Archives of Dermatological Research (2018) 310:759–767
https://doi.org/10.1007/s00403-018-1857-1

ORIGINAL PAPER

The investigation of the relationships of demodex density with inflammatory response and oxidative stress in rosacea

Tuğba Falay Gur[1] · Aslı Vefa Erdemir[2] · Mehmet Salih Gurel[2] · Abdurrahim Kocyigit[3] · Eray Metin Guler[3] · Duygu Erdil[4]

Received: 25 April 2018 / Revised: 5 August 2018 / Accepted: 21 August 2018 / Published online: 27 August 2018

Abstract

The relationships of demodex density with systemic oxidative stress, inflammatory response, and clinical severity in rosacea are not clear. This study aimed to (a) analyze the levels of systemic oxidative stress, antioxidant capacity, inflammatory parameters, and matrix metalloproteinases (MMPs) in systemic circulation in patients with rosacea, (b) identify the relationship between mite density and both oxidative stress and inflammation, and (c) investigate the role of photoaging and sebum secretion in etiopathogenesis. Forty patients with rosacea and 40 age-, sex-, and skin phenotype-matched healthy volunteers were included in the study. Clinical disease severity of the patients was determined. Sebum levels were measured in both the groups, and photoaging was evaluated. Reflectance confocal microscopy was used to calculate demodex density. Serum total antioxidant capacity (TAC), total oxidant capacity (TOC), myeloperoxidase (MPO), MMP-1, MMP-9, arylesterase (ARES), interleukin-1β (IL-1β), paraoxonase-1 (PON-1), and tumor necrosis factor-α (TNF-α) levels were also analyzed. The patients with rosacea had significantly higher serum TOC and lower TAC levels ($p < 0.001$). The serum ARES and PON-1 levels were significantly lower ($p = 0.045$ and $p < 0.001$, respectively); however, the serum levels of MMP-1, MMP-9, IL-1β and MPO were higher in the patient group. Demodex parameters were higher in the patient group compared to the control group. There was no significant correlation between the number of mites and disease severity. In addition, the number of mites was not correlated with the serum levels of TAC, TOC, OSI, MPO, MMP-1, MMP-9, ARES, PON-1, TNF-α, and IL-1β. However, sebum levels were directly proportional to the number of mites. Photoaging severity was similar between the patients and control subjects. The changing sebaceous microenvironment in rosacea leads to an increase in the number of demodex mites. However, increased demodex density does not alter disease severity, level of oxidative stress, or inflammation. Although none of the patients with rosacea had any underlying systemic disease, patients' systemic oxidative stress and inflammation parameters were found high in systemic circulation. It is assumed that the patients with rosacea are more prone to systemic diseases.

Keywords Demodex · Inflammation · Oxidative stress · Photoaging · Rosacea · Sebum

Introduction

Rosacea is a common, chronic inflammatory disease of the skin, which can cause prominent redness and sometimes permanent deformations. Genetic factors, immune system, microorganisms, such as *Helicobacter pylori* and *Demodex folliculorum*, ultraviolet (UV) light, neurovascular disorder, disruption of skin barrier, and various environmental factors are considered to play a role in the etiopathogenesis of this disease [1, 11, 15, 18, 29]. The proinflammatory effect of UV and the role of oxidative stress and immune response to demodex mite are considered to contribute to the development of inflammation in rosacea [3, 25, 30, 31].

✉ Tuğba Falay Gur
tugbafalay@hotmail.com

[1] Department of Dermatology, Sultan Abdulhamid Han Training and Research Hospital, Health Science University, Usküdar, Istanbul, Turkey

[2] Department of Dermatology, Göztepe Training and Research Hospital, Istanbul Medeniyet University, Istanbul, Turkey

[3] Department of Biochemistry, Bezmialem Vakif University Medical School, Istanbul, Turkey

[4] Department of Dermatology, Ağrı State Hospital, Ağrı, Turkey

Demodex mites are a member of the human skin flora. While the quality and amount of sebum are held responsible for the increased number of demodex mites in rosacea, the exact cause of mite proliferation is not fully understood [17, 23, 26]. The increase in the number of mites can contribute to rosacea pathogenesis by triggering inflammatory response, causing mechanical obstruction in follicles, or functioning as bacterial vectors [5, 8, 10]. However, the relationship of mite density with disease severity, systemic oxidative stress and inflammatory response in rosacea is not clear. This study aimed to (a) evaluate systemic oxidative stress and antioxidant capacity in patients with rosacea, (b) to determine the levels of inflammatory parameters and matrix metalloproteinases (MMPs) in systemic circulation, (c) identify the relationship of mite density with oxidative stress and inflammation, and (d) explore the role of photoaging and sebum secretion in the etiopathogenesis of rosacea.

