Linking skin barrier improvement to underlying molecular mechanisms using a multiOMICS approach.

Arbey Eric[a*], Baltenneck Carine[a], Rayée Chrystelle[a], Lynch Barbara[a], Sahuc Florent[b], Benas Damien[b], Valentin Tanguy[b], Ovigne Jean-Marc[b], Gregoire Sebastien[a], Potter Anne[a], Billoni Nelly[a], Bernard Dominique[a], Foucher Aude[a]

a- L'Oréal Research and Innovation, Aulnay-sous-Bois, France
b- Episkin, Lyon, France

* Arbey Eric, 1 avenue Eugene Schueller, 93600 Aulnay sous Bois, France. +33148689137 Eric.arbey@rd.loreal.com

Key words: Reconstructed Skin; Barrier Function; Lipidomics; Proteomics; Mechanical Properties

Abstract (Maximum of 250 words)

The link between the mechanical and physical properties of skin with proteins and lipids, were evaluated in reconstructed skins supplemented or not with FBA, a mix of free fatty acids, cholesterol and vitamin E. Mechanical and physical properties of the reconstructed skins in presence of FBA or the vehicle were evaluated using TEER, TEWL and reference chemical penetration. Molecular entities associated with the improved barrier function and mechanical strength of reconstructed skin were determined using targeted lipidomics and untargeted proteomics approaches. This mechanical and physical improvement was associated with increased representation of bound ceramides and modulation of enzymes involved in the metabolism of ceramides.

Introduction.

The skin, the human body's largest organ, provides a protective barrier against multiple threats: penetration of molecules, water loss, harmful effect of environmental challenges (i.e: UV, ozone). Additionally, it enables the detection of signals such as temperature through its

sensory receptors and contains immune cells enabling the detection and responses to infections. Reconstructed human epidermal (RHE) models have been developed to reproduce in vitro skin functions for research and evaluation purposes. The commercial availability of the SkinEthic™ RHE and EPISKIN™ RHE models in the early 1990s and their validations for use in skin irritation and corrosion assays [1, 2]. Despite many advances in skin engineering and skin model reconstruction, there are still challenges remaining for the production of models with all *in-vivo* functions. One of the main essential skin's functions which require further improvements in reconstructed models is the barrier function [3]. The barrier function *in-vivo* provides a- protection against the external environment (OUT-IN barrier, against physical, chemical and biological aggressors) and b- retention of moisture (IN-OUT barrier).

The *in-vivo* barrier function is supported mainly by the stratum corneum (SC), a multilayer tissue composed of flattened anucleate corneocytes, surrounded by multiple planar lamellae layers, enriched in ceramides, cholesterol and free fatty acids (FFA) [4].

In this study, we evaluated the effect of adding a mix of lipids to the culture media of reconstructed skins on the physical aspects of barrier function, both OUT-IN and IN-OUT, as well as the mechanical properties of the models. We then determined the molecular entities that were associated with the improved function.

1- Materials and Methods.

a. Reconstructed skin model

The reconstructed skin model (SkinEthic Episkin Co, Lyon, France) was reconstructed following the state-of-the-art proprietary epidermal reconstruction protocol in defined culture medium containing either ethanol (vehicle) or a mix of lipids: free fatty acid+ cholesterol +Vitamin E (=FBA) for 17 days. After reconstruction was achieved, histology (HES) was performed to provide a holistic information on the quality of the model. Reconstructed skins were fixed in 10% neutral-buffered formalin, dehydrated in graded alcohols then xylene and embedded in paraffin blocks. Sections 4 micron thick were collected and rehydrated in xylene then alcohols. The hematoxylin-eosin-saffron staining was performed on paraffin sections

using the Sakura Tissue-Tek® Prisma® automated slide stainer, according to the manufacturer's protocol.

b. Histological analyses

Formol fixation and short cycle dehydration Ethanol – Isopropanol Inclusion in paraffin blocks. Paraffin sections at 5 µm, HES staining and image acquisition with Nanozoomer slide scanner (same acquisition parameters for all images).

c. Barrier function mechanical and physical measurements

The barrier function was measured through complementary measurements: chemical (Bodipy) penetration for the OUT-IN barrier, TEWL (trans-epidermal water loss) for the IN-OUT barrier, as well as Trans Epithelial Electrical Resistance (TEER), epidermal thickness (through OCT measurements), and mechanical properties.

