Ultrastructural observation by TEM of tape-stripped skin explants

Germon Goncalves Alexya[1], Abadie Sophie[1], Grosjean Sarah[1], Saito Maï[1], Leduc Claire[1] (presenting author), Philippe Bedos[1].
[1] Syntivia, Toulouse, France.

Introduction:
The *stratum corneum* is the uppermost layer of the skin. It is composed of corneocytes keratin-filled embedded in a lipid matrix characterized by membrane-bound organelles called lamellar bodies that contain a variety of lipids and proteins. Upon reaching the skin surface, lamellar bodies release their contents into the extracellular space, contributing to the formation of the lipid matrix in the *stratum corneum*.
This lipid matrix interspersed between corneocytes allows to reduce trans epidermal water loss (TEWL) and protects against external irritants and pathogens. The organized lipid matrix created by the lamellar bodies is essential to maintain skin hydration and overall integrity.
The purpose of this study is to observe the biological changes after tape stripping on human skin explant. This experimental model consists of removing most of the *stratum corneum* to mimic a superficial injury. It can be used to assess the superficial re-epithelialization, in order to test dermo-cosmetics compounds.

Materials and methods:
A piece of abdominoplasty is stripped using a specific adhesive and then biopsies are created, embedded in a nourishing matrix and disposed in an insert. Barrier alteration and the progress of repair on 7 days, is monitored by histological analyses, ceramides expression and ultrastructural analysis by TEM (Transmission Electron Microscopy).

Results:
On tape-stripped condition, the *stratum corneum* was completely reconstituted after 7 days of culture. The thickness difference between untreated and tape-stripped condition is not significant (-6.0% ns). Ceramides expression significantly decreases after the tape-stripping compared to the untreated condition (- 84.0% ***).
For the ultrastructural analysis by TEM, the main observation is that there are very few lamellar bodies in the analysed skin. Nevertheless, there is an accumulation of lipids droplets in the *stratum corneum*.

Discussion:
Ultrastructural analysis by TEM shows a more detailed skin alteration after tape stripping although reconstruction the *stratum corneum* is complete.
These observations support the hypothesis that the *stratum corneum* reconstruction during the 7 days after tape stripping is accelerated and shows an alteration in the lipidic mechanism.
It seems that 7 days after tape stripping may be too late to observe the early stages of lamellar bodies formation.

Introduction

The skin is the first barrier against exogenous physical and chemical aggression. Skin is composed of different layers. The *stratum corneum* (SC) is the most superficial layer of the epidermis and contains around 20 layers (10µm thickness) [1]. It is composed of flattened, anucleated cells called corneocytes. These keratinocytes are considered as "dead" because they have lost their nuclei and organelles. The plasmic membrane also disappears to create the corneal envelope, a rigid protein shell, composed of loricrin and proteins rich in prolines. This envelope also serves as an anchoring support for the lamellar bodies which will deliver their lipid content into the intercorneocyte space thus serving as intercellular cement to consolidate the cohesion of the cells. Its lipid coating (coming from the lamellar bodies and the appendages of the skin) will also protect the skin from bacteria and maintain its hydration. The cytoplasm of corneocytes is composed of a dense keratin matrix and the subunits of filaggrin. Thanks to the action of transglutaminases, the cytoskeleton will be covalently bound to the corneal envelope, thus playing a major mechanical resistance role[2]. This process of morphological change is called cornification. To maintain a constant epidermal thickness, cells are eliminated by desquamation. During this process, the corneodesmosome, the intercellular junctions of the corneocytes, are degraded by enzymes, the kallikreins, which come from the lamellar bodies. The cells are then detached by mechanical friction. The main functions are to maintain the homeostatic balance, hydration and protection of the body.[3]. Historically, barrier studies and drug penetration have been performed on animals or volunteers using the tape stripping technique. This technique consists of removing several layers of corneocytes with an adhesive tape applied to the skin surface[4]. The procedure is noninvasive, given that only dead cells are removed. So, at day zero the skin has no more barrier, and the restoration of new barrier can be followed the next days. Tape stripping is used to study product penetration, barrier homeostasis on skin diseases and to mimic skin damage. The biological consequences are evaluated by monitoring gene and protein expression, skin morphology, lipid composition, hydration and drug penetration on biopsies[5]. The result of this process depends on anatomical site, sex and age of donor, skin temperature and pH and so the protocol used (number of layers removed, duration, pressure, etc.). Since 2009, animal testing for cosmetics has been banned in UE. It is possible to use human volunteers, but these tests are very expensive and complex to set up to protect human integrity. Consequently, the use of three-dimensional human skin models is required to develop novel active ingredients to repair and maintain the skin barrier. The most used model is reconstructed skin. Models are formed by cultivating human keratinocytes under air-liquid interface conditions to form a stratified epithelium on a matrix containing fibroblasts to mimic the dermis [6]. Its disadvantage is the fragility of the barrier. Human skin equivalents have a poor cells cohesion, and the 3D structure differs slightly from normal human skin. Moreover, to examine the skin barrier, it is better to use a human skin explant [7]. Following aesthetic surgery, skin is exercised and kept alive in specific culture conditions for several days. The native 3D structure, appendages, cellular environment and metabolism are maintained. The skin barrier is complete, allowing topical application of dermo-cosmetic products. To address these issues and avoid the use of volunteers, we have developed a robust cutaneous barrier repair model to evaluate new dermo-cosmetic compounds. For this study, the SC is removed from human skin explants by tape stripping. After stripping, biopsies were cultured to follow the regeneration of SC. To monitor SC formation, we examined skin morphology, ceramide expression and barrier integrity between untreated and tape stripped skin explants. Skin morphology was examined by transmission electron microscopy to observe the lamellar body.

