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"A multiscale analysis of ceramide's alterations in atopic dermatitis skin highlights enzymes as a possible target for treatment"

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1. Introduction

Atopic dermatitis (AD) is a multifactorial and complex skin inflammatory disease, and its prevalence is increasing among years affecting up to 10% of adults and 15% to 20% of children worldwide [1]. An epidemiological study declared that approximately 23.42 million people would be affected by AD in the European Union. This large study reported the importance to raise awareness about skin conditions as they become a potential public health problem especially in developed countries [2]. The disease is characterized by the appearance of eczema flare-ups followed by remission periods. The flare-ups can be triggered by different factors such as weather, pollution, or psychological stress [3-5].

The pathophysiology of AD is complex and involves the immune system, the cutaneous microbiome, and the skin barrier of the stratum corneum [6]. The implication of the immune system in the disease is already well studied and established [7-9] but the alteration of the skin barrier has been shown to play an important role in the mechanism of AD [10].

The stratum corneum (SC), the outermost layer of the epidermis is crucial to skin health. Its barrier function is primarily maintained by a lipidic matrix composed of ceramides (~50%), cholesterol (~28%), and free fatty acids (~10%) and surrounding corneocytes, analogous to bricks and mortar. Within this matrix, ceramides, a class of lipids, play a pivotal role in maintaining SC integrity, cohesion, and hydration. They form a crucial part of the lamellar structures that regulate water loss and prevent the entry of harmful substances. To our knowledge, 12 classes of free ceramides have been identified in the human stratum corneum's lipidic matrix. Ceramides are classified based on the composition of their fatty acids (FA) and long chain sphingosine base. To produce these 12 classes, there are 3 different moieties of fatty acid (Non-hydroxyl, α-hydroxyl and esterified ω-hydroxyl) which can be combined with 4 long chain sphingosine base (sphingosine, dihydrosphingosine, phytosphingosine and 6-hydroxy-sphingosine). In each of these classes, there is a wide distribution of their chain length. Also, in the cornified envelope, ceramides are found to be linked to structural proteins attached to the

corneocytes. Their structure and organization is essential to ensure the protective properties of the stratum corneum.

Alteration in ceramides composition and reduction of their total amount and chain length have been associated with atopic dermatitis [11]. Despite that, there is only a few information available on the mechanism preceding this change and even less on protein-bound ceramides in AD.

In this study, we aimed to characterizing the structure, organization and composition of SC ceramides by an in vivo multiscale approach in lesional, non-lesional atopic skin and healthy skin.

2. Materials and Methods

Clinical Design & Scores

A prospective, monocentric, open-label, controlled clinical study was conducted comparing subjects with atopic dermatitis presenting active lesions to matched healthy subjects. Recruitment was performed through the Bioclinical Research Center and the Dermatology Department of Saint Louis Hospital in Paris. This study followed the Declaration of Helsinki Principles and was approved by the French Ethic Committee "Comité de protection des personnes Sud-Méditerranée V" (IDRCB: 2023-A00227-38). A written informed consent was obtained from all participants.

10 subjects with mild-to-severe atopic dermatitis and 10 matched healthy subjects were enrolled in this study. We investigated 2 lesional zones (ADL) and 2 adjacent non-lesional zones (ADNL) on AD subjects and 2 matching healthy zones (HVH) on healthy subjects. Several clinical scorings were performed: the Eczema Area and Severity Index (EASI), Pruritus Numerical Rating Scale (NRS), Sleep quality NRS and the validated Investigator Global Assessment (vIGA). Only lesions without oozing or crusting and non-treated for the past seven days were investigated.

TEWL

The TransEpidermal Water Loss (TEWL) quantifies the water evaporated through the skin (expressed in g.h-1.m-2) and one measurement per skin zone was performed using a portable, closed condenser-chamber device (AquaFlux model AF200, Biox Systems, UK).

Raman spectroscopy

In vivo Raman spectra were acquired using a RiverD International B.V. gen2-SCA Skin Composition Analyzer. Employing a 50 µm pinhole, Raman scattering was recorded for six profiles per test area (500 x 500 µm), ranging from the skin surface to depths of 28 µm (fingerprint region: 400-1800 cm^{-1}) and 40 µm (high wavenumber region: 2500-3600 cm^{-1}). Total lipid content and lateral packing order were calculated using an in-house Python script based on the method described by ChunSik et al [12].

