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Exploratory Research for Pathogenesis of Papulopustular Rosacea and Skin Barrier Research in Besancon and Shanghai

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CONTENT OF THESIS

I –Introduction

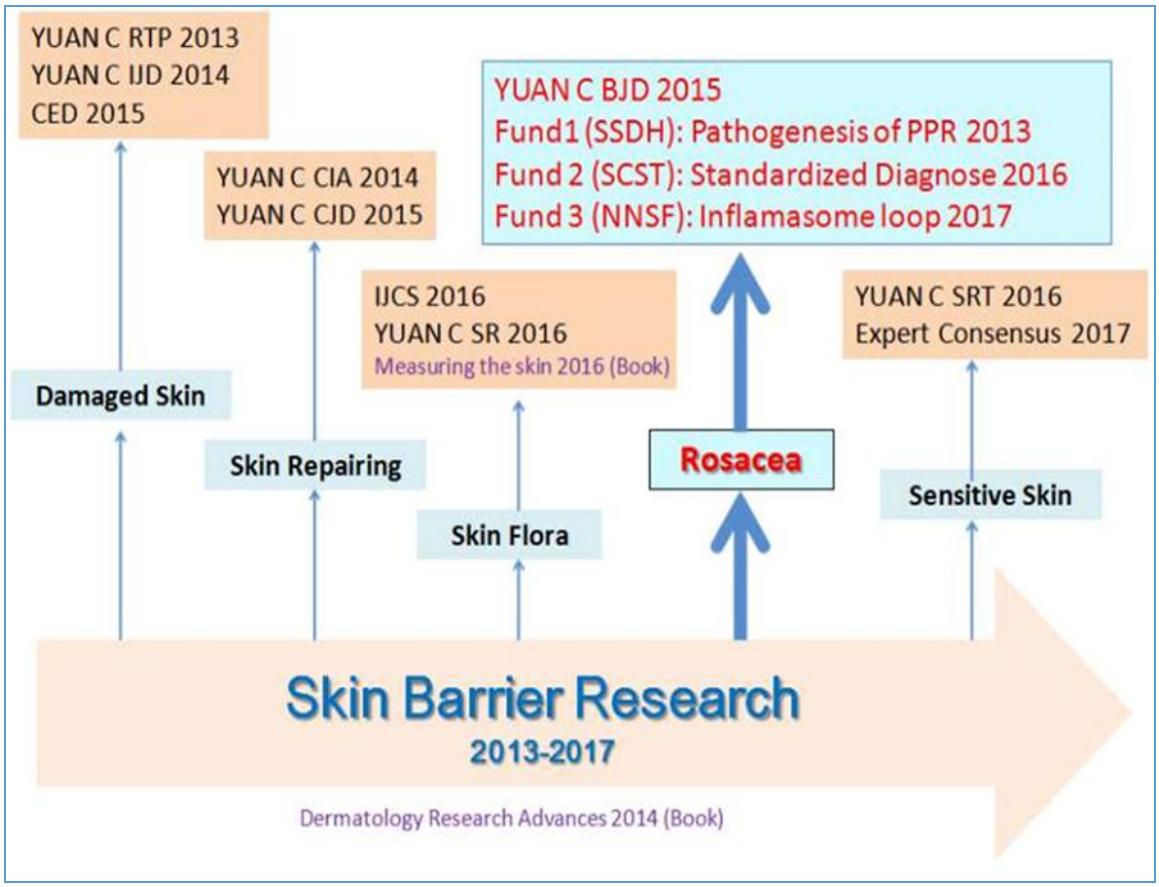
In 1975, Michaels et al. suggested a schematic model to explain the permeability of the stratum corneum (SC), which was called “brick & mortar” (1). The corneocytes are the bricks and the lipids are the mortar. Today this is viewed as the most appropriate model for the understanding of skin permeability (2).

In the early point of view, skin barrier consisted of two important structures: one is the SC (corneocytes), which functions as a physical barrier against both percutaneous penetrations of harmful substances and excessive trans-epidermal water and salt loss (3); the other is the corneocytes-bound intercellular hydrophobic matrix, mainly composed of ceramides, fatty acids, and cholesterol, which functions as a chemical barrier against pathogens (4).

In the past several years, advances in human measurement capabilities (5), in particular non-invasive methodologies, have not only reinforced the fact that the stratum corneum is incredibly responsive and adaptive, but also continue to provide new insights and opportunities for all the dermatologists (6).

From 2013 to 2017, I devoted myself to the research of skin barrier, and paid more attention to the pathogenesis of rosacea (See fig.1). From 2013 to 2017, I published 9 SCI articles (among them, I am the first author or corresponding author for 6 articles), 7 articles in Chinese Core Journals, and 3 funded projects were awarded (among them, one is from the National Science Foundation of China).

Fig.1. the research lines and achievements from 2013 to 2017

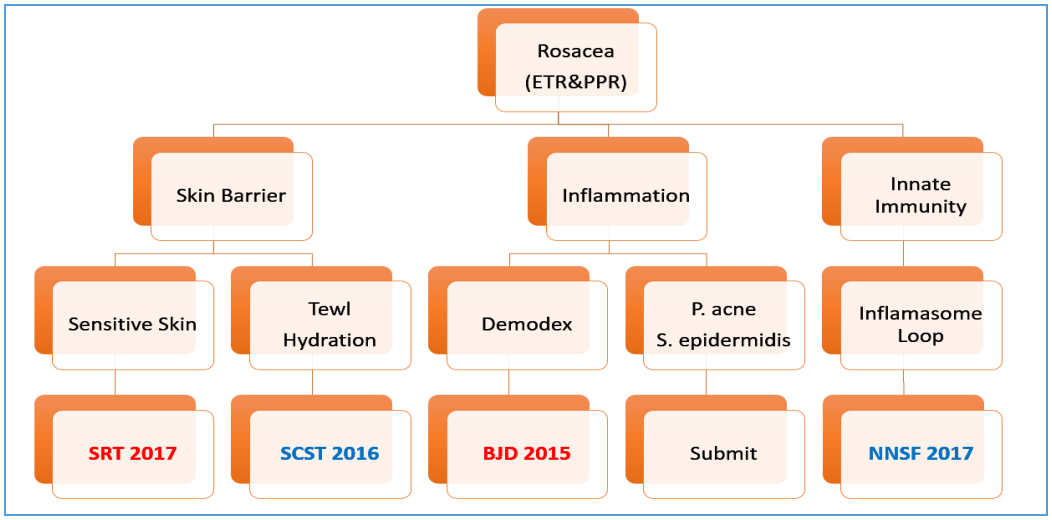


The SC is under a constant barrage of environmental insults such as temperature, humidity, pathogens, pollution, and solar UV. First, many researches were focused on the different damaged skin from the environment. For example, we did the Human Repeated Insult Patch Test (HRIPT) testing in Shanghai and Mumbai and we found that meteorology and ethnicity are critical factors in skin reaction (published in RTP 2013)(7). I was also interested in UV-damaged skin, and we explored differences in phototest and photopatch test results, and in skin color‐related parameters between healthy subjects and patients with chronic actinic dermatitis (CAD). Skin photobiological testing plays a critical role in the diagnosis of CAD (published in CJD 2016) (8).

How to repair the damaged skin barrier? After these first tests, I began to investigate more precisely on different ways to repairing the skin barrier. Moisturization is not the most dramatic or exciting effect of skin care products. Tranexamic acid (TA) is a traditional plasmin inhibitor, and its role in the renovation of damaged skin has become the topic of a lot of research. Two kinds of damaged skin models were set up and TA was then used to repair the skin. TA can accelerate skin barrier recovery and upregulate occludin induced by physicochemical damages of human skin (published in IJD 2014)(9). N-palmitoylethanolamine (PEA) and N-acetylethanolamine (AEA) are both endogenous lipids used as novel therapeutic tools in the treatment of many skin diseases. A monocentric, randomized, double-blind, comparative trial was conducted in 60 Asteatotic eczema (AE) patients to evaluate and compare the efficacy of the two emollients. Compared with traditional emollients, regular application of a topical PEA/AEA emollient could improve both passive and active skin functions simultaneously (published in CIA 2014) (10).

After an improved understanding of the physiology of the skin barrier as well as enhanced methodologies to measure skin barrier quality, I set out to choose rosacea as the target skin disease to do more pervasive research from 2013 in Besancon (See fig.2).

Fig.2. the Exploratory Research for Pathogenesis of Rosacea



The exact pathogenesis of rosacea remains unclear. But microorganisms, such as Demodex folliculorum, Staphylococcus epidermidis, and others may also contribute to the pathogenesis of rosacea by stimulating the innate immune system. It remains controversial whether this dysbiosis triggers rosacea, or whether the dysbiosis is a response to changes in the skin microenvironment resulting from rosacea's underlying pathophysiology.

Firstly, I practiced using the Standardized Skin Surface Biopsy (SSSB) in patient's lesions and forehead to check the number of demodex in the skin. During the application of SSSB, I found an interesting patient who had a lot of demodex in her face even though it was not rosacea. She was diagnosed with pityriasis folliculorum ultimately. There was a significant difference between Reflectance Confocal Microscopy (RCM) and SSSB in the assessment of D numbers per cm^2 (published in BJD 2015) (11).

Secondly, I practiced using RCM to find characteristic phenomenon in

rosacea. As we all know, rosacea is a classic skin disease in patients with sensitive skin. But the diagnosis for sensitive skin relies on subjective assessment or on the combination of subjective and objective evaluation. Through our study, we found that epidermal honeycomb structure' and 'spongiform edema' may be used as new skin signs of RCM evaluation of sensitive skin effectively (published in SRT 2017)(12). We used the skin signs of RCM evaluation of sensitive skin in the rosacea patient's grading and it was easy to find the difference with normal skins (ready to submit the article).

Thirdly, we studied the microorganisms present on lesions and control areas in Papulopustular Rosacea in Besancon. Our results indicate that the physiology features of rosacea are closely associated with the interactions between the host and the microorganisms. First of all, skin barrier rebuilding is one of the key points for the treatment of rosacea. Secondly, the results also suggest that adjusting the balance of the bacteria on the skin, particularly by enhancing Propionibacterium acnes (P. acne) and suppressing Staphylococcus epidermidis (S. epidermidis), might be a potential solution to lessen rosacea symptoms (Submitted to Medicine in May, 2017).

In the future, we will do more research on the changed innate immunity in rosacea patients. As we all know, rosacea is a kind of refractory skin disease which has serious influence in people's appearance and health. It is of paramount important to find that innate immune response is over-activated in rosacea.

Recent research reveals that the excreted ASC-Speck could be internalized by bystander cells (13), which leads to NLRP3 activation and the auto-amplification mechanism of inflammasome (14). In our previous clinical study, it was found that the severity of inflammatory papulopustular rosacea was negatively related with the numbers of demodex folliculorum, while positvely related with ASC-Speck status. It was also demonstrated that in vitro experiments, the macrophage drived ASC-Speck and then internalized by fibroblast 3T3 cells, which directly activates inflammasome and IL-1β production (15). Based on these findings, our research team proposes the hypothesis that there is an inflammation amplification system which transduces epidermal microorganism infection into dermal inflammation. In other words, demodex folliculorum induces first an inflammation response in epidermis, which could be transduced into dermis by internalization of ASC-Speck. In consequence, our research team are planning to investigate the NLRP3-medicated inflammasome and its amplification system for rosacea's development.

The research process includes four levels: clinical assessments, mice rosacea model testing, cell function in vitro and signal transduction assay. Our research will provide theoretical basis for rosacea treatment and open new ways for rosacea's medicine design.

The idea behind this thesis and the aims of this manuscript are therefore:

- **To consider rosacea as the research emphasis, through the point of inflammation to find the probable pathogenesis of rosacea. To learn more about the connection between the rosacea and the skin barrier function.**
- **To evaluate the different damage types of skin barrier and set up the repairing model for the skin. To try to use these models in clinical application.**
- **Through the research of skin microbial distribution and skin biophysical parameters in Chinese females, we concluded that maintaining healthy skin requires selective microbial shifts or permeability barrier changes, inhibiting the growth of pathogenic bacteria and promoting the growth of symbiotic bacteria.**

II –Pathogenesis Study of Papulopustular Rosacea

A- Background of Rosacea Pathogenesis

Rosacea is a common condition that predominantly affects the central regions of the face characterized by transient or persistent facial erythema, vascular abnormalities, and often papules or pustules (16). Rosacea affects up to 5%-20% of the world's population and a number of subtypes are recognized (17). It is a high morbidity disease in France and has been increasing in China lately (18). It affects people of all ages but is most common in middle-aged and older adults, with women being more often affected (19). Because the facial skin is the predominantly involved site, many patients sense that rosacea alters their social and professional interactions, leading to problems in their job, in their marriage, or in meeting new people (20).

Until now, our understanding of the pathophysiology of rosacea has not progressed substantially (21). Generally speaking, in many previous studies, researchers showed that the pathogenesis of rosacea involves skin barrier defects and dysregulation of innate immunity (22). Microorganisms, such as Demodex folliculorum, S. epidermidis, P. acne and others may also contribute to the pathogenesis of rosacea by stimulating the innate immune system (23). In this study, we pay close attention to the impact from microorganisms in the pathophysiology for rosacea. By using skin biometrology measuring methods, we evaluated the rosacea patient’s skin barrier functions.

In Europe, a lot of studies all showed that Demodex folliculorum covers a wide range of clinical features (24): pityriasis folliculorum (PRF), papulopustular rosacea (PPR), granulomatous rosacea and so on (25). Demodex is a 0.3-mm long transparent mite that parasitizes the normal skin with a prevalence of 100% and a density$\leqslant$5D/cm^2 in adult population (26). A definite diagnosis of demodicosis requires both a compatible clinical picture and the presence of high density of mites (27). The threshold usually used in the literature is >5D/cm^2 in lesional skin (28), measured using standardized skin surface biopsy (SSSB). In addition, many articles in literature show that good effects are obtained with acaricidal treatments for PPR patients. (29). On the other hand, there are few reports about Demodex in China. We have seldom used the SSSB to test the Dd. In some reports, Chinese investigators tested the Demodex by direct microscopic examination of fresh secretions from sebaceous glands. In this study, the major objective is to study the Demodex in PPR by the SSSB from two different countries.

A. 1 History of Rosacea

The earliest reference about “rosacea” found in Pubmed Web was reported by Robinson T in the British Medical Journal in 1885 (30). He said this pre-eminent supply of arterial blood manifests itself in the red cheeks of the white race. And this disease was always heralded by the flushing of the regions attacked, which is much increased after food, or by an injudicious diet.

More and more doctors and researchers pay close attention to rosacea. They found that rosacea affects all ages and has different subtypes (19). It primarily affects people of northwestern European descent and has been nicknamed the "curse of the Celts" by some in Britain and Ireland (31), although such a connection has been questioned. Rosacea is almost three times more common in women. It is commonly found in people between the ages of 30 and 50, and is more common in Caucasians (32).

A. 2 Rosacea definition and subtypes

Rosacea is a chronic skin disease characterized by transient or persistent central facial erythema, inflammatory papules and pustules, and often telangiectasia. Its main features include burning or stinging sensations, facial dryness, and edema. In 2002, the National Rosacea Society Expert committee developed a classification system for rosacea to help standardize its diagnosis. The committee divided rosacea's diagnostic criteria into primary and secondary characteristics, with the presence of ≥1 primary feature being indicative of the diagnosis (31).

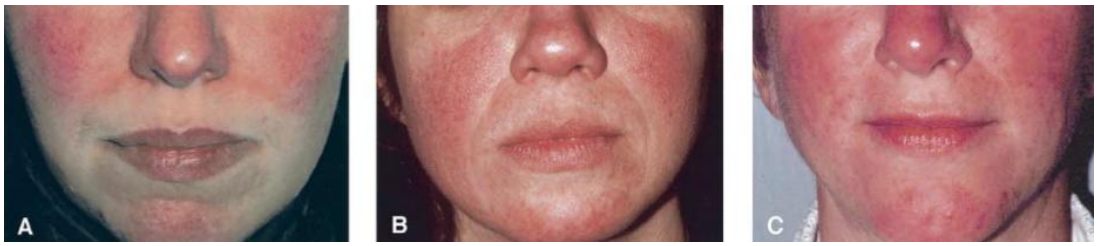
The committee then described 4 rosacea subtypes. Many of the histopathologic findings in the different rosacea subtypes are nonspecific. It is not necessary to obtain a skin biopsy specimen in order to reach a diagnosis of rosacea. These diagnostic criteria are now used by all Chinese and French dermatologists.

Subtype 1- Erythematotelangiectatic rosacea (ETR) (16)

ETR is characterized by nontransient episodes of flushing and persistent central facial erythema. Telangiectases are also common in ETR, but they are not required for diagnosis even if they are always together with flushing and erythema.

In our viewpoint, ERT is the common subtype of rosacea in Shanghai. Many Chinese dermatologists are always puzzled to identify the diagnosis of ETR and other facial allergic dermatitis.

Fig.3. Three different Degrees of ERT

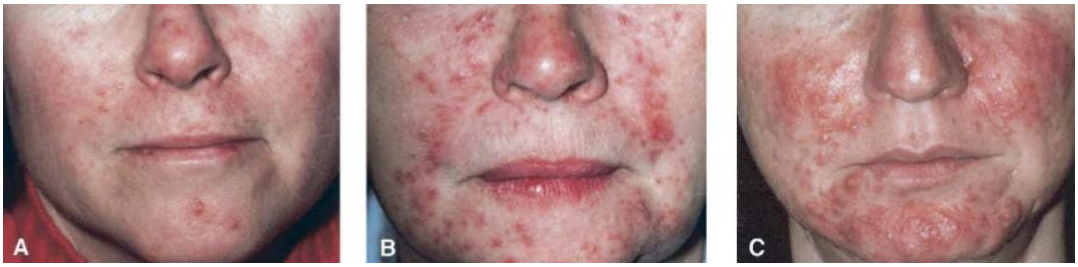


Note: A mild, B moderate and C severe.

Subtype 2- Papulopustular rosacea (PPR) (16)

Rosacea patients with PPR experience transient papules or pustules in a central facial distribution. PPR is the severe inflammation subtype among the four and we particularly focus on its pathogenesis.

Fig.4. Three different Degrees of PPR

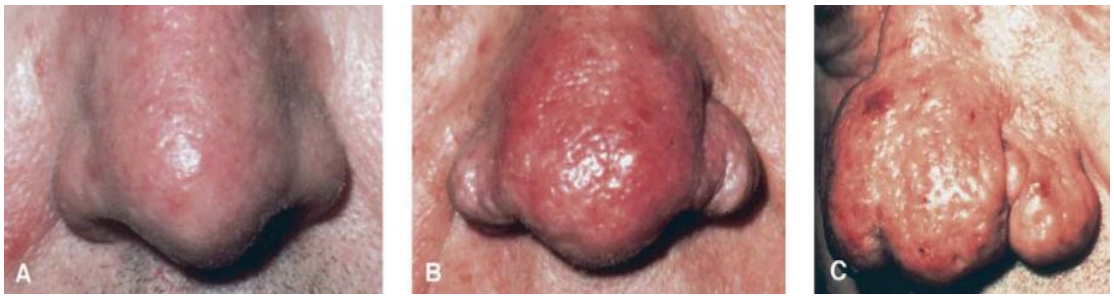


Note: A mild, B moderate and C severe.

Subtype 3- Phymatous rosacea (PR) (16)

Phymatous rosacea is characterized by thickened, enlarged skin with irregular surface nodularities. Males are more commonly affected by this subtype than females. In China, the oldest diagnosis for rosacea is concentrated on this subtype.

Fig.5. Three different Degrees of PR

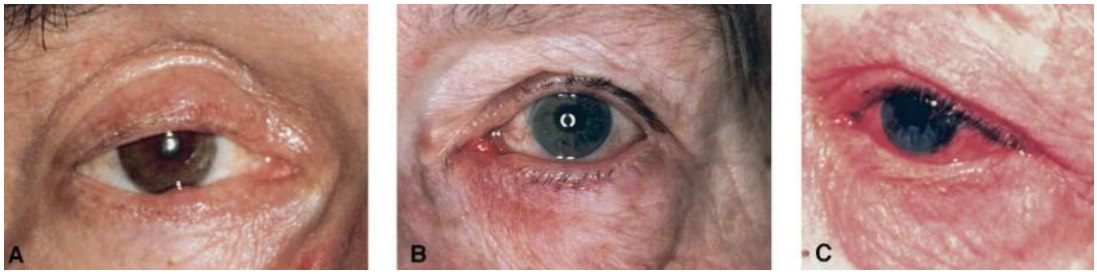


Note: A mild, B moderate and C severe.

Subtype 4- Ocular rosacea (OR) (33)

Ocular rosacea was defined by the National Rosacea Expert Committee as having ≥1 of the following signs or symptoms: watery or bloodshot appearance, foreign body sensation, burning or stinging, dryness, itching, light sensitivity, blurred vision, telangiectases of the conjunctiva and lid margin, or lid and periocular erythema. But this subtype has the lowest incidence rates among the four types, especially in Shanghai.

Fig.6. Three different Degrees of OR

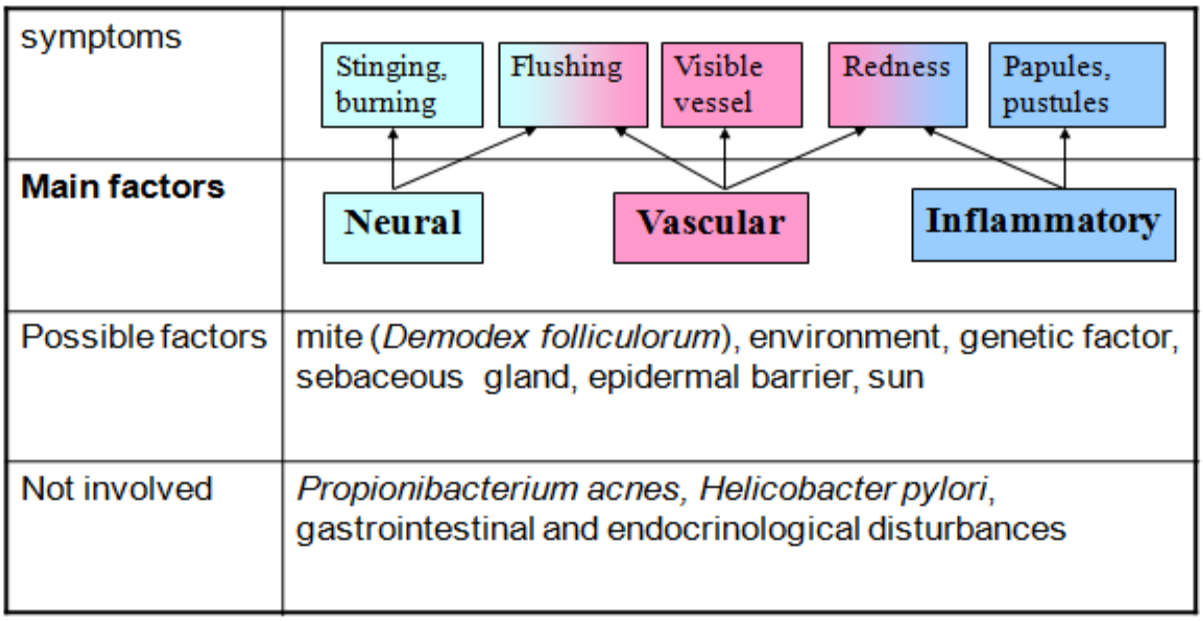


Note: A mild, B moderate and C severe.

B- Pathogenesis of Rosacea from retrospect references

The etiology of rosacea remains unknown. Both genetic and environmental factors are thought to have an impact on the pathogenesis of rosacea (34). Genetic, environmental, vascular, inflammatory factors and microorganisms such as Demodex folliculorum and Helicobacter pylori have been considered (35). And each symptom of rosacea would find the possible change in pathological manifestation (fig.7).

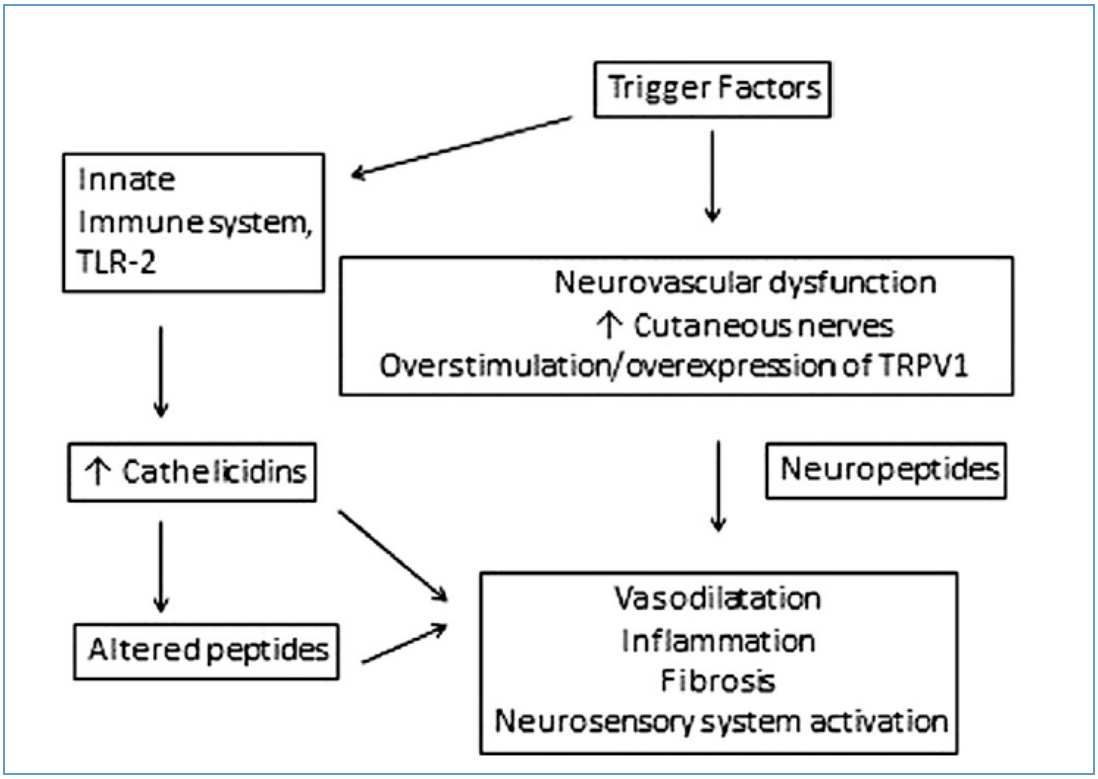
Fig.7. Rosacea's symptoms and possible factors



More and more researches showed us the schematic of proposed interactions among clinical, immunologic, neurovascular, and molecular characteristics of rosacea (36). Recent molecular studies suggest that an

altered innate immune response is involved in the pathogenesis of the vascular and inflammatory disease seen in patients with rosacea. The innate immune systems would be enhanced by cytokine, ROS, antimicrobial peptide, and proteases, which lead histological changes observed in rosacea. The pathophysiology of rosacea appears to be complex, as virtually all cutaneous cells, including immune cells, appear to have roles. Ultimately, rosacea is a good ‘‘model’’ through which investigators can learn more about the complexities of neuroimmune communication, inflammation and immunity, as well as about chronic inflammation and the development of fibrosis (Fig.8).

Fig.8. Clinical, immunologic, neurovascular, and molecular characteristics of rosacea



Note: TLR, Toll-like receptor; TRPV1, transient receptor potential channel vanilloid receptor 1.

B.1 Controversy of Rosacea Pathogenesis

The cause of rosacea remains unknown and the clinical course likely varies from patient to patient. It is becoming increasingly clear that the pathophysiology of rosacea is quite complex, involving a large variety of cell types and molecules in the skin (37). Several factors have been implicated in its pathogenesis, some based on the evidence of scientific investigation, others on clinical observation. Whether the dense presence of sebaceous glands in this area (cheeks, nose, chin, and forehead) or a specific physiology of the nerve innervation and vascular composition is crucial for this aspect is still a matter of debate (34).

Based on rosacea's clinical features (flushing, chronic inflammation, and fibrosis) and trigger factors, a complex pathobiology involving different regulatory systems can be anticipated.

Triggers for flushing events and aggravation of rosacea include a wide variety of physical, chemical, psychological and emotional factors including temperature changes (heat, noxious cold), UV irradiation, ingredients of spicy food (e.g. mustard oil or capsaicin), certain alcoholic beverages, increased body temperature (exercising), certain cosmetic formulations or emotional stress.

