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Immunohistochemical characteristics of inducible nitric oxide synthase and estrogen receptors alpha expression in patients with keratoderma climactericum

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

Aim. To examine the characteristics of immunohistochemical expression of inducible nitric oxide synthase and estrogen receptors alpha in patients with keratoderma climactericum compared to postmenopausal women with clinically intact skin, to reveal possible correlation between these markers' expression levels and pathomorphogenesis of menopausal keratoderma.

Materials and methods. The pathomorphological and immunohistochemical analysis was performed on the biopsy material of 22 patients with keratoderma climactericum, who constituted the study group, and on the autopsy material from 32 women in a postmenopausal period without any signs of climacteric keratoderma, who were considered as a control group.

Results. A significant difference was found in the area and intensity of inducible nitric oxide synthase expression between the study group diagnosed with keratoderma climactericum and the control group with unaffected skin, with increasing expression parameters among patients with keratoderma. A significant difference was also observed in the area and intensity of estrogen receptors alpha expression between the study and the control group, this time with increasing expression parameters among the control group.

Conclusions. Patients with keratoderma climactericum demonstrate statistically significantly higher levels of nuclear and cytoplasmatic expression of inducible nitric oxide synthase in epidermal and dermal cells than individuals from the control group, which may indicate acute inflammation as a part of climacteric keratoderma pathogenesis. The area and intensity of estrogen receptors alpha expression in material from patients diagnosed with menopausal keratoderma are lower than in the material from the control group, which demonstrates a correlation between estrogen-deficient state in postmenopausal period and the development of skin changes in women with keratoderma climactericum.

Key words: palmoplantar keratoderma, climacteric, inducible nitric oxide synthase, estrogen receptor alpha, pathomorphological and immunochemical studies.

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Імуногістохімічна характеристика експресії індуцибельної синтази оксиду азоту та альфа-рецепторів естрогену в пацієнтів із клімактеричною кератодермією

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Мета роботи – вивчити особливості імуногістохімічної експресії індуцибельної синтази оксиду азоту та рецепторів естрогену альфа в пацієнтів із клімактеричною кератодермією порівняно з жінками в постменопаузі з клінічно інтактною шкірою, виявити можливу кореляцію між рівнями експресії цих маркерів і патоморфогенезом менопаузальної кератодермії.

Матеріали та методи. Патоморфологічний та імуногістохімічний аналіз здійснили на біопсійному матеріалі 22 пацієнток із клімактеричною кератодермією (група дослідження) та на аутопсійному матеріалі 32 жінок у постменопаузальному періоді без ознак клімактеричної кератодермії (контрольна група).

Результати. Виявили значущу різницю за площею та інтенсивністю експресії індуцибельної синтази оксиду азоту між групою дослідження (з діагнозом клімактерична кератодермія) та контрольною групою (з неураженою шкірою) зі збільшенням параметрів експресії в пацієнтів із кератодермією. Суттєву різницю також визначили за площею та інтенсивністю експресії рецепторів естрогену альфа між групою дослідження та контрольною з підвищенням параметрів експресії в препаратах від обстежених без ознак клімактеричної кератодермії.

Висновки. У пацієнтів із клімактеричною кератодермією виявили статистично вірогідно вищі рівні ядерної та цитоплазматичної експресії індуцибельної синтази оксиду азоту в епідермальних і дермальних клітинах, ніж в осіб контрольної групи. Це може вказувати на запалення як частину патогенезу клімактеричної кератодермії. Площа й інтенсивність експресії рецепторів естрогену альфа в шкірі пацієнток із менопаузальною кератодермією менші, ніж в осіб контрольної групи; це показує кореляцію між естроген-дефіцитним станом у постменопаузі та розвитком шкірних змін у жінок із клімактеричною кератодермією.

Ключові слова: долонно-підошовна кератодермія, клімактерій, індуцибельна синтаза оксиду азоту, альфа-рецептори естрогену, патоморфологічне та імунохімічне дослідження.

