

MIB1 expression in basal cell layer: a diagnostic tool to identify premalignancies of the vulva

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Lichen sclerosus, high-grade usual vulvar intraepithelial neoplasia (VIN) and differentiated VIN have a different malignant potential. The objective of this study was to quantify the proliferative activity in the basal region of the epithelium of vulvar premalignancies. Furthermore, we investigated whether MIB1 expression in the basal region of vulvar epithelium can be helpful in diagnosing differentiated VIN, which may be hard to discern from normal epithelium. MIB1 was used to immunohistochemically visualise proliferating cells within formalin-fixed, paraffin-embedded, archival tissue sections of different vulvar premalignancies (N=48) and normal vulvar epithelium (N=16). Automatic digital image analysis software was developed to quantify the proliferating fraction in different parts of the epithelium (MIB1 positivity index). MIB1 expression differed among the various vulvar premalignancies; a MIB1-negative basal cell layer was a distinct feature of normal vulvar epithelium. No MIB1-negative basal cell layer was noted in differentiated VIN or other vulvar premalignancies. Owing to this negative cell layer, the MIB1 proliferation index in normal vulvar epithelium was significantly lower than in vulvar premalignancies. In conclusion, MIB1 expression can be a helpful tool in diagnosing a premalignancy and has additional value especially to distinguish differentiated VIN neoplasia from normal vulvar epithelium, but cannot explain the differences in malignant potential.

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Vulvar cancer is the fourth most common gynaecologic cancer and comprises 5% of all malignancies of the female genital tract. On the basis of clinical and pathological features, vulvar squamous-cell carcinoma can be subdivided into two different types, which seem to develop from their own associated premalignancies.

The most frequent type of vulvar carcinoma occurs mainly as a unifocal lesion in elderly women and is related to lichen sclerosus and/or differentiated vulvar intraepithelial neoplasia (VIN). This type of tumour is probably not associated with human papillomavirus (HPV) infection.^{1–4} The less common type, accounting for about one-third of all

vulvar squamous-cell carcinomas, is associated with HPV, predominantly HPV types 16 and 18. This type of tumour often occurs as a multifocal lesion in relatively young women and is usually preceded by high-grade usual VIN lesions (also referred to as classic VIN lesions).^{3,5,6} Recently, the International Society for the Study of Vulvovaginal Disease (ISSVD) has proposed a new nomenclature for premalignant vulvar lesions (see Table 1).⁷

In usual VIN, the epithelium is thickened and accompanied by hyperkeratosis and/or parakeratosis. Although a spectrum of architectural abnormalities may be seen, the lesion is readily recognisable as an intraepithelial neoplasm by the pathologist due to cytological abnormalities of the epithelial cells throughout the whole thickness of the epithelium.⁸ Differentiated VIN is clinically and pathologically more difficult to recognise. There is little or no atypia above the basal or parabasal layers and it has a high degree of cellular differentiation, which combined with an absence of widespread architectural disarray hinders recognition.^{2,3,9} Differentiated

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Table 1 Old and new nomenclature for vulvar lesions⁷

Old nomenclature	New nomenclature
VIN1 (Classic) VIN2/3	No cancer precursor High-grade VIN, usual type/ usual VIN
(Well-)differentiated VIN(3)/ VIN simplex	Differentiated VIN

VIN, vulvar intraepithelial neoplasia.

VIN is relatively infrequently diagnosed in its pure form and is often seen adjacent to lichen sclerosus and/or rapidly growing invasive vulvar squamous-cell carcinoma.^{3,8,10} Besides the difficulties in clinical and histological recognition of differentiated VIN, it is suggested that this form of VIN is highly proliferative and might be more likely to progress to an invasive neoplasm than lichen sclerosus and HPV-related VIN lesions.^{2,3,11} Currently, no biomarker or diagnostic tool to predict possible invasive behaviour of premalignant vulvar lesions is available. Because of the risk of malignant progression it is current practice that all patients with vulvar premalignancies undergo regular check-ups. However, there is no evidence that this follow-up prevents the development of cancer or results in earlier detection of a malignancy.¹²

