Patients with Allergic and Irritant Contact Dermatitis are Characterized by Striking Change of Iron and Oxidized Glutathione Status in Nonlesional Area of the Skin

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To assess the consequences of oxidative stress in allergic and irritant contact dermatitis, we compared the iron level, unsaturated iron-binding capacity, total iron binding capacity, the percentage saturation of iron-binding capacity, the amount of diene conjugates as well as the amounts of total glutathione, reduced glutathione, oxidized glutathione, and the oxidized glutathione/reduced glutathione ratio in skin homogenate from lesional and nonlesional skin. Lesional skin samples were obtained from positive patch test sites to 5% $NiSO_4$ in five subjects, and from chronic contact dermatitis lesions on the hands, which had exacerbated over 3–9 wk in six subjects. Contact dermatitis caused at least a 4-fold increase in the iron level in the lesional skin area compared with the nonlesional skin area ($p < 0.02$). The increase in the iron level depended on the duration of contact dermatitis and was accompanied by high unsaturated iron-binding capacity and total iron-binding capacity values in the positive patch test sites ($p < 0.05$), and by a high percentage saturation value in the chronic contact dermatitis lesions ($p < 0.05$). We found high indices for iron, total iron-binding capacity and diene conjugates in the apparently healthy skin of the patients with persistent contact dermatitis that significantly ($p < 0.05$) exceeded the corresponding values in the patients with only patch test reactions. In summary, we have succeeded in providing evidence that generalized oxidative damage of the skin occurs as a consequence of contact dermatitis in a restricted area.
Key words: diene conjugates/iron-binding capacity/reduced glutathione. J Invest Dermatol 116:886–890, 2001

The skin functions as a biobarrier against hazardous environmental physical, chemical, and biologic agents. Inflammatory reactions triggered by exogenous and endogenous factors, including free radicals, are involved in the pathogenesis of dermatologic diseases (Harman, 1992; Janssen *et al*, 1993; Darr and Fridovich, 1994; Picardo *et al*, 1996; Lopez-Torres *et al*, 1998; Halliwell and Gutteridge, 1999).

In allergic and irritant contact dermatitis (ACD, ICD) an array of reactive oxygen species (ROS) is produced. ROS are set free by inflammatory mediators, generated directly by irritants or allergens (Corsini and Galli, 1998; Willis *et al*, 1998), and released during free radical chain reactions. The main source of ROS is inflammatory cellular infiltrate. Stimulated monocytes produce superoxide, the respiratory burst of infiltrating PMN in inflamed skin will produce high local levels of superoxide anion and hydrogen peroxide (Trenam *et al*, 1992a; Darr and Fridovich, 1994). Production of nitric oxide by nitric oxide synthase in keratinocytes also occurs (Halliwell and Gutteridge, 1999). Excessive production of ROS results in peroxidation of cell membrane lipids and damages of proteins and DNA (Bunker, 1992; Pantopoulos and Hentze, 1995; Picardo *et al*, 1996).

Iron and oxygen are at a metabolic crossroad where any mismanagement leads to toxicity, tissue damage, and severe inflammation. Therefore, biochemistry both of ROS and iron are closely related to each other (Trenam *et al*, 1992a; Morris *et al*, 1995; Halliwell and Gutteridge, 1999). ROS can release iron from intracellular iron storage protein, ferritin, and trigger a rapid reduction of ferritin synthesis (Trenam *et al*, 1992b; Pantopoulos and Hentze, 1995); and *vice versa*, iron promotes the formation of ROS that contribute to lipid peroxidation (LP) (Morris *et al*, 1995; Pantopoulos and Hentze, 1995; Van Lenten *et al*, 1995).

The skin possesses considerable endogenous protection against oxidative damage, as it is equipped with several antioxidants (Vessey *et al*, 1995; Kerb *et al*, 1997; Thiele *et al*, 1997; Kohen, 1999). Reduced glutathione (GSH) has gained attention as the central cellular antioxidant in the skin (Vessey *et al*, 1995; Picardo *et al*, 1996; Hirai *et al*, 1997; Shvedova *et al* 2000) that also participates in the metabolism of xenobiotics and leukotrine synthesis (Halliwell, 1994; Hanada *et al*, 1997). The protective role of cutaneous GSH against UV injury has been thoroughly investigated on cultured human skin cells (Punnonen *et al*, 1991; Vile and Tyrrell, 1995), in the skin of mice (Hanada *et al*, 1997; Lopez-Torres *et al*, 1998), and in human volunteers (Kerb *et al*, 1997). The

Manuscript received July 17, 2000; revised February 1, 2001; accepted for publication March 12, 2001.