Materials and methods

This research was planned as an observational, case–control study to investigate the roles of systemic oxidative stress, MMPs, inflammatory markers, demodex infestation, photoaging, and sebum level in rosacea pathogenesis, and to identify potential correlations between these parameters. Ethical approval was obtained and the study was conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from each participant.

Patient selection

The patient group consisted of 40 individuals who were admitted to the Istanbul Training and Research Hospital Dermatology Clinic and were diagnosed with rosacea based on the criteria of the National Rosacea Society of the United States [35]. The patients that had any systemic disease which could affect systemic oxidative stress or inflammatory parameters and those with inflammatory dermatoses were excluded. The age range was 18–70 years, and all patients were non-smokers. Other exclusion criteria were undergoing systemic or topical treatment or using sunscreen on the face that would change the systemic oxidative stress or sebum level within the last month. The control group consisted of 40 age-, sex-, and skin phenotype-matched healthy volunteers. Similar to the patient group, the volunteers were non-smokers, did not have any systemic disease, and did not use any medication within the past month. Furthermore, rosacea patients with only eye involvement and patients and volunteers having one or more of the following conditions were excluded: Diabetes mellitus, hypertension, metabolic syndrome, cardiovascular disease, renal disease, liver disease, neurodegenerative disease, active infection, thyroid disease, cancer, and other inflammatory dermatoses that could increase systemic oxidative stress.

Calculation of disease severity

The patients diagnosed with rosacea were divided into three clinical types: Type 1—erythematotelangiectatic (ETR), Type 2—papulopustular rosacea (PPR) and Type 3—fimatous rosacea (FR). Disease severity was evaluated using a modified rosacea clinical severity scorecard based on the rosacea clinical scorecard of the National Rosacea Society of the United States [35]. Transient erythema, nontransient erythema, papules-pustules, and telangiectasia were recorded as primary features; and burning-stinging, plaque formation, dry appearance, edema, ocular manifestations, and fimatous change were recorded as secondary features. Each feature was scored depending on the severity (0 none, 1 mild, 2 moderate, 3 severe). The physician's rating by subtype and the patient's global assessment score were added to obtain the total score. Disease severity classification was based on the total scores of 0–12 for mild, 13–24 for moderate, and 25–36 for severe forms of the disease.

Measurement of sebum level

A sebumeter (Sebumeter® SM 815, Courage + Khazaka Electronic GmbH, Köln, Germany) was used to measure sebum levels on skin, and the results were expressed as µg/cm^2. The measurements were taken at room temperature (20 °C) and a relative humidity of 40–60%.

Two hours prior to sebum measurement, the patients and control subjects were instructed to clean their faces with a mild cleanser, and not to apply foundation, talc or any other cream to their faces. Sebum measurement was performed on two different regions of the face: the midpoint of the vertical line passing through the glabellar region and 1 cm below the midpoint of the orbital rim in the right infraorbital region.

Evaluation of photoaging

FotoFinder (TeachScreen Software GmbH) digital dermoscopy and reflectance confocal microscopy (RCM) (VivaScope® 3000, Lucid, Rochester, NY, USA) were used to evaluate photoaging in patients and control subjects.

Forehead, left and right cheeks, and jaw regions were evaluated with dermoscopy. In each region, the presence of yellowish discoloration, white linear scars, ephelide/lentigo, hypo/hyperpigmented macules, telangiectasia, yellowish papules, actinic keratosis, senile comedones, deep wrinkles, superficial wrinkles, and crisscross wrinkles were examined. Each region received a score of 1 for the presence of a given parameter and 0 for the absence of the parameter.

Dermoscopic photoaging scale (DPAS) was obtained as the sum of the scores of each region [14]. The maximum score in this scale is 44.

