The changes in melanocyte number and melanin density occurring in vitiligo patches during 180 days of narrow band-ultraviolet B therapy

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Background
Narrow band-ultraviolet B (NB-UVB) has been used in the treatment of vitiligo for years, but the sequence of events of repigmentation, occurring throughout the period of NB-UVB treatment for restoring melanocytes and melanin density, is not clear.
Objective
This study aimed to follow up the changes in melanocyte number and melanin density in vitiliginous patches during NB-UVB therapy for vitiligo patients.
Patients and methods
This study included 25 patients with nonsegmental vitiligo. NB-UVB therapy was given twice weekly for 6 months. Four skin biopsies were obtained from each patient at days 0, 30, 90, and 180. Biopsies were stained using hematoxylin and eosin stain, Masson–Fontana stain, and human melanoma black-45 (HMB-45) antibody. Qualitative and quantitative measurements were determined for both melanocyte number and melanin density.
Results
There was a significant increase in the number of HMB-45-negative melanocytes ($P<0.000$) at day 30 of therapy compared with day 0. At day 90, there was a significant increase in the number of HMB-45-positive melanocytes ($P<0.001$) and melanin ($P<0.001$) compared with days 0 and 30. As regards HMB-45-negative cells at day 90, there was a significant increase in its number compared with day 0 ($P<0.001$) with no significant increase compared with day 30 ($P=0.13$). At day 180, there was a significant increase in the number of HMB-45-positive melanocytes ($P<0.001$) and melanin ($P<0.001$) but with a significant decrease in HMB-45-negative melanocytes ($P<0.001$) compared with days 30 and 90.
Conclusion
We concluded that the sequence of events of repigmentation started with the appearance of inactive melanocytes, which were first detected at day 30, followed by the appearance of active melanocytes, melanin, and clinical repigmentation at day 90, which reached its highest levels at day 180. Moreover, we concluded that the presence of inactive melanocytes at day 30 might represent an index of favorable prognosis.

Keywords:
melanin, melanocytes, narrow band, vitiligo

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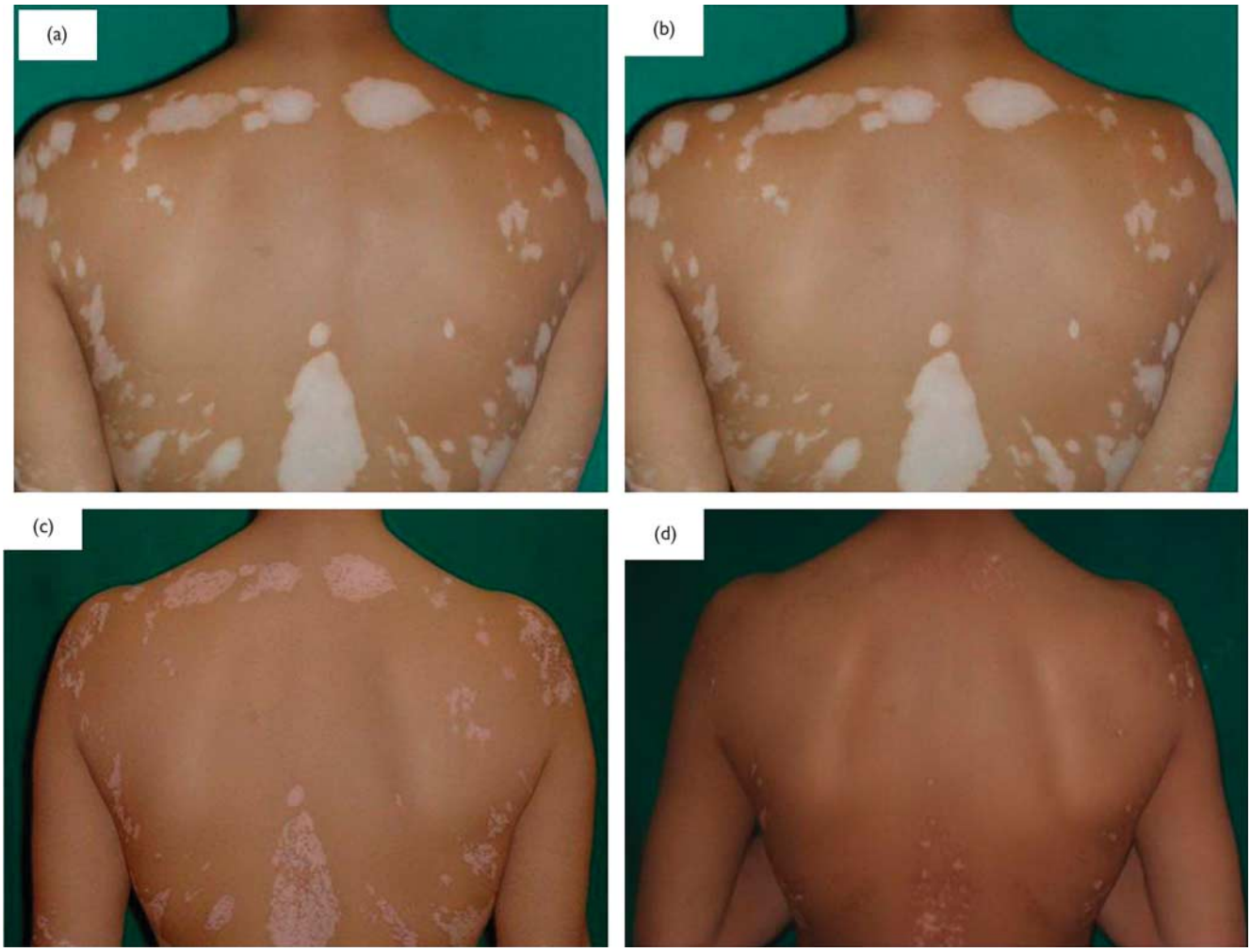
Introduction

Vitiligo is an acquired idiopathic pigmentary disorder of skin and hair, characterized by well-circumscribed asymptomatic white macules [1]. A variety of therapeutic approaches have been described in the literature and are directed toward reversing the progression of melanocyte loss and reconstituting normal skin coloration, but none is uniformly effective [2].

