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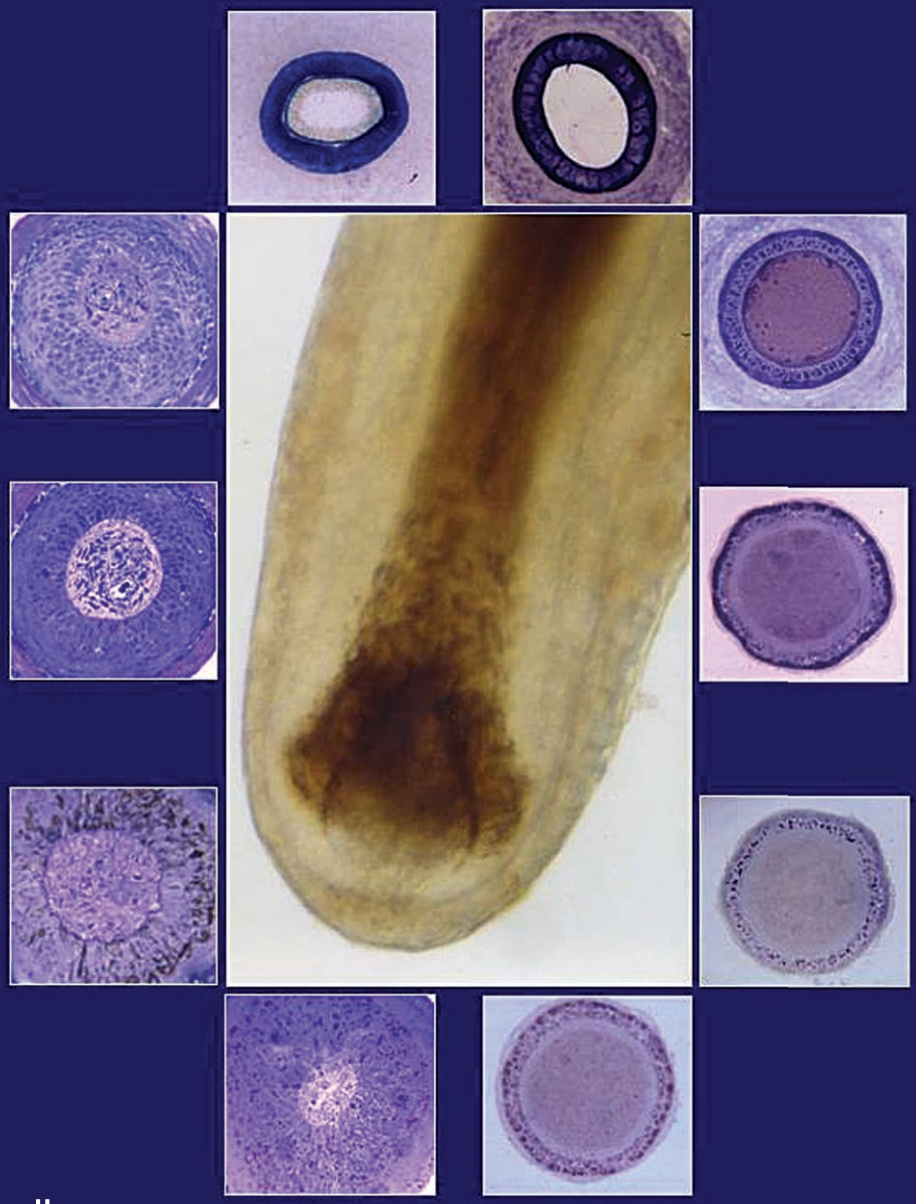

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Detection of Tumor Necrosis Factor-alpha in Nonlesional Tissues of Alopecia Areata Patients: A Prove for a Systemic Disease