Current concepts suggest that known clinical trigger factors of rosacea such as UV radiation, heat, cold, stress, spicy food, and microbes modulate Toll-like receptor signaling, induce reactive oxygen species, as well as enhance antimicrobial peptide and neuropeptide production (34). (Fig.9.

Fig.9 Trigger factors of rosacea

Histologically (38), rosacea findings include dilated blood and lymphatic vessels leading to erythema and edema; a perivascular infiltrate consisting of increased T cells, macrophages, and mast cells; and often but not exclusively solar elastosis, edema, and tortuous, dilated vessels.
Many researchers agree with this points: during rosacea manifestation and early stage, the innate immune system and neurovascular dysregulation seem to be driving forces in rosacea pathophysiology (39). Dissection of major players for disease progression and in advanced stages is severely hampered by the complex activation of the innate and adaptive immune systems, enhanced neuroimmune communication, profound blood vessel and possibly lymphatic vessel changes, and activation of almost every resident cell in the skin.

B.2 Augmented immune response

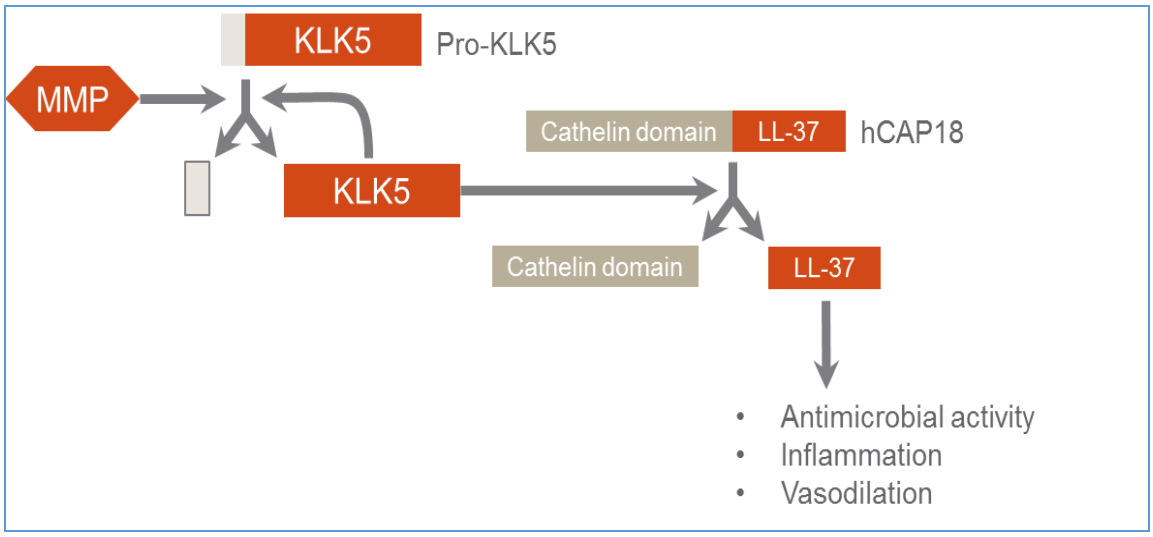
The innate immune system plays a pivotal role because keratinocytes are

equipped with sensors and are capable of communication with each other upon microbial challenge or encountering danger signals (40). Innate immune mechanisms and dysregulation of the neurovascular system are involved in rosacea initiation and perpetuation, although the complex network of primary induction and secondary reaction of neuroimmune communication is still unclear.

The most promising preliminary evidence for a pathophysiological impact for the induction of erythema and vasodilation demonstrates an involvement of increased baseline expression of cathelicidin and kallikrein 5 (41), the predominant serine protease responsible for cleaving cathelicidin into its active form (42).

Upregulation of both adaptive and innate immune response genes has been demonstrated in patients with ETR, PPR and PhR; thus, a contribution of the adaptive immune system is likely, although it is still poorly understood (19). Increased stratum corneum permeability is probably structurally and functionally linked to an aggravated innate immune response in patients with rosacea, resulting in increased trans-epidermal water loss and an increase in mRNA expression levels, as well as secretion of cathelicidin peptides (LL-37)(43). Increased levels of Toll-like receptor 2, which activates kallikrein 5, are seen in patients with rosacea (44) (Fig.10).

Fig.10. aggravated innate immune response in rosacea patients



B.3 Microorganisms and demodex

The active contribution of a microbial agent in the development or progression of rosacea continues to be debated (35). To better understand the microbiology of rosacea, we must evaluate not only the microorganisms themselves but also the microenvironments and macroenvironments in which they reside.

Several microorganisms have been shown to be increased or immunoreactive in patients with rosacea (35). Six microorganisms are discussed for the mechanism of rosacea: Demodex folliculorum (45)，Bacillus oleronius (46)，Helicobacter pylori (47)，Staphylococcus epidermidis (48)，Propionibacterium acnes (49) and Chlamydophila pneumoniae．More and more researches give a new view through skin microbial barrier to elaborate the possible pathogenesis of rosacea (50)． Although

Microorganisms may not be central causative factors in rosacea they are likely to be altered in multiple subtypes of rosacea and may act as trigger factors or potentiators of inflammation in an undefined subset of predisposed patients.

In our clinical study, we focus on the microorganism conditions of PPR patients and find the relationship between skin flora and physical skin parameters by using non-invasive testing technology.

B.4 Abnormalities in Cutaneous Vascular and Neurogenic dysregulation

Transient or persistent diffuse vascular erythema is the most common symptom of rosacea and has been a considerable unmet need in many patients with rosacea. Inherent abnormalities in cutaneous vascular homeostasis might be the most-cited pathogenic theories about rosacea (51). That flushing in ETR and PPR is visibly concentrated on the face can be explained by the fact that baseline blood flow is increased in the face and that facial vessels are larger, more numerous, and nearer to the surface than in other areas of the body (52). Consequently, both neural mechanisms and circulating humoral agents produce flushing reactions that may be visibly limited to the face.

The sensory axon reflex of primary afferents in the dermis and epidermis releases vasoactive neuropeptides such as pituitary adenylate cyclase-activating polypeptide or vasoactive intestinal peptide (VIP) into the microenvironment (25). Binding of neuromediators to high affinity neuropeptide receptors on arterioles or venules leads to vasodilatation (flushing, erythema) or plasma extravasation (edema) (53). Activation of T cells, macrophages, and mast cells by neuropeptides results in activation or aggravation of inflammatory responses. It is unknown to what extent neuromediators may also exert anti-inflammatory capacities in human skin diseases. Bi-directional communication between the innate immune and nervous systems may aggravate early rosacea leading to chronic disease.

Four vanilloid receptors and one ankyrin receptor within the transient receptor potential family of cation channels have been shown to be active in rosacea. TRPV1 is expressed by sensory nerves and other nonneural cells, such as keratinocytes, where it is activated by capsaicin, heat, and inflammatory states, and ultimately plays a role in vasoregulation and nociception (54). Recent evidence has also shown that TRPA1 may function as an oxidant sensor for vasodilator responses in vivo, providing yet another mechanism through which ROS may contribute to the pathogenesis of rosacea (55). TRPV2, -3, and-4 have also been identified on both neuronal and nonneuronal cells, such as keratinocytes, endothelial cells, and immune cells.

C – Non-invasive testing in Rosacea

C.1 Standardized Skin Surface Biopsy use in Acne

(Accepted by the Journal of Dermato-Endocrinology in June 26, 2017)

Until now, the etiology of acne vulgaris has been uncertain (56). Demodex mites are normal colonizers of the skin around the sebaceous glands, and these mites have been associated with several other skin conditions, including Papulopustular Rosacea (57) and Pityriasis Folliculorum (11). Whether Demodex infestation plays a part in acne pathogenesis is therefore quite a relevant question. Although clinicians usually deny the association between Demodex infestation and acne vulgaris, Zhao et al confirmed the association between them through a meta-analysis from 63 publications (58). However, to obtain more convincing data, it was necessary to conduct a clinical trial with a large sample size to confirm the relationship between Demodex infestation and acne vulgaris. In this study, Standardized Skin Surface Biopsy (SSSB) was used to measure Demodex Folliculorum density (Dd)(28).

This monocentric, prospective, double-blind study enrolled 132 participants with mild to moderate vulgaris acne on their faces. Individuals with nodules or cysts in the face or active psoriasis or a history of psoriasis, active allergic skin responses, or severe eczema were excluded. In addition, subjects that had been under treatment for any type of cancer within the last 6 months, or

subjects that had used anti-inflammatory drugs, anti-acne drugs, immunosuppressive drugs, or antihistamine medications were also excluded. There were 92 females and 40 males, and the average age was 23.4 years with a standard deviation of 3.3 years. Each subject had signed a copy of the informed consent before joining the study. The study was conducted strictly in accordance with the instructions governed by the Ethic Committee of Shanghai Skin Disease Hospital (No. 2014-005).

For the test, we used a cleanser without any pesticide or antimicrobial agent, which was widely available on the market (POND's intensive moisture cleanser). Each subject was exposed to the cleanser twice a day only for 7 days, once in the morning, once in the evening. The first application of the products was done at the testing facility under the supervision of the testing facility staff. Subjects subsequently self-administered the cleanser at home according to instructions and kept a diary for the product use.

Two well-trained dermatologists counted the number of lesions according to their types, including non-inflamed lesions (black comedones and white comedones) and inflamed lesions (papules and pustules), at baseline and 7 days later. All data were recorded on individual subject data forms.

Firstly, the patient's cheek was cleaned with water before the test. Then cyanoacrylate glue (3S BIOKIT) was dropped on a marked 1 cm2 area on a glass slide. The adhesive bearing surface of the glass slide was applied for 1 min on the cheek. The specimen was examined by light microscopy at ×40 and ×100 magnifications. After one week, the same SSSB procedure was repeated on the other cheek.

The SPSS13.0 software (Inc., Chicago, IL, USA) was used in the calculation of statistical data, which are expressed as Median (Q1, Q3), Mean±SD and assessed for statistical significance. We used Wilcoxon Signed Ranks Test to compare the clinical scoring and Dd values at different times. McNemar Test was used to compare the Positive Ratio (PR), Relevance Ratio (ReR) and Zero Ratio (ZR) for the two different times. We used Pearson's correlation coefficient and conducted a linear regression analysis to determine correlations between Dd and acne lesions. A value of $P < 0.05$ was considered as being statistically significant.

Clinical Scoring of different types of acne

The whole face of the acne patients was evaluated twice: before using the cleanser and after using it for 7 days. Two experienced Chinese dermatologists were in charge of the evaluation and counted the different types of acne lesions. Black heads, white heads, papules and pustules were counted respectively. There was no significant difference between the two times for each type of acne ($P > 0.05$) (Table 1).

Table.1. Number of lesions for each type of acne at both assessment times (Median (Q1, Q3))

	Baseline	After 7 days
Black Heads	2 (0, 6.75)	2 (0, 5.75)
White Heads	4 (2, 8)	4 (2, 7)
Papules	14 (11, 20)	16.5 (11, 22)
Pustules	1 (0, 3)	1 (0, 2)
Number of Inflammatory Lesions[1]	16 (12, 22.75)	18 (11.25, 24)
Number of Non-Inflammatory Lesions[2]	7 (3, 13)	6 (3, 12.75)
Total Number of lesions[3]	24.5 (17, 34.5)	26 (16.25, 34)

Notes: 1: Inflammatory lesions = papules and pustules; 2: Non-inflammatory lesions= black heads and white heads; 3: Total number = adding numbers of lesions for all types of acne.

Dd in SSSB

Through SSSB, we assessed the Dd in each patient's cheek at both times, and we also calculated the Average number of DF, Positive Ratio (Dd$\geqslant$ 5DF/cm^2 was regarded as a positive case),

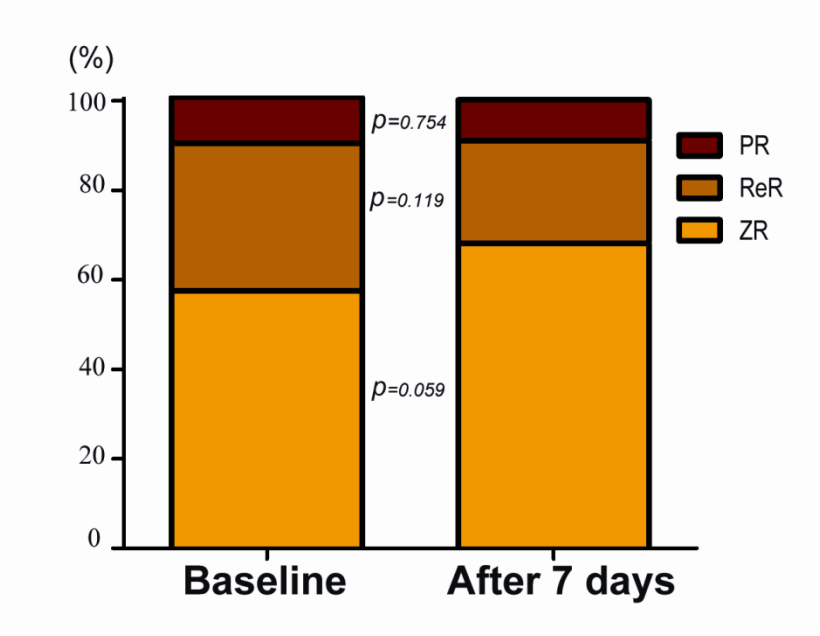
Relevance Ratio (Dd$\geqslant$1DF/cm^2 was regarded as a relevance case) and Zero Ratio (Dd=0 DF/cm^2). After using the cleanser for 7 days, the number of DF was significantly decreased compared with baseline ($P<0.05$). There was no statistical change for the Positive Ratio, but the Zero Ratio (ZR) was significantly increased after using the cleanser for 7 days only ($P<0.10$). (Please see Table 2 and Figure 11).

Table.2. Changes in Dd after using cleanser

	Baseline	After 7 days	P value
Average number of DF(Mean±SD)	1.77±3.60*	1.50±4.13*	**0.022**
Dd≥5DF/cm^2 (n)	14	12	
PR (%)	10.6	9.1	0.754
Dd≥1DF/cm^2	56	42	
ReR (%)	42.4	31.8	0.119
Dd=0 DF/cm^2(n)	76	90	
ZR (%)	57.6	68.2	**0.059**

Note: There was a statistical decrease in the Average number of DF after using the cleanser for 7 days only (P<0.05). PR : Positive Ratio; ReR : Relevance Ratio; ZR : Zero Ratio.

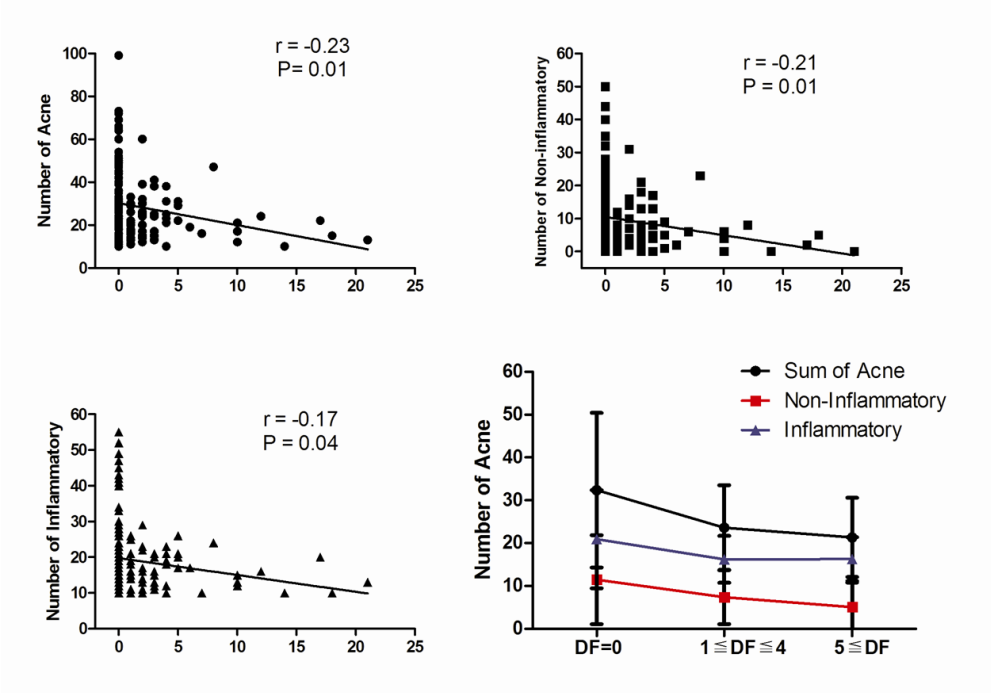
Fig.11. Three different rates for after using 7 days



Relationship between the number of acne lesions and Dd at Baseline

Pearson's correlation coefficient showed that there was no relationship between Dd and the total number of acne lesions (r=-0.23, P<0.05) or inflammatory lesions (r=-0.17, P<0.05) or non-inflammatory lesions (r=-0.21, P<0.05). Please see figure 12.

Fig.12. Relationship between the number of acne lesions and Dd at Baseline



The pathophysiology of acne is complex and multifactorial, and its etiology is considered as related to factors such as heredity, androgen, increase of sebum secretion, dyskeratosis of pilosebaceous duct, follicular orifice block up and proliferation of Propionibacterium. Recently further research has focused more on Demodex infestation and the development of acne. Zhao performed a meta-analysis of 63 studies, among them 43 found an association and 20 found no association between Demodex infestation and the development of acne. The Odd Ratio (OR) of an association between Demodex infestation and the development of acne was significant at P=2.80 (95% CI 2.34-3.36). Four different methods were described in the 63 studies: Cellophane Tape Method (CTP) over a short period of time, CTP overnight, extruded sebum smear microscopy and scraping method. Among the assessment methods for Demodex Folliculorum, the most efficient is

Standardized Skin Surface Biopsy (SSSB), but no reference used SSSB in these 63 studies. SSSB is a non-invasive sampling method by which it is possible to collect the superficial part of the horny layer and the contents of the pilosebaceous follicle.

Demodex Folliculorum (DF) is a 0.3-mm long mite that parasitizes healthy skin with a density $\leqslant$5Dd/cm^2 in adult population (59). In many skin diseases, such as Pityriasis folliculorum, Papulopustular rosacea (PPR), DF multiples and penetrates the dermis. There is a significant difference in the density of DF(28) between healthy skin and skin affected by disease. In this study, we tested DF's presence and mean density in vulgaris acne by SSSB, which helps to compare the different inflammation levels in various skin conditions (Table 3). Compared with healthy skin, assessments using SSSB show that DF's Presence and Mean Density in vulgaris acne are between healthy skin and PPR. It is well-known that the etiologies of rosacea and acne are completely different (60), now these findings show that the DF infestation situation is different.

Table.3. DF results by SSSB in different skin conditions

	Presence (%)	Mean Density (DF/cm^2)
Healthy Skin	11.9	0.7
Rosacea	/	10.8
Papulopustular Rosacea	90.2	36
Pityriasis Folliculorum	/	61
Vulgaris Acne	**42.4**	**1.77**

In the study of risk factors of demodex infestation, questionnaires were given to healthy subjects and then a logistic regression analysis was performed. The results for the subjects using the facial cleanser had no statistical relationship regarding the Demodex infestation (P=0.170). Through our study, we found that using the cleanser could decrease the average number of DF in only days in acne patients. These two conclusions are not contradictory because two different test subjects could not compare directly. Many clinical studies have shown that the combined use of a facial skin cleanser and a moisturizer is safe and effective for the care of acne without skin irritation by intensive washing (61), and that an acne cleanser could reduce both inflammatory and non-inflammatory acne lesions (62). The use of a cleanser is recommended in the treatment of acne vulgaris. We also found that there was statistically no improvement in the Positive Ratio of DF within 7 days, only the Zero Ratio was increased (P=0.059). These phenomena suggest that the infestations of DF in acne patients were at different levels, and that mild infestation (DF<5D/cm^2) is easy to clear in just 7 days. But if the infestation is more than 5 D/cm^2, more time may be necessary to wipe out Demodex. For the future study, we need longer follow-up time to see the details.

Regarding the relationship between Demodex and acne lesions number, we reached the same conclusion as many clinical dermatologists (63). However, more clinical research is needed to determine whether the Demodex infestation situation could have an impact on the skin barrier function, and then have some impact on skin sensitivity in acne patients.

C. 2 SSSB and Confocal Laser Scanning Microscopy use in rosacea

The skin surface biopsy was standardized with a red waterproof marker to analyze a standardized surface of 1 cm^2 and to obtain the Dd (number of mites/cm^2).

In this study, the dermatologist initially cleaned the slide and the skin of the rosacea patient with water before performing the SSSB, to improve and to standardize the adherence. Only D folliculorum clearly identified on the basis of its own anatomic characteristics were taken into consideration. If Dd was <5 D/cm^2 at the first SSSB, a second SSSB was performed, at the same place, to avoid false-negative results.

The Vivascope 1500 (CLSM) gives researchers and users in the fields of medicine and cosmetics the opportunity to study the skin in real time (64). This non-invasive method is used to see through the epidermis and the dermis down to the reticular stratum in vivo (65). A near infrared laser beam (830 nm) is directed toward and reflected by the investigated skin areas. Melanin and keratin are like natural contrast agents because of their relatively high reflection index. The cell microstructures of the skin can be represented cell by cell in horizontal cross sections (thickness: 5 µm). Black and white images of each cutaneous layer are produced and visualized instantly

Confocal microscopy is a particularly well adapted tool in many medical and

cosmetic applications (lesions, burns, dermatitis, inflammation, hyper- and hypopigmentation, quantification of melanocytes, analysis of capillaries). In this year, there is an interesting controversy about using confocal laser scanning microscopy (CLSM) to test Dd between Professor Fonton and Professor Sattler (66). Professor Sattler suggested that CLSM offers a non-invasive and quick method (67). Professor Fonton considered that CLSM could not accurately detect absolute numbers of mites in human skin, but the SSSB is likely to have a higher yield than the images of follicular orifices using the CLSM (68). So it is necessary to confirm these two methods in a third group. In this study, we could do some comparing between these two methods.

In our testing, we compared the PPR patients in Besancon and Shanghai respectively. Healthy PPR patients were recruited at the Dermatology Clinic in the University Hospital of Besançon, France and at the Dermatolgy Clinic in Shanghai Skin Disease Hospital. All diagnoses were confirmed by at least two dermatologists and each patient had at least three lesions (papules or pustules) on the face. Subjects undergoing topical treatment within 4 weeks prior to the enrollment and subjects on an antibiotic treatment scheme were excluded from the study. Finally, 25 PPR patients in each clinic center were enrolled in this study, and included 2 men and 23 women, with ages of 28 to 60 years. The mean duration of rosacea was 11.8 ± 9 years in Besancon and 9.7±3.8 years in Shanghai, and none of the patients had taken medications during the past 3 months. Please see the subject’s details in Table 4.

Inclusion criteria

a) Females or males in good general health
b) Age: 18~65 years
c) The course of rosacea was no more than 18 years in PPR subtypes
d) At least four or five lesions of PPR
e) Individuals with Fitzpatricks skin type I, II and III only
f) Individuals free of any dermatological of systemic disorder which would interfere with the results, at the discretion of the investigator
g) Individuals willing to refrain from using any new cosmetic products when they attend this study
h) Able to follow all study requirements, undergo all the skin examination
i) Signed the informed consent.

Exclusion criteria

a) Pregnant or nursing female
b) Individuals with a history or current disease or condition of the skin that the study investigator deems inappropriate for participation (atopic skin disease, eczema, psoriasis etc.)
c) Have a history of a disease/condition or a concurrent illness that could interfere with the outcome of the study
d) Subjects with a history of any form of skin cancer, melanoma, lupus, psoriasis, connective tissue disease, diabetes or any disease that would increase the risks associated with study participation
e) Individuals currently taking medications, which, in the opinion of the Investigator, may interfere with the study (e.g., prescription steroids, prescription anti-inflammatory drugs, prescription vasoactive agents, topical drug, etc.) or increase the risk to the subject
f) Individuals receiving or have received any facial or body beauty treatments like laser treatments, Botox, hyaluronic acid injections (or

equivalent), thermage (or equivalent) within the last 6 months or having facial cosmetic surgery within the last 12 months

g) Individuals participating in any other clinical studies in recently 3 months.

Table.4. PPR patients in Besancon and Shanghai

	Female(N)	**Male(N)**	**Age(yr)**	**Course(yr)**
Besancon	23	2	45.1±10.1	11.8±9.0
Shanghai	22	3	46.1±10.5	9.7±3.8

Note: the course means the time from the first break out of rosacea.

This study was approved by the Ethics Review Committee of the University Hospital of Besançon. Written informed consent was obtained from all subjects participating in the study, following a detailed description of the purpose of the study. All experiments were performed in accordance with the Declaration of Helsinki.

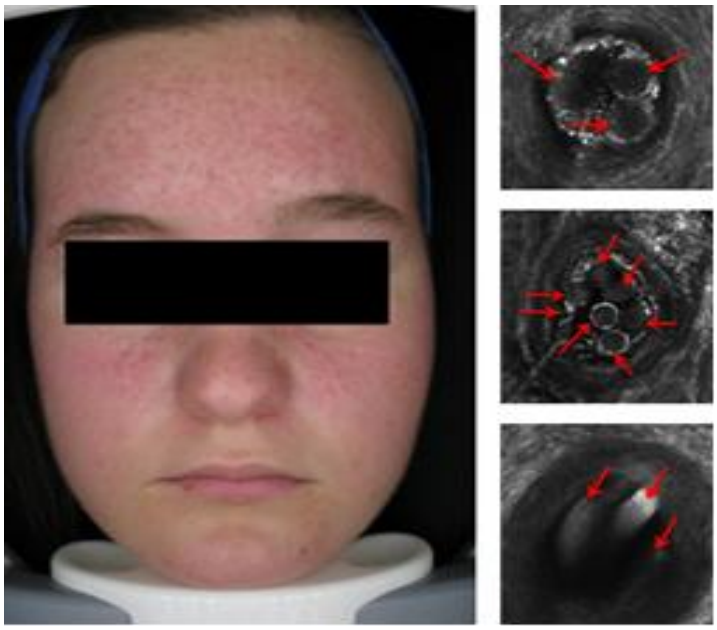
We compared the Domedex in PPR patients in these two cities. It was showed that Besancon patients were much easier to be tested the positive for Domedex than in Shanghai regardless for the SSSB or CLSM, and the demodex number was much higher than Shanghai PPR patients. Please see the Table 5 and Figure 13.

Table.5. difference of demodex testing between Besancon and Shanghai in SSSB and CLSM

	SSSB		CLSM	
	PR(%)	Domedex(N)	PR(%)	Domedex(N)
Besancon	96*	28.8±17.8*	100*	307.3±313.5*
Shanghai	52*	7.6±14.8*	68*	56.3±43.2*

*Note: PR means the Positive Rate. The * means that there was significant difference between these two cities (P<0.01).*

Fig.13. PPR patient's VISIA pictures and Domedex in CLSM



C. 3 CLSM and SSSB use in a pityriasis folliculorum patient

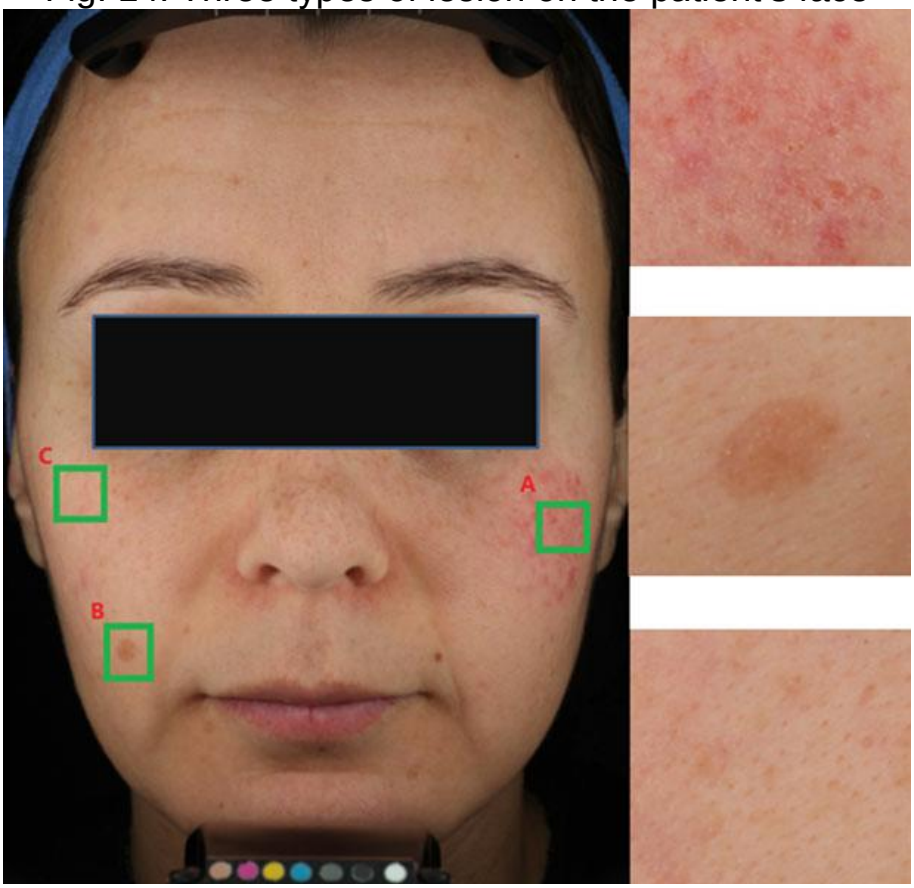
(Published in BJD 2015)

Pityriasis folliculorum (PF) is a human demodicosis caused by the proliferation of Demodex folliculorum (D); Chen and Plewig have proposed that it is one of the primary forms of the condition (69). PF consists of very small, discrete and regularly dispersed follicular scales, involving sebaceous hair follicles, often without visible inflammation (70). Patients may complain about pruritus and dry, sensitive, irregular or rough skin. Forton et al. report that PF is the most frequent demodicosis, is commoner than papulopustular rosacea and has a higher demodex density (D = 61 cm^{-2}, n = 45) tested by standardized skin surface biopsy (SSSB)(71). An alternative method of diagnosis, reflectance confocal microscopy (RCM) now shows promising results (72).