Ceramides extraction and analysis of human stratum corneum

The human stratum corneum was collected using D-squame tape strips (22 mm in diameter, Monaderm, Monaco, France). 4 tape-strips were applied on each skin areas for 30 seconds by hand pressurizing and then stored in 1.8 mL tubes at -30°C before ceramides extraction.

A targeted lipidomics approach focusing on free ceramides extracted from tape strips was performed by mass spectrometry using an IDX Tribrid ™ orbitrap instrument. Free lipids were extracted from each tape strip using Methanol. Each sample was vortexed (5sec), heated (1h30min at room temperature) and finally sonicated (5min). The 4 extracts obtained for each skin zone were then pooled and the pooled extract was evaporated using a speedvac.
To extract the lipids bound (to the envelope), a gentle alkaline hydrolysis on the same samples was performed after free lipids extraction as follows: first NaOH was added (1 mol/L in methanol/H2O mixture (19/1), incubation 1h at 45°C) The extract was recovered and treated with HCl (2 mol / L in the mixture methanol / H2O 19 / 1) to which was added chloroform. After shaking, the organic phase was recovered and evaporated using a speedvac.
All samples were then resuspended in MeOH/Iso (2/1), filtered, and injected on UPLC coupled with Orbitrap High Resolution Mass Spectrometry. Sample extracts were injected on analytical column (Acquity BEH C8 2.1mm*100). Free lipids and bound lipids were eluted on a linear gradient from 0 to 100% Isopropanol (in 0.1% HCOOH) in 25min and 30 min respectively. The Orbitrap™ IDX tribrid™ was run in “data dependent acquisition” mode, with a survey scan acquired on Orbitrap using a mass range of 150 to 2000 m/z and a resolution of 70 000. Electrospray ionization was performed in positive mode.

Statistics
In this paper, boxplots are presented, where the bold line represents the median, the diamond the average, and the box limits the first and third quartiles. For lipidomics results, all values of ceramides area under the curve were normalized on cholesterol area under the curve and then transformed in Log10. For statistical tests, the t-test or Wilcoxon test with a significance level of 5% were used depending on the following of normal distribution or not by the data.

3. Results

3.1. Clinical evaluation and altered TEWL in AD lesional skin

Table I summarizes the characteristics of the two groups (AD and control), in terms of age, sex, clinical scores (EASI, Pruritus NRS and Sleep quality NRS), as well as TEWL values. The groups were matched in terms of age and sex. By design the study included various degrees of AD severity (2 severe, 5 moderate and 3 mild - EASI first quartile = 6.25 and EASI third quartile = 17.25), to validate if the results found hold in all cases. The Pruritus and Sleep quality NRS scores were found to be strongly correlated with the EASI score (Spearman scores 0.914 and 0.844 respectively), revealing a significant impact of the severity of AD on the quality of subjects’ life.

Table I. Clinical and subclinical characteristics of the AD and control groups: age, sex, clinical scores (EASI, Pruritus NRS and Sleep quality NRS) and TEWL values.

Variables	AD Group (n=10)	Control Group (n=10)	p-value
Age* (years)	27 (22.75 – 29)	24 (23 – 26.25)	n.s.
Sex (%)	Female = 70 Male = 30	Female = 70 Male = 30	n.s.
EASI*	13.5 (6.25 – 17.25)	0	n.s.
Pruritus NRS*	4.5 (4 – 6.75)	0	n.s.
Sleep quality NRS*	5.5 (3.25 – 7)	NA	n.s.
vIGA	ADL: 3 (2 – 3); ADNL: 0	0	n.s.

