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ORIGINAL PAPER

Time-kinetic study of repigmentation in vitiligo patients by tacrolimus or pimecrolimus

L. J. Lubaki · G. Ghanem · P. Vereecken · E. Fouty · L. Benammar · J. Vadoud-Seyedi · M. L. Dell'Anna · S. Briganti · M. Picardo · M. Heenen

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Abstract New topical immunomodulators have been reported to cause repigmentation of vitiligo lesions. However, time-kinetics of such repigmentation in different anatomic locations is not well known. We performed a randomized double-blind placebo control study with tacrolimus versus the vehicle and a nonrandomized control study with pimecrolimus to evaluate the time to reach significant pigmentation, its duration and extent in treated areas. Antioxidant status of serum was also assessed. Twenty patients, in the tacrolimus study, had one pair of lesions on different localizations, and 20 on face and/or upper limbs for pimecrolimus. The extent of repigmentation was evaluated by slides and mapmakings at baseline and every 4 weeks during 7 months. Adverse events were recorded. The derivatives of oxygen metabolites, the ferric reducing ability of serum and vitamin E were assessed. Three groups of patients were identified with the tacrolimus study. Eight had no significant change in response characterized by a parallel increase of repigmentation or none in treated and control areas. Nine had a better repigmentation to tacrolimus at fifth month of treatment. Three had a marked repigmentation in control areas at the end of treatment. Repigmentation was significant on the face compared to upper-limbs with pimecrolimus from fourth to seventh month. A significant reduction of oxidative stress and an increase in antioxidant capacity in serum of patients treated with topical tacrolimus was observed, while those treated with pimecrolimus did not show any significant changes but an increase in vitamin E. Our work defines three periods in repigmentation, triggering during the first 4 months, increase in pigmentation with tacrolimus and a plateau or a sustained repigmentation. The continuity of the treatment seems necessary to ensure a prolonged repigmenting effect and even an enhanced one, such as the one we observed on the face with pimecrolimus. The extent of repigmentation was more significant on the face compared to other locations probably due to differences in melanocyte density. Furthermore, we did not find any relationship between repigmentation and the duration of vitiligo. Tacrolimus was able to reduce the systemic oxidative stress independently from its repigmenting capacity. Both drugs were well tolerated.

Keywords Vitiligo · Tacrolimus · Pimecrolimus · Time-kinetics

L. J. Lubaki (✉) · P. Vereecken · J. Vadoud-Seyedi · M. Heenen
Department of Dermatology, Hôpital Erasme,
Université Libre de Bruxelles, 808 Route de Lennik,
1070 Brussels, Belgium
e-mail: klubaki@ulb.ac.be

L. J. Lubaki · G. Ghanem · P. Vereecken
LOCE, Institut J. Bordet,
Université Libre de Bruxelles, Brussels, Belgium

E. Fouty
Public Health School,
Université Libre de Bruxelles, Brussels, Belgium

L. Benammar
Department of Pharmacy, Hôpital Erasme,
Université Libre de Bruxelles, Brussels, Belgium

M. L. Dell'Anna · S. Briganti · M. Picardo
Instituto Dermatologico San Gallicano, Rome, Italy

Introduction

Vitiligo is an acquired and common depigmenting disease of the skin with loss of melanocytes. Several authors reported an impairment of redox system even in nonepidermal compartments, including red blood cells and peripheral

lymphocytes, suggesting that systemic oxidative stress can play a role in the disease [1, 8, 16]. One hypothesis is the existence of a constitutive defect capable of inducing a persistent imbalance of the systemic redox system [9]. On the other hand, some authors indicate the imbalance of some epidermal biochemical pathways and the subsequent accumulation of reactive oxygen species (ROS) in the vitiligo skin as responsible for the alteration of antioxidant capacity in other compartments [29]. The primary goal of therapy is to restore the lack of melanocytes [10].