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Abbreviations: ACD, allergic contact dermatitis; CD, contact dermatitis; DC, diene conjugates; GSH, reduced glutathione; GSSG, oxidized glutathione; ICD, irritant contact dermatitis; LP, lipid peroxidation; OS, oxidative stress; ROS, reactive oxygen species; TGSH, total glutathione; TIBC, total iron-binding capacity; UIBC, unsaturated iron-binding capacity.

Table I. Characteristics of OS in inflamed and apparently healthy skin

	Patients with positive patch test reactions[a]			Patients with chronic CD on hands[b]		
	Apparently healthy skin	Patch test site	p[f]	Apparently healthy skin	Lesional skin	p[f]
Iron[c]	104 ± 9	741 ± 396	<0.02	322 ± 183	1038 ± 493	<0.02
UIBC	157 ± 16	888 ± 443	<0.05	440 ± 226	314 ± 233	NS[g]
TIBC	256 ± 21	1629 ± 815	<0.05	800 ± 203	1319 ± 649	NS
% saturation	39 ± 1	45 ± 8	NS	45 ± 22	74 ± 11	<0.05
DC[d]	266 ± 97	306 ± 102	NS	541 ± 156	533 ± 210	NS
TGSH[e]	627 ± 400	791 ± 209	NS	1442 ± 1194	1527 ± 1246	NS
GSSG	169 ± 61	320 ± 121	<0.05	431 ± 292	463 ± 221	NS
GSH	457 ± 442	470 ± 191	NS	1011 ± 931	1064 ± 1156	NS
GSSG/GSH	0.76 ± 0.44	0.86 ± 0.49	NS	0.65 ± 0.33	1.37 ± 1.25	NS

[a]Data represent the mean ± SD in positive patch test sites to 5% NiSO4 in five patients.
[b]Data represent the mean ± SD in six patients with chronic CD on hands with signs of exacerbation within the last 3–9 wk.
[c]Iron, unbound iron-binding capacity (UIBC), and total iron-binding capacity (TIBC), are expressed as μm per g skin.
[d]Diene conjugates (DC) are expressed as μm per g skin.
[e]Total glutathione (TGSH), oxidized glutathione (GSSG), and reduced glutathione (GSH) are expressed as mg per g skin.
[f]Significantly different ($p < 0.05$) by Student's t test.
[g]NS, not significant.

inhibitory role of GSH in contact dermatitis (CD) in mice has been exemplified by Hirai *et al* (1997).

This study was performed to compare the iron status and glutathione redox status in ACD and ICD lesions with the values of apparently healthy skin of the same subjects.

MATERIALS AND METHODS

Patients With permission of the local ethics committee, two 4 mm skin punch biopsies were taken from 11 study participants under lidocaine + epinephrine local anesthesia. Lesional skin was biopsied in six patients (four female, two male, mean age 46.5 y, within the range of 22–84 y) with chronic ICD (three patients) or ACD (three patients) on their hands, which had exacerbated over 3–9 wk, and at the time of investigation was characterized by marked erythema, edema, and vesiculation. Simultaneously, the skin of symmetrical or functionally comparable unaffected sites was biopsied. These analyzes served to provide control values. Skin from positive patch test sites to 5% nickel sulfate was obtained from the backs of five female patients (mean age 35.4 y, within the range of 19–67 y) on the second to the fifth day after the application. Positive test reactions were graded as +++ (extensive infiltrated erythema with coalescing vesicles) or as ++ (erythema, edema, and discrete vesicles). Their control biopsies were taken from the back or flexor surface of the forearm. For patch testing, the European standard series (Chemotechnique Diagnostics AB, Sweden) was used. The investigation was carried out in the wintertime. None of the patients had received any systemic remedies. The treatment with topical corticosteroids was stopped 1 wk before skin sampling. No changes in blood albumin, bilirubin, urea, and hemoglobin content were established.