In 1997, Westerhof and Nieuweboer-Krobotova [3] were the first to study the effect of narrow band-ultraviolet B (NB-UVB) in vitiligo. After that, many researchers have confirmed the clinical effect of NB-UVB on generalized nonsegmental vitiligo (NSV) [4–6] and segmental vitiligo [5]. Several patterns of clinical repigmentation are seen at the beginning of the process, including perifollicular, in which the predominant repigmentation was follicular, marginal, in which the predominant repigmentation was from the borders of vitiliginous patches, diffuse pigmentation, in which generalized darkening occurred across the patches of vitiligo, and combined, in which more than one pattern contributed to the pigmentary process [7].

On a review of the literature, it was found that the clinical effect of NB-UVB on vitiligo was confirmed by

Figure 1.



(a) Vitiligo patient had milky-white patches at baseline state. (b) No clinical improvement after 1 month. (c) Follicular hyperpigmentation started to appear after 3 months. (d) Coalescence of this pigmentation led to complete repigmentation after 6 months.

histopathological and immunohistochemical studies, which proved the recovery of melanogenesis at the end of treatment [8,9]. Meanwhile, the sequence of events of repigmentation, occurring throughout the period of NB-UVB treatment for restoring melanocytes and melanin density, is not clear.

This study aimed to follow up the changes in melanocyte number and melanin density during NB-UVB therapy for vitiligo patients.

An informed consent was taken from each patient for treating with NB-UVB and taking biopsies.

Patients and methods

Patients

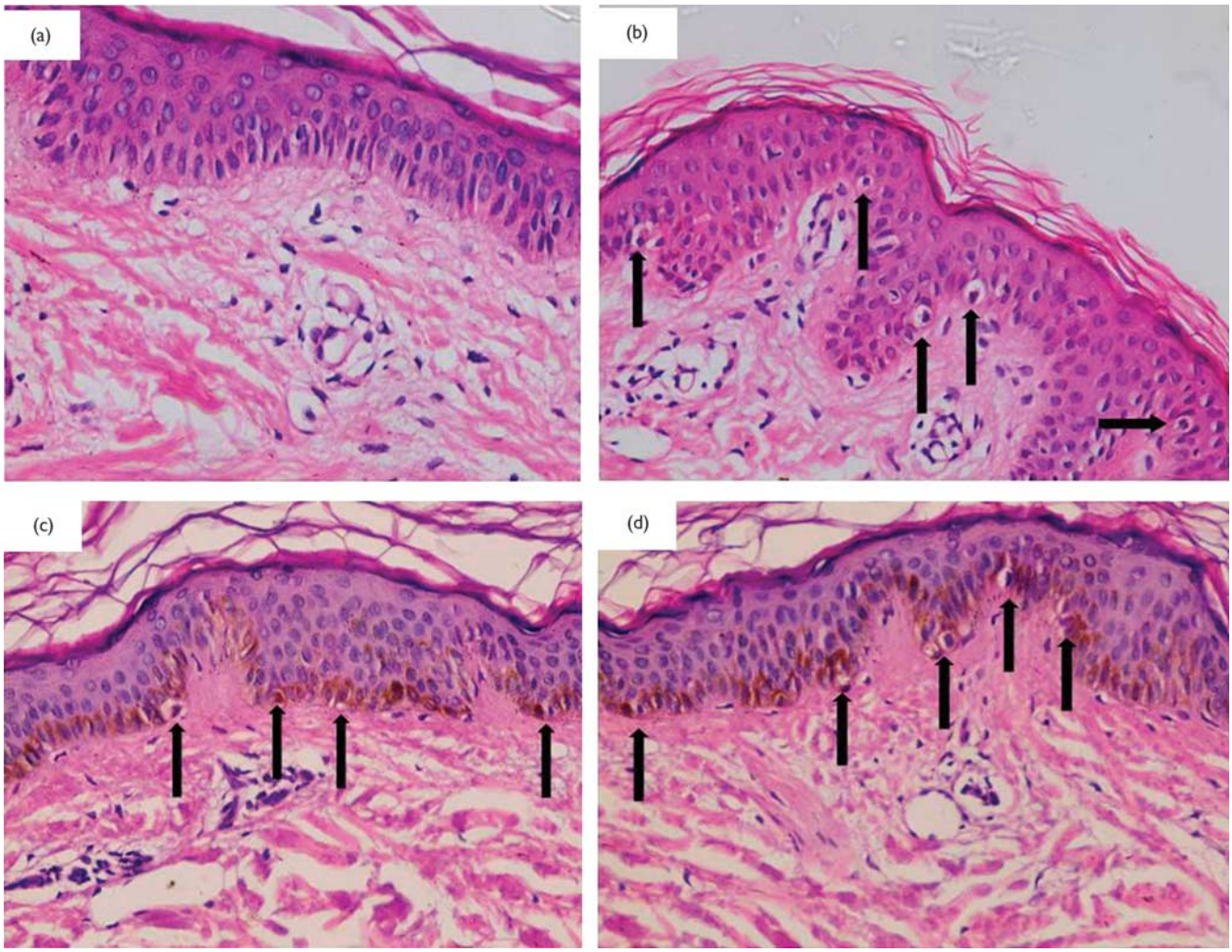
This study was conducted on 25 patients with stable NSV, attending the Dermatology Outpatient Clinic, Al-Minya University Hospital, over a period of 2 years. It was approved by the Committee for Studies and Research of the Dermatology Department, Al-Minya University. All patients were subjected to full history taking including personal history (age, sex, and occupation), present history (onset, course, and duration of the disease), past history (previous medications), and family history for vitiligo. Two dermatologists independently examined each patient to reach the diagnosis and type of vitiligo. Patients with segmental vitiligo or active vitiliginous lesions and patients who received topical and systemic therapies and phototherapy in the last 3 months before starting the study were excluded.

