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ORIGINAL ARTICLE

Interlaboratory variability of MIB1 staining in well-differentiated pancreatic neuroendocrine tumors

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Abstract Neuroendocrine tumors (NET) are routinely graded and staged to judge prognosis. Proliferation index using MIB1 staining has been introduced to assess grading. There are vivid discussions on cutoff definitions, automated counting, and interobserver variability. However, no data exist regarding interlaboratory reproducibility for low proliferation indices which are of importance to discriminate between G1 and G2 NET. We performed MIB1 staining in three different university hospital-based pathology laboratories on a tissue micro array (TMA) of a well-characterized patient cohort, containing pancreatic NET of 61 patients. To calculate the proliferation index, number of positive tumor nuclei was divided by the total number of tumor nuclei. Labeling index was compared to mitotic counts in whole tissue sections and to clinical outcome. Linear regression analysis, intraclass comparison, and log-rank analysis were performed. Intraclass correlation showed moderate-to-fair agreement. Especially low proliferating tumors were affected by interlaboratory differences. Log-rank analysis was performed for each lab and resulted in three different cutoffs (5.0, 3.0, and 0.5 %). Every calculated cutoff stratified the patient cohort to a significant extent for the underlying stain ($p < 0.001$, <0.001, and <0.001) but showed no or lesser significance when applied to the other stains. Significant and relevant interlab differences for MIB1 exist. Since the MIB1 proliferation index influences grading, local cutoffs or external standardization should urgently be introduced to achieve reliability and reproducibility.

Keywords Interlaboratory variability · MIB1 · Ki67 · Neuroendocrine · Grading

Introduction

Pancreatic neuroendocrine tumors (pNET) have a wide range of biological behavior which is difficult to predict [1–4]. Metastasis is the most important predictor of survival, but also among metastasized tumors, the survival time is still very variable [2, 5–7]. In the absence of metastases, prediction of relapse is also challenging. To date, pNET are stratified mainly according to stage and grade. Grading has been introduced as an important parameter by the WHO 2004 classification [8] and has formally been proposed by the European Neuroendocrine Tumor Society (ENETS) [6]. Since its proposal in 2006, grading has been implicated in decision-making and in choosing treatment options in unresectable neuroendocrine neoplasms (NEN) [9]. Grading can be based on H&E sections by counting mitotic figures in an area of 2 mm^2 (10 high power fields (HPF)). In the last decade, grading using immunohistochemical staining of the proliferation marker Ki67, an antigen expressed in G1, G2, S, and M-phase of the cell cycle, has been considered to be more precise [10, 11]. The immunohistochemical staining is performed using the monoclonal antibody MIB1 [6, 7, 12].

Grading using MIB1 labeling has indeed been shown to be a good prognostic marker in many retrospective studies [3–7, 13, 14]. According to the actual WHO 2010 classification, two approaches exist to determine tumor grade: counting

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mitosis in an area of 2 mm^2 on H&E and counting positive cells in 2000 tumor cells using MIB1 staining. Although the latter is considered difficult to apply in routine practice, some studies suggest a better stratification by using the labeling index [15, 16].

The optimal method to assess the MIB1 index is topic of ongoing discussion. Counting 2000 tumor nuclei is a time-consuming process suitable for study purposes and is considered the gold standard. In daily practice, pathologists frequently choose to estimate the proliferation index by "eyeballing" or computer-assisted automated analysis to avoid this time-consuming procedure [15]. While some studies reported all methods equally suitable [17], important differences between the three methods were instead reported by others [18–21].

Interobserver and interlaboratory comparisons have been performed for MIB1 staining for other tumor entities as well, and evidence indicating some variability has been reported [21 22, 23]. Interlaboratory differences for MIB1 staining have been stated as of minor importance [21, 23]. However, these studies focused on highly proliferative lesions and differences in the 1 to 20 % range, which are crucial for NET grading, have not been examined. Studies regarding interlaboratory differences in this low range of MIB1 index have not been performed in general and for NET more specifically.

To assess potential interlaboratory differences in NET, we performed Ki67 staining in three different university hospital-based pathology laboratories on one well-characterized patient cohort of pancreatic NET (pNET). We did not want to assess interobserver differences, so scoring of all stained slides was performed by one person using a clearly defined approach. To assess reliability of the different stains, we performed intraclass correlation as well as log-rank analysis, considering tumor-specific survival.