Sample preparation Epidermis and dermis together were frozen and stored at –80°C until used. Biopsies were kept for 5 d in liquid nitrogen to produce supernatants whose homogeneity was comparable with supernatants produced by sonification (Lopez-Torres *et al*, 1998). Biopsies were carefully homogenized in 1.15% KCl solution with special homogenizer and centrifuged at 10,000 × *g* for 10 min. The supernatants were kept on ice and used for antioxidant assays.

Level of iron, the unsaturated iron-binding capacity (UIBC), the total iron binding-capacity (TIBC), and the percentage saturation of iron-binding capacity For the assessment of these indices a special kit (Sigma 565, Sigma, St. Louis, MO) was used. All procedures were performed in triplicate. Briefly, for determination of the tissue iron level 0.250 ml of the tissue homogenate and 1.250 ml Iron Buffer Reagent (hydroxylamine hydrochloride, 1.5% wt/vol in acetate buffer, pH 4.5) were mixed, centrifuged for 10 min at 3000 × *g* and initial absorbance was measured at 560 nm. Then 0.025 ml Iron Color Reagent, which forms water-soluble magneta complex with iron (Ferrozine 0.85% wt/vol in hydroxylamine hydrochloride solution) was added, mixed, incubated for 10 min at 37°C and centrifuged for 10 min at 3000 × *g*. Absorbance was measured at 560 nm *versus* water blank and the iron concentration was calculated by using a kit formula. Iron content was expressed as μM per g skin. To determine UIBC of the tissue 1.000 ml UIBC buffer reagent (TRIZMA, 0.5 M per liter, pH 8.1), 0.25 ml of the tissue homogenate and 0.25 ml iron kit standard (0.500 mg per dl) were mixed, centrifuged for 10 min at 3000 × *g* and the initial absorbance was measured at 560 nm. The procedure was completed as described for total iron determination. UIBC (expressing the number of free iron-binding sites) was presented in μM per g skin. TIBC is the sum of iron and UIBC values, and the percentage saturation indicates the percent of bound iron.

Lipid peroxidation The level of diene conjugates (DC) was measured as previously described (Ristimäe *et al*, 1999). Briefly, 0.15 ml of sample and 0.15 ml 0.9% NaCl (reagent blank contains isotonic saline) were incubated at 37°C for 30 min. Then 0.25% BHT (0.015 ml) was added, the samples were extracted with heptane/isopropanol (1:1, whole volume 1.8 ml) and acidified by 0.5 ml 5 N HCl. After extraction with cold heptane (1.6 ml), samples were centrifuged (for 5 min at 3000 × *g*) and absorbance of heptane fraction was measured at 234 nm.

Determination of total glutathione (TGSH), oxidized glutathione (GSSG), and reduced glutathione (GSH) TGSH and GSSG were measured by the method of Griffith (1980), which we had slightly modified. The samples were deproteinated with 10% metaphosphoric acid (Aldrich, Cat. 43157). To measure glutathione content 0.005 ml 4 M triethanolamine (Aldrich, Steinheim, Germany, Cat T5830-0) in water was added to the 0.1 ml of protein free sample and immediately mixed. The sample was diluted with 0.895 ml of 0.2 M sodium phosphate buffer (pH 7.5) containing 0.01 M ethylenediaminetetraacetic acid (buffer 1). For determination of GSSG, GSH was derivatized by adding 0.01 ml of 1 M 2-vinylpyridine (Aldrich, Cat 13229–2) in ethanol, mixed, and kept at room temperature for 1 h. To assay TGSH or GSSG, the samples were mixed with 0.5 ml of buffer 1 containing 0.5 U glutathione reductase (Sigma Cat G4751), and 0.3 mM NADPH (Sigma Cat N7505). Reaction was initiated by the addition of 0.1 ml of 1 mM 5,5,-dithio-bis-(2-nitrobenzoic acid) in buffer 1. The change in optical density was measured after 10 min at 412 nm. TGSH, GSH, and GSSG are expressed as μg per g skin. Glutathione redox status was expressed as GSSG/GSH.

Statistical analysis Statistical analysis was performed using the Student's t test for unpaired samples. A difference was considered significant if $p < 0.05$.