New topical immunomodulators (TIMs), tacrolimus (FK506) and pimecrolimus (SDZ ASM 981) are macrolides with immunosuppressant properties. TIMs were developed and launched to the indication of atopic dermatitis [3, 21]. They modulate immune-cell function by inhibiting calcineurin-dependent dephosphorylation-activation of specific nuclear factors, thus preventing transcription of pro-inflammatory cytokines [19]. Furthermore, tacrolimus has shown protective effects in experimental in vitro models of oxidative stress by increasing glutathione levels [31], inhibiting arachidonic acid release by cytosolic phospholipase A_2 [11], and increasing cellular antioxidant capacity.

Some authors have reported potent repigmenting effects and safe use of TIMs in vitiligo [12, 20, 32, 33], with a preferential repigmentation on face and neck. However, time-kinetics of repigmentation in different anatomic locations of skin surface are not well known in the literature. We hypothesized that properties of TIMs may provide an effective response on different localizations of skin surface with good tolerance. We performed two prospective studies each conducted during 7 months in different locations: with tacrolimus ointment 0.1%, a randomized, double-blind, placebo-controlled and with pimecrolimus cream 1%, a non-randomized control study. In order to evaluate the time of response, its extent and duration by comparing tacrolimus to its vehicle (petrolatum) in the same localization and within the same patient, and pimecrolimus-associated kinetics in different localizations within the same patient. At the same time, we investigated the antioxidant capacity in patient serum samples.

Materials and methods

Subjects

Forty out-patients, with generalized vitiligo, were recruited for two prospective studies. Twenty were selected in a randomized study, with tacrolimus ointment 0.1% versus placebo and 20 in an uncontrolled study with pimecrolimus cream 1%, each during 7 months. Ethical approval was obtained from the hospital ethical committee. A written consent form, after understanding all the information, was obtained from each patient. Before starting the treatment, they have been invited to a screening visit. Patients included in both studies had observed a washout period of 12 weeks. There were 25 females and 15 males, mean age 44 years (range 14–68 years), median disease duration 13 years (range 1–39 years). Patients had at least one pair of symmetric lesions, of 3 cm^2 in size, in nine different localizations of the body including face to treat, left/right (tacrolimus/petrolatum, the vehicle) for inclusion in the tacrolimus study. The presence of face and/or upper limb lesions were required for inclusion in the pimecrolimus study. All patients, except 3, had previously experienced partial repigmentation with some therapeutic modalities for vitiligo: 17 in NB-UV-B, 12 in NB-UV-B + topical steroids, 3 in NB-UV-B + calcipotriol, 2 in surgical autologous minigraft, 1 in local steroids, 1 in calcipotriol + local steroids, 1 in psoralen-UVA.

Venous blood samples were drawn before the first administration and every 4 weeks during therapy with tacrolimus or pimecrolimus. Serum was isolated at room temperature and, after centrifugation (400*g*, 15 min), it was kept frozen at −80°C until the analysis.

Treatment

Patients were instructed to apply twice daily a thin layer of topical tacrolimus or pimecrolimus on target lesions. Tacrolimus ointment 0.1% and its vehicle (petrolatum, the base of the ointment) were conditioned in identical containers by a person unaware of the study. One of the symmetrical lesions was assigned to topical tacrolimus and the other to placebo twice daily. Pimecrolimus cream 1% was applied twice daily on face and/or upper limbs.

Assessment

Repigmentation

Its extent on treated lesions, previously depigmented was evaluated at baseline and every 4 weeks. Adverse events were recorded. Color slides of lesions using digital photographs in a standard pose and serial mapmakings were analyzed visually by two physicians, one involved in the two studies and one independent. Depending on the extent of the repigmentation, response to treatment was classified into five categories: none (0%); mild (1–25%); moderate (26–50%); good (51–75%); complete (76–100%).

D-Roms measurement in serum

The derivatives of oxygen metabolites (D-Roms) were evaluated in serum samples by the "D-Roms test" (Diacron s.r.l., Grosseto, Italy). This test is based on the concept that

the amount of organic hydroperoxides present in serum is related to the free radicals from which they are derived [13]. The hydroperoxides are able to oxidize an additive (*N*,*N*-diethyl-*p*-phenylendiamine) to the corresponding radical cations, whose concentration can be spectrophotometrically detected by measuring their absorption at 550 nm. The intensity of the developed color is directly proportional to the concentration of D-Roms, according to Lambert–Beer's law. The normal values of the test are between 250 and 300 U. CARR. (Carratelli Units), arbitrary units calculated according to a formula (U. CARR. = $F \times (\Delta abs/min)$, where F is a correction factor with an assigned value. 1 U.CARR. corresponds to 0.8 mg/l H_2O_2. Values outside this range indicate a modification of the prooxidant/antioxidant ratio. Values >300 U.CARR. indicate a condition of oxidative stress. A change of at least 15% from the baseline value was taken as the cut-off value for a significant variation in oxidative stress.