Proliferative activity in tissues can be visualised using a proliferation marker like MIB1, which is a monoclonal antibody against the Ki-67 antigen, a nuclear antigen present in human proliferating cells in all stages of the cell cycle, but not in the G₀ phase.^{13,14} In many (pre)malignant lesions, qualitative MIB1 expression is used for grading and estimating prognosis.^{10,15–18} Bulten *et al*¹⁵ used a method in which epithelial MIB1 expression patterns in cervical lesions were digitally quantified. The method proved to be an objective, reproducible and reliable method of classification for dysplastic changes in cervical epithelium. Systematic quantitative evaluation of MIB1 expression in the basal region of premalignant vulvar lesions has not yet been performed.^{10,19–23}

The primary objective of this study was to quantify the proliferative activity in the epithelial cell layers in differentiated VIN and other vulvar premalignancies with the aim to find an explanation for the differences in malignant progression. Furthermore, we investigated whether MIB1 expression in the basal regions of the vulvar epithelium may be helpful in diagnosing differentiated VIN.

Materials and methods

Specimens

Sixty-four specimens from vulvar biopsies, vulvar excisions or vulvectomies, diagnosed as non- or premalignant vulvar lesions between 1992 and 2002,

were retrospectively obtained from the archives of the Department of Pathology at the Radboud University Nijmegen Medical Centre (Nijmegen, the Netherlands). All specimens were routinely fixed (4% buffered formalin) and paraffin-embedded. Standard 4- μ m thick haematoxylin and eosin (H&E)-stained sections were used for the classification of the lesions. Two expert pathologists (JB and JMMG), specialised in gynaecological pathology, blindly and independently reexamined the slides and classified them according to current WHO criteria and the recent modification of the ISSVD.^{7,24} They agreed on all diagnoses.

The 64 tissue specimens in this study consisted of 22 usual type VIN lesions, 14 differentiated VIN lesions, 12 lesions with lichen sclerosus and 16 normal epithelium samples (10 patients with vulvodynia who were treated with a partial resection of the vulvar vestibule and six normal epithelium samples from free tumour margins in vulvectomy specimens of patients treated for vulvar cancer).

Immunohistochemistry

Four-micrometer-thick paraffin sections were mounted onto polylysine-coated slides and dried overnight at 58°C. The sections were dewaxed in xylene and endogenous peroxidase was blocked using H₂O₂ in methanol for 15 min and the slides were rinsed three times in phosphate-buffered saline (PBS; pH 7.4) for 5 min. The slides were placed in a citrate buffer (0.01 M; pH 6.0) and heated in a household microwave oven (3 min at 850 W until boiling; followed by 10 min at 180 W). The sections were allowed to cool down to room temperature (RT) and were briefly washed in PBS (10 min). Subsequently, the slides were preincubated with 20% normal goat serum and incubated with the primary antibody Ki-67 (clone MIB1, Dakocytomation, Denmark) 1:100 in PBS with 1% BSA (60 min, RT). Subsequently, the slides were rinsed in PBS (10 min) and post-antibody blocking was done for 15 min (powervision plus). This was followed by incubation with polymeric horseradish peroxidase goat anti-mouse/rabbit/rat IgG (30 min, RT). The slides were developed with diaminobenzidine (mixed with H₂O₂), counterstained with Mayer's haematoxylin, dehydrated in ethanol and xylene and finally mounted. In each run, a buffer only and a vulvar squamous-cell carcinoma served as negative and positive controls.

Quantification of Immunohistochemical Staining/ Image Analysis

Quantitative analysis of MIB1 staining was achieved using digital image analysis of microscopic images. Image acquisition was performed using a 3CCD colour video camera (Sony DXC-950P, Sony Corp.,