Materials and methods

Human skin tissue collection

Normal human skin samples were collected from donors who underwent abdominoplasty procedures and had given informed consent. Full ethical approval for the study protocol was obtained and all studies were conducted according to the Declaration of Helsinki protocols. The experiments in this study were conducted on three skin donors (women, 33, 35 and 44 years old, phototype II, II and III respectively) originating from abdominoplasty. Skin samples were collected immediately after surgery and stored at 4°C until processed. On the day following surgery and skin sample collection, hypodermis excision was performed and 8 mm diameter round biopsies were punched, stripped and included in nourishing matrix. The human skin explant is maintained in culture at 37°C, 5% CO_2, in an incubator for 7 days with specific medium provided by the supplier, renewed daily.

Tape stripping process

At day zero, the skin explants were stripped with an adhesive tape (3M, Minnesota, USA). One adhesive was used for pressures. In total, 50 pressures have been applied to remove the SC.

Histological analysis

Human skin biopsies were fixed in 4% neutral-buffered formalin (Sigma) at room temperature (RT) 24h after the 0 or 7 days. Skin explants were dehydrated, paraffin-embedded and sectioned at 5 µm thick using a Microtome (Leica, Germany).

Hematoxylin and eosin (HE) staining was performed to identify skin components. Transmitted-light images of the staining were acquired with a Zeiss Axio vert microscope. The mature SC was measured on 10 pictures by conditions. one donor has been evaluated (n=10).

Immunostaining

Skin sections were deparaffinised and hydrated. Ceramids were detected using antibody anti-Ceramides (Glycobiotech GmbH). A secondary antibody, Alexa Fluor 594 anti-mouse IgM (Invitrogen Life Technologies, Carlsbad, USA), was then added for detection. Nuclei were stained with 4,6-diamidino-2-Phenylidole (DAPI, Sigma-Aldrich) and slides were observed with a fluorescence microscope Zeiss Axio vert. The specific mean fluorescence intensity was determined by image analysis (Image J) from 10 images by condition (n=10).

TEM (Transmission Electronic Microscopy)

