



Variation in the activities of late stage filaggrin processing enzymes, calpain-1 and bleomycin hydrolase, together with pyrrolidone carboxylic acid levels, corneocyte phenotypes and plasmin activities in non-sun exposed and sun-exposed facial stratum corneum of different ethnicities

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Abstract

Background

Knowledge of the ethnic differences and effects of photodamage on the relative amounts of natural moisturizing factor (NMF) together with filaggrin (FLG) processing enzymes in facial stratum corneum (SC) is limited. Our aim was to characterize the activities of calpain-1 (C-1), bleomycin hydrolase (BH) and the levels of pyrrolidone carboxylic acid (PCA) as a marker for total NMF levels and to relate them to plasmin activities and corneocyte maturation.

Methods

Enzyme activities, PCA levels and corneocyte maturation were determined from facial tape strippings of photoexposed cheek and photoprotected post-auricular areas (PA) of healthy Caucasian (C), Black African (BA) and Albino African (AA) female subjects living in South Africa.

Results

PCA concentration levels were of the order AA > BA > C subjects and the highest activities of BH were present in the AA subjects. BH activities were greater on the photoexposed sites for the BA and C subjects but they were only numerically elevated in the AA subjects. Photoprotected sites had an increase in C-1 activity in pigmented groups (C and BA) whereas in the AA subjects the opposite was measured. Plasmin activities were greater on the cheek compared with the PA site for the AA and C subjects but the activity was low in the BA subjects, In both sites. In both test sites, the AA, but not the BA and C subjects, had smaller, parakeratotic and less mature corneocytes.

Conclusion

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4 Variation in PCA levels has been found for different ethnic groups in this study (AA
5 > BA > C subjects). The values in the AA subjects are surprising as one might
6 expect that the lack of pigmentation, and thereby increased photodamage, might
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8 lead to lower levels. Increased BH, but not C1 activity, was observed in the AA
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10 subjects indicating that BH is associated with PCA production to a greater extent.
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12 Surprisingly, corneocyte maturation is still impaired with elevated PCA levels in AA
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14 subjects. The higher levels of plasmin and BH activities on the cheeks, especially
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16 for AA and C subjects, suggest that they can be used as markers for epidermal
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18 photodamage.
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Introduction

The epidermis is the primary barrier of the body to solar radiation which is mainly provided by melanocytes that transfer melanin into keratinocytes to protect their nuclear DNA from irradiation [1, 2]. However, the protective role of the SC in photoprotection is often underestimated [3]. In this respect, a study comparing subjects with vitiligo and normally-pigmented skin showed that photoprotection is a property of SC in both groups [4] and that a thicker SC acts as a physical barrier to light in an attempt to compensate for the absence of pigmentation and it becomes thicker [2, 5]. In support of this Thomson *et al.* found that photoprotection in Albino African (AA) skin was similar to minimally pigmented European skin [2]. Moreover, in the most recent report on facial skin pigmentation and photodamage that dissects the relationship of SC basal transepidermal water loss (TEWL), barrier integrity and epidermal barrier repair to SC thickness, it was shown that although AA SC was thicker it was less protective in terms of basal TEWL and skin hydration [6]. These studies offer an insight into the compensation mechanisms that the skin develops in these subjects but the biochemical differences in their thickened SC have not been fully evaluated.

In other studies the superior facial barrier function exhibited by African Americans was shown to be due to be related to a more complete maturation of forearm corneocytes even though their associated levels of SC ceramides were low [7]. However, these corneocyte maturation results are inconsistent with those of Hirao *et al.* who found no differences on facial SC [8]. Nevertheless, corneocyte surface area is reported to be similar between different ethnicities despite differences in intracellular cohesion [9]. Additionally, one study shows Asian skin has lower NMF