Reflectance confocal microscopy photoaging assessment was performed on the horizontal sections from the most protrusion of the zygomatic bone. Epidermal disarray, epidermal hyperplasia and collagen alteration scores were calculated for each section. RCM skin aging score was calculated as the sum of three parameters [19]. In this scale, the maximum scores for epidermal disarray, epidermal hyperplasia, and collagen alteration are 9, 9, and 12, respectively.

Calculating mite density

A RCM hand instrument (VivaScope® 3000, Lucid, Rochester, NY, USA) was used to calculate mite density. Ten non-overlapping images of 1 mm^2 (1000 × 1000 µm) sections were acquired from the regions described in sebum measurement. The sections were taken from epidermis to dermis, where mites were best visualized and counted (Fig. 1). The number of mites, follicles, and infested follicles was counted for each image. These values were used to calculate the number of mites per follicle and the number of mites per infested follicle [32].

Laboratory examinations

After overnight fasting, blood samples were obtained from the cubital vein into test tubes containing gel additive. The samples were centrifuged at 3500×*g* for 5 min to separate plasma. The plasma samples were aliquoted into microcentrifuge tubes and were stored at − 80 °C.

Enzyme-linked immunosorbent assays (ELISA) were used to measure serum total antioxidant capacity (TAC), total oxidant capacity (TOC), myeloperoxidase (MPO), matrix metalloproteinase-1 (MMP-1), MMP-9, arylesterase (ARES), interleukin-1β (IL-1β), paraoxonase-1 (PON-1), and tumor necrosis factor-α (TNF-α) levels. Absorbance values at 450 nm wavelength were recorded on a microplate reader (ThermoScientific™ Varioskan™ Flash Multimode Reader). Oxidative stress index (OSI) was calculated as the ratio of TOC to TAC.

Statistical analysis

SPSS v. 15.0 for Windows was used for the statistical analysis. Descriptive statistics were obtained as numbers and percentages for the categorical variables, and mean and standard deviation values for numerical variables. For the

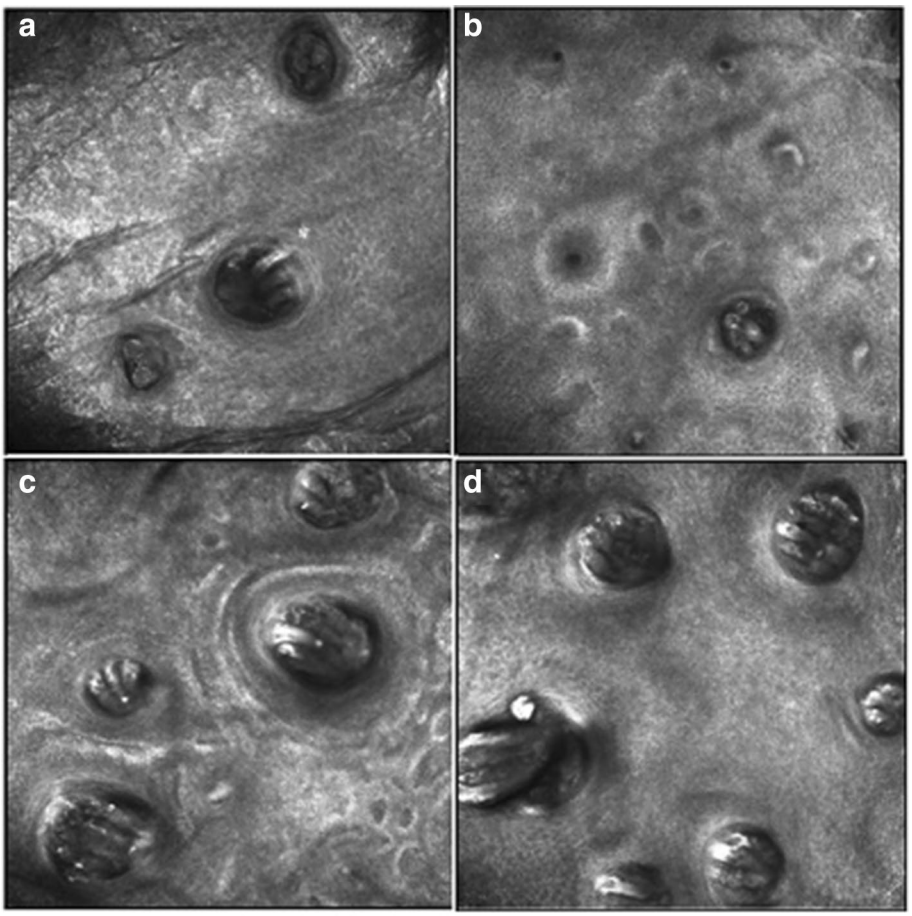

Fig. 1 Imaging of demodex mites on 1 mm × 1 mm sectioning via RCM. When the head of RCM is vertical, the mites are seen as bright round objects in the hair follicles (**a, b**); when the head of RCM is inclined, the mites are seen as thin, long, and conical objects (**a, c, d**)

