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Original Article

Quantification of *Demodex folliculorum* by PCR in rosacea and its relationship to skin innate immune activation

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Abstract: The aim of this study is to quantify *D. folliculorum* colonisation in rosacea subtypes and age-matched controls and to determine the relationship between *D. folliculorum* load, rosacea subtype and skin innate immune system activation markers. We set up a multicentre, cross-sectional, prospective study in which 98 adults were included: 50 with facial rosacea, including 18 with erythematotelangiectatic rosacea (ETR), and 32 with papulopustular rosacea (PPR) and 48 age- and sex-matched healthy volunteers. Non-invasive facial samples were taken to quantify *D. folliculorum* infestation by quantitative PCR and evaluate inflammatory and immune markers. Analysis of the skin samples show that *D. folliculorum* was detected more frequently in rosacea patients than age-matched controls (96% vs 74%, $P < 0.01$). *D. folliculorum* density was 5.7 times higher in rosacea patients than in healthy volunteers. Skin sample analysis showed a higher expression of genes encoding pro-inflammatory cytokines (Il-8, Il-1b, TNF-a) and inflammasome-related genes (NALP-3 and CASP-1) in rosacea, especially PPR. Overexpression of LL-37 and VEGF, as well as CD45RO, MPO and CD163, was observed, indicating broad immune system activation in patients with rosacea. In conclusion, *D. folliculorum* density is highly increased in patients with rosacea, irrespective of rosacea subtype. There appears to be an inverse relationship between *D. folliculorum* density and inflammation markers in the skin of rosacea patients, with clear differences between rosacea subtypes.

Key words: biomarkers – inflammation – mites – Rosacea

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Introduction

Rosacea is a chronic inflammatory skin disease characterised by a combination of facial telangiectatic erythema and inflammatory papules and pustules. Although four subtypes are usually described clinically, the question remains as to whether these subtypes truly represent different stages of the same pathology or are merely part of a syndrome, with some patients presenting with only one subtype (1).

The pathophysiology of rosacea is complex and multifactorial, and the association between *D. folliculorum* colonisation and rosacea has long been advocated (2). Indeed, several clinical studies have shown that modest increases in *D. folliculorum* colonisation could be considered a trigger factor for rosacea (2–7). According to recent experimental data, the development of rosacea lesions may result from an excessive pro-inflammatory skin response induced by *D. folliculorum* in the pilosebaceous unit. The role of innate immunity, particularly cathelicidin (LL37), allied to an increase in inflammation mediators, has been demonstrated in rosacea patients (8).

In addition, LL-37 appears to play a pro-angiogenic role, leading to neovascularisation (9). The resultant vascular hyperactivity would promote the development of *D. folliculorum* (10), which in turn could revive the inflammatory reaction, amplifying the mechanisms and producing a vicious circle.

There has been considerable debate regarding the exact role of *D. folliculorum* in rosacea. Increased numbers of *D. folliculorum* have been demonstrated in rosacea (2,3,7), but the classical skin surface biopsy technique used to quantify *D. folliculorum* on facial skin in these studies is limited in terms of standardisation and reproducibility (11–13). Moreover, this technique is cumbersome and requires the direct counting of mites, which become difficult to distinguish from cellular debris after a few hours.

The purpose of this study was to quantify *D. folliculorum* colonisation using a new polymerase chain reaction (PCR)-based method and to analyse the relative expression of biomarkers involved in skin inflammatory response in the two most common forms of rosacea: erythematotelangiectatic (ETR) and papulopustular rosacea (PPR). To determine the clinical relevance of the findings, patients were assessed in parallel with a sex- and age-matched control population.

Material and methods

Study design and patient selection

This exploratory, multicentre, case–control study was carried out at the Dermatology Department, Paul Sabatier University, in the private dermatology practice of Dr E. Durbise, Toulouse and at the Centre de Recherche sur la Peau Pierre Fabre (CRP), Toulouse (France), in accordance with the ethical principles of the Declaration of Helsinki and the guidelines for Good Clinical Practice.