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3 levels compared with African American and Caucasian skin [10]. As it has
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5 previously been shown that pigmentation/ethnicity has no effect on facial barrier
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7 skin function we were interested if an ethnic difference in NMF production and
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9 corneocyte maturation is present in Black African (BA), Caucasian (C) and AA
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11 subjects [6].
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18 Filaggrin (FLG) is a major protein found in the SC and is involved in its barrier and
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20 moisturisation properties [11]. The final degradation of FLG gives rise to a group of
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22 hygroscopic molecules in the upper SC (stratum disjunctum) responsible for
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24 effective SC hydration and its water-holding capacity [12] together with SC
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26 plasticisation [13]. These hygroscopic protein breakdown products (free amino acids
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28 (FAA) and associated products) together with lactate, urea, inorganic ions and
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30 sugars are collectively termed natural moisturizing factor (NMF) [14]. Urocanic acid
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32 (UCA) and pyrrolidone carboxylic acid (PCA), two transformed FLG breakdown
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34 products are often used as markers for NMF and together with FAA have been
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36 used to assess SC moisturisation in different dry skin types [15-18]. Their
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38 production by the enzymatic processing of profilaggrin/FLG has been of recent
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40 interest with several groups examining the role of calpain -1 (C-1) and bleomycin
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42 hydrolase (BH) in the later stages of FLG degradation [17, 19-25].
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51 Takeda *et al.* first identified BH as an aminoacyl hydrolase in rat and human SC to
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53 be involved in the keratinization process [19, 20]. Schwartz *et al.* also demonstrated
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55 that BH was essential for epidermal integrity [21]. Later Kamata *et al.* identified BH
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57 in the upper layers of SC as a late stage NMF generating enzyme by monitoring its
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59 citrulline-releasing activity [22].
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3 In contrast, C-1 is a heterodimeric calcium-activated cysteine protease comprised of
4 a large subunit (80kDa) and a small subunit (30kDa). C-1 is known to be present in
5 human and porcine epidermis [23] and has been reported to play an important role
6 in the generation of NMF [24] [25]. It also has a direct role in epidermal
7 differentiation by activation of epidermal transglutaminases [26]. Thus, C-1 is
8 involved not only in the processing of profilaggrin to FLG but also corneocyte
9 maturation [24, 27]. In fact, C-1 inhibitors have been shown to reduce corneocyte
10 envelope (CE) formation by inhibiting the proteolytic processing of
11 transglutaminases [28].
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28 Kamata *et al.* [29] identified the sequence of FLG degradation by these two
29 enzymes and determined their relationship to peptidyl arginine deiminases (PADs)
30 together with caspase-14. In this sequence of events, arginine residues in FLG
31 monomers are deiminated by PADs and although caspase-14 participates in the
32 processing of deiminated FLG to limited fragments, C-1 degrades them to smaller
33 peptides. BH then eventually degrades these peptides into FAA. However, recent
34 studies have demonstrated that BH but not C-1 activities that are decreased in the
35 SC of aged and sensitive skin, are associated with lower NMF levels and elevated
36 TEWL [30, 31]. Additionally, BH was found to be decreased in parakeratotic but
37 increased in hyperkeratotic, skin disorders [31-33]. Most recently Son *et al.* has
38 shown that BH and NMF levels but not C-1 and filaggrin expression was decreased
39 in dry volar forearm SC [30].
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3 The induction of CE maturity by transglutaminases is key for barrier function and
4 their phenotypes are assessed microscopically through Nile red lipid staining and
5 involucrin antigen binding [8]. There is a specific distribution pattern of more
6 rigid/polygonal shaped, mature CEs in the superficial layers of SC, whereas, fragile/
7 asymmetrical immature CEs are found predominantly in the deeper layers of SC.
8 Increased levels of immature CEs are known to occur in barrier compromised and
9 dry skin conditions [8, 34, 35]. Moreover, as there appears to be an inverse
10 relationship between FLG degradation and CE maturation we were interested to
11 examine CE phenotypes in different ethnic groups together with the activities of
12 FLG degrading enzymes (C1 and BH) and NMF levels [18].
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31 In normal epidermis, plasminogen is expressed mostly in the basal cell layer of the
32 epidermis but it is expressed suprabasally in compromised skin conditions [36, 37].
33 Indeed, elevated SC plasmin levels are associated with elevated TEWL [38]. To
34 date there has been no investigation of differences in the activities of this enzyme in
35 the SC of different ethnic groups.
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61 The present study was therefore designed to characterize the ethnic differences in
62 the activities of the FLG processing enzymes (C1 and BH) and the levels of NMF in
63 facial regions of 3 ethnic groups (C, BA and AA) living in the same region with
64 varying degrees of pigmentation and photodamage. Moreover, as there are few
65 studies examining the effect of photodamage on facial SC, we chose to examine
66 cheek and post-auricular skin testing sites in the three different ethnic groups [6, 39,
67 40]. Also to bring some clarity to the discussion on corneocyte maturity we also