comparison of numerical variables between two independent groups, the Student's *t* test was used when the normal distribution condition was satisfied and the Mann–Whitney *U* test was applied if this condition was not met. One-way ANOVA was undertaken to compare more than two groups in terms of independent numerical variables that followed a normal distribution, and Kruskal Wallis test was utilized if the assumption of normality was not met. Subgroup analyses were undertaken using Tukey as a parametric test and Mann Whitney *U* as a nonparametric test, and interpreted by the Bonferroni correction. The dependent groups were compared using the paired samples *t* test when the differences between the variables satisfied the conditions of normal distribution. For variables that were not normally distributed, Spearman's correlation analysis was performed to determine the relationship between two continuous variables. The ratios of the categorical variables among the groups were tested by Chi square analysis. The statistical significance level of alpha was accepted as $p < 0.05$.

Results

The groups were similar with respect to the mean age, sex, and Fitzpatrick skin types (Table 1). Seventeen patients (42.5%) had ETR, 15 patients (37.5%) had PPR, and 8 patients (20%) had FR. The mean rosacea clinical severity score was 16.8 ± 5.9 in the patient group. With respect to disease severity, 14 patients (35%) had mild disease, 19 patients (47.5%) had moderate disease, and 7 patients (17.5%) had severe disease. Eye involvement was present in 50% of the patients.

There were no significant differences in the forehead sebum level and the right cheek sebum level between the groups (Table 1). The serum TOC and OSI level was higher in the patient group. The serum TAC, ARES and PON-1 levels were significantly lower in the patient group (Table 2). The serum IL-1β, MPO, MMP-1 and MMP-9 levels were significantly higher in patients compared to control subjects (Table 2). MMP-1 and MMP-9 levels were higher in PPR patients compared to the other rosacea groups (Table 3).

The number of mites on forehead and cheek, mites per follicle, infested follicles, and mites per infested follicles were higher in the patient group; however, these parameters did not significantly differ between the cheek and forehead regions in the patient group (Table 4). The disease severity was not correlated with the number of mites on forehead and cheek, follicles, mites per follicle, infested follicles, and mites per infested follicle. Similarly, there was no significant difference in the number of demodex mites on forehead and cheek with respect to the rosacea type.

The results of TAS, TOS, OSI, MPO, MMP-1, MMP-9, ARES, IL-1b, PON-1 and TNF a according to disease characteristics are shown in Table 3. No significant correlations were found between the number of mites on forehead and cheek, infested follicles, and mites per follicle, and serum TAC, TOC, OSI, MPO, MMP-1, MMP-9, ARES, PON-1, TNF-α, and IL-1β levels.

The mean dermoscopic aging severity was higher in patients with severe disease ($p = 0.024$). However, there was no correlation between RCM aging severity and rosacea severity. Since the RCM photoaging scale is not affected by

Table 1 Demographic features of patients and control subjects

	Patients	Control subjects	
Age	44.0 ± 12.8	44.0 ± 12.8	1.000
Sex			
Male	13 (32.5)	13 (32.5)	1.000
Female	27 (67.5)	27 (67.5)	
Fitzpatrick skin type			
Type 2	16 (40)	16 (40)	1.000
Type 3	19 (47.5)	19 (47.5)	
Type 4	5 (12.5)	5 (12.5)	
Forehead skin type			
Dry	15 (37.5)	13 (32.5)	0.859
Normal	24 (60)	25 (62.5)	
Oily	1 (2.5)	2 (5)	
Sebum level on forehead	124.1 ± 54.5	130.2 ± 55.8	0.621
Right cheek skin type			
Dry	23 (57.5)	24 (60)	1.000
Normal	14 (35)	13 (32.5)	
Oily	3 (7.5)	3 (7.5)	
Sebum level on cheek	77.0 ± 55.0	67.1 ± 51.2	0.419