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Keratoderma climactericum (KC) is a term, firstly proposed by Haxthausen in 1934, who described a condition developing in postmenopausal women with appearance of non-pruritic circumscribed hyperkeratosis of the palms and soles. Haxthausen's disease is one of the most common forms of acquired palmoplantar keratoderma, which occurs in 10–15 % of females in the climacteric period, often in pressure and abrasion sites, and is associated with hypertension and obesity [1].

Clinical manifestation of this dermatosis is presented by hyperkeratotic papules with clear margins, which do not rise above the skin surface and tend to coalesce into plaques

with appearance of skin fissures, localized on palms and soles exclusively. Hyperkeratotic lesions on the plantar surfaces are usually larger, than on the palmar surface, and gradual development of deep, sometimes even bleeding fissuring, may cause painful walking. The disease is prone to progression with the growth of lesion areas, loss of edges' clarity, increase in degree of infiltration and a number and depth of fissures [2].

Histological examination of the skin in patients with KC usually observes the following changes: a thickening of the epidermis, moderate acanthosis, pronounced hyperkeratosis, in some cases – small focal parakeratosis and spongiosis, moderate papillomatosis, edema and slight, predominantly perivascular lymphohistiocytic infiltration of derma [3,4].

Estrogen deficiency is considered to be the major reason of KC development. Estradiol levels rapidly decline after menopause, which adversely affects many cellular and homeostatic skin mechanisms, as well as other significant physiological functions. The changes include loss of collagen and elastin, decrease in vascularity, resulting in cellular and extracellular degradation which leads to dryness, wrinkles, atrophy, impaired barrier function and decreased antioxidant capacity of skin [5].

Two estrogen receptors (ERs), α and β, are the predominant transducers of estrogenic signals in the skin. Their relative expression levels start to decrease as women enter an estrogen-deficient state after menopause, however, the role of this decline as well as of estrogen deficiency in general in the development of KC is still not completely identified [6].

The inflammatory component of KC pathogenesis, manifested by lymphohistiocytic dermal infiltration, observed in histological examinations of patients with KC, requires thorough assessment in order to understand the nature of this disorder. Inducible nitric oxide synthase (iNOS) is the enzyme, involved in a formation of nitric oxide (NO), which can be expressed by immune (macrophages, neutrophils, monocytes) as well as by non-immune (fibroblasts, keratinocytes) cells. iNOS can be strongly induced by proinflammatory stimuli, and thus be an indicator of inflammation. A large amount of NO, produced by iNOS is toxic, and may cause oxidative damage, nitrosative stress and apoptosis, contributing to the subsequent pathological skin changes [7,8].

The pathogenesis of KC still remains insufficiently researched as well as the role of iNOS expression and the mechanisms of estrogen deficiency effects on skin in postmenopausal women.

Aim

To examine the characteristics of immunohistochemical expression of iNOS and ERα in patients with KC compared to postmenopausal women with clinically intact skin in order to reveal possible correlation between these markers' expression levels and pathomorphogenesis of KC.

Materials and methods

The pathomorphological and immunohistochemical analysis was performed on the biopsy material of 22 patients with KC. The study group consisted of 22 women aged 46–60 years, in a climacteric period, who were diagnosed with KC. The autopsy material from 32 women aged 46–60 years, in a postmenopausal period without any signs of KC, obtained during surgical dermatology interventions, was used as a control group.

Biopsy and autopsy material of the skin was fixed in neutral 10 % buffered formalin (pH 7.0) for 24 hours, then the standard method of paraffin section preparation of 4 μm thick was used for further histological and immunohistochemical examination (IHC analysis).

Microscopy of skin samples of the study and control groups was evaluated using histological staining with hematoxylin and eosin.