Bodipy®FL permeability - A solution of Bodipy®FL 1,5 mM (LifeTechnologies, D3834) was applied in triplicate on the surface of reconstructed skins for 2h at 37°C. 100 µl of basal culture medium were used to measure the Bodipy dye permeability with a spectrofluorometer apparatus (Infinite200 Pro, TECAN).

Permeability coefficient (Kp) on reference chemicals: An aqueous solution containing a mixture of three chemicals (Caffeine, Minoxidil and Oxybenzone) was applied in triplicate at infinite dose for 4 hours. The receptor media, corresponding to the usual media used with reconstructed skin, was sampled over the time, and amount of chemicals was quantified by LC/MS/MS to plot the cumulated permeated amount as a function of time. The slope of curve defined the flux at steady state dived by the concentration to measure permeability coefficients. The assay was conducted directly on the reconstructed skin set up in Petri dish with a magnet bar. Magnetic agitation guaranteed sink conditions. The chemicals were chosen to cover a set of physic-chemicals parameters (log P from -0.07 to 4.03) in a single assay.

Trans-epidermal water loss (TEWL) - The TEWL was measured directly on the reconstructed skin models using a closed-chamber device, the AQUAFLUX (BIOX), with a custom-made measurement head.

Trans Epithelial Electrical Resistance (TEER) - Trans Epithelial Electrical Resistance measurement (TEER) reconstructed skin was topically treated with Triton X-100 1%. Tissues were then rinsed with PBS and TEER was measured using EVOM2 device according to the manufacturer's instructions (World Precision instruments).

Epidermal thickness using Optical Coherence Tomography - Optical coherence tomography (OCT) is a powerful non-invasive optical imaging technique (Ganymede, Thorlabs) that acquires real-time cross-sectional images.

Mechanical properties – The stratum corneum was isolated from the reconstructed skins using tweezers and a classical trypsin-treatment. After drying, the samples were stretched using a uniaxial tensile device (Bose), up to rupture. The slope of the linear part of the stretch-stretch curve was taken as the stiffness modulus of the samples.

d. OMICS analysis

Untargeted bottom-up proteomics, using a label free approach on a Fusion™ mass spectrometer, was undertaken on total cell extracts of reconstructed skin treated with either the vehicle (ethanol, N=6) or a mix of lipids (FBA cocktail, N=6). Briefly, reconstructed skins were grinded via inox beads beating (3min, 30 Hz) in presence of DOC buffer (0,5% sodium deoxycholate, 50 mM ammonium bicarbonate, 50 mM DTT, 1µM Pepstatin, and cocktail of inhibitors) followed by sonication (20 pulses of 1sec on/1sec off at intensity 3). Supernatant was filter (0.45µm, 12000g, 2min), then precipitated with 5 vol of ice-cold acetone. After resuspension in 1% sodium deoxycholate/50mM ammonium bicarbonate, proteins were quantified using the Bradford method at 595nm. Finally, 20 µg were subjected to denaturation (5min at 95°C), reduction (0.2mM DTT for 30min at 37°C), alkylation (0.8mM iodoacetamide for 30min at 37°C in the dark), and digestion via Trypsin (400ng trypsin, overnight at 37°C). Digestion was stopped with 5µl of 50% formic acid and centrifugation (16000g 5min) was performed to remove deoxycholate. Samples were cleaned up on a stage tip and then dried in a speedVac. Tryptic digest analyzed via a Dionex Ultimate™ 3000 nano RSLC coupled to mass spectrometer Orbitrap™ Fusion™. Samples were loaded onto a pre-column (300µmx5mm) to be desalted and concentrated (5min) before injection on an analytical column (15µx500mm C18 3µ 100A). Peptides were eluted using a linear gradient from 5 to 40% of solvent B for 270min. The Orbitrap™ Fusion™ was run in