Japan) mounted on a conventional light microscope (Axioskop 2 plus, Carl Zeiss AG, Germany) and attached to a personal computer with frame grabber card (Matrox Meteor-II Multichannel, Matrox Imaging, Dorval, Canada). Images were acquired using a $\times 20$ objective (Plan Neofluar, NA = 0.5, resulting specimen level pixel size $0.39 \mu\text{m}^2$). Before analysis of the immunohistochemical staining, an image of an empty microscopic field was acquired, which was used for correction of unequal illumination. In each tissue section, we aimed to measure eight microscopic fields, representative of the lesion. Image acquisition and analysis were performed using a custom written macro in KS400 image analysis software (Carl Zeiss AG, Germany). For each digitised red green blue (RGB) image, the following procedure was performed. The operator interactively defined the location of the basement membrane by drawing a line with a mouse cursor. From this line, 20 strata of each $5 \mu\text{m}$ thickness were automatically determined, covering the basal region of the epithelium. Within these areas, pixels with a ratio between the red and green RGB component of >1.03 and a red intensity under 180 were labelled as belonging to MIB1-positive nuclei. In the same way, pixels with a ratio between blue and green intensity over 1.07 and red camera channel under 210 were labelled as belonging to haematoxylin-stained, MIB1-negative nuclei. See Figure 1a and b for the different steps in the process. Thresholds were determined from a set of training slides and were found adequate for almost all slides analysed in this study. When the initial thresholds led to unrealistic patterns, adjustment was performed by the operator (data not shown).

For each measured field, the total area of positive nuclei per stratum of $5 \mu\text{m}$ and the total area of all nuclei per stratum was automatically recorded. For each stratum, the ratio between the MIB1-positive nuclear area and total nuclear area was calculated (single layer). This ratio, $\times 100\%$, was used as a measure of MIB1 positivity: the MIB1 positivity index. In addition, the cumulative MIB1 positivity indices were calculated by dividing the sum of the MIB1-positive nuclear area in multiple strata by the sum of the total nuclear area in the same strata ($\times 100\%$).

In normal vulvar epithelium, the presence or absence of inflammatory cells directly underlying

the basement membrane was recorded for each measured field.

Statistical Analysis

All measured fields were averaged to calculate mean values per patient. These values were used to calculate mean values per type of lesion.

All analyses were performed using SPSS 12.0.1 software (SPSS Inc., Chicago, IL, USA). To test whether the positivity index in the lowest $5 \mu\text{m}$ of normal vulvar epithelium differed between the fields with and without inflammatory cells underneath the basal membrane, the nonparametric Mann-Whitney-*U*-test for unpaired observations was used. The Kruskal-Wallis one-way analysis of variance by ranks was used to test whether the different positivity indices were different for at least one of the four diagnostic groups. When a significant difference was found, distribution-free all-treatments multiple comparisons based on pairwise rankings with correction for tied observations were used to disclose which of the diagnostic groups differed significantly.²⁵

Results

Pathology

In Figure 1c, e and g, H&E-stained sections of differentiated VIN, usual VIN and lichen sclerosis are shown.

The epithelial cells of *usual VIN* (Figure 1c) have a high nuclear: cytoplasmic ratio and lack cytoplasmic maturation above the basal and parabasal layers. Mitotic activity is present above the basal layer with mitotic figures that are often abnormal in appearance and reach the upper cell layers. Multinucleation and dyskeratosis, including formation of intraepithelial squamous pearls, may be seen. Nuclear pleomorphism and hyperchromasia are present; however, nucleoli are uncommon.