A 33-year-old woman from Algeria with recalcitrant rosacea-like lesions presented with a 2-year history of persistent facial stinging, mild pruritus, dryness and a sandpaper-like texture of her left cheek. Eight months before our assessments, a brown flat macula with follicular scales had appeared on her right cheek, and had grown and spread. Previous treatment with oral metronidazole had been stopped because of the adverse side-effect of a severe headache. Her left cheek had multiple papulopustular lesions and mild telangiectasia, covering an area of 25 * 23 mm (Lesion A). The brown flat macula without inflammation was located lateral to her right nasolabial fold and covered an area of 4* 5 mm (Lesion B). Fine desquamation and

follicle orifices were present on this lesion which was not sun-induced or sun-aggravated over the 8 months. Although the skin of her right cheek looked normal, there were also many small grey spike changes (Lesion C) (Fig. 14).

Fig. 14. Three types of lesion on the patient's face



Lesion a: rosacea-like lesions of left cheek. Lesion b: brown flat macula. Lesion c: numerous small grey spike changes, looking like normal skin.

Noninvasive examination of the three lesions was performed with RCM (VivaScope 1500, Lucid Inc., Rochester, NY, U.S.A.). Then SSSB was carried out on the same areas. This method consists of collecting 1 cm^2 of the superficial part of the horny layer and the follicle content. RCM over a 25 mm^2 area in lesions A, B and C showed 90, 297 and 226 D 25 mm^{-2}, respectively (equal to 360, 1188 and 904 D cm^{-2}). The highest percentage of follicle infestation (90.7%) was in lesion B and the lowest (49.5%) was in

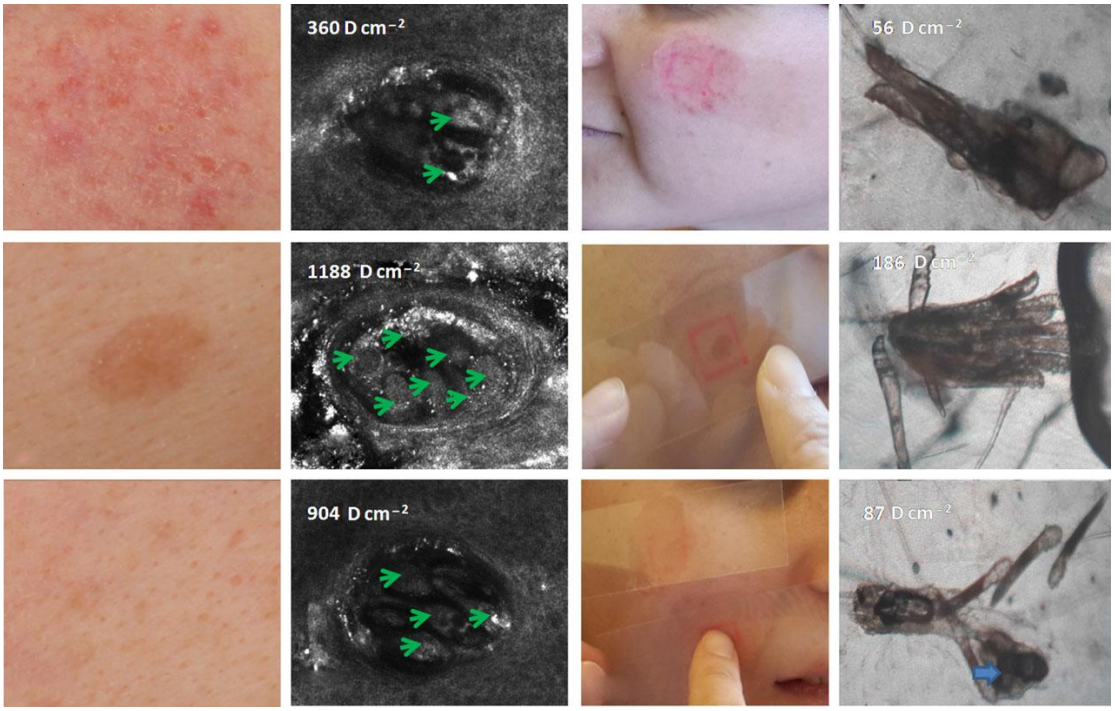
lesion A.

With RCM, we also observed that lesion B had more mites in infested follicles (3.4 D per infested follicles, 2.0 D in lesion A and 2.7 D in lesion C). For the individual lesions, the best image of D was taken at depths of 55.8, 28.8 and 16.0 lm respectively, corresponding to the epidermis (Fig. 11). With SSSB, we observed 56, 186 and 87 D cm^{-2} in lesions A, B and C, respectively.

According to SSSB, the presence of five or more mites per 1 cm^2 is the cut-off limit for the diagnosis of demodicosis.5 Our patient shows a 10–37 times higher demodex density than this limit; nevertheless, SSSB results were all lower than RCM results, suggesting that the cut-off level might be higher for RCM.6

The patient was given combination therapy with benzyl benzoate 12% lotion applied nightly for 4 weeks and a single dose of oral Ivermectin (12 mg). She experienced complete resolution of symptoms after 4 weeks.

Fig. 15. Dermoscopy, RCM, SSSB and normal microscopy images of the three lesions.



Top, lesion A: papulopustular with telangiectasia. (a-1) Dermoscopy; (a-2) two D appear as long cone-shaped bodies in a follicle under RCM; (a-3) SSSB sampling; (a-4) 2 D inside a follicle (original magnification 9 40);

Middle, lesion B: brown flat macula without inflammation. (b-1) Dermoscopy; (b-2) 7 D appear as clusters of bright, roundish structures in RCM images taken perpendicularly in the lesion; (b-3) SSSB sampling; (b-4) 7 adult D inside a follicle and 2 larvae outside the follicle (original magnification 9 40).

Bottom, lesion C: many small grey spike changes. (c-1) Dermoscopy; (c-2) 4 D inside a follicle under RCM; (c-3) SSSB sampling; (c-4) 4 D leaving the follicle (original magnification 9 40). Each green arrow indicates one mite inside the follicle under RCM.

The difference between RCM and SSSB in the assessment of D numbers per cm^2 is not surprising. With SSSB, superficial layers of the skin are taken from the patient, then viewed under microscope ex vivo. But a single SSSB may fail to collect all the mites from the skin(28). Kligman recommends

performing two sequential SSSB from precisely the same site (59); more, but probably not all, mites would be extracted. Secondly, it was difficult to extract elongated and hyperkeratotic follicles; therefore we did not collect each follicle in the test areas. In terms of scientific relevance regarding investigations in PF patients or other skin diseases, RCM may be a better choice than SSSB because of its accuracy, completeness and as an in vivo noninvasive painless procedure.

Our findings are based on the observation of one patient. In addition the diagnosis of lesion B could not be ascertained: demodicosis with lentigo, or unknown type of lesion in pityriasis folliculorum? However, to some extent, they support a previous observation by Forton on the development of PF: first, the appearance of small grey spike changes due to D proliferation on superficial skin, then as infestation spreads to more follicles, spikes merge as a macule with brown scales and proliferation reaches the top of the epidermis. Inflammatory lesions appear, looking like papulopustular rosacea, with D at a deeper level but in decreasing numbers in comparison with noninflammatory lesions.

To our knowledge, demodicosis is a very frequent disease (69) and SSSB, even a single one, is wholly sufficient in daily clinical practice for diagnosis and follow-up. A second SSSB performed at the same place would highly increase the sensitivity of this method. However, RCM appears to be a more sensitive method which could be used more in research or clinical studies or to follow up treatment or recurrence.

D- Skin flora situation in PPR patients

(Submitted to Medicine in May, 2017)

Rosacea is a chronic skin disease characterized by transient or persistent central facial erythema, inflammatory papules and pustules, and often telangiectasia (73). Its main features include burning or stinging sensations, facial dryness, and edema (16). This disorder affects people of all ages but is most common in middle-aged and older adults, with women being more frequently affected (74). Although several hypotheses have been proposed, the exact pathogenesis of rosacea remains unclear until now (75). Inflammation plays a prominent role in the pathophysiology of rosacea (40); however, there is increasing evidence showing that skin barrier defects and dysregulation of innate immunity are involved in the pathogenesis of rosacea (20). Microorganisms (76), such as Demodex folliculorum (77), Staphylococcus epidermidis (78) and Propionibacterium acnes, may also contribute to the pathogenesis of rosacea by stimulating the innate immune system (25). There are some important links between skin physiological conditions and skin microbiota in multiple specific skin diseases, including rosacea (79). However, the correlation between microbial dysbiosis and rosacea remains controversial like a “chicken-or-the-egg” conundrum; in other words, it has not been fully demonstrated that whether microorganisms triggers rosacea, or dysbiosis is a response to changes in the skin microenvironment resulting from rosacea.

Skin physiological conditions could affect the microbial flora living on the face by affecting the skin microenvironment (80). Increased transepidermal

water loss (TEWL) is reported to correlate with an increase in the skin disease severity (81). The skin is susceptible to Demodex mite infection in patients with rosacea, notably papulopustular rosacea (PPR)(71). It is plausible to hypothesize that a disrupted skin barrier may promote skin colonization by microbes, and that the damaged skin barrier and microbial colonization may both trigger and aggravate skin diseases. In this study, we aimed to examine the associations of the density of microbial colonization with the severity and skin barrier function in patients with rosacea.

D.1 Materials and methods

D.1.1 Subjects

Healthy PPR patients were recruited at the Dermatology Clinic in the University Hospital of Besançon, France. All diagnoses were confirmed by at least two dermatologists and each patient had at least three lesions (papules or pustules) on the face. Subjects undergoing topical treatment within 4 weeks prior to the enrollment and subjects on an antibiotic treatment scheme were excluded from the study. Finally, 25 PPR patients were enrolled in this study, and included 2 men and 23 women, with ages of 28 to 60 years. The mean duration of rosacea was 11.8 ± 9 years, and none of the patients had taken medications during the past 3 months.

This study was approved by the Ethics Review Committee of the University Hospital of Besançon. Written informed consent was obtained from all subjects participating in the study, following a detailed description of

the purpose of the study. All experiments were performed in accordance with the Declaration of Helsinki.

D.1.2. Clinical assessments

We utilized a broad subjective scoring system, based on a 4-point scale, with 0 = normal, 1 = mild, 2 = moderate, and 3 = severe (16). The primary features included frequent flushing, persistent erythema, papules/pustules and telangiectasia. Other symptoms included burning or stinging, plaque, dryness, edema, ocular manifestations or phymatous changes. Final assessments were made based on rosacea subtypes and subjects' self-assessments.

D.1.3. Measurement of physical conditions

Skin samples were taken from the rosacea lesions and the rosacea-surrounding areas (controls), and TEWL and skin hydration (water content) were measured by using the Tewameter TM210® (Courage & Khazaka Electronic GmbH; Koln, Germany) and the Corneometer 820® (Courage & Khazaka Electronic GmbH; Koln, Germany), respectively. Rosacea patients were at rest for at least 20 minutes in an environment-controlled room: relative humidity of 40% to 60% and ambient temperature of 20 to 22°C, prior to the measurements,

D.1.4. Bacterial colonization

Bacterial samples were taken from the rosacea lesions (at least three

lesions for each patient) and the surrounding control areas with two different dry swabs, and each site was wiped 20 times with the same time and pressure. Specimens were then incubated in Columbia agar supplemented with 5% of sheep blood (Thomas Scientific; Swedesboro, NJ, USA) at 35°C containing 5% CO2 for 5 d. Each colony was identified by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) with a log value of ≥ 2 according to the manufacturer's recommendations (Bruker Daltonik GmbH; Bremen, Germany).

D.1.5. Colonization condition

In this study, three methods were used based on the data of bacterial colonization, with two groups defined in each method. Method 1 was based on the presence of identical dominant germs in lesions and the control areas, and 25 rosacea patients were assigned into the bacterial colonization balance (Group A) and the bacterial dysbiosis group (Group B). Methods 2 and 3 were based on the presence of P. acnes or S. epidermidis in lesions and control areas. In Method 2, P. acnes as the dominant microorganism was assigned into Group A, while non-P. Acnes was assigned into Group B. In Method 3, S. epidermidis as the dominant microorganism was assigned into Group A, while non-S. epidermidis was assigned into Group B.

D.1.6. Statistical analysis

Diversity indices for lesions and controls were calculated using vegan in R package version 3.3.0, and the distances were compared using Bray-Curtis distance measurements. Calculations of Bray-Curtis dissimilarities were

done between datasets and hierarchical clustering using the R package. Differences between groups were tested for statistical significance with the Wilcoxon-Mann-Whitney rank sum test. All statistical analyses were performed using the statistical software SPSS version 16.0 (SPSS, Inc.; Chicago, IL, USA), and a P value < 0.05 was considered statistically significant.

D.2 Results

D.2.1. Water content and TEWL

In all subjects enrolled in this study, the water content detected in the lesions was lower than in the control areas (43.5 ± 12.4 vs. 57.2 ± 11.6 U, P < 0.05), and TEWL measured in the lesions was higher than in the control areas (17.2 ± 5.9 vs. 14.2 ± 4.1 g/(m2·h), P < 0.05).

There were no significant differences detected in the clinical assessment between groups A and B using methods 1, 2 or 3 (P > 0.05). In Method 1, higher TEWL was found in the lesions in Group A compared to Group B (P = 0.016), and there were significant differences in both water content and TEWL between the lesions and controls in Group B (P < 0.001) (Table 6). In Method 2, there were significant differences in both water content (P < 0.001) and TEWL between the lesions and controls in Group B (P = 0.01), and there was a significant difference detected in the TEWL between lesions and controls in Group A (Table 7). In Method 3, significant

differences were seen in the water content and TEWL between the lesions and controls in both groups A and B ($P < 0.05$) (Table 8).

Table. 6. Comparison of intrinsic skin features in Method 1.

Method 1	Clinical assessment	Water content		TEWL	
		Lesions	Controls	Lesions	Controls
P value (A vs. B)	0.697	0.125	0.278	0.016	0.16
P value (A)		0.052		0.375	
P value (B)		< 0.001		< 0.001	

A, the bacterial colonization balance group, B, the bacterial dysbiosis group.

Table.7. Comparison of intrinsic skin features in Method 2.

Method 2	Clinical assessment	Water content		TEWL	
		Lesions	Controls	Lesions	Controls
P value (A vs. B)	0.826	0.393	0.203	0.451	0.303
P value (A)		0.09		0.04	
P value (B)		< 0.001		0.01	

Group A, *P. acne* is the dominant microorganism; Group B, non-*P. acne* is the dominant microorganism.

Table .8. Comparison of intrinsic skin features in Method 3.

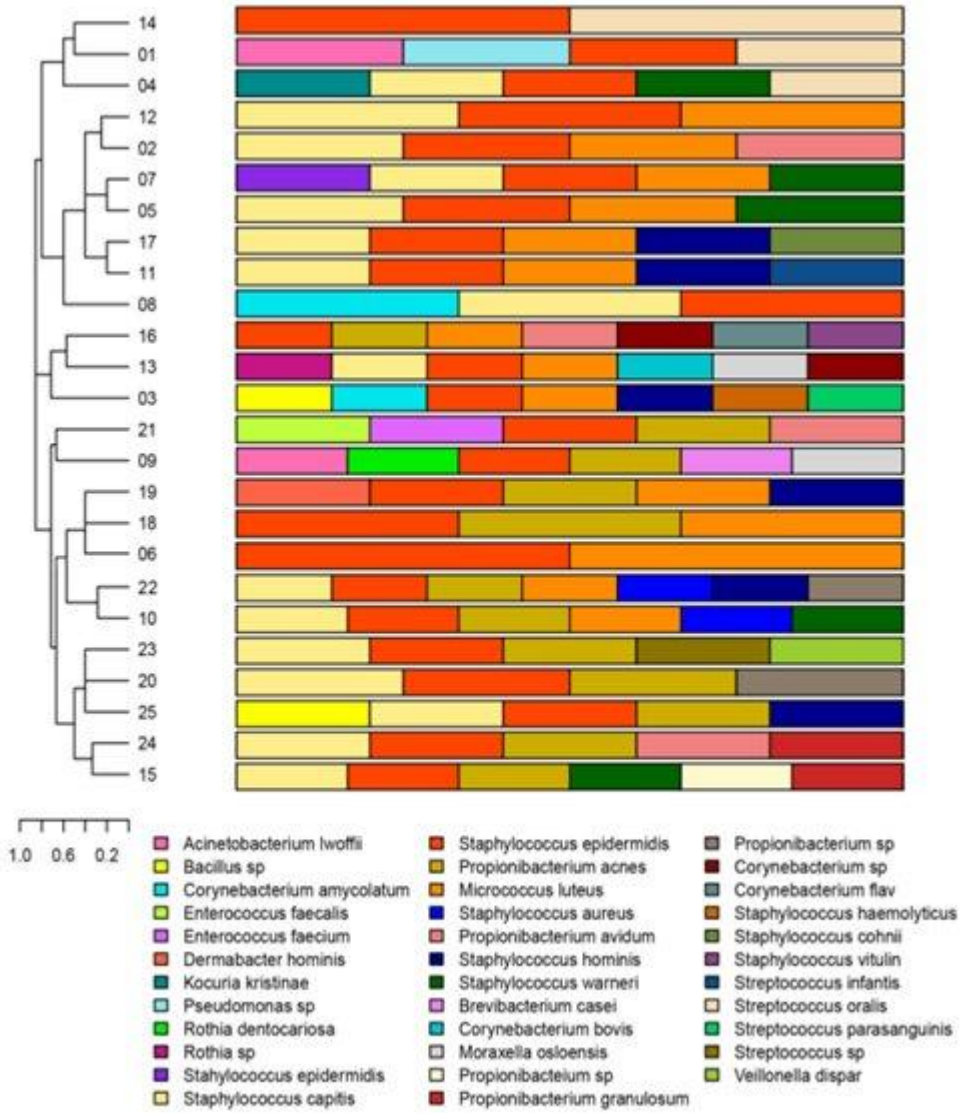
Method 3	Clinical assessment	Water content		TEWL	
		Lesions	Controls	Lesions	Controls
P value (A vs. B)	0.857	0.643	0.504	0.377	0.394
P value (A)		0.04		0.011	
P value (B)		0.001		0.034	

Group A, *S. epidermidis* is the dominant microorganism; Group B, non-*S. epidermidis* is the dominant microorganism.

D.2.2. Composition and diversity of microbial communities

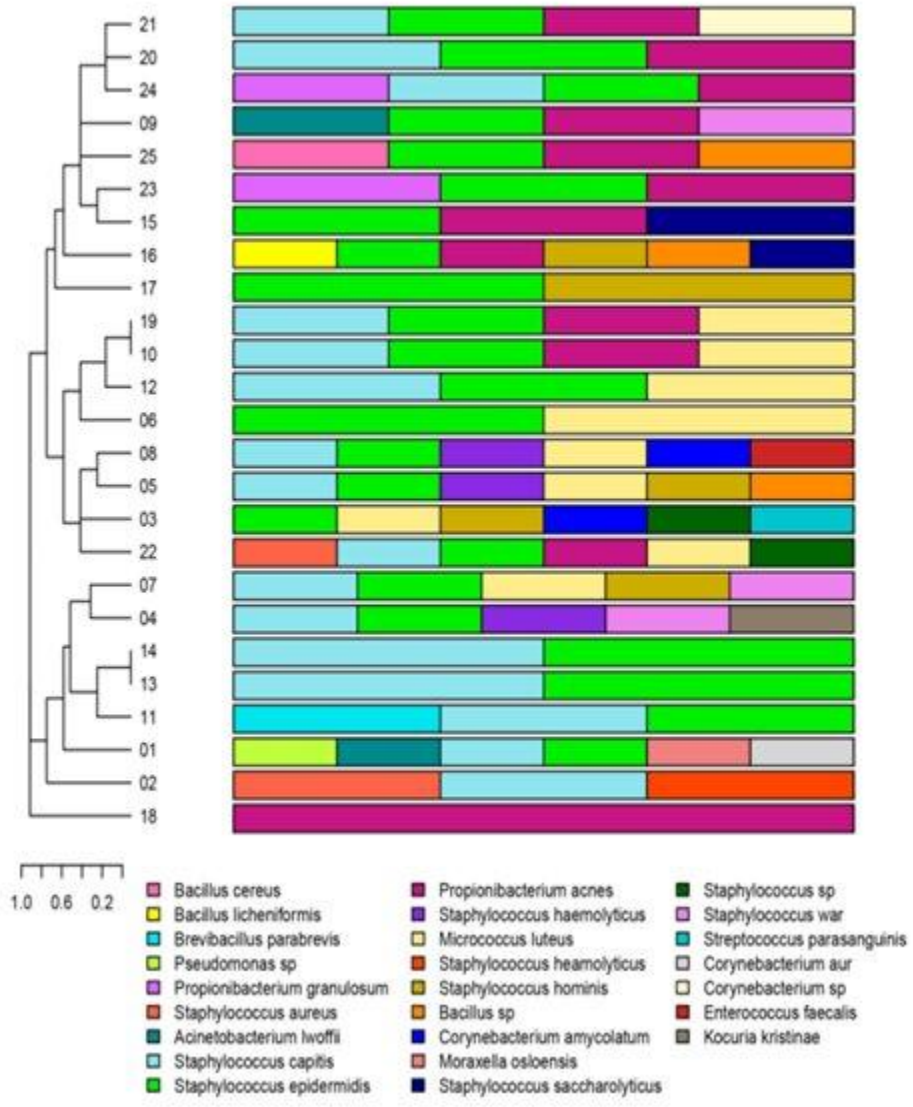
The sequences of microorganisms were aligned with cmalign 1.0.2. On the left is the sample room based on the hierarchy of colony clustering analysis, on the right is the histogram colony structure of sample. The similarities and differences of multiple samples in strains are expressed through colors in Figure 16.

Fig.16. Relative abundance of the most predominant microorganisms in the lesions in 25 patients



Each color piece represents a species, and each color represents a sample of species abundance. Clustering is based on the similarity of species abundance, and various combinations between samples are made according to the strains of mesh, clustering, species and genera, to reflect the multiple samples of colony at the species level.

Fig.17. Relative abundance of the most predominant microorganisms in the control areas in 25 patients



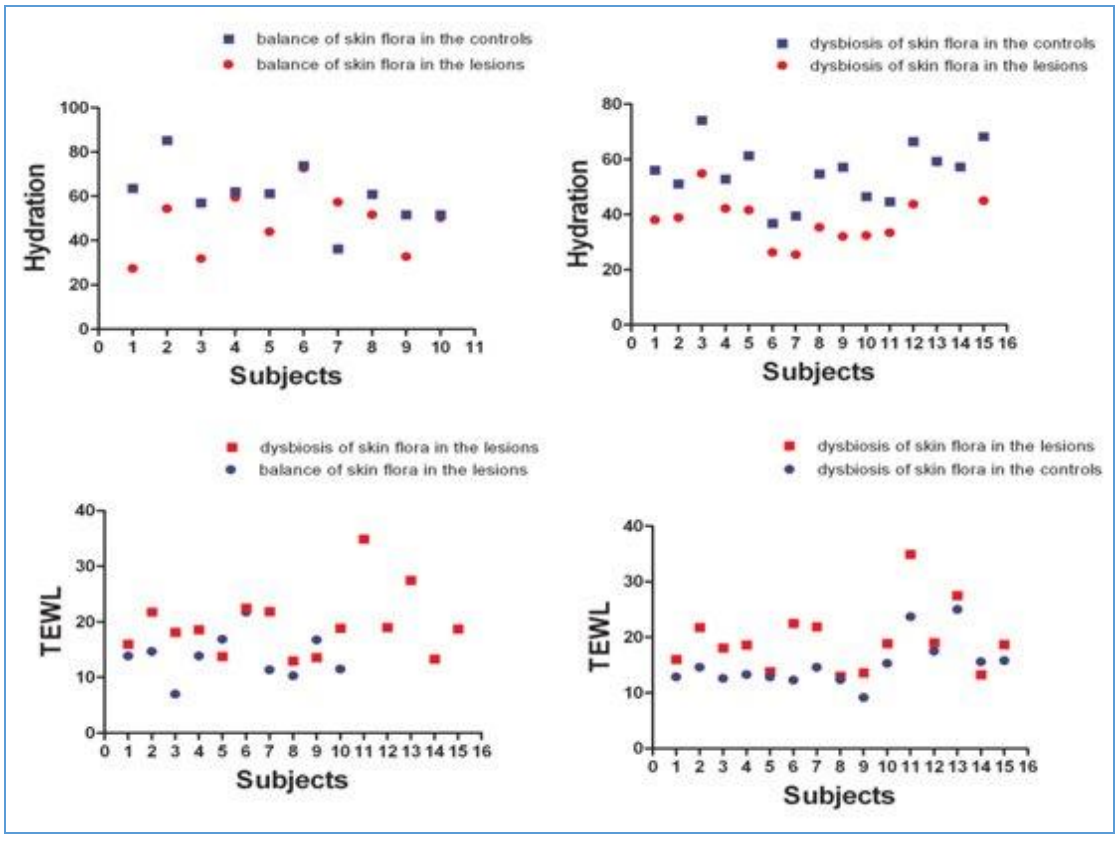
Each color piece represents a species, and each color represents a sample of species abundance. Clustering is based on the similarity of species abundance, and different combinations between samples are made according to the strains of mesh, clustering, species and genera, to reflect the multiple samples of colony at the species level.

There were 41 and 30 types of microorganisms identified in the lesions and control areas respectively, with 17 types of common microorganisms

identified in both lesions and controls (Figures 16 and 17). Comparison of the dominant microorganisms in the lesions and controls showed S. epidermidis, P. acnes and S. capitis as the three most prevalent microorganisms.

Figure 18 shows the water content and TEWL in the lesions and controls in groups A and B, and there were significant differences in the skin physiological features between lesions and controls in Group A relative to Group B ($P < 0.001$).