Evaluation of serum antioxidant capacity with BAP-test

The biological antioxidant potential (BAP) test (Diacron s.r.l., Grosseto, Italy) measures the ferric reducing ability of serum [5]. In this test, a solution of ferric ions is complexed to form a chromogenic compound and an intense red-purple color with a maximum absorption at 505 nm develops. In the presence of serum, a reduction of complex absorbance at 505 nm related to the serum antioxidant potential can be detected. BAP assay has been performed in accordance with the manufacturer's instructions. Briefly, 1 ml of chromogenic substrate (R_1) and 50 µl of $FeCl_3$ (R_2), were mixed with 10 µl of serum. A blank reagent, obtained by replacing serum with distilled water, was included for each assay. After 5 min of incubation at 37°C, absorption of solutions were detected and the entity of color decrease is directly related to the amount of Fe^{3+} ions reduced to ferrous ions, and consequentially to the redox capacity of serum. The results of BAP-test are expressed as µmol/l of ascorbic acid, as index of physiological reduction capacity of serum against ferric ions.

Vitamin E measurement

To measure vitamin E**,** serum samples (0.1 ml) were extracted three times in hexane/ethanol (3/1; v/v) with 1 ml of sodium dodecyl sulfate as detergent in the presence of 1.25 µg of δ- and γ-tocopherols, as internal standards. The solvent was evaporated to dryness under a nitrogen stream. Vitamin E (α-tocopherol) was derivatized with *N*,*O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% of trimethylchlorsilane as catalyst. Tocopherol was analyzed by gas chromatography/mass spectrometry (GC/MS) on a capillary column (RTX5 Restex 30 m × 0.20 µm ID, 0.25 mm) by a selected ion monitoring (SIM) technique as previously described [26].

Statistical analysis

McNemar (χ^2) paired *t* test was used to compare the effectiveness of tacrolimus in the repigmentation of vitiligo lesions versus placebo. Kruskal Wallis test for continuous variables, analyses the relationship between the response (tacrolimus/placebo) and the duration of vitiligo. It compares the medians of duration of the disease inside the three new categories of repigmentation (none, 1–25%, >25%), redefined according to our sample. In the pimecrolimus study, means of four classes (or categories) of repigmentation were used to analyze data. Fisher test for trend compare treatment responses of different localizations in pimecrolimus study. Data analyses were performed using Epinfo6, SPSS and Prism statistical softwares and figures were built by Prism.

Results

Patients achieved varying levels of repigmentation on treated lesions. No repigmentation has been observed on untreated lesions.

Tacrolimus study: the effectiveness of tacrolimus and the relationship between duration of vitiligo and repigmentation

Among 20 vitiligo lesions treated with tacrolimus, 16 (80%) achieved some degrees of pigmentation versus 11 (55%) of 20 assigned to the vehicle. The effectiveness of tacrolimus was significantly higher ($P < 0.05$) than placebo, McNemar paired *t* test (Fig. 1). In addition, Kruskal Wallis test (Table 1), did not show a relationship between the duration of vitiligo and repigmentation with tacrolimus p50 exact test = 0.714 (NS), and placebo p50 exact test = 0.281 (NS).