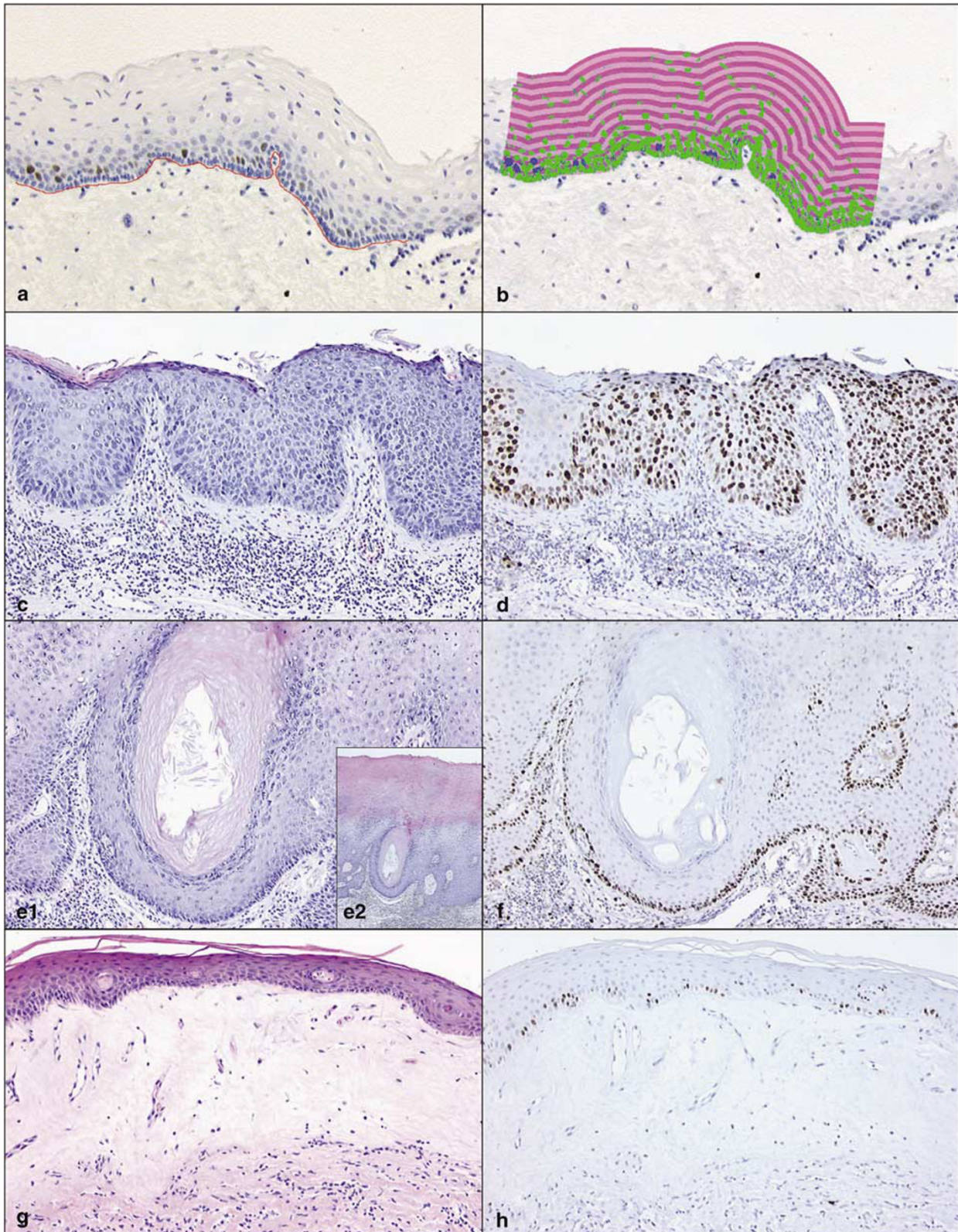
The atypia in *differentiated VIN* lesions (Figure 1e) is strictly confined to the basal and parabasal layers of the epithelium, where the cells have abundant cytoplasm and form abortive pearls. The nuclei are relatively uniform in size and contain coarse chromatin and prominent nucleoli leading to

Figure 1 (a and b) Photos of MIB1 staining and the digital image analysis process in normal vulvar epithelium. (a) A red line under the basement membrane, drawn by the operator. (b) An $100 \mu\text{m}$ wide area covering part of the epithelium was identified. Twenty strata of each $5 \mu\text{m}$ width, in which MIB1-negative (green) and MIB1-positive (blue) nuclei are shown. Note the negative MIB1 cell layer above the basement membrane. (d–h) H&E and MIB1 staining of $4 \mu\text{m}$ paraffin sections of a usual VIN lesion, differentiated VIN and lichen sclerosis. The peroxidase-labelled immunohistochemical complexes were visualised with diaminobenzidine (DAB) (brown-black). (c) H&E-stained section of a usual VIN lesion, showing atypical cells throughout the whole thickness of the epithelium. (d) In usual VIN, MIB1-positive cells can be seen in all layers of the epithelium. (e1) An abortive pearl that can be found in differentiated VIN. (e2) The atypia in differentiated VIN is confined to the basal layers of the epithelium. (f) In differentiated VIN, the MIB1-positive cells are confined to the basal layers. (g) In lichen sclerosis little atypia can be seen. (h) In lichen sclerosis, the MIB1-positive cells are confined to the basal layers. Original magnifications: (a and b) $\times 20$; (c, d, e1, f, g, h) $\times 10$; (e2) $\times 5$.

paradoxical maturation abutting on the epithelial-stromal junction. The superficial layers of the epithelium have a normal maturation, exhibit hyperkeratosis and do not show koilocytosis.²⁴

Furthermore, in differentiated VIN, the epithelium exhibits elongation of rete pegs.