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ABSTRACT

Introduction: The pathogenesis of alopecia areata (AA) remains incompletely understood. Different cytokines may play a role in AA. Tumor necrosis factor-alpha (TNF-α) has been shown to be inhibitory to hair follicle growth in *in vitro* studies suggesting that it may play an important role in AA. This study was conducted to assess the presence of TNF-α in lesional and nonlesional skin of AA, to review its possible role in AA, and to show whether AA is a systemic or localized disease by comparing the level of TNF-α between lesional and nonlesional skin biopsies of the patients. **Materials and Methods:** Thirty patients with AA and thirty age- and sex-matched healthy controls were included in the study. A 4 mm punch skin biopsy was taken from lesional and nonlesional skin of every patient, as well as from the normal skin of each individual in the control group for immunohistochemical analysis of TNF-α. **Results:** The level of TNF-α in lesional skin biopsies was significantly higher than in nonlesional skin biopsies of patients as well as controls' biopsies. Furthermore, TNF-α level in nonlesional biopsies of patients was significantly higher than the level in controls' biopsies. **Conclusions:** We concluded that skin of AA has a high level of TNF-α (a normal inhibitor of hair follicle growth *in vitro*). This high level may point to the important role of TNF-α in AA. Further studies should be conducted to detect the level of TNF-α in long-standing AA and the more severe cases of AA.

Key words: Alopecia areata, lesional, nonlesional, tumor necrosis factor-alpha

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INTRODUCTION

Alopecia areata (AA) is a common cause of reversible loss of hair affecting approximately 1%–2% of general population.[1]

The cause of AA is unknown although the presence of evidences suggesting the link between lymphocytic infiltration of the hair follicle and the disruption of the hair cycle provided by a combination of multiple factors including cytokine release, cytotoxic T-cell activity, and apoptosis.[2]

The persistence of AA lesions is attributed to disequilibrium in the production of cytokines, with a relative excess of pro-inflammatory and Th1 types cytokines versus anti-inflammatory cytokines, as shown in human scalp biopsies.[3]

Tumor necrosis factor-alpha (TNF-α) is synthesized in epidermal keratinocyte is known to be a very potent inhibitor of proliferation. Furthermore, *in vitro* studies showed that TNF-α, along with interleukin 1α (IL-1α) and IL1-β, causes vacuolation of the matrix cells within the hair follicle bulb and a decrease in the size of the matrix and also causes a disorganization of follicular melanocytes and abnormal differentiation and keratinization of the precortical cells and the inner root sheath.[4]

A limited number of studies in the literature were done to evaluate the serum levels of TNF-α in patients with

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AA. Teraki *et al.* reported that serum levels of TNF-α in patients with AA were significantly higher than those in patients with alopecia universalis (AU).[5]

On the other hand, in the study that was done by Koubanova and Gadjlgoroeva, serum levels of TNF-α in patients with AA did not reveal any difference from that in controls.[6]

In addition, Lis *et al.* found that serum level of TNF-α receptors Type I was significantly elevated in patients with AA in comparison with healthy controls.[7]

Aim of the work

Since TNF-α has known to be an inhibitor to proliferation and its effect on hair follicle growth in the *in vitro* studies with observed changes in hair follicles incubated with TNF-α and the controversial data about the elevated serum level of TNF-α in AA, we decided to detect the tissue levels in lesional and nonlesional skin of AA patients.

MATERIALS AND METHODS

A controlled study was carried out on thirty patients suffering from AA and thirty healthy volunteers served as controls. Patients and controls were recruited from Outpatient Clinic of Dermatology in Beni-suef University Hospital, Beni Suef, Egypt.

The patients' ages ranged from 3 to 41 years. As regards the sex, 13 patients were females (43.3%) and 17 were males (56.7%). Disease duration varied from 1 week to 2 years.

Thirty healthy volunteers, age and sex matched, served as controls. Eighteen were males (60%) and 12 were females (40%) with ages ranging from 3 to 43 years.

Every patient was subjected to the following

Full informed consent

the purpose of the study was explained for each patient. A written informed consent was taken from each patient. Local research ethics approval was taken before starting data collection. With respect to patients' confidentiality, code numbers changed all personal data of patients. All personal data were concealed. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a prior approval by the Institution's Human Research Committee.

History taking

History taking included name, age, sex, occupation, and special habits. History of the present illness included the onset, course and duration of the disease, predisposing factors, and previous treatment. Past history of similar conditions and of other skin diseases, as well as family history of skin and systemic diseases were also checked for.

Physical Examination

Full general and dermatological examination was done at the time of presentation.

Exclusion criteria

We excluded patients who received any systemic medication or topical treatment of AA 3 months before sample collection.