IHC analysis using monoclonal and polyclonal antibodies were performed on serial paraffin sections placed on adhesive slides "SUPER FROST PLUS" by "DAKO" company (Denmark). Deparaffinization and rehydration with simultaneous high-temperature unmasking of antigens were performed by heating in a RT module using Dewax & HIER buffer H by "Thermo Fisher Scientific", USA (pH = 9.0), endogenous peroxidase activity was inhibited with 3 % H_2O_2 solution. Incubation with primary antibodies was performed according to the instructions of the manufacturers, visualization of the IHC reaction was performed using the detection system UltraVision Quanto HRP + DAB System ("Thermo Fisher Scientific", USA). The sections were stained with Mayer's hematoxylin and sealed in Canada balsam.

Polyclonal antibody Rb a-Hu iNOS kit ("Thermo Fisher Scientific", USA) was used to identify enzyme that catalyzes the formation of NO in epitheliocytes.

Immunohistochemical reactivity for iNOS was defined as the proportion score multiplied by the intensity score. The proportion score was defined as 0 – <5 %; 1 – 6–25 %; 2 – 26–75 %; or 3 – >76 % positive cells. The staining intensity score was defined as 0 – negative; 1 – weak; 2 – moderate; 3 – strong. The total score ranged from 0 to 9. The immunoreactivity scores were used to classify the samples into one of the following three groups based on the final score: negative immunoreactivity, defined as a total score of 0; low expression, defined as a total score of 1–3; moderate expression, defined as a total score of 4–6; and high expression, defined as a total score of >6.

To determine estrogen-dependent cells and their localization, a monoclonal antibody Mo a-Hu Estrogen Receptor-Alpha, Clone SP1 kit ("Thermo Fisher Scientific", USA) was used.

Semi-quantitative analysis was performed following the Allred morphological scoring system for estrogen receptors (Er). In the Allred scoring system, cells are assigned a score from 0 to 5 depending on the proportion of stained cells (Proportion Score [PS]): 0 – no cells are Er positive, 1 – <1 % cells are Er positive, 2 – 1–10 % of cells are Er positive, 3 – 11–33 % of cells are Er positive, 4 – 34–66 % of cells are Er positive, 5 – 67–100 % of cells are Er positive, and a score from 0 to 3 is assigned depending on the intensity of staining (Intensity Score [IS]): 0 – negative expression, 1 – weak expression, 2 – moderate expression and 3 – strong expression.

Microphotographs of histological and IHC skin samples of the study and control groups were recorded in an Axioplan 2 microscope ("Carl Zeiss", Germany) using a digital camera Camedia C5060WZ Olympus (Japan).

Statistical processing of the results was performed on a personal computer in the program Statistica® for Windows 13.0 (StatSoft Inc., USA, license No. JPZ804I382130ARCN10-J). The hypothesis of the distribution normality for the studied indicators was tested using the Shapiro–Wilk test. Data with a different from normal distribution were presented using the median and interquartile range of Me (Q1; Q3), and the data obtained in the study and control groups were compared using the nonparametric Mann–Whitney U test for two independent groups. For all types of analysis, differences were considered significant at a P level <0.05.

Results

Pathohistological examination of skin biopsies of patients with menopausal keratoderma showed signs of diffuse acanthosis, mainly of the spinous layer of the epidermis, with the presence of single eosinophilic apoptotic Civatte bodies and morphological manifestations of severe hyperkeratosis (*Fig. 1*).

In isolated cases, there were weak signs of epitheliocyte spongiosis, local parakeratosis of the stratum corneum with the preservation of nuclei in single keratinocytes. Signs of balloon degeneration of single epitheliocytes were detected in the cells of the basal and spinous layers, and single microvesicular lipid inclusions were visualized in the cytoplasm of the cells in the spinous and granular layers. Examination of the papillary dermis revealed signs of weak interstitial edema and the presence, mainly perivascularly, of foci with moderate lympho-macrophage inflammatory infiltration (*Fig. 2*).