Tacrolimus study: difference of scores between tacrolimus and placebo repigmentation within the same patient

Among 40 target lesions to treat (half for tacrolimus and half for placebo), 14 were located on hands, 8 on abdomen, 4 on face, 4 on elbows, 2 on forearms and arms, 2 on legs, 2 on upper back, 2 on feet, 2 on thighs and buttocks. Figures 2, 3 and 4 illustrated the repigmentation, scored for each patient over 7 months of treatment. Scores, ranged from 0 to ±2, were obtained by the difference of the extent of repigmentation (defined in methods) between tacrolimus and vehicle treated areas at a given time and within the

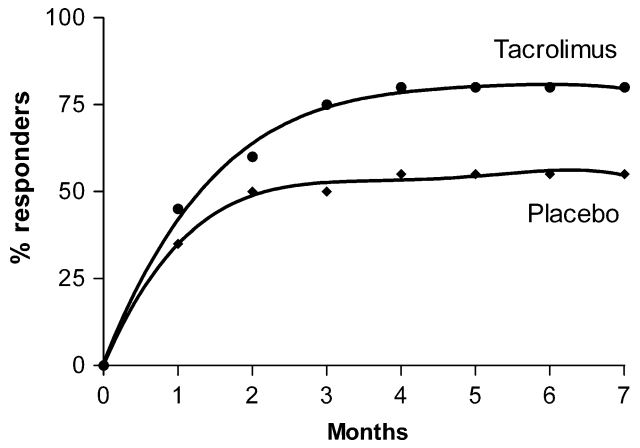

Fig. 1 Percentage of patients achieving repigmentation on paired treated lesions within the same patient over 7 months of treatment. Sixteen (80%) lesions among 20 have repigmented with tacrolimus and 11 (55%) from the other 20 lesions with placebo

Table 1 Number of patients and their median duration of vitiligo into different categories of responses to tacrolimus/placebo

Repigmentation	Tacrolimus (median duration of vitiligo) (n = 20)	Placebo (median duration of vitiligo) (n = 20)
None (0%)	4 (23.50)	9 (14)
Mild (1–25%)	9 (14)	7 (16)
Moderate, good, complete (>25%)	7 (9)	4 (8.50)

Comparison of the median duration of the illness into the three categories of repigmentation in patients treated with tacrolimus versus placebo

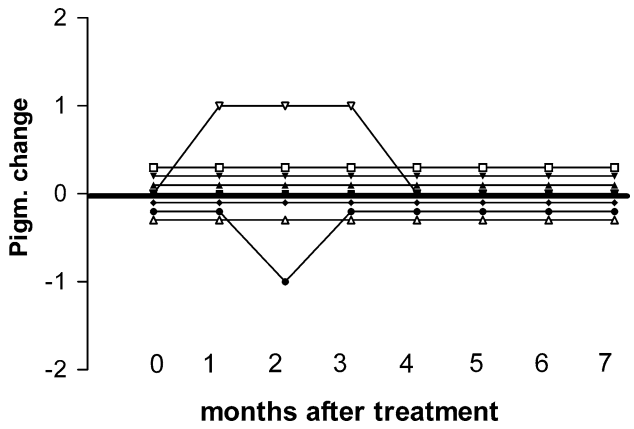

Fig. 2 First group of patients with no significant change in pigmentation between paired lesions treated each either by tacrolimus or by vehicle. Out of eight patients, five did not repigment either with tacrolimus, or with the vehicle on hands, buttocks and chest, three have shown a similar (1–25%) repigmentation to tacrolimus and the vehicle on hands and chest

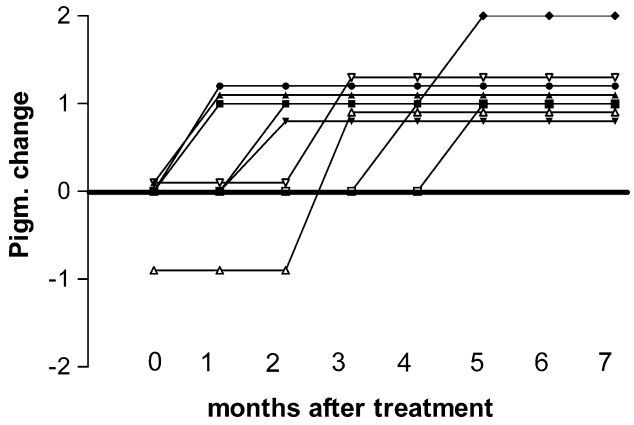

Fig. 3 Second group of patients with beneficial effect of tacrolimus over the vehicle. A cascade of responses over 7 months of treatment in nine patients on hands, elbows, buttocks, upper-back, forearms and face with initial repigmentation (1–25%) between the first and fourth months with tacrolimus and vehicle. Good responses (51–75%) were achieved in two patients at fourth and fifth months on upper-back and face with tacrolimus versus knee and face at sixth and seventh months in two patients with placebo