Narrow-band therapy

Eight NB fluorescent tubes (Philips TL100W/01; Philips B.V, Eindhoven, the Netherlands), with a spectrum of 310–315 nm and a maximum wavelength of 311 nm, were installed in a Waldmann UV-1000 unit (Waldmann Gmb, Schwenningen, Germany). We started with 0.30 J/cm^2, independent of the skin type, and we increased the dose by 20% every session until we reached the minimal erythema dose, which caused mild erythema, which disappeared the next day of the session [5]. During treatment, the eyes were protected with ultraviolet-blocking goggles.

The patients were examined during each visit for erythema, progress of therapy, and the presence of side

Figure 2.



Hematoxylin and eosin (H&E)-stained biopsies revealed the following: (a) at day 0, no melanocytes or melanin could be detected; (b) at day 30, clear basal cells (black arrows) started to appear but without melanin or basal pigmentation; (c) at day 90, melanin pigmentation started to appear; (d) at day 180, clear basal cells and melanin became more evident (H&E, $\times$ 200).

effects. The pattern of repigmentation was determined within the trunk and proximal extremities excluding acral areas. The number of sessions and duration to induce this clinical response were reported. Every patient was photographed before the start of treatment and then repeatedly at days 30, 90, and 180 of NB-UVB therapy.

Biopsy

From each patient, a skin biopsy specimen, using a 3-mm punch probe, was obtained from the centre of a vitiliginous area within the trunk and proximal extremities excluding acral areas at day 0. At days 30, 90, and 180 of NB-UVB therapy, the nearest point to the previous biopsy showing pigmentation was chosen. If no pigmentation occurred, biopsy was taken from a nonpigmented site close to the previous biopsy. Each biopsy was fixed in 10% neutral-buffered formalin, routinely processed, embedded in a paraffin block, and sectioned using an ordinary microtome into 5-µm-thick sections. The resulting sections were mounted on glass slides to be subjected to the following:

(1) Staining with hematoxylin and eosin (H&E) to evaluate the presence or absence of melanocytes.
(2) Staining with Masson–Fontana (MF) according to Masson [10] for subjective detection of melanin in keratinocytes. Morphometric analysis for assessment of melanization in skin biopsy was performed according to the method of Anbar *et al.* [11] using the software FIVE (Olympus Soft Imaging Solutions GmbH, Münster, Germany). A scanning power of microscope was used to examine sections, each containing 10 fields. Then, using a $\times$ 40 objective lens, alternate fields were measured, that is, five fields per section, and the average value was calculated. In the examined fields, the epidermal surface area (ESA) was measured in mm^2 using the software Closed Polygon (Olympus Soft Imaging Solutions GmbH, Münster, Germany), in which the epidermal boundaries were outlined forming a closed polygon. The melanin particles were outlined using the software Magic Wand (Olympus Soft Imaging Solutions GmbH, Münster, Germany), which can accurately select an object of any shape and size. The

melanin particles surface area (MPSA) in the epidermis was measured in mm^2 and summed up. The MPSA/ESA percentage was calculated in the odd five fields of each section. The difference in the mean ± SD of MPSA/ESA ratio of all biopsies at days 0, 30, 90, and 180 of NB-UVB irradiation was tested for significance.

(3) Immunohistochemical examination for evaluation of human melanoma black-45 (HMB-45) premelanosomal protein expression and thus detection of active melanocytes in the epidermis. We stained sections according to the manufacturer's instructions with monoclonal mouse anti–HMB-45 protein (code no: 364S207, ready to use; NeoMarkers, Fremont, California, USA). An UltraVision detection system, antipolyvalent, HRP/DAB, was used for the procedure (code no: TP-015-HD, ready to use; LabVision Corporation, Fremont, California, USA). The immunohistochemical stain was evaluated by means of a qualitative method, whereby the staining of melanocytes was defined as negative or positive. Further analysis of the HMB-45-stained biopsies was carried out quantitatively by counting the number of both negative (inactive clear) and positive (active) melanocytes in 10 high-power fields with measurement of the mean value for each biopsy. A light microscope [Accu-Scope #3025 five headed (A3025-5); Olympus, Tokyo, Japan] with a built-in camera (digital camera E-330 SLR; Olympus) was used to examine and photograph the sections.