“data dependent acquisition” mode, with a survey scan acquired on Orbitrap using a mass range of 350 to 1800 m/z and a resolution of 120000. The most intense precursors were then selected during 3sec (TopSpeed) for fragmentation HCD, and fragments ions were detected in the ion trap. Dynamic exclusion was applied to precursor for 20 sec with a mass tolerance of 10ppm. Lock Mas son siloxane ion m/z 445.12005 was used for internal mass calibration. Bioinformatic analysis was performed using MaxQuant 1.6.7.0 against the Human Uniprot database, using as search parameters: Trypsin P, 2 miss cleavages, methionine oxidation and acetylation of proteins in N-terminal as variable modifications, carboxymethylation of cysteines as fixed modifications and a FDR fixed to 1%. Proteins are selected as regulated by the treatment if they fit the following criteria: |Z-score|>1.96, Log2(ratio)>0 (up-regulated) or <0 (down-regulated) and Limma q-value<0.05.

Targeted lipidomics approach focused on free omega fatty acids, free ceramides, bound ceramides extracted from models was performed by mass spectrometry using a Q-Exactive™ orbitrap instrument.

Lipidomic Sample preparation

Free lipids extraction: a serial extraction form the reconstructed skin was undertaken using the following solvent mixtures: 1-MeOH / H_2O: 4/1: 2- CHCl3 / MeOH: ½; and 3 CHCl3 / MeOH : 2/1 o CHCl3. For each fraction, samples were vortexed (5sec), sonicated (5min), heated (30min at 37°C) and finally sonicated once again (10sec). After collection of the organic phase, KCl was added (2.5%), the sample was then vortex before adding 1/10 of MeOH / H_2O (3/2). After decanting, the chloroform phase was recovered and evaporated using a speedvac. Samples were then resuspended in MeOH/IO (2/1), filtered, and injected on HPLC/MS.

Bound lipids: To extract the lipids bound (to the envelope), a gentle alkaline hydrolysis was performed as follows: first NaOH was added (1 mol/L in methanol/H_2O mixture (19/1), incubation 1h at 45°C) The extract was recovered and treated with HCl (2 mol / L in the mixture methanol / H_2O 19 / 1) to which was added chloroform. After shaking, the organic phase was recovered, evaporated and resuspended in MEOH/ISO 2/1. LCMS: Injection on analytical column (Acquity™ BEH C8 2.1mm*100). Lipids were eluted on a linear

gradient from 0 to 100% Isopropanol (in 0.1% HCOOH) in 30 min. 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.

2- Results.

a. Evaluation of reconstructed skins' barrier properties

Multiple physical and mechanical parameters were measured to evaluate the impact of the FBA on the barrier function of the reconstructed skins. We evaluated first the effect of FBA addition to culture medium on epidermal barrier function by studying permeation to 3 reference chemicals with different structures and penetration potentials: caffein, minoxidil and oxybenzone. FBA treated reconstructed skin (FBA) has a significant lower kp that the non-treated (NT) control for all 3 chemicals (Figure 1.A). For example, we detected a kp for caffein decreased by over 2 folds following addition of FBA in the culture medium. In addition, penetration of Bodipy was also significantly reduced after FBA treatment (Figure 1.B), with a decrease of over 3 folds. These results show that the FBA treated reconstructed skin has an improved OUT-IN barrier function compared to the non-treated reconstructed skin.

The TEWL, the quantity of condensed water that diffuses across a fixed area the skin surface per unit time, was significantly decreased in reconstructed skins treated with FBA (Figure 1.C), suggesting a better retention of water (IN-OUT). Finally, the Transendothelial Electrical Resistance (TEER) measurements were increased following FBA treatment (Figure 1.D), pointing towards a better integrity of the tight junctions.

All together, these data demonstrate the global physical and mechanical improvement of the barrier function of reconstructed skins by the FBA both in the OUT-IN and IN-OUT directions.