Our study shows significant interlaboratory differences in intensity and number of MIB1-stained cells, which highlights the need for standardization.

Materials and methods

Patients and tumor specimens

We included 61 patients with well-differentiated primary pNET and available follow-up data, who underwent surgical excision (26 males, 35 women) from 1974 to 2004, as previously described in Schmitt et al. [24]. The study was approved by the local ethics committee (StV 40-2005).

Only G1 and G2 tumors were included, classified based on mitotic count according to the WHO guidelines in 2010 and ENETS guidelines 2006. Mitoses were evaluated on whole slide sections in an area of 2 mm^2 (A.P., A.S.).

A tissue micro array (TMA) comprising randomly punched areas of these 61 primary tumors [24] was used for interlaboratory comparison. Punch diameter was 0.6 mm comprising an area of 0.28 mm^2.

Immunohistochemistry

Four-micrometer sections of the tissue micro array were stained in three different university hospital-based pathology laboratories with MIB1 antibody, according to their current protocols for diagnostic staining (Table 1). A MIB1 staining-based proliferation index was established in random tumor areas. In a first step, all tumor cell nuclei per punch were counted with a counting grid and counting device (mean 1702, range 267 to 5520 tumor cells) followed by evaluating all positive nuclei. Tumor cells with faint and dot-like staining were considered positive as well, because the Ki67 staining pattern varies during different phases of the cell cycle [10, 11]. Labeling index was calculated by dividing the number of positive nuclei by the total number of tumor nuclei. Immunohistochemically stained slides were evaluated blinded to the mitotic count and clinicopathological data. Examples of immunohistochemical staining are given in Fig. 1.

Statistical analysis

Time interval from surgical intervention to tumor-induced death (TTD) was assessed by log-rank test, using GraphPad Prism4 software (GraphPad Software, Inc., San Diego, CA). p values <0.05 were considered to indicate statistical significance. Linear regression analysis was performed with SPSS version 16.0.1 (SPSS® software, Chicago, IL, USA). Intraclass correlation was performed with SAS version 9.2 (SAS institute, Cary, NC, USA).

Results

Patients and tumor specimens

Patient age ranged from 14 to 83 years (mean 56.4 years; median 56 years). Male to female ratio was 0.74:1. Follow-up ranged from 1 to 323 months (mean 106.3 months; median 86 months). Tumor size ranged from 0.4 to 15 cm with (mean diameter 3.7 cm; median diameter 3.0 cm). Tumor relapse occurred in 24/61 patients (39 %; mean time interval 93.9 months, median 72.5 months). Of the patients, 14/61(23 %) died of tumor (mean time interval 106.3 months; median 85.5 months). Lymph node metastases were found in 15/61 patients (25 %); 46/61 patients had no lymph node metastases documented at time of surgery (75 %). Distant metastases occurred in 9/61 patients (15 %); 52/61 patients

Table 1 Comparison of MIB1 staining conditions for institutes 1, 2, and 3

Institute	Antibody/clone	Pretreatment/incubation time	Dilution	Detection system
1	MIB1, Dako	CC1, pH 8.4 Heat-pretreatment, 30 min	1:20	Ventana BenchMark
2	MIB1, Immunotech S.A.	EDTA, pH 8.8 Heat-pretreatment, 20 min	1:50	Dako Envision
3	MIB1, Dako	Tris/EDTA-buffer, pH 9.0 Heat-pretreatment, 30 min	1:100	Dako Real Detection system

