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The importance of 12R-lipoxygenase and transglutaminase activities in the hydration-dependent *ex vivo* maturation of corneocyte envelopes

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Abstract

BACKGROUND: Terminally differentiated keratinocytes acquire corneocyte protein envelopes (CPE) complexed with corneocyte lipid envelopes (CLE). These two structural components of the corneocyte envelopes (CEs) undergo maturation by gaining in hydrophobicity, rigidity and surface area. Linoleoyl acylceramides are processed by 12R-lipoxygenase (12R-LOX) and other enzymes before transglutaminase (TG) attaches ω-hydroxyceramides to involucrin in the CPE. Concurrently, structural proteins are cross-linked by TG that has been activated by cathepsin D (CathD).

OBJECTIVES: The primary aim of this work was to demonstrate the impact of relative humidity (RH) during *ex vivo* CE maturation. Low, optimal and high RH were selected to investigate the effect of protease inhibitors (PIs) on CE maturation and TG activity; in addition, 12R-LOX and CathD activity were measured at optimal RH. Finally, the effect of glycerol on *ex vivo* CE maturation was tested at low, optimal and high RH.

METHODS: The first and ninth tape strip of photo-exposed (PE) cheek and photo-protected (PP) post-auricular sites of healthy volunteers were selected. *Ex vivo* CE maturation was assessed via the relative CE maturity (RCEM) approach based on CE rigidity and hydrophobicity. The second and eighth tapes were exposed to RH in the presence of inhibitors.

RESULTS: Irrespective of tape stripping depth, CEs from PE samples attained CE rigidity to the same extent as mature CEs from the PP site, but such improvement was lacking for CE hydrophobicity. 70% RH was optimal for *ex vivo* CE maturation. The inhibition of 12R-LOX activity resulted in enhanced CE rigidity which was reduced by the TG inhibitor. CE hydrophobicity remained unchanged during *ex vivo* maturation in the presence of TG or 12R-LOX inhibition. CE hydrophobicity was enhanced in the presence of glycerol at 44% RH and 100% RH but not at 70% RH. Furthermore, TG activity was significantly diminished at 100% RH compared to the commercial inhibitor LDN-27219. However, a protease inhibitor mix reversed the negative effect of overhydration.

CONCLUSION: The study adds to the understanding of the roles of 12R-LOX and TG activity in CE maturation and gives further insight into the effect of glycerol on the SC.

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Résumé

CONTEXTE: Les kératinocytes à différenciation terminale acquièrent les enveloppes protéiniques des cornéocytes (ECP) complexées aux enveloppes lipidiques des cornéocytes (ELC). Ces deux composants structurels des enveloppes cornéocytaires (EC) subissent un processus de maturation en gagnant en hydrophobicité, en rigidité et en surface. Les linoléoyl-acyle-céramides sont traités par 12R-lipoxygénase (12R-LOX) et d'autres enzymes avant que la transglutaminase (TG) ne fixe les x-hydroxy-céramides à l'involucrine dans les ECP. Les protéines structurelles sont simultanément réticulées par la TG qui a été activée par la cathepsine D (CathD).

OBJECTIFS: L'objectif principal de ces travaux visait à démontrer l'impact de l'humidité relative (HR) pendant la maturation de l'EC *ex vivo*. Des humidités relatives faible, optimale et élevée ont été retenues pour étudier l'effet des inhibiteurs de la protéase (IP) sur la maturation de l'EC et l'activité de la TG ; l'activité de CathD et 12R-LOX a également été mesurée à une HR optimale. Finalement, l'effet du glycérol sur la maturation de l'EC *ex vivo* a été testé à des humidités relatives faible, optimale et élevée.

MÉTHODES: La première et neuvième bandes adhésives sur un site à l'arrière de l'oreille protégé de la lumière (photo-protégé, PP) et sur une joue exposée à la lumière (photo-exposée, PE) de volontaires sains ont été sélectionnées. La maturation de l'EC *ex vivo* a été évaluée par l'approche de la maturité relative d'EC (RCEM) reposant sur l'hydrophobicité et la rigidité de l'EC. Les deuxième et huitième bandes ont été exposées à l'humidité relative en présence d'inhibiteurs.

RÉSULTATS: Indépendamment de la profondeur de bande adhésive, les EC des échantillons EP ont atteint la rigidité d'EC de la même manière que les EC matures du site PP, mais ces améliorations faisaient défaut en ce qui concerne l'hydrophobicité des EC. Une HR à 70 % était optimale pour la maturation de l'EC *ex vivo*. L'inhibition de l'activité du 12R-LOX a entraîné une rigidité accrue de l'EC, laquelle était réduite par l'inhibiteur de la TG. L'hydrophobicité des EC est restée inchangée pendant la maturation *ex vivo* en présence de l'inhibition de la TG ou du 12R-LOX. L'hydrophobicité des EC a été améliorée en présence de glycérol à une HR de 44 % et à une HR de 100 %, mais non à une HR de 70 %. L'activité de la TG a par ailleurs significativement diminué à une HR de 100 % par rapport à l'inhibiteur commercial LDN-

27219. Cependant, un mélange inhibiteur de la protéase a inversé l'effet négatif de la surhydratation.
CONCLUSION: L'étude renforce la compréhension des rôles de l'activité de la TG et du 12R-LOX dans la maturation de l'EC et donne de plus amples détails sur l'effet du glycérol sur la couche cornée (stratum corneum, SC).

Introduction

Keratinocytes develop into corneocytes during the epidermal differentiation process and formation of the stratum corneum (SC). Progressive cross-linking of a range of structural proteins in the internal side of the keratinocyte cell membrane creates an insoluble

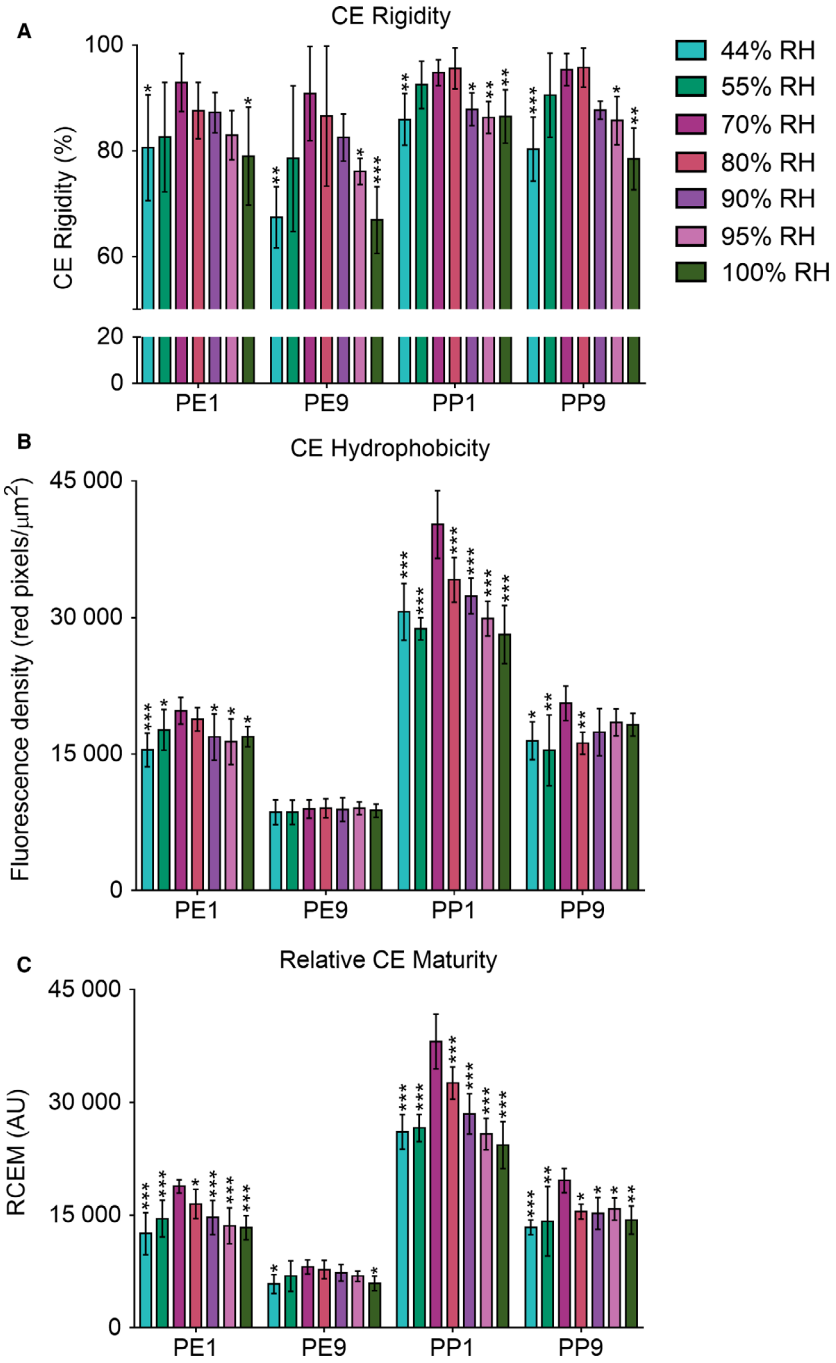

Figure 1 CE rigidity (a) and CE hydrophobicity (b) and RCEM (c) are shown in mean ± SD (n = 6) and the statistical differences compared with ex vivo matured at 70% RH which showed more efficient enhancement via one-way ANOVA with Holm–Sidak's post-test; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ or $^{***}P \leq 0.001$.