same patient. Score 0 represents no repigmentation or a similar response to tacrolimus and vehicle (no change), while ±1 and ±2 represent the lower/higher scores of repigmentation to tacrolimus (positive score) or the vehicle (negative score). On the basis of the three figures showing responses to the treatment compared to the control, we could clearly identify three groups of patients. In group 1 (Fig. 2), 8 patients had no change in pigmentation: 5 did not repigment either to tacrolimus, or to the vehicle on hands, buttocks and chest, 3 have shown a similar (1–25%) repigmentation to tacrolimus and to the vehicle on hands, and chest. In group 2 (Fig. 3), 9 patients had a pigmentation benefit to tacrolimus between the first and the fifth months on hands, elbows, buttocks, upper-back, forearms and face with good responses (51–75%) reached at fourth and fifth months (1 on upper-back and 1 on face). In group 3 (Fig. 4), 3 patients had a higher repigmentation in control areas to the vehicle between the first and the seventh months with good responses (51–75%) reached at sixth and seventh months (1 knee and 1 face). Initial repigmentation (mild) occurred between the first and the fourth months with tacrolimus and vehicle. In all patients, acquired pigmentation was sustained or gradually increasing over the 7 months of treatment. These responses lasted until the end of the study.

Pimecrolimus study: percentage of repigmented areas during 7 months treatment of different localizations

Among 20 patients, 17 lesions on upper limbs, 14 on the face, 8 on lower limbs, 7 on chest, 3 on abdomen, 2 on back, 2 on genitalia have been recorded. For statistical reasons, we only considered two frequent localizations. Figure 5 shows the extent of repigmentation between face

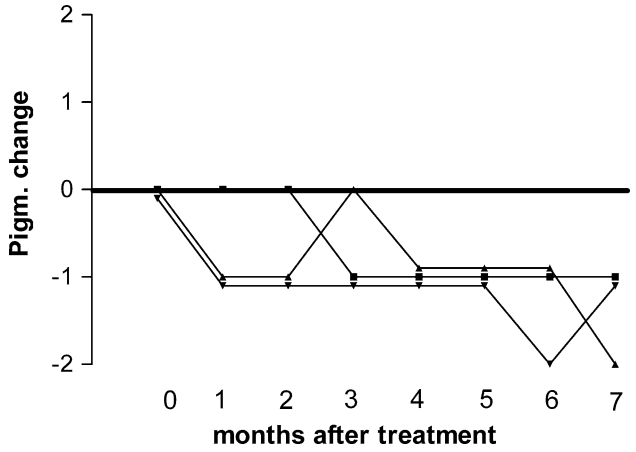

Fig. 4 Third group of patients with vehicle performing better than tacrolimus. Three subjects had an unexpected effect of the vehicle outscoring tacrolimus between the first and seventh months on knee (51–75%), face (51–75%), and foot (26–50%)

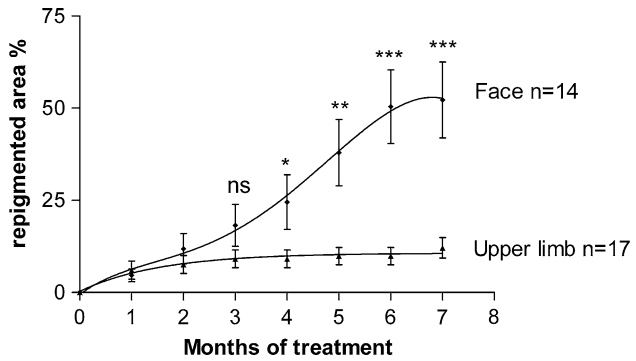

Fig. 5 Repigmenting effect of pimecrolimus in two different localizations (values are means ± SEM). Seven lesions achieved 76–100% of repigmentation with pimecrolimus after the fourth month of treatment and seven others, from 1 to 50%, on the face. Lesions of upper-limbs show low levels of repigmentation ranging from none to 26–50%

and upper limbs within the same patient over 7 months of treatment in 11 patients. The tendency to a substantial repigmentation was significantly greater on the face compared to upper-limbs ($P < 0.001$, Fischer test for trend). Significant repigmentation difference between the two localizations occurred 4 months after the treatment and increased steadily with time ($P = 0.040$, Fischer test for trend).