RESULTS

Patients with CD are characterized by increased iron level in lesional areas of skin One of the features of OS in skin inflammation is altered iron metabolism (Trenam *et al*, 1992a; Morris *et al*, 1995). To evaluate the iron alteration in CD, comparative studies were carried out in lesional and nonlesional skin from 11 patients. As shown in **Table I**, both skin patch testing

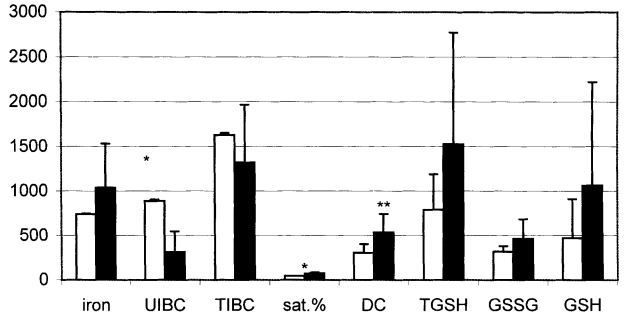

Figure 1. Changes in iron status of CD lesions depending on the duration of inflammation. Skin punch biopsies were taken from positive to 5% $NiSO_4$ patch test sites in one group of the patients (*open bars*), and from chronic CD lesions on the hands in another group of patients (*closed bars*). We identified a significantly higher UIBC value in the positive patch test sites and higher percentage saturation values of iron-binding capacity in the patients with chronic exacerbated CD. The difference in the DC quantities was statistically not significant. The data shown are mean ± SD. * $p < 0.05$,** $p < 0.1$.

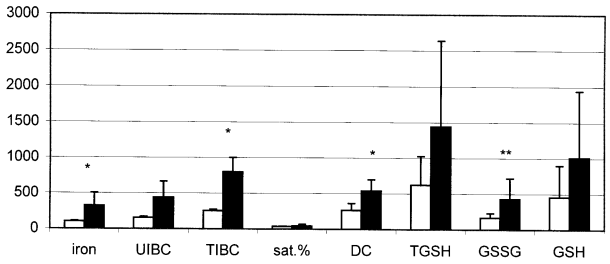

Figure 2. Chronic restricted CD influencing the iron status of apparently healthy skin. Control biopsies were obtained from apparently healthy skin of patients with positive to 5% $NiSO_4$ test reactions (*open bars*), and from apparently healthy skin of patients with chronic CD that had become exacerbated during the last 3–9 wk (*closed bars*). In the normal skin of the patients with chronic CD, the iron concentration, the TIBC level, and the DC quantities were significantly higher in comparison with the normal skin values in the patients with only patch test reactions. The difference in the GSSG values was not significant. The data shown are mean ± SD. *$p < 0.05$, **$p < 0.1$.

and spontaneous CD caused at least a 4-fold increase in iron level in lesional skin compared with nonlesional area ($p < 0.02$). In patients biopsied from positive patch test sites the level of iron in lesional region was 741 ± 396 μM per g skin and in apparently healthy skin 104 ± 9 μM per g skin. UIBC and TIBC values were about six times higher in the patch test sites compared with healthy skin ($p < 0.05$). In chronic CD lesions the iron level was enhanced compared with the level in uninvolved skin (1038 ± 493 and 322 ± 183 μM per g skin, respectively, $p < 0.02$). There were no significant differences in UIBC and TIBC values, but the percentage saturation was higher in lesional skin ($p < 0.05$). All of these results are presented in **Table I**.

Increase of iron level in lesional and nonlesional skin depends on duration of dermatitis The effect of disease duration on iron status in inflammatory skin condition caused by ACD or ICD is given in **Fig 1**. The iron level and the percentage saturation value in lesional skin were higher in the patients with chronic exacerbated CD in comparison with the data, obtained from the positive patch test sites. The difference between the saturation indices was significant ($p < 0.05$). The UIBC value, on the contrary, was significantly higher in the patch test areas ($p < 0.05$). Surprisingly, in nonlesional skin of the patients with chronic CD the indices for iron and TIBC were about three times higher ($p < 0.05$) as in nonlesional skin of the patients whose skin symptoms were caused by patch testing and thus were of a shorter duration (**Fig 2**). The measured iron was nonhaem iron, as the control test with red blood cells, treated under the same conditions as the skin samples, did not result in detectable iron.