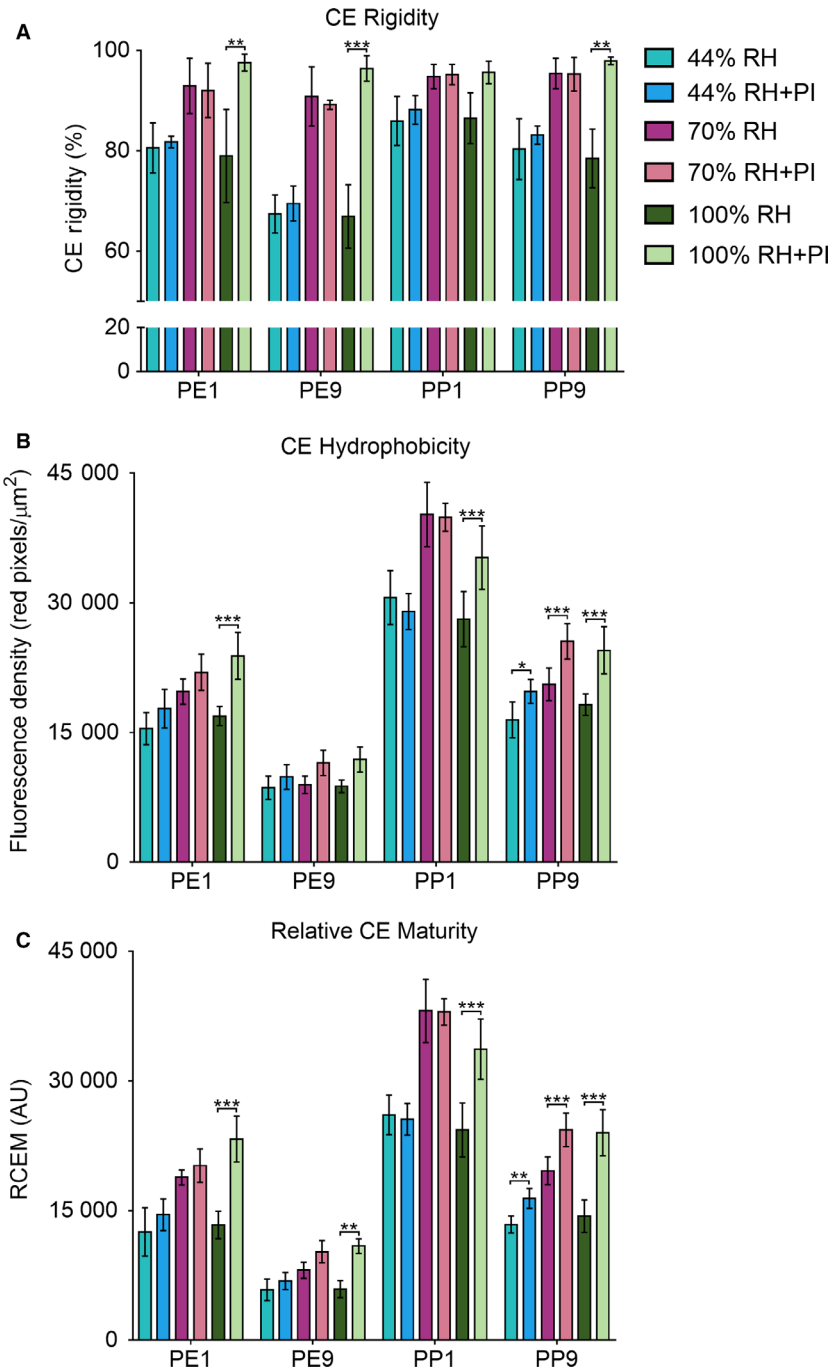

Figure 2 CE rigidity (a) and CE hydrophobicity (b) and RCEM (c) in CEs ex vivo matured at 44% RH, 70% RH and 100% RH. Protease inhibitors enhanced ex vivo CE maturation in 100% RH. Mean ± SD (n = 6); one-way ANOVA with Holm–Sidak's post-test; *$P \leq 0.05$, **$P \leq 0.01$ or ***$P \leq 0.001$.

corneocyte protein envelope (CPE) [1]. At the same time, phospholipases degrade the phospholipid bilayer of the cell membrane resulting in the formation of free fatty acids [2]. Concomitantly, covalent attachment of ω-hydroxyceramides to the involucrin-exposed CPE external surface occurs to begin to form the corneocyte lipid envelope (CLE). Linoleoyl acylceramides are processed to ω-hydroxyceramides by a cascade of enzymes namely 12R-lipoxygenase (12R-LOX), epidermal hydro peroxide isomerase eLOX3, epoxide hydrolases and esterases [3,4]. The isodipeptide cross-linking of proteins in the CPE and the attachment of the CLE are believed to

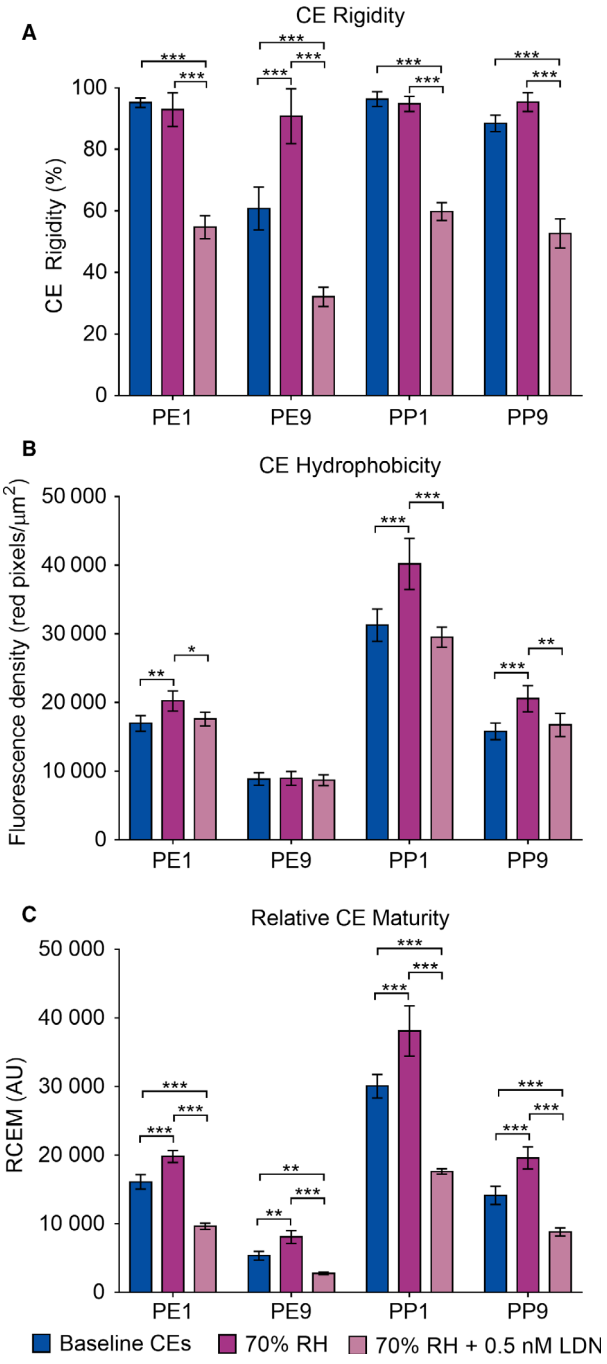

Figure 3 The effect of TG inhibition (by 0.5 nM LDN-27219) on CE rigidity (a), CE hydrophobicity (b) and RCEM (c) of corneocytes ex vivo matured at 70%. Data are shown in mean ± SD (n = 6) with statistical analysis via one-way ANOVA with Holm–Sidak's post-test; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ or $^{***}P \leq 0.001$.

be mediated by transglutaminases (TG) 1, 3 and 5 [5]. These two processes contribute to the gain in rigidity and hydrophobicity of corneocyte envelopes (CE) that occurs during SC maturation [6–8].

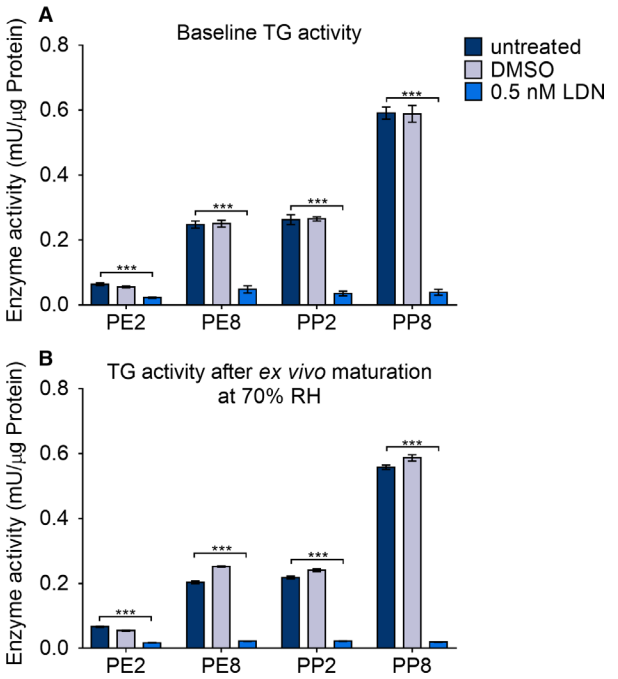

Figure 4 TG activity at baseline and ex vivo matured samples in the presence of 0.5 nM LDN-27219, its solvent DMSO and the untreated reaction buffer. Data are shown in mean ± SD (n = 12) with statistical analysis via one-way ANOVA with Holm–Sidak's post-test; $^{***}P \leq 0.001$.

Mature corneocytes subsequently contribute to SC integrity [9] and SC cohesion [10] in order to support SC barrier function [11]. A recent study demonstrated low protein levels of 12R-LOX in photo-damaged facial SC using mass spectrometry [12]. A lower 12R-LOX activity has also been reported in photo-damaged skin [13] along with a lower CE hydrophobicity [8].

Reports have shown that changes in relative humidity (RH) affect different aspects of skin physiology [14,15]. Murine models demonstrated that the skin barrier is formed more efficiently in mice maintained at conditions of 50–75% RH compared to higher RH levels (85%) [16]. A later study demonstrated that hairless mice developed a thicker epidermis with a thicker SC at dry RH (10%) compared to 40–70% RH [17]. External humidity has also been shown to play a regulatory role in proteases involvement in filaggrin degradation [18–20], skin hydration [21,22], skin integrity [17] and desquamation [23]. However, aside from these studies, little is known about the effect of RH on corneocyte maturation [14,15,24].