In *lichen sclerosis* (Figure 1g), the loss of rete ridges is clearly visible. There is little or no cellular



or nuclear atypia. A zone of homogeneous collagenised subepithelial oedema of variable thickness is present as is a band of lymphocytic infiltration beneath this zone. The epithelium is flattened and/or thinned, and mild hyperkeratosis is present.

MIB1 Expression Pattern

In all lesions, MIB1 staining was present in the basal and/or parabasal regions of the epithelium and the basal membrane was always clearly identifiable. As can be seen in Figure 1d, f and g, MIB1 expression varied in the different vulvar premalignancies. In usual VIN all cell layers were positive for MIB1 (see Figure 1d). In differentiated VIN (Figure 1f), the majority of the epithelium was MIB1-negative with a thin layer of MIB1-positive cells parabasally. MIB1 positivity was restricted to the lower one-third of the epithelium in lichen sclerosus (Figure 1h). Furthermore, normal epithelium had a distinct MIB1 expression pattern. Like in lichen sclerosus, the lower one-third of the normal vulvar epithelium was MIB1-positive, but a MIB1-negative cell layer directly above the basement membrane was visible (Figure 1a and b). A simplified schematic representation of the different expression patterns is depicted in Figure 2.

MIB1 Positivity Index

In Figure 3, an overview of the single (Figure 3a) and cumulative (Figure 3b) MIB1 positivity indices in different areas of the epithelium are shown. The thick line represents the median positivity index, boxes represent quartiles and the lines indicate

extreme values. The MIB1 positivity indices per patient were based on a median number of 6 (range 4–10) measurements. In normal epithelium, there was no statistically significant difference in MIB1 positivity index in the lowest 5 μm of the epithelium between the measured fields *with* or *without* inflammatory cells underneath the basal membrane (data not shown, Mann–Whitney–U-test, $P > 0.05$). The MIB1 positivity indices in normal epithelium from vulvar vestibulitis patients and from

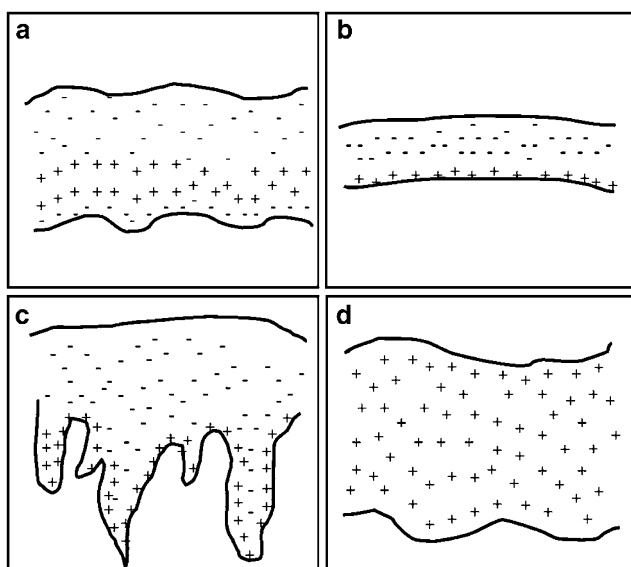


Figure 2 Simplified schematic representation of the MIB1 expression in (a) normal vulvar epithelium, (b) lichen sclerosus, (c) differentiated VIN and (d) usual VIN.