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3 included this measure in our study and plasmin activity was chosen as a marker of
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5 barrier damage [8, 34-38].
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10 11 Materials and methods

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13 Standard D-Squame[®] tapes, a D-Squame[®] pressure applicator and D-Squame[®] disc
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15 racks were obtained from Cuderm Corporation (Dallas, US). The Orbital incubator
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17 SI50 was purchased from Stuart Scientific (Staffordshire, UK) and the infrared
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19 densitometer, model SquameScan[™] 850A was obtained from Heiland electronic
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21 (Wetzlar, Germany). Sodium dodecyl sulphate (SDS) ACS reagent > 99.0%
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23 (436143), ethylene diamine tetra-acetic acid, Triton X-100, acetic acid and Nile red
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25 were obtained from Sigma-Aldrich (Dorset, UK). High performance liquid
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27 chromatography (HPLC) analytical grade water and acetonitrile were obtained from
28
29 Fisher Scientific (Hertfordshire, UK). A Waters Symmetry[®] C₁₈ HPLC column (5 µm,
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31 150 x 4.60 mm) (Waters, Milford MA, US) was used for the determination of AMC
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33 and a Synergi HPLC column (4 µm POLAR-RP-80A, 150 x 4.60 mm) (Phenomenex,
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35 USA) for the determination of PCA. DL-dithiothreitol, trifluoro acetic acid and Tris-
36
37 HCl (0.1M) were purchased from Fluka (Buchs, Switzerland). Dimethylsulphoxide
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39 (DMSO) was obtained from VWR International Ltd (Bedford, UK) and phosphate
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41 buffered saline (PBS) tablets were obtained from Oxoid (Hampshire, UK). The
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43 primary monoclonal antibody, anti-human involucrin (clone SY5) was purchased
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45 from Cambridge Scientific Products (Watertown, MA, USA). The rabbit anti-mouse
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47 IgG labelled with fluorescein isothiocyanate (FITC) was purchased from Abcam
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49 (Cambridge, UK). Aminomethyl coumarin (AMC) and all fluorogenic peptide
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51 substrates were donated by DSM Nutritional Products Ltd. (Kaiseraugst,
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53 Switzerland). A water bath was purchased from Benchmark Scientific (New Jersey,
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3 USA) and a Canon 70D 20.2 megapixel camera was obtained from Canon USA Inc.
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5 (New York, USA). IMAGEJ™ software was downloaded from the; National Institutes
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7 of Health website (Bethesda, MD, US).
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14 **Study subjects**

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17 The study was a cross-sectional study and was approved by the School of Health
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19 Care Sciences Research and Ethics committee (SREC) together with the Medunsa
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21 Campus Research and Ethics Committee (MREC) and was conducted in
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23 accordance with the Declaration of Helsinki Principles. Written informed consent
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25 was obtained from all participants before enrolment. 60 healthy female volunteers,
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27 living in Pretoria, South Africa participated in this observational study which took
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29 place from the end of November to early December 2013. There were three age-
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31 matched groups composed of 20 AA (40.3 ± 2.9 years old), BA (38.2 ± 2.3 years
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33 old, Fitzpatrick skin phototype V) and C (44.6 ± 3.1 years old, Fitzpatrick skin
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35 phototype II/III) subjects each. The subjects did not apply any dermatological or
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37 cosmetic products to their faces for 3 days before expert grading and evaluation of
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39 their facial skin. For the 3-day conditioning phase subjects cleansed the face with
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41 distilled water in the morning as well as in the afternoon. Before tape stripping the
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43 skin was cleaned by gently swabbing with a cotton pad soaked in distilled water at
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45 ambient temperature, allowed to dry for 20 min and then acclimatized for 30 min at
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47 $21 \pm 1^\circ\text{C}$ and $35 \pm 10\%$ relative humidity.
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Sample collection and SC protein evaluation

D-Squame[®] disks with a diameter of 2.2 cm and an area of 3.8 cm² were placed on the right cheek (3 cm vertically beneath the outer edge of the eye) and post-auricular area opposite to right earlobe). D-Squames were applied with 225g cm⁻² of pressure with a pressure device for 5 sec and then removed by a single stroke movement. In order to minimize variation, the procedure was conducted by the same technician for all volunteers throughout the study. The interval between the tape strippings was 20±5 seconds [41]. The SC protein content of the tape strippings was quantified by infrared absorption measurements at 850 nm with a SquameScan[™] 850A. This non-destructive method of SC protein quantification permits the further use of tape stripping's for biochemical assays and enables normalisation of the SC protein content [42,43]. For SC protein quantification, the following equation was used [42]:

$$C_{\text{protein}}[\mu\text{g cm}^{-2}] = 1.366 \times \text{Absorption} [\%] - 1.557 \quad \text{Equation 1}$$

Tape strips 1, 4 and 6 were used for the biochemical and morphological studies.