Collection of specimens and immunohistochemical staining

A 4 mm punch skin biopsy was taken from lesional skin of every patient as well as from a nonlesional area (the forearm). Furthermore, two biopsies were taken from each individual in control; one from the scalp and the other was taken from the forearm.

Serial sections from each tissue block were cut at 5 μ thickness. One section stained by hematoxylin and eosin, for histopathological evaluation.

Immunostaining was done using monoclonal rabbit antihuman TNF-α antibody (MyBioSource, USA) and ultra ision detection system. Immunopositivity was selected and captured at a magnification ×200, using a digital video camera mounted on a light microscope (CX21, Olympus, Japan). Images were then transferred to the computer system for analysis.

All steps for immunohistochemical evaluation were carried out using image analysis software (Image J, 1.46a, NIH, USA). Images manually corrected for brightness and contrast are converted to 8-bit monochrome type, color threshold was then performed automatically. Area fraction was then calculated automatically representing the area percentage of TNF-α immunopositive cells to the total area of the microscopic field.

Statistical analysis

Fisher's exact and Chi-square tests were used in the analysis to estimate the correlation between TNF-α immunoreactivity

and clinicopathological data for each case. The significance of the results assessed by determining the probability factor "*P*" value. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical characteristics of participants

This study included thirty AA patients and thirty healthy controls. Seventeen (56.7%) AA patients were males and 13 (43.3%) were females. Their ages ranged from 3 to 41 years with a mean of 20.1 ± 13.3 years.

The control group included thirty apparently healthy dermatologically free individuals 18 (60%) were males and 12 (40%) were females. Their ages ranged from 3 to 43 years with a mean of 21.50 ± 12.85 years.

All lesional biopsies were taken from the scalp whereas nonlesional biopsies were taken from the forearm skin. In control, all biopsies were taken from the forearm.

Eight patients presented with temporal lesion, eight with parietal lesion, five with occipital lesion, five with frontal lesion, two patients with both frontal and temporal lesions, one with frontal and occipital lesions, and one with alopecia totalis (AT).

Tumor necrosis factor-alpha tissue level in patients (lesional and nonlesional)

All patients (100%) expressed TNF-α in both lesional and nonlesional biopsies and the level varied from 2.4–29.9 to 2.2–20.8, respectively [Table 1], with a mean level of 13.7 ± 7.2 and 8.7 ± 4.6 lesional and nonlesional, respectively.

Tumor necrosis factor-alpha tissue level in controls

All controls (100%) expressed TNF-α in both scalp and forearm biopsies; the level varied from 1.3–1.9 to 1.0–1.5, respectively, with a mean level of 1.6 ± 0.1 and 1.1 ± 0.1 scalp as well as forearm, respectively.

Relations and correlations

Relation between tumor necrosis factor-alpha tissue level and clinical data of patients

Regarding age, sex, family history, disease duration, history of other autoimmune diseases, previous history of AA, site, and size of lesion, there was no significant correlation between TNF-α tissue level in both lesional and nonlesional skin biopsies.

Table 1: Values of lesional and nonlesional tumor necrosis factor-alpha tissue level in patients

Number	Lesional TNF-α	Nonlesional TNF-α
1	15.2	20.8
2	8.0	6.4
3	5.5	5.1
4	16.7	12.9
5	14.0	9.0
6	29.9	11.0
7	15.5	14.9
8	4.2	5.7
9	7.1	15.0
10	10.5	9.9
11	12.0	4.8
12	2.4	11.5
13	5.5	5.6
14	10.3	9.9
15	8.7	4.1
16	18.1	15.1
17	11.8	2.3
18	22.7	14.1
19	9.9	9.3
20	8.9	7.8
21	26.8	6.0
22	25.5	7.5
23	19.3	7.2
24	14.9	8.1
25	12.5	3.3
26	24.8	2.6
27	6.1	2.2
28	9.7	9.1
29	12.5	3.3
30	23.6	15.5

TNF-α – Tumor necrosis factor-alpha

Relation between tumor necrosis factor-alpha tissue level in alopecia areata patients and in controls

Regarding lesional tumor necrosis factor-alpha tissue level in alopecia areata patients and controls

Mean value of lesional TNF-α tissue levels in AA cases was significantly high compared with their levels in scalp of the controls, $P < 0.0001$ [Table 2 and Figures 1-5].

