a reduction of score 1 cases was observed as shown in table 2.

We observed that the majority of 100 G2 cases with PPH3 stain shifted to a higher mitotic score. Only 1 case with PPH3 shifted from score 2 to score 1. Probably in this case, the MC on H&E section was overestimated for the presence of hyperchromatic, karyorrhetic or apoptotic nuclei.

Finally, we found that re-grading the 100 cases with PPH3 the G2 category was halved. This result demonstrates that PPH3 can re-stratify the G2 cases, reducing the number of this category and identifying cases with different prognosis. In fact we found that 68% of G3 cases identified by PPH3 have Ki-67≥20% and 72% of cases are HER-2 positive.

We re-classified G2 early breast cancers according to the 2013 St Gallen guidelines (Goldhirsh et al., 2013).

Interestingly, we observed that 14 of the 40 luminal A cases (35%) initially diagnosed as G2 were G3 with PPH3 stain and of these 13 (33%) cases were pN0. Although these observations do not cause upgrading of the molecular type they could have some therapeutic and prognostic consequences. In fact, the luminal A-like subtype tumours are indolent, with better prognosis and generally endocrine sensitive but cases with a high grade and pN0, as established by the 2013 St Gallen guidelines can benefit from chemotherapy.

In conclusion the main results of our study are:
- PPH3, improving mitotic count, allows identification of more cases with mitotic score 3,
- new grading system based on PPH3 can improve, consequently, categorization of G2 cases, halving this group,
- the better categorization of the G2 group, using a simple immunohistochemical analysis, allows identification within the surrogate luminal A subtype, cases that could benefit from adjuvant cytotoxic chemotherapy.

These results may suggest the routine use of PPH3 immunostaining to evaluate MC in breast cancer although this must be confirmed with larger case studies with longer follow-up.

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