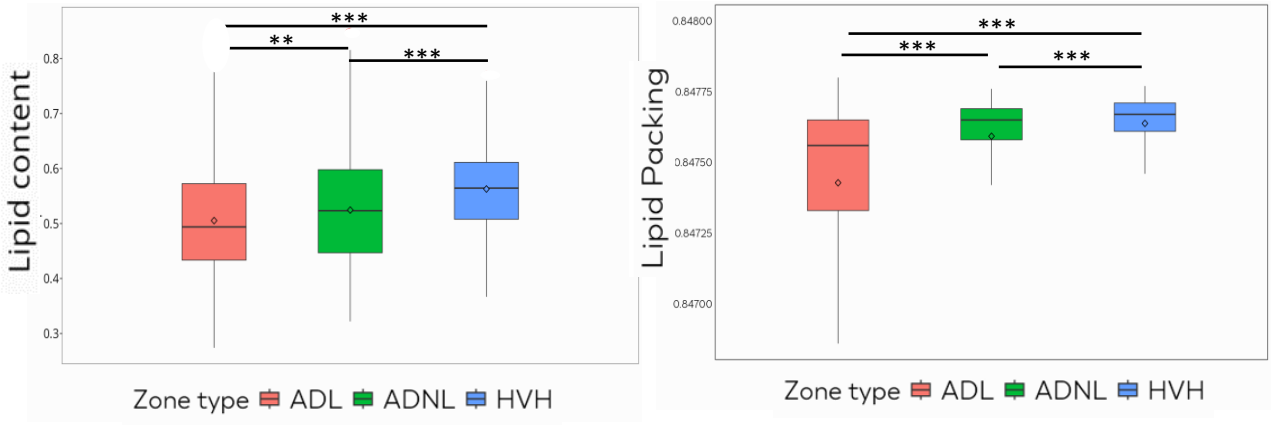
TEWL ($g.m^2.h^{-1}$)	ADL 59.5 ± 21.7	ADNL 26.7 ± 7.5	20.4 ± 11.8	ADL – ADNL < 0.001 ADL – HVH < 0.001 ADNL – HVH = 0.035

*Data provided as median (first quartile – third quartile); NA: Non applicable; n.s.: non significant

The TEWL (Table I) was found to be much higher on ADL skin compared to adjacent ADNL skin (p-value < 0.001), translating a much higher water flux through the SC, as expected [13]. ADNL skin, although clinically healthy, still exhibited TEWL values significantly higher than HVH skin from matched healthy subjects (p-value = 0.035).

3.2. Deficient lipid structure in SC of AD lesional and non-lesional skin using Ramn spectroscopy

In Raman spectroscopy, the total lipid content, which comprises cholesterol, ceramides and free fatty acids amount, was found to be lower in ADL skin compared to ADNL skin, as shown in Figure 1. ADNL skin also had less total lipid than HVH healthy skin. Raman spectroscopy revealed a significantly lower SC lipid density at the microscopic scale of AD skin. Again, the ADNL skin from AD patients was mid-way between ADL skin and skin from healthy volunteers (Figure 1b). This looser lipid packing is believed to compromise barrier function, facilitating both transepidermal water loss and the penetration of external aggressors.



(**a**) (**b**)

Figure 1. Stratum corneum composition and structure revealed using Raman spectroscopy: (a) Total lipid content ; (b) Lipid lateral packing. The stars indicate the p-value (***: p-value < 0.001 ; **: p-value < 0.01 ; *: p-value < 0.05). In red: AD lesional skin (ADL), in green: AD non-lesional skin (ADNL) and in blue: healthy skin (HVH).

To better understand this less dense lipidic organization in AD skin, we investigated the SC ceramides composition.

3.3. Dysfunctional ceramide composition in AD lesional and non-lesional skin using HPLC-MS lipidomics analysis

The analysis of the free ceramides in the skin revealed a significant decrease of most free ceramide classes in ADL skin compared to ADNL and HVH skins, and ADNL skin compared to HVH skin, specifically in classes AH, NH, NP, AP where the drop is very important but also

in the less major classes ADS, EOH, EOS, EODS and EOP (Figure 2). Only the classes AS, ADS and NS are not significantly different between AD skin and healthy skin.