Fig. 1 Example of two tumor cores with MIB1 staining of each institute: patient aP129 important differences, institute 1 (**a**), institute 2 (**b**), and institute 3 (**c**); patient aP081 minor differences, institute 1 (**d**), institute 2 (**e**), and institute 3 (**f**)
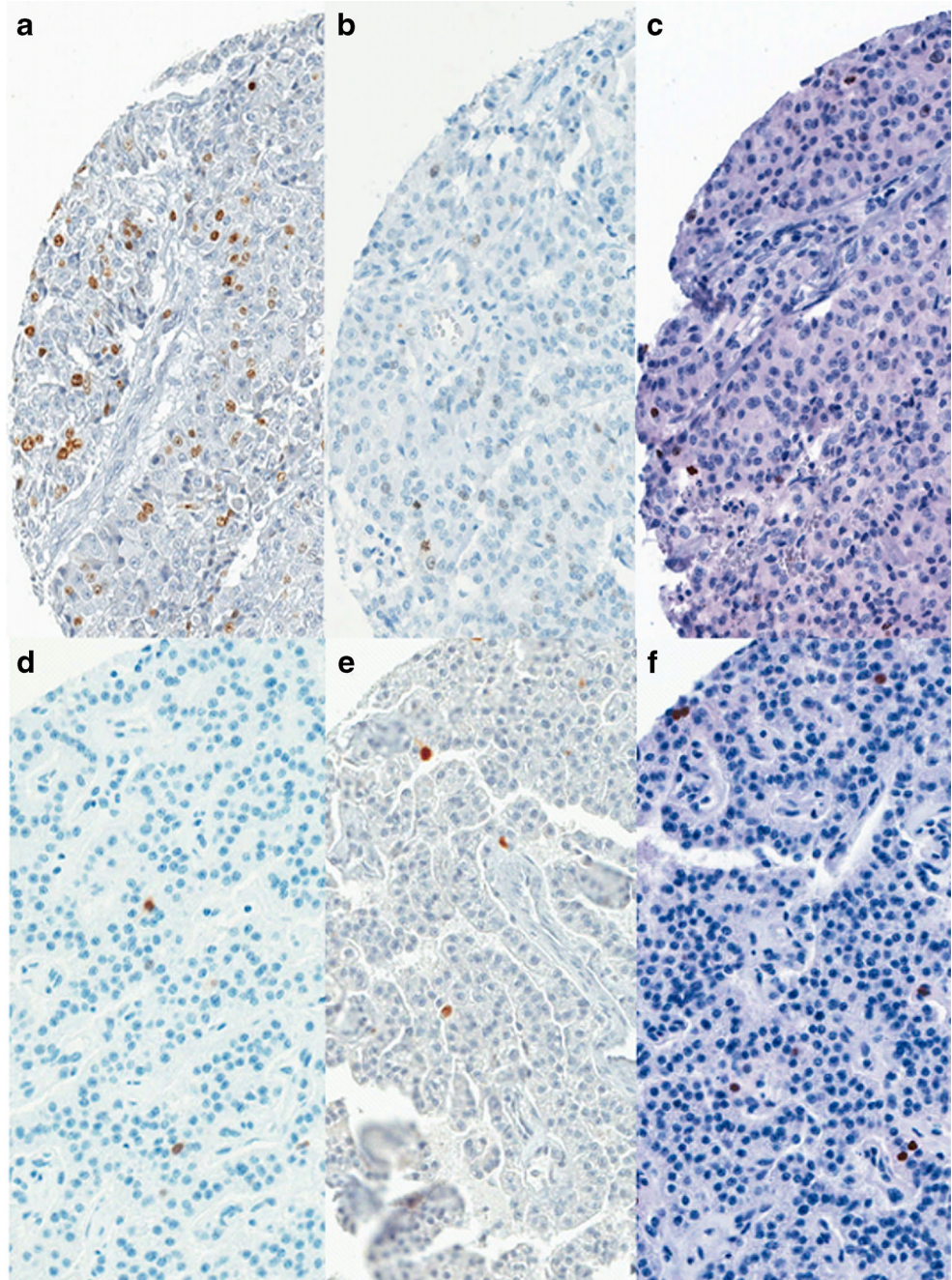
had no distant metastases documented at time of surgery (85 %) (Table 2).

MIB1 staining-based proliferation index

Significant differences in intensity as well as proportion of positive tumor nuclei were seen, even at low magnification. Highest staining intensity was provided by institute 1, whereas that from institute 3 was weakest. Log-rank analysis for calculated proliferation indices rounded to 0.5 % was performed. Chi-square values and p values were recorded. The highest chi-square value correlated with the lowest p value and indicated the best cutoff value for the separation between G1 and G2 tumors [25]. Regarding survival, a prognostic cutoff of 5.0 % was found for institute 1, 3.0 % for institute 2, and 0.5 % for institute 3 for the differentiation between G1 and G2 tumors (Fig. 2a–c).