DC level does not differ in lesional and nonlesional skin areas but is increased time-dependently ROS-mediated OS may induce an increase in iron-caused LP (Bunker, 1992; Trenam *et al*, 1992a, b; Morris *et al*, 1995). To assess LP level in patients suffering from ACD or ICD, the amount of DC was measured. The increase of DC level in the positive patch test sites to 5% $NiSO_4$ in comparison with uninvolved skin of the same patients was insignificant (**Table I**). Likewise, the comparison of DC levels in the chronic CD lesions and in the positive patch test sites did not reveal any significant differences (**Fig 1**); however, the increase of DC in nonlesional skin areas was dependent on the duration of CD ($p < 0.05$, **Fig 2**). It is noteworthy that the level of DC in nonlesional skin area of patients with dermatitis of longer duration was significantly higher ($p < 0.05$) in comparison with the values in the positive patch test sites where the duration of inflammation was only 2–5 d (**Table I**).

Oxidized glutathione level in CD is increased both acutely and time-dependently GSSG and glutathione redox status (GSSG/GSH) are well-known indices for cellular OS. GSSG and redox status values were increased in the lesional area of skin compared with the nonlesional skin area (**Table I**); however, due to significant individual variability of glutathione amounts a statistically significant difference was established only for GSSG in patients biopsied from positive to 5% $NiSO_4$ patch test sites during the early stage of inflammation (**Table I**). Nonetheless, it should be underlined that the level of GSSG in the nonlesional skin area of patients suffering from chronic CD was even higher than in the positive to 5% $NiSO_4$ patch test sites.

DISCUSSION

In this study, we have provided evidence that CD, restricted to a small area, is not a local problem as it is often thought to be, but its consequences are present in whole skin.

CD causes a potent increase of iron level not only in acute or chronic lesional area, but also in apparently healthy skin. In prolonged duration of inflammation the increase is even more evident.

Iron is a biologically important trace element. As iron catalyzes the formation of highly toxic hydroxyl radical, and peroxidation of lipids, excessive iron is stored in Fe(III) form. In cells, iron is bound to ferritin, extracellularly to transferrin and lactoferrin, and in circulation to hemoglobin (Halliwell, 1994; Morris *et al*, 1995; Pantopoulos and Hentze, 1995; Van Lenten *et al*, 1995). The iron measured in our samples was nonhaemic (mainly ferritin iron) as the control test with red blood cells, treated under the same conditions as skin samples, did not result in detectable iron. The possibility that some of the iron is of hemosiderin and low-molecular weight chelators origin, in chronic inflammation in particular, cannot be excluded.

The high iron content in lesional skin might be explained by inflammation-induced OS. A chronic exposure of hairless mice to low levels of UVB has been reported to have increased the nonhaem iron content of the skin (Bissett *et al*, 1991). Trenam *et al* (1992b) have shown that intradermal injection of H_2O_2-producing enzyme glucose oxidase rapidly produces a cellular infiltrate of monocytes and neutrophils, and significantly increases the levels of iron in the skin of rats both with or without prior iron loading. Despite not being species-specific, these is the best evidence of inflammation-induced changes in iron content of the skin so far.

In CD at least four potential mechanisms involved in storage and reduction of Fe(III) to Fe(II) may contribute to the rise of iron. (i) The mobilization of redox active iron from storage protein, e.g., ferritin, is related to the oxidative burst of infiltrating PMN with

superoxide radicals formation, and degradation of ferritin in macrophages (Trenam *et al*, 1992a; Morris *et al*, 1995). (ii) Inflammatory mediators (e.g., TNF-α and IL-1) generated by monocytes and keratinocytes under the influence of irritants and allergens (Barker *et al*, 1991; Howie *et al*, 1996) or staphylococcal superantigens in chronic lesions (Leung *et al*, 1998), and ROS themselves bring about an increase in the iron-containing acute phase reactants such as ferritin and lactoferrin (Morris *et al*, 1995). (iii) Acidic pH in the activated phagocytes and on the skin may contribute to the release of active iron (Trenam *et al*, 1992a). (iv) Inflammation-induced hyperproliferation of keratinocytes (Barker *et al*, 1991) may increase the epidermal excretion of iron (Trenam *et al*, 1992a; Morris *et al*, 1995).

In acute inflammation, the values reflecting sequestered iron (UIBC) and possible total binding capacity of sequesters (TIBC) were high in the tissue samples in our study. Furthermore, the increase of iron in the patch test sites concurred with the increase in UIBC, indicating a rapid response to inflammation. Chronic CD was characterized by an elevated percentage saturation value showing that more iron-binding sites are occupied with iron. In addition, restricted to the hands chronic CD influenced the iron status in whole skin, evidenced by high TIBC values in the apparently healthy skin of our patients. Therefore, it can be stated that the iron level response to dermatitis involves whole skin, i.e., it is of a generalized nature.