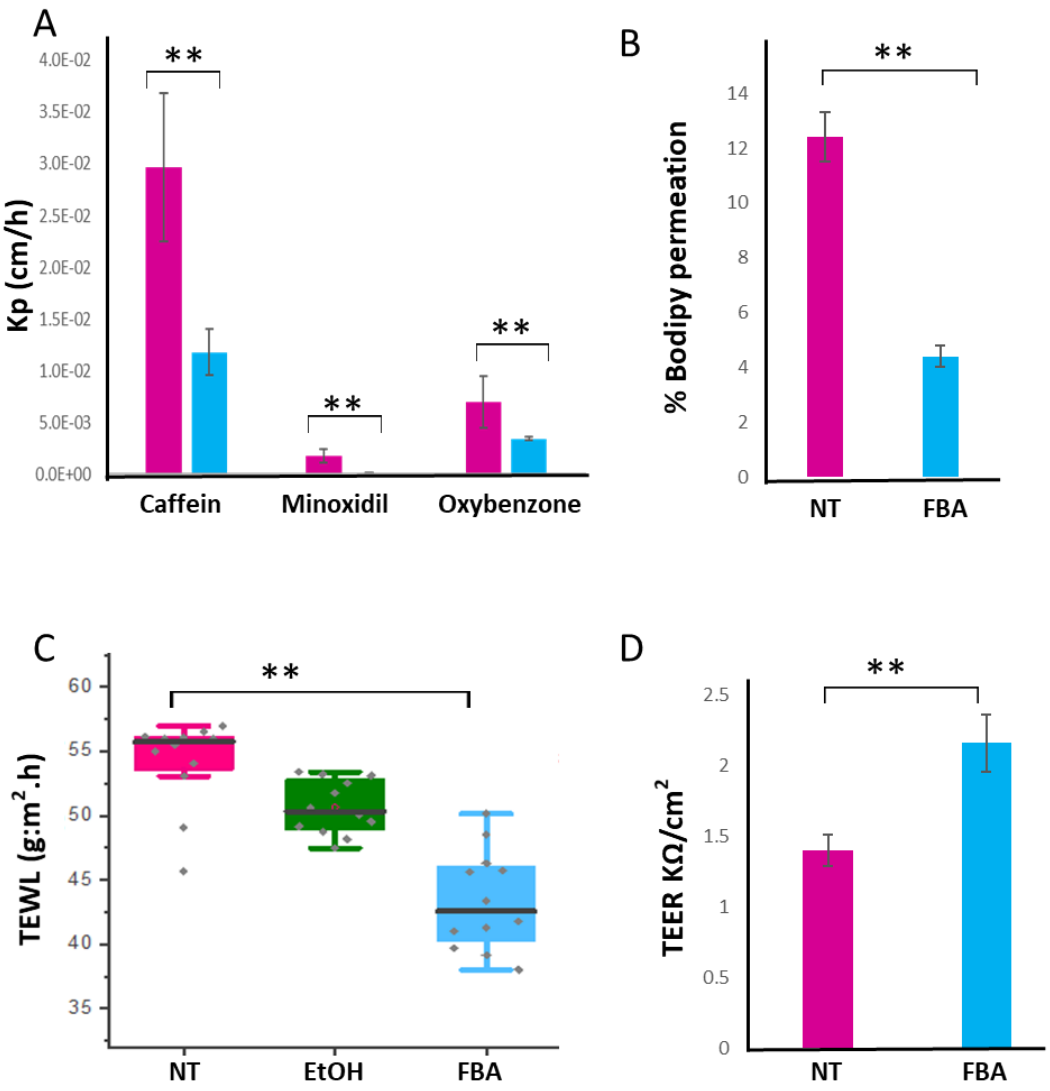

Figure 1: Reconstructed skin barrier function measurements. A) coefficient of penetration kp (cm/h) of 3 reference chemicals (caffein, minoxidil and oxybenzone). B) BODIPY penetration. C) Evaluation of Transepidermal water loss (TEWL) measurements. D) Transendothelial Electrical Resistance (TEER) measurement of barrier integrity as an indication of tight junction formation. *= $P<0.05$, N=3 for each condition.

b. Protein bound ceramides is the main lipid associated with improved physical and mechanical properties

In order to identify the molecular entities associated with the improved IN-OUT and OUT-IN barrier function of the FBA reconstructed skin we performed a multi-OMICS analysis, targeting lipids and proteins. A large panel of molecules known to be present and essential for skin barrier function were analyzed, including 1-free fatty acids, 2- free ceramides, 3-bound ceramides, and 4-cholesterol, covering over 200 entities. In parallel, untargeted proteomics via label free technology enabled the analysis of the proteome, representing over 4000 proteins present in the reconstructed skins. Differential analysis revealed 13 lipids and 158 proteins differentially represented (4% of the proteome) after FBA treatment, with 19 lipids up regulated and 26 proteins up/132 proteins down regulated. From these multi-OMICS analyses, we focused here on 1- lipids and their metabolism and 2- tight junction proteins.