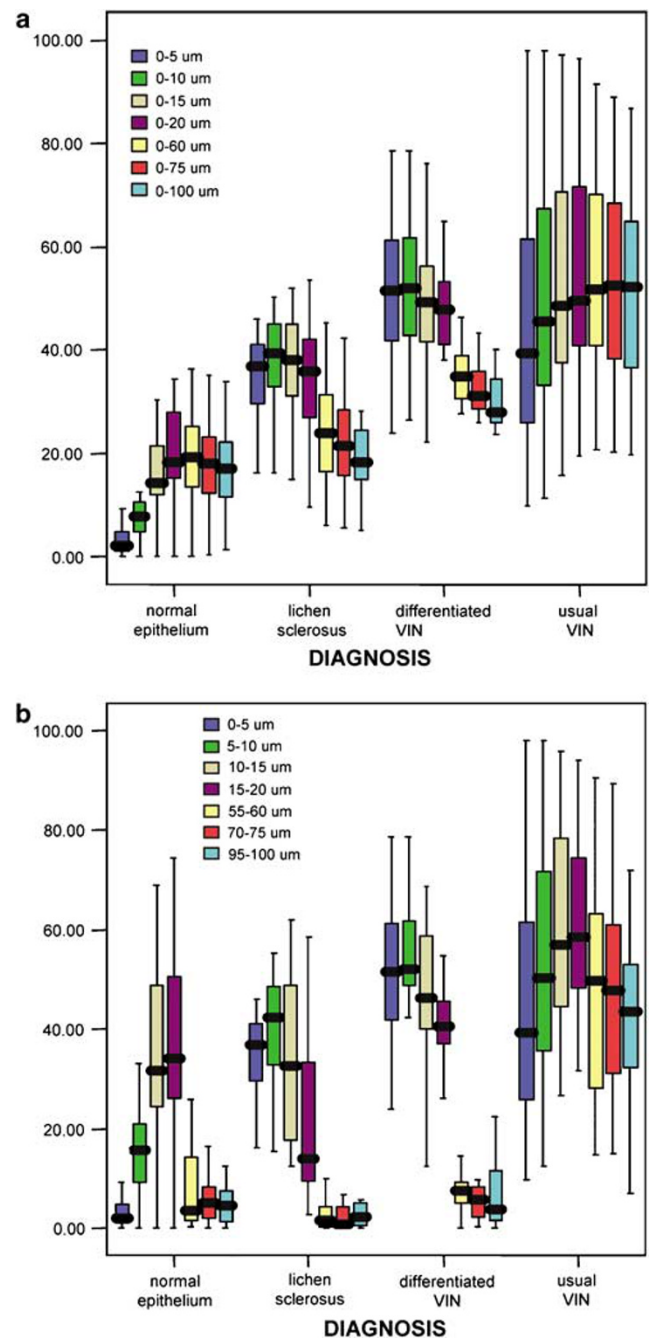


Figure 3 Boxplots of MIB1 positivity indices. The thick line represents the median positivity index, boxes represent quartiles and the lines indicate extreme values. (a) Single layer MIB1 positivity indices, (b) Cumulative MIB1 positivity indices.

patients with a carcinoma did not differ from one other (data not shown, Mann–Whitney-*U*-test, all *P*-values > 0.05).

In Table 2, an overview of *P*-values (distribution-free all-treatments multiple comparisons based on pairwise rankings with correction for tied observations) can be found, comparing cumulative and single layer MIB1 positivity indices between the four diagnostic groups.

In normal vulvar epithelium, the lowest 10 μm of the epithelium was almost negative for MIB1. The cumulative MIB1 positivity indices at 5 and 10 μm in normal epithelium were significantly lower than the corresponding areas in any of the other lesion types (all *P*-values < 0.001). In the higher strata,

single layer MIB1 positivity indices in normal vulvar epithelium were comparable to the single layer MIB1 positivity indices in differentiated VIN. Owing to the big difference in the lowest two layers, the difference between the cumulative MIB1 positivity indices remained statistically significant. Comparing the MIB1 positivity indices between lichen sclerosis and normal vulvar tissue revealed differences in only the layers up to 15 μm .

In usual VIN lesions, a MIB1 positivity index of almost 40% or more was observed in all layers of the epithelium, causing the cumulative MIB1 positivity indices to be around the same values. In none of the other epithelia, single layer MIB1 positivity indices (at 55 μm or higher) were as high as those in usual

