ORIGINAL ARTICLE

MALASSEZIA COLONIZATION CORRELATES WITH THE SEVERITY OF SEBORRHEIC DERMATITIS

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ABSTRACT

The aim: To compare the number of fungi of the genus *Malassezia* on inflated and healthy areas of the skin and to correlate them with the severity of seborrheic dermatitis.

Materials and methods: 168 patients with typical manifestations of seborrheic dermatitis on the scalp and face and 30 healthy individuals were recruited. SD severity was assessed by SEDASI. Samples from lesions on scalp, face and intact chest skin were cultivated and/or stained with methylene blue or cotton and inoculated onto *Malassezia* Leeming & Notman Agar Modified (MLNA).

Results: A statistical difference in colonization intensity between all body zones (Dwass-Steel-Critchlow-Flinger pairwise comparisons $p \leq 0{,}001$). Face zone with lesions of SD patients was two times more colonized with funguses than in the control group (38,5 vs 16,5 $p=0{,}003$). The sternal area with no skin lesions was more colonized in the SD group (25,0 vs 9,0 $p=0{,}013$). The SEDASI was positively correlated with the amount of CFU on the face (Spearman's rho 0,849; $p \leq 0{,}001$) and trunk (0,714; $p \leq 0{,}001$).

Conclusions: Our results demonstrate that inflamed seborrheic areas are more colonized with *Malassezia* fungi than intact areas. The intensity of *Malassezia* growth is correlated with the severity of the symptoms of seborrheic dermatitis. The level of colonization may be a potential biomarker to indicate the efficiency of new treatment approaches

KEY WORDS: seborrheic dermatitis, *Malassezia spp*, SEDASI

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INTRODUCTION

The prevalence of clinically significant manifestations of seborrheic dermatitis (SD) in the population is approximately 3% [1], and it's even higher in immunocompromised patients [2]. Each fifth human has minor peeling of the skin described as dandruff [3]. Persistent itching, redness, and peeling of the skin, especially on the face and near the ears significantly reduces the quality of life and confidence of patients with SD [4].

Malassezia lipid-dependent commensal fungus is an important resident of the skin microbiome. Different *Malassezia* species were mentioned to cause desquamation, hypopigmented macules, and eczematous dermatitis [5]. Despite all previous studies the fact of *Malassezia* colonization of seborrheic areas requires more proof. Different studies show that the prevalence of *Malassezia spp*. in Canada is 82% [6], in Sweden 88% [7], Greece 85% [8]. The pathogenetic role remains unclear for people not colonized by *Malassezia*.

In addition, few commensalism mechanisms are described. In healthy skin, *Malassezia* interacts with keratinocytes and the immune system, as it inhabits superficial layers of the skin and follicular infundibulum [9]. Skin fungal microbiome, mainly *Malassezia*, protects the skin through its large expansion and competition with bacteria. *Malassezia* metabolites azelaic acid, which is known to have antibacterial and anti-mycotic properties [10]. Also, *Malassezia* generates ethyl ester derivatives with in vitro show antimycotic properties [11]. *Malassezia* secreted aspartyl protease 1 may disrupt Staphylococcus aureus biofilms via hydrolysis [12]. In atopic dermatitis, a significant reduction of *Malassezia* was described resulting in a potentially decreased protective function against *S. aureus* [13].

Malassezia produces lipase to hydrolyze sebum triglyceride and releases unsaturated fatty acids such as oleic acid and arachidonic acid [14] These acids insult keratinocyte differentiation, which leads to stratum corneum abnormalities such as parakeratosis and corneocyte damage. These metabolites also induce keratinocyte production of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, interferon-gamma, and tumor necrosis factor [15]. At the same time *M. furfur, M. globosa*, and *M. restricta* enhance expression

of toll-like receptor 2 IL-8, human beta-defensin 2, which benefits skin protection [16]. These cytokines recruit immune cells to skin sites with a compromised barrier, for example, follicular infundibulum where tissue-resident dendritic cells, macrophages, and myeloid cells can directly meet *Malassezia* [17]. So *Malassezia* may be a commensal and a trigger of inflammation.

THE AIM

The aim of the study was to compare the number of fungi of the genus *Malassezia* on inflamed and healthy areas of the skin and to correlate them with the severity of seborrheic dermatitis.