Statistical analysis

Data were statistically analyzed using SPSS (version 16.0.1; SPSS Inc., Chicago, Illinois, USA) for Windows. Statistical analysis included descriptive analysis such as mean ± SD and paired sample *t*-test for the results. Significance was expressed in terms of a *P* value, which was considered significant when 0.05 or less.

Results

The study included 25 patients with NSV (17 female and eight male). There was complete concordance (100%) of the observations made by two blinded dermatologists with respect to the diagnosis of NSV. The patients' ages ranged between 15 and 67 years, with a mean ± SD of 35.52 ± 14.51 years. The duration of the disease before NB-UVB therapy ranged from 5 to 52 months, with a mean of 19.76 ± 14.25 months. The maintenance dose of NB-UVB ranged from 0.65 to 1.2 J/cm^2, with a mean of 0.95 ± 0.15 J/cm^2. The cumulative NB-UVB dose ranged from 30.55 to 57.60 J/cm^2, with a mean of 44.82 ± 7.21 J/cm^2. As regards skin type, 17 (68%) patients were of skin type IV, three (12%) patients were of skin type III, and the remaining five (20%) patients were of skin type II. Family history was positive in three (12%) patients.

Three modes of repigmentation – namely, perifollicular, mixed perifollicular pigmentation with marginal pigmentation, and marginal pigmentation alone – were observed in 12, seven, and one patient, respectively. The number of sessions to induce the start of repigmentation ranged from 8 to 23, with a mean of 15.64 ± 8.35. The duration of treatment to induce this repigmentation ranged from 28 to 84 days, with a mean of 54.60 ± 30.31 days. Five (20%) patients showed no improvement until the end of the study.

Clinically, there was no difference between baseline state (Fig. 1a) and after 1 month of phototherapy (Fig. 1b). Pigmentation started to appear after 3 months (Fig. 1c) and reached its maximum at 6 months of NB-UVB phototherapy (Fig. 1d).

The results of histopathological, histochemical, and immunohistochemical examination

Before treatment (day 0), vitiliginous skin biopsy specimens stained with H&E did not reveal clear cells at the basal layer of the epidermis (Fig. 2a). In biopsies carried out at days 30, 90, and 180 of therapy, these clear basal cells were present in 19 (76%), 20 (80%), and 20 (80%) patients, respectively (Fig. 2b–d).

On staining with MF, biopsies taken at days 0 and 30 demonstrated the absence of melanin in 21 out of 25 (84%) biopsies and in 20 out of 25 (80%) biopsies, respectively (Fig. 3a and b). Melanin was present in all biopsies carried out on repigmented skin (20 patients) at

Table 1. Comparison between the mean melanin density (melanin particles surface area/epidermal surface area)/high-power field in biopsies taken at days 0, 30, 90, and 180

	Day 0	Day 30	Day 90	Day 180
MPSA/ESA				
Range	0–0.0049	0–0.0120	0–0.2149	0–0.6085
Mean ± SD	0.0006 ± 0.0014	0.0011 ± 0.0027	0.0786 ± 0.0569	0.1476 ± 0.1304
P value		0.34*		
			$<0.001^{\triangle}$	
				$<0.001^{\infty}$
			$<0.001^{\square}$	
				$<0.001^{\diamond}$
				$0.004^{\blacksquare}$

ESA, epidermal surface area; MPSA, melanin particles surface area.
**P* value between day 0 and day 30 using paired *t*-test.
$^{\triangle}$*P* value between day 0 and day 90 using paired *t*-test.
$^{\infty}$*P* value between day 0 and day 180 using paired *t*-test.
$^{\square}$*P* value between day 30 and day 90 using paired *t*-test.
$^{\diamond}$*P* value between day 30 and day 180 using paired *t*-test.
$^{\blacksquare}$*P* value between day 90 and day 180 using paired *t*-test.

Table 2. Comparison between the mean number of human melanoma black-45-positive and negative melanocytes/high-power field in human melanoma black-45-stained biopsies taken at days 0, 30, 90, and 180

	Day 0	Day 30	Day 90	Day 180
HMB-45-positive melanocytes				
Range	0–0	0–0.2	0–1	0–3.1
Mean ± SD	0 ± 0	0.008 ± 0.039	0.360 ± 0.274	1.196 ± 0.966
P value		0.33*		
			$<0.001^{\triangle}$	
				$<0.001^{\infty}$
			$<0.001^{\square}$	
				$<0.001^{\diamond}$
				$<0.001^{\blacksquare}$
HMB-45-negative melanocytes				
Range	0–0	0–0.7	0–0.5	0–0.2
Mean ± SD	0 ± 0	0.26 ± 0.200	0.208 ± 0.157	0.044 ± 0.060
P value		<0.001*		
			$<0.001^{\triangle}$	
				$<0.001^{\infty}$
			$0.13^{\square}$	
				$<0.001^{\diamond}$
				$<0.001^{\blacksquare}$

HMB-45, human melanoma black-45.
**P* value between day 0 and day 30 using paired *t*-test.
$^{\triangle}$*P* value between day 0 and day 90 using paired *t*-test.
$^{\infty}$*P* value between day 0 and day 180 using paired *t*-test.
$^{\square}$*P* value between day 30 and day 90 using paired *t*-test.
$^{\diamond}$*P* value between day 30 and day 180 using paired *t*-test.
$^{\blacksquare}$*P* value between day 90 and day 180 using paired *t*-test.

day 90 (Fig. 3c). Meanwhile, at day 180, all of these patients showed increase in melanin density (Fig. 3d).