Table 2 Comparisons of single layer and cumulative MIB1 positivity indices

Lesion types compared	P-value single layer MIB1 positivity indices		P-value cumulative MIB1 positivity indices	
Normal vs lichen sclerosis	0–5 μm	<i>P</i> < 0.001	5 μm	<i>P</i> < 0.001
	5–10 μm	<i>P</i> < 0.01	10 μm	<i>P</i> < 0.001
	10–15 μm	NS	15 μm	<i>P</i> < 0.01
	15–20 μm	NS	20 μm	NS
	55–60 μm	NS	60 μm	NS
	70–75 μm	NS	75 μm	NS
	95–100 μm	NS	100 μm	NS
Normal vs differentiated VIN	0–5 μm	<i>P</i> < 0.001	5 μm	<i>P</i> < 0.001
	5–10 μm	NS	10 μm	<i>P</i> < 0.001
	10–15 μm	NS	15 μm	<i>P</i> < 0.001
	15–20 μm	NS	20 μm	<i>P</i> < 0.001
	55–60 μm	NS	60 μm	<i>P</i> < 0.01
	70–75 μm	NS	75 μm	<i>P</i> < 0.01
	95–100 μm	NS	100 μm	<i>P</i> < 0.05
Normal vs usual VIN	0–5 μm	<i>P</i> < 0.001	5 μm	<i>P</i> < 0.001
	5–10 μm	<i>P</i> < 0.001	10 μm	<i>P</i> < 0.001
	10–15 μm	<i>P</i> < 0.05	15 μm	<i>P</i> < 0.001
	15–20 μm	<i>P</i> < 0.01	20 μm	<i>P</i> < 0.001
	55–60 μm	<i>P</i> < 0.001	60 μm	<i>P</i> < 0.001
	70–75 μm	<i>P</i> < 0.001	75 μm	<i>P</i> < 0.001
	95–100 μm	<i>P</i> < 0.001	100 μm	<i>P</i> < 0.001
Lichen sclerosis vs differentiated VIN	0–5 μm	<i>P</i> < 0.05	5 μm	<i>P</i> < 0.05
	5–10 μm	NS	10 μm	<i>P</i> < 0.05
	10–15 μm	NS	15 μm	NS
	15–20 μm	NS	20 μm	NS
	55–60 μm	NS	60 μm	NS
	70–75 μm	NS	75 μm	NS
	95–100 μm	NS	100 μm	NS
Lichen sclerosis vs usual VIN	0–5 μm	NS	5 μm	NS
	5–10 μm	NS	10 μm	NS
	10–15 μm	<i>P</i> < 0.05	15 μm	NS
	15–20 μm	<i>P</i> < 0.001	20 μm	NS
	55–60 μm	<i>P</i> < 0.001	60 μm	<i>P</i> < 0.001
	70–75 μm	<i>P</i> < 0.001	75 μm	<i>P</i> < 0.001
	95–100 μm	<i>P</i> < 0.001	100 μm	<i>P</i> < 0.001
Differentiated VIN vs usual VIN	0–5 μm	NS	5 μm	NS
	5–10 μm	NS	10 μm	NS
	10–15 μm	NS	15 μm	NS
	15–20 μm	<i>P</i> < 0.05	20 μm	NS
	55–60 μm	<i>P</i> < 0.001	60 μm	<i>P</i> < 0.05
	70–75 μm	<i>P</i> < 0.001	75 μm	<i>P</i> < 0.05
	95–100 μm	<i>P</i> < 0.001	100 μm	<i>P</i> < 0.01

VIN, vulvar intraepithelial neoplasia; NS, nonsignificant (*P* > 0.05).

VIN (all P -values < 0.001). Comparing the cumulative MIB1 positivity indices of usual VIN with the other lesions gave the same results (all P -values < 0.05). The differences with normal epithelium were most striking, as all cumulative and single layer MIB1 positivity indices were significantly higher in usual VIN.

The cumulative MIB1 positivity indices up to $10\ \mu\text{m}$ in lichen sclerosis were significantly lower than these strata in differentiated VIN. The single and cumulative MIB1 positivity indices higher in the epithelium were not significantly different and the pattern was comparable (see Figures 2 and 3).