The protocol was approved by the Sud Ouest et Outre Mer III Ethics Committee and the French Agency for the Safety of Health Products (AFSSAPS). Each volunteer signed a written informed consent.

Patient inclusion and exclusion criteria

Subjects had to have a diagnosis of facial rosacea performed by a licensed dermatologist with the presence of persistent centrofacial or diffuse erythrosis. Patients with ETR or PPR were included. Patients with ETR had to have telangiectasia or acute episodes of flushing, and patients with PPR had to have inflammatory papules and pustules of the facial region (nose, cheek, chin). They were recruited from two centres: a tertiary care centre and a dermatology practice.

The control group of healthy volunteers was recruited from the Centre de Recherche sur la Peau Pierre Fabre (CRP). Volunteers had to be free of any facial dermatosis and were matched for age and sex with the rosacea patients included in the study.

Subjects exhibiting the following characteristics were not included: isolated rhinophyma, isolated ocular rosacea, granulomatous rosacea, fulminant rosacea, steroid-induced rosacea, any facial dermatosis other than the condition studied that might interfere with clinical evaluation (atopic dermatitis, psoriasis, disseminated lupus erythematosus, seborrhoeic dermatitis, etc.), or inflammatory or cicatricial skin lesion other than rosacea in the areas of the face from which the samples were taken.

The exclusion criteria relating to treatment were any systemic or topical antiparasitic treatment in the 4 weeks preceding the study; any antibiotic treatment active against *Bacillus oleronius* in the week or 2 weeks preceding sampling for the topical facial route and the oral route, respectively; any local antiseptic treatment applied to the face in the week preceding the study. Pregnant and lactating women were excluded from the study.

Fifty patients in the rosacea group (32 PPR and 18 ETR) and 50 control subjects were planned to be recruited.

Selection of patients was performed by experienced certified dermatologists, and particular attention was devoted not to include patients with chronic photodamaged skin that may resemble rosacea.

Sampling protocol

Different types of samples were taken from subjects' faces.

For the samples intended to quantify *D. folliculorum*, an amount of hydrophilic wax (sugar wax – Laurence Dumont brand) sufficient to cover a surface of 5 cm^2 was heated for 30 s in a microwave and then removed with an appropriate strip. Samples were stored at −20°C for 1 week and then at −80°C for subsequent analysis by quantitative PCR (qPCR).

The sample for Il-8 protein evaluation was taken using a swab (VWR, #710-0457) impregnated with a mixture of PBS buffer containing 1% Triton x100. After swabbing of the lesional area, the tip of the swab was snapped off and stored at −80°C.

To obtain epidermal mRNA from lesional areas, skin samples were taken by scratching the skin surface (five times back and forth) with a sterile micro-abrasive tool (Vitry, France) approved by the French Ethics Committee in Nice (CCPPRB No CCP 08.013). Cell aggregates were transferred from the tool to a microtube containing 500 μL of RNA-protect Cell Reagent (Qiagen, Courtaboeuf, France) and placed at −80°C.

Samples performed by swabbing and micro-abrasion have always been performed on a cheek lesional area, which differed from the area used for wax sampling, but was directly bordering. The use of a tracing pattern has allowed sampling the same areas in apparied healthy subjects.

Marker evaluation

- *Demodex folliculorum* quantification as primary study outcome

A specific target sequence of *D. folliculorum* 18S rRNA (EU 861211) was first determined, and a primer couple and probe for the qPCR assay were then sought in the sequence identified. The design was produced using the Clone Manager 9 software (Scientific & Educational Software, Cary, NC, USA), and the specificity of the amplicons validated by BLAST against the NCBI nucleotide database. The fluorescent probe chosen was synthesised in accordance with TaqMan criteria. A 'FAM' fluorochrome was positioned at the 5′ extremity, and a fluorescence quencher added at 3′ (black hole quencher 'BHQ1'). The predictive three-dimensional structure of the oligos and probes identified was validated using Clone Manager 9 and Oligonucleotide Properties Calculator software. The selected primer sequences and probe were as follows (5′>3′): sense: CTCGTAGTTGTATCTCAGTTCAT; antisense: ACCCGGTAAAGAGCATCAGA; probe: [6FAM]TCAATTGGTCAATCA CTTAATA[BHQ1a].