Previous work has demonstrated that corneocytes were able to mature *ex vivo* using a test solution containing calcium and dithiothreitol to enhance TG activity. An autofluorogenic exogenous substrate, dansyl cadaverine, was inserted into the CEs by TG. Tape strips from the cheek were immersed in the test solution and exposed to 100% and 70% RH in the presence of glycerol as a humectant. CE maturation was enhanced at 100% RH but suppressed at 70% RH. The diminished CE maturation at 70% RH was enhanced in the presence of glycerol [24,25]. The CE maturity was determined via immunostaining for involucrin and lipid staining [24], an approach that has been recently demonstrated to have

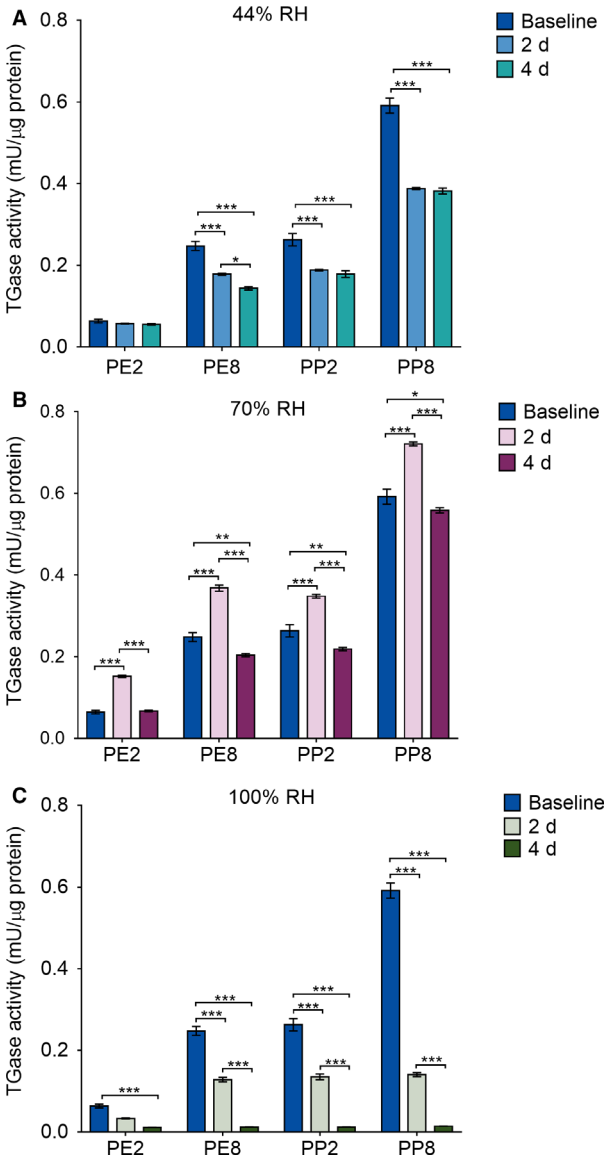

Figure 5 TG activity at baseline, second and fourth days of ex vivo maturation at 44% RH (a), 70% RH (b) and 100% RH (c) is shown in mean ± SD (n = 6). TG activity enhances in 70% RH at the second day while decreases in 44% RH and a steeper decrease in 100% RH. One-way ANOVA with Holm–Sidak's post-test; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ or $^{***}P \leq 0.001$.

methodological limitations [8]. This may explain why 100% RH was optimal for corneocyte maturation but 70% RH inhibited the maturation process, reversible only in the presence of glycerol [25]. This is surprising as glycerol was observed to improve corneodesmolysis at 80% RH [22] and CE maturation *in vivo* [7] in line with other *in vivo* benefits associated with this humectant [25].

In this current study, the *ex vivo* maturation of corneocytes on SC tape strippings from photo-exposed (PE) cheek and photo-protected (PP) post-auricular sites of Chinese subjects was assessed. A range of relative humidities (44, 55, 70, 80, 90, 95 and 100% RH) was screened to identify the optimal humidity for CE maturation. This was followed by investigating the effect of protease inhibition (PI), before *ex vivo* maturation at low, optimal and high RH, on CE maturation. The TG and 12R-LOX activities were assessed with and without inhibition at optimal RH for *ex vivo* CE maturation. Finally, the impact of glycerol on *ex vivo* CE maturation was determined at low, optimal and high RH. In all sets of experiments, the changes in CE rigidity and CE hydrophobicity were assessed using the recently published relative CE maturity (RCEM) approach [8].

Materials and methods

Recruitment of participants

Ethical approval was granted by the UCL Research Committee and the NHS London-Bromley Research Committee (Reference: 16/LO/1672). Healthy Chinese volunteers were recruited, and the participants were six females and six males (26 ± 3 years old, median age 25 years). Subjects were advised not to use any cosmetic products for at least 15 days before the tape stripping the SC.

SC sampling

Nine consecutive standard D-Squame® tapes (diameter: 2.2 cm; area: 3.8 cm^2; CuDerm Corporation, Dallas, TX, USA) were sampled from the PE cheek (3 cm below the outer edge of the eye) and the PP post-auricular sites (close to the earlobe). Tape stripping was performed on acclimatized skin (19 ± 2°C; 44 ± 7% RH). A pressure device (CuDerm Corporation, Dallas, TX, USA) was used to apply 225 g cm^{-2} pressure to the skin and tape for 5s with intervals of 20 ± 5s between each tape which were removed in a single movement [26]. SC integrity was determined via transepidermal water loss (TEWL) measurement with an Aquaflux AF102 instrument (Biox Systems Ltd., London, UK) at baseline and 30 ± 5s after the third, sixth and ninth tape strippings. SC protein content on tape strips was measured using a SquameScan™ 850A device (Heiland Electronics GmbH, Wetzlar, Germany) [27]. Inverse TEWL values and cumulative SC protein amounts were plotted in order to evaluate SC thickness [28,29].

Ex vivo CE maturation

The first and ninth tape strip from the cheek (PE1/PE9) and post-auricular (PP1/PP9) sites were selected to determine the RCEM based on CE rigidity and hydrophobicity. All tapes were cut in half and placed in humidified chambers for four days at 37°C to allow corneocytes to mature at humidities established with saturated salt solutions: potassium carbonate (44%), ammonium nitrate (55%), sodium nitrate (70%), ammonium sulphate (80%), potassium sulphate (90%), barium chloride (95%) and water (100%). Protease activities on tape strips were blocked with Pierce™ Protease Inhibitor Tablets (containing 4-benzenesulfonyl fluoride hydrochloride (AEBSF), aprotinin, bestatin, E-64, leupeptin and pepstatin A) – EDTA free in 1/10 mL PBS (Thermo Fisher Scientific, Hertfordshire) before incubation at low, optimal and high RH. 12R-LOX was inhibited before *ex vivo* CE maturation at optimal RH with 1:500 anti-12R-LOX polyclonal antibody (0.32 mg mL^{-1}, Thermo Fisher Scientific, Hertfordshire, UK; Cat# PA5-23608, RRID: AB_2541108) diluted in 0.5% bovine serum albumin (BSA) in PBS. TG activity was inhibited by immersing the tapes in TG blocking solution containing LDN-27219 (Sigma-

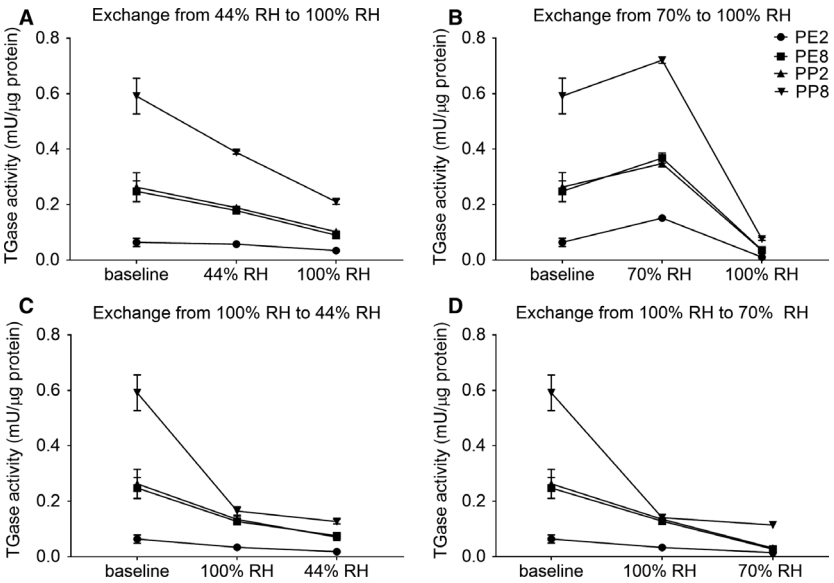

Figure 6 Change in RH after two days of ex vivo CE maturation has an impact on TG activity. TG activity is shown when RH is changed from 70% RH to 100% RH (a) and vice versa (b) with 100% RH being the limiting factor. Similar changes in TG activity were seen when changed from 44% RH to 100% RH (c) and in reverse (d) shown in mean ± SD (n = 6).

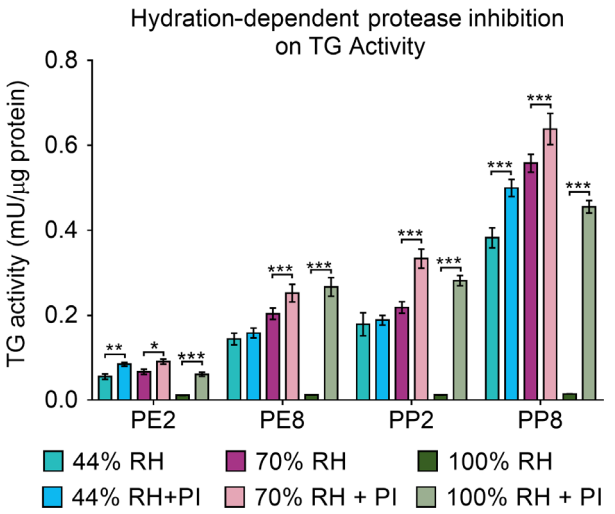

Figure 7 A cocktail of protease inhibitor prior to ex vivo maturation shows a humidity-dependent effect on TG activity with the biggest impact on samples ex vivo matured at 100% RH. Data are shown in mean ± SD (n = 6) with one-way ANOVA with Holm–Sidak's post-test; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ or $^{***}P \leq 0.001$

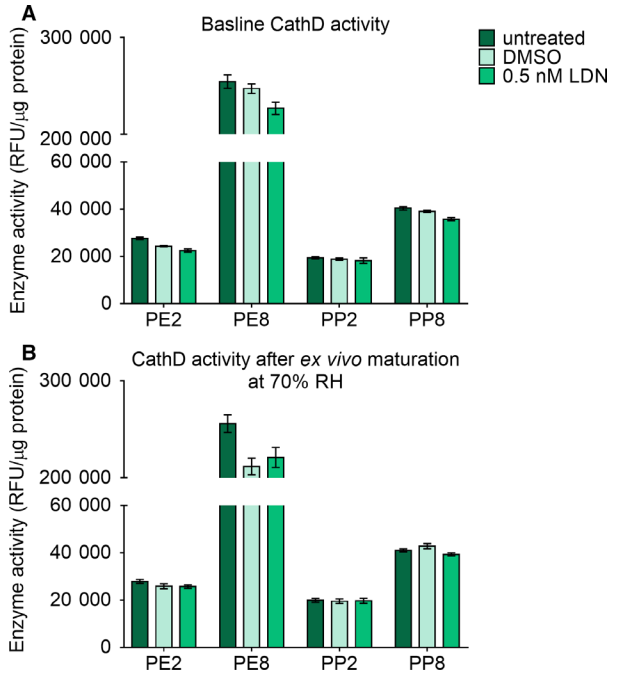

Figure 8 Cathepsin D activity at baseline and ex vivo matured samples in the presence of 0.5 nM LDN-27219, its solvent DMSO and the untreated reaction buffer. Data are shown in mean ± SD (n = 12) with statistical analysis via one-way ANOVA with Holm–Sidak's post-test.