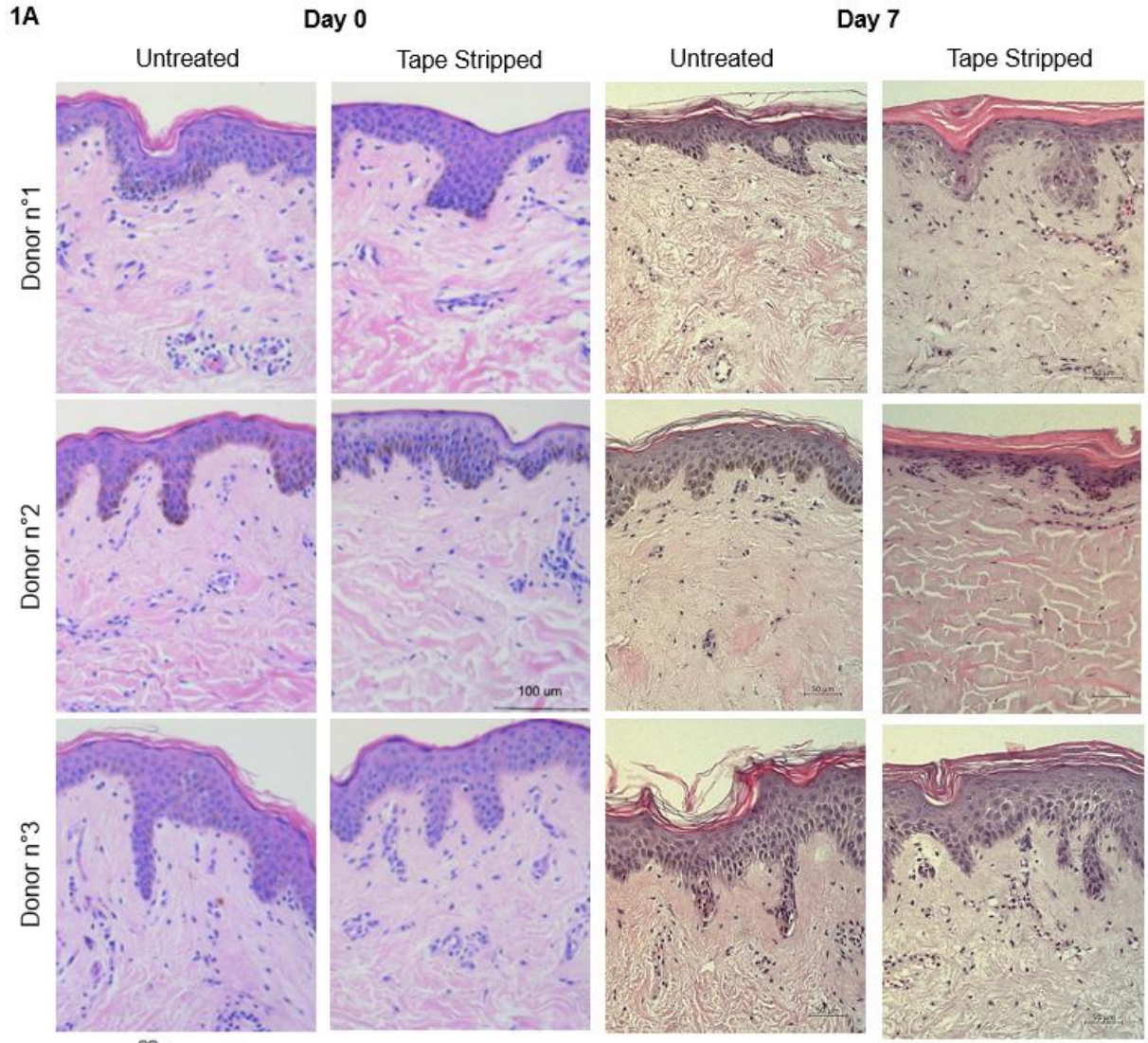
Following the treatments, explants were fixed, rinsed and post-fixed. Explants were then dehydrated and embedded in EMbeb 812 resin. Afterwards, the samples were sliced into 70 nm-thick sections and were mounted on a copper grid coated with Collodion 100 before being stained with uranyl acetate and Reynolds Lead Citrate. The samples were imaged using a Hitachi HT7700 transmission electron microscope (TEM) at an acceleration voltage of 80 kV. 10 images per sample were captured and analysed qualitatively. The accumulation of lipid droplets in the SC was quantified on 10 images per conditions and was reported to 100µm².

Statistical analysis

Data represent the mean ± standard error of mean. Significant differences between averages were analysed with a Mann-Whitney test at a p-value of 5% (*p-value<0.05, **p-value<0.01, ***p-value<0.001, ns non-significant). The comparisons were performed in relation to respective donors between control and treated skin.

Results

Measure of SC reconstruction



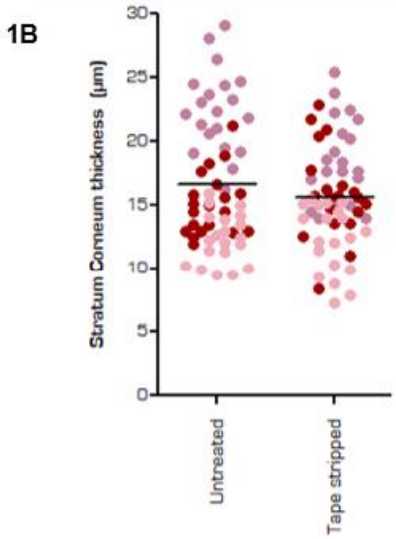


Figure 1: *Stratum corneum* size stripped Human skin explant.
(1A) Representative images of H&E staining acquired from sections of paraffin-embedded
human skin explants: untreated and tape-stripped (scale bars 100 µm),
(1B) Quantification of SC thickness on 3 skins donors

- **Donor 1**
- **Donor 2**
- **Donor 3**

In normal skin, the keratinocytes are well-connected in the epidermis (Figure 1A). The nuclei and the cytoplasm are stained in purple. The *SC* is visible as a pink layer. The upper layers

of the *SC* are composed of disjoined corneocytes, A normal *SC* measures around 10 µm of thickness. The extracellular matrix of dermis is composed of fibers that appear in pink. In this matrix, we can see small, purple-stained nuclei, identified as fibroblasts. Fiber's density normally varies between the papillary and the reticular dermis.
On tape-stripped explants, the *SC* was completely reconstituted after 7 days of culture. The thickness of the epidermis is heterogeneous and is sometimes thinner than that of the normal skin. *SC* appears mainly mature and well reconstituted, but it may present an immature formation like in donor number 2 with some area containing parakeratosis (persistence of nuclei).
The thickness of the *SC* was measured (pink and continuous stratum).
For the global result, data from all donors are pooled (Figure 1B). The thickness of the *SC* is not modified after the tape-stripping. The reconstruction is complete.