IHC analysis with monoclonal antibodies to iNOS among patients with keratoderma revealed moderate intensity of nuclear expression, mainly of nucleoplasmic type, with an area of 35.02 (28.04; 41.32) %. The highest expression of iNOS was associated with the nuclear membranes of epitheliocytes in the basal and parabasal layers of the epidermis with a gradual decrease in the number of immunopositive nuclei in cells in the spinous and granular layers of the epidermis (*Fig. 3*). At the same time, cytoplasmic expression of iNOS was observed in epitheliocytes, the degree and intensity of which varied considerably among patients of this clinical group.

A study of the dermal complex showed that in the papillary layer of the dermis, iNOS expression was observed among vascular endothelial cells and foci of immunocompetent cells.

Cytoplasmic expression of iNOS in the epidermis of clinically unaltered skin in the vast majority of cases was negative or was detected only in single apical cells within the spinous layer of the epidermis (*Fig. 4*).

When comparing the results of iNOS expression among the control group, a mild intensity of nuclear expression was observed in the cells within the spinous and granular layers, mainly of the nuclear type, the area of which was 23.84 (15.00; 28.00) %.

In the study group of patients with keratoderma and clinically unaffected epidermis, via the statistical analysis of the data using the Mann–Whitney U-test, a significant difference was found between the area and intensity of iNOS expression (U = 22.000; P < 0.05) with increasing expression parameters among patients with keratoderma.

IHC analysis of epitheliocytes to estrogen alpha-receptors (ERα) in the group of patients with keratoderma in all cases had a negative expression, low intensity of nuclear expression was observed in single cambial cells of the sebaceous glands, the area of which was 4.61 (3.15; 6.35) %; among the glandulocytes of the excretory and terminal ducts of the sweat glands and among the cells of the hair follicles, the expression of ERα was also absent (*Fig. 5*).

The area of ERα expression among the control group was 11.62 (9.65; 13.06) %, the morphogenesis of immunohistochemical changes was mainly localized only in the nuclei of cells in the basal layer of the epidermis (*Fig. 6*).

During examination of the dermal complex, nuclear expression of ERα among the cambial cells of the sebaceous glands, in single differentiating sebocytes and dermal fibroblasts was observed, immunopositive reaction was revealed in the glandulocytes of the excretory ducts of the sweat glands. During microscopy of hair follicles at different levels of section, immunopositive coloring of ERα was observed only in cells of the infundibular zone of a hair papilla and among basal cells of a hair bulb (*Fig. 7*).

Decreased number of α-estrogen receptors among the pilosebaceous skin complex did not exclude secretory dysfunction of sebaceous and sweat glands and caused changes in protein and lipid metabolism of the skin, which was manifested in the balloon degeneration of keratinocytes and the appearance of lipid vacuoles in the cytoplasm of single epitheliocytes, which was not observed in the control group.

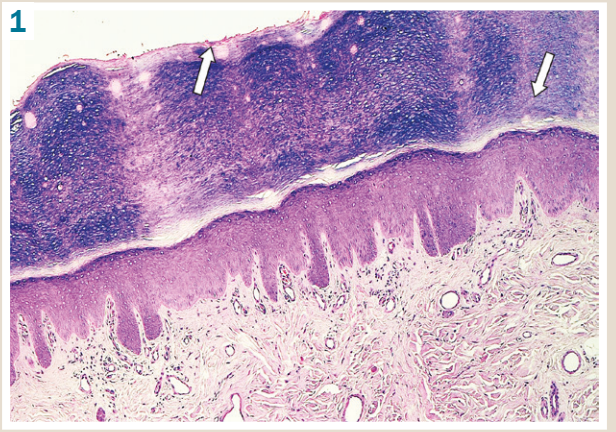
Statistical analysis using the Mann–Whitney U-test, in the material of the study group of patients with keratoderma and clinically unaffected epidermis found a significant difference between the area and intensity of ERα expression (U = 1.000; P < 0.05) with increasing expression parameters among the control group.