Regarding nonlesional tumor necrosis factor-alpha tissue level in alopecia areata patients and in controls

Mean value of nonlesional TNF-α tissue levels in AA cases was significantly high compared with their levels in normal skin of the controls, $P < 0.0001$ [Table 2 and Figures 1-4].

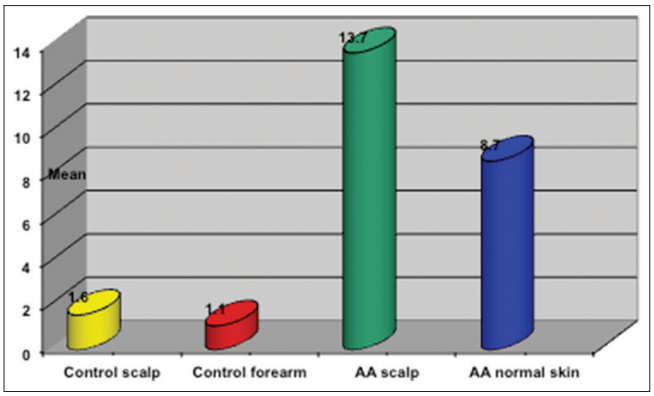

Figure 1: Mean value of tumor necrosis factor-alpha tissue level in (lesional and nonlesional) alopecia areata patients compared to controls

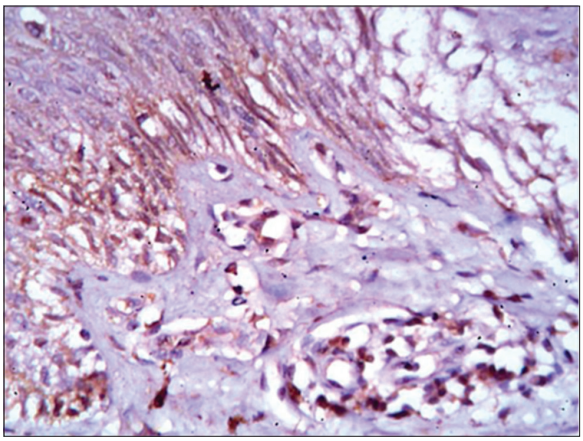

Figure 3: A case of alopecia areata with late stage (lesion) with no inflammatory cells, showing tumor necrosis factor-alpha in telogen germinal unit of hair follicle

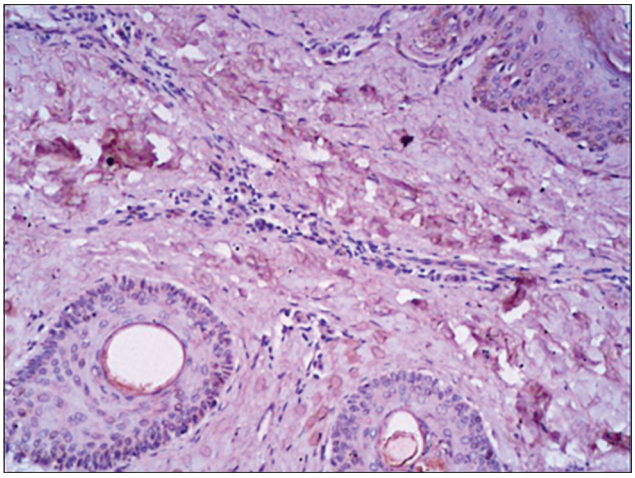

Figure 5: Control case inflammatory cells showed negative reaction for tumor necrosis factor-alpha

Relation between lesional and nonlesional tumor necrosis factor-alpha tissue level in alopecia areata patients

Level of TNF-α expression in the lesional AA biopsies was statistically higher than the level of TNF-α in the nonlesional skin ($P < 0.0001$).