Aldrich, Dorset, UK) dissolved in DMSO and diluted in PBS to a final concentration of 0.5 nM prior to *ex vivo* CE maturation at optimal RH. A further set of samples collected with the first and ninth tape of both anatomical sites was immersed in either water or 5% glycerol solution (Sigma-Aldrich, Dorset, UK) before *ex vivo* maturation at low, optimal and high humidity. All samples were stored along with control samples at −80°C before determination of RCEM [30].

CE isolation and determination of relative CE maturity

Corneocyte envelopes were extracted as previously described by incubating the tapes in 750 µL dissociation buffer (20 mM Tris-

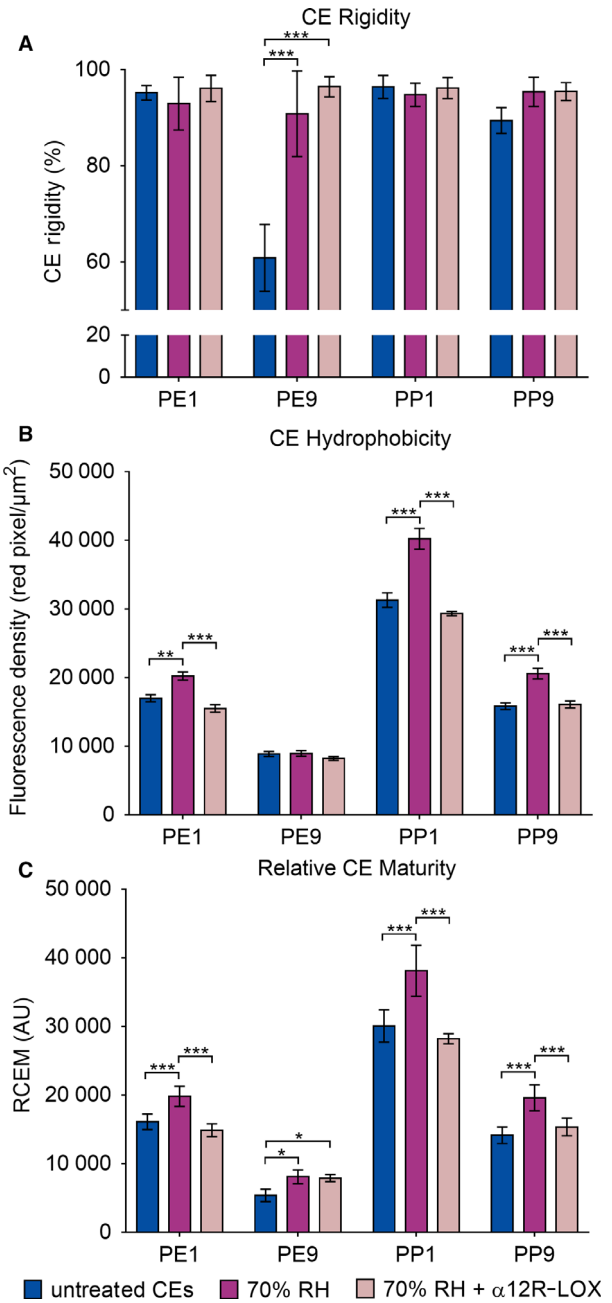

Figure 9 Blocking of 12R-LOX via antibody leads to increased CE rigidity (a), whereas CE hydrophobicity (b) and RCEM (c) were suppressed at 70% RH ex vivo maturation. Data are shown in mean ± SD (n = 6) with statistical analysis via one-way ANOVA with Holm–Sidak's post-test; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ or $^{***}P \leq 0.001$.

HCl pH 8.0, 5 mM EDTA, 2% SDS and 10 mM DL-dithiothreitol; Sigma-Aldrich, Dorset, UK) for 10 min at 75°C. This was followed by 3-min shaking at 1000 rpm and centrifugation at 5000 ***g*** for 10 min. The insoluble CE pellet was washed in 20 mM Tris-HCl pH 8.0, 5 mM EDTA, 0.2% SDS and 10 mM DL-dithiothreitol, and the

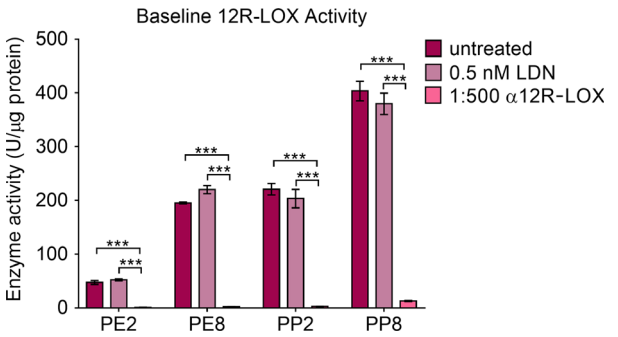

Figure 10 12R-LOX activity at baseline and ex vivo matured samples in the presence of antibodies against 12R-LOX, 0.5 nM LDN-27219 and untreated reaction buffer. Data are shown in mean ± SD (n = 12) with statistical analysis via one-way ANOVA with Holm–Sidak's post-test.

washing process was repeated three times before suspending CEs in 50 µL of 1x PBS (Thermo Fisher Scientific, Hertfordshire, UK) [8].

Corneocyte envelope rigidity was assessed by subjecting 20 µL of the CE sample to sonication (44 kHz) for 10 min at 4°C (Grant Instruments Ltd., Cambridgeshire, UK), whereas untreated CEs were kept at 4°C for 10 min before placing samples (n = 3) on polysine-coated microscope slides (VWR International Ltd, Leicestershire, UK). CE hydrophobicity was visualized with Nile red staining (20 µg mL^{-1} in 75% glycerol solution). Fluorescence images were taken at 10x objective magnification and analysed via ImageJ® version 1.51j8 (National Institutes of Health, Bethesda, MD, USA).

Corneocyte envelope rigidity was evaluated according to morphological appearance and expressed as the percentage of rigid CEs after sonication (equation 1).

$$\text{CE Rigidity } (\%) = \frac{\text{sonicated CEs}}{\text{control CEs}} \times 100 \quad (1)$$

Images of non-sonicated CEs were measured for CE hydrophobicity (fluorescence per surface area) as shown in equation 2 [8].

$$\text{Relative CE Maturity (AU)} = \text{CE Hydrophobicity}\left(\frac{\text{red pixels}}{\mu m^2}\right) \times \text{CE Rigidity} \quad (2)$$

Transglutaminase activity assay

The TGase activity assay was performed with samples from the second and eighth tape strips taken from the PE cheek and PP postauricular area at baseline and at various conditions of *ex vivo* maturation. The baseline samples represent the conditions at the point of sample collection before any treatment. Tapes were incubated for four days at 44% RH, 70% RH or 100% RH. One set of tapes was exposed to 44% or 70% RH for two days and then maintained at 100% RH for two days. Another set of samples was incubated at 100% RH for two days, followed by incubation at 44% RH or 70% RH for two further days. The tapes that were cut in half were incubated in 500 µL of 0.5 % Triton X-100 in 0.1 M Tris-HCl buffer for 20 min on a shaker at 600 rpm and at 4°C (Eppendorf, Stevenage, UK). The assay was performed as described by Slaughter [31] with 100 µL/well of sample and substrate solution, which were incubated for 30 min at 37°C. The reaction was stopped before

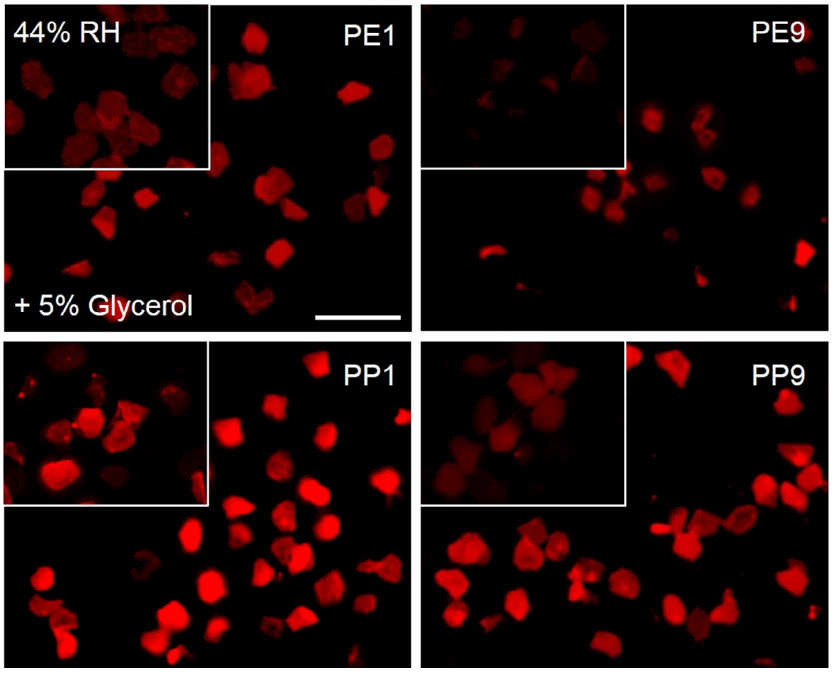

Figure 11 Nile red staining of corneocytes ex vivo matured at 44% (inlays) and with additional 5% glycerol prior to ex vivo maturation. Scale bar = 100 μm.

adding streptavidin–alkaline phosphatase which was incubated for 1 h at RT. The unbound enzyme was washed off before adding the phosphatase substrate solution containing magnesium chloride. The enzyme kinetics were measured at 405 nm in a SpectraMax iD3 (Molecular Devices, Wokingham, UK) over a period of 1 h at intervals of 1 min. The TGase assay value is expressed as mU min^{-1} normalized to the total SC protein amount on the corresponding tape strip.

Cathepsin D activity assay

Cathepsin D (CathD) is a protease that activates TG; hence, CathD activity was measured with the Fluorometric Assay Kit (BioVision, Milpitas, CA, USA) as a proof of concept for the impact of DMSO and LDN-27219 (LDN) on CathD. The CathD activity was measured at baseline and *ex vivo* matured CEs at optimal RH for both tested areas of the skin and depths. Samples were lysed in 200 μL of cell lysis buffer chilled at 4° on ice for 10 min and centrifuged for 5 min at 14 500 *g* (Eppendorf, Stevenage, UK). The assay was prepared with 25 μL sample, 75 μL reaction buffer and 2 μL substrate solution, and the resulting fluorescence was measured in a Corning® clear flat-bottom black 96-well plate (Sigma-Aldrich, Dorset, UK) at an interval of 1 min for 1 h at 37°C with Ex/Em = 328/460 nm in a SpectraMax iD3 (Molecular Devices, Wokingham, UK). The results are expressed as relative fluorescence unit (RFU) after 10 min of reaction as some samples reached a fluorescence maximum after 15 min; the results were normalized to the total protein amount on the corresponding tape strips.