Subsequently, total DNA was extracted from wax strips using the QIAamp DNA micro kit (QIAGEN) following the supplier's recommendations. Given that the quality of spreading and, as a result, of sampling depends upon the experimenter, we reduced the standardisation flaw by normalising the amount of *D. folliculorum* rDNA measured by PCR after total DNA extraction by the number of cells (DNA) in the living layers submitted to extraction. Real-time PCR quantification of human DNA in the sample was performed using a Human DNA Quantification Kit (Quantifiler - Applied Biosystems, Foster City, CA, USA) following the supplier's instructions.

To allow absolute quantification of *D. folliculorum* rDNA, a synthetic DNA sequence identical to the 18S rDNA target amplified through PCR was sub-cloned in a *Escherichia coli* plasmid. This plasmid was purified, quantified and used to define a standard curve for absolute quantification of *D. folliculorum* rDNA.

- Il-8 and protein quantification

Il-8 was quantified in the skin extract using the R&D kit (DuoSet ELISA/DY-208; R&D, Minneapolis, MN, USA). The protein concentration used to weight the Il-8 values was determined with the BioRad kit (BIORAD # 500-0113/500-0114/500-0115, Hercules, CA, USA). The results were expressed in pg/mg of protein.

- mRNA analysis

mRNA extraction and qPCR analysis on skin samples obtained by micro-abrasion were performed as previously described (14). RPLP0 was chosen as housekeeping gene to standardise gene expression.

Primers and probes used for preamplification and TLDA coating were designed by Applied Biosystems: IL8 Hs99999034_m1, CASP1 Hs00354836_m1, PTGS2 (COX-2)

Hs00153133_m1, MPO Hs00165162_m1, CD163 Hs00174705_m1, 18S Hs99999901_s1, NLRP3 (NALP-3) Hs00918082_m1, CAMP (LL-37) Hs00189038_m1, VEGFA Hs00900055_m1, TNF Hs00174128_m1, IL1B Hs01555410_m1, RPLP0 Hs99999902_m1, PTPRC (CR45Ro) Hs00894734_m1.

Results are expressed as $2^{(Ct\ RPLP0\text{-}Ct\ target\ gene)}$, where Ct corresponds to the number of cycles needed to generate a fluorescent signal above a predefined threshold.

Statistical analysis

The calculation of the size of the population required for the study was based on the hypothesis that the average density of *D. folliculorum* per cm^2 as determined by facial skin biopsy in a group of patients with rosacea is higher than the density in the control group with a difference of 10.1 (7).

Comparison of the markers between control and rosacea groups and between subtypes was performed using Wilcoxon's test because of a lack of normality (Shapiro-Wilks test).

Fisher's exact test was used to determine the difference in the prevalence of *D. folliculorum* between control and rosacea groups and for each subtype versus the control group (ETR versus control and PPR versus control). $P < 0.05$ was considered statistically significant without adjustment for multiple comparisons.

Results

Ninety-eight male and female volunteers aged 19 years or older were included. The rosacea group consisted of 50 patients: 18 patients suffering from ETR and 32 patients suffering from facial PPR. Two cases were not matched to control subjects.

High *Demodex folliculorum* density is associated with rosacea, with differences according to clinical classification

The probe and primers designed to determine the density of *D. folliculorum* allowed for absolute quantification of *D. folliculorum* densities ranging from 2×10^2 to 2×10^6 molecules of mite with a PCR efficacy close to 100%.

Prevalence calculations showed that *D. folliculorum* was found in at least one of the two samples in 96% of rosacea patients. *D. folliculorum* detection was positive in 88% of ETR patients, and 100% of PPR patients, compared with 74% of age- and sex-matched control subjects. *D. folliculorum* prevalence was significantly higher in subjects with rosacea ($P = 0.0032$), especially those with the PPR form ($P = 0.0011$), than in control subjects.