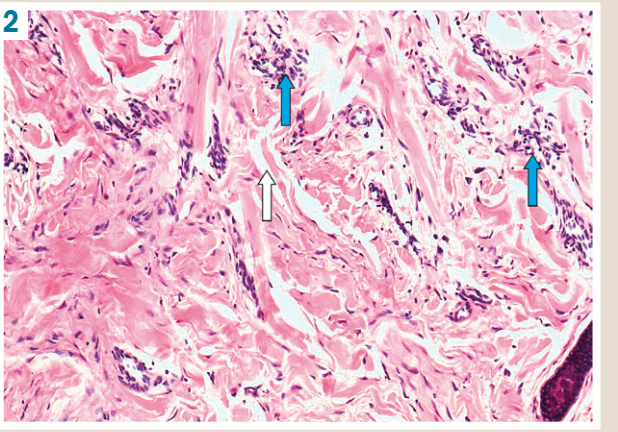
Discussion

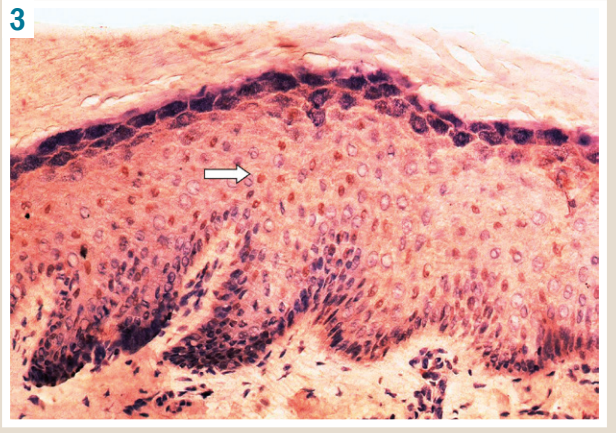
The study of iNOS expression in the skin in an animal experiment several decades ago proved that as a very mobile and indicative substance. Thus, in the study of injured skin of mice, the expression of iNOS and eNOS was determined both in and around the skin lesion and subsequently in granulation tissues, which may serve as a marker of inflammatory activity and duration of the lesion [9]. At the same time, it was noted that with the skin aging, age-related changes were accompanied by a decrease in L-arginine, a substrate for NO synthesis, and a decrease in iNOS expression, which reduced the bioavailability of L-arginine [10].

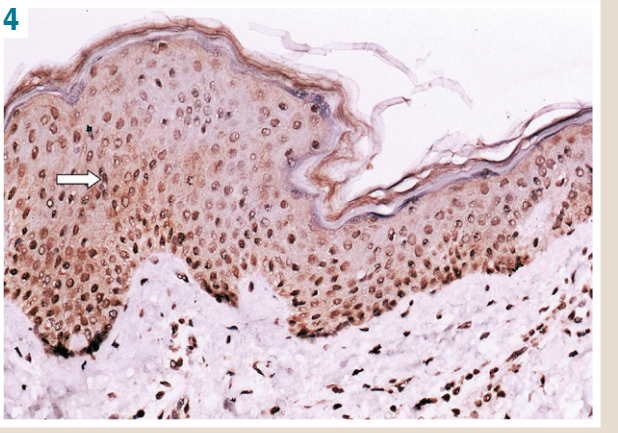
In practical medicine, the study of iNOS was conducted in non-inflammatory and inflammatory dermatoses. Thus, in melasma, there was a direct correlation between the increase in iNOS expression in the affected areas of the skin and the degree of activity in the disease clinical manifestation [11]. Examination of patients with psoriasis and concomitant hypertension showed an increase in iNOS expression in psoriatic plaques, which was accompanied by marked structural changes in the skin and was associated with systemic disorders of NO metabolism [12].