First, a focus is made on the impact of addition of FBA in the culture medium onto the lipid metabolism pathways in the reconstructed skins. No significant modulation was observed on the free fatty acids (70 molecules), the free ceramides (111 molecules) and the cholesterol by the supplementation with FBA.

Free ceramides were recovered in the non-saponified fraction and show no significant modulation. Protein bound ceramides, recovered after saponification, were found significantly increased in the FBA treated reconstructed skin (Figure 2.A, 2.B) with a fold change of 3.4.

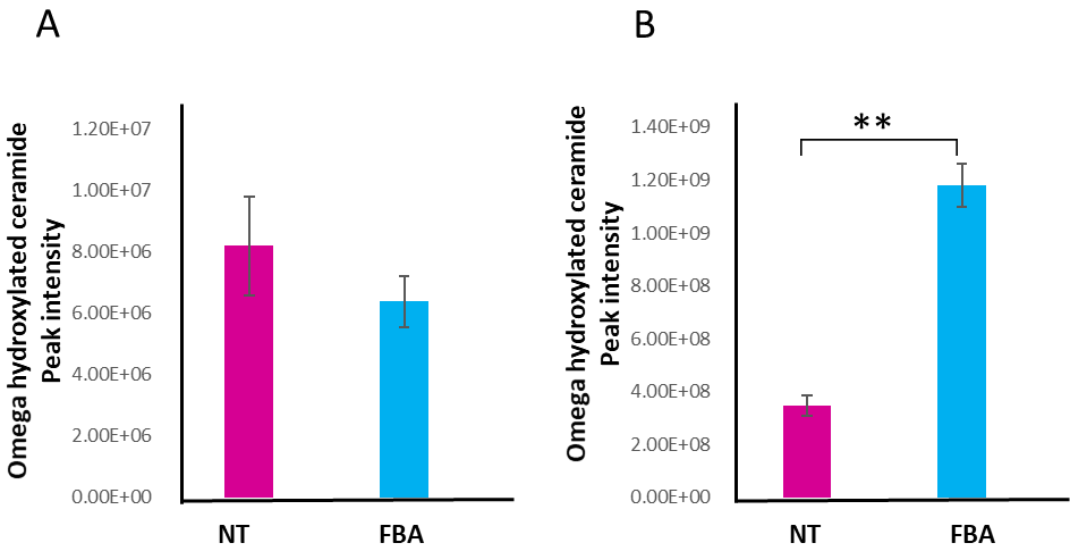

Figure 2: Evaluation of Omega hydroxylated ceramides (C30 to C34). Free ceramides (A) and protein bound ceramides (B) were measured. Five Omega hydroxylated ceramides from chain length C30 to C34 were detected. Histogram representation of the mean of all 5 omega hydroxylated ceramides is represented (N=6 for each condition) with the standard deviation. **=p <0.05.

To understand this difference in amount of protein bound ceramide, we examined the proteins implicated in the metabolism of ceramides. Key enzymes implicated in the production of protein bound ceramides are 1- ceramidase synthase 3 (CERS3, not detected), 2- Omega-hydroxyceramide transacylase (PNPLA1, not detected), 3- Arachidonate 12-lipoxygenase, 12R-type (12RLOX, significantly reduced), 4- Hydroperoxide isomerase (eLOX3, significantly reduced), 5- Short-chain dehydrogenase/reductase family 9C member 7 (SDR9C7, not significantly regulated) and finally 6- Protein-glutamine gamma-glutamyltransferase K (TGM1, not significantly regulated) (Figure 3).