Lipids analysis through Ceramides expression

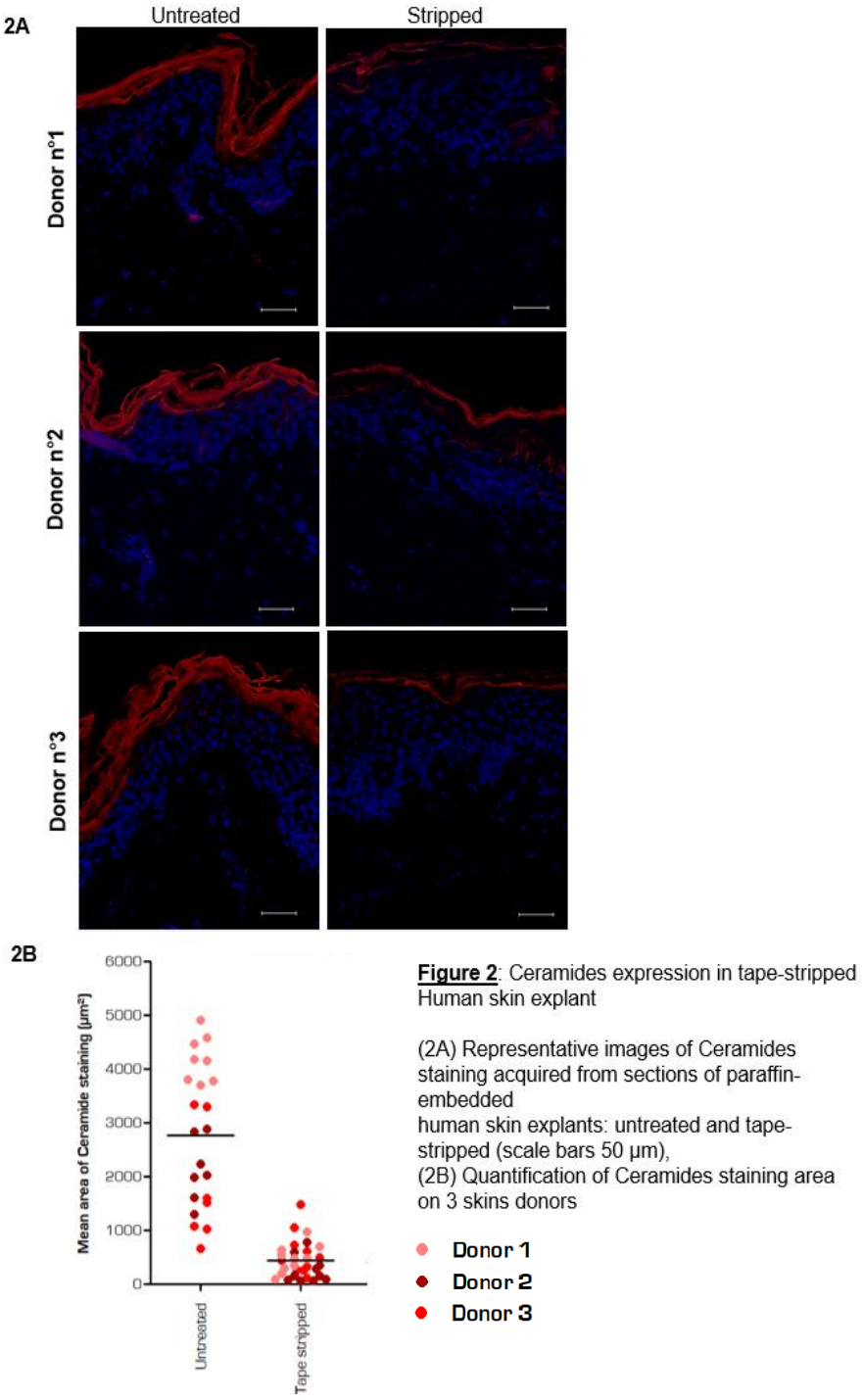


Figure 2: Ceramides expression in tape-stripped Human skin explant

(2A) Representative images of Ceramides staining acquired from sections of paraffin-embedded
human skin explants: untreated and tape-stripped (scale bars 50 µm),
(2B) Quantification of Ceramides staining area on 3 skins donors

Ceramides are particularly present in the *SC* (Figure 2A). The mean area of ceramides staining was calculated.
For donors 1, 2 and 3, Ceramides expression significantly decreases after tape-stripping compared to untreated explants (- 88.6% ***, - 87.4% *** and - 67.4% ** respectively). For the global result (Figure 2B), data from all donors are pooled. Ceramides expression significantly decreases after the tape-stripping compared to untreated explant (- 84.0% ***).