12R-LOX activity assay

A recently developed 12R-LOX assay was used to analyse the 12R-LOX activity at baseline and activity in *ex vivo* matured samples at optimal RH [13]. The tapes were immersed for 20 min on a shaker at 600 rpm at 4°C in 0.1% NP40 in 50 mM Tris (Sigma-Aldrich, Dorset, UK) and supplemented with Pierce™ Protease Inhibitor Tablets (1 per 10 mL of buffer, Thermo Fisher Scientific, Hertfordshire). The reaction buffer was prepared with 50 mM Tris, 4 mM $CaCl_2$, 4 mM EDTA, 4 μM ethyl linoleic acid (Sigma-Aldrich, Dorset, UK), 5 μM ATP (New England Biolabs, Hitchin, UK) and 5 μM 2′,7′-dichlorodihydrofluorescein diacetate (Thermo Fisher Scientific, Hertfordshire). Each component of the reaction buffer was purged with argon. The assay was prepared with 50 μL of sample and purged with 150 μL reaction buffer in a Corning® clear flat-bottom black 96-well plate (Sigma-Aldrich, Dorset, UK) with an interval of 1 min for 20 mins at 37°C with Ex/Em: 495/527 nm in a SpectraMax iD3 (Molecular Devices, Wokingham, UK). The 12R-LOX antibody was diluted (1:500) in argon-purged 0.5% bovine serum albumin (BSA) and used as a blocking agent for 12R-LOX activity. In addition, the effect of 0.5 nM LDN was tested on 12R-LOX activity. The 12R-LOX activity is expressed as the difference in resulting fluorescence per unit time and is normalized for the protein content on the corresponding tape strip.

Statistics

All statistical analysis was performed with GraphPad Prism (version 7). The data passed the D'Agostino & Pearson normality test; therefore, one-way ANOVA with the Sidak–Holm post-hoc test were chosen to determine statistical differences. Results are expressed as mean ± SD ($n = 6$ for *ex vivo* experiments and $n = 12$ for enzyme assay), whereas statistical significance is represented as $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ or $^{***}P \leq 0.001$. Furthermore, the Pearson correlation was determined for CE properties and the influence of RH on TG activity (Table S4) as well as the impact of glycerol (Tables S1-S3) on *ex vivo* CE maturation.

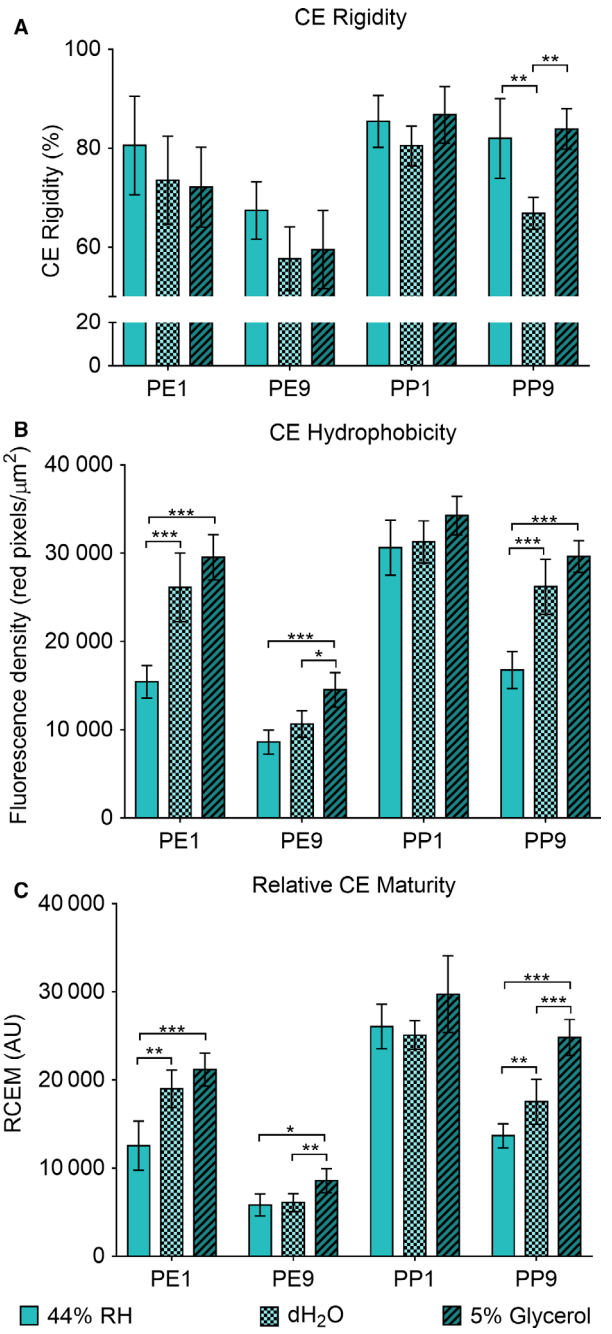

Figure 12 CE rigidity (a), CE hydrophobicity (b) and RCEM (c) of corneocytes ex vivo matured at 44% RH and with pre-treatment of water or 5% glycerol which enhances CE hydrophobicity at low RH. Data are shown in mean ± SD (n = 6) with statistical analysis via one-way ANOVA with Holm–Sidak's post-test; $^{*}P \leq 0.05$ $^{**}P \leq 0.01$ or $^{***}P \leq 0.001$.

Results

The effect of relative humidity on ex vivo CE maturation

Baseline characteristics of CE properties were determined for both photo-exposed-cheek (PE) and photo-protected post-auricular areas (PP) for tape 1 and tape 9 samples (Fig. S2). The CE from the deeper SC of both anatomical sites has a lower mechanical resistance than those from the superficial SC layer. CE rigidity for PP9 samples was significantly weaker than for the PE1 site ($P \leq 0.05$) and PP1 ($P \leq 0.001$), whereas CEs from PE9 were mechanically weaker than PP9 samples ($P \leq 0.001$) (Fig. S2A). Nile red staining indicated higher lipid content in the superficial SC samples compared to those from the deeper SC layers ($P \leq 0.001$). PP1 samples have a higher lipid content per unit surface area than PP9 ($P \leq 0.001$), whereas the CE hydrophobicity in PP9 and PE1 samples is similar (Fig. S2B). These findings indicate that CEs from the PP site are more mature than those from the PE site, whereas CEs from the deeper SC layers are less mature ($P \leq 0.001$; Fig. S2C).

Humidity clearly influenced CE rigidity together with hydrophobicity and hence RCEM. A bell-shaped tendency was seen in CE rigidity with rising RH, reaching a peak at 70% RH, with a decrease in CE rigidity at 100% RH. Samples from the ninth tape stripping of the PE cheek site showed a stronger response to the tested range of RHs than the other tested samples ($P \leq 0.001$). However, CE rigidity in PE9 samples reached maximal mechanical resistance to sonication at 70% RH and showed no change at 100% RH (Fig. 1a) compared to the untreated control CEs (Fig. S2A). The only significant increase in CE hydrophobicity was observed at 70% RH for PP1 ($P \leq 0.001$) and PP9 ($P \leq 0.01$) samples. PE cheek samples showed an enhancement in CE rigidity but a less marked enhancement in CE hydrophobicity in PE1 ($P \leq 0.05$), whereas PE9 samples showed no enhancement in CE hydrophobicity at any RH condition. All samples showed an overall improvement in RCEM at 70% RH in samples from PP1 ($P \leq 0.01$), whereas PP9 samples ($P \leq 0.001$) had a more significant improvement than PE1 ($P \leq 0.05$) and PE9 ($P \leq 0.01$) samples compared to baseline CE maturity. 70% RH was more beneficial for the *ex vivo* RCEM value than 44% RH, PE1 ($P \leq 0.001$), PE9 ($P \leq 0.05$), PP1 ($P \leq 0.001$) and PP9 ($P \leq 0.001$). Furthermore, the *ex vivo* RCEM index was higher at 70% RH than samples kept at 100% RH. All samples showed an enhancement at 70% RH ex vivo maturation. The CEs from the deeper SC of the PE cheek ($P \leq 0.05$) are less mature than the PE1 samples ($P \leq 0.001$). CEs from the SC surface of the PP post-auricular site were more mature ($P \leq 0.001$) than those from PP9 ($P \leq 0.01$; Fig. 1c).

The influence of protease inhibition at low, optimal and high RH on ex vivo CE maturation

Ex vivo maturation with protease inhibition had no impact on CE rigidity at 44% or 70% RH, whereas the mechanical resistance was restored at 100% RH with PIs for PE1, PE9 and PP9 samples (Fig. 2a). Samples from the SC surface of both anatomical sites showed an improvement in CE hydrophobicity in the presence of PI at 100% RH ($P \leq 0.001$), but no effects on samples were evident at 44% or 70% RH. PE9 samples showed no significant changes in CE hydrophobicity at all RH conditions in the presence of PI. Interestingly, PP9 samples showed an enhancement in CE hydrophobicity ($P \leq 0.001$) at low, optimal and high RH in the presence of PI (Fig. 2b). The RCEM reflects the improved CE rigidity and CE hydrophobicity when treated with PI at 100% RH in CEs from the first tape strips of both anatomical sites. *Ex vivo* CE maturation was enhanced in the presence of PI in CEs from PP9 at low, optimal and high RH (Fig. 2c).