Fig.18. Dominant microorganism's difference (same species and different species) for intrinsic skin features

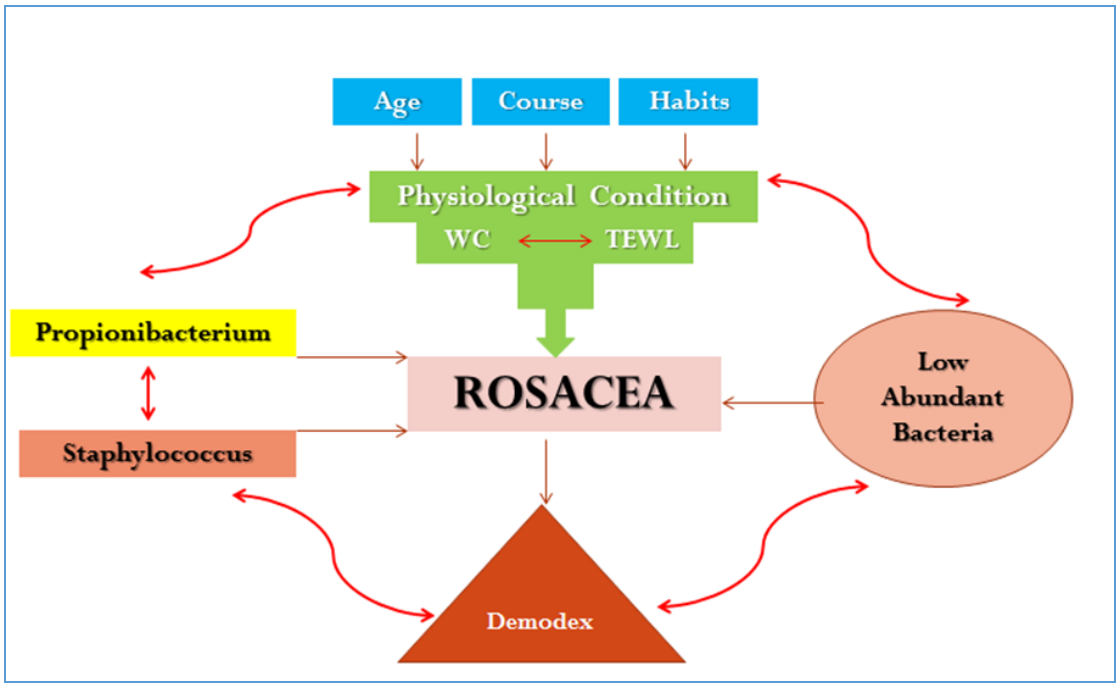


A significant difference is found in skin physiological features (skin hydration and TEWL) in the bacterial dysbiosis group between lesions and controls ($P < 0.001$) compared with the bacterial colonization balance group.

D.3 Discussion for the skin flora condition for rosacea

The diagnostic criteria of rosacea include primary features, such as flushing erythema, permanent erythema, papules, pustules, telangiectasias and other inflammatory lesions (31). Although the exact pathogenesis of rosacea is unknown, there are several factors implicated in the pathophysiology of rosacea (82). These influencing factors, which may include age, medical course, sun, life habit, Demodex and microorganisms, may affect skin physiological features such as water content and TEWL (76). The main microorganisms include Propionobacterium, Staphylococcus and low abundant bacteria (83). These stimuli induce episodes of flushing with progressive damage to the endothelium and angiogenesis, as well as inflammatory changes in the dermis with production of vasoactive substances, worsening of the vascular framework which will have repercussions in the epidermis (84). In addition, these influencing factors may interact with physiological features and microorganisms interact with each other (Figure19). The role of microorganisms in the development of rosacea has been extensively investigated. However, the exact pathogenic role of microorganisms in rosacea has not been fully demonstrated until now, and continues to be debated (85).

Fig.19. Overview of the relationships among rosacea, host demographics, physiological conditions and microorganisms



The primary function of human skin, one of the largest and most versatile organs in human body, is to protect the host from stimuli by external agents, including chemical, physical and microbial factors (57). As the first barrier to environmental exposure, it is composed of dozens of distinctive and diverse microenvironments for colonization by a variety of microorganisms. Skin microbiota has been widely accepted to be of high importance for human health and well-being (60). Multiple pathways and events that contribute to rosacea pathophysiology have recently been defined; however, the presence of a microorganism as a contributing agent remains controversial to date (86). The response of the microbiome to inflammation and to the changes in microenvironments and macroenvironments is supposed to play a possible role in the

pathophysiology of rosacea (34). The microbes inhabiting a given microenvironment are diversified, based on the suitability of these conditions for growth of each individual species. During normal skin homeostasis, the microbes inhabiting the microenvironment keep a balance; however, a disorder of the microenvironment may occur if factors affecting the growth or survival of microorganism change. In addition, changes in microbiota may be due to individual, environmental or behavioral factors, such as age, gender, climate, hygiene, antibiotic consumption, humidity, temperature, pH and lipid composition, which may cause dysbiosis (87). It is therefore of great importance to examine the correlation between microenvironments and rosacea, which may interact with each other.

Human skin provides a great living environment for the growth of microbes. P. acnes, a major commensal of the human skin, colonizes the lipid-rich sebaceous glands of the skin (86), and is presented as an opportunistic pathogen via bacterial seeding causing invasive infections. It has been shown that P. acnes exhibits a strong proinflammatory activity and targets molecules involved in the innate cutaneous immunity, keratinocytes and sebaceous glands of the pilosebaceous follicle. Our data indicated that P. acnes correlated with the physiological features of rosacea, which was inconsistent with the previous study reporting no link between P. acnes and rosacea. Further studies are required to examine the exact correlation between P. acnes and rosacea.

S. epidermidis is the most important member of the coagulase-negative staphylococci and one of the most abundant colonizers of human skin (88). As a biofilm-producing commensal found ubiquitously on human skin and mucous membranes, S. epidermidis has been shown to be involved in rosacea (48). S. epidermidis strains isolated from patients with rosacea

have previously been shown to secrete more proteins and were more consistently beta-hemolytic than those from control subjects (89). Its ability to cause disease is linked to its presence as a natural resident on human skin and its ability to attach and form biofilm on foreign bodies. In the study, S. epidermidis was found to affect the skin barrier.

As a metabolically active structure that has adaptive features, stratum corneum may play a regulatory role in the process of inflammatory response (90). In the current study, we measured lower water content and higher TEWL in the lesions that in the control areas, which is consistent with previous studies demonstrating that the deficient stratum corneum has a low ability to attract and retain water. However, mild cleansers/moisturizers have been found to improve the stratum corneum barrier function and can help relieve symptoms.

Prevention of rosacea is very important, which may be achieved by avoiding specific triggering factors, increasing skin water content and decreasing TEWL (79). In the process of complex treatments, manifestations of impaired barrier function of the skin are observed and the protection and restoration of the damaged stratum corneum are necessary. The treatment, with consideration of morphological and functional features of facial skin, may help improve the outcomes of therapy in patients with rosacea.

Our study has several limitations. Firstly, the study suffered from a small sample size. Secondly, no 16S rRNA gene-based analysis was performed. Thirdly, only two physiological features were investigated. Fourthly, innate immunity, a main factor contributor to the pathogenesis of rosacea, was not noted. For the better understanding of the microbiology of rosacea, more studies are needed to help illustrate the mechanism of rosacea and

contribute to provide more therapeutic approaches based on the controversial studies and opinions expressed in the literature.

In summary, the results of this study demonstrate that the physiological features of rosacea are strongly associated with the interactions between the host and microorganisms, and our data indicate the importance of the bacterial colonization balance on the skin surface. It is important to realize that the management of rosacea involves not only the choice of appropriate medication and treatment for daily skin care, but also a careful attention to the skin bacterial protection.

E- Sensitive Skin and Rosacea

E.1 Reflectance confocal microscopy for the evaluation of sensitive skin

(Published in SRT 2017)

Sensitive skin is a clinical condition characterized by the occurrence of unpleasant sensations, tightness, tingling, pruritus, burning and pain which are the commonest complaints. These symptoms may occur in response to various factors, including cosmetics (91), water, cold, heat, dry, sun screens, food, menstruation or other physical and/or chemical factors. A lot of studies have reported that the prevalence of sensitive skin was increasing in many areas and many studies also have shown that sensitive skin is more frequent in women (92). The demand for sensitive skin research was also increasing (93). This so-named sensitive skin represents a syndrome of physiological reactions rather than a disease entity (94). Sensitive skin may occur in individuals with healthy skin or with skin barrier disturbance (95). Nevertheless, a part of the symptoms can be associated with inflammation diseases such as rosacea (96), atopic dermatitis, and psoriasis.

The most widely used methods of self-assessment questionnaire and lactic acid sting test were applied to test subjects' skin sensitivity in our study. So far, lots of methods have been used to evaluate sensitive skin, which

include subjective, a half subjective and objective evaluation (97). Subjective evaluation mainly means self-assessment questionnaire. The half a subjective evaluation refers to the chemical probe test such as lactic acid stinging tests (98). The objective assessment refers to the use of the instruments to detect biological physical parameters and related cutaneous functions molecular of the skin, such as TEWL and hydration (99).

Reflectance confocal microscopy (RCM) is a non-invasive, real-time, high-resolution and repetitive imaging tool (100). It was widely used in many cutaneous disorders, such as pigmentary disorders, inflammatory dermatoses and skin cancers (72). RCM can provide in vivo imaging with a horizontal axis and a cellular-level resolution of the skin and allows skin examination from the superficial layers to the dermis repeatedly (101). It also provides images of cell and tissue structures and dynamics in situ, without the need for ex vivo tissue samples. The aim of this study was to explore the suitable parameters of RCM which could evaluate the skin sensitivity in practice. In this study, we explored the structure of the skin epidermis and dermal-epidermal junction (DEJ): parakeratosis, honeycomb structure, spongiform edema, and dermal papilla, thickness of epidermis and honeycomb structure depth (102). Our goal was to research whether sensitive skin has structural changes in RCM level and to explore whether the impaired RCM structures correlated with the skin sensitivity.

E.1.1 Participants and methods

Participants

The study consisted of 166 female healthy subjects who lived in Shanghai

more than 5 years and aged from 18 to 55 years old, with a mean age of 37.8 ± 10.7 years. No participants were under systemic treatment and topical treatments before the recruitment. All participants provided written informed consents. The protocol had passed the review of the Ethics Committee of the Shanghai Skin Disease Hospital and all subjects gave their informed consent.

Self-Perception Sensitive Skin questionnaire The Self-Perception Sensitive Skin (SPSS) questionnaire consisted in 28 related influence factors of sensitive skin (103), including environmental factors, contacted materials, foods, seasonal changes, menstruation and scalp sensitivity conditions. The positive self-perception was SPSS positive group and the negative self-perception was SPSS negative group.

Reflectance confocal microscopy

Reflectance confocal microscopy imaging was performed using a commercially available RCM system for in vivo imaging (Vivascope 1500; Lucid Technologies, Henrietta, NY, USA) which uses a diode laser with a low-power wavelength of 830-nm. RCM imaging was performed according to a standardized protocol. This system provides high-resolution horizontal mapping (8 mm*8 mm), and was performed at the level of the stratum corneum, the stratum granulosum and the DEJ. Vertical mapping was obtained by capturing a series of images in depth (0.5 mm * 0.5 mm), with steps of 4.54 μ m down to a depth of 150 μ m in vivo (from the epidermis down to the dermis) taken from the center of the area examined with RCM in order to measure the thickness of the epidermis and the depth of honeycomb structure and get clear structure images. After imaging of the

face, the fossa cubitalia was examined as well. The structures of fossa cubitalia are used as the control group to remove the unhealthy subjects.

The reading for RCM

Parakeratosis

In the stratum corneum, granular cells appear as very large and polygonal structures, with an evident grainy cytoplasm, the transition between spinous and granular cells was usually clearly recognizable (104). Presence of refractile as well as dark, nucleated structures at the level of stratum corneum, visualized as retainment of bright nuclei in dark corneocytes, highly refractile round cellular structures in the stratum corneum, and this layer can be easily distinguished from the spinous layer (105).

Honeycomb pattern

Normal honeycomb pattern of the granular and spinous layers formed by bright polygonal outlines of keratinocytes (cytoplasm and intercellular borders) with dark central nuclei and cells are regular in size and shape (106).

Spongiform edema

Darker areas relative to the surrounding epithelium of the stratum spongiform edema with enlarged intercellular spaces (72) led to semiquantification of spongiform edema,starting from the evaluation of a 0.5 mm* 0.5 mm block taken from the stratum spongiform edema and classified as <5% and $\geqslant$5% group.

Dermal ring pattern

A low-magnification pattern composed of bright thin rim of cells surrounding dark dermal papillae (107) was seen when there was predominance of ‘edged papillae’ at the EDJ with similar size.

Epidermal thickness

Evaluated using ‘stack analysis’ as an increased number of single 4.54 μ m step, from the top of the first cellular layer to the first dermal papillae appeared.

Lactic acid stinging test

Subjects rested for 30 min in an environment controlled room (temperature 20 ± 2°C, relative humidity 40–60%) after cleaning their face with water and drying it gently with paper towels. Then, we used a solution of 10% aqueous lactic acid (50 lL) on one nasolabial fold through two layers of filter paper (0.8 cm diameters) randomly (23, 24). Another solution with distilled water was used on the other side. Stinging sensation was recorded at three time points (30 s, 2.5 min, and 5 min) on a 4-point scale (0 = no stinging; 1 = slight stinging; 2 = moderate stinging; 3 = strong stinging). The stinging scores at 2.5 and 5 min were recorded. When the total score of stinging was ⩾3, the subject was assigned to the lactic acid sting test (LAST) positive group; If the sum score was <3, the subject was in the LAST negative group.

Statistical analysis

The mean values and standard deviations were calculated for each of the outcomes measured. Visualization of single RCM features and the

association between sensitive skins were evaluated using SPSS software (SPSS version 16.0; SPSS Inc., Chicago, IL, USA) and Origin Pro 9.0 (OriginLab, Northampton, MA, USA). Difference between two groups was compared by Chi-square test, Wilcoxon rank sum test and Students's t-test. Results were expressed as mean ± SD and $P < 0.05$ was considered statistically significant.

E.1.2 Results

Self-Perception Sensitive Skin and lactic acid sting test.

According to the results of SPSS questionnaire and LAST, all subjects were divided into six groups. The positive subjects were as assigned to the sensitive skin group and the negative subjects to the healthy control group. According to the results of the questionnaires, subjects were divided into positive- group SPSS (+) and negative-group SPSS (-). Similarly, the LAST group was also divided into positive-group LAST (+) and negative-group LAST (-) (Table 9).

Table.9. Numbers and frequency of each group

Groups	N (%)
Sensitive skin	76(45.78)
Healthy control	38(22.89)
SPSS (+)	109(65.66)
SPSS (-)	57(34.34)
LAST (+)	95(57.23)
LAST (-)	71(42.77)

SPSS, Self-Perception Sensitive Skin; LAST, lactic acid sting test.

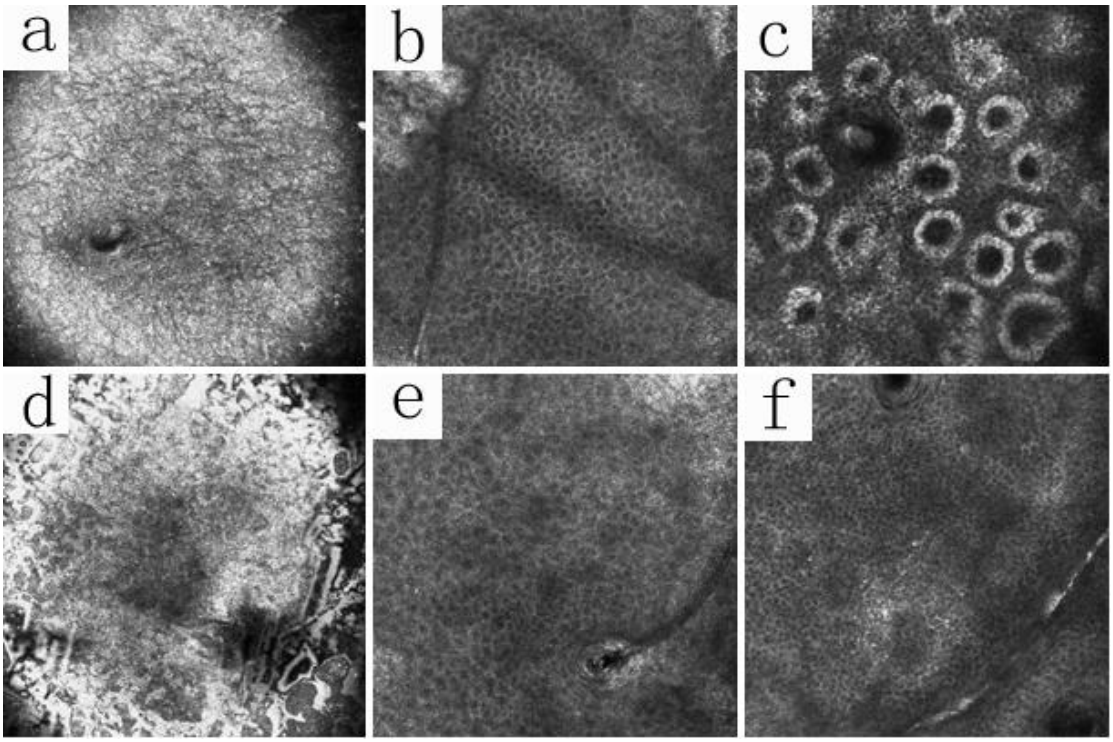
The result of RCM structures in these groups:

Figure 20 shows all the RCM images considered, with normal and disarranged structures. There are four qualitative data of the RCM structures: parakeratosis, honeycomb pattern, spongiform edema, and dermal papillae. The difference between each group in the above four parameters is shown in Table 10. ‘Sensitive’ refers to the comparison between sensitive skin group and healthy control group. ‘LAST’ means the comparison between LAST (+) group and LAST (-) group, and ‘SPSS’ refers to the comparison between SPSS (+) group and SPSS (-) group. According to the results, significant differences were observed between the positive-group and the negative-group at each group ($P < 0.05$) of honeycomb pattern and spongiform edema. There were no significant differences in parakeratosis and dermal ring pattern ($P > 0.05$) of each group (Table 10).

Table.10.The difference between each with RCM **qualitative** structures

Group	Parakeratosis	Honeycomb pattern	Spongiform edema	Dermal papillae
Sensitive	0.252	0.005*	0.003*	0.888
LAST	0.129	0.004*	0.044*	0.349
SPSS	0.110	0.040*	0.006*	0.686

Fig. 20. RCM images considered, with normal and disarranged structures



Reflectance confocal microscopy images (0.5mm x 0.5 mm), (a)normal structure of stratum corneum with anucleate cell, (b)normal honeycomb pattern regular in size and shape, (c)normal papillary rings in the EDJ, (d)Parakeratosis with little cell nucleus of stratum corneum, (e)disarranged honeycomb pattern and spongiform edema, (f) absence of papillary rings in the EDJ.

At the same time, we divided the RCM image of spongiform edema into 400 equal pieces and counted the proportion of spongiform edema.

Based on the reading data, we classified two groups according to the proportion of the image <5% and⩾5%. The following table shows the number and the area ratio of each group. We can see that the proportion of spongiform edema was more than 30% in all positive groups (Table 11).

Table.11. Area ratio of spongiform edema in positive group

	＜5%（N）	≥5%（N）	All（N）	≥5%（%）
SPSS（+）+LAST（+）	6	3	9	33.3
LAST（+）	8	4	12	33.3
SPSS（+）	11	6	17	35.3

In addition, we also compared the honeycomb pattern depth and epidermal thickness of each group. The epidermal thickness of the sensitive skin group was 38.88±6.81 lm and in the healthy control group it was 40.31± 9.37 lm ; there were no significant statistical differences between the positive-group and the negative-group in each group (P > 0.05). The honeycomb structure depth of the sensitive skin group was 20.57±4.86 lm and in the healthy control group it was 23.27±6.38 lm ;significant statistical difference was found between the two groups (P < 0.05), but there were no significant statistical differences between the positive-group and negative-group of the LAST and SPSS groups (P > 0.05). (Table 12, Fig 21 and 22).

Table.12. Correlation between skin groups with RCM quantitative structures

	Honeycomb pattern thickness	Epidermal thickness
Sensitive	0.005*	0.314
LAST	0.034*	0.319
SPSS	0.004*	0.623

*Correlation is significant *P*＜0.05.

Fig.21. Epidermal thickness comparison of the positive-group and the negative-group in each group

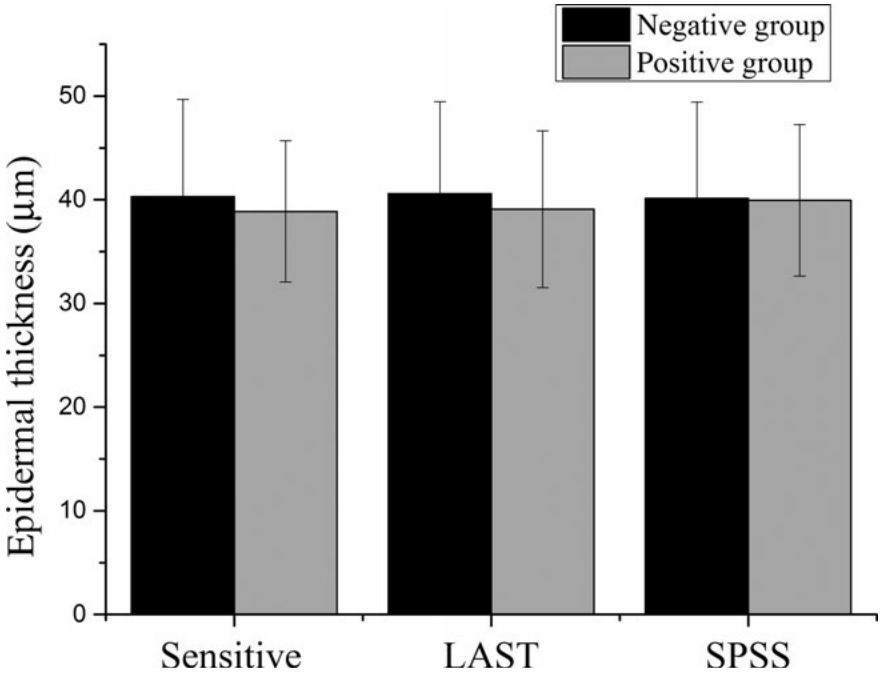
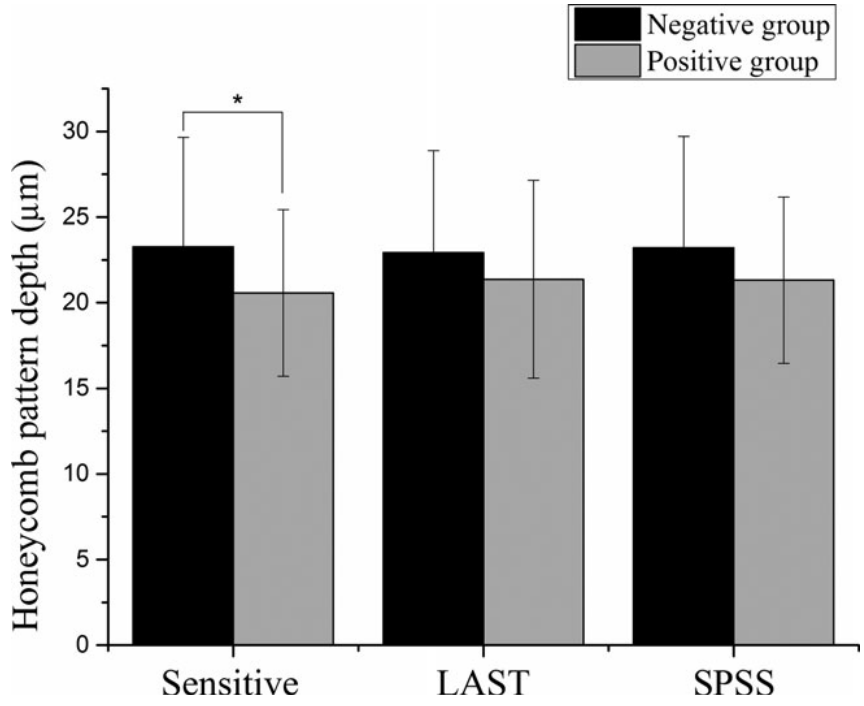


Fig.22.Honeycomb pattern depth of subjects and comparison of the positive-group and the negative-group in each group.



According to the questionnaire, the six correlation factors were compared with the disarranged honeycomb pattern and spongiform edema; the results are shown in the following table (Table 13), we can see that there is no statistically significant correlation with disarranged honeycomb pattern (P>0.05) between the positive-group and the negative-group.

Table.13. Correlation factors with the positive results

	environment	**contact material**	food	**seasonal variation**	Menstruation	scalp sensitivity
Honeycomb pattern	0.869	0.404	0.537	0.696	0.979	0.068
Spongiform edema	0.154	0.238	0.835	0.619	0.661	0.014*

*Correlation is significant $P<0.05$.

E.1.3 Discussion

The evaluation methods mainly include subjective, semi-subjective and objective evaluation. Subjective evaluation is mainly provided by self-assessment questionnaires (108). It is applicable for clinical practice in order to predict one's propensity to experience sensitive skin. But it may be impacted by some factors such as education level and cognitive degree, lacking objectivity, and may not be well-correlated with other testing results. Semi-subjective evaluation methods meaning chemical probe test, many sensory testing methods, such as stinging tests with lactic acid, capsaicin, and dimethyl sulfoxide (109). Initially, it was regarded as the most commonly used and effective method to evaluate sensitive skin and it also can be used to detect the severity of sensitive skin. But, unfortunately, lack of objectivity,

sensitivity, and specificity was found after the application process for a long time. The objective evaluation assessment refers to the use of the instruments to detect the biological physical parameters of the skin, such as TEWL, hydration, sebum, and pH and so on (91). These methods are simple, non-invasive, objective, and have now more and more the favor of dermatologists and cosmetic companies (110). But the only respond to the status of healthy skin and are not well-associated with skin sensitivity, and thus are not suitable for evaluating the severity of sensitive skin. To our knowledge, there was no real-time cellular and structural level detection method for sensitive skin.

Nowadays, lots of studies have been carried out to research sensitive skin mechanisms, pathophysiology, and skin barrier (111). Mechanisms in signal transduction involve cytokines and neurotransmitters, but the exact pathways are unknown. The pathophysiology of sensitive skin consists of an inflammatory reaction resulting from the abnormal penetration in the skin of potentially irritating substances, due to skin barrier dysfunction and changes in the production of local neuromediators (91). The results of many researches showed significant lower sensory perception value of sensitive skin group compared with healthy control group (112). Several studies have suggested a link between sensitive skin and a disruption of the epidermal barrier function, resulting in the perception of skin discomfort (113). Impaired skin barrier function plays a role in the pathophysiological mechanism of sensitive skin and needs to be investigated in depth (114). The skin of sensitive subjects was described as less supple, less hydrated and more erythematous with some telangiectasias with respect to the skin of normal subjects.

Disarranged honeycomb pattern was shown with focal or diffuse loss of the normal patterns of the granular and spinous layers characterized by unevenly distributed bright cells and granular particles (115). These structures vary in thickness and have holes that vary in sizes, reflecting the disarranged keratinocytes. Such aspects can be seen in a lot of dermatoses, such as skin cancer and photoaging (116). Spongiform edema shows darker areas relative to the surrounding epithelium of the stratum, with enlarged intercellular space and may correlate with epidermis layer integrity and inflammation state. The proportion of spongiform edema is less, but there are statistical differences, and it has been demonstrated that at the site of edema, allergy or inflammation occasionally occurs, such as allergic contact dermatitis and irritant contact dermatitis. Interestingly, the results are in concordance with previous reports and data of sensitive skin observed with skin barrier: a trend toward an increase in TEWL (117), pH, epidemic blood flow and erythema, and a decrease in ceramides (118). Sebum, hydration, and capacitance of subjects with sensitive skin were compared with respect to the skin of normal subjects, these studies implied that an impaired skin barrier and a dryer skin underlie sensitive skin (110).

Parakeratosis means there are still remaining nucleus in the horny layer, often seen in psoriasis (119) and inflammatory diseases. Sensitive skin subjects in this experiment did not see an obvious difference compared with the healthy control group. Impaired ring pattern means rings with irregular shape contours, elongated, and partially anastomosing structure (120) or absence of rings, mostly seen in nevi with histopathologically identified lentiginous or small-nested junctional proliferation of melanocytes. Our results could conclude that the dermal ring pattern was not obviously

damaged in sensitive skin. The results of epidermal thickness did not disclose significant differences and confirm the earlier results that did not find any significant difference between sensitive group and control group (121). Nevertheless another study could conclude on adverse results (122), so research with more methods and samples is needed to study differences in the skin epidermal thickness in sensitive skin. The honeycomb pattern depth was reduced of sensitive skin, but until now there was no more evidence to support the results. Thus, this may be a new research direction in sensitive skin.

The ability of RCM could potentially enable more accurate typical histopathologic features for a best assessment of sensitive skin. The obvious advantages of RCM over the majority of other research methods and tools is that it is noninvasive, repeatable, and provides a more accurate picture of the in vivo situation because imaging is performed in real time and provides cellular and structural level evaluation. This method also could be used to combine the skin sensitivity with dermatoses. The results of our study using RCM for the evaluation of sensitive skin demonstrates that non-invasive RCM imaging may be a new possible method to explore sensitive skin confocal features and patterns.