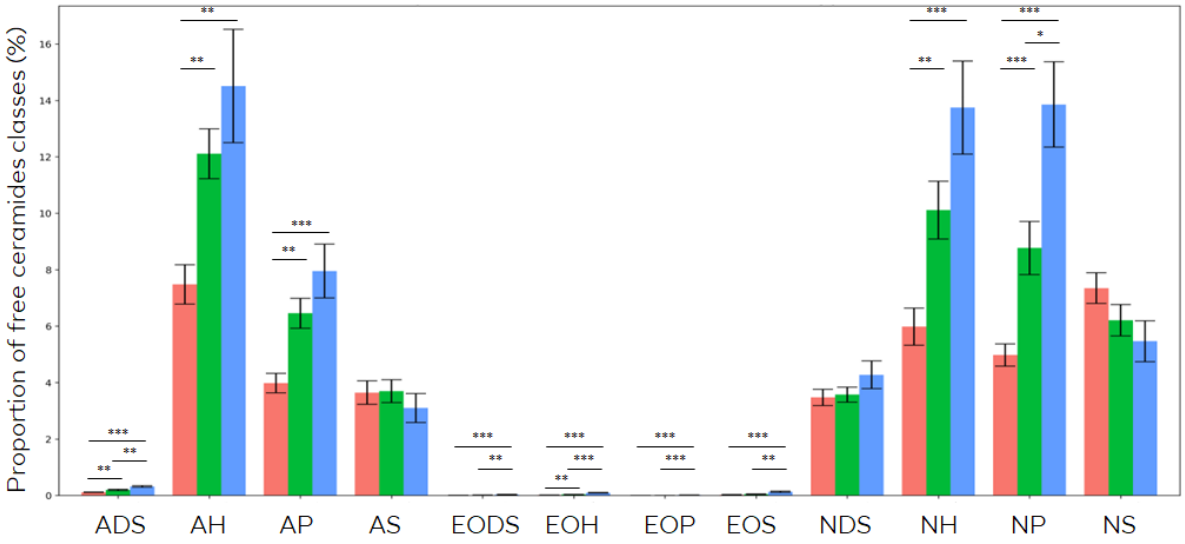
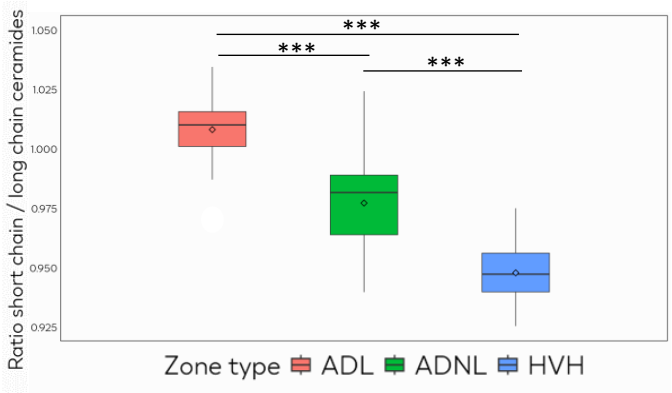


Figure 2. Proportion of stratum corneum free ceramides classes in each skin zone type. The stars indicate the p-value (***: p-value < 0.001 ; **: p-value < 0.01 ; *: p-value < 0.05). In red: AD lesional skin (ADL), in green: AD non-lesional skin (ADNL) and in blue: healthy skin (HVH).

Regrouping the free ceramides by their chain length, ADL skin was found to have a shift of chain length towards short-chain ceramides rather than long-chain ceramides, in line with previous studies [11], compared to ADNL skin and HVH. Again, ADNL skin was found to be midway between ADL and HVH (Figure 3a). Looking at hydroxyl groups in free ceramides, we observe for the first time a significant decrease of trihydroxylated and tetrahydroxylated ceramides in ADL skin compared to ADNL and HVH skins (Figure 3b). No difference was found in the dihydroxylated ceramides. We know that hydroxyl groups enable molecules to create high-intensity intermolecular hydrogen bonds that enhance barrier function and orthorhombic organization of lipidic lamellae in SC [14]. Thus, the decreased amount of long-chain and tri- and tetrahydroxylated ceramides in AD lesion could be partly explain this loss of density and rigidity in lipidic lamellae observed with Raman spectroscopy (Figure 1b).