Ultrastructural observation and lipid analysis by Transmission Electron Microscopy

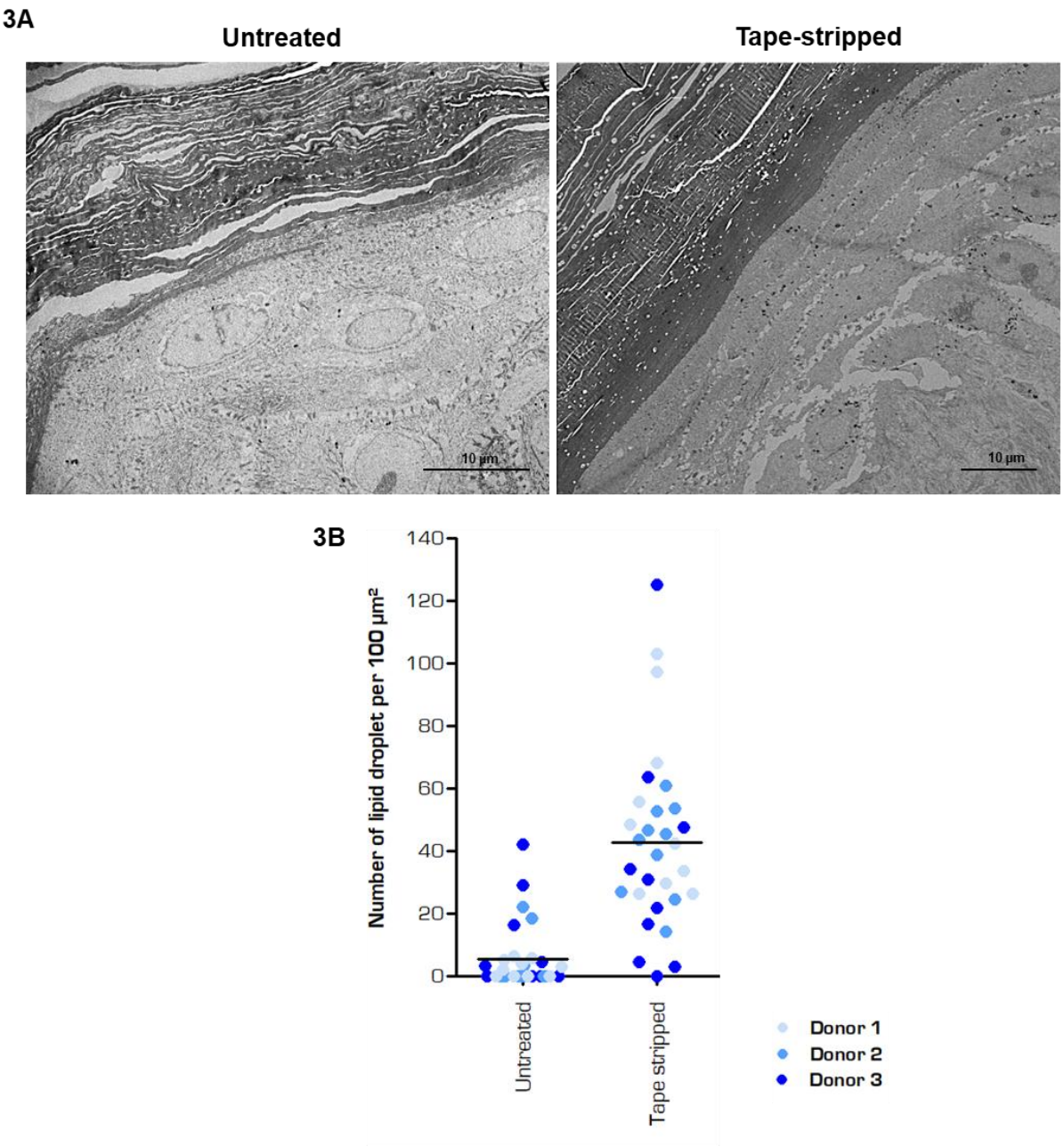


Figure 3: Global view of Human skin explant by TEM
(3A) Representative images of skin explant ultrastructure acquired by TEM human skin explants: untreated and tape-stripped (scale bars 10 µm),
(3B) Quantification of lipid droplets in SC on 3 skins donors

In tape stripped condition, the intercellular junctions between epidermal cells are stretched (black arrows Figure 3A). In contrast, in the untreated unstriped condition, epidermal cells are well connected by desmosomes, and the *SC* and *SG* are attached (Figure A).
Additionally, in tape stripped conditions, there is an accumulation of lipid droplets in the *SC*. In contrast, in untreated conditions, there is much less lipid persistence. This suggests that the accumulation of lipids droplets in the tape-stripped condition is not normal.
These observations support the hypothesis that the *SC* reconstruction during the 7 days after tape stripping is accelerated and presents a defect in the lipidic mechanism.