E.1.4 Conclusion

From the study results, we can see that RCM can detect in sensitive skin epidermal damaged structures, including parakeratosis, disarranged honeycomb pattern and reduced honeycomb pattern depth. It could be used as a new kind of auxiliary method in the detection and diagnosis of sensitive

skin, providing new paths for the diagnosis and treatment of sensitive skin and direction. This could potentially enable better assessment of sensitive skin and the effect of cosmetics for sensitive skin. It could be used to develop rational interventions for each individual with sensitive skin, implementing personalized medicine. RCM is a possible technique which might contribute to new insights into the pathogenesis and mechanisms sensitive skin. Further well designed, large population would be needed to validate the use of RCM for sensitive skin.

E.2 Sensitive Skin and Rosacea in Reflectance confocal microscopy

For the clinical diagnosis of rosacea, until now many Chinese dermatologists have been confused with differential diagnosis. On the other hand, the pathogenesis of sensitive skin also has a relationship with enhanced immune responsiveness, blood vessel hyperactivity and altered mechanisms for inflammatory mediator release (123), which has some resemblance with the rosacea's pathogenesis. We designed this study to find if there is some method to easily distinguish sensitive skin and rosacea with RCM.

E.2.1 Participants and methods

Participants

Three groups were set up in this study: 60 normal skin (negative stinger test and negative SPSS questionnaire), 40 SS skin (positive stinger test and positive SPSS questionnaire) and 40 rosacea patients (only ETR and PPR according to the diagnostic criteria of rosacea). The subjects' details are listed in table 14. There was no significant difference in these three groups for subjects' demography distribution ($P>0.05$). No participants were under systemic treatment and topical treatments before the recruitment. All participants provided written informed consents.

The protocol had passed the review of the Ethics Committee of the Shanghai Skin Disease Hospital and all subjects gave their informed consent.

Table.14. Subjects' demography distribution

	Total (N)	Female(N)	Male(N)	Average Age(yr)
NS	60	56	4	39.07±9.16
SS	40	34	6	39.18±9.54
RP	40.	37	3	38.17±8.66

Note: NS means normal skin; SS means sensitive skin; RP means rosacea patients.

E.2.2 Results

E.2.2.1 Qualitative data of the RCM structures

Here are four qualitative data of the RCM structures: parakeratosis, honeycomb pattern, spongiform edema, and dermal papillae. The difference between each group in the above four parameters is shown in Table 16.

According to the results, significant differences were observed between the Normal skin and the Sensitive skin ($P < 0.01$) of honeycomb pattern and spongiform edema. There were no significant differences in parakeratosis and dermal ring pattern ($P > 0.05$) of these two groups (Table 10). Significant differences were also observed between the Normal skin and Rosacea patients for honeycomb pattern ($P < 0.01$), parakeratosis ($P < 0.05$) and spongiform edema ($P < 0.01$). For the sensitive skin and rosacea patients, we could find the significant differences between them in parakeratosis ($P < 0.01$) and Spongiform edema ($P < 0.05$) (Table 15).

Table. 15. Four qualitative data of the RCM structures in three groups

	Honeycomb pattern		Parakeratosis		Spongiform edema		Dermal papillae	
	+	-	+	-	+	-	+	-
NS	10	50	5	55	3	57	20	40
SS	18	22	2	38	12	28	13	27
RP	24	16	10	30	21	19	17	23
NS-SS	P<0.01		P>0.05		P<0.01		P>0.05	
NS-RP	P<0.001		P<0.05		P<0.001		P>0.05	
SS-RP	P>0.05		P<0.01		P<0.05		P>0.05	

Note: NS means the Normal skin, SS means the sensitive skin, and RP means the rosacea patients; + means the positive results and – means the negative results.

E.2.2.2 Quantitative data of the RCM structures

In addition, we also compared the honeycomb pattern depth and epidermal thickness of each group. The epidermal thickness in the sensitive skin group was 38.18±6.57μ m, in the normal skin group it was 39.77±6.30μ m and in rosacea patients it was 39.66±5.04μ m. But there were no significant statistical differences between each group ($P > 0.05$).

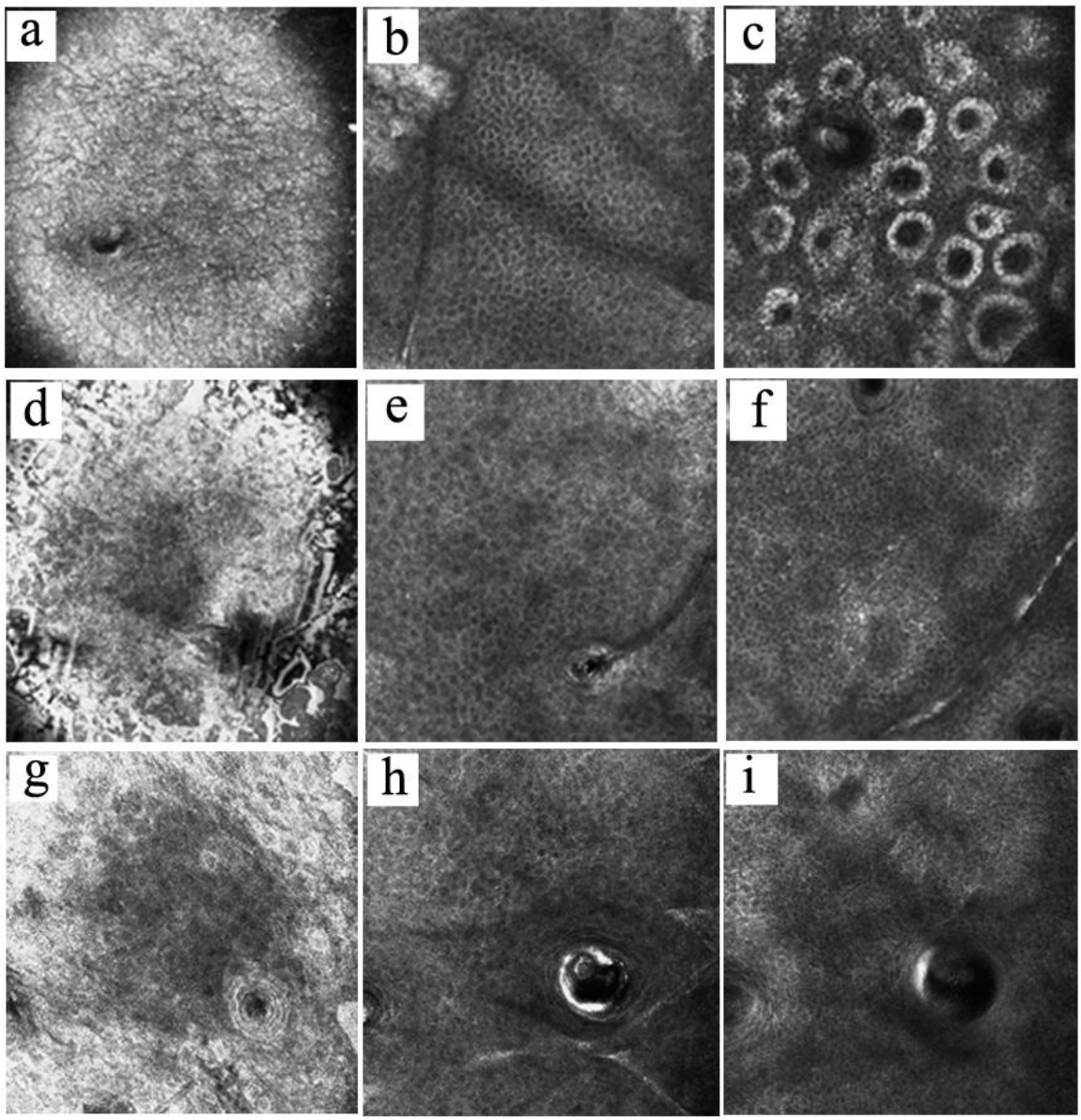
The honeycomb structure depth of the sensitive skin group was 20.34±4.60 μ m,.18±4.79μ m in the normal skin group, and 22.50±3.98 μm in the rosacea patients. There was a significant statistical difference between the normal skin group and sensitive skin group ($P < 0.01$) and sensitive skin and rosacea patients groups ($P < 0.05$), but there was no significant statistical differences between normal skin and rosacea patients groups ($P > 0.05$) (Table 16).

Table.16. the quantitative data of the RCM structures in three groups

	Ages(yr)	Epidermal thickness(μm)	Honeycomb pattern thickness(μm)
NS	39.07±9.16	39.77±6.30	23.18±4.79
SS	39.18±9.54	38.18±6.57	20.34±4.60
RP	38.17±8.66	39.66±5.04	22.50±3.98
NS-SS	P>0.05	P>0.05	P<0.01
NS-RP	P>0.05	P>0.05	P>0.05
SS-RP	P>0.05	P>0.05	P<0.05

Note: NS means the Normal skin, SS means the sensitive skin, and RP means the rosacea patients.

Fig.23.RCM images considered, with normal and disarranged structures



Reflectance confocal microscopy images (0.5× 0.5 mm).

(a-c) Normal skin group: Normal structure of stratum corneum with anucleate cell, normal honeycomb pattern regular in size and shape, normal papillary rings in the EDJ; (d-f) SS group: parakeratosis with little cell nucleus of stratum corneum; disarranged honeycomb pattern and spongiform edema; absence of papillary rings in the EDJ. (g-i) Rosacea group: obvious parakeratosis; disarranged honeycomb pattern and spongiform edema and the demodex folliculus; incompleted dermis papillary rings in EDJ.

E.2.3 Conclusion

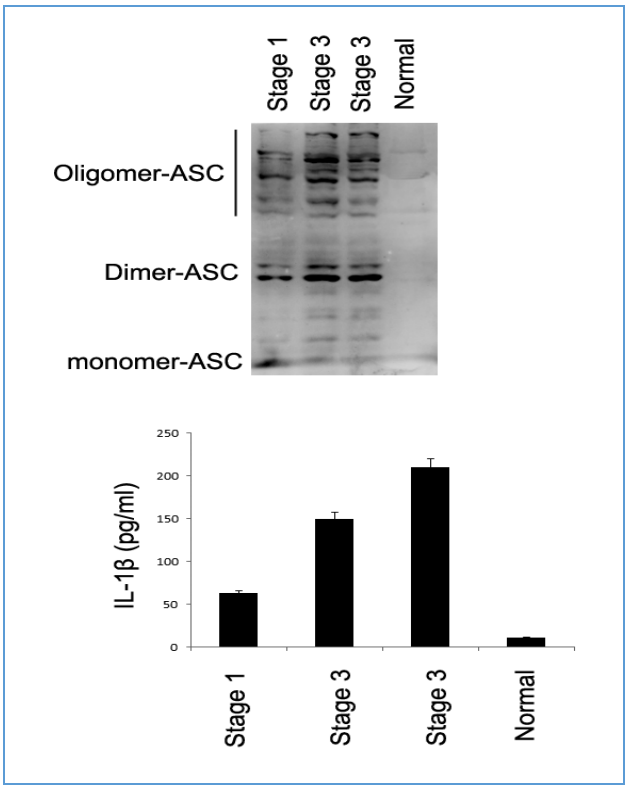
From the study results, we can see that RCM can detect in sensitive skin and rosacea patients their epidermal damaged structures, including parakeratosis, disarranged honeycomb pattern and reduced honeycomb pattern depth. We also found that the Honeycomb pattern thickness had interesting differences in these three groups. The normal skin had the deepest location for honeycomb pattern, the rosacea patient was the second deepest location, and the sensitive skin was the most superficial for honeycomb pattern. Just as we found that the sensitive skin has the lowest epidermal thickness among the three groups, the honeycomb pattern was also the most superficial andhas some relationship with the lowest epidermal thickness.

It could be used as a new kind of auxiliary method in the detection and differential diagnosis of sensitive skin and rosacea patients, providing new paths for the diagnosis and treatments. RCM is a possible technique which might contribute to new insights into the pathogenesis and mechanisms rosacea not only in demodex testing, but also in other inflammation signs. Further well designed, large population would be needed to validate the use of RCM for rosacea, especially in neutrophil changes in different levels of the skin.

F-Future ideas for the research of rosacea

In the future, we will do more research for the changed innate immunity in rosacea patients. As we all know, rosacea is a kind of refractory skin disease which has serious influence in people's appearance and health. It is of paramount important to find that innate immune response is over-activated in rosacea.

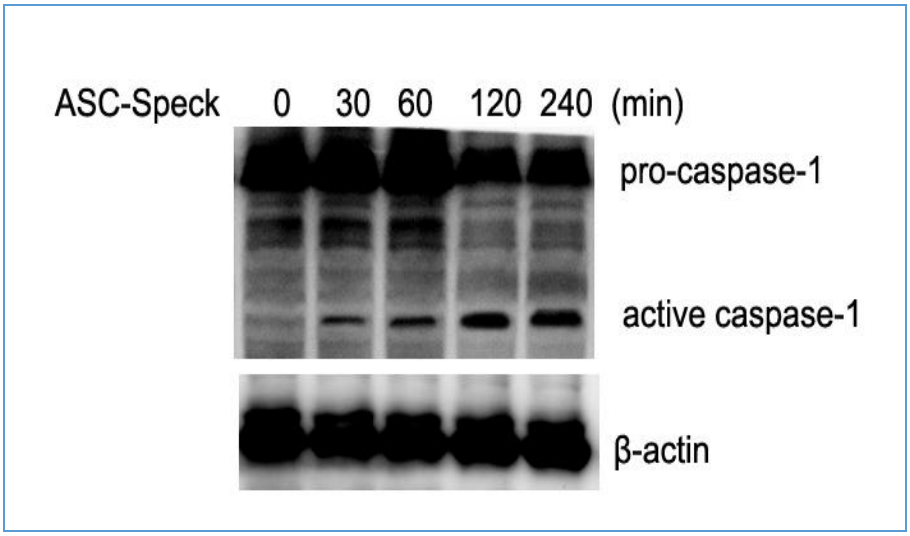
Fig.20 ASC-Speck and IL-1β has positive correlation in rosacea lesions



Recent research reveals that the excreted ASC-Speck could be internalized by bystander cells, which leads to NLRP3 activation and the

auto-amplification mechanism of inflammasome (15). In our previous clinical study, it was found that the severity of inflammatory papulopustular rosacea was negatively related with the numbers of demodex folliculorum, while positively related with ASC-Speck status. It was also demonstrated that in vitro experiments, the macrophage drived ASC-Speck and then internalized by fibroblast 3T3 cells, which directly activates inflammasome and IL-1β production (124) (Fig 24).

Fig.24. Macrophage drived ASC-Speck and then internalized by fibroblast 3T3 cells



Based on these findings, our research team proposed a hypothesis that there is an inflammation amplification system, which transduces epidermal microorganism infection into dermal inflammation. In other words, demodex folliculorum induced first inflammation response in epidermis, which could be transduced into dermis by internalization of ASC-Speck. In consequence, our research team plans to investigate the NLRP3-medicated inflammasome and its amplification system for rosacea's development.

Four levels will be preceded in this research: clinical assessments, mice rosacea model testing, cell function in vitro and signal transduction assay. Our research will provide theoretical basis for rosacea treatment and supply the novel candidate for rosacea’s medicine design.

Ⅲ－Skin Microbial distribution and Biophysical Parameters in Chinese female

A-Microbiology of Skin Surface

One of the chapters of the book “Measuring the Skin”

A.1 Introduction

The human skin is the largest organ of the human body. It protects the underlying tissues and plays an important role as a frontline defense system against invading pathogens and external environmental factors (125). It is colonized by a unique and complex microbial ecosystem, including bacteria, fungi, and bacteriophages, some of which could become pathogenic under certain conditions. The skin microbiome is very complex (23). Hundreds of different microbial species reside on the whole surface of the skin. Its composition and distribution are uniquely different from the flora of other organs. There are always differences among different people and different areas of the skin on the same person. Recent 16S rRNA-based methods revealed similar situations (126).

Skin flora are usually nonpathogenic. They are not harmful to their host and offer benefits. The benefits bacteria can offer include preventing transient pathogenic microorganisms from colonizing the skin surface, either by competing for nutrients, secreting chemicals against them, or stimulating the skin’s immune system. The microbial composition and distribution of the human skin microbiota have been associated with multiple skin diseases,

including atopic dermatitis (AD), acne vulgaris (AV), psoriasis vulgaris (PV), rosacea, dandruff, seborrheic dermatitis, etc.

A.2 The Skin Flora

The human skin flora, more properly referred to as the skin microbiome, are the microorganisms which reside on the whole human skin. Most studies have been carried out on those that reside upon the $2m^2$ of human skin (cf. the human microbiome). Many of them are bacteria of which there are around 1,000 species from 19 phyla upon the human skin. The total number of bacteria on an average human has been estimated at one trillion. Most are found in the superficial layers of the epidermis and the upper parts of hair follicles.

Staphylococcus epidermidis and other coagulase-negative staphylococci have been regarded as the primary bacterial colonizers of the human skin (127). Other microorganisms that are generally regarded as skin colonizers include coryneforms of the phylum Actinobacteria, for example, the genera Corynebacterium, Propionibacterium, and Brevibacterium and the genus Micrococcus. Gram-negative bacteria, with the exception of some Acinetobacter spp., are generally not isolated from the skin, but are thought to arise in cultures owing to contamination from the other organs, such as the gastrointestinal tract. Non-bacterial microorganisms have also been isolated from the skin. The most commonly isolated fungal species are Malassezia spp., which are especially prevalent in sebaceous areas (128). The Demodex mites, such as Demodex folliculorum, Demodex brevis, etc., are regarded as part of the normal skin flora. The living of Demodex mites

depend on the amount of sebum. They are much more prevalent following puberty and reside at sebaceous areas of the face. Demodex mites may also feed on epithelial cells lining the pilosebaceous unit or even on other microorganisms (such as Propionibacterium acnes) that inhabit the same space (129). The role of commensal viruses has not been published, and investigations are limited by the available molecular and microbiological means to identify and characterize viruses. Historically, culture-based approaches have been the standard for characterizing microbial diversity. It is now evident that only a minority of bacteria are able to thrive in isolation (130). Furthermore, hair follicles and sebaceous glands are the typical anoxic environments where some of the anaerobic microorganisms reside.

A.2.1 Healthy Skin

The skin represents an interesting human habitat in which life style and environmental factors shape the microbial community of different specific body sites. No taxa are ubiquitously present in every subject and body site, although targeted studies reveal that specific body sites are generally dominated by certain de fining taxa.

The human skin is mainly comprised of Actinobacteria, Proteobacteria, and Formicates, some studies finding that more than 90 % of the microbiota of the forearm belonged to these phyla (131). The volar forearms of different subjects were found to only share 2 % of species level operational taxonomic units (OTUs), whereas the hands share 13 % of OTUs (132). Estimates of species level OTUs for skin sites include the volar forearm, more than 150 for the palms, and the inner elbow. More than 50 % of sequences obtained from arm skin sites belong to Propionibacterium,

Corynebacterium, Staphylococcus, Streptococcus and Lactobacillus species (133).

A.2.2 Special Area Skin

The skin microbial community also plays an important role in the formation of body odor in, for instance, the vulva, axillae, etc. Few molecular-based researches were done on the axillary microbiome. Callewaert et al detected the axillary microbiome of a group of 53 healthy subjects (134). A profound view was obtained of the interpersonal, intrapersonal, and temporal diversity of the human axillary microbiota. Denaturing gradient gel electrophoresis and next generation sequencing on 16Sr RNA gene region were combined and used as extent to each other. Two important clusters were characterized, where Staphylococcus and Corynebacterium species were the abundant species. Females predominantly clustered within the Staphylococcus cluster (87 %, n = 17), whereas males clustered more in the Corynebacterium cluster (39 %, n = 36). The axillary microbiota was unique to each individual. Left-right asymmetry occurred in about half of the human population.

The inter- and intra-individual differences in bacterial flora on the vulvar skin are known to exist. Aly et al. used a cultivation method to show that microbial counts are higher on the vulvar skin than on the forearm skin and that S. aureus normally inhabits the vulvar area (135). Brown et al. detected bacteria, such as S. epidermidis, S. aureus, P. acnes, Lactobacillus spp., Prevotella spp., etc., identified on the vulvar skin using the16SrRNA gene-based clone library method. The number of total bacteria and the predominant species, such as S. epidermidis and Lactobacillus spp., were

higher in the labia than those at other sites. There were only 60 % of subjects with S. aureus detected (136). Prevotella spp. were more predominant in the labial skin than in the vaginal skin (137). The species of the genus Lactobacillus are predominant in the labia minora of Japanese women, identified using the 16S rRNA gene-based clone library method (138). Prevotella spp. were detected in the labia and groin of 95% of Japanese subjects by another study, so it is indicated that Prevotella spp. play a key role in vulvar skin conditions (139).

A.3 Diseased Skin

A.3.1 Atopic Dermatitis

Atopic dermatitis (AD) is a common, multifactorial, fluctuating, chronic inflammatory skin disease with a genetic predisposition. AD is often associated with atopic conditions such as asthma and IgE-mediated food allergy, which can be triggered by different allergens and various environmental factors. The relevance of the colonization of the skin with bacteria, such as S. aureus, and fungi, such as Malassezia furfur, Pityrosporum orbiculare, and Candida albicans, to AD has been controversial over the past few decades. Children with AD often have infective exacerbations which are treated with antibiotics and/or antiseptics.

The most common infective cause is S. aureus with a trend toward antibiotic resistance. S.aureus is one of the important microorganisms of normal skin flora. Bacterial skin flora of patients with AD differ from that of healthy people.

AD skin provides a favorable environment for colonization and proliferation of S. aureus. Skin colonization with S. aureus is more in the lesional skin than in non-lesional skin and is minimal in the skin of healthy children. All of these published data have shown that there are significant differences between S. aureus in the lesional skin and non-lesional skin ($P < 0.01$) (Table 17).

Haslund et al. confirmed the important role of colonization with S. aureus as an aggravating factor in AD, as there was a significant correlation between the severity of AD and S. aureus skin colonization (140). The results of their study were in agreement with other results (Table 18). Several studies demonstrated that the colonization of the skin with super antigen-producing Staphylococcus aureus is associated with increased severity of AD. It has been shown that AD may be aggravated by the direct biological action of bacteria or their products or by an immunological reaction to bacterial antigens or super antigens.

Table 17 Colonization of S. aureus in AD skin (%)

Reference	Lesional skin	Nonlesional skin t1
Miyamoto et al. (2013)	65.0	30.0
Gomes et al. (2011)	57.0	43.0
Al-saimary et al. (2005)	69.7	30.3
Pezesk et al. (2007)	42.5	57.5
Hon et al. (2005)	48.5	51.5
Matsui et al. (2005)	86.0	14.0
Guzik et al. (2005)	100.0	0

Table 18 Relationship between severity of AD and skin colonization (%)

Severity	Gomes et al. (2011)	Haslund et al. 2009
Mild	46.0	48.0
Moderate	73.0	0.0
Severe	100.0	77.5

The studies had shown association between lesional skin S. aureus colonization rates with increasing age. The colonization rate in this study was 41.4 % (12 out of 29) in the youngest group, 81.8 % (18 out of 22) in the second group (>2–12 years), and 100 % (9 out of 9) in the third group (>12 years) (141). The study had also shown that the colonization rate was 61.1 % (22 out of 36) in females and 70.8 % (17 out of 24) in males. There are no differences on the skin colonization rate between the two sexes.

Malassezia species are confirmed to be involved in the development of skin lesions in AD; sometimes the response of adult AD to anti-inflammatory treatments is poor. Takahata et al. collected scale samples from skin lesions of 58 patients with AD in the head and neck regions (28 males and 30 females; 31 children and 27 adults), and fungal DNA was extracted from the samples directly (142). The number and identities of the Malassezia species were analyzed with high accuracy using a polymerase chain reaction-based culture-independent method. The in vivo level of anti-Malassezia IgE antibody was also assayed. The results had shown that Malassezia restricta was the predominant species in the children with AD, while both M. restricta and M. globosa predominated in the adults. The increased sensitization in terms of anti Malassezia-specific IgE responses in the sera to both M. globosa and M. restricta from adults was comparable to that from children.

There are some differences of the cutaneous Malassezia flora between adults and children with AD.

AD skin lesions are characterized by a Th-2 cell-mediated response to environmental antigens (143). The increasing prevalence and severity of atopic diseases including AD over the last three decades have been attributed to decreased exposure to microorganisms during early life, which may result in an altered Th-1/ Th-2 balance and/or reduced T cell regulation of the immune response (144). The patients with AD exhibit defects in innate and acquired immune responses resulting in a heightened susceptibility to bacterial, fungal, and other microorganism colonization, most notably colonization by S.aureus. Toxins produced by S. aureus exacerbate disease activity by both the induction of toxin-specific IgE and the activation of various cell types including Th-2 cells, eosinophils, and keratinocytes. Allergens expressed by the Malassezia furfur have also been implicated in disease pathogenesis in some of AD patients. Microorganisms play an influential role in AD pathogenesis, interacting with disease susceptibility genes to cause initiation and activation of disease activity.

The relevance of specific IgE antibodies for AD is still under discussion. Several experimental studies focus on a link between allergens and AD by IgE-independent mechanisms. There is increasing evidence of a relationship between microorganisms and the deviation of immune responses. A high correlation between a positive patch test with milk and delayed-onset reactions due to milk provocation tests in children was found, while milk-specific IgE antibodies were relevant only for immediate type reactions. Some clinical studies had shown that the IgE-mediated

sensitization to P. ovale for the prediction of a therapeutic effect of ketoconazole in the treatment of AD using patch tests with P. ovale may be more useful. Positive patch test reactions to P.orbiculare have been demonstrated in atopic patients (145). In the case of positive patch test results with food and mite allergens, patients may find relief by avoiding allergen exposure. The treatment of children with AD with specific probiotic bacteria strains reduces the eczema severity.

Many researchers found that some clinical features are the special features of AD, such as miliaria of AD, which causes the itching, the most common symptom of this dermatitis, which occurred in areas of sweating, even in areas where patients did not realize they perspired (146). The epidemiological study results found that sweating was a significant factor in exacerbating eczema. Miliaria arises from blockage of the eccrine sweat ducts. The material causes the blockage of duct. It is the film-like materials, which contain film-producing S. epidermidis, extracellular polysaccharide substance, and filaggrin-deficient stratum corneum. So the sweating and sweat retention are the important information of flare factors. The subclinical miliaria provoked by film-producing S. epidermidis as part of a “double-hit” phenomenon fits well in the pathogenesis of AD.

The microbiology of AD skin is dealt with the causes, mechanism, treatment, and prevention. This is the new insight to help us research and formulate the treatment strategy for AD.

A.3.2 Acne Vulgaris

Acne vulgaris (AV) is the most common, multifactorial, chronic inflammatory follicle disorder affecting much more individuals all over the world. It is a global disease that has no predilection for a specific race or gender. More than 60 % of the population suffers from AV at some point in their life. Four basic mechanisms contributing to acne are hormones, increased sebum production, changes inside hair follicles, and bacteria. The most commonly cited theory regarding the pathogenesis of acne states that increased sebum production leads to alterations in the lipid composition of hair follicles.

P. acnes is a species of bacteria that is implicated frequently and acts as the key player of the acne genic microbes, not only eliciting inflammatory lesion response but also the important pathogenesis of the whole mechanism of the disease (147). P. acnes can be recovered from skin surface as well as follicles on both normal and acne skin. There is also no correlation between the number of P.acnes within a lesion and the clinical features of acne, both the types of lesion and severity of disease. But P.acnes is able to metabolize triglycerides into free fatty acids and glycerol, which is an immunological stimulant and/or a cytotoxic agent that leads to breakage in the follicular epithelium (148). Other proposed P. acnes virulence factors include enzymes that are involved in adherence and colonization of the follicle (149). Other bacterial species detected frequently from pilosebaceous units and acne lesions are S. epidermidis and Propionibacterium species. They are often significantly less abundant than P. acnes in acne lesions, but their abundance appears to correlate with clinical severity of acne. In addition to bacterial species, another microbial group is the fungal genus Malassezia,

which has been implicated in the pathogenesis of seborrheic dermatitis and dandruff; their link to acne remains far more speculative (150).

A.3.3 Psoriasis Vulgaris

Psoriasis vulgaris (PV) is a common, chronic, relapsing/remitting, immune-mediated skin disease characterized by red, scaly patches, papules, and plaques, which usually itch. The skin lesions seen in psoriasis may vary in severity from minor localized patches to complete body coverage. The prevalence of psoriasis is around 2-4 % of the general population. The clinical characteristics are red, scaly patches on the skin. The manifestation of psoriasis includes hyperkeratosis, hyper proliferation of keratinocytes, infiltration of the skin by immune cells, and angiogenesis. The most commonly affected skin areas are elbows and knees.

Several bacterial species, including S. aureus and Streptococcus pyogenes, have been suggested to play a role in the pathogenesis of psoriasis (151). Fungi, including M. furfur and Candida albicans, have also been linked with the development of psoriatic skin lesions and play a role in the pathogenesis of PV (152).