In positive biopsies for melanin taken at days 0, 30, and 90 of NB-UVB therapy, the established melanin was restricted to the basal cell layer with increase in its density of staining from day 0 to days 30 and 90. At day 180 of therapy, it reached its highest density and acquired basal and suprabasal locations (Fig. 3 and Table 1). Figure 4 demonstrates an example of quantitative measurements of melanin using the morphometric technique.

In pretreated vitiliginous biopsies stained with HMB-45 antibody, both HMB-45-positive melanocytes and HMB-45-negative clear cells were absent in the basal layer of the epidermis (Fig. 5a). In biopsies carried out at days 30, 90, and 180 of therapy, one (4%), 20 (80%), and 20 (80%) patients showed HMB-45-positive melanocytes, respectively (Fig. 5b–d). Meanwhile, HMB-45-negative cells appeared in 19 (76%), 18 (72%), and eight (32%) patients at days 30, 90, and 180 of therapy, respectively (Fig. 5b–d).

The total number of both HMB-45-positive and negative melanocytes is shown in detail (Table 2).

In the only patient who had repigmentation at day 30 of therapy we detected simultaneous appearance of HMB-45-positive and negative melanocytes and melanin at day 30; in contrast, in the only patient who showed marginal pigmentation only, biopsies from central vitiliginous area at days 0 and 30 showed neither clear basal cells in H&E and HMB-45-stained sections nor HMB-45-positive melanocytes.

The five patients who did not show any clinical improvement until the end of the study did not show melanocytes, melanin, or HMB-45-positive or negative cells in any of their biopsies.

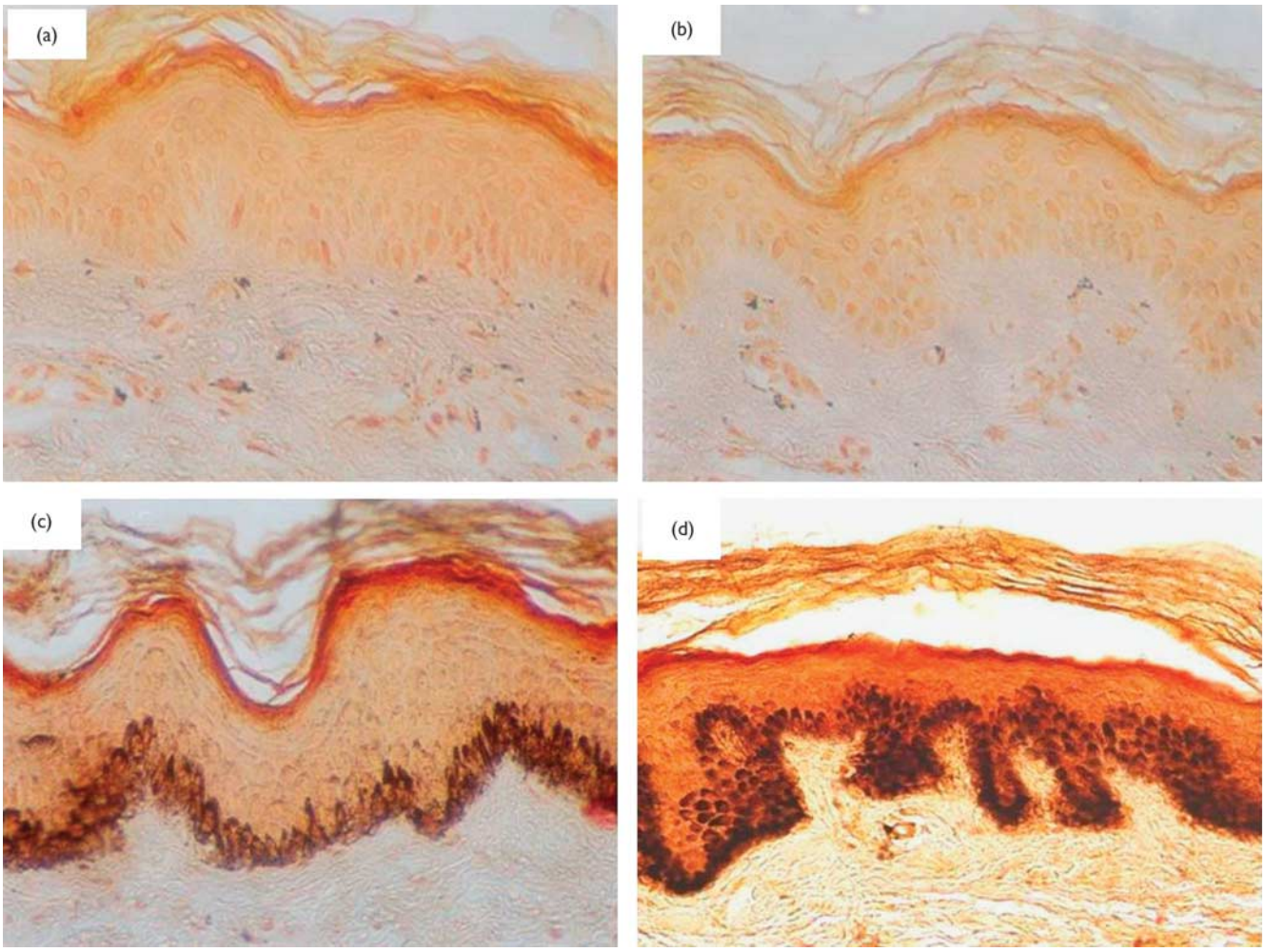
Discussion

NB-UVB phototherapy is an effective and safe therapeutic method for treatment of vitiligo, considering both the good clinical and histological results obtained and the absence of important side effects [9]. To the best of our knowledge, there are no reports in the literature about the sequence of events occurring throughout the period of NB-UVB treatment for restoring melanocytes and melanin.