Table 2 Comparison of serum TAC, TOC, OSI, MPO, MMP-1, MMP-9, ARES, IL-1β, PON-1 and TNF-α levels

	Patients	Control subjects	*p* value
TAC	1.8 ± 0.9	2.5 ± 0.8	< 0.001
TOC	13.2 ± 3.7	3.3 ± 1.9	< 0.001
OSI	8.5 ± 3.8	1.4 ± 0.9	< 0.001
MPO	14.4 ± 7.0	10.8 ± 5.9	0.001
MMP-1	37.2 ± 23.1	28.6 ± 20.1	0.023
MMP-9	4781.5 ± 2654.8	3342.8 ± 1801.8	0.005
ARES	900.4 ± 181.1	981.9 ± 176.5	0.045
IL-1β	1537.2 ± 443.9	862.0 ± 301.1	< 0.001
PON-1	121.6 ± 38.5	182.8 ± 51.2	< 0.001
TNF-α	13.9 ± 14.6	10.3 ± 9.1	0.189

Values are mean ± SD

TAC total antioxidant capacity, *TOC* total oxidant capacity, *OSI* oxidative stress index, *MPO* myeloperoxidase, *MMP-1* matrix metalloproteinase-1, *MMP-9* matrix metalloproteinase-9, *ARES* arylesterase, *IL-1β* interleukin-1β, *PON-1* paraoxonase, *TNF-α* tumor necrosis factor-α

Table 3 The results of TAS, TOS, OSI, MPO, MMP-1, MMP-9, ARES, IL-1b, PON-1 and TNF-a according to disease characteristics

	Severity of rosacea		Duration of disease		Age at onset		ETR	PPR	FR	*p*	DPAS		RCM aging severity	
	Rho	*p*	rho	*p*	rho	*p*	Ort. ± SD	Ort. ± SD	Ort. ± SD		Rho	*p*	rho	*p*
TAS	0.313	0.050	0.324	0.041	0.203	0.208	1.6 ± 0.5	1.8 ± 0.8	2.5 ± 1.5	0.26	0.205	0.204	0.333	0.036
TOS	0.062	0.706	−0.057	0.728	−0.054	0.743	12.6 ± 4.0	13.3 ± 3.4	14.1 ± 3.7	0.569	−0.089	0.584	0.051	0.754
OSI	−0.233	0.147	−0.282	0.078	−0.169	0.298	8.7 ± 3.8	8.7 ± 3.6	7.7 ± 4.5	0.735	−0.156	0.335	−0.212	0.19
MPO	0.054	0.74	0.047	0.771	−0.075	0.645	12.8 ± 6.6	17.4 ± 7.7	12.2 ± 4.6	0.164	−0.157	0.332	−0.183	0.259
MMP-1	−0.033	0.839	0.044	0.789	−0.042	0.797	26.4 ± 17.8	51.3 ± 25.3	33.8 ± 15.9	0.003	−0.128	0.43	−0.208	0.199
MMP-9	−0.199	0.217	0.046	0.779	−0.156	0.336	3948.2 ± 2375.6	6221.1 ± 2866.8	3853.0 ± 1679.0	0.028	−0.161	0.32	−0.33	0.037
ARES	−0.44	0.005	0.068	0.675	−0.399	0.011	900.9 ± 149.0	895.3 ± 205.4	908.9 ± 217.7	0.986	−0.308	0.053	0.102	0.532
IL-1b	−0.311	0.051	0.046	0.779	−0.1	0.541	1633.7 ± 337.8	1580.5 ± 580.3	1251.1 ± 207.8	0.117	−0.129	0.428	−0.242	0.133
PON-1	−0.214	0.185	−0.126	0.44	0.015	0.925	123.1 ± 28.8	114.0 ± 32.6	132.9 ± 62.8	0.845	0.024	0.883	0.166	0.306
TNFa	0.043	0.798	−0.083	0.619	0.282	0.086	13.9 ± 7.7	15.3 ± 21.8	11.3 ± 10.9	0.312	0.326	0.046	0.005	0.975

TAC total antioxidant capacity, *TOC* total oxidant capacity, *OSI* oxidative stress index, *MPO* myeloperoxidase, *MMP-1* matrix metalloproteinase-1, *MMP-9* matrix metalloproteinase-9, *ARES* arylesterase, *IL-1β* interleukin-1β, *PON-1* paraoxonase, *TNF-α* (tumor necrosis factor-α, *ETR* erythematotelangiectatic rosacea, *PPR* papulopustular rosacea, *FR* fimatous rosacea, *DPAS* dermoscopic photoaging scale, *RCM* reflectance confocal microscopy

disease features, such as erythema or telangiectasia, it is a more reliable tool for patients with severe rosacea, compared to the DPAS.