The overall bacterial diversity of the microbiota in the psoriatic lesions is greater than in normal skin samples. There are significant differences in the distribution of the three major bacterial phyla in the human skin biota: Actinobacteria, Firmicutes, and Proteobacteria. Firmicutes are overrepresented in the lesion whereas the other two are underrepresented. The distribution of dominant bacterial phylum in lesional skin and nonlesional skin of patient with PV (%) is shown in Table 19 (131).

Table 19 Distribution of dominant bacterial phylum in patient with PV (%) (Gao et al. 2008)

Bacteria	Nonlesional skin	Psoriatic lesional skin
Actinobacteria	47.6	37.3
Firmicutes	39.0	46.2
Propionibacterium	21.9	11.4

A.3.4 Others

The genus Malassezia (Pityrosporum), recognized as a member of microbiological flora of the human skin, has been recently revised to include Malassezia species. The pityriasis versicolor (PV) is one of the common skin diseases caused by the Malassezia infection (153). The results of Salah's study had shown Malassezia globosa was the predominant species in lesional skin of PV (65 %)(154). It was isolated alone in 47 % of cases and associated in 18 % with M. furfur (13 %) or M. sympodialis (5 %). In the healthy skin, M. globosa was found alone in 7.77 % and associated in 15.54 % with M. furfur (4.44 %), M. sympodialis (4.44 %), M. restricta (3.33 %), and M. slooffiae (1.11 %). M. globosa presents the main species implicated in the pathogenicity of PV and M. furfur as the second agent of importance.

The vulvar skin is the special area of females. The bacterial population of vulvar skin is characterized by a high density of microorganisms that are related to flora of the vagina and urethra, such as Lactobacillus spp., or are common on other areas of the skin, such as Staphylococcus epidermidis and Staphylococcus aureus. Miyamoto et al. studied the vulvar skin of healthy Japanese women and understood microbes of the stratum corneum

(139). A total of 40 subjects were quantified. The detection ratio and number of skin bacteria at the three test sites, labia and groin, mons pubis, and inner thigh, were taken. The labia and groin had significantly (>10-fold) more bacteria than the other sites. Lactobacillus spp. and S. epidermidis were the predominant species at all sites, followed by S. aureus. Propionibacterium acnes was present in almost all subjects but was less abundant than S. aureus, which was present in about 50 % of subjects. Prevotellaspp. were detected in the labia and groin in almost all subjects but not in other sites.

Gardnerella vaginalis is one of the common skin diseases at the genital area (155). Myhre et al took the samples from 278 (99 boys and 179 girls) out of 3,773 children, with a mean age of 5.63 years (range: 5.13–6.73), and found that at least one bacterial species was isolated from the genitals of 59 (33.9 %) girls (156). Most isolates (39 out of 99) were bacteriare presenting skin flora (staphylococci and coryneform organisms), with viridans streptococci and related organisms as the second most common group of isolates (31 out of 99). S. anginosus was the single most frequent bacterial species identified (17 isolates). Streptococcus pyogenes was isolated from the genitals of two girls, Streptococcus pneumoniae from one girl, and Haemophilus influenzae from eight girls. G. vaginalis was not isolated from the genitals in any girl, but the organism was isolated from the anal canal in three children. The results had shown that a large number of different aerobic organisms from children were identified from the genital area. G. vaginalis was rare and only isolated from the anal canal.

The skin flora is influenced by some system diseases (157). Mean colony forming units were 160.6, forearm, and 229.4, sternum ($P < 0.000$). In

logistic regression analysis, patients in the medical intensive care unit were significantly more likely to have high counts on the arm (odds ratio, 2.48; 95 % confidence interval, 1.34–4.43; P = 0.004), and blacks were significantly more likely to have higher counts on the sternum when compared with other ethnic groups (odds ratio, 1.92; confidence interval, 1.18–3.11; P = 0.009). No differences were noted between inpatients or outpatients in prevalence of methicillin-sensitive Staphylococcus aureus, but inpatients were more likely to carry methicillin-resistant Staphylococcus aureus (arm, P = 0.007; sternum, P = 0.02). Outpatients had a higher prevalence of micrococci and gram negative bacteria at both skin sites (all P < 0.01) and yeast at the sternal site (P = 0.007). This comparison provides data to differentiate between effects of hospitalization and effects of chronic illness on skin flora.

A.4 Skin Flora Influence by Skin Surface pH

The acidic pH of the horny layer, measurable on the skin surface, has long been regarded as a result of exocrine secretion of the skin glands (158). The “acid mantle” was thought to regulate the bacterial skin flora and to be sensitive primarily to skin-cleansing procedures. The pH of the deeper layers of the stratum corneum changes with the influence of physiological and pathological factors (159). The central role for the acidic milieu as a regulating factor in stratum corneum homeostasis is now emerging. This has relevance to the integrity of the barrier function, from normal maturation of the stratum corneum lipids to desquamation. Changes in the pH and the organic factors influencing it appear to play a role, not only in the

pathogenesis, prevention, and treatment of irritant contact dermatitis but also of atopic dermatitis and ichthyosis and in wound healing (160). On the basis of these findings, a broader concept, exceeding the superficial “acid mantle” theory, has been formulated.

Microbiology of skin surface deals with skin health care and skin disease treatment and prevention. So healthy skin, abnormal skin and skin diseases, and the skin microbiome and its diversity are all being the hot topics of dermatology.

B-Evaluation of Skin Surface Flora

One of the chapters of the book “Measuring the Skin”

B.1 Introduction

The microorganisms, such as bacteria, fungi, etc., on the skin have been identified mostly by culture based on the sampling methods. The composition and distribution of the microorganisms on the skin were not extensively described until culture independent molecular methods were used. The main techniques in sampling the skin microorganisms include impression methods, swabbing methods, and washing methods and punch biopsy. Both swabbing and taping are simple, quick, and noninvasive. Other two methods are scraping and punch biopsy which are applied under certain situation. Scraping usually comprises significant amount of skin cells. Punch biopsy is invasive, and it can cover all layers of the skin microbiota. There are two kinds of specific sampling techniques for follicle of the skin. Each category included several techniques. For example, the impression methods included contact plates, pads, sell tape stripping, etc. The washing methods included the detergent scrub technique and the sterile bag technique. About these three different sampling techniques, such as swabbing, scraping, and punch biopsy, the results showed that there was no difference of detection at all depths of the skin (161). There are special methods for follicular sampling methods. They are comedone extractor and cyanoacrylate glue.

B.2 Methods of Sampling

There are several sampling methods for microorganism of skin surface. How to choose the method for applying it depends on the numbers and types of bacteria. For all surface sampling methods, adequate time is needed after sample collection to allow the bacterial flora to reestablish before a further sample can be taken from the same site. Another way is sampling bacteria from adjacent sites or from identical sites on the right and left side.

B.2.1 Impression Methods

B.2.1.1Contact Plates

The contact plates are specialized Petri dishes that are filled with different appropriate culture media until the agar surface is slightly concaved. The different bacteria should be under different culture conditions in suitable media. The common culture medium is fresh blood agar.

The contact plates are pressed firmly onto the skin to remove surface bacteria. One study was used to sample the medial surface of the forearm and midsternum because they are representative of the “dry” areas of the skin surface that are readily accessible and in contact with the environment (162). These are also frequent sites of intravenous (IV) catheterization (arm) or surgery (sternum). Generally the dominant arm was sampled, but in inpatients, attempts were made to avoid obtaining cultures near IV sites.

When a mediastinal incision was present, the upper back was sampled rather than the sternum.
It is limited to one recovery medium. The facilitated colony counting is mainly a method to assess the results of this test. It only gives the estimation of the number of the micro colonies. The number of colonies (colony-forming units) is counted with a grid printed or glued on its base. It is not a quantitative way because there is no dispersal step to break down aggregates of cells into smaller colony-forming units.

However, these have severe limitations and should only be used if a specific organism is being sought or if low numbers of bacteria ($\leqslant$10 cm^2) are expected and they can all be cultured on the same medium. The method is suitable for isolating S. aureus from suspected infected eczema. It is easy and quick to use on intact and broken skin. The method of contact plates can be employed for routine patient sampling. But it is not quantitative. Since these colonies are not dissociated, the density of obtained bacteria does not correspond to the density of bacteria on the skin.

B. 2.1.2 Pads
The velvet pads are used to remove bacteria from the skin surface. The main advantage of it is that the sufficient microorganisms are removed to serially inoculate a number of different culture media. The method is very inefficient too, and only a small proportion of microorganisms are success fully transferred from the pad to the medium. Since mechanical rinsing could improve bacterial recoveries, the pads are no longer used directly to inoculate culture media. The quantitative estimate of bacterial numbers could be obtained.

B.2.1.3 Tape Stripping

Tape stripping is a common method for skin microbiological study since it could sample not only skin surface but also aerobic bacteria residing in the upper part of the epidermis. Mostly all areas of the skin contain numerous pilosebaceous follicles. Some microorganisms are pulled out from the upper portion of each duct so that the numbers of bacteria may not decline in subsequent strips, as they do in areas with few pilosebaceous units. The tape strips are inverted onto the surface of the culture medium. If the tape is removed, not all microorganisms are transferred to the culture media successfully. The combination of tape stripping and contact plates is often used for skin sampling. The tape is used to remove successive sheets of epidermal cells, and the contact plates are used to remove the exposed bacteria.

B.2.2 Swabbing Methods

The swabbing methods include dry swabs and moist swabs. The dry swabs are associated with poor recoveries of viable organisms. The moist swabs are among the most versatile of skin sampling techniques. Normally the swab, fixed to an applicator, is soaked in phosphate buffer saline containing 2 % Tween 80 and 0.3 % lecithin or Williamson-Kligman washing fluid. A template delimiting the sampling area is put on the skin. The swab is vigorously rubbed inside the template. Then the buffer is inoculated to appropriate culture media. The number of colonies in each culture can be counted. If density is too high, it makes counting impossible, and serial dilutions are done before inoculation. The sensitivity limit is 4 CFUs per template. The accurate quantitative results cannot be obtained by the

swabbing methods. There is no doubt that moist swabbing is the method of choice for skin surface sampling. It remains the most commonly used method for routine sampling of patients with diseases, infections, or wounds of the skin. It can detect the unknown pathogen of the above skin disease. They are semi-quantitative. But the method can be used when few microorganisms are present or when the swabbed area can be accurately defined and the bacteria of interest are known to reside superficially. In other words, it inoculates several culture media with a single swab and thus makes it possible to detect the entire range of skin flora present, and finally bacterial counting is possible since the original colonies have been dissociated by shaking in the buffer and flora collection has been improved by the friction. Usually skin swabs could be collected both from skin lesions and non-lesional areas from patients with skin diseases (e.g., AD) and also from healthy control skin (Petry et al. 2014). Two skin swabs were taken from each patient for culture and sensitivity, one from the worst area of atopic dermatitis and the other from non-lesional skin. Also 15 skin swabs were taken from the skin of healthy children. Specific swabs for this method exist in various materials, such as polyvinyl alcohol foam, cotton, rayon, calcium alginate, etc. And they are commercially available. There are severe limitations, and for seeking the specific microorganism or the lower numbers of bacteria ($\leqslant$10 cm^2), they can be cultured on the same medium. They depend on the types of swab used, and the procedures are used to transfer microorganisms to the culture media. Several recovery media can be inoculated immediately, both in the clinic or laboratory. All types should be moistened by phosphate-buffered saline. The area of the sample site can be standardized by holding a template onto the skin surface. The swabs rub the studied area of skin surface firmly and repeatedly for several seconds to

ensure adequate removal of microorganisms. For semiquantitative work, the swabs transfer to 1 ml of half-strength wash fluid and decimally diluted in the same. A fixed volume (usually 100 ml) of each dilution and the undiluted sample is then plated onto one or more suitable recovery media and spread with a sterile glass spreader. The correct use of swabs is the best or only possible methods of skin sampling. They are used for routine clinical or research sampling from any sites, e.g., the back, chest, forehead, shoulders, etc. The inoculum is put on the suitable medium for culture.

For research the swabbing techniques can be standardized, whether in intertriginous areas or on damaged skin whichever is interesting. It can be an identification of components of the aerobic skin flora of premature neonates.

B.2.3 Washing Method

B.2.3.1The Detergent Scrub Technique

The detergent scrub technique is the most widely used for research purposes since this method could be standardized, quantitative, reproducible, and efficient (i.e., removes over 95 % of the aerobic bacteria present at the sample site). Several modifications and adaptations have appeared in different studies. A metal ring is held firmly against the skin surface and the procedures are standardized, including the wash fluid, the amount, the time of rubbing, the collection of wash fluid, etc. There are several features of the scrub wash technique that are worthy of further comment. The wash fluid contains a mild detergent in order to facilitate dispersal of clumps of bacteria. Various modifications ford the survival of different skin bacteria in wash fluid within conclusive results. It is best for

individual investigators to estimate for themselves the survival time in wash fluid of those organisms of special interest to them. The sample site is chosen because the subungual space harbors large numbers of bacteria and is one of the most difficult sites to disinfect. Several recovery media can be employed. The detergent scrub technique is more efficient in terms of number of microorganisms recovered or in reproducibility. For research purposes, and when quantitative data are required, the detergent scrub technique should be chosen. The major limitation of this technique is that it is fairly aggressive and cannot be used on sensitive or damaged skin, although several groups have used it to sample bacteria from eczema lesions. It is suitable for determining the proportion of the resident staphylococcal flora resistant to an antibiotic.

B.3. Follicular Sampling Methods

Some researchers with an interest in the pathogenesis of pilosebaceous follicle use methods that facilitate sampling of intrafollicular organisms. The methods that will be described below can be used to sample non inflamed lesions only and are not suitable for use with normal follicles or inflamed lesions.

3.1 Comedone Extractor

Open comedones are removed non traumatically using a comedone extractor. It is the best method of sampling intrafollicular bacteria. Microcomedones are more common, but it is not easier than rapidly polymerizing cyanoacrylate glue. Both open comedones and

microcomedones can be obtained noninvasively. This method for diseased follicles is not similar to the method for normal follicles. The standard procedure is as follows. The first step is sterilizing the skin surface with an isopropanol swab. Then the entire comedone is removed by the extractor. It is transferred by a sterile needle into a preweighed microcentrifuge. The amount of wash fluid is pipetted into the tube and the bacteria are dispersed from the comedone with the micro tissue grinder. The fluid according to the different treating procedure is then plated onto one or more selective or nonselective media as required.

Bacteria can be counted and expressed as CFU per milligram wet weight of comedonal material. The detection limit is 4 CFU/comedo. However, it should been ensured that the whole of the comedone is removed from the skin because the distribution of bacteria in follicular ducts varies with depth. The density and composition of the microflora on the face. It is a simple and quick procedure. It can study micro flora of a single pilosebaceous unit. It can inoculate several recovery media.

B.3.1.1 Cyanoacrylate Glue

Rapidly polymerizing cyanoacrylate glue is used to remove thin sheets of stratum corneum. It can be quickly realized since follicular plugs are extracted from pilosebaceous follicles as the glue is pulled away from the skin surface. This method is very easy. It is not used for normal follicles. The first drop of glue is spread over an area of the skin and left to polymerize for 1 min. The second drop is pressed on a glass slide for 1 min and then the slide from the skin is slowly removed. It is then applied on top of the area of the skin and spread uniformly by inverting a glass slide over it and pressing down firmly. After several minutes, the slide is removed from the surface of

the skin with the adherent sheet of both adhesive and follicular casts, which represent the contents of microcomedones and consist of a mixture of corneocytes, sebum, and microbes. The more standardized procedure uses a sterile glass sampler of known surface area instead of a slide and a sterile Teflon ring to delineate the target area, which is extracted twice with glue. It can inoculate several recovery media. Normal pilosebaceous follicles cannot be studied by either of these two methods, but only microdissected follicles from biopsies. It can collect the entire comedo or cyst because the flora of the follicular duct varies according to depth. It can counter analyze several samples because the flora can be very different from one follicle to another.

One commercial kit, such as Exolift®, includes a patented dermal tape and cyanoacrylate glue (163). It is easier to use than glass slides or samplers, but is much more expensive. Whichever procedure is followed, only follicular bacteria will be enumerated since surface organisms are sequestered between the glue and the thin sheet of stratum corneum. The main problems with the use of cyanoacrylate glue are the high frequency of incomplete takes, when the glue fails to polymerize properly over part of the sample site, and the uncertainty of removing entire follicular casts. For obvious reasons, the method should not be used near the eyes.

B.3.1.2 Impact Factors

There are some important factors in determining the choice of sampling method (164), location of bacteria, type of the skin, type of bacteria, choice of sample site, efficacy of technique, reason for sampling, etc. The different growth media are suitable for recovery of resident skin bacteria and primary

pathogens. For example, the brain heart infusion or reinforced clostridial agar containing 6 mg/l furazolidone is suitable for Propionibacteria; heated blood agar for coagulase-negative staphylococci; mannitol salt agar and cysteine lactose electrolyte deficient (CLED) medium for 350 S. aureus; fresh blood agar containing 0.2 % w/v glucose,0.3%w/v yeast extract, 0.2%v/vTween 35280, and 6 mg/l furazolidone for aerobic coryne forms ; and fresh blood agar containing 0.0002 % 354crystal violet or 7.5 mg/l nalidixic acid and 17 units/ml polymyxin B for group A beta-hemolytic streptococci . Antibiotic sensitivity test can be done according to the Clinical and Laboratory Standards Institute (CLSI, 2011), using vancomycin, erythromycin, gentamicin, penicillin, ampicillin, fusidic acid, and flucloxacillin antibiotics (r1).

B.3.1.3 Application of New Techniques

Recently the molecular characterizations of the human surface skin microorganisms based on 16S ribosomal RNA analysis have been carried out on a large scale (131). For the fungi, such as yeast, the specific techniques of culture are used and the 18S ribosomal DNA analysis has been done. Molecular analyzing was used for some studies, not only qualitative but also quantitative. The polymerase chain reaction restriction fragment length polymorphism method (PCR-RFLP) of Malassezia species, a part of the skin micro flora of neonates, was applied. The results supported that neonates acquire Malassezia flora through direct contact with their mothers or hospital personnel. For quantitative analysis of Malassezia, the real-time polymerase chain reaction (PCR) assay could be used also (165). The dominant operational taxonomic units (OTUs)

conducted often are captured by all these methods although the rare OTUs are different. Commensal bacteria play a crucial role in the development of the immune system in humans (166).

The objectives of the evaluation of skin surface flora are to study the pathogenic flora carriage in patients, the effects of antibiotics and antiseptics on skin flora, the efficiency of skin disinfection methods, and the physiology of resident skin flora under different environmental conditions. So understanding the methods of evaluation, knowing their impact factors, and applying new techniques are the means of achieving these aims.

C-Skin Microbial distribution and skin Biophysical Parameters in Chinese female

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C.1 Introduction

The intricate structure of the cutaneous system represents an ecosystem harbouring a multitude of microorganisms including bacteria, fungi, viruses, and mites, collectively referred to as the human microbiome (126). These microflora are in equilibrium with the host innate immune system and maintain homeostasis (167), which when altered directly impacts skin health (164). Skin microbial imbalance or a shift in the abundance of resident microbial taxa may be a determining factor in various disorders such as acne (168), atopic dermatitis (169), and psoriasis (152).

The skin is a critical barrier between the body and the external milieu comprising a “physical barrier” (environment, surface pH, lower temperature, acidic nature, timely desquamation, and tight junction proteins) (170) and a “chemical barrier” (host defense molecules released by keratinocytes such as anti-microbial peptides [AMPs] [e.g. defensins, cathelicidin LL-37, and dermcidin], cytokines, proteases, lysozymes, and chemokines) (171). These barriers safeguard against pathogen invasion and colonization (172). The human microbiome is dynamic and exhibits diversity within and across

individuals, which is attributable to genetic and demographic properties, age, gender, ethnicity, skin type, lifestyle, hygiene, geographical differences, environmental stress (temperature, moisture, seasonal variation, radiation exposure) and cohabitation with other animals (173). Studies have also suggested that biophysical parameters (such as surface pH, hydration, sebum content, transepidermal water loss [TEWL] and barrier function) vary with the age, gender, and body site, which in turn influence microbial composition (174). Bacterial colonization relies on the physiology of skin and is influenced by invaginations, appendages, and the skin micro-environment (e.g., humid, dry or sebaceous, exposure to macro environment) and has an impact on skin health (175). Also, commensal microbes, which prevent colonization of opportunistic or pathogenic organisms, produce AMPs and play a critical role in modulating both innate and adaptive immune response1 (176).

Comprehensive understanding of the topographical and temporal diversity of the skin microbiome and associated biophysical parameters may unveil the relationship between skin health and disorders. Furthermore, it can also aid in understanding subclinical skin changes, which may help in identifying the role of prebiotics and/or probiotics in skin disorders (e.g. acne), wound healing, and photoprotection (177). In addition, dermatological treatment should be tailored to population-specific approach, avoiding extrapolation from global studies or from dissimilar populations.

Previous studies have reported that the skin of Chinese populations has distinct microbiomes and Actinobacteria (Propionibacterium, Corynebacterium and Micrococcus), Firmicutes (Staphylococcus and

Lactobacillus), Proteobacteria (Pseudomonadaceae), and fungi (Malassezia) as commonly occurring microbial phyla with varying relative abundance at different skin sites (178). There are few studies that evaluated association of predominantly-occurring microorganisms with biophysical parameters, biomarkers, and distinct skin environments. Hence, the present study was conducted in Chinese women to evaluate cutaneous microflora distribution and their co-occurrence in different body sites, as well as in different skin environments, and association of this microbial distribution with biomarkers and skin biophysical parameters.

C.2 Methodology

Study participants

Healthy women between 20-60 years who resided within the city area were included in this study conducted in Shanghai Skin Disease Hospital, China from February 2012 to March 2012 (during winter season). The average high and low temperatures during the study period ranged between 8-13°C and 1-4°C, respectively, with ~79% relative humidity. Participants who had not received antibiotics three months before the sampling and who were willing to avoid any other medicine during the test period were recruited. Participants were asked to select a test time-point that did not overlap with their menses. Key exclusion criteria included involvement with other clinical research in the last three months, pregnant or lactating women, presence of any skin ailments (atopic dermatitis, psoriasis and stasis eczema), scar,

inflammation or tattoos, which might interfere with findings of the current study.

Before the study, participants were asked to complete a self-assessment questionnaire that included basic information, habits of life, family medical history and their perception on skin situation (itches, stinging, burning, dryness, and scaling) at the selected body sites. Included participants were instructed to bathe with only water and to avoid using any personal hygiene products during the two day wash-out period. Moreover, washing the body sites chosen for the study was not allowed for 12 h (except 4 h for hands) before sampling. Swimming in chlorinated pools, or use of hot water/sauna/tanning bed was avoided.

The study protocol was approved by the Scientific and Ethical Committee at the Shanghai Skin Disease Hospital and the study was conducted in accordance with the Declaration of Helsinki Principles. All participants provided informed consent to participate in the study.

Study Design

This observational single-centre study evaluated cutaneous microbial distribution of six microorganisms within the family, genera or species of Propionibacterium acnes [P. acnes], Staphylococcus aureus [S. aureus], Staphylococcus epidermidis [S.epidermidis], Lactobacillus, Pseudomonadaceae, and Malassezia furfur [M. furfur] on six selected skin sites (glabella, GL; hand-back, HB; interdigital web space IS; antecubital

fossa, AF; volar forearm, VF; back, BA) representative of different skin type (classified as normal, oily and dry) and exposure status (based on seasonal apparel). The sites were clinically assessed (skin aesthetics and dermal tolerance-related assessment grading) and evaluated for biophysical parameters TEWL, skin pH, sebum and hydration levels and surface evaluation of living skin [SELS] parameters) and biomarkers (AMPs such as: LL-37, β- defensins [HBD-2, HBD-3] and claudin-1) as detailed below.

Dermatological assessments

Aesthetic conditions and skin tolerance of the selected six skin sites were clinically evaluated by an independent dermatologist. Skin aesthetic conditions included tone, glossiness, hydration, sagging, and smoothness. Skin tolerance-related assessments included scales, dryness, redness, hemangiectasis, skin integrity, and skin lesions such as acne or spots. Clinical evaluations were conducted using 9-point scales (0 = most positive response and 9 = most negative response).

Evaluation of biophysical parameters

The measurement of biophysical parameters of the skin was performed using 6 different instruments (Courage and Khazaka electronic GmbH, Cologne, Germany): pH meter, skin-Glossymeter GL 200, Sebumeter SM 820 (measures the amount of sebum), Corneometer CM 820 (to assess epidermal hydration), Tewameter (to evaluate TEWL) and Visioscan® VC 98 (for qualitative and quantitative direct analysis of skin surface

topography)(179).

Assessment of cutaneous microflora diversity

Sample collection was performed at 6 skin sites (3 exposed sites: GL, HB, IS and 3 unexposed sites: AF, VF, BA) from each participant (left or right sides were chosen randomly). The sampling was carried out as three replicate swabs of six identified body sites from each participant during a week with a 1-day interval between each sampling (Monday-Wednesday-Friday). The sampling regions were swabbed for approximately 50 swabs each time with physiological saline in back-and-forth motion with firm pressure in a temperature and humidity controlled environment (18-22° C; relative humidity of 40-60%) and stored at 4° C to avoid organism growth post-sampling. DNA was extracted following the manufacturer's protocol (QIAamp DNA Microbiome kit 2016, Qiagen, CA, USA). To quantify the total skin bacteria and fungi, real-time quantitative polymerase chain reaction (RT-qPCR) testing was performed for all specimens by amplification of extracted DNA using specific primers and the Applied Biosystems 7000 Sequence Detection System (Foster City, CA). Primer and cycle details are given in the supplementary file (Tables 20). DNA sequencing was used to verify the PCR result.

Assessment of anti-microbial peptide (AMP) biomarkers

For evaluation of AMP biomarkers, specimens of stratum corneum were obtained from the skin of identified test areas of healthy participants by tape-stripping (5 times in same region and using last 4/5 tapes stored at −

20° C) with Corneofix® (F 20, Courage and Khazaka, Germany). Venous blood samples were collected from all the participants for detection of claudin-1.

Tape-strippings were analysed for the presence of biomarkers of AMPs (LL-37, β- defensins [HBD-2, HBD-3]). For quantification, LL-37 and β-defensins were extracted from last 4/5 tapes using 15 mL Tris buffered saline and the extract was kept at 4° C overnight. Next, each extract was filtered through polytetrafluoroethene (PTFE) membrane and the trapped corneocytes in the membrane filter were analysed using chemiluminescence immuno-detection method (VECTASTAIN® Universal Elite ABC Kit, Vector Laboratories, CA, USA). For detection of claudin-1, venous blood samples were evaluated with an ELISA kit (Immundiagnostik Bensheim, Germany).

Statistical analysis

Statistical analysis was performed with SPSS-17.0 (SPSS Inc., Chicago, IL, USA). Descriptive statistics by site was applied for demographic information, skin conditions, skin tolerance and aesthetics scores. Selected skin sites were differentiated as normal, dry, and oily by two-step clustering (SPSS 17.0) based on the best subsets of three skin physiology parameters [moisture, sebum, and trans-epidermal water loss (TEWL), which are considered the most representative parameters of skin micro-environment]15,25. In the cluster analysis, the pre-clusters were clustered using the hierarchical clustering algorithm based on the similarity in their pattern of correlation. Microbial diversity at different sites was

calculated using alpha diversity index (Shannon and Simpson index). Paired/independent T-test and ANOVA (Dunnett's comparison) and/or Friedman/Wilcoxon Test (for non-parametric data) were performed for each parameter to determine significant differences of subsequent readings between different sites.

Microbiome detection consistency was visualized to demonstrate microbial co-occurrence between different pairs of microorganisms. Consistency between two microorganisms was defined as the percentage of sites growing both or neither of them per total number of sites and is represented as a matrix in a scatter diagram depicted as a point-size. Cochran–Mantel–Haenszel (CMH) test was applied for each pair of microorganisms present in the matrix to test consistency in exposed or unexposed skin sites and oily, normal or dry skin type. Change in consistency (point-size) estimates and CMH p-value depicts the variation in microbial co-occurrence by skin type.