Side effects

Both treatments were well tolerated during 7 months trials with no systemic side effects except a transient pruritus in the treated areas of 4 tacrolimus and 2 pimecrolimus patients.

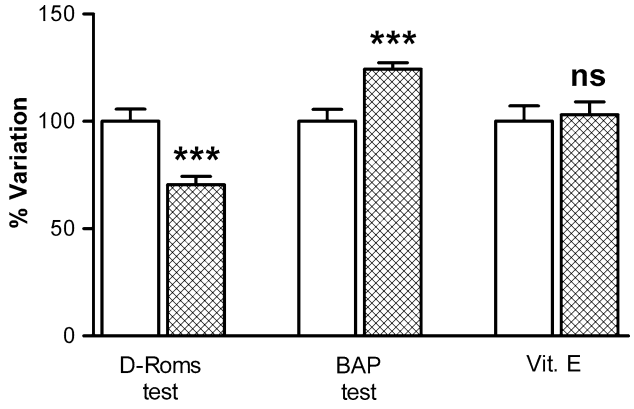

Fig. 6 Effect of tacrolimus therapy on plasma oxidative stress

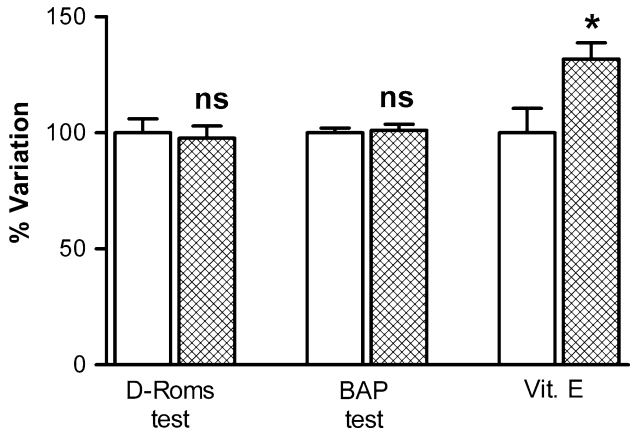

Fig. 7 Effect of pimecrolimus therapy on plasma oxidative stress

Oxidative stress evaluation in blood

Mean values of serum oxygen derived metabolites and vitamin E levels have been compared for each patient before and after treatment in both studies. A significant reduction of oxidative stress in serum of patients treated with topical tacrolimus is demonstrated by 30% decrease in oxygen derived metabolites (D-Roms test) and 20% increase of the serum antioxidant capacity (BAP test, Fig. 6), while a significant increase of vitamin E (30%) was observed after the treatment with pimecrolimus (Fig. 7).

Discussion

Both trials pointed out the repigmenting effect of tacrolimus and pimecrolimus in a long-term therapy for vitiligo. Our work was focused on the time-kinetics of repigmentation of vitiligo lesions at a given time. We compared the response in the same location and within the same patient with tacrolimus versus placebo and the response between two

frequent locations (face and upper limbs) within the same patient with pimecrolimus. In our findings, patients did not achieve complete repigmentation with tacrolimus in any localization compared to patients who had undergone pimecrolimus. Repigmentation was also observed on control lesions. We did not note any spontaneous repigmentation of untreated lesions in different anatomic locations. Repigmentation is mild in the first 4 months with tacrolimus and the vehicle. In both studies, acquired pigmentation was either sustained or gradually increasing at different levels in face and body localizations during the 7 months of treatment. These responses lasted until the end of the study. Good repigmentation has been observed on upper-back and face with tacrolimus, on knee and face with placebo while complete on the face with pimecrolimus. The comparison of two frequent localizations underlined predominant repigmentation on the face, a daily sun exposed site as compared to upper-limbs in the same patient in accordance with previous studies [12, 14, 30, 32].