On slides stained in institute 1, survival of patients with a proliferation index >5.0 % was significantly poorer ($p < 0.001$). A cutoff of 3.0 % showed a significant difference in survival but with lower significance ($p = 0.001$), whereas a cutoff of 0.5 % did not show a significant difference in survival ($p = 0.361$).

On slides stained in institute 2, survival of patients with a proliferation index >3.0 % was significantly poorer ($p < 0.001$). A cutoff of 5.0 % showed the same significant difference ($p < 0.001$); a cutoff of 0.5 % also showed a significant difference.

On slides stained in institute 3, survival of patients with a proliferation index >0.5 % was significantly poorer ($p = <0.001$). A cutoff of 5.0 and 3.0 % showed no significant difference in survival ($p = 0.800$ and 0.198) (Table 3).

Linear regression analysis showed a significant positive correlation between mitotic counts and MIB1 staining-based proliferation index for institutes 1 and 2 ($p = 0.000$, $R^2 = 0.383$ and $p = 0.000$, $R^2 = 0.416$), whereas no significant correlation was found for institute 3 ($p = 0.260$, $R^2 = 0.033$) (Fig. 3a–c).

Strong positive correlation was found between MIB1 staining-based proliferation index from institutes 1 and 2 ($p = 0.000$, $R^2 = 0.358$) (Fig. 3d). Borderline significance was found between institutes 1 and 3 ($p = 0.049$, $R^2 = 0.93$), and no correlation was found between institutes 2 and 3 ($p = 0.508$, $R^2 = 0.11$) (data not shown).

Intraclass correlation found a correlation coefficient of 0.51 for the proliferation index from institutes 1 and 2 and a correlation coefficient of 0.32 for institutes 1, 2, and 3.

Grading shifts

To differentiate between G1 and G2 tumors, a cutoff of 2.0 % was applied to the proliferation indices. For slides stained in institute 1, this resulted in 50 G1 and 11 G2 tumors, whereas

Table 2 Clinical characteristics of 61 patients with pancreatic neuroendocrine tumors; TNM-classification according to Rindi et al. [1]