The association between skin microbiome, physiology, biomarkers with skin exposure status and skin type classification as dry, normal, oily was determined by regression analysis and the level of association was depicted by regression coefficient. For exposed and non-exposed skin sites, logistic regression analysis was used to identify factors among 6 skin physiology parameters and 4 biomarkers that potentially contribute to the occurrence of each of the 6 microorganisms (indicated as positive and negative association). For skin type classification (normal, oily and dry), linear regression was used to identify factors (defined as any other index, biomarker or microorganism) to every skin physiology parameters. Based

on the results from above regression analyses, influence of biophysical parameters and biomarkers, occurrence of microorganisms, site groups (model-based and exposed or unexposed groups) were visualized (R package igraph) as directed social network analysis diagrams to demonstrate all relationships together, wherein $p<0.05$ was considered as statistically significant.

C.3 Results

In total 100 Chinese women were enrolled in the study and the average age was 39.6 ± 11.9 years. All participants completed the study.

Clinical assessment at different skin sites

A total of 97 participants completed the questionnaire. Of these, as reported by participants, a higher frequency of skin problems were observed for unexposed skin sites (BA [56/97; 57.73%], AF [15/97; 15.46%], and VF [27/97; 27.83%]) compared to exposed skin sites (GL [2/97; 0.02%], IS [4/97; 0.04%], and HB [2/97; 0.02%]) (Fig. 24). Clinical assessment at different skin sites showed that the measurement scores (mean±SD) of skin aesthetic grading was lowest at unexposed BA region (1.19±1.19) and the highest in exposed HB region (4.40±1.45, Fig. 25.A). Scores of skin tolerance grading, where lower score denotes better dermal health, were overall lower for the exposed sites (IS: 0.81±0.98; HB: 0.68±0.94; and GL: 0.40±0.67) and higher for the unexposed sites (BA: 1.19±1.19; AF: 0.84±1.04; and VF: 1.20±1.11) (Fig. 25.B).

Evaluation of biophysical parameters

Biophysical parameters were evaluated to ascertain skin barrier properties which may have an impact on microbial distribution. The GL had significantly ($p<0.05$) higher sebum levels compared with AF, IS, VF and HB; higher stratum corneum hydration compared with VF and HB; and skin gloss compared with AF and IS. The mean pH of the skin was 5.3 (range: 5.26 GL to 5.63 HB) and was not significantly different among the sites. TEWL was significantly higher in IS ($p<0.05$) compared with AF and VF. Skin pH, skin roughness (SEr) and skin scaliness (SEsc) showed no significant differences between the 6 selected sites (Table 17).

Skin site cluster analysis and associated microflora

Based on the similarity in cluster analysis (Fig. 24) of core biophysical parameters (moisture, sebum, and TEWL) and the number of samples in each cluster, skin sites were classified into 3 clusters. ‘Normal’ (cluster 1; moisture, non-oily and strong barrier) representing: HB (95%), BA (87%), AF (95%), and VF (93%) sites; ‘dry’ (cluster 2; dry, non-oily and weak barrier): IS (99%); and ‘oily’ (cluster 3; moisture, oily and strong barrier): GL (90%).

Diversity of skin microflora at different skin sites

The occurrence of P. acnes, S. aureus, S. epidermidis, Lactobacillus,

Pseudomonadaceae, and M. furfur species varied with different skin sites. The lowest detection rate was observed for S. aureus in all 6 sites compared with other microorganisms, whereas, the highest detection rate was observed for Pseudomonadaceae, with a highest occurrence in IS region ($p<0.05$, compared with other sites, Table 18). A similar pattern was observed in the exposed as well as unexposed sites. Bacterial alpha diversity was higher in exposed sites (HB, IS, and GL) compared with the unexposed sites (BA, AF and VF). Shannon diversity index (H) progressively decreased in the order of IS>GL>HB>AF>VF>BA with diversity index value, H = [0.88 to 1.01] for exposed sites and H = [0.64 to 0.75] for the unexposed sites. Simpson index, a measure of dominance (D) of the species increased in the order of IS<GL<HB<AF<VF<BA (Table 18). As per the cluster analysis, S. epidermidis predominantly occurred in the oily and dry clusters, followed by Pseudomonadaceae and P. acnes, whereas Pseudomonadaceae occurrence was higher in the normal cluster (Table 18).

Evaluation of biomarkers

It was of interest to determine the abundance of AMPs, whose antibacterial properties might modulate bacterial populations, at each of the 6 skin sites. Significantly higher distribution of HBD-3 and LL-37 was observed in GL followed by BA compared with all other sites ($p<0.05$). With HBD-2, a similar pattern was noted except for BA site, which did not differ significantly with GL region (Table 19).

Association between skin microbiome, AMP biomarkers and skin physiological microenvironment

Site exposure status (exposed or unexposed)

In exposed sites, sebum secretion appeared to correlate with P. acnes occurrence but not Pseudomonadaceae. Also, M. furfur occurrence in the exposed region was associated with less hydrated skin. In unexposed sites, occurrence of P. acnes was prominent in skin sites with less scaling and higher levels of AMP LL-37. Also, the occurrence of S. epidermidis was associated with less skin glossiness. Higher TEWL in unexposed sites was correlated with occurrence of S. aureus; however, lower TEWL was associated with occurrence of Pseudomonadaceae. A higher level of claudin-1 biomarker was associated with higher occurrence of M. furfur in unexposed sites. In addition, AMP HBD-2 did not appear to prevent the occurrence of S. epidermidis in both exposed and unexposed sites; however, a negative association was observed with the growth of M. furfur in unexposed sites.

Skin type (normal, oily and dry)

Higher TEWL was associated with the presence of S. aureus in normal sites and with Pseudomonadaceae in dry sites, whereas lower TEWL was associated with P. acnes in dry sites. Glossiness correlated with the growth of Lactobacillus in dry sites. In addition, skin scalinesss was supportive of M. furfur growth in normal sites. TEWL was positively associated with AMP HBD-2 in normal site and negatively associated with AMP LL-37 in the dry

site. A higher sebum level in normal sites was associated with the presence of P. acnes and LL-37.

Microbial populations in different skin environment

Site exposure status (exposed or unexposed)

Co-occurrence of microorganisms varied depending on skin exposure status. For instance, in exposed skin sites, co-occurrence of S. aureus with other microorganisms was lower than the unexposed sites. A similar trend was observed for M. furfur. However, co-occurrence of Pseudomonadaceae with S. epidermidis was higher in exposed sites compared to unexposed sites.

Skin type (normal, oily and dry)

Microbial co-occurrence also differed based on the skin type. Co-occurrence of S. aureus with all the other microorganisms was lower in both dry and oily skin types as compared with normal skin. Co-occurrence of M. furfur with Lactobacillus in dry skin is lower in both normal, and oily skin and P. acne with M. furfur in oily skin is lower in both normal, and dry skin. However, for other microorganism pairs a clear trend was not observed. For example, co-occurrence of S. epidermidis at dry and oily sites was higher with some microorganisms (P. acnes and Pseudomonadaceae) and lower with others (M. furfur and Lactobacillus) as compared with normal skin type.

C.4 Discussion

The current study demonstrates for the first time, an association between specific skin microbiome, AMP biomarkers, biophysical environment and skin types in a female Chinese population. The skin sites chosen belonged to distinct niches, which are known to be affected by different microorganisms associated with skin disorders such as atopic dermatitis, acne vulgaris, psoriasis etc (180).

Previous studies have documented that Propionibacterium species mostly occur in sebaceous sites, Staphylococcus species in sebaceous and moist sites and a mixed population was observed in dry skin sites (181). Earlier studies in Chinese individuals reported Propionibacterium, Malassezia, and Staphylococcus as commonly occurring genera in different skin sites. In the current study, in all 6 skin sites monitored, S. aureus occurrence was lowest among all bacteria species examined, whereas S. epidermidis and Pseudomonadaceae were found to have high occurrences. In all skin sites classified as dry, oily or normal, S. epidermidis, Pseudomonadaceae and P. acnes predominated over other species. A significantly higher diversity of skin microbiota was noted in the exposed sites compared with the unexposed regions. The Simpson index, a measure of dominance, was found to be higher for unexposed sites compared to exposed, confirming lesser diversity in unexposed sites. The GL, a common exposed site, showed lowest skin tolerance score but higher aesthetic grading (better

dermal health) whereas BA, an unexposed site, showed highest tolerance score and lowest skin aesthetic grading score. The greater microbial diversity of exposed skin sites (compared to unexposed) is likely due to its higher interaction with the external environment, and exposure to diverse microflora (126).

Furthermore, exposure could also modulate resident microflora by encouraging evaporation of water, reducing the accumulation of secretions and maintaining the skin pH (182). These factors may contribute to increasing the tolerability of the skin to the external environment, leading to better dermal health in exposed compared with unexposed sites.

Topographical variations in microbial distribution are associated with the physicochemical properties of the skin (183). The present findings demonstrated that sebum-rich sites and exposed skin surfaces supported the lipophilic anaerobe P. acnes in this population of Chinese women (184). Similar findings were reported in earlier studies. Previous studies have also reported reduced lipid production and impaired barrier function (reduced hydration and increased TEWL) in winter season. This may possibly explain the negative association between the lipophilic P. acnes, and TEWL in the dry sites during winter season in the current study. In agreement with a study conducted in China, a positive association between S. aureus and TEWL at all sites was observed in the current study, indicating a strong relation between skin barrier impairment and S. aureus colonization. The beneficial role of Lactobacillus has been confirmed in a preliminary clinical study wherein hydration and glossiness were improved, thereby delaying signs of early aging. Consistent findings were noted this study, as

Lactobacillus demonstrated positive association with hydration in oily and normal sites and glossiness in dry sites. As well, TEWL and sebum levels were observed to be negatively associated with Pseudomonadaceae whereas TEWL was positively associated with S. aureus colonization, possibly implying that the differences in the host skin physiological environment affect bacterial colonization.

The AMPs constitute a first line of defence of the innate immune response against bacteria, viruses and fungi, and thus play a critical role in animals and humans to control the infection before the advent of symptoms. Thus, it is necessary to consider AMPs during assessments related to skin barrier43. A positive association between LL-37 and P. acnes was found in the present results. An earlier study had shown increased HBD-2 and LL-37 levels along with other proinflammatory cytokines, due to the release of proteases by P. acnes (185). Similarly, a positive association between S. epidermidis and HBD-2 in both exposed and unexposed sites was noted in this study, which is consistent with previous studies. Furthermore, a negative association between HBD-2 and M. furfur was noted in unexposed sites in the current study; in contrast, HBD-3 did not appear to be significantly associated with any of the microorganisms examined. The findings in this study reveal a positive association of claudin-1, a tight junction protein, with M. furfur, Pseudomonadaceae and S. aureus. Additionally, claudin-1 was found to be negatively associated with sebum at both oily and dry sites (186). This is consistent with earlier observations suggesting a strong link between reduced claudin-1 levels (less tight junction proteins), skin dryness and a weak barrier, which alters microflora occurrence. Furthermore, decreased claudin-1 expression in tight junctions is found to be associated with reduced immune response and skin diseases such as atopic dermatitis

and psoriasis. Higher levels of claudin-1 in the blood can be correlated with higher occurrence of M. furfur in unexposed sites, however, as claudin-1 is present on several tissue linings, the observed higher levels in blood needs further evaluation.

Overall, co-occurrence of most of the microorganism pairs was lower in the oily and dry sites as compared with the normal site. However, exceptions to this were co-occurrence of P. acnes with S. epidermidis in the oily region, P. acnes with Lactobacillus in the dry region, and Pseudomonadaceae with S. epidermidis in both dry and oily regions, which was higher compared with the normal sites (187). Additionally, with the exposure of the skin, co-occurrence of most of the microorganisms was found to be lower. Taken together, these findings suggest that co-occurrence of micro-organisms may be affected by different skin micro-environment properties, which may be due to differences in skin adaptability and barrier function. The microbial co-occurrence could also be associated with varying nutrients and metabolites provided by the different skin micro-environments of the host.

A limitation of the present study sample is that it represents only a section of the Chinese population and therefore is specific to this cohort of Chinese women. Hence, the generalisation of results may have limitations owing to differences in lifestyle, diet, environmental exposure and seasonal variations. The current study is associative and an exact causative mechanism of microbial association needs further exploration. Future large studies are required to confirm current findings. In addition, age may have an important bearing on overall skin health, texture and barrier function, and future studies will address the influence of age on association between

microorganisms, AMP biomarkers and biophysical parameters.

C.5 Conclusion

The present findings suggest that the skin exposure, biophysical and barrier profile and biomarkers are associated with the bacterial distribution and co-occurrence. This underlines the importance of comprehensive understanding of the association of microorganisms, skin biophysical parameters, microenvironment and skin barrier function including physical, chemical and microbial barriers, which is essential for designing skin care products and anti-microbial drugs. Maintaining healthy skin requires selective microbial shifts or permeability barrier changes, inhibiting the growth of pathogenic bacteria and promoting the growth of symbiotic bacteria. Hence, an alteration in the skin microbiome in certain disorders by selective modulation of microbiome (pre- and/or probiotics) could be a promising treatment strategy in clinical and sub-clinical skin conditions.

C.6 Appendix

Table 17. Distribution of biophysical parameters at different skin sites (Mean±SD)

Parameters	Instrument	GL	IS	HB	BA	AF	VF
Water content/epidermal hydration	Corneometer	68.92±10.71[a]	10.21±11.12[a]	45.87±12.02	53.91±8.78[a]	49.88±10.48[a]	44.02±9.59
TEWL	Tewameter	10.65±4.58[b]	31.45±11.22[b]	7.54±3.08[b]	4.66±2.89[b]	2.38±2.05	3.01±2.45
Sebum	Sebumeter	106.00±75.14[c]	4.06±11.98	3.27±5.84	31.24±34.62[c]	8.91±21.46	12.33±38.00
Gloss	Glossmeter	10.69±2.60[d]	4.99±1.29	6.78±2.42[d]	7.33±1.79[d]	4.82±0.65	8.57±1.56[d]
pH	pH meter	5.26±0.46	5.36±0.56	5.63±0.52	5.43±0.53	5.30±0.43	5.56±0.49
SEr	Visioscan	3.64±1.29	3.76±1.73	3.08±1.46	2.95±1.87	2.11±0.82	3.49±1.53
SEsc		0.87±0.35	1.44±0.65	0.99±0.55	0.62±0.15	1.04±0.43	1.46±0.66

[a, b, c, d]: $p < 0.05$; [a], compared with VF and HB; [b], compared with AF and VF; [c], compared with AF, IS, VF and HB; [d], compared with AF and IS.
Abbreviations: AF, antecubital fossa; BF, back; GL, glabella; HB, hand-back; IS, interdigital web space; SEr, skin roughness, SEsc, skin scaliness; TEWL, transepidermal water loss; VF, volar forearm.

Parameter description

SEsc: Scaling calculated as a portion of light pixels (gray level higher than established threshold)

SEr: Roughness calculated as a portion of dark pixels (gray level is below established threshold)

Table 18: Relative occurrence of each microorganism at different skin sites (n = 100)

Skin site	*Staphylococcus aureus* (n)	*Staphylococcus epidermidis* (n)	*Lactobacillus* (n)	*Malassezia furfur* (n)	*Propionibacterium acnes* (n)	Pseudomonadaceae (n)	Shannon index	Simpson index
GL	4	85[b,c,f]	35[b,c,f]	16	83[b,c,d,e,f]	74[d,f]	1.00 ±0.43[b,c,f]	0.39 ±0.19[b,c,f]
IS	3	86[b,c,f]	47[b,c]	26[b,c,f]	70 [a,c]	87[a,b,c,e,f]	1.01 ±0.50[b,c,f]	0.38 ±0.21[b,c,f]
HB	3	81[b,c,f]	43[b,c]	20[d]	67 [a,c]	74[d,f]	0.88 ±0.51[c,f]	0.43 ±0.25[b,c,f]
BA	1	53[a,b,d,e]	12 [a]	14[d]	61 [a]	60 [a,d,e]	0.64 ±0.50[a,d,e]	0.57 ±0.29[a,d,e]
AF	1	67 [a,d,e,f]	19 [a,d,e]	14[d]	59 [a]	70[d]	0.75 ±0.50[a,d]	0.51 ±0.27[a,d,e]
VF	3	55 [a,d,e]	19 [a,d,e]	10	48 [a,d,e]	65[d]	0.67 ±0.52[a,d,e]	0.55 ±0.28[a,d,e]

[a,b,c,d,e,f] $p<0.05$; [a], compared with GL; [b], compared with AF; [c], compared with VF; [d], compared with IS; [e], compared with HB; [f], compared with BA
Exposed sites, GL,HB,IS; unexposed sites, AF,BA,VF; normal sites, AF,BA,VF,HB; oily site, GL; dry site, IS
Abbreviations: AF, antecubital fossa; BF, back; GL, glabella; HB, hand-back; IS, interdigital web space; VF, volar forearm.

Table 19. Distribution of biomarkers at different skin sites (Mean±SD)

Skin sites	HBD-2	HBD-3	LL-37	Claudin-1
GL	$0.27 \pm 0.51^{b,c,d,e}$	$0.47 \pm 0.63^{b,c,d,e,f}$	$0.84 \pm 0.90^{b,c,d,e,f}$	14.72 ± 6.64
IS	0.12 ± 0.24^{a}	$0.07 \pm 0.09^{a,f}$	$0.12 \pm 0.12^{a,f}$	
HB	0.14 ± 0.25^{a}	$0.11 \pm 0.18^{a,f}$	$0.17 \pm 0.22^{a,f}$	
BA	0.20 ± 0.37	$0.22 \pm 0.37^{a,b,c,d,e}$	$0.45 \pm 0.53^{a,b,c,d,e}$	
AF	0.13 ± 0.26^{a}	$0.08 \pm 0.15^{a,f}$	$0.17 \pm 0.13^{a,f}$	
VF	0.14 ± 0.29^{a}	$0.08 \pm 0.11^{a,f}$	$0.17 \pm 0.15^{a,f}$	

^{a,b,c,d,e,f} $p<0.05$; ^{a}, compared with GL; ^{b}, compared with AF; ^{c}, compared with VF; ^{d}, compared with IS; ^{e}, compared with HB; ^{f}, compared with BA

Abbreviations: AF, antecubital fossa; BF, back; GL, glabella; HB, hand-back; IS, interdigital web space; VF, volar forearm.
Claudin-1 values were obtained from blood sample and hence, the values are not site-specific.

Figure legends:

Figure 24: Frequency of dermal problems classified by skin site as per participants' perception
Abbreviations: AF, antecubital fossa; BF, back; GL, glabella; HB, hand-back; IS, interdigital web space; VF, volar forearm.

Figure 25: Comparison of dermal health grading of exposed and unexposed sites by dermatologist. A) Skin aesthetics, and B) Skin tolerance
Abbreviations: AF, antecubital fossa; BA, back; GL, glabella; HB, hand-back; IS, interdigital web space; VF, volar forearm.
Boxed sites represent exposed skin sites.

Figure 26: Differentiation of skin sites by cluster analysis based on moisture, sebum and transepidermal water loss
Abbreviations: AF, antecubital fossa; BF, back; GL, glabella; HB, hand-back; IS, interdigital web space; TEWL, transepidermal water loss; VF, volar forearm.

Figure 27: Microbial co-occurrence based on A) Exposure of skin
The estimate of co-occurrence percentage between microorganisms was depicted by point-size. The difference of co-occurrence of in different skin type (exposed or unexposed) was visualized by change in point-size. For e.g., co-occurrence between S. aureus and S. epidermidis was estimated as 19.333% at exposed sites and 43.333% at unexposed sites.
B) Skin type and biophysical parameters
The estimate of co-occurrence percentage between microorganisms was depicted by point-size. The difference of co-occurrence of in different skin type (exposed or unexposed) was visualized by change in point-size. For example, co-occurrence between S. aureus and S. epidermidis was 38 at normal sites, 17 at dry sites and 19 at oily sites.

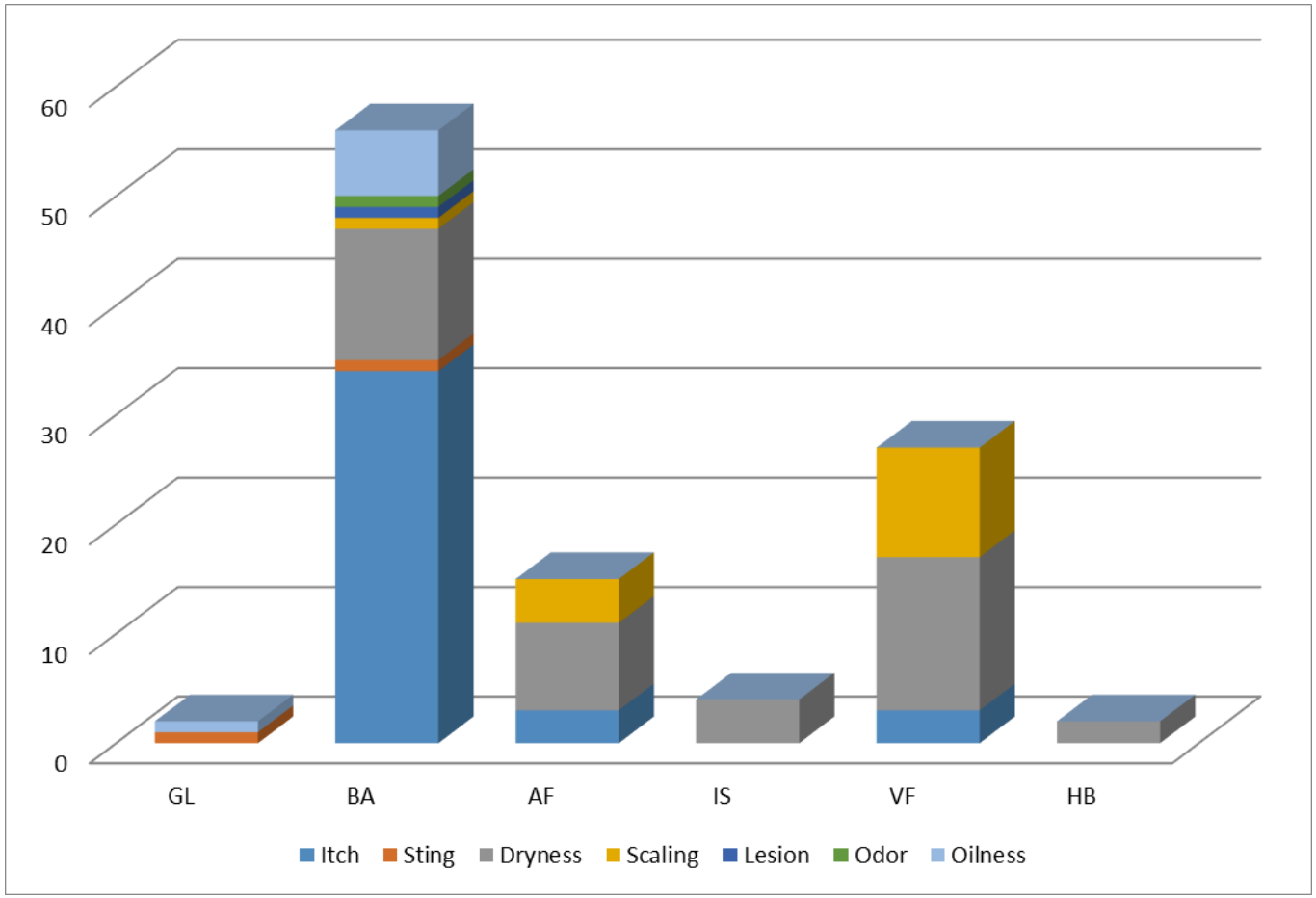
Figure 28: Association between A) site exposure dependent skin microbiome distribution, skin physiology environment and biomarkers (by logistic regression)
Each node shows one bacteria/fungi, the colour of the nodes corresponds to the unexposed site (cyan) or exposed site (pink). Bacteria/microorganism are represented as circle. The size of the

node corresponds to the square root of (positive rate) *30. The colour of the edges corresponds to the positive (green) or negative (purple) regression estimated coefficient. The length of the edges has no meaning. Solid line represents $p<0.05$ and dotted line represents $0.05 < p < 0.1$. Star shape represents biophysical parameters, triangles correspond to biomarkers.
Abbreviations: Malass, Malassezia furfur, TEWL, transepidermal water loss.

B) Physiology dependent with other physiology parameters, microbiome, and biomarkers in every skin type (by linear regression)
Each node represents bacteria/fungi, the colour of the nodes corresponds to the normal skin site (dark blue), oil skin site (red), and dry skin site (yellow). The size of the node corresponds to the square root of (positive rate) *30. The colour of the edges corresponds to the positive (green) or negative (purple) regression estimated coefficient. The length of the edges has no meaning. Solid line represents $p<0.05$ and dotted line represents $0.05 < p < 0.1$ Star shape represents biophysical parameters and skin texture index, triangles correspond to biomarkers.

Abbreviations: Lactob, Lactobacillus; Malass, Malassezia furfur, SEsc, scaliness; SEr, roughness; TEWL, transepidermal water loss.

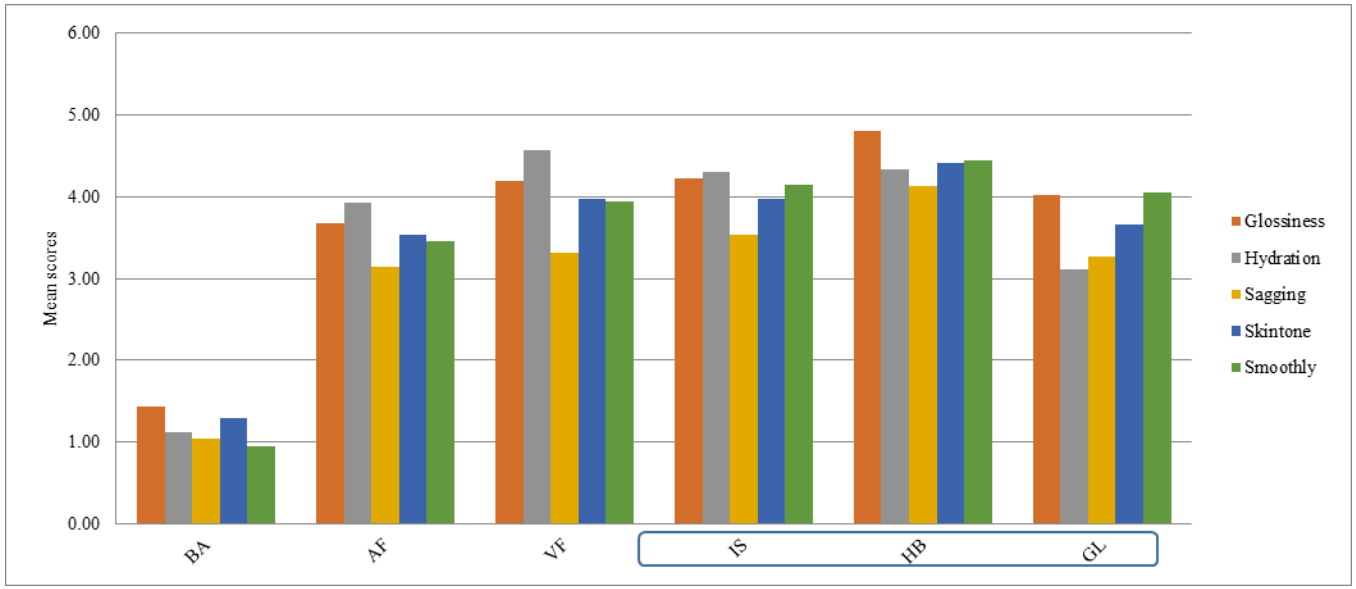
Figure 24: Frequency of dermal problems classified by skin site as per participants' perception



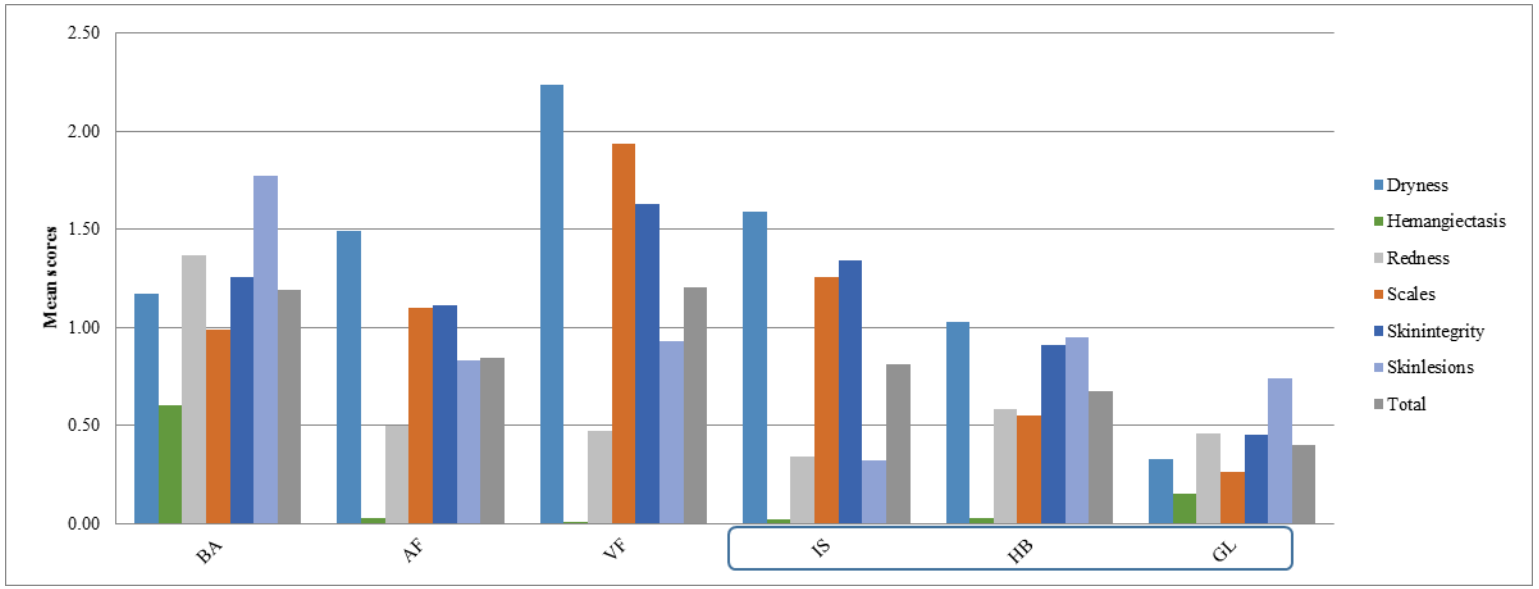
Abbreviations: AF, antecubital fossa; BF, back; GL, glabella; HB, hand-back; IS, interdigital web space; VF, volar forearm.