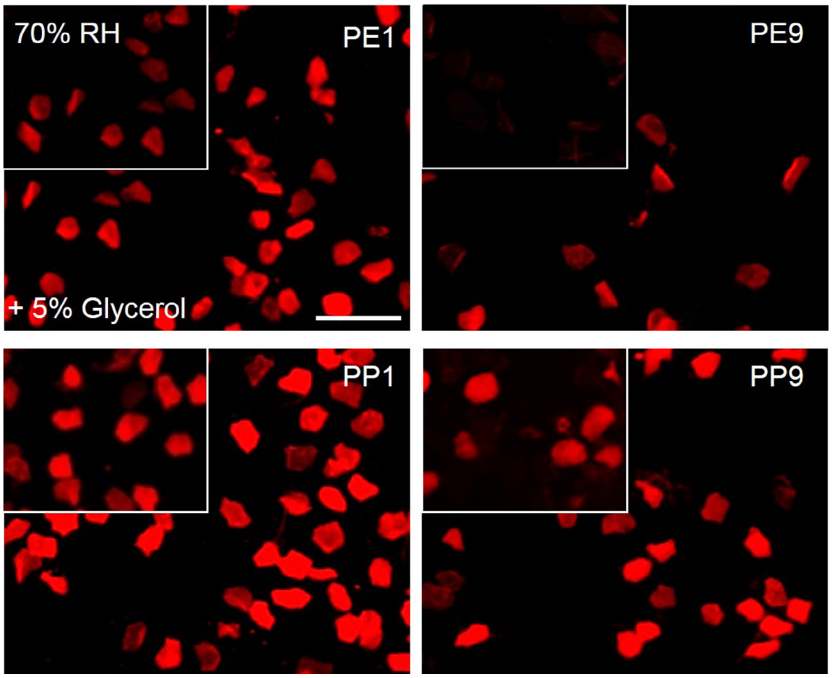

Figure 13 Nile red staining of corneocytes ex vivo matured at 70% (imbeds) and with a 5% glycerol treatment before ex vivo CE maturation. Scale bar = 100 μm

The importance of TG in ex vivo CE maturation at optimal RH

Ex vivo CE maturation was diminished considerably in the presence of the TG inhibitor (Fig. 3). CE rigidity was significantly decreased in all tested samples ($P \leq 0.001$) compared to baseline and *ex vivo* matured CEs at 70% RH. This impact is especially marked in PE9 samples that have a baseline CE rigidity of 60.9 ± 6.9%, increasing to a value of 90.8 ± 8.9% in *ex vivo* matured CEs, and levelling off at 32.1 ± 3.2% in samples exposed to the TG inhibitor (Fig. 3a). The markedly reduced CE rigidity and unchanged CE hydrophobicity (Fig. 3b) result in significantly lowered RCEM values in all samples compared to baseline CEs ($P \leq 0.001$) and samples matured under *ex vivo* conditions (Fig. 3c). This is consistent with the results for the enzyme assay at baseline (Fig. 4a) and in *ex vivo* matured samples (Fig. 4b) in the untreated reaction buffer, in the presence of the solvent DMSO and the TG inhibitor LDN-27219. TG activity at 70% RH and DMSO was unchanged during *ex vivo* maturation as shown in Fig. 3, whereas incubation with LDN-27219 significantly reduced TG activity.

TG activity in ex vivo matured CEs

The TG activity was measured on protein samples extracted and collected at baseline and corneocytes matured *ex vivo* at 44, 70 and 100 % RH for two and four days of the *ex vivo* maturation protocol. The enzymatic activity was reduced ($P \leq 0.001$) from the second day of *ex vivo* maturation and remained constant for the remaining days of *ex vivo* maturation at 44 % RH (Fig. 5a). At 70 % RH, there was a significant rise in TG enzyme activity ($P \leq 0.001$) which decreased over time ($P \leq 0.001$) to baseline levels (Fig. 5b). Interestingly, those samples investigated at 100 % RH showed a dramatic decrease in TG activity after two days of *ex vivo* maturation ($P \leq 0.001$) which decreased even further ($P \leq 0.001$; Fig. 5c). The TG activity was significantly reduced ($P \leq 0.001$) when the samples were taken from 44% RH (Fig. 6a) to 100% RH as well as from 70% RH (Fig. 6b) to 100% RH ($P \leq 0.001$). Samples that were kept at 100% RH for two days and then changed to 44% RH (Fig. 6c) or 70% RH (Fig. 6d) showed little change in TG activity. Corneocytes exposed to protease inhibitors prior to *ex vivo* CE maturation had a significantly higher TG activity than those matured in the absence of protease inhibitors. However, the effect of protease inhibitors on TG activity shows a hydration-dependent tendency where the most drastic rise in TG activity is seen in samples kept at 100% RH ($P \leq 0.001$). The TG activity was significantly increased at 44% RH and 70% RH ($P \leq 0.001$; Fig. 7). CathD activity was measured at baseline (Fig. 8a) and in *ex vivo* matured samples at 70% RH (Fig. 8b), and values were not affected by LDN-27219 nor its vehicle DMSO. Interestingly, corneocytes from the PE cheek showed a higher CathD activity than samples from the PP post-auricular site especially for those collected on the eighth tape.

Pearson's correlations show a clear positive correlation between TG activity and CE rigidity for untreated baseline CEs ($r = 0.91$, $P \leq 0.001$) and *ex vivo* matured CEs at 44% RH ($r = 0.84$, $P \leq 0.001$), 70% RH ($r = 0.93$, $P \leq 0.01$) and 100% RH ($r = 0.67$, $P \leq 0.05$). Furthermore, a positive correlation was demonstrated between baseline TG activity and CE hydrophobicity ($r = 0.90$, $P \leq 0.001$) and *ex vivo* CE maturation at 44% RH ($r = 0.90$, $P \leq 0.001$), 70% RH ($r = 0.96$, $P \leq 0.001$) and 100% RH ($r = 0.74$, $P \leq 0.05$). Interestingly, the positive correlation between TG activity at baseline ($r = 0.89$) and 100% RH ($r = 0.69$) was apparent but not as strong as for those samples *ex vivo* matured at 44% RH ($r = 0.96$) and 70% RH ($r = 0.95$; Table S4).

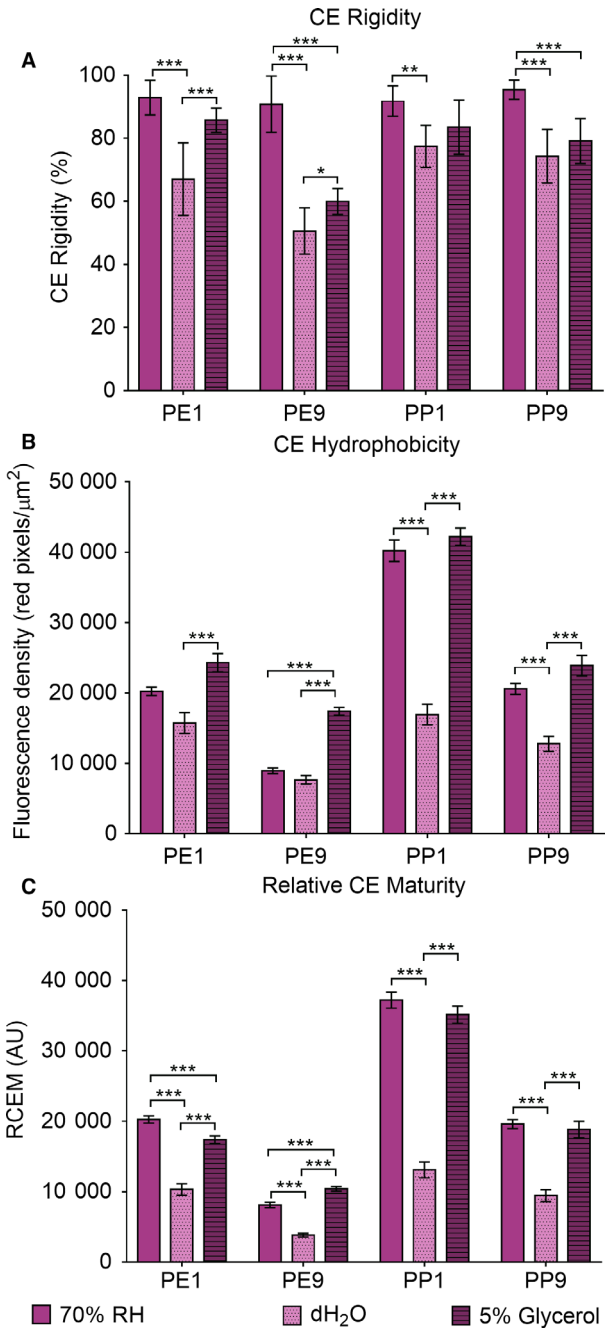

Figure 14 CE rigidity (a), CE hydrophobicity (b) and RCEM (c) measurements show that 5% glycerol maintains CE hydrophobicity at 70% RH. Data are shown in mean ± SD ($n = 6$) with statistical analysis via one-way ANOVA with Holm–Sidak's post-test; $^{*}P \leq 0.05$ $^{**}P \leq 0.01$ or $^{***}P \leq 0.001$.

The impact of 12R-lipooxygenase blockage during ex vivo CE maturation at 70% RH

The more mature CEs (PE1 and PP samples) remained more resistant to mechanical challenge by sonication as for those matured at 70% RH and in the presence of the 12R-LOX blocking antibodies. The improvement in CE rigidity in PE9 samples was comparable for *ex vivo* matured CEs in 70% RH ($P \leq 0.001$) and samples treated with 12R-LOX antibodies ($P \leq 0.001$) prior to *ex vivo* maturation (Fig. 9a). The enhancement in CE hydrophobicity during *ex vivo* maturation at 70% RH was not observed in the presence of 12R-LOX antibodies; in fact, CE hydrophobicity remained in the same range as untreated control CEs (Fig. 9b). The improved CE rigidity and constant CE hydrophobicity are reflected in an enhanced RCEM in PE9 ($P \leq 0.05$) although PE1 and the PP samples remained at baseline RCEM (Fig. 9c). The 12R-LOX assay confirmed that the enzyme activity at baseline (Fig. 10a) declined significantly in samples after *ex vivo* maturation at 70% RH (Fig. 10b). 12R-LOX was not affected by LDN-27219 although the presence of anti-12R-LOX antibody reduced the activity markedly in samples collected at baseline and *ex vivo* matured samples ($P \leq 0.001$).

The influence of glycerol on ex vivo CE maturation at 44% RH

Low relative humidity resulted in a decrease in CE rigidity and had no effect on CE hydrophobicity, but immersing the tapes in glycerol before the *ex vivo* CE maturation at 44 % RH resulted in a clear visual difference compared with untreated CEs, following Nile red staining (Fig. 11). The image analysis confirms reduced CE rigidity in the samples taken from the superficial SC layers from the cheek ($P \leq 0.001$) and post-auricular ($P \leq 0.01$) site (Fig. 12a). On the other hand, glycerol increased CE hydrophobicity to a greater extent than water ($P \leq 0.001$). CE hydrophobicity increased significantly in PE cheek samples from the first tape ($P \leq 0.001$) as well as the ninth tape ($P \leq 0.001$). CEs from PP1 have reached a plateau in CE hydrophobicity, whereas corneocytes from PP9 ($P \leq 0.001$) have a similar CE hydrophobicity as CEs from the superficial SC layers in the presence of glycerol (Fig. 12b). The RCEM for CEs from PE cheek at both depths shows an enhancement in the presence of glycerol and in corneocytes collected of PP9. Alternatively, CEs matured *ex vivo* only at 44% RH showed a significantly higher RCEM than CEs treated with glycerol before *ex vivo* maturation at the same humidity, PE1 ($P \leq 0.001$), PE9 ($P \leq 0.01$), PP1 ($P \leq 0.05$) and PP9 ($P \leq 0.001$; Fig. 12c).