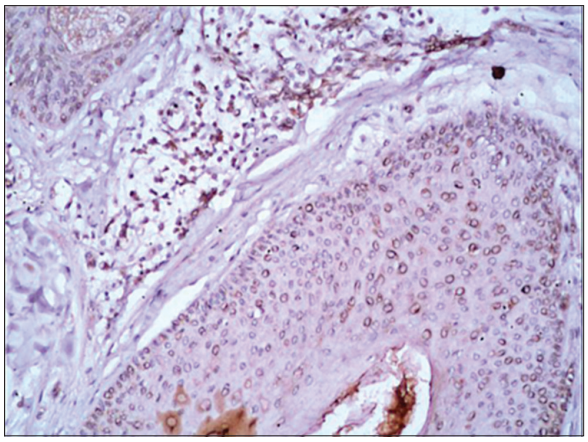

Figure 2: A case of early acute stage of alopecia areata (lesion) showing positive immunostaining reaction for tumor necrosis factor-alpha

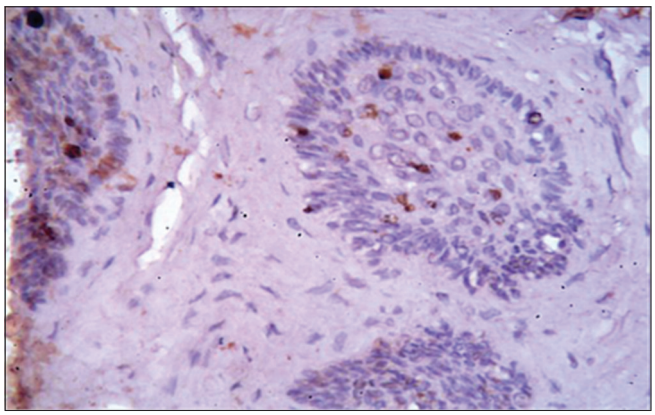

Figure 4: A case of alopecia areata (nonlesional) showing immunoreactions for tumor necrosis factor-alpha

DISCUSSION

AA is a dermatologic disease characterized by nonscarring hair loss on the scalp or any hair-bearing surface. A wide range of clinical presentations can be presented ranging from a single patch of hair loss up to complete loss of hair on the scalp; AT or the entire body, and AU.[2] Etiology of AA is unknown although both genetic factors and environmental agents are thought to contribute to the immune dysregulation leading to the final pathways of the disease.[8]

Basic researches have established AA as a T-cell-mediated autoimmune disease. Peri- and intra-follicular mononuclear cell infiltrates directed to anagen hair bulbs are characteristic histological features.[9] The inflammatory infiltrate is composed of activated CD4+ and CD8+ T-cells, together with macrophages and Langerhans cells.[10] This inflammation requires actions of multiple cytokines, especially the helper T-cell Type 1 cytokines such as TNF-α, which may be a central core to the pathophysiology of this disease.[11]

Table 2: Comparison between lesional tumor necrosis factor-alpha tissue levels in alopecia areata cases and in healthy controls

Cases	Mean±SD		*P*	Significant
	Patients	Controls (scalp)		
Lesional TNF-α	13.7±7.2	1.6±0.1	<0.0001	HS
Nonlesional TNF-α	8.7±4.6	1.1±0.1	<0.0001	HS

TNF-α – Tumor necrosis factor-alpha; HS – Highly significant; SD – Standard deviation

The role of TNF-α in the pathogenesis of AA is still controversial. Its role appears complex; on the one hand, it is considered important in the hair loss as it inhibits hair growth *in vitro*, also TNF-α producing cells can be found in the mononuclear infiltrate surrounding the hair follicle.[4] On the other hand, treatment with etanercept (an anti-TNF-α) showed no improvement in 17 cases with AA.[11] Thus, this study was conducted to assess the presence of TNF-α in lesional skin of AA and to review its possible role in AA.

In our study, we found that TNF-α levels in lesional skin were higher than nonlesional skin of patients and this difference was statistically significant (lesional vs. nonlesional = 13.8 ± 7.2 vs. 8.7 ± 4.6, $P = 0.001$). The TNF-α level in lesional biopsies of patients was higher than that of controls' biopsies which was also statistically significant (lesional vs. control = 13.8 ± 7.2 vs. 1.6 ± 0.1, $P = 0.0001$).

Furthermore, TNF-α level in nonlesional biopsies of patients was significantly higher than the level in controls' biopsies which was also statistically significant (nonlesional vs. control = 8.7 ± 4.6 vs. 1.1 ± 0.000, $P = 0.000$).