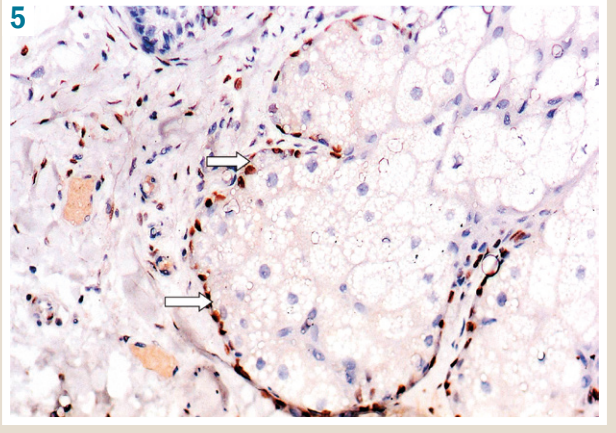
Recent studies on the role of iNOS in patients with melanoma suggested that elevated levels of its expression were a negative indicator of life expectancy and duration [13].

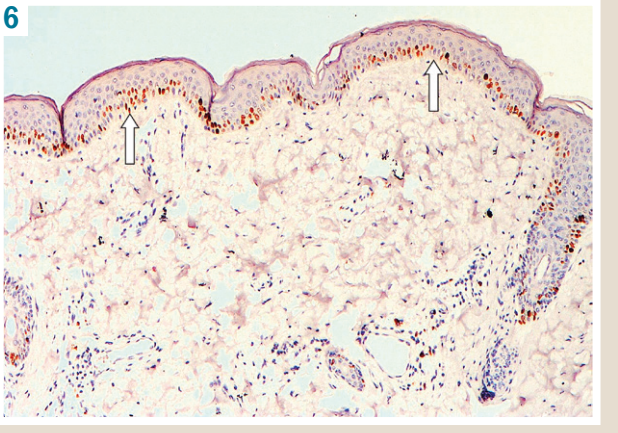












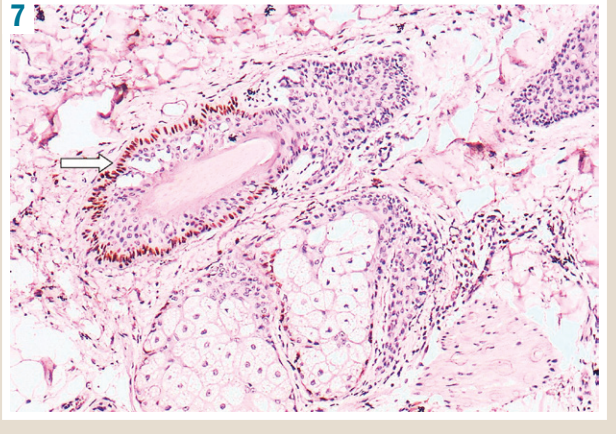


Fig. 1. Keratoderma, signs of severe hyperkeratosis of the integumentary epithelium (shown by arrows). Hematoxylin and eosin staining. Mag: ×200.

Fig. 2. Interstitial edema (shown by white arrow) with the formation of foci with moderate perivascular lympho-macrophage inflammatory infiltration (shown by blue arrow), keratoderma. Hematoxylin and eosin staining. Mag: ×200.

Fig. 3. Moderate nuclear expression of nucleoplasmic type among epitheliocytes in different layers of the epithelium (shown by arrow), keratoderma. Rb a-Hu iNOS. Mag: ×400.

Fig. 4. Mild nuclear expression of the nuclear type in the epitheliocytes within the spinous and granular layers (shown by arrow), control group. Rb a-Hu iNOS. Mag: ×400.

Fig. 5. Mild intensity of nuclear expression in single cambial cells of the sebaceous glands (shown by arrows), keratoderma. Mo a-Hu Estrogen Receptor-Alpha, Clone SP1. Mag: ×400.

Fig. 6. Local mild nuclear expression among epitheliocytes of basal layer (shown by arrow), control group. Mo a-Hu Estrogen Receptor-Alpha, Clone SP1. Mag: ×200.

Fig. 7. Local, moderate nuclear expression of the nucleoplasmic type in the pilosebaceous complex (shown by arrows), control group. Mo a-Hu Estrogen Receptor-Alpha, Clone SP1. Mag: ×200.

iNOS expression can be induced by cytokines and other agents in almost any cell type, which leads to a massive production of NO. High levels of NO not only provide a cytotoxic effect on pathogens or tumor cells, but also damage to healthy tissues, resulting in a subsequent cascade of inflammatory reactions [14].