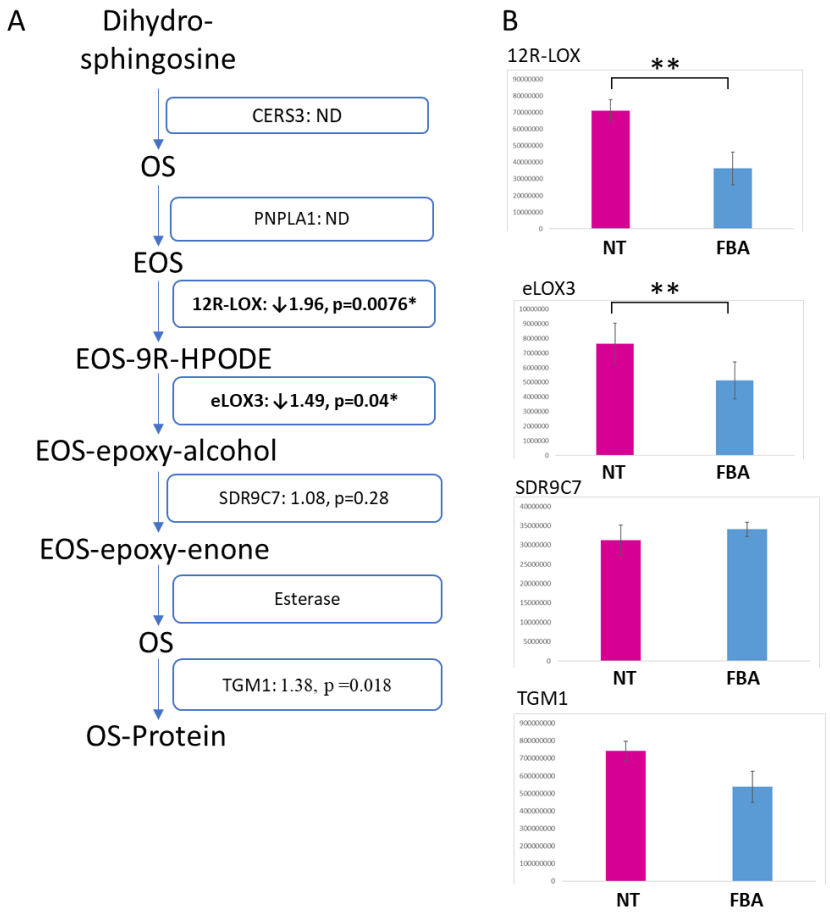

Figure 3: Simplified metabolic pathway leading to protein bound ceramides. A) The main metabolic pathway with the enzymes implicated are presented with the fold change, sens of modulation (↓=down regulation) and adj. p. values when the enzymes were detected in the proteomics analysis. ND= not detected. B) Histogram representation of the protein abundance based on mean peak intensity (N=5) in the reconstructed skin not treated (NT) and treated with FBA (FBA).

As protein bound ceramides are linked to proteins of the cornified envelope via ester linkage through the action of TGM1, we differentially analyzed proteins of cornified envelope identified in the cellular component classification of GO term (GO:0001533). This GO term contains 62 genes, was searched against the proteome of the reconstructed skins in order to reveal potential modulations of proteins associated with the cornified envelope. From this, 13 proteins of belonging to the cornified envelope were found significantly down regulated (FLG2, RPTN, KLK6, KRT16, FLG, SERPINB12, KRT78, KRT1, KLK7, KRT10, TGM3

KRT2, HRNR). Involucrin, one of the main targets of protein bound ceramide was not modulated (fold change = 1.05, adj. P. value=0.822). Another important structural protein of the cornified envelope, Loricrin was analyzed and found not modulated (fold change = 1.20, adj. P. value=0.23).

Discussion.

To understand and characterize the link between physical and mechanical properties of epidermis and its composition in molecular entities such as proteins and lipids, we reconstructed skins with FBA, a mix of fatty acid, cholesterol and vitamin E.