This study showed perifollicular repigmentation, mixed perifollicular pattern with marginal pigmentation, and marginal pigmentation alone in 12, seven, and one patient, respectively. According to Yang *et al.* [12], the most frequent repigmentation pattern was the perifollicular type in both groups treated with NB-UVB and excimer laser, followed by marginal, diffuse, and combined in that order. Meanwhile, Njoo *et al.* [13] observed perifollicular repigmentation only in vitiligo lesions treated with NB-UVB therapy. Falabella [14] explained these patterns and described three possible sources of melanocytes, including the hair follicle unit, which is the main provider of pigment cells, the border of vitiligo lesions, and unaffected melanocytes within depigmented areas.

At day 0, pretreated vitiliginous skin biopsy specimens did not reveal clear cells at the basal layer of the epidermis in H&E-stained and HMB-45-stained sections. This is in agreement with the results of many previous studies [15,16]. Meanwhile, some authors showed clear melanocytes in pretreated vitiliginous biopsies in vitiligo patients but they were functionally dormant or inactivated [8,9]. Even in a previous study conducted by almost the same team of this present study, the results were different, as we demonstrated clear cells in three out of 30 vitiligo (10%) patients [17] and none were

Figure 3.



Masson–Fontana (MF)-stained biopsies showed the following: at day 0 (a) and day 30 (b), melanin pigmentation was absent; (c) at day 90, melanin started to appear in the basal layer, whereas at day 180 there was significant increase in the density of melanin pigmentation, reaching suprabasal layers (MF, × 200).

found in the present study. The difference may be because of variations in disease duration and activity. Accordingly, the long-standing controversy about the presence of melanocytes in vitiligo lesions is still present.

As regards melanin in pretreated vitiliginous biopsies, it was found to be present in four vitiligo patients in whom the duration of disease was 5, 6, 8, and 9 months. This was explained by the presence of melanin in keratinocytes injected from functioning melanocytes before their loss of function or disappearance.

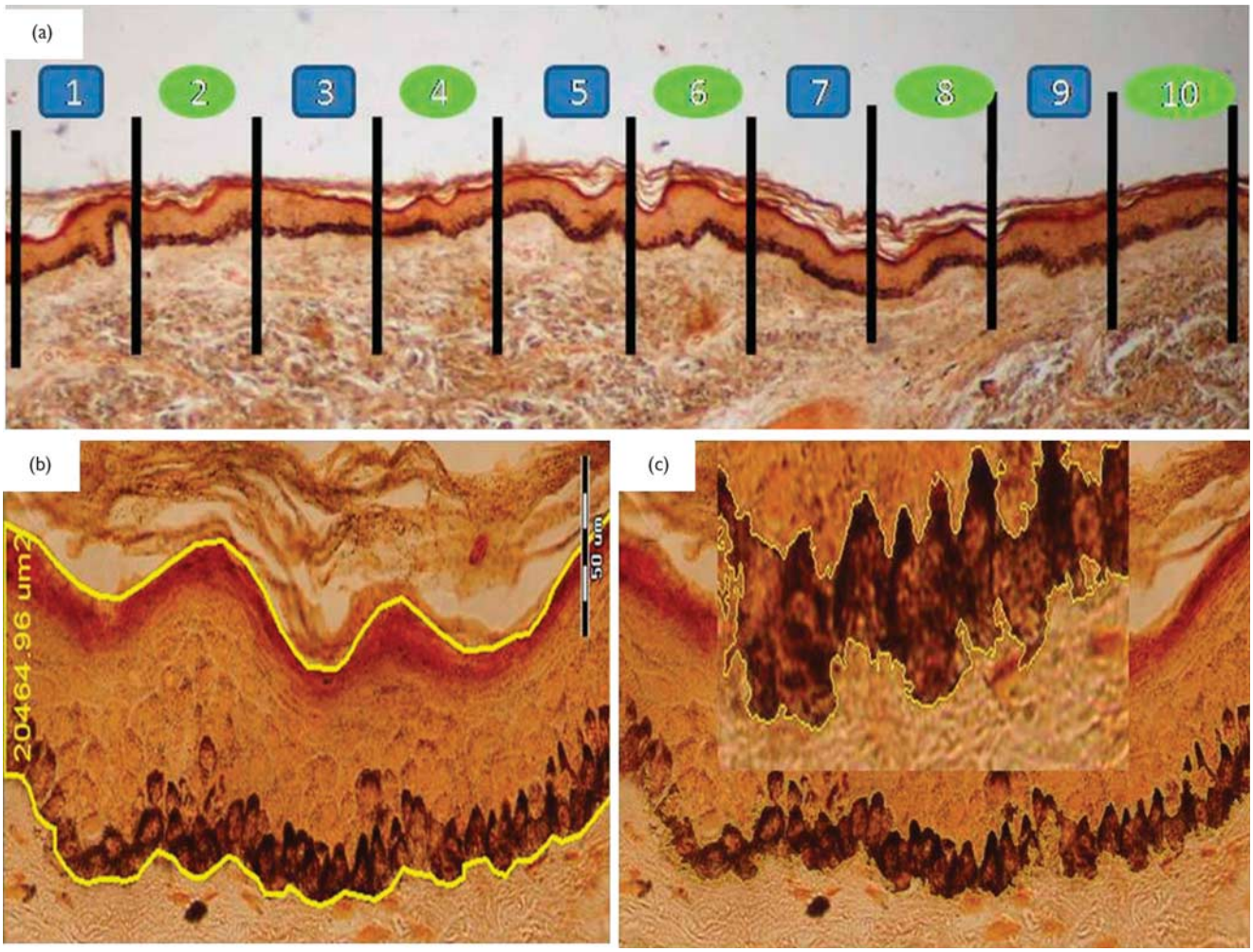
Absence of melanin in 21 out of 25 (84%) pretreated vitiliginous biopsy specimens was similar to the findings of De Francesco *et al.* [9], who showed absence of tyrosinase, melanosomes, and melanin in 11 out of 14 (78,6%) patients, as assessed by tyrosinase antibody, HMB-45 antibody, and MF staining, respectively.