To the best of our knowledge, no previous studies were done to detect the level of TNF-α in lesional skin of AA patients. However, few studies were done on the serum level. Barahmani *et al.,* 2010,[12] studied the sera of 269 patients with AA, in which the levels of Th1 cytokines (including TNF-α), IL-1 receptor antagonist, and IL-8 levels were found to be significantly higher than in the control group.

In the present study, when we correlate TNF-α level in lesional, nonlesional, and controls' biopsies, it was neither affected by the participant's age and gender nor the duration, site, and extent of lesion. Furthermore, Barahmani *et al.,* 2010,[12] found that increased serum levels of Th1 cytokines are associated with AA with no correlation with participant's age and gender or disease severity, which matches our study.

The significantly higher level of TNF-α in the lesional skin of AA patients of the present study suggests that TNF-α has a role in AA supported by the inhibitory effects of TNF-α on hair follicles *in vitro*,[4] and the high level of TNF-α in serum of AA patients regardless of disease severity[12] is in agreement with our results.

The significantly higher level of TNF-α in nonlesional biopsies of our patients than in the controls' biopsies is in agreement with the concept that AA is a systemic disease. Tobin, 2003,[13] also had revealed circulating IgG autoantibodies to the HF inner and outer root sheaths, matrix, and precortex using indirect immunofluorescence, which matches our results.

There are increasing reports of failure of TNF-α inhibitors in controlling AA which makes the role of TNF-α in AA doubted and thus needs further investigations. Etanercept was ineffective in treating participants with treatment refractory, moderate-to-severe AA, AT, or AU. Seventeen otherwise healthy adults with moderate-to-severe AA were given etanercept (50 mg subcutaneously twice weekly). Significant regrowth of hair was not shown in any of the participants treated.[11]

The lack of efficacy suggests that the dose of etanercept used was not adequate to block the pro-inflammatory activity of TNF-α. Many of the participants in this study had long-standing, severe, and treatment refractory disease, thus may represent a population not amenable to most therapies for AA. However, the negative results of this study suggest that another therapeutic approach should be considered when treating patients with long-standing, severe, and treatment refractory, moderate-to-severe AA. Furthermore, anti-TNF-α therapy for less severe and newer onset AA may yield positive results.

To the best of our knowledge, 16 patients developed AA whereas on TNF-α inhibitor treatment. When the anti-TNF-α was discontinued in 7 of the 16 patients, only two cases showed improvement with complete regrowth of hair. Drug-induced alopecia should be diagnosed if improvement of the alopecia occurs after cessation of the suspected drug. Hence, the immune-mediated diseases induced by anti-TNF-α agents do not follow the patterns of most adverse drug reactions and do not fit the classic criteria for adverse effect.[14]

One explanation of the paradoxical aggravation or occurrence of AA with anti-TNF-α agents reported when anti-TNF-α antibodies (i.e., infliximab and adalimumab) were used, where antinuclear antibodies were detected in up to two-thirds of patients.[15]

It is possible that TNF-α inhibitor-induced autoimmunity is mediated by the inhibition of the suppressive functions of the regulatory T-cells which play an important role in maintaining immune tolerance and prevent autoimmunity.[16]

Another explanation is that biological therapy may affect the normal cytokine pattern. This could result in a relative increase of INF-γ that is well known to be increased in AA patients.[17]

A further proposal for explaining this paradoxical effect of anti-TNF-α is that switching off the primary disease inflammatory pathway and can move the unblocked inflammatory response into an alternative signaling pathway. This depends on the individual's genetic susceptibility, so the alternative pathway can clinically manifest as one or another immune-mediated disease states such as psoriasiform paradoxical eruptions or AA.[14]

Based on the results of our study on AA patients, we can conclude that skin of AA has a high level of TNF-α (a normal inhibitor of hair follicle growth *in vitro*). This high level may point to the important role of TNF-α in AA as the changes in hair follicles incubated with TNF-α are similar to those reported in AA pathology.

CONCLUSIONS AND RECOMMENDATIONS

Furthermore, we concluded that AA is a systemic disease not only a localized one, as we found that TNF-α was also present in relatively high levels in nonlesional skin biopsies of AA patients so that we should give systemic treatment for treating AA patients and do not wait for good results by only using the usual topical options.

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Conflicts of interest

There are no conflicts of interest.

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