| TMA	Total	Men	Women
Patients	61 (100 %)	26 (42.6 %)	35 (57.4 %)
Tumor-related death	14 (23.0 %)	8 (13.1 %)	6 (9.8 %)
No tumor-related death/alive	47 (77.0 %)	18 (29.5 %)	29 (47.5 %)
Follow-up			
Range (month)	1–323	3–285	1–323
Mean (month)	106.3	86.08	121.31
Median (month)	85.5	73	107
T stage			
T1	16 (26.2 %)	5 (8.2 %)	11 (18.0 %)
T2	24 (39.3 %)	11 (18.0 %)	13 (21.3 %)
T3	14 (23.0 %)	5 (8.2 %)	9 (14.8 %)
T4	1 (1.6 %)	1 (1.6 %)	0
Unknown	6 (9.8 %)	4 (6.6 %)	2 (3.3 %)
N stage			
N0	14 (23.0 %)	4 (6.6 %)	10 (16.4 %)
N1	15 (24.6 %)	10 (16.4 %)	5 (8.2 %)
Unknown	32 (52.5 %)	12 (19.7 %)	20 (32.8 %)
Mitoses per 2 mm^2			
<2	46 (75.4 %)	17 (27.9 %)	29 (47.5 %)
2–20	11 (18.0 %)	7 (11.5 %)	4 (6.6 %)
>20	0	0	0
Unknown	4 (6.6 %)	2 (3.3 %)	2 (3.3 %)
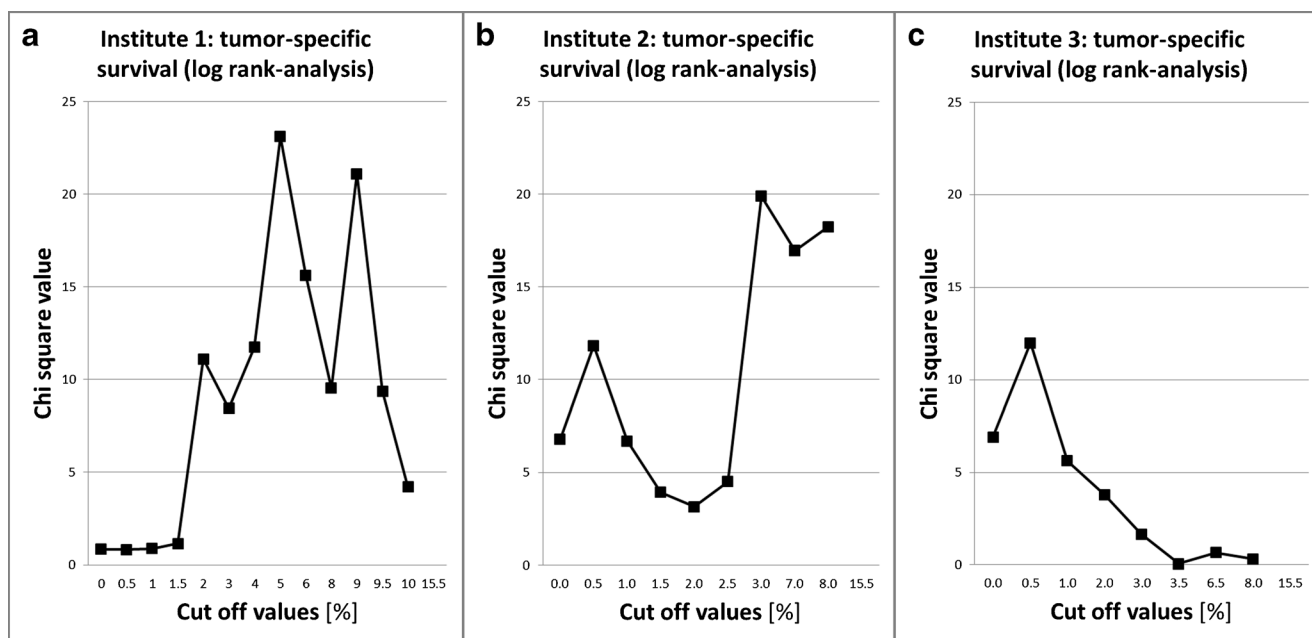
Fig. 2 Chi-square values for rounded proliferation indices: the higher the chi-square value, the better the stratification between G1 and G2 according to survival; staining institute 1 (**a**), staining institute 2 (**b**), and staining institute 3 (**c**)

for slides stained in institute 2, this was 53 G1 and 8 G2 tumors. Grading shifts were observed in nine cases (15 %). Even though staining in institute 1 was generally stronger, grade shifts went in both directions: three tumors shifted from G1 to G2 on slides stained in institute 1, while six tumors shifted from G2 to G1. Because of missing correlation between immunohistochemical MIB1 staining and mitotic count in institute 3, we considered this staining protocol as unreliable and performed no analysis of grading shifts for this institute.

Discussion

Tumor grade and stage are to date the most reliable factors used in clinical practice to predict prognosis of pNET [2 7]

Table 3 Prognostic value of different cutoffs, estimated by log-rank analysis

	Staining institute 1	Staining institute 2	Staining institute 3
≤5.0 %	55/61(90.2 %)	58/61 (95.1 %)	39/42 (92.9 %)
>5.0 %	6/61 (9.8 %)	3/61 (4.9 %)	3/42 (7.1 %)
p value	<0.001	<0.001	0.800
≤3.0 %	51/61 (83.6 %)	58/61 (95.1 %)	38/42 (90.5 %)
>3.0 %	10/61 (16.4 %)	3/61 (4.9 %)	4/42 (9.5 %)
p value	0.001	<0.001	0.198
≤0.5 %	25/61 (41.0 %)	37/61 (60.7 %)	30/42 (71.4 %)
>0.5 %	36/61 (59.0 %)	24/61 (39.3 %)	12/42 (28.6 %)
p value	0.361	0.001	<0.001

and are supported by WHO and ENETS [6 26, 27]. Intriguingly, not much attention has been paid to potential interlaboratory differences, even though for the analysis of parameters such as chemical (electrolytes) and biological variables (e.g., Chromogranin-A measurement in the serum), each laboratory needs to indicate local reference values [28, 29]. For MIB1 staining, the antibody batch used will be different and different laboratories use different staining protocols. In addition, pre-analytical variables such as transport times, fixation times, and tissue processing procedures are not standardized. Few data exist regarding interlaboratory variability of Ki67 proliferation index [21], and only one study from 2002 reported this, among other tumors, in pNET [23].