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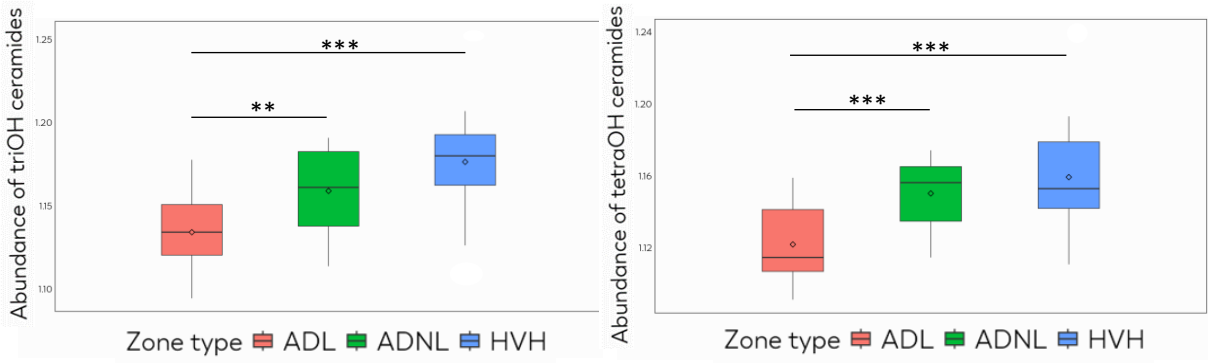
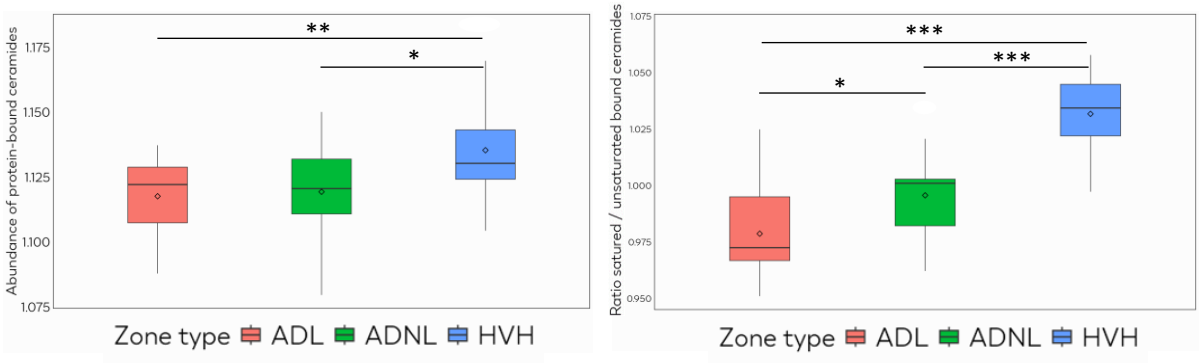


(**b**)

Figure 3. Stratum corneum free ceramides structure showed a loss of rigidity in lipidic lamellae : (a) Decreased long chain ceramides and increased short chain ceramides in AD skin ; (b) Decreased tri and tetrahydroxylated ceramides in AD lesions. The stars indicate the p-value (***: p-value < 0.001 ; **: p-value < 0.01 ; *: p-value < 0.05). In red: AD lesional skin (ADL), in green: AD non-lesional skin (ADNL) and in blue: healthy skin (HVH).

Bound ceramides (to proteins of the cornified envelope) were also found to be decreased in AD skin, whether lesional or non-lesional, compared to healthy skin (Figure 4a). When looking at the fatty acid chain, the ratio of saturated over unsaturated ceramides shifted towards less saturated ceramides in AD skin (Figure 4b), as already shown in the literature [15]. This decrease of saturated bound ceramides weaken the cornified envelope as prevalence of saturated ceramides in healthy skin allows to increase the hydrophobicity and then the membrane rigidity. Looking at the ratio of trihydroxylated over dihydroxylated bound ceramides ((OiH+OH)/(OiS+OH)), we also show that the base of protein-bound ceramides tended to have less hydroxyl groups in ADL skin as for free ceramides (Figure 4c). Thus, less hydrogen bonds are created between molecules and the cornified envelope is weaker than in healthy skin.