The effect of glycerol on ex vivo CE maturation at 70% RH

A visual comparison shows an increased fluorescence signal in glycerol-treated *ex vivo* matured PE cheek CEs and PP9 compared to *ex vivo* matured CEs at 70% RH. PP1 samples showed no distinct difference between *ex vivo* matured CEs at 70% RH and CEs treated with glycerol prior to *ex vivo* maturation at 70% RH (Fig. 13). Corneocytes collected from the deeper SC layer have a lower CE rigidity in the presence of glycerol compared to *ex vivo* matured CEs solely at 70% RH ($P \leq 0.001$). Untreated CEs have a comparable CE rigidity to glycerol-treated *ex vivo* matured CEs. All samples show a significant decrease in CE rigidity ($P \leq 0.001$) with *ex vivo* matured CEs treated with water (Fig. 14a). CE hydrophobicity was enhanced noticeably for both anatomical sites and depths for CEs treated with glycerol compared to untreated CEs (Fig. 14b). *Ex vivo* maturation in glycerol-treated CEs at 70% RH and ex vivo matured CEs in solely 70% RH showed an enhancement in CE hydrophobicity in CEs from PE9 ($P \leq 0.001$) and PP9 ($P \leq 0.05$). CE rigidity and hydrophobicity are reflected in the RCEM showing that *ex vivo* matured CEs at 70% had significantly higher RCEM values compared to untreated CEs (Fig. 14c). However, glycerol-treated CEs

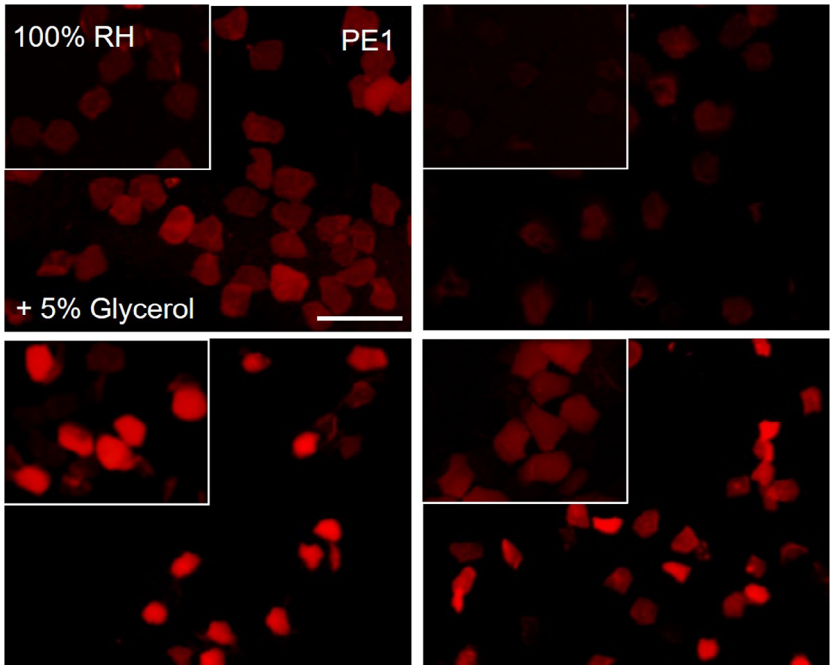

Figure 15 Nile red staining of corneocytes ex vivo matured at 100% (inlays) and corneocytes treated with 5% glycerol before ex vivo CE maturation. Scale bar = 100 μm.

show a significantly improved RCEM for PE cheek, whereas PP post-auricular sites show no further increase in RCEM compared to *ex vivo* matured CEs at 70% RH. RCEM declines with water treatment prior to *ex vivo* maturation compared to untreated CEs and *ex vivo* matured CEs (70% RH).

The impact of glycerol on ex vivo CE maturation at 100% RH

The Nile red staining indicates a positive impact of glycerol on CEs in the deeper SC layers of both anatomical sites compared to *ex vivo* matured CEs at 100% RH (Fig. 15). Image analysis showed that CEs from the SC surface of both anatomical sites show a decrease in CE rigidity but an increase in PE9 ($P \leq 0.01$). Corneocytes showed no change in CE rigidity in untreated CEs and glycerol-treated CEs from PP9 (Fig. 16a). The fluorescence density originating from the lipid staining shows enhanced CE hydrophobicity in glycerol-treated CEs collected from the deeper SC layer of PE cheek ($P \leq 0.05$) as well as PP post-auricular sites ($P \leq 0.001$). However, water had no impact on *ex vivo* CE maturation at 100% RH (Fig. 16b). Glycerol-treated CEs at 100% RH showed little enhancement in RCEM compared to *ex vivo* maturation at 100% RH. Water-treated CEs showed a reduced RCEM value PE9 ($P \leq 0.001$), and PP9 ($P \leq 0.01$) samples showed an enhancement in RCEM with glycerol at 100% RH compared to untreated CEs and *ex vivo* matured CEs (Fig. 16c). Nevertheless, glycerol-treated CEs were more mature than *ex vivo* matured CEs at 100% RH. Glycerol-treated CEs were enhanced in RCEM at 100% RH in the PE cheek, samples PE1 ($P \leq 0.01$) and PE9 ($P \leq 0.001$). A similar enhancement in RCEM is observed in PP post-auricular CEs from the first tape strip ($P \leq 0.05$) and CEs from the deeper SC layer ($P \leq 0.05$).

Discussion

The present work investigated facial CE maturation from Chinese subjects using the recently introduced RCEM method which was originally reported for a study of Caucasian volunteers [8]. The TEWL in PE cheek (Fig. S1A) and PP post-auricular sites (Fig. S1B) showed no difference between previously tested Caucasian and Chinese subjects in this study. CE cohesiveness is stronger in PE cheek samples (Fig. S1C) from Chinese participants; however, PP post-auricular samples (Fig. S1D) showed no differences between the ethnic groups. The thickness estimation approach demonstrated a thinner SC (Fig. S1E) in Chinese PE cheek sites compared to Caucasians [8]. Previous studies, on the inner upper arm, have been conducted in Asian subjects, including a large pool of Chinese participants, and have reported a high prevalence for sensitive skin compared to Caucasians [32]. A later study indicated that Chinese participants have a lower skin barrier function and a thinner SC compared to Caucasians [33]. These studies did not focus on the differences of Chinese and Caucasian skin and anatomical sites; however, Chinese participants had a thinner SC than Caucasians. Understanding the underlying mechanisms of CE maturation is essential for the development of new topical formulations. As a result, we focussed on TG, CathD and the linoleoyl-acylceramide-processing enzyme 12R-LOX. Photo-damaged SC has been shown to have a reduced 12R-LOX protein mass level [12] as well as reduced 12R-LOX activity [13]. An abundant amount of TG was suggested to be a compensatory mechanism in impaired skin barrier to support the SC integrity by enhancing CE hydrophobicity [34].

The skin is constantly exposed to varying environmental conditions that can change its physiological properties and architecture.

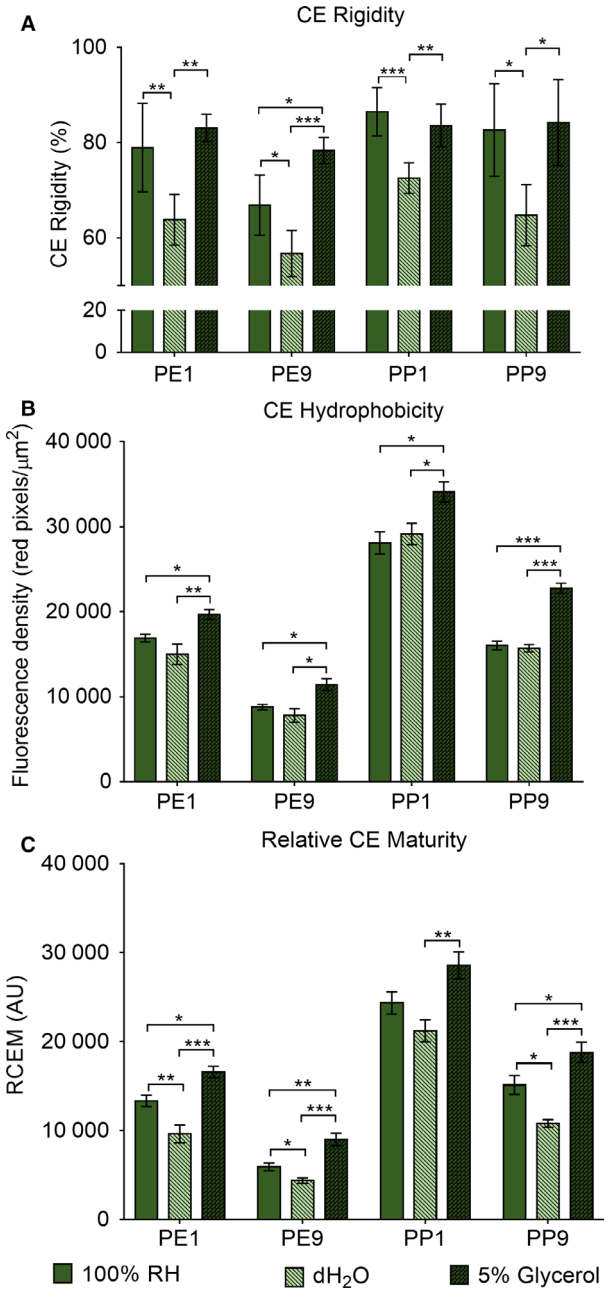

Figure 16 Glycerol counteracted to the harm during ex vivo CE maturation at 100% in terms of CE rigidity (a), CE hydrophobicity (b) and RCEM (c). Data are shown in mean ± SD (n = 6) with statistical analysis via one-way ANOVA with Holm–Sidak's post-test; $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ or $^{***}P \leq 0.001$.