To assess the extent of interlaboratory Ki67 staining variability, we assessed staining of 61 tumors on TMA slides stained in three different university hospital-based pathology laboratories, all regularly participating in quality assurance assessments with good results. We found significant and relevant differences in staining intensity and proportion of MIB1 positive cells in immunohistochemically stained slides impacting on the proliferation index, notably in low proliferating tumors. This calls for external standardization.

Because of missing correlation between immunohistochemical MIB1 staining and mitotic count in institute 3, we considered this staining protocol as unreliable. We therefore focused statistical analysis on a comparison of institutes 1 and 2. In fact, on stained slides from both institutes, clear prognostic stratification could be attained. Considering staining results from these two institutes, a shift in grade was observed in nine cases, accounting for 15 % of tumors. Proliferation indices from both correlated well with mitotic counts. Using
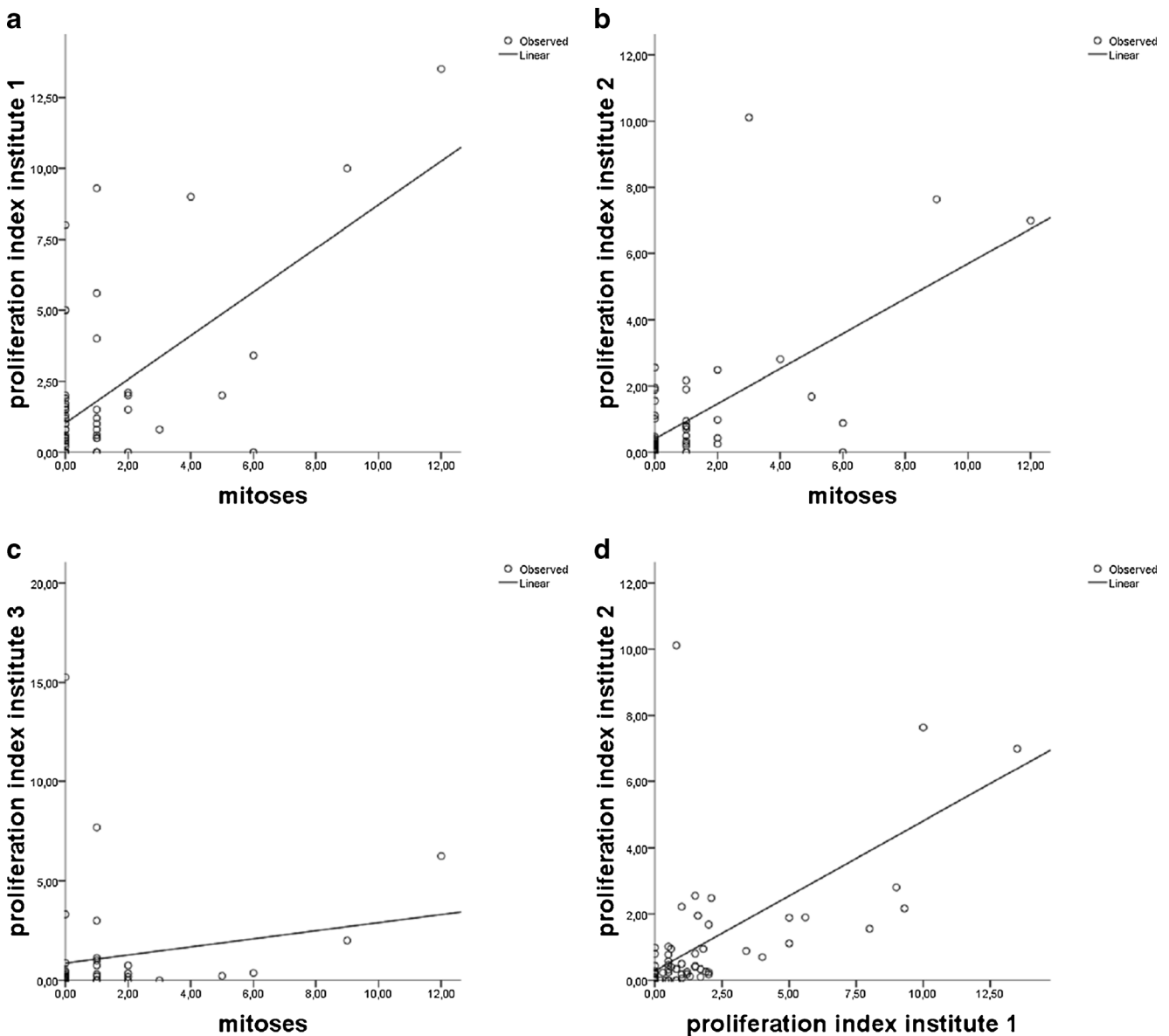
Fig. 3 Regression analysis: proliferation index (institute 1) and mitoses, $p = 0.000$, $R^2 = 0.383$ (a); proliferation index (institute 2) and mitoses, $p = 0.000$, $R^2 = 0.416$ (b); proliferation index (institute 3) and mitoses, $p = 0.260$, $R^2 = 0.033$ (c), proliferation index institutes 1 and 2, $p = 0.000$, $R^2 = 0.358$ (d)