MATERIALS AND METHODS

168 patients with seborrheic dermatitis and 30 healthy individuals were examined. The study included patients aged 18-55 years from 2020 to 2022 who were treated at the clinical departments of the medical faculty of the State Higher Educational Institution "Uzhhorod National University"; Regional clinical skin and venereological dispensary and private dermatological clinic. Before the examination, the patients were informed about the research design, developed within the framework of the Helsinki Declaration of the World Medical Association "Ethical principles of medical research with the participation of a person as an object of research", the Convention of the Council of Europe on human rights and biomedicine, and the legislation of Ukraine, and signed the informed consent.

Patients with typical manifestations of seborrheic dermatitis on the scalp and face were included in the study after consulting a dermatologist and verifying the diagnosis. A mandatory condition was the absence of SD-associated treatment for the last month and the absence of other inflammatory skin diseases. Severity was assessed using the seborrheic dermatitis area and severity index (SEDASI) scale [18] and divided into groups: mild 1-14 points, medium severity 15-29 points, and severe 30 or more points. Medical workers with healthy skin were included in the control group.

Subjects were asked not to shower or wash in the evening and morning before sampling. Samples were taken from 3 areas: the first from the hairy part of the head, the second from the face (in places where there is inflammation, such as the mustache, nasolabial fold, and bridge of the nose), and the third from the sternum. Patients with manifestations of inflammation in the chest area were excluded from the study. First, smears were taken with a cotton swab dipped in a sterile isotonic solution, and transferred to the transport medium. Then skin flakes were collected by the atraumatic scraping of a 1 cm2 area. The scraped material was mixed with an isotonic sodium chloride solution, applied to a glass slide, and dried. 96% ethyl alcohol and direct heating were used to fix the sample. Methylene blue was applied for 1 min and washed. To count the number of yeasts, a light microscope was used at a magnification of 40 and 100 with the addition of immersion oil. The results were evaluated as follows: 1–5 yeasts per field = +; 6–10 = ++; > 10 = +++.

Swab material was inoculated directly onto *Malassezia* Leeming & Notman Agar Modified (MLNA) Kairosafe. Petri dishes were kept in a thermostat for 72 hours at a temperature of 37 °C. Gram staining was used for verification. Microscopy and lactophenol cotton blue dye were used to count the number of CFUs (colony-forming units) of fungi. The obtained results were divided into 4 groups according to the number of CFUs: no fungi, 1-sample with 1-25 CFUs, 2 - 26-50 CFUs, 3 - 51, and more CFUs.

The chi-square test was used to determine the difference in gender distribution between the SD and control groups. The Mann-Whitney t-test for independent samples was used to determine the difference in age between groups and to compare the distribution of the number of CFUs between groups. Fisher's exact test to compare the mean number of CFUs in groups. Spearman's correlation was used to determine the relationship between the severity of SD and the number of CFUs in the sample. Kruskal-Wallis test was used to compare fungi colonization in healthy and SD patients. A correlation was considered significant at $p \leq 0.05$. Dwass-Steel-Critchlow-Flinger pairwise comparisons test was used to compare CFU quantity between different skin sites.

RESULTS

According to the microscopy of skin scrapings, the prevalence of *Malassezia spp.* in patients with SD was 53% (89/168), and in healthy individuals 27% (8/30). A difference between prevalence in SD and control group is significant ($p \leq 0{,}05$). Both groups showed the largest number of fungi on the scalp and face, and the lowest on the trunk in the area of the sternum. A significant difference was found between the number of fungi in samples with seborrheic plaques and samples without skin inflammation (7.44; 4.51; $p \leq 0.05$) and compared with healthy individuals (2.83; $p \leq 0.05$) (Fig. 1).

Comparing the growth of colonies on modified Leming-Notman agar taken from skin swabs (Fig. 2), the prevalence of *Malassezia spp.* in patients with SD was 87% (113/168). Culture result was considered positive

Table I. Quantity of CFU *Malassezia spp.* cultivated from typical seborrheic zones in patients and healthy controls.

Skin zone	SD patients (total n=168)	Control (total n=30)	Statistic*	p
Scalp with seborrheic plaques	53,5 (42,8;58,5) n=36	0		
Scalp without seborrheic plaques	27,0 (16,0;42,0) n=113	16,5 (7,25;44,8) n=18	859	0,291
Face	38,5 (22,0;47,0) n=146	16,5 (8,75;40,8) n=18	744	0,003
Trunk	25,0 (19,0;36,0) n=109	9,0 (4,0;34,0) n=17	580	0,013

*Mann-Whitney U test; n=number of swab samples with *Malassezia spp.* growth on MLNA.