The presence of considerable amounts of PUFA in the skin makes it particularly vulnerable to ROS (Trenam *et al*, 1992b), especially in the case of inflammation when excessive formation of ROS occurs. Some studies have reported signs of LP in a model of human skin (Podda *et al*, 1998), in the skin of mice with ICD and ADC (Hirai *et al*, 1997), and after UV irradiation of cultured keratinocytes (Punnonen *et al*, 1991; Stewart *et al*, 1996). Lipoperoxidation of skin surface lipids has also been described (Giralt *et al*, 1996). We could not establish a statistically significant difference in the quantities of DC between the inflamed and the apparently healthy skin of the same subject; however, the level of DC was dependent on the duration of the disorder. This was less obvious between lesions of acute *versus* chronic CD, but statistically significant in apparently healthy skin. As the consequence of chronic inflammation, the level of DC in the unaffected skin of patients suffering from chronic CD was significantly higher even in comparison with the values in lesions of acute CD (patch test sites). It refers to the fact that an increased iron level is accompanied by increased LP and continuous inflammation, even if inflammation is restricted to such a small area as the hands, will result in potently oxidatively stressed whole skin.

ROS biochemistry and both iron (possible pro-oxidant) and glutathione (scavenger of ROS) biochemistry are known to be intimately related. The observation that localized CD causes a generalized OS is further supported by our data about glutathione. Glutathione is the most abundant low molecular weight antioxidant in the skin (Fuchs *et al*, 1989; Hirai *et al*, 1997). It is postulated that in most tissues the amount of GSH greatly exceeds the level of GSSG (Hall, 1999), but the exact relationship of GSH and GSSG in the skin remains to be established. Many factors (physical stress, consumption of supplements, etc.) influence the GSSG/GSH ratio but in the skin, the outer cover of the body, it is influenced additionally by many exogenous factors. On exposure to externally applied oxidizing agents or thiol-reactive toxins, the consumption of GSH may increase dramatically (Hall, 1999). Exposure of human keratinocytes to paraphenylene diamine decreases the level of GSH in a dose- and time-dependent manner (Picardo *et al*, 1996). Hirai *et al* (1997) have provided evidence that a decrease of glutathione and free sulfydryl-groups in the skin of mice with experimentally induced ICD and ACD after challenge with dinitrochlorobenzene may occur because of the conjugation of this allergen with GSH. Recently, Shvedova *et al* (2000) reported a decrease of the GSH content in cultured human keratinocytes exposed to phenol and suggested that the decrease of GSH may either be due to direct oxidation by phenoxyl radicals or result from consumption in GSH peroxidase-catalyzed reactions. For these reasons we believe that the detected 2–4-fold difference in favor to GSH in our study reflects the real situation in the nonlesional skin of CD patients.

During chronic inflammation, the generation of ROS may become damaging (Halliwell, 1994). In this context, the low GSSG/GSH ratio in our patients may be accounted for by the study groups compared, both consisting of CD patients: an asymptomatic group and a symptomatic group. Besides, the level of glutathione reductase in the course of differentiation of cultured human keratinocytes is known to decrease (Vessey *et al*, 1995), reducing the GSH regeneration.

Nevertheless, the glutathione system reacts efficiently with ROS generated by dermatitis. (i) We found a significant increase of the GSSG level only in the positive to 5% $NiSO_4$ patch test sites, i.e., in short-time CD. (ii) Although chronic CD caused an increase of GSSG levels in lesional as well in apparently healthy skin indicating to the severity and generalized nature of OS, these differences were because of a concomitant increase of the GSH values below statistical significance.

Skin disease, especially when restricted to a small area, is often considered to be a local problem. For that reason, it may be of particular interest to evaluate the degree of OS in urticaria, where the ability of the skin to respond to the stimulus as a uniform organ is much more obvious. Further research may be directed to the improvement of the skin antioxidative capacity by the adding of topical and systemic antioxidants and iron chelators to the treatment schedule of patients with CD that might facilitate a faster and more complete improvement of the patient's state. In summary, we have provided evidence that generalized severe OS of the skin is expressed as a consequence of CD in a restricted area.

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