Figure 25: Comparison of dermal health grading of exposed and unexposed sites by dermatologist. A) Skin aesthetics, and B) Skin tolerance

A.

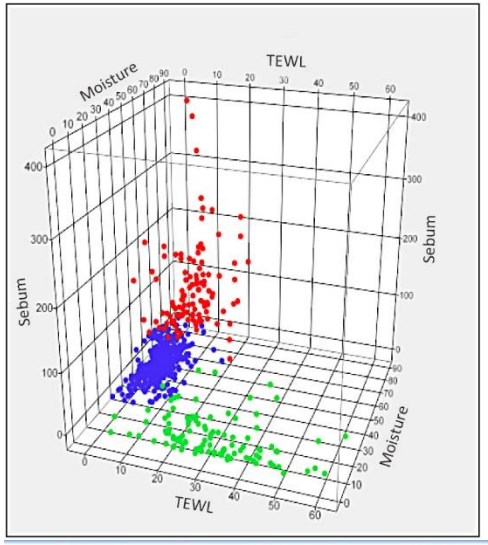


B.



Abbreviations: AF, antecubital fossa; BA, back; GL, glabella; HB, hand-back; IS, interdigital web space; VF, volar forearm. Boxed sites represent exposed skin sites.

Figure 26: Differentiation of skin sites by cluster analysis based on moisture, sebum and transepidermal water loss



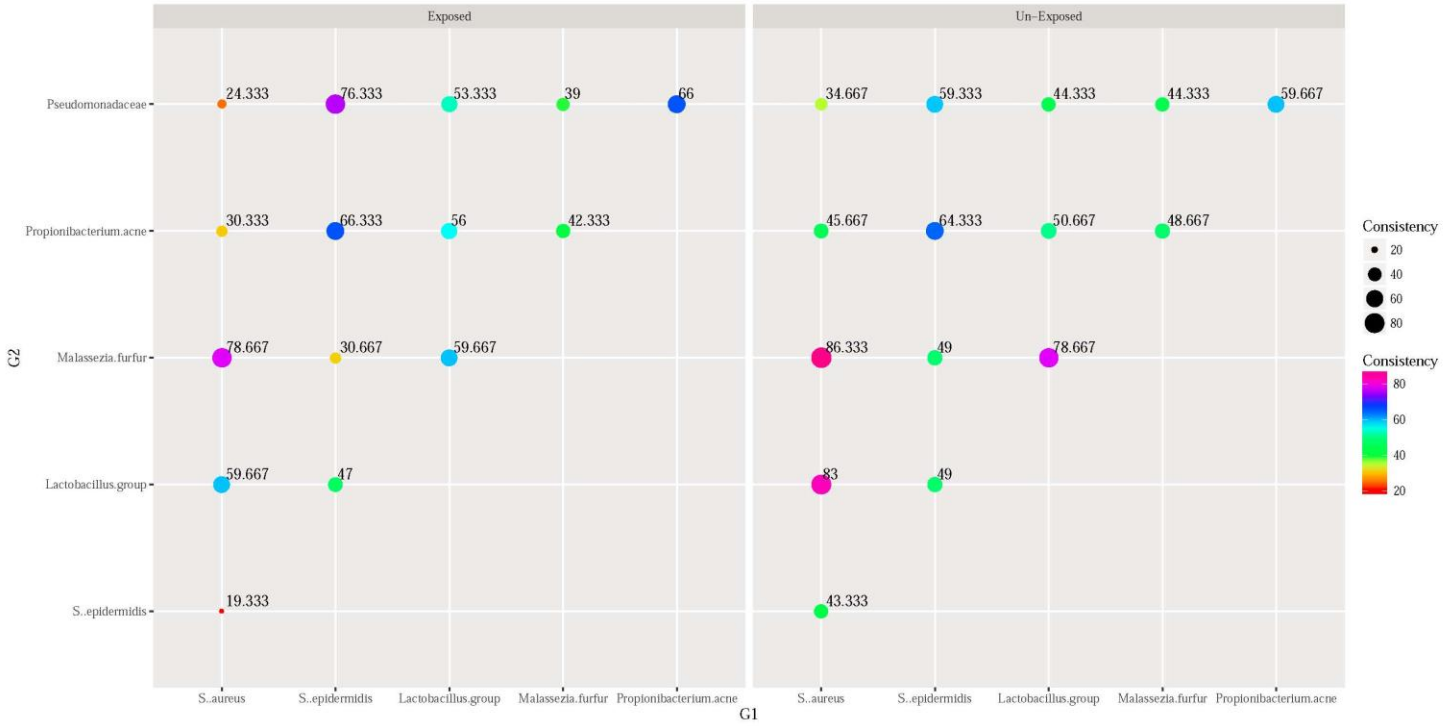
Site	Moisture+Sebum+TEWL		
	1	2	3
BA	87.00%	0.00%	13.00%
VF	93.00%	2.00%	5.00%
HB	95.00%	4.00%	1.00%
IS	1.00%	99.00%	0.00%
GL	8.00%	2.00%	90.00%
AF	95.00%	0.00%	5.00%
Total	63.2%	17.8%	19.0%

Site	Dimension	Exposed	Un-exposed
Normal	Moisture, non-oily and strong barrier	hand back (HB)	back (BA) antecubital fossa (AF) volar forearm (VF)
Dry	Dry, non-oily and weak barrier	interdigital web space (IS)	/
Oil	Moisture,oily and strong barrier	glabella (GL)	/

Abbreviations: AF, antecubital fossa; BF, back; GL, glabella; HB, hand-back; IS, interdigital web space; TEWL, transepidermal water loss; VF, volar forearm.

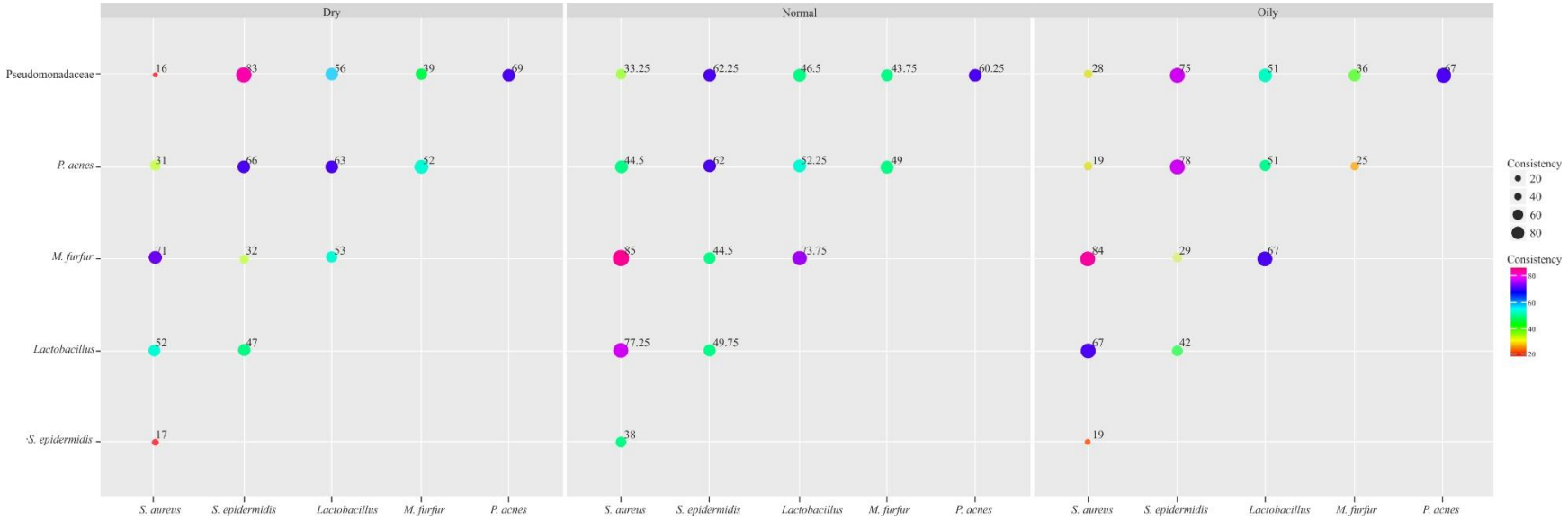
Figure 27: Microbial co-occurrence based on
A) Exposure of skin, and B) Skin type and biophysical parameters

A.



The estimate of co-occurrence percentage between microorganisms was depicted by point-size. The difference of co-occurrence of in different skin type (exposed or unexposed) was visualized by change in point-size. For e.g., co-occurrence between *S. aureus* and *S. epidermidis* was estimated as 19.333% at exposed sites and 43.333% at unexposed sites.

B.



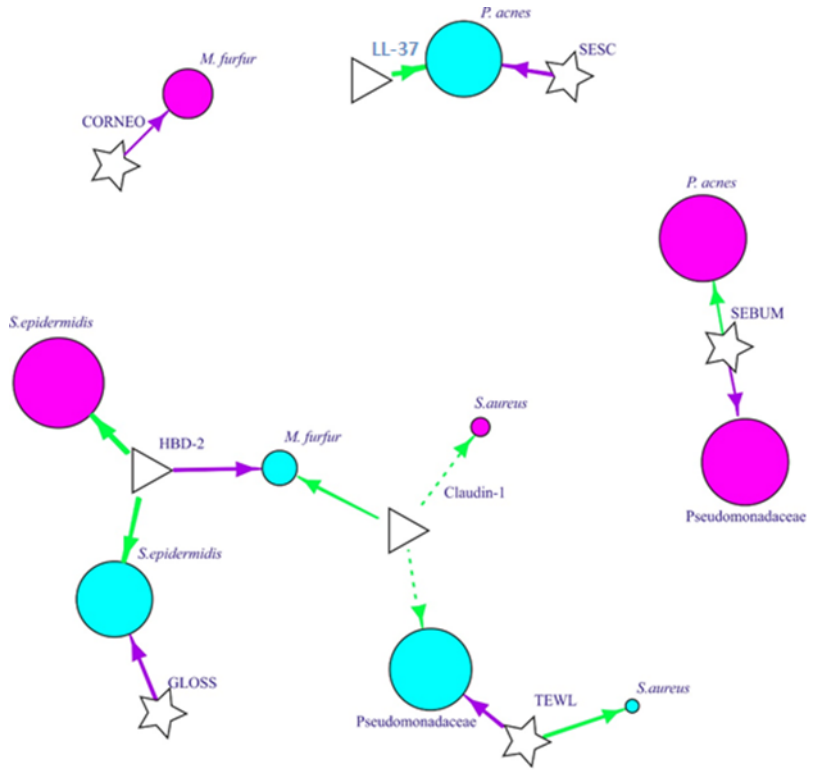
The estimate of co-occurrence percentage between microorganisms was depicted by point-size. The difference of co-occurrence of in different skin type (exposed or unexposed) was visualized by change in point-size. For example, co-occurrence between S. aureus and S. epidermidis was 38 at normal sites, 17 at dry sites and 19 at oily sites.

Figure 28: Network analysis

A) Association between site exposure dependent skin microbiome distribution, biophysical parameters and biomarkers (by logistic regression);

B) Physiology dependent with other physiology parameters, microbiome, and biomarkers in every skin type (by linear regression)

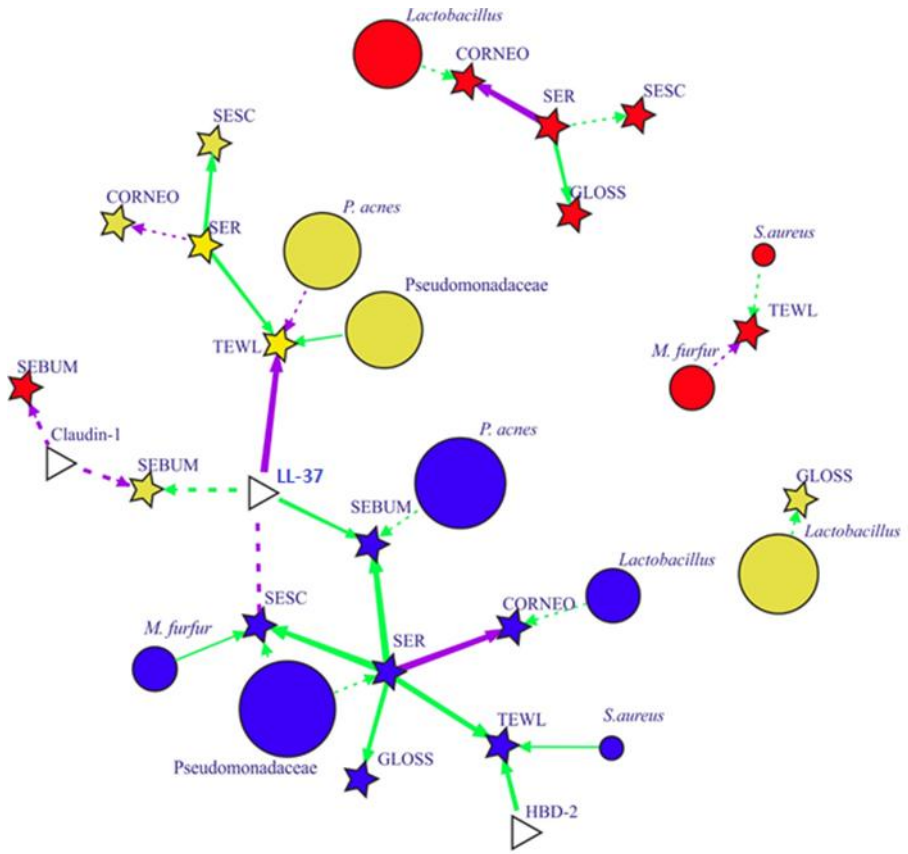
A.



Each node shows one bacteria/fungi, the colour of the nodes corresponds to the unexposed site (cyan) or exposed site (pink). Bacteria/microorganism are

represented as circle. The size of the node corresponds to the square root of (positive rate) *30. The colour of the edges corresponds to the positive (green) or negative (purple) regression estimated coefficient. The length of the edges has no meaning. Solid line represents $p<0.05$ and dotted line represents $0.05 < p < 0.1$. Star shape represents biophysical parameters, triangles correspond to biomarkers.
Abbreviations: Malass, Malassezia furfur, TEWL, transepidermal water loss.

B.



Each node represents bacteria/fungi, the colour of the nodes corresponds to the normal skin site (dark blue), oil skin site (red), and dry skin site (yellow). The size of the node corresponds to the square root of (positive rate) *30. The colour of the edges corresponds to the positive (green) or negative (purple) regression estimated coefficient. The length of the edges has no meaning. Solid line represents $p<0.05$ and dotted line represents $0.05 < p < 0.1$ Star shape represents biophysical parameters and skin texture index, triangles correspond to biomarkers.
Abbreviations: Lactob, *Lactobacillus*; Malass, *Malassezia furfur*, SEsc, scaliness; SEr, roughness; TEWL, transepidermal water loss.

Supplementary Content

Table 20: Specific primers for each microorganism

Target germ	Primer	Sequence (5'–3')	Size (bp)
Lactobacillus spp	lac-F	AGCAGTAGGGAATCTTCCA	345
	lac-R	ATTCCACCGCTACACATG	
Propionibacterium acnes	PA-F	GCGTGAGTGACGGTAATGGGTA	131
	PA-R	TTCCGACGCGATCAACCA	
Staphylococcus epidermidis	Se705-F	ATCAAAAAGTTGGCGAACCTTTTC	125
	Se705-R	CAAAAGAGCGTGGAGAAAAGTATC	
Staphylococcus aureus	femA-F	AACTGTTGGCCACTATGAGT	306
	femA-R	CCAGCATTACCTGTAATCTCG	
Pseudomonadaceae	PsI-F	GGTGGGCACTCTAAGGAGAC	173
	PsI-R	TGCGATCCGGACTACGAT	
Malassezia furfur	Mala-F	CTCGCGTACAACGTCTCTGG	226
	Mala-R	CGCTGCGTTCTTCATCGA	

F, forward primer; R, reverse primer.

Ⅳ-Conclusions

Rosacea is a common chronic inflammatory skin disease that almost exclusively affects the central facial skin. In these years, the morbidity of rosacea in China has increased significantly. Characteristic clinical signs or symptoms of rosacea include transient to persistent facial erythema, telangiectasia, papules, pustules, edema, or a combination of these; some individuals with rosacea also experience pain, stinging or burning, and, albeit rarely, pruritus. And we all know that each clinical sign is related by the pathogenesis of this skin disease, and rosacea pathophysiology is very complex, involving various cell types and molecules in the skin, and various subtypes. According to these viewpoints, we chose the ERT and PPR patients, and focused on microorganisms and skin barrier to know more about the pathogenesis of rosacea.

One of the objectives of this thesis was to know more about whether the skin dysbiosis is a response to changes in the skin microenvironment resulting from rosacea's underlying pathophysiology. And we were also interested in the difference between the French rosacea patients and the Chinese patients regarding the skin barrier function.

Another objective was to find a practical non-invasive testing technology to evaluate the rosacea patients' skin barrier damage condition and the treatment efficacy. Through these assessments, we can know more about the skin barrier situation of the patient, which will help us to choose the more suitable therapy approach for the long time treatment of rosacea patients.

Through the 4 years research of this thesis, we have shown that:

- ✓ Standardized Skin Surface Biopsy is a good practical method to measure Demodex Folliculorum density in rosacea and acne patients in clinical experience.
- ✓ RCM may be a better choice than SSSB because of its accuracy, completeness and as an in vivo noninvasive painless procedure. RCM appears to be a more sensitive method which could be used more in research or clinical studies or to follow up treatment or recurrence.
- ✓ According to the results of testing demodex number in lesions of PPR patients, we found that it was much higher in Besancon than Shanghai even if we used the same method.
- ✓ The physiological features of rosacea are strongly associated with the interactions between the host and microorganisms, and our data indicate the importance of the bacterial colonization balance on the skin surface. In the pathogenesis of rosacea, we should focus more on the skin dysbiosis with the enhanced immunity response.
- ✓ RCM can detect in sensitive skin and rosacea patient epidermal damaged structures, including parakeratosis, disarranged honeycomb pattern and reduced honeycomb pattern depth. It could be used as a new kind of auxiliary method in detection and diagnosis, providing new mentality for the diagnosis and treatment.
- ✓ It is important to take into consideration that the association of microorganisms, skin biophysical parameters, and microenvironment and skin barrier function including physical, chemical and microbial barriers even in normal skin, which is essential for designing skin care products and anti-microbial drugs.

In the future, we want to concentrate our studies on whether there is an

inflammation amplification system, which transduces epidermal microorganism infection into dermal inflammation. Our research team will plan to investigate the NLRP3-medicated inflammasome and its amplification system for rosacea’s development.

V - Acknowledgements

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Ⅶ--List for the Published Papers in English

First Author or Corresponding（IF=16.629）

1) Ma YF, Yuan C#, Jiang WC, et al. Reflectance confocal microscopy for the evaluation of sensitive skin. Skin Res Technol, 2017, 23(2): 227-34.（IF=1.776）

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6) Yuan C#, Wang XM, Galzote C, et al. Meteorology and ethnicity as critical factors in HRIPT: comparing the response between Chinese and Indian ethnicities. Regul Toxicol Pharmacol, 2013, 66(1): 59-65.（IF=2.031）

The Second Author（IF=1.092）

1) Qian CY, Yuan C#, Tan YM, et al. Comparing performance of Chromameter®, Mexameter® and full-field laser perfusion imaging for measurement of ultraviolet B light-induced erythema, Clin Exp Dermatol,

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1) Tan YM, Wang XM, Yuan C#, et al. Skin sensitivity and intolerance in Shanghai: cumulative influence of different meteorological parameters, Cutan Ocul Toxicol, 2015, 34(2):132-8. （IF=1.122）

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1) Guichard A, Ma L, Tan Y, Yuan C#, et al. What if scalp flora was involved in sensitive scalp onset? Int J Cosmet Sci, 2016, 38(4): 429-30.（IF=1.377）

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1. Evaluation of Skin Surface Flora, Measuring the Skin. Springer International Publishing Switzerland, 2016.

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3. Skin Barrier and Transdermal Therapy, Dermatology Research Advances. Nova Biomedical, 2014.

Ⅷ--List for the Published Papers in Chinese

First Author or Corresponding

1. Yang Lijie, Hu Weiyi, Ma Yafeng, Yuan Chao#. Establishment of positive control for benchmark in human single patch test. Chin J Lepr Skin Dis, 2017, 33(2): 88-91.

2. Li Xiuling, Yuan Chao#, Yang Lijie, et al. Photobiological responses in patients with chronic actinic dermatitis and their relationship with themelanocortin-1 receptor gene Arg163Gln variant: a preliminary study. Chin J Dermatol, 2016, 49(10): 712-716.

3. Yuan Chao#, Wang xuemin.The skin aging research progress of middle-aged and old women. Practical Geriatrics, 2015, 29(8): 625-627.

4. Yuan Chao#, Wang Xuemin. Role of Microorganisms in the Pathogenesis of rosacea. Chin J Dermatol, 2015, 29(5): 521-523.

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6. Yuan Chao#, Wang Xuemin, Tan Yimei, et al. Tranexamic Acid renovate skin texture in damaged skin. Chin J Med Aesth & Cosmet, 2013, 22(2): 267-271.

7. The expert consensus of diagnosis and treatment of Chinese skin sensitivity. 2017.

Ⅸ--List for the Congress Presentation

2017.05.14 Chongqing, China

The 23rd Annual Meeting of Chinese Society of Dermatology and venereal diseases.

Presentation: “Epidemiology and Pathogensis of Rosacea”.

2017.05.12 Chongqing, China

The 23rd Annual Meeting of Chinese Society of Dermatology and venereal diseases.

Presentation: “How to prevent baby’s irritated diaper Dermatitis?”

2017.05.12 Chongqing, China

The 23rd Annual Meeting of Chinese Society of Dermatology and venereal diseases.

Presentation: “Rosacea and Sensitive Skin”

2017.05.05 Shanghai, China

The 14th Annual Medical Aesthetics Meeting of Chinese Society of Dermatology and venereal diseases

Presentation: “Baby Skin Barrier Research Progress”.

2016.09.12 Shanghai, China

National Acne Summit Forum in Renji Hospital.

Presentation: “Microorganism Skin Barrier and Rosacea”.

2016.11.27 Hangzhou, Zhejiang Province China.

The 1st Forum for skin management and facial rejuvenation.

Presentation: “The application for evaluation of sensitive skin in Chinese female”.

2016.09.08 Shanghai, China
3rd International Conference of Sebaceoous Gland, Acne, Rosacea and Related Disorders.
Presentation: “Rosacea is associated with the conjoined interactions between intrinsic features and microorganisms: a pilot study”.

2016.06.19 Shanghai, China
Department of Dermatology, Shanghai Medical Association.
Presentation: “Reflectance Confocal Microscopy for the evaluation of sensitive skin”.

2016.05.27 XiaMen, FuJian Province,
The 22nd Annual Meeting of Chinese Society of Dermatology and venereal diseases.
Presentation: “The sensitive skin in Chinese female”.

2016.05.15 Shanghai, China
Shanghai Medical Association Medical Aesthetics and cosmetology branch 2016
Presentation: “Progress in Diagnosis and Treatments for Rosacea”.

2015.09.12 Shanghai, China
National Acne Summit Forum in Renji Hospital..
Presentation: “Progress in Diagnosis and Treatments for Rosacea”.

2015.07.17 Hefei, Anhui Province, China

The 21th Annual Meeting of the Chinese society of Dermatology and venereal diseases.

Presentation: “The role of cosmetics and evalution elements in human efficacy testing”

2015.05.15 Shanghai, China

Cosmetics R&D and Technology Cooperation 2015

Presentation: “Strategies to improve skin barrier homeostasis”

2014.05.29 Hangzhou, Zhejiang Province China.

The 20th Annual Meeting of the Chinese society of Dermatology and venereal diseases.

Branch Presentation: “Rosacea and Demodex Folliculorum” .

2014.04.17 Paris, French.

Societe Francaise de Dermatologie,Groupe Imagerie Cutanee Non Invasive.

Presentation: “Interest of confocal laser scanning microscopy for three different lesions in a pityriasis folliculorum patient: a case report”.

2013.11.08 Besancon, French.

Besançon University Hospital, University of Franche-Comté.

Presentation: “Evaluation of Demodex Folliculorum as a Risk Factor for Rosacea”.

X --List for the Relevant Investigation Fund

- 2017.01-2019.12 Young of National Natural Science Funds：The effect and machanism of inflammasome amplificatic loop in promoting rosacea： (No: 81602778);
- 2016.07-2019.06 Shanghai Committee of Science and Technology Funds： Standardized diagnose technology's clinical application in rosacea (No: 16411961400);
- 2013.06-2016.05 Shanghai Skin Disease Hospital Outstanding Youth Funds: Exploratory research for pathogenesis of papulopustular rosacea in Besancon and Shanghai.

XI—Resume

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EDUCATION

2013.12 Ph.D. candidate, Franche-Comte University

2010.7 M.S. in Dermatology, Second Military Medical University

2001.7 B.S. in Dermatology, Second Military Medical University

PRESENT OCCUPATION

- Committee Member in Cosmetology Group, Chinese Society of Dermatology (2016)
- Committee Youth Member in Allergy Group, the Shanghai medical association branch of Allergy (2016)
- Committee Member in Allergy Group, the Shanghai medical association branch of dermatology (2015)
- Principal Investigator in Skin & Cosmetic Research Dept, Shanghai Skin Diseases Hospital
- Associate Chief Dermatologist

RESEARCH EXPERIENCE

2016.02-present

Principal Investigator

In charge of the clinical trials in Skin & Cosmetic Research Dept

2014.5-2016.02

Head of Clinical Application and Translational Medicine

2013.6-2014.5

- Medical Visitor in Besancon Franche-Comte University in France
- Ph.D. candidate, Franche-Comte University

2010.7-2013.5

- As head of Technology Department, I am in charge of all the clinical research projects for the Skin & Cosmetic Research Division
- Studying the reflection and restoration of skin protection function after chemical or physical damage
- Exploring the influences of Tape Striping Test on human different body parts.
- Observing and defining sensitive skin from traditional Chinese medical view.

2009.6-2010.7

- Working for an administrative licensed testing institution which is directly owned by Chinese Ministry of Health, responsible for testing sunscreen products SPF & PA value and studying the research methodology
- Leading Human Patch Test (including Human Repeat Insult Patch Test and 12-day Cumulative Irritancy Study)

- Carrying out Skin Photo Test and Photopatch Test on patients having lesions in exposure areas
- Studying the effects of different wavelength of UV on healthy skin biological characters.

2003.5-2009.6

- Studying and measuring the skin color of different body parts of Chinese people in different seasons, and skin color changing after exposure under different wave bands of UV
- Applying skin no-invasive technique to detect efficiency and safety of cosmetics
- Studying contact dermatitis caused by cosmetics

2001.7-2003.5

Training as resident