Disease severity was not found to be correlated with sebum level on forehead ($p = 0.696$) or right cheek ($p = 0.733$). Positive correlations were identified between sebum levels on forehead and cheek, and the number of mites, mites per follicle, and infested follicles (Table 5).

The mean dermoscopic aging severity and the mean RCM aging severity did not significantly differ between the groups. A positive correlation between dermoscopic aging severity and RCM aging severity was found in both groups.

Discussion

Chronic inflammation is the key factor in rosacea pathogenesis and emergence of clinical symptoms. The specific stimulus responsible for the dysregulation of normal immune signaling pathway and increased levels of inflammatory mediators is not known. The proinflammatory effect of UV on skin and immune response to demodex mites are considered to trigger inflammation [11, 12].

The role of free oxygen radicals in the etiology of rosacea has been explored in several studies. Low superoxidedismutase activity and high malondialdehyde (MDA) activity are seen in cases of severe rosacea, and elevated levels of reactive oxygen species (ROS) decrease in response to treatment [2, 25]. It has also been shown that oxidative stress is increased in systemic circulation in rosacea [3, 30, 31]. Similar to previous studies, we found that rosacea patients had high TOC and OSI levels, but low TAC, paraoxonase, and arylesterase levels. In addition, these parameters were independent of duration, type, and severity of the disease, excluding ARES and severity of rosacea and TAS and duration of disease. Taken together, these findings provide evidence for the elevated levels of systemic oxidative stress and free radicals in rosacea, which lead to a decrease in the activity of paraoxonase and arylesterase, thereby indirectly increasing lipid peroxidation. In other words, a high level of oxidative stress in rosacea was not limited to the disease area, but was also visible in systemic circulation.

Myeloperoxidase level is a specific indicator of neutrophil activation, and we found that the serum MPO levels were higher in patients with rosacea. Based on this finding, it is possible that a bacterial agent or a structural component of demodex mites triggers neutrophil activation, and MPO-derived oxidation products overcome the cellular antioxidant defense mechanism and contribute to oxidative stress.

Metalloproteinases are endopeptidases responsible for the degradation of the extracellular matrix to its components. In normal physiological conditions, their levels are maintained at a certain equilibrium [34]. Elevated TNF-α, IL-1β, and

Table 4 Comparison of demodex parameters on forehead and cheek

	Patients	Control subjects	*p* value
Mite count on forehead	47.8 ± 41.6	22.2 ± 14.5	0.009
Follicle count on forehead	44.6 ± 7.3	49.7 ± 8.5	0.006
Number of mites per follicle	1.1 ± 1.0	0.5 ± 0.3	0.006
Number of infested follicles on forehead	14.5 ± 10.9	8.8 ± 5.7	0.037
Number of mites per infested follicles on forehead	3.0 ± 0.9	2.4 ± 0.8	0.002
Mite count on cheek	50.5 ± 40.9	19.7 ± 15.9	< 0.001
Follicle count on cheek	35.4 ± 5.9	42.6 ± 8.4	< 0.001
Number of mites per follicle	1.4 ± 1.1	0.5 ± 0.4	< 0.001
Number of infested follicles on cheek	15.6 ± 10.6	8.5 ± 5.8	0.002
Number of mites per infested follicles on cheek	3.1 ± 0.7	2.0 ± 0.8	< 0.001

Values are mean ± SD

Table 5 Correlation between forehead and cheek sebum level and demodex parameters

	Sebum level in forehead	
	Rho	*p* value
Mite count on forehead	0.466	0.002
Follicle count on forehead	− 0.013	0.935
Number of mites per follicle	0.446	0.004
Number of infested follicles on forehead	0.425	0.006
Number of mites per infested follicles on forehead	0.217	0.179
	Sebum level in cheek	
	Rho	*p* value
Mite count on cheek	0.419	0.007
Follicle count on cheek	− 0.278	0.083
Number of mites per follicle	0.482	0.002
Number of infested follicles on cheek	0.377	0.017
Number of mites per infested follicles on cheek	0.423	0.007

ROS levels are known to stimulate MMP production [11]. In the current study, we found that the serum MMP-1, MMP-9, and IL-1β levels were higher in patients with rosacea.