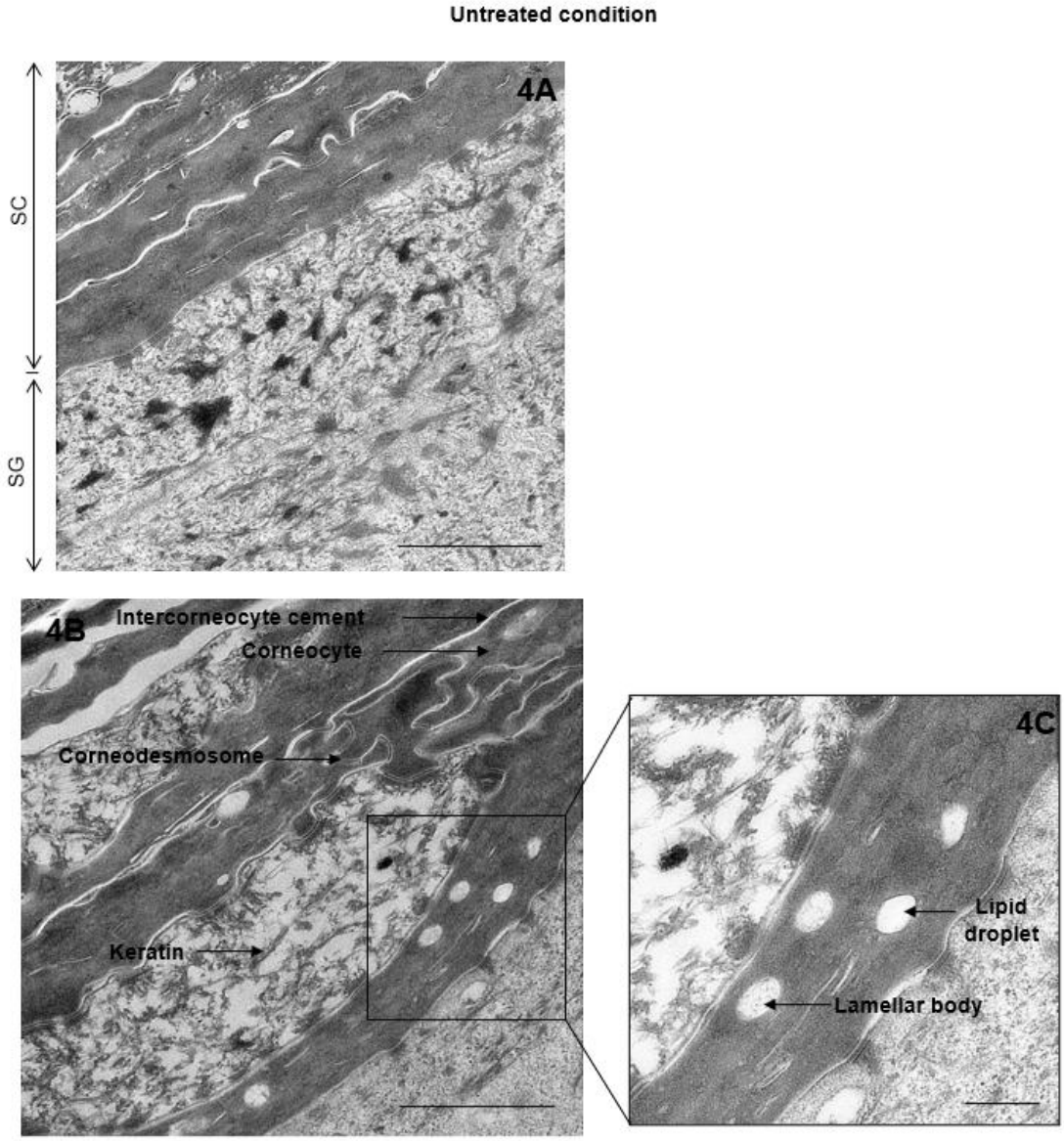


Figure 4: Analysis of skin morphology by TEM in Human skin explant
(4A) Representative images of global SC ultrastructure acquired by TEM human skin explants: untreated (scale bar 2 µm),
(4B) Focus of SC ultrastructure acquired by TEM human skin explants: untreated (scale bar 2 µm),
(4C) Focus of SC ultrastructure acquired by TEM human skin explants: untreated (scale bar 500 nm),