Low relative humidity and cold temperature may cause dry and flaky skin in individuals without explicit skin condition whereas atopic dermatitis patients may experience a worsening of the symptoms [14,33]. However, sun-exposed skin has been demonstrated to show inflammatory traits [35] with elevated protease expression [12] and especially increased serine protease activities [27]. Water has been shown to promote proteolytic activity [36] by breaking down profilaggrin to filaggrin and further into natural moisturizing factor (NMF) in order to prevent the swelling of CEs [18]. Conversely, high and low humidities can prevent filaggrin degradation. Moreover, the degradation of corneodesmosomes is enhanced in high humidity thus suggesting a hydration-driven proteolysis [22,26]. The protease inhibitor mix used in the current study is composed of broad-spectrum protease inhibitors including AEBSF which irreversibly inactivates serine proteases such as kallikreins and plasmin. Caucasian subjects with increased TEWL, thinner SC and increased CE cohesiveness in the PE cheek site were shown to have elevated protease activity such as plasmin [36] which might apply to the findings here in Chinese participants.

At the tested humidity values, the SC retained the capacity to improve CE hydrophobicity and rigidity up to 70% RH for all samples except for hydrophobicity in the PE9 samples whereas *ex vivo* maturation in samples incubated above 70% RH was reduced. Interestingly, PE cheek samples showed a poor improvement in CE hydrophobicity compared to the enhancement in the PP post-auricular samples, indicating differences in enzymatic capacity. An earlier *ex vivo* study found optimal CE maturation at 100% RH with little improvement at 70% RH. This may be due to differences in methodology as discussed previously [8]. CE rigidity and hydrophobicity increased significantly in the presence of PIs at 100% RH, resulting in similar RCEM values as observed for 70% RH. This indicates an insufficiency in natural protease inhibition for subjects living in high humidity climates.

High hydration is associated with improved CE maturation [25], but the present study proved otherwise which supports the findings where high humidity enhances corneocyte corneodesmolysis and desquamation [23,37]. However, filaggrinolysis is impeded at both low and high humidities. The protease inhibition in this study was performed in order to understand whether the protease activity was involved in suppressing potential *ex vivo* CE maturation. In fact, TG activity is reduced at high humidity levels and recovered in the presence of a protease inhibitor. However, the protease of interest still needs to be identified which could be included in moisturizing formulations to improve skincare products. Structural changes to the corneocytes, for example swelling, may contribute to enzyme–substrate co-localization when considering hydrated SC [20,38]. An *in vitro* study has previously characterized the conformational changes in ceramides at different RH conditions via Raman spectroscopy [39]. A clear change in ceramide conformational freedom, weakening of hydrogen bonds and lateral packing was observed at 88% RH [39]. This could suppress the accessibility and affinity between TG and ceramides which may explain less improvement in CE hydrophobicity from 80 to 100% RH compared to 70% RH for both anatomical sites. The findings show the detrimental impact of high humidity on CE maturation and the skin barrier function. Filaggrin may be processed at higher water levels in the SC compared causing structural changes [40]. High environmental humidity may impair the skin barrier by reducing CE maturation which has been reported in individuals living in humid climates [41].

Two subtypes of TGs are expressed mainly in the corneocytes: membrane-bound TG1 and cytosolic TG3. These enzymes have two crucial actions during CE maturation. First, they form isopeptide cross-linkages between lysine moieties and glutamine between structural proteins thereby replacing the cell membrane of keratinocytes with a CE. This processing leads to a progressive gain in CE rigidity. Secondly, TG1 is believed to covalently connect the glutamine residues of CE proteins and the ω-hydroxyl group of

acylceramides after the hydrolysis of the linoleic acid domain that is modified by 12R-LOX, eLOX3, epoxide hydrolase and an unknown esterase [1]. In our experiments, inhibition of TG activity at optimal RH showed that TG was also responsible for the increase in hydrophobicity during *ex vivo* maturation which was missing in the presence of the TG inhibitor. However, there was a marked decrease in CE rigidity following TG inhibition. The CPE is known for its insoluble proteins; hence, the decrease in CE rigidity in all samples was unexpected. This decrease in mechanical resistance combined with an unchanged CE hydrophobicity resulted in a dramatically reduced RCEM. We cannot explain the decrease in CE rigidity following TG inhibition, but it may point to isodipeptide bond dissolution.

Interestingly, CathD activity was higher in PE cheek than PP post-auricular sites which reflects the increased CathD expression in the mass spectrometry analysis in photo-damaged facial skin [12]. This suggests a higher potential for TG activation in PE cheek; however, this study proved evidence for limited enhancement in CE maturation in the sun-exposed cheek. In fact, the PP post-auricular site has a higher TG activity than the PE cheek site. The lower CathD activity in PP post-auricular site activates TG sufficiently to facilitate CE maturation. In theory, LDN could have an impact on CathD activity but the experiment set-up in this study has proven that LDN only blocks TG activity.

The epidermal lipoxygenase, 12R-LOX, catalyses the dioxygenation of the linoleic acid domain in the acylceramides, in the first step of processing these lipids for their subsequent de-esterification to free the ω-hydroxyceramide, to then be attached to the CE by TG. Thus, if 12R-LOX is insufficiently expressed or inactive, this could result in poor skin barrier function. Autosomal recessive congenital ichthyosis has been linked to loss of function mutation in the 12R-LOX gene with associated changes in CLE composition; however, the condition can vary in severity [42,43]. 12R-LOX knockout mice lacked an appropriate skin barrier that led to excessive dehydration and ultimately post-natal death [4]. The CEs in knockout mice were fragile and had reduced CE hydrophobicity [4], similar to the PE cheek samples in the present study at baseline that were also shown to have a lower 12R-LOX activity [13]. A recent proteomics study also showed that 12R-LOX was significantly reduced in PE cheek sites compared with control PP post-auricular sites [12], and the enzyme activity was shown to be lower in cheek samples. Inhibitors for 12R-LOX are still unknown; hence, a polyclonal 12R-LOX antibody was used to block activity in our experiments and we clearly demonstrated that 12R-LOX activity within the SC is essential for maturation of the lipids of the corneocytes. However, blocking the enzyme activity had no effect on CE rigidity in contrast to the genetic knockout study of this enzyme. 12R-LOX activity was significantly higher at baseline compared to *ex vivo* matured CEs at 70% RH. The CLE is enhanced in hydrophobicity, and it is possible that the lipids may cause spatial hindrance to the substrate as well as the polyclonal antibody. Moreover, 12R-LOX has the property of suicidal inactivation [44]; therefore, this may have contributed to an overall lower 12R-LOX activity at the end of the *ex vivo* maturation.

The effect of glycerol has been investigated at low, optimal and high RH during *ex vivo* maturation. Many studies have been conducted with glycerol which is a common component of cosmetic products [45]. Low humidity has been shown to have a negative impact on CE rigidity; however, neither glycerol nor water were beneficial to *ex vivo* CE maturation compared to CEs kept at 44% RH. More interestingly, CE hydrophobicity improved significantly in the presence of glycerol or water for samples matured *ex vivo* at 44% RH for PE cheek and PP9 sites compared to untreated baseline CEs. Glycerol has been shown to have softening effects on CEs [15] which may simulate reduced cross-linkage of structural proteins in corneocytes. This might result in artefactual fragility which might be masking any possible improvement in CE rigidity. Alternatively, glycerol might promote the lipid attachment due to its influence on lipids [3,39] and TG activity [46]. The decreased CE rigidity and enhanced CE hydrophobicity are reflected in a significantly improved RCEM. Glycerol promoted *ex vivo* maturation at low RH although water was less effective in improving the RCEM which is in line with previously reported differences between water and glycerol for skin hydration [47]. On the other hand, *ex vivo* maturation with hydration, at the optimal RH, resulted in suboptimal CE maturation in the presence of glycerol or water. Decreased CE rigidity and unchanged CE hydrophobicity point towards a moderate enhancement in TG activity without further improvement in the RCEM in both anatomical sites. Glycerol seems to compensate for the high humidity at 100% RH thereby providing a more beneficial effect than water. Glycerol affects corneocyte maturation positively in terms of CE hydrophobicity especially in the PP post-auricular sites and at different depths. This hints to a major difference between PE and PP skin as CE rigidity for PE cheek samples showed a significant improvement from the deeper SC layers whereas CE hydrophobicity only improved in the presence of glycerol. Earlier work [12] revealed differences in proteomics between these two anatomical sites, and TG expression is elevated in PE cheek sites. Increased TG should overcome and enhance CE maturation in the PE cheek; however, CE rigidity was enhanced only at optimal RH**.** Samples from the SC surface of the PP post-auricular seem to have reached a maturation maximum due to either changes in TG activity during *ex vivo* maturation or substrate availability. The decreased 12R-LOX protein level in PE cheek samples [12] hints towards a limitation in enhancing CE hydrophobicity and was reported with a reduced 12R-LOX activity in the PE cheek [13]. At high humidity, glycerol was shown to absorb moisture, whereas at low humidity, it provides hydration in CE maturation [23]. This is in line with the demonstrated findings where glycerol seems to be more effective at low and high humidity values but not at intermediate humidity.

The present work demonstrates the importance of RH as an environmental factor affecting CE maturation while indicating the optimal conditions to perform *ex vivo* CE maturation following tape stripping. This has obvious implications for SC maturation *in vivo*. The importance of TG and especially 12R-LOX activities in *ex vivo* CE maturation was also shown. Furthermore, this work suggests that glycerol as a humectant has limitations at 70% RH while being beneficial at low and high RH, especially for improving CE hydrophobicity. Moreover, the importance of protease inhibition for CE maturation has been identified especially at high humidity. This study provides new insights for CE maturation and demonstrates the effect of 12R-LOX on CE maturation in the SC for the first time.

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Conflicts of interests and disclosures

RV is an employee of DSM Nutritional Products Ltd. AVR is a consultant to DSM Nutritional Products Ltd.

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Supporting Information

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

Figure S1. Comparison of SC properties of PE cheek and PP post auricular sites between Chinese and Caucasian participants.

Figure S2. CE rigidity (A) and CE hydrophobicity (B) and RCEM (C) of Caucasian and Chinese participants

Table S1. One way ANOVA analysis of CE rigidity and hydrophobicity and RCEM of ex vivo matured CEs at 44% RH followed with Holm-Sidak's multiple comparison post-test. $P < 0.05$*; $P < 0.01$**; $P < 0.001$***.

Table S2. One way ANOVA analysis of CE rigidity and hydrophobicity and RCEM of ex vivo matured CEs at 70% RH with Holm-Sidak's multiple comparison post-test. $P < 0.05$*; $P < 0.01$**; $P < 0.001$***.

Table S3. One way ANOVA analysis of CE rigidity and hydrophobicity and RCEM of ex vivo matured CEs at 100% RH with Holm-Sidak's multiple comparison post-test. $P < 0.05$*; $P < 0.01$**; $P < 0.001$***.

Table S4. Pearson Correlation between TG activity at different RH conditions and CE maturation. Student t-test, $P < 0.05$*; $P < 0.01$**; $P < 0.001$***.