We could clearly identify three periods during repigmentation: a triggering, a rapid increase and a plateau. Repigmentation is triggered during the first 4 months. The second period is characterized by a rapid increase of repigmentation in a month. The third period corresponds to a sustained repigmentation in any localization. However, in an open trial, Kanwar et al. [14] had reported areas of depigmentation over repigmented sites in 4 of 22 children treated with tacrolimus ointment 0.03%.

Petrolatum, the vehicle of tacrolimus, performed better in three patients on face, upper-back and foot. The two studies were carried out from September to April (autumn to spring), prescribing protection of vitiligo lesions by clothes, avoidance of sun exposure and use of sunscreens. However, UV-radiation can play a role especially in sun-exposed areas. And, as it is already known, vehicles enhance penetration of ultraviolet radiation into the skin [4]. Another explanation could be the higher hair follicle density in these areas [14, 20, 24] that may also influence the penetration and absorption of topically applied drugs. In addition, epidermal melanocyte density is variable in anatomical site [10, 35]; thus, migrating melanocytes into the depigmented skin may come from contiguous pigmented skin areas. Moreover, petrolatum plays an occlusive role and might prevent access of molecular oxygen to a rapidly metabolising damaged tissue [7].

Antioxidant administration has been found to have a co-therapeutic effect, leading to the improvement of effectiveness of vitiligo treatments [2]. A similar degree of improvement of the redox system has been observed in patients in which tacrolimus was found to be able to induce repigmentation, as well as in those that did not show any significant clinical effects. However, in patients treated with pimecrolimus a good repigmentation has been induced, while no improvement of serum antioxidant capacity has been observed.

These data indicate that topical tacrolimus is able to effectively reduce the systemic oxidative stress independently from its repigmenting capacity. Induction of repigmentation is strongly related to the presence of residual melanocytes in skin sites surrounding the vitiligo lesions and it can be also mediated by direct action of tacrolimus on keratinocytes to induce enhancement of melanocyte proliferation [17, 18], while the observed antioxidant effect seems to be related with tacrolimus penetration through skin layers. Data in the literature reported in fact that pimecrolimus has a lower potential for percutaneous absorption, and consequently a lower systemic exposure to pimecrolimus after topical application, as compared to tacrolimus [6]. Useful tacrolimus drug concentrations reached in the blood with 0.5% of formulation applied to the skin [21] and up to 4 ng/mL could be measured [25]. Long-term intermittent treatment of adult patients with extensive atopic dermatitis with pimecrolimus cream 1% was associated with minimal systemic exposure (<0.5 ng/mL) with no evidence of drug accumulation [34]. Pimecrolimus is structurally similar to tacrolimus but binds to macrophilin 12 with a threefold lower affinity compared with tacrolimus [28], leading to a better inhibition of cytokines including TNFα [27] by a mechanism involving the inhibition of both iNOS mRNA expression and the activation of NF-*k*B [15].

Some authors believe that spontaneous repigmentation is a rare phenomenon [22, 23], and when it occurs, it is probably UV-induced**.** TIMs were safe and well tolerated in long-term vitiligo therapy. The effectiveness of tacrolimus was statistically significant compared to placebo ($P < 0.05$). We did not find any relationship between duration of vitiligo and the repigmentation with tacrolimus compared to placebo (NS).

In conclusion the outcomes support a 3-month period to reach some levels of repigmentation; otherwise the treatment has to be stopped for nonresponders. The continuity of the treatment may sustain and enhance the repigmenting effect as observed on the face with pimecrolimus.

Topical tacrolimus and pimecrolimus could be considered as alternatives in the treatment of localized vitiligo and small lesions over the face [20, 30, 33] in monotherapy, as well as in specific cases of contraindication or resistance to conventional therapies. Pimecrolimus was more cosmetically appreciated by our patients because of its creamy formulation [20]. The extent of repigmentation was significantly higher on the face ($P < 0.001$). Unlike pimecrolimus, tacrolimus antioxidant capacity could be demonstrated in serum also probably related to its formulation leading to better skin absorption and penetration properties.

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