Discussion

In general, it is suggested that differentiated VIN has a higher malignant potential than other vulvar premalignancies.^{8,26} This is not explained by our quantitative analysis of proliferative activity. The MIB1 positivity indices in the epithelium of differentiated VIN were not statistically different from the MIB1 positivity indices in well-established other precursors of vulvar carcinoma, that is usual VIN. The proliferative activity in the lower strata was comparable, whereas the MIB1 positivity indices covering a higher part of the epithelium were significantly higher in usual VIN, which is thought to be of lesser malignant potential. Several other investigators calculated a MIB1 positivity or labeling index, measuring the entire thickness of the epithelium or only the basal cell layer, but none of the groups used digital quantitative image analysis and mostly no distinction was made between differentiated and usual VIN, or only usual VIN was analysed.²⁷ Only scoring the MIB1 pattern or using a semiquantitative scoring method is less accurate.²⁸ Owing to deep rete pegs the measurement of the entire epithelium, as previously published by Bulten *et al*¹⁵ for cervical lesions, is technically not possible in differentiated VIN lesions. We calculated the MIB1 positivity index by dividing the measured MIB1-positive nuclear area by the total nuclear area. In this manner, the thickness of the epithelium that can be highly variable among different vulvar premalignancies and among different patients was no confounder in the measurements. When it would be possible to measure the entire epithelium, the differences in cumulative MIB1 positivity indices between differentiated VIN and usual VIN would probably be more prominent.

We noticed a remarkable difference in the MIB1 staining pattern of vulvar premalignancies vs normal vulvar epithelium. In normal vulvar epithelium, a MIB1-negative cell layer above the basal membrane was observed, which was absent in differentiated VIN. There was no MIB1-negative cell layer in other vulvar premalignancies and therefore the presence of a negative MIB1 layer seems to be a

characteristic of normal vulvar epithelium and the absence of such a layer above the basal membrane, indicates the presence of a premalignancy. Scurry *et al*²¹ describe similar results in their qualitative analysis of Ki-67 of lichen sclerosis and normal epithelium. Although they did not digitally quantify their measurements and did not specifically mention a MIB1-negative basal cell layer, they describe that the MIB1 staining in normal epithelium was localised more parabasally and in lichen sclerosis more basally. The lack of effect of inflammatory cells beneath normal vulvar epithelium on the presence of a MIB1-negative basal cell layer and the absence of a MIB1-negative basal cell layer in vulvar premalignancies can be seen as disturbance of cell growth in vulvar premalignancies.

Lichen sclerosis and usual VIN lesions are readily recognisable as they have distinct characteristics at histopathological examination. Differentiated VIN is often very difficult to recognise and sometimes hard to discern from normal epithelium.^{2,3,9} Using MIB1 expression to detect a proliferating basal cell layer in combination with the characteristic MIB1 staining pattern with only positive cells in the rete pegs and little positivity in the upper layers of the epithelium, might improve the early recognition of differentiated VIN. A simplified schematic representation of the different expression patterns is depicted in Figure 2. Further investigations have to provide evidence whether this feature of MIB1 is applicable in daily practice.

Despite its atrophic clinical aspect, lichen sclerosis has a relatively high proliferative activity, which was significantly higher than in normal vulvar tissue, which might be responsible for its malignant potential. This is in contrast with Tan *et al*,²⁹ who found a lower MIB1 positivity index in vulvar lichen sclerosis compared to normal controls. However, they did not use digital imaging techniques to quantify MIB1 positivity and only counted the basal and suprabasal cell layers. Lichen sclerosis and differentiated VIN showed comparable patterns of MIB1 positivity indices, both single and cumulative. This attributes to the assumption that lichen sclerosis and differentiated VIN belong to the same entity that might proceed to HPV-negative vulvar carcinoma.^{1-3,30} Differentiated VIN might develop from lichen sclerosis and/or squamous hyperplasia, through loss of apoptosis regulation, like the development of squamous-cell carcinoma in oral lichen planus lesions.³¹ The role of the regulator of apoptosis, p53, and its mutations, in differentiated VIN and HPV-negative vulvar carcinoma remains to be elucidated.^{8,32-34}

In conclusion, the use of MIB1 in the diagnosis of premalignant vulvar lesions might prove helpful, especially to discern differentiated VIN from normal vulvar epithelium. In all vulvar premalignancies, the relationship between basal and parabasal cell layers seems to be disturbed. How-

ever, the MIB1 positivity index was not higher in differentiated VIN lesions, which are presumed to be more likely to become invasive, than in usual VIN lesions.

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Disclosure/conflict of interest

The authors state that concerning this manuscript, neither conflict of interest nor funding exists.

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