Comparison of *Demodex folliculorum* densities showed a 5.7-fold higher *D. folliculorum* density in the rosacea group than in the control group ($P < 0.0001$) (Table 1/Fig. 1). Comparison of *D. folliculorum* density in each rosacea subgroup showed a higher density of *D. folliculorum* in both the ETR and the PPR subgroups than in the control group ($P = 0.0013$ for the ETR subtype and $P < 0.0001$ for the PPR subtype).

Mean *D. folliculorum* density was higher in facial skin surface biopsies of patients with ETR than in those of patients with PPR, although the difference between the two subtypes was not statistically significant owing to the high variability of *D. folliculorum* density in patients with ETR.

Table 1. Quantification of *Demodex folliculorum* density in each group and in the two subtypes compared with the control group. The density of *D. folliculorum* is given as the number of DNA copies coding for 18S rRNA of Demodex per ng of human gDNA ($\times 10^{-6}$).

	Density of *D. folliculorum*	
Population group (*n* =)	**Mean ± SD**	**Median [min-max]**
Rosacea group ($n = 50$)	4.9 ± 11.2	15.3 (0.00–73.8)
ETR subtype ($n = 18$)	9.1 ± 17.8	1.5 (0.00–73.8)
PPR subtype ($n = 32$)	2.4 ± 2.6	1.5 (0.02–10.8)
Control group ($n = 47$)	0.84 ± 1.9	0.08 (0.00–9.9)

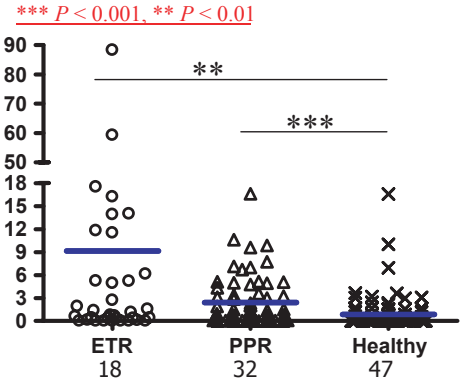

Figure 1. Graphical representation of the distribution of *Demodex folliculorum* density found in the different groups. The density is given as the number of DNA copies coding for 18S rRNA of *D. folliculorum* per ng of human genomic DNA (gDNA). The blue line represents the median. ***$P < 0.001$, **$P < 0.01$.

IL-8, IL-1, TNF-alpha and Cox-2 are highly expressed in rosacea lesions

Quantification of Il-8 protein from swab sampling of facial skin showed a significant increase in the rosacea group compared with control subjects ($P = 0.0015$). Of the two subtypes, the PPR group displayed the highest levels of Il-8 protein ($P = 0.0351$) (Table 2). As the messengers coding for this protein could be recovered in the epidermis by scrape sampling, we were able to confirm the stronger transcription of the Il-8 gene in rosacea patients than in control subjects ($P < 0.0001$). The IL-8 messenger is more highly expressed in patients with PPR (x10) than in patients with the ETR subtype ($P = 0.0081$) (Table S1).

Il-1b, TNF-a and COX-2 gene expression was determined from the epidermal scrape sample taken. Generally, stronger expression was observed for each of these genes in the rosacea group than in the control group. This was especially evident in facial skin from patients with PPR for Il-1b ($P < 0.0001$), COX-2 ($P < 0.0001$) and TNF-a ($P < 0.0001$), which were highly expressed, and between the ETR subtype and the control group for Il-1b ($P < 0.001$) and COX-2 ($P = 0.0039$). COX-2 was the only gene showing a slightly significant difference between the two subtypes ($P = 0.046$) (Table S1).

Other mediators involved in the inflammatory reaction were detected in surface skin scrape samples and showed higher expression in rosacea, especially in the PPR form. CD45R0, a marker of memory T lymphocytes, was more strongly expressed in both rosacea subtypes than in control subjects ($P < 0.001$ for PPR and $P = 0.018$ for ETR); myeloperoxidase (MPO) and CD163 were significantly increased in PPR subjects compared with control subjects ($P = 0.0013$ and $P < 0.0001$, respectively) (Table S1).