log-rank analysis, an optimal cutoff of 5.0 % was established for institute 1 and of 3.0 % for institute 2. As we used a TMA composed of randomly chosen tumor areas, these cutoff values are expected to be different from those in studies based upon counting in hotspots. Our cutoff values can therefore not be taken as generally applicable. A random area rather than a hotspot approach has been shown to result in lower proliferation indices [30].

A potential limiting factor of our study is the use of consecutive sections, a few micrometers apart, which could account for slight differences. This however is compensated by the relatively large number of tumors

included in the study. We conclude that the observed interlaboratory differences are a result of variations in staining procedures between the participating laboratories. Our findings are concordant with other investigations regarding interlaboratory variability, which emphasized the role of processing and fixation processes [31–33], different reagents and pretreatment for immunohistochemical staining [34]; [35, 36], while antibody dilution was found to be less important [37] [23].

Our results indicate that differences in MIB1 staining-based proliferation indices are likely to be higher in tumors with low proliferative activity. This finding is of importance in

NET because the cutoff values for G1 and G2 tumors are in a very low proliferation range. This must be accounted for using potential external calibrators and must be checked in quality control measures.

We used log-rank analysis to define a cutoff value for the proliferation index based upon the slides stained in each of the three institutes. The cutoff value obtained for each institute appropriately stratified the patient cohort on the stained slide set on which it was calculated but was less significant when applied to the slides stained in one of the other institutes. This result might explain why in the literature different cutoff values have been published [2, 7, 24]. While significant interlaboratory differences were found, all had prognostic value, which underlines the prognostic importance of Ki67 staining-based proliferation index as has been repeatedly reported [3–7, 13, 14]. Our results, however, also indicate that minimizing interlaboratory variability is a priority. Grading based on proliferative activity may impact on therapeutic decisions, and consistent and reproducible grading is mandatory to maintain comparability of clinical studies. Two approaches might be considered to attain this goal. The first is standardization of staining intensity but to this end, an external benchmark needs to be artificially defined. This could be the immunohistochemical staining result obtained in a reference institute, allowing application of a "correction factor" for other institutes based on interlaboratory comparison. Alternatively, an external calibrator could be introduced such as a slow growing cell line, of which proliferative activity can be defined using BrDU incorporation over a defined timespan. Whichever the chosen solution, intralaboratory reproducibility has to be monitored by regular quality control measures such as round robin tests.

Our data contribute to the ongoing discussion on the optimal approach to establish proliferative activity, to the definition of the cutoff value to be used for NET grading [3–7, 13, 14, 16] and to the need for standardization of MIB1 staining. Since proliferation index-based grades impact on therapy decisions and follow-up intervals [14], reliability and reproducibility of their assessment are essential. This is not limited to pNET but holds true for a variety of tumors including breast cancer [21, 38], melanoma [39], and bronchopulmonary carcinoids [40, 41].

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