On day 30 of NB-UVB therapy, clear basal cells in H&E and HMB-45-stained sections started to appear and were detected in 19 (76%) patients; however, the start of clinical repigmentation did not appear at that time. Meanwhile, the mean duration of treatment to induce this repigmentation response was 54.60 ± 30.31 days, indicating that the clinical response started to appear between days 30 and 90 of NB-UVB therapy after the appearance of HMB-45-negative clear melanocytes. As regards melanin, there was no significant increase in melanin density on day 30 of NB-UVB therapy compared with day 0.

The presence of clear cells in H&E and HMB-45-stained sections concomitant with nonsignificant increase in melanin density on day 30 of NB-UVB therapy is explained by the migration of melanocytes, from its reservoir, either in the hair follicle or in the nearby healthy epidermis, to the lesion; however, they were still inactive and were not capable of producing melanin. This is in agreement with the study by Falabella and Barona [18], who proposed that immature melanocytes at different stages of development must be present somehow in vitiligo lesions to induce repigmentation during therapy.

Only one patient showed clinical repigmentation on day 30 of therapy, and this is concomitant with the appearance of melanin and both HMB-45-positive and

Figure 4.



Morphometric measurement of melanin density in the biopsy specimen stained with Masson–Fontana (MF). (a) Schematic illustration showing that the section is divided into 10 fields by imaginary lines perpendicular to the epidermis (MF, ×40). (b) Measurement of epidermal surface area in field no. 2, in which the epidermal boundaries were outlined (MF, ×400). (c) Measurement of melanin particles surface area in the same field, in which melanin particles were outlined in yellow (MF, ×400).

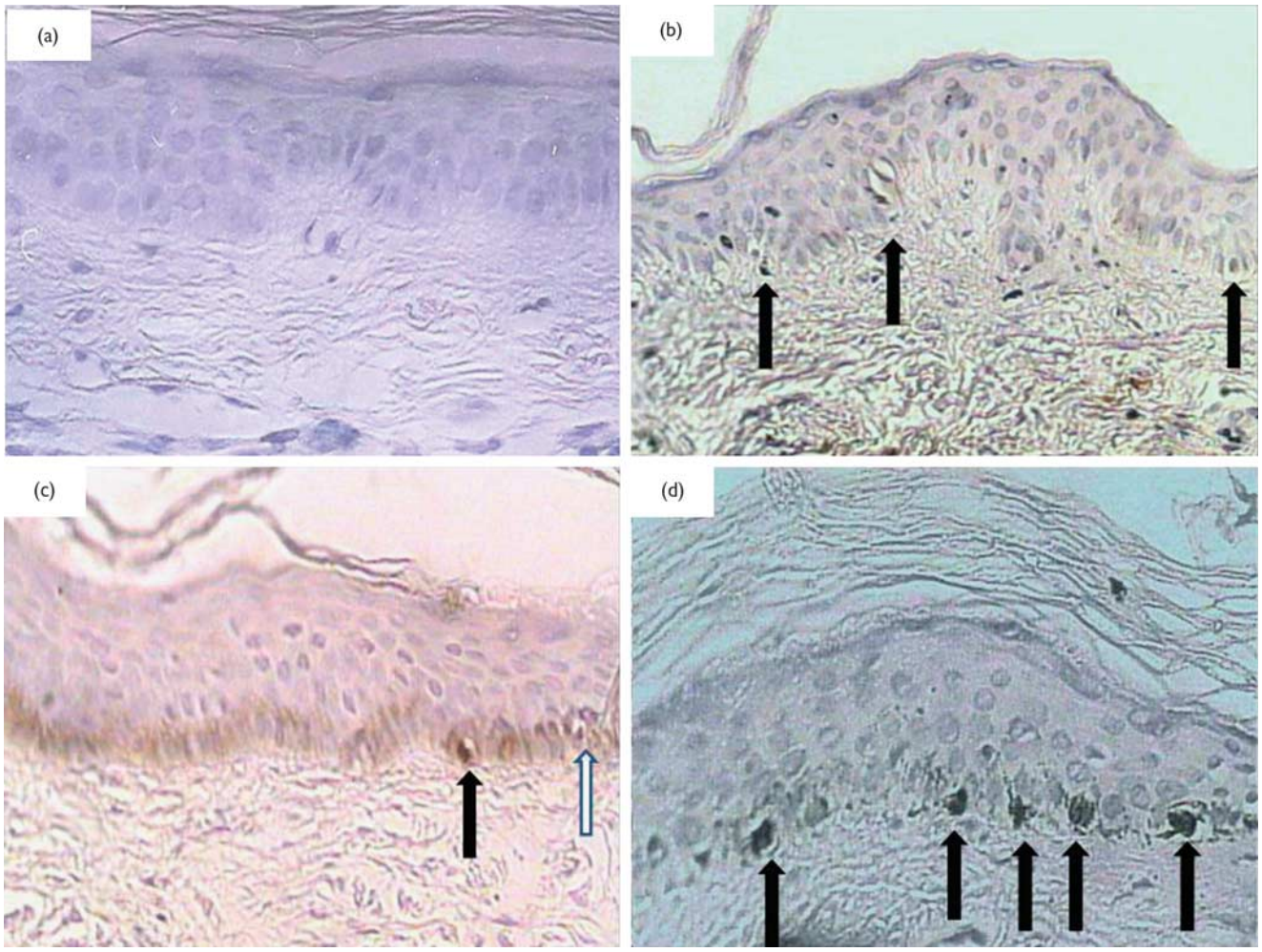
negative melanocytes. This may be due to spontaneous recovery as previously reported [19] or due to the effect of sunny climate on this outdoor worker. In both speculations, the migration of inactive melanocytes, which was considered the first step of repigmentation dynamics, preceded the onset of NB-UVB therapy in the study. Accordingly, there was earlier transformation to active melanocytes, which was evident in histopathological sections and was reflected clinically as early-onset repigmentation.