Genes coding for the inflammasome are overexpressed in rosacea

The mRNAs coding for the NALP-3, CASP-1, ASC and Il-18 proteins involved in the inflammasome were also analysed. The increased expression of NALP-3 and CASP-1 markers in the rosacea group was confirmed by comparison with the PPR subtype with the control group ($P < 0.0001$ for both genes). NALP-3 was expressed 10 times more in the PPR subtype than in the control group and only four times more in the ETR subtype. In the case

Table 2. Inflammatory Markers protein expression profile: The results are expressed in pg of Il-8 per mg of total protein. Wilcoxon's test was used.

Marker Il–8	Population group			
	Rosacea group (n = 48)	PPR (n = 31)	ETR (n = 17)	Control group (n = 48)
Mean ± SD	441.01 ± 624.71*	532.36 ± 702.74**	274.44 ± 418.09	164.85 ± 280.17
Median [min–max]	165.65 [0.00–3041.92]	209.38 [0.00–3041.92]	92.14 [0.00–1642.21]	91.27 [0.00–1725.04]

*P < 0.01 versus control.
**P < 0.05 ETR versus PPR rosacea subtypes.

of CASP-1, this gene is overexpressed with the same intensity in both subtypes compared with the control group (4–4.5 times more) (Table S1). For ASC and Il-18, we found decreased expression in the rosacea group, which was statistically significant for each subtype compared with the control group.

Gene encoding for cathelicidin LL-37 is upregulated in rosacea

Expression of the gene encoding cathelicidin LL-37 (CAMP) was significantly increased in the rosacea group compared with the control group (P < 0.0001). This overexpression was significant in the ETR and PPR subtypes relative to the control group (P = 0.002 and P < 0.0001, respectively) (Table S1). A significant increase was also noted in the expression of the gene encoding VEGF in the rosacea group and in the PPR subtype compared with the control group (P < 0.0001 and P = 0.00012, respectively) (Table S1).

Discussion

In this work, we show that clinical quantification of *D. folliculorum* with PCR is feasible and revealed a nearly 6-fold increase in *D. folliculorum* density in the rosacea population compared with age-matched controls. There appears to be a trend to a higher *D. folliculorum* density in ETR versus PPR. In terms of inflammation markers, a general overexpression of the genes may be noted in the rosacea population compared with the age-matched group, linked to overexpression of LL-37 and VEGF, as well as CD45RO, MPO and CD163, indicating broad immune system activation in the skin of rosacea patients.

To our knowledge, this is the largest clinical study to have analysed *Demodex folliculorum* and biological markers involved in rosacea. In the case of *Demodex folliculorum,* many authors had found increased numbers in rosacea patients versus a control population using skin surface biopsies (2,7), and an association between *D. folliculorum* and rosacea now seems clearly demonstrated (3). However, the traditional surface biopsy technique for collecting *D. folliculorum* has been subject to criticism (12,13) and has only very recently been standardised (11). Very recently Sattler et al. (15) have demonstrated the interest of *in vivo* confocal microscopy to detect and quantify Demodex in patients with rosacea and Ravera et al. (16) showed the possibility of *D. canis* detection by qPCR from dog hair follicles and skin biopsies. Based on this last approach, we have developed a new assay allowing us to estimate *Demodex folliculorum* density by qPCR from non-invasive skin samples. Analysis of our results on *D. folliculorum* obtained with this new methodology confirms those of previous studies where *D. folliculorum* had been quantified on traditional skin surface biopsies, that is, a significantly increased concentration in rosacea patients compared with control subjects, irrespective of rosacea subtype.

Moreover, to obtain data on the cascade of events occurring during rosacea, we have used assay methods that enabled us to collect mRNA of key genes from an epidermal extract obtained after micro-abrasion (14) and proteins on the skin surface (as IL-8) by swabbing (17).