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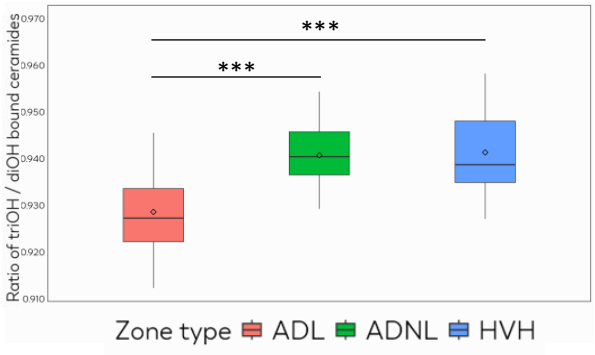

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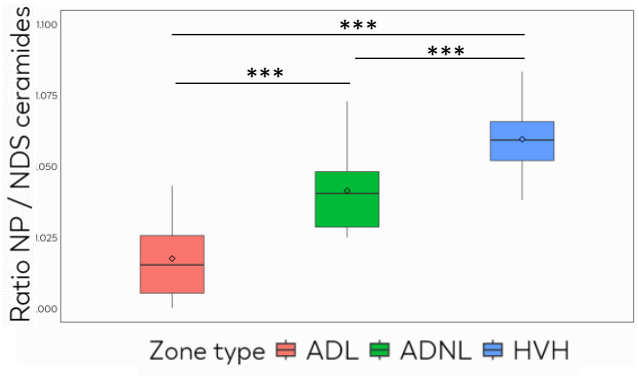
Figure 4. Stratum corneum protein-bound ceramides structure showed a loss of rigidity in cornified envelope: (a) Decreased amount of total protein-bound ceramides in AD skin ; (b) Ratio of saturated over unsaturated bound ceramides showed a decrease in saturated protein-bound ceramides in AD skin ; (c) Ratio of tri over dehydroxylated bound ceramides showed a decrease in trihydroxylated ceramides in AD lesions. The stars indicate the p-value (***: p-value < 0.001 ; **: p-value < 0.01 ; *: p-value < 0.05). In red: AD lesional skin (ADL), in green: AD non-lesional skin (ADNL) and in blue: healthy skin (HVH).

Finally, the lipidomics results can be looked at from the perspective of the ceramides metabolism. In figure 5a, The NP/NDS ratio decreased in ADL compared to ADNL, and ADNL compared to HVH. Thus, the DEGS2 enzyme, transforming NDS into NP by adding hydroxyle group on C4, could have a lower activity or its gene expression could be decreased in AD skin, even when looking clinically normal. Similarly, the NS/NDS ratio increased in ADL skin compared to ADNL skin, but also in ADNL skin compared to HVH skin. However, the NS/NH ratio decreased in ADL and ADNL compared to HVH (Figure 5b). This could be explained by an accumulation of the ceramide NS due to a decreased activity or gene expression of cytochrome P450 responsible of ceramide NS transformation into NH by adding hydroxyle group on C6. Thus, the DEGS1 enzyme activity or gene expression would remain intact in AD skin.



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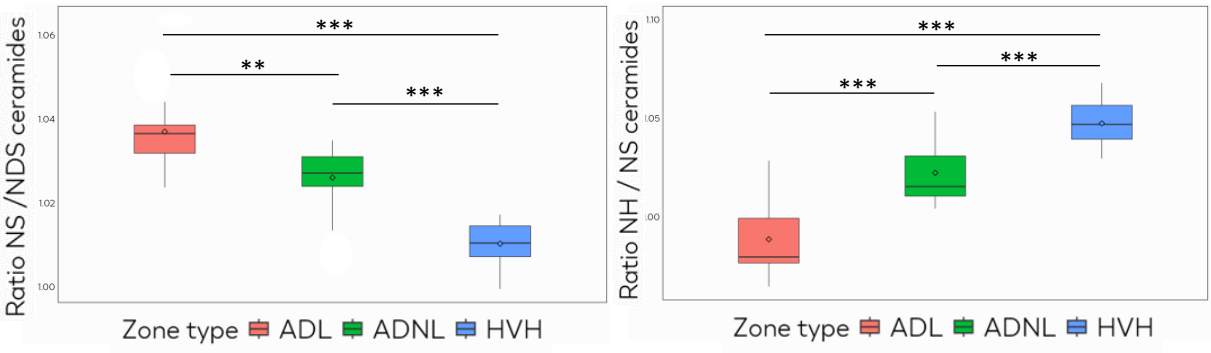

(**b**)

Figure 5. Decreased amount of specific classes of free ceramides showed a change in ceramides metabolism in AD skin : (a) Ratio of NP / NDS could be explained by an alteration of NDS transformation into NP by the enzyme DEGS2 ; (b) Ratios of NS / NDS and NH / NS could be explained by an alteration of NS transformation into NH by the cytochrome P450. The stars indicate the p-value (***: p-value < 0.001 ; **: p-value < 0.01 ; *: p-value < 0.05). In

red: AD lesional skin (ADL), in green: AD non-lesional skin (ADNL) and in blue: healthy skin (HVH).