PCA quantification

The fourth tapestrip was used for PCA quantification. Tapes were transferred into a 1.5 ml Eppendorf tubes and extracted at 1000 rpm in 750 μl , 0.1 mol L⁻¹ KOH solutions for 2 h at 32°C with continuous shaking. The alkaline extracts were neutralized with 4.5 μL of perchloric acid (12 M), shaken again for 2 h and filtered through a 0.2 μm membrane. HPLC with UV detection at 210 nm was used to analyse the PCA content of the tape stripping extracts, at room temperature. The volume of injection was 10 μL and the flow rate 0.4 mL min⁻¹. The mobile phase

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3 consisted of 20 mmol L⁻¹ ammonium formate, containing 1.5 mmol L⁻¹
4 tetrabutylammonium hydroxide and 3% acetonitrile at pH 7.3 [44]. The retention
5 time was 7.5 min. The total SC protein determined by infrared densitometry was
6 used to normalize the PCA amounts.
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12 13 14 15 16 **Protease activities**

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18 Protease activities were quantified using the methods previously reported [38, 45-
19 47] and the sixth tape strip was used for analysis. Tapes were transferred into 1.5
20 ml Eppendorf tubes and extracted for 25 min at 25°C and 1000 rpm in 750 µl of 0.1
21 M Tris-HCl and 0.5% Triton X-100 buffer at pH 7.5. The extracts (250 µl) were
22 combined with 1.25 µl fluorogenic peptide substrates, namely for BH (H-Cit-AMC),
23 C-1 (Suc-Leu-Leu-Val-Tyr-AMC) and for plasmin (MeOSuc-Ala-Phe-Lys-AMC). The
24 mixtures were shaken at 1000 rpm at 37°C. The reaction was stopped after 2 hours
25 by adding 250 µl of acetic acid (1%). The wavelength for emission was 442 nm and
26 for excitation it was 354 nm. The elution was conducted using reverse phase HPLC
27 with a mobile phase of 75% water, 25% acetonitrile, 0.01% trifluoroacetic acid
28 (TFA). The flow rate was 1 mL min⁻¹, the injection volume 20 µl and the retention
29 time of AMC was 6.5 minutes. The total SC protein determined by infrared
30 densitometry was used to normalize activity values for the proteases.
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52 53 **Corneocyte maturity and surface area measurement**

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55 Corneocyte maturity was assessed by differential Nile red and immunostaining of
56 involucrin for the first tape strip, using a modification of the method described by
57 Mohammed *et al.* [45]. Tapes were transferred to Eppendorf tubes and extracted
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3 using 750 μl of dissociation buffer (2% SDS, 20 mmol L^{-1} DL-dithiothreitol, 5 mmol
4 L^{-1} EDTA, 0.1 M Tris HCl buffer adjusted to pH 8). Using a dry water bath, the tubes
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6 were heated at 75°C for 10 minutes, centrifuged and washed with dissociation
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8 buffer (3 times). The corneocytes were separated as pellets, which were then
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10 suspended in dissociation buffer. The suspension was applied on a microscopic
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12 slide and dried. Primary monoclonal antibody was added and incubated overnight at
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14 4°C in a humidity chamber. The next day FITC-labelled 1:50 rabbit polyclonal to
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16 mouse antibody IgG (H&L) was added and incubated for 1 hour in the dark. The
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18 slides were rinsed with PBS and incubated with Nile red for 10 min. The slides were
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20 subsequently rinsed with PBS and covered with a slide cover. Fluorescence was
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22 measured with a fluorescence microscope equipped with a Canon 70D, 20.2
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24 megapixel camera (2.85 $\text{pixels } \mu\text{m}^{-1}$). IMAGEJ™ image analysis software was used
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26