In our opinion, the increase in the area of iNOS expression in epitheliocytes among patients with keratoderma is one of the indicators of the acute phase of inflammation, as evidenced by perivascular increase in immunopositive lymphocytes and macrophages. Significantly activated synthesis of abnormal forms of NOSs amid pathological process in morphologically altered skin may play a role in the structural changes of the skin in this group of patients and serve as one of the diagnostic parameters of dermatosis.

The triggers and molecular mechanisms of KC development are not fully understood, though the main factor – estrogen deficiency in climacteric period – is considered to underly this disorder.

Estrogens are proved to play a significant role in skin aging, their deficiency results in decreased collagen content and elasticity, reduced vascularity, increased wrinkling and dryness. Estrogen effects on skin are realized through a complex system of interactions involving estrogen receptors alpha and beta [15,16].

Our study has shown the significant reduction in the ERα expression level among patients with KC. We consider this decrease in the area of α-estrogen-dependent cells in the epithelial-dermal complex to be related to the histological appearance of acanthosis signs and hyperkeratosis in patients with keratoderma, which may indicate imbalance between desquamation of keratinocytes and proliferative activity of basal epidermal cells. Thus, we can see a possible correlation between estrogen deficiency and the development of KC.

Thus, the revealed association between decreased levels of ERα expression in skin of patients with KC and increased levels of iNOS expression, accompanied by histological features of inflammation in foci of damage, can highlight one more mechanism of disease development and subsequent search of new therapeutic strategies of dermatosis correction.

Conclusions

1. Patients with keratoderma climactericum demonstrate statistically significantly higher levels of nuclear and cytoplasmatic expression of inducible nitric oxide synthase in epidermal and dermal cells than individuals from the control group which may be an indicator of inflammation as a part of keratoderma climactericum pathogenesis.

2. The area and intensity of estrogen receptors alpha expression in material from the patients diagnosed with keratoderma climactericum are lower than in the material from the control group which demonstrates a correlation between estrogen deficient state in postmenopausal period and the development of skin changes in women with climacteric keratoderma.

Future research perspectives. The obtained results can serve as a basis for further research in the direction of NO metabolism and the corresponding therapeutic correction of patients with menopausal keratoderma.

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References

[1] Has, C., & Technau-Hafsi, K. (2016). Palmoplantar keratodermas: clinical and genetic aspects. *JDDG: Journal Der Deutschen Dermatologischen Gesellschaft*, *14*(2), 123-140. https://doi.org/10.1111/ddg.12930
[2] Bolick, N. L., Trivedi, L. E., & Elston, D. M. (2021). Thick Hyperkeratotic Plaques on the Palms and Soles. *Cutis*, *107*(3), 128, 134-135. https://doi.org/10.12788/cutis.0200
[3] Sinach, O. K., & Fedotov, V. P. (2017). Osobennosti kliniko-morfologicheskikh i endokrinnykh sdvigov u bol'nykh klimaktericheskoi keratodermiei [Features of the clinical and morphological and endocrine