The FBA, was used in systemic application onto reconstructed skin during 17 days before being characterized for its permeability and mechanical properties. Significant decrease in permeability to all reference chemicals tested (Bodipy, caffein, minoxidil and oxybenzone) and improvement of the transepithelial/transendothelial electrical resistance (TEER) was observed in reconstructed skin following FBA supplementation. The improvement was quantified with a decreased of 3 folds for BODIPY and 3 folds for caffein. The FBA being composed mainly of lipids (fatty acids and cholesterol) and the permeability of the reconstructed skins being decreased, the content of the reconstructed skins in 1-free fatty acids, 2-cholesterol, 3-free ceramides and 4-bound ceramides was analyzed in a targeted lipidomic approach covering over 200 molecules. Surprisingly, FBA supplementation had no significant impact on contents of free fatty acids, cholesterol and free ceramides. Only protein bound ceramide were found significantly modulated, with an increased representation in the reconstructed skin model showing improved IN-OUT and OUT-IN barrier function properties. Protein bound ceramides in the *stratum corneum* have been previously found decreased in pathologies in which skin barrier function is impaired, such as atopic dermatitis [5]. Protein bound ceramides are bound via ester linkages to proteins of the cornified envelope (CE). While the exact protein targets for ceramide attachment is still unknown, involucrin has been identified as enriched in protein bound ceramide fractions [6]. Involucrin and loricrin, two main components of the cornified envelope, were not found modulated in the reconstructed skin analyzed. To dig deeper into understanding the molecular mechanisms

linked to the improved barrier function we also analyzed the enzymes responsible for the synthesis and binding of these ceramides to the proteins of the cornified envelope. Two essential enzymes in the metabolism pathway, 12R-LOX and eLOX3 are found significantly down regulated. The proteins, 12R-LOX and epidermal LOX-3 (eLOX-3), act in sequence to convert fatty acid substrates via R-hydroperoxides to specific epoxy alcohol derivatives during epidermal barrier formation. Loss of function of both these enzymes have been described autosomal recessive congenital ichthyosis. Additionally, 12R-LOX and eLOX3 resulted in severe barrier defects [7]. While the data suggest a down regulation of the metabolic pathway leading from the free fatty acids to the free ceramide and finally to the bound ceramides, once must keep in mind that all these analyses were carried out at day 17 of reconstruction. The increase quantities of bound ceramides is not match with an increased amount of 12R-LOX and eLOX3 suggesting that either 1- the timing for looking at these enzymes is not optimum and the up-regulation might have been much earlier, which is then counter balance by a down regulation to keep the equilibrium, 2- the activity might be increased while the amount of enzyme is lower, with some compartmentalization involve or 3- that other enzymes, not yet identified might participate and in some condition even take over 12R-LOX and eLOX3.

Conclusion. Using a multi-parameter analysis, we were able to determine the molecular entities linked to improved physical and mechanical improvement of barrier function of reconstructed skin. At day 17, both IN-OUT and OUT-IN barrier function of the model is significantly improved, and this improvement is associated with a significant increased representation of protein bound ceramides.

Acknowledgments.

Conflict of Interest Statement. L'Oréal funded this study.

References.

1. Green H., Kehinde O.,Thomas J. Growth of cultured human epidermal cells into multiple epithelia suitable for grafting. *Proc. Natl. Acad. Sci. USA.* 1979;76:5665–5668..

2. Prunieras M. Epidermal cell cultures as models for living epidermis. *J. Investig Dermatol.* 1979;73:135–137.

3. J van Smeden , M Janssens , G S Gooris , J A Bouwstra The important role of stratum corneum lipids for the cutaneous barrier function . *Biochim Biophys Acta. 2014 Mar;1841(3):295-313*

4. Schmitt T., Neubert R. State of the art in stratum corneum research. Part II: Hypotehtical stratum corneum lipid matrix models. *Skin Pkarmacol Physiol*, 2020,33(4):213-230.

5. Macheleidt O., Kaiser H.W., Sandhoff K. Deficiency of epidermal protein-bound omega hydroxyceramides in atopic dermatitis. *J Invest Dermatol.* 2002;119(1):166-173.

6. Marekov L., Steinert P. Ceramides are bound to structural proteins of the human foreskin epidermal cornified envelope. *J of biol chem.* 1998;273(28):17763-17770.

7. Krieg P., Rosenberg A., de Juanes S. et al. Aloxe3 knowout mice reveal a function of epidermal lipoxygenase-3 as hepoxilin synthase and its pivotal role in barrier formation. *J invest Dermatol.* 2013;133(1):172-180.