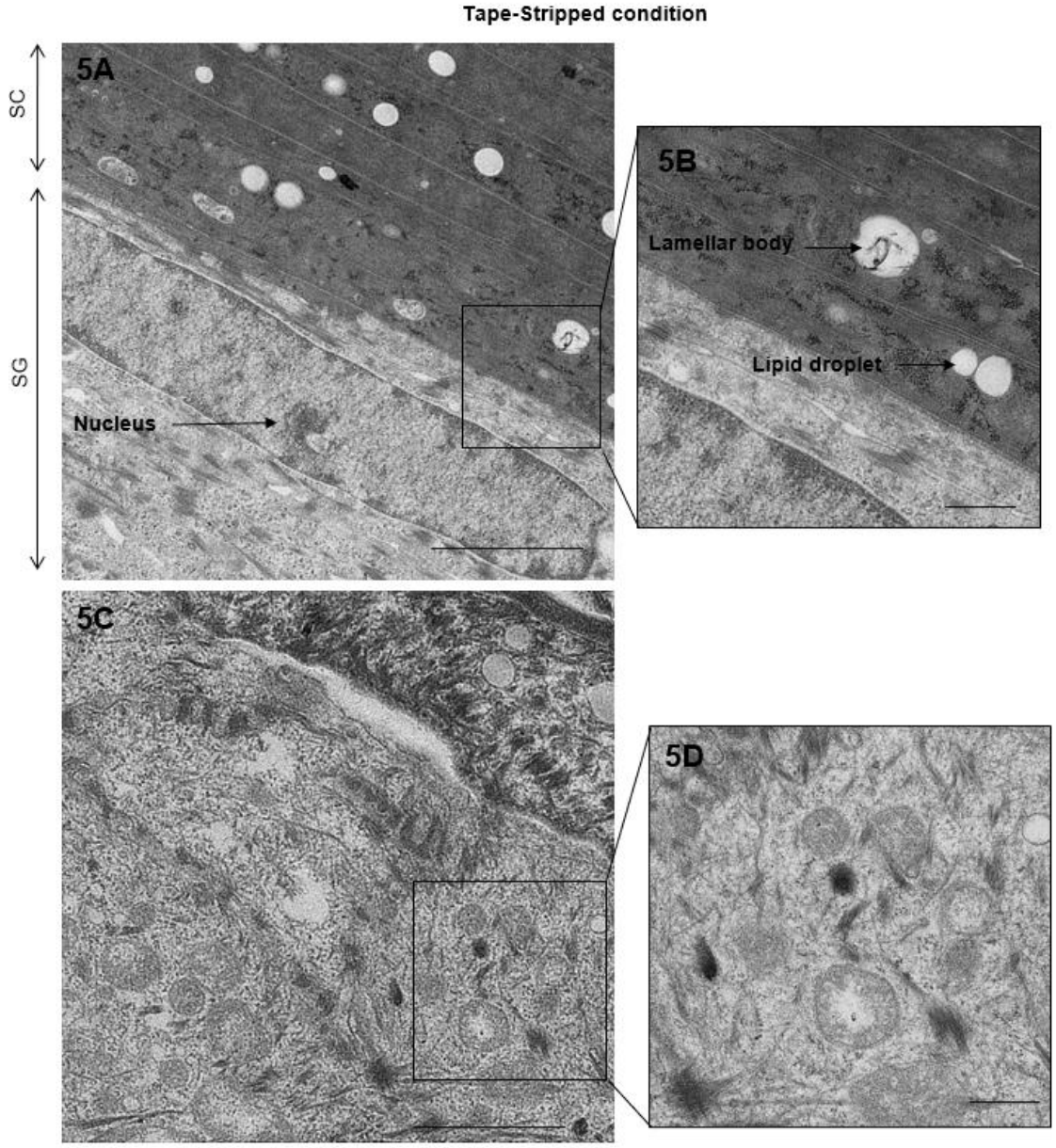


Figure 5: Analysis of skin morphology by TEM in tape-stripped Human skin explant
(5A/5C) Representative images of global SC ultrastructure acquired by TEM human skin explants: tape-stripped (scale bar 2 µm),
(5B/5D) Focus of SC ultrastructure acquired by TEM human skin explants: tape-stripped (scale bars 500 nm),

To analyse the *SC* reconstruction, each condition was observed and described qualitatively. We focus our observations on the upper layers between the *SC* and the *SG*. In untreated conditions (Figures 4), corneocytes are well connected, corneodesmosomes and the intercorneocyte cement are intact and linked (Figure 4A). Inside corneocytes there are keratin mesh, some lipid droplets and lamellar bodies (Figures 4B/4C).
In tape stripped condition, the *SC* is dense and compact. The intercorneocyte cement is less visible. Lipid droplets accumulate in the *SC* (Figure 5A). This lipid persistence suggests an alteration in the lipid metabolism and cells differentiation. A few lamellar bodies filled with lipids are presents in the *SC* and in the *SG* (Figure 5B).