Dyslipidemia, hypertension, coronary arterial diseases, allergies, gastrointestinal diseases, and respiratory diseases have been linked to rosacea [13, 27, 33]. Decreased levels of PON-1, a high-density lipoprotein-associated antioxidant enzyme, and structural changes in lipoproteins due to systemic inflammation may be risk factors for atherosclerosis and coronary heart disease in rosacea [4, 7, 33]. MPO-induced oxidative stress and inflammation are known to contribute to the formation of sclerotic plaques, their progression, and impairment of stabilization [21]. In addition to MPO, MMPs are considered to be another mediator responsible for vascular events. A retrospective review has argued that the incidence of vascular events in rosacea is reduced upon tetracycline treatment as a result of the anti-inflammatory effect of tetracycline and subsequent inhibition of the elevated MMP levels [6, 13]. In the current study, we found that patients without any risk factor had a high level of systemic oxidative stress, low serum PON-1 levels, and high serum MPO and MMP levels. These findings may explain the relationship between rosacea and systemic diseases, particularly cardiovascular diseases.

While UV is considered to play a role in rosacea pathogenesis, considering the higher prevalence of rosacea in individuals with light skin and UV-induced rosacea attacks, the number of studies investigating the relationship between UV and rosacea is limited. Photodamage scale has been used to investigate the relationship between exposure to UV and prevalence in patients with PPR [20]. We used photoaging to evaluate UV exposure, but did not find a significant difference in the level of photoaging between the patients and control subjects. In addition, the higher serum levels of MMP-1, MMP-9 and IL-1β were not correlated with photoaging severity. Thus, we hypothesized that another mechanism, rather than UV exposure, was responsible for the elevated levels of these cytokines and MMPs. Except UV, microorganisms are also suspected as a potential cause of inflammation in rosacea. Demodex mites, despite their controversial pathogenicity in rosacea, are considered to be the most important factor.

Demodex mites play a role in pathological changes in rosacea; the increase in the number of mites can contribute to rosacea pathogenesis by triggering inflammatory response, causing mechanical obstruction in follicles, or functioning as bacterial vectors [5, 8, 10, 22]. A high number of demodex mites in patients with rosacea, and the reversal of symptoms and number of mites following acaricide treatment indicates the indirect pathogenic role of mites [8, 9]. In recent years, RCM has gained popularity as a fast, non-invasive, in vivo method to detect demodex mites and determine their density. Different studies have shown that RCM-based mite counts are considerably higher compared to those of superficial skin biopsies, which fall short of calculating the actual number

of mites due to a high false negative rate [28, 32]. For these reasons, we used RCM to calculate demodex density; the mean total number of demodex mites in a $10mm^2$ area on the cheek was calculated as 50.5 ± 40.9 in the patient group, and 19.7 ± 15.9 in the control group. Consistent with previous studies on patients with rosacea and healthy volunteers, the actual number of demodex mites was considerably higher compared to that calculated by superficial skin biopsy, and patients with rosacea had a significantly higher number of demodex mites, compared to the healthy control subjects.

After demonstrating that mite density was higher in patients with rosacea, we investigated the effect of number of mites on disease severity. First, we compared the demodex mite densities between the cheek region (where primary disease symptoms, such as erythema or telangiectasia, are more severe) and forehead (where symptoms are less severe compared to cheek). Our results showed that the total number of mites, infested follicles, and mites per infested follicles on forehead and cheek regions did not differ significantly between the groups. We did not find a correlation between clinical severity and mite density. Then, we aimed to identify possible correlations between demodex-associated parameters, systemic oxidative stress, and inflammatory response. However, no such correlation existed between the number of mites on forehead and cheek, the number of infested follicles and the number of mites per follicle, and the serum TAC, TOC, OSI, MPO, MMP-1, MMP-9, ARES, PON-1, TNF-α, and IL-1β levels. Therefore, we suspected that demodex-associated allergens or pathogens that penetrated into the dermis and caused inflammatory response, rather than the number of demodex mites, were responsible for the increased level of inflammation in rosacea.