Table II. Quantity of CFU *Malassezia spp.* on different skin zones in patients with different SD severity.

	mild	moderate	severe	χ2*	df	p
Scalp with lesions (total n=36)	0	46,5(38,3;54,3) n=24	57,5(54,8;62,3) n=12	8,84	1	0,003
Scalp without lesions (total n=113)	15,0(9,0;19,0) n=55	42,0(32,0;52,3) n=58	0	79,07	1	≤0,001
Lesions on face (total n=146)	18,0(13,8;19,0) n=52	44,0(38,0;47,8) n=82	57,0(53,8;62,5) n=12	104,36	2	≤0,001
Trunk with no lesions (total n=109)	5,0(4,0;11,0) n=15	26,0(22,0;33,0) n=82	52,0(44,8;54,3) n=12	52,57	2	≤0,001

* Kruskal-Wallis test

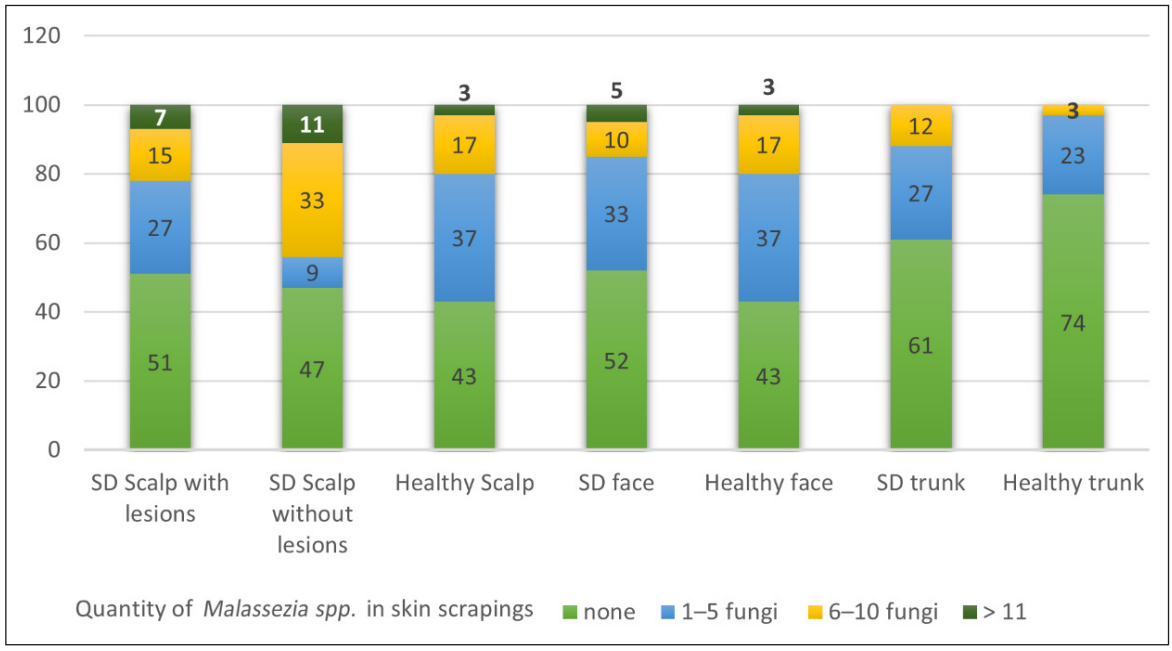

Fig. 1. Distribution of patients with SD and healthy individuals according to the number of *Malassezia* fungi in the skin scrapings.

if at least one skin zone showed positive fungi growth. The minimum number of CFU was 2, the maximum was 68. In the group of healthy individuals, the prevalence of *Malassezia spp.* was 60% (18/30), the minimum number of CFUs was 2, the maximum was 65.

Similar body zones of healthy and SD people were compared to estimate the density of fungi colonization. 21% of SD patients had seborrheic plaques on the scalp. The Mean of CFU in samples taken from inflamed skin regions was 53,5. These patients were excluded from the comparison. SD patients without scalp lesions had no statistical difference in the number of CFU compared with healthy people (p=0,291). In particular, the face zone with lesions of SD patients was two times more colonized with funguses than in the control group (38,5 vs 16,5 p=0,003). The sternal area with no skin