Discussion

The aim of this project is to study the alteration of the barrier function by tape-stripping on human skin explant.
In this way, human skin explants were tape stripped to remove the *SC* to assess barrier alteration, we analysed the skin morphology by TEM and Ceramides expression.

After 7 days of culture, the *SC* of tape-stripped skin explants was reconstructed. However, the Ceramides expression was significantly decreased (- 84.0% ***).
The analysis of skin morphology shows a balance between proliferation and differentiation.
For tape stripped explants, the thinner epidermis is associated with low proliferation. However, the reconstruction of the *SC* thickness is representative of the differentiation.

To analyse the lipid production, an observation was performed by Transmission Electron Microscopy (TEM).
The main observation is that there are very few lamellar bodies in the analysed skin. Some of them contained lipids, while others were empty, depending on the stage of their exocytosis in the intercorneocyte cement. We wonder why a significant number of lamellar bodies have not been observed. One hypothesis is that 7 days after tape stripping may be too late to observe the early stages of lamellar bodies formation. After 7 days, the *SC* is completely reconstructed as the untreated condition. Moreover, the mechanism of lipid discharge into the intercorneocyte cement could have slowed down, resulting in fewer lamellar bodies. It might be interesting to observe the process earlier after the tape stripping, during the early stage of *SC* reconstruction. At this point, we would expect to observe a higher quantity of lamellar bodies.
Nevertheless, in the tape-stripped condition, a particularity was observed: a persistence of lipids in the *SC*. There is a high number of lipid droplets. In the literature, this accumulation suggests an alteration in the lipid metabolism and a high rate of triglyceride synthesis [8]

This still represents an altered skin model that could be used to test dermo-cosmetics products that could improve lipid metabolism and enable better skin repair.

Conclusion

This observation aligns with the findings from the measure of *SC* thickness and morphological analysis: We observed incomplete differentiation and accelerated maturation of the horny layer after tape stripping. The use of TEM has enabled us to visualize the ultrastructure of the *SC* and the dispersal of lipids in the tissue.

1. Danso, M.O., Berkers, T., Mieremet, A., Hausil, F., et Bouwstra, J.A. (2015) An *ex vivo human* skin model for studying skin barrier repair. *Exp. Dermatol.*, **24** (1), 48-54.
2. Bonté, F. (2011) Skin moisturization mechanisms: New data. *Ann. Pharm. Fr.*, **69** (3), 135-141.
3. Elias, P.M., Gruber, R., Crumrine, D., Menon, G., Williams, M.L., Wakefield, J.S., Holleran, W.M., et Uchida, Y. (2014) Formation and Functions of the Corneocyte Lipid Envelope (CLE). *Biochim. Biophys. Acta*, **1841** (3), 314-318.
4. Keurentjes, A.J., Jakasa, I., et Kezic, S. (2021) Research Techniques Made Simple: SC Tape Stripping. *J. Invest. Dermatol.*, **141** (5), 1129-1133.e1.
5. Escobar-Chávez, J.J., Merino-Sanjuán, V., López-Cervantes, M., Urban-Morlan, Z., Piñón-Segundo, E., Quintanar-Guerrero, D., et Ganem-Quintanar, A. (2008) The tape-stripping technique as a method for drug quantification in skin. *J. Pharm. Pharm. Sci. Publ. Can. Soc. Pharm. Sci. Soc. Can. Sci. Pharm.*, **11** (1), 104-130.
6. Jung, K.-M., Lee, S.-H., Jang, W.-H., Jung, H.-S., Heo, Y., Park, Y.-H., Bae, S., Lim, K.-M., et Seok, S.H. (2014) KeraSkin^{TM}-VM: A novel reconstructed human epidermis model for skin irritation tests. *Toxicol. In Vitro*, **28** (5), 742-750.
7. Lebonvallet, N., Jeanmaire, C., Danoux, L., Sibille, P., Pauly, G., et Misery, L. (2010) The evolution and use of skin explants: potential and limitations for dermatological research. *Eur. J. Dermatol. EJD*, **20** (6), 671-684.
8. Vicanová, J., Boelsma, E., Mommaas, A.M., Kempenaar, J.A., Forslind, B., Pallon, J., Egelrud, T., Koerten, H.K., et Ponec, M. (1998) Normalization of epidermal calcium distribution profile in reconstructed human epidermis is related to improvement of terminal differentiation and SC barrier formation. *J. Invest. Dermatol.*, **111** (1), 97-106.