alterations in patients with climacteric keratoderma]. *Dermatovenerologiya. Kosmetologiya. Seksopatologiya*, (1-4), 79-83. [in Russian].
[4] Zapolsky, M. Ye., Lebedyuk, M. M., Frolova, A. I., Kvitko, L. P., & Nerubaschenko, S. V. (2015). Porazhenie kozhi kistei i stop pri ostrykh i khronicheskikh dermatozakh. Terapevticheskie vozmozhnosti na sovremennom etape [Skin lesions of hands and feet in acute and chronic dermatoses. Therapeutic possibilities at present stage]. *Ukrainskyi zhurnal dermatolohii, venerolohii, kosmetolohii*, (3), 101-108. [in Russian].
[5] Lephart, E. D., & Naftolin, F. (2021). Menopause and the Skin: Old Favorites and New Innovations in Cosmeceuticals for Estrogen-Deficient Skin. *Dermatology and Therapy*, *11*(1), 53-69. https://doi.org/10.1007/s13555-020-00468-7
[6] Rzepecki, A. K., Murase, J. E., Juran, R., Fabi, S. G., & McLellan, B. N. (2019). Estrogen-deficient skin: The role of topical therapy. *International journal of women's dermatology*, *5*(2), 85-90. https://doi.org/10.1016/j.ijwd.2019.01.001
[7] Cinelli, M., Do, H., Miley, G., & Silverman, R. (2019). Inducible nitric oxide synthase: Regulation, structure, and inhibition. *Medicinal Research Reviews, 40*(1), 158-189. https://doi.org/10.1002/med.21599
[8] Xue, Q., Yan, Y., Zhang, R., & Xiong, H. (2018). Regulation of iNOS on Immune Cells and Its Role in Diseases. *International journal of molecular sciences*, *19*(12), 3805. https://doi.org/10.3390/ijms19123805
[9] Zhao, R., Guan, D. W., & Lu, B. (2005). Immunohistochemical study on expression of iNOS and eNOS during skin incised wound healing in mice. *Fa Yi Xue Za Zhi*, *21*(3), 161-164.
[10] De Souza, Á. Do P. B., de Oliveira, M. M. R., de Andrade, R. R., de Amorim, R. F. B., Bocca, A. L., & Borin, M. de F. (2017). The in vivo effect of L-arginine on skin elasticity in mice. *Brazilian Journal of Pharmaceutical Sciences*, *53*(3). https://doi.org/10.1590/s2175-97902017000300045
[11] Samaka, R. M., Bakry, O. A., Shoeib, M. A., & Zaaza, M. M. (2014). Expression of iNOS and NF-κB in melasma: an immunohistochemical study. *Analytical and quantitative cytopathology and histopathology*, *36*(5), 245-257.
[12] Makurina, G. I., & Baudarbekova, M. M. (2016). Osoblyvosti aktyvnosti iNOS u vohnyshchakh urazhenoi shkiry khvorykh na psoriaz iz suputnoiu arterialnoiu hipertenziieiu [Features of iNOS activity in the skin lesions of patients with psoriasis and concomitant hypertension]. *Pathologia,* (2), 40-46. [in Ukrainian]. https://doi.org/10.14739/2310-1237.2016.2.80990
[13] Ding, Z., Ogata, D., Roszik, J., Qin, Y., Kim, S. H., Tetzlaff, M. T., Lazar, A. J., Davies, M. A., Ekmekcioglu, S., & Grimm, E. A. (2021). iNOS Associates With Poor Survival in Melanoma: A Role for Nitric Oxide in the PI3K-AKT Pathway Stimulation and PTEN S-Nitrosylation. *Frontiers in oncology*, *11*, 631766. https://doi.org/10.3389/fonc.2021.631766
[14] Lind, M., Hayes, A., Caprnda, M., Petrovic, D., Rodrigo, L., Kruzliak, P., & Zulli, A. (2017). Inducible nitric oxide synthase: Good or bad?. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie*, *93*, 370-375. https://doi.org/10.1016/j.biopha.2017.06.036
[15] Lephart, E. D. (2018). A review of the role of estrogen in dermal aging and facial attractiveness in women. *Journal of cosmetic dermatology*, *17*(3), 282-288. https://doi.org/10.1111/jocd.12508
[16] Liu, T., Li, N., Yan, Y. Q., Liu, Y., Xiong, K., Liu, Y., Xia, Q. M., Zhang, H., & Liu, Z. D. (2020). Recent advances in the anti-aging effects of phytoestrogens on collagen, water content, and oxidative stress. *Phytotherapy research : PTR*, *34*(3), 435-447. https://doi.org/10.1002/ptr.6538