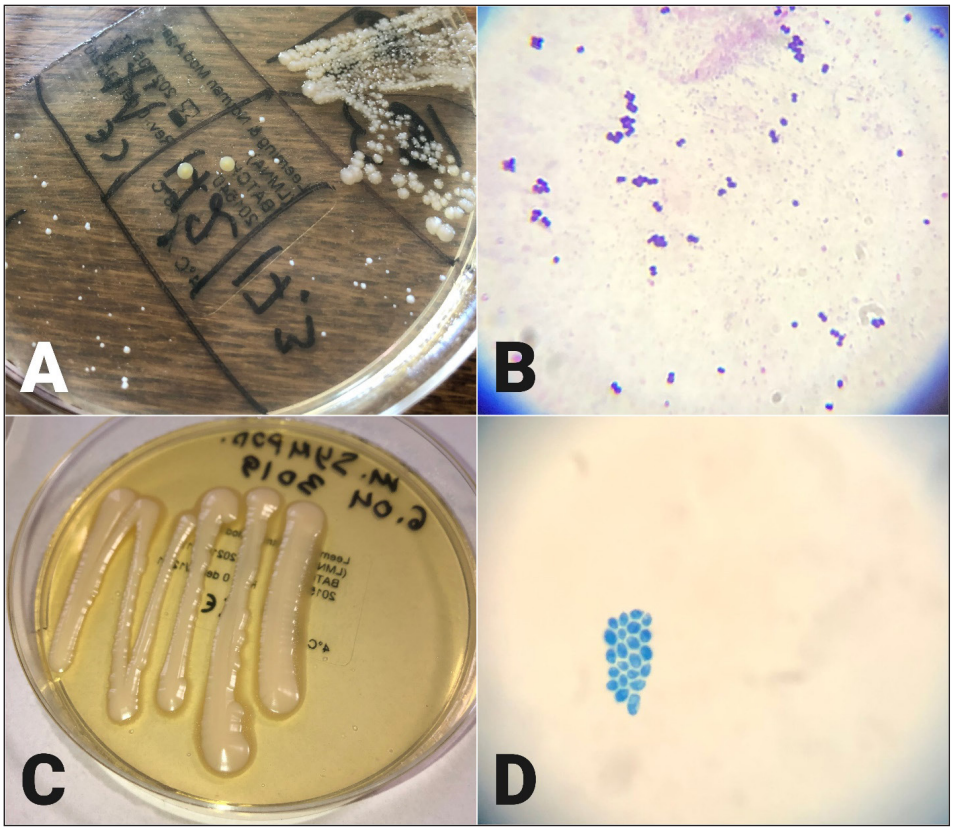

Fig. 2. (A) Representative image of direct culture on modified Leming-Notman agar from skin swab of SD patient (kept in thermostat for 3 days at 37°C). (B) Representative image of *Malassezia spp*. under a light microscope (original magnification x400) after Gramm staining for species identification. (C) Representative image of purified colony of *Malassezia sympodialis* on modified Leming-Notman agar cultured at 37°C for 7 days. Clear zone around the colony indicates a lipolytic activity (D) Representative image of *Malassezia spp*. stained with lactophenol cotton blue dye under a light microscope (original magnification x400).

lesions was more colonized in the SD group (25,0 vs 9,0 p=0,013) (Table I). Was found a probable difference in the number of CFU depended on the skin area in patients with SD and healthy individuals (Kruskal-Wallis test $\chi 2$ 15,9; df 2; p≤0,001). The control group had no statistical difference in the number of CFU between the face and scalp and a significant difference compared with the trunk. Dwass-Steel-Critchlow-Flinger pairwise comparisons: trunk vs face (W 5.72; p≤0,001); trunk vs scalp (W 3.93; p=0.15); face vs scalp (W -1.32; p=0,618).

We have found a statistical difference in colonization intensity between all body zones (Dwass-Steel-Critchlow-Flinger pairwise comparisons p≤0,001). As shown in table II, the quantity of CFU *Malassezia spp*. has increased according to the severity of seborrheic dermatitis symptoms. Patients with a mild form of SD had a mean of 15,0 CFU on the scalp, 18 on the face, and 5 on the trunk. The mild form was associated with moderate growth of fungi, per 42 on the scalp, 44 on the face, and 26 on the trunk. Patients with severe form had the most intensive *Malassezia spp*. grows. Lesioned areas on the face and scalp were colonized more intensively than non-inflamed areas typical for SD occurrence.

Based on the severity of symptoms and percentage of inflamed skin patients were divided into having severe 7% (12/168), moderate 49 % (82/168), and mild 44% (74/168) symptoms. All patients with severe form had lesions on the scalp and face and tested positive for *Malassezia*. The moderate symptom group also tested positive for *Malassezia* and the third part had scalp and face lesions. A quarter part of patients with mild symptoms tested negative for *Malassezia*. Half patients in the mild symptom group had positive cultivation results on the scalp and face but no growth on the trunk.