4. Discussion

Atopic dermatitis is one of the most prevalent skin pathologies, affecting millions of people worldwide with sometimes severe consequences on quality of life. One of the key elements of the physiopathology of AD, along with disruptions in the immune system and imbalance of the cutaneous microbiome, is an altered barrier function. In this study, lesional and adjacent non-lesional skin from AD patients were compared with skin from healthy volunteers with regards to the barrier function, with a focus on the stratum corneum lipids. The lipids were studied from a multiscale perspective using lipidomics techniques, Raman spectroscopy, TEWL measurements and clinical scoring.

The disruption of the barrier function in AD has been described previously in the literature, and in the present study the hallmarks of this disruption were found, as expected. From a functional point of view, the trans-epidermal water loss was indeed higher in AD skin, as is well known [13]. From a compositional point of view, the overall free ceramide content was decreased, while the free ceramide's chain length shifted towards shorter chains [11].

Other results have been partially described and are strengthened by the present study. This is the case for the lower content of saturated protein-bound ceramides [15] and lower DEGS2 enzymatic activity or gene expression leading to a decreased NP ceramide content [16]. The looser lipid packing in the stratum corneum had been previously hypothesized based on the changes in ceramide chain-length and as a way to explain the higher water permeability and described using Raman spectroscopy in only one previous study [17]. In this study, we strengthened these previous results by confirming them.

Finally, in this study, new biomarkers of AD skin were described for the first time, such as the specific decrease in number of OH groups in the base of the free and protein-bound ceramides (reduced triOH and tetraOH free ceramides), a lower activity or expression of the P450 cytochrome, leading in a decreased ceramide NH content, and an accumulation of ceramide NS in AD skin.

These findings at a multiscale level enable to understand the alteration of the skin barrier function in AD by investigating its structure, organization and composition. In this study, specific ceramides classes (free and protein-bound ceramides) and enzymes implicated in ceramides metabolism have been identified as potential new targets to treat AD within medical treatment or emollients. In fact, new formulas of emollients enriched in specific ceramides classes (long-chain ceramides, tri or tetrahydroxylated ceramides) or enzymes (DEGS2 and cytochrome P450) lacking in AD skin could be more efficient to repair the barrier function between eczema flare-ups.

The results highlighted in this study will be useful to investigate specific AD problems that suffer from insufficient understanding and therapeutic options, such as chronic lesions, or the prevention of relapses. Additionally, the suite of techniques used in the current study, by their multiscale complementarity, represent an interesting tool to investigate other barrier-related skin problems, whether pathological (senile xerosis, psoriasis) or more cosmetic.

5. Conclusion

In this study, we investigated AD skin (lesional and non-lesional) compared to healthy skin on clinical, functional, structural and compositional levels. This multiscale approach enabled us to present new findings on the alteration of skin barrier function in AD. The alteration seen as an increased water permeability on a functional level was explained by a looser lipid packing in stratum corneum lipidic lamellae. These lipidic lamellae were weakened by a decrease in long-chain and tri or tetrahydroxylated ceramides. The cornified envelope was also altered due to a lack in saturated and ctrihydroxylated protein-bound ceramides. Finally, these changes in ceramides composition could be explain by the enzymatic activity involved in their metabolism. This study highlights the need of further investigation on how the skin barrier function in healthy-looking skin on AD patients is different from healthy skin on healthy subjects.

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Conflicts of interest

S.B., J.A., E.A., G.R., C.B, E.Rx, and B.L. are L'Oréal employees. E.Rd and A.S. are L'Oreal Research and Innovation dermatologist consultants. J-D.B. received grants from L'Oréal.

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