Bacillus oleronius is considered to be a part of the demodex gut flora, and antigenic proteins of this bacterium causes a significant increase in the proliferation of mononuclear cells in peripheral blood of patients with rosacea. When neutrophils isolated from healthy volunteers are exposed to Bacillus proteins, the level of neutrophil migration increases, MMP-9 and cathelicidin secretion occurs together with neutrophil degranulation, and the level of IL-8 and TNF-α biosynthesis in neutrophils is increased [16, 24]. Healthy individuals have a lower number of demodex mites, and thus demodex-associated antigens; therefore, the antigenic load is not sufficient to induce an immune response. On the other hand, increased number of mites in rosacea is considered to cause an increase in the antigenic load, which in turn bypasses the follicles and induces an inflammatory response in neutrophils [24].

The antigenic nature of demodex mites results in increased neutrophil accumulation, which in turn is responsible for elevated MPO levels in patients with rosacea. The antigens can stimulate the synthesis of proinflammatory cytokines, particularly IL-1β, in neutrophils. Considering that the lethal effects of neutrophils on microorganisms depend on ROS production, it can also affect systemic oxidative stress, and MMP levels can increase as a result of neutrophil degranulation. On the other hand, the observation that inflammatory response and oxidative stress do not depend on the number of mites might be explained by disrupted epithelial barrier and receptor hyperactivity in patients with rosacea; as a result, antigens, even at low levels, can bypass the follicle wall and bind to the receptors, thereby inducing inflammatory response [12]. Further research is necessary to identify the potential correlations between antigen levels, systemic oxidative stress, and inflammatory parameters.

In the present study, we found that sebum levels on forehead and cheek did not show significant differences between the patients and control subjects, which initially suggested that rosacea is not a seborrhea-associated disease. However, we also identified that the number of (a) mites, (b) mites per follicle, and (c) infested follicles increased as the sebum levels on forehead and cheek increased. The fact that the healthy control subjects with the same sebum level had lower mite density suggests other factors, such as sebum quality, may be key to underlie the increased proliferation of mites in rosacea.

This study has certain limitations. When calculating the number of mites with RCM, inexperienced researchers can confuse hair shafts in the hair follicle with mites. We aimed to overcome this potential limitation by using the mobile VivaScope 3000 instrument, rather than RCM VivaScope 1500. Secondly, RCM is unable to distinguish (a) *Demodex folliculorum* from *Demodex brevis* and (b) live mites from dead mites. It is possible that disease severity, oxidative stress, and inflammatory condition are only associated with live mites or detachment of antigens from follicles once the mites are dead. Telangiectasia and erythema, two primary features of rosacea, can lead to false positive and false negative results during dermoscopic evaluation of photoaging, and this represents another limitation of the current study. Telangiectasia is a symptom of photoaging that can be visualized with dermoscopy. This false positivity might explain the high level of dermoscopic aging in patients with severe rosacea. On the other hand, intense erythema can mask other dermoscopic features of photoaging in rosacea. RCM-based evaluation of photoaging is similar to histopathological examination; therefore, we used RCM to evaluate photoaging, and to overcome false positive and negative results.

In conclusion, rosacea is a chronic inflammatory disease, and the etiology of this disease has not yet been fully clarified. Except for UV-induced attacks, the contribution of chronic UV exposure to disease etiology and inflammation is limited. The changing sebaceous microenvironment in rosacea results in an increased number of demodex mites. Yet, this increase does not change disease severity, oxidative stress, and inflammation. It is possible that oxidative stress

and inflammation are related to demodex-associated bacterial antigens. Moreover, inflammation and oxidative stress were not limited to disease area, also they were increased in systemic circulation too. Considering that none of the patients with rosacea had any systemic disease, the high levels of systemic oxidative stress and inflammation parameters suggest that patients with rosacea are more susceptible to systemic diseases.

Funding This research was supported by Istanbul Training and Research Hospital, Education Planning and Coordination Unit.

Compliance with ethical standards

Conflict of interest There are no conflicts of interest to declare.

Ethical approval was obtained from Bezmialem University Clinical Research Ethics Committee and the study was conducted in accordance with the principles of the Declaration of Helsinki.

Informed consent A written informed consent was obtained from each participant.

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