The correlation between the SEDASI score and quantity of CFU was counted only for the patients with positive *Malassezia spp*. grows and the absence of any lesions on the scalp. A larger inflamed area would have a logical impact on the increase in severity measured by SEDASI. To avoid false statistical evidence, we compared inflamed areas on the face with intact areas on the trunk. The severity of SD measured by SEDASI is positively correlated with the amount of CFU on the face (Spearman's rho 0,849; p≤0,001) and trunk (Spearman's rho 0,714; p≤0,001). The results suggest that the intensity of fungi growth has a strong impact on the severity of symptoms.

DISCUSSION

This study reports a difference in the prevalence of *Malassezia spp*. as a result of counting by different methods. According to the results of inoculation on modified Lemming-Notman agar, the prevalence was 87%, which coincides with the results of studies in different countries [6,7,8]. When counting the number of CFU by microscopy

after staining with methylene blue, the prevalence was 60%. Only yeast forms of fungi were counted, as they are the most recognizable. Short filaments were considered accidental finds. It is described that about a third of all fungi are in the form of hyphae and predominate on seborrheic plaques. Researchers suggest using fluorescence microscopy to count CFUs, as the dye delineates the polysaccharide particles of hyphae [19].

We found a positive correlation between the number of CFUs of *Malassezia* fungi and the severity of seborrheic dermatitis. Studies that adhere to the hypothesis that *Malassezia* fungi are a main factor in pathogenesis indicate a strong correlation between the intensity of fungi colonization and SD severity [19-21]. It has been described that there is a significant difference in the intensity of fungal growth on inflamed and healthy areas of the skin in patients with SD [22]. An additional argument for this theory is the reduction of symptoms after the use of ketoconazole. Antifungal drugs inhibit the growth of *Malassezia* so they shed together with the dead epithelium particles. Inhibition of the growth of fungi leads to a decrease in redness and desquamation of the skin [23]. It was hypothesized that the triggers of inflammation are directly the hyphae and not the yeast form of the fungus. Hyphae grow into the depths of the derma, changing the immune response and metabolism of the skin [24]. Excessive colonization by fungi and the transformation of yeast into hyphae are considered novel factors of pathogenesis [25].

However, some studies consider sebum metabolism or other homeostasis changes as the main trigger factors of seborrheic dermatitis [26]. Lately described the theory of the pathogenesis of SD points to the leading role of immune and neuroendocrine factors that change the composition and amount of sebum. Such changes create favorable conditions for the growth of lipophilic *Malassezia* fungi over time inhibit the growth of other commensals and damage the integrity of the skin barrier with their waste products. Skin defects trigger a nonspecific inflammatory response that triggers the release of inflammatory cytokines, which further destroys the skin barrier. Thus, excessive colonization by fungi starts a pathological circle of inflammatory reactions [27].

In contrast, sebum secretion, skin pH, and trans-epidermal water loss (TWEL) of SD patients were analyzed. There was no statistical difference between the normal and lesion sites on the scalp. But *Staphylococcus*, *Pseudomonas*, *Malassezia*, and *Aspergillus* were proposed as potential biomarkers for SD [28]. SD lesions were reported to have reduced *Corynebacterium spp*. amount, and were dominated by *Firmicutes*, *Pseudomonas spp*., *Staphylococcus spp*., and *Micrococcus spp*. at the genus level. Bacterial alterations were found to be predominating factors of SD development [29]. Another study stated that TWEL is 3 times more increased in the SD group, decreases after ketoconazole treatment but still does not reach the level of the control group. Increased *Malassezia*, *Staphylococcus*, and decreased *Cutibacterium* are treatment goals as they represent disturbed skin microbial diversity [30].

Promising results were reported in the first-ever conducted study of the identification of differentially expressed miRNAs (DEMs). Skin lesions of elderly male patients with SD had several up and downregulated miRNAs. They were predicted to be significantly associated with typical dermatological pathogenesis like immune response, cell proliferation, and apoptosis [31].

Furthermore, skin biopsy of SD patients revealed sebaceous gland atrophy that opposes the idea of greasy skin and high colonization of lipid-depended fungi [32].

CONCLUSIONS

Our results demonstrate that inflamed seborrheic areas are more colonized with *Malassezia* fungi than intact areas. The intensity of *Malassezia* growth is correlated with the severity of the symptoms of seborrheic dermatitis. The level of colonization may be a potential biomarker to indicate the efficiency of new treatment approaches.

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Conflict of interest:
The Authors declare no conflict of interest.

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