We showed that the expression of a number of genes conventionally associated with vascular hyperactivity and inflammation is modified in rosacea. Indeed, an increase was observed in the transcripts of pro-inflammatory mediators encoding for TNF-α and IL-1β, and also for other mediators such as Il-8, Cox-2 and VEGF, irrespective of rosacea subtypes. In addition, we found an increased expression of genes encoding for LL-37 (cathelicidin), MPO (neutrophil marker), CD163 (macrophage marker) and CD45Ro.

These results highlight the role of cathelicidin, which has been shown to trigger cutaneous inflammation by increasing protease activity within the epidermis (8). Similarly, it is known that histological examination of rosacea lesions reveals a polymorphic inflammatory infiltrate composed of neutrophils and lymphocytes. In this last respect, we have demonstrated a higher expression of memory T lymphocyte markers (CD45RO) in rosacea patients.

In addition, our observations show that certain markers of inflammatory response, such as IL-8 production and those relating to IL-8 and COX-2 transcripts and the leucocyte markers CD163 and CD45RO, differentiate between papulopustular and telangiectatic rosacea. This persistence of IL-8 production with the exacerbation of this disease suggests a relationship between leucocytic infiltrates and the clinical manifestations of papulopustular rosacea, IL-8 being the main chemokine responsible for neutrophil recruitment.

Expression of genes associated with danger signals and potentially induced by mite colonisation, such as NLRP3, has been identified. NLRP3 belongs to the family of NOD-like receptors identified as intracellular receptors of danger signals capable of activating a protease, caspase-1. Caspase-1 induces maturation of IL-1β and IL-18 by proteolysis. The capacity of mite allergens to activate the NLRP3 inflammasome has recently been demonstrated in keratinocyte cultures (18). We have found increased Nalp3, pro-caspase-1 and pro-IL-1β expression in patients with rosacea. Our results suggest that NLRP3 plays a stimulatory role in IL-1β production, which in turn might be responsible for accelerating the inflammatory response by activating a large panel of inflammatory response genes. Reduced expression of pro-IL-18 and ASC adaptor was shown in the same subjects, allowing the recruitment of caspase-1. This work might substantiate the hypothesis whereby the reduction in IL-18 or ASC adaptor expression has consequences for the microbial composition of the pilosebaceous units. In fact, it has recently been shown that IL-18−/− and ASC−/− mice are immunodeficient and that this causes a major imbalance

of the intestinal microbiota (19). In addition, it is noted in this paper that IL-1β−/− mice exhibit no change in intestinal flora. For this reason, the microbial imbalance promotes infection, which can itself re-initiate the inflammatory response, thus instigating a vicious cycle mechanism.

In conclusion, our study shows that rosacea is associated with elevated *D. folliculorum* density and a generalised increase in inflammation markers. These results are consistent with mechanisms depicting neutrophilic activation induced by bacterial proteins associated with Demodex in rosacea patients (20). In addition, our results suggest that the NLRP3 inflammasome might be a new factor in the pathogenesis of rosacea by promoting excessive secretion of IL-1β. Our results also suggest that IL-18 underexpression, together with that of the ASC adaptor, might be responsible for a loss of microbial homoeostasis.

The genes identified in this way appear to be particularly interesting candidates for genes involved in the different phenotypes observed. Those genic results will need to be confirmed at the protein level, in particular for IL-1ß and IL-18, in a complementary clinical study. Lastly, our study provides the clinician with more detailed information about the existing links between the degree of *D. folliculorum* colonisation and the agents involved in the inflammatory cascade of rosacea.

Acknowledgements

CC, DR, CP, AD and AMS designed the study; MDs, ML and CL included volunteers; MDs, CSM and ED included volunteers and performed sampling; OL and SAG performed the experiments; HG developed Demodex quantification by qPCR; CC, DR and VM analysed the data, and CC, DR and CP reviewed the literature and wrote the manuscript. All authors revised the paper critically and approved the submitted and final versions. We are indebted to Pascale Lachaize, research nurse, Department of Dermatology, Toulouse for sampling volunteers, and we thank Marielle Romet who provided editorial assistance on behalf of Pierre Fabre Group.

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Conflicts of interests

The authors declare that there are no conflicts of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Gene expression profiles.