On day 90 of therapy, HMB-45-positive melanocytes were observed in 20 patients concomitant with a significant increase in melanin compared with day 0. As regards HMB-45 clear cells at day 90 of therapy, there was significant increase in its number compared with day 0 and no significant increase compared with day 30. This shows that there is a continuous arrival of inactive melanocytes into the epidermis, leading to its highest level at day 30. After that, there is transformation of these cells to active melanocytes, preventing its further accumulation. This was followed by significant decrease in the number of inactive melanocytes at day 180 compared with both day 30 and day 90. This indicates that the migration of inactive melanocytes to the epidermis decreases at a point between day 90 and day 180 after supplying the epidermis with sufficient populations of cells, which were further changed or in the process of changing into active melanocytes.

At day 180, there was a statistically significant increase in both the number of HMB-45-positive melanocytes and the concentration of melanin compared with days 0, 30, and 90 of therapy. These results were concomitant with the distribution of melanin in the epidermis, which was restricted to basal cell layer at days 30 and 90 and reached suprabasal layers at day 180 of therapy. We surmised that the new location of melanin-containing keratinocytes was due to their upward migration to reach the final destination.

The clear cells detected by H&E and HMB-45 could be melanocytes and/or Langerhans cells. Their absence on day 0, their appearance on day 30 of NB-UVB, their

Figure 5.



Staining the biopsies using human melanoma black-45 (HMB-45) antibodies revealed no HMB-45-positive or negative melanocytes in the basal layer at day 0 (a), whereas at day 30 (b) HMB-45-negative clear cells (melanocytes) appeared in the basal layer (black arrows). At day 90 (c), HMB-45-positive melanocytes (black arrows) appeared, besides HMB-45-negative clear basal cells (white arrow). The number of HMB-45-positive melanocytes (black arrows) significantly increased in biopsies performed at day 180 with disappearance of the HMB-45-negative clear melanocytes (HMB-45, × 200).

increase in number on day 90, and their presence preceding the detection of HMB-45-positive melanocytes indicate that they were inactive melanocytes. Clear cells in basal and suprabasal locations were proved by electron microscopy to be melanocytes and Langerhans cells, respectively [17]. It is noteworthy that all clear cells detected in the present study were in basal locations.

In the five patients in whom there was no clinical response until day 180 of NB-UVB therapy, HMB-45-positive and negative melanocytes and melanin did not appear on days 0, 30, 90, and 180 of therapy. This may be due to the long duration of disease in these patients causing exhaustion of melanocyte stores in the hair follicles, as was previously proved by Njoo *et al.* [13] and Anbar *et al.* [20].

In conclusion, the sequence of events of repigmentation started with the appearance of inactive melanocytes, which were first detected at day 30, followed by active melanocytes at day 90. This point indicates the presence of two subsets of melanocytes: the inactive melanocyte, which is most probably concerned with migration and is the precursor of the second type, and the active melanocyte, which is concerned with melanin production. This was also supported by increase in melanin density at day 90 concomitant with the appearance of active melanocytes, which reached its highest level at day 180 with the highest level of active melanocytes. The migration of inactive melanocytes is a continuous process throughout repigmentation, which declines at a point between day 90 and day 180 after they have supplied the epidermis with the needed population density. The clinical appearance of repigmentation coincided with the increase in melanin particles at day 90 and both reached their maximal levels at day 180. Moreover, we concluded that the absence of inactive melanocytes at day 30 might represent an index of unfavorable prognosis without the need for waiting for 50 or 60 NB-UVB sessions to take a decision for cessation of treatment.

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Conflict of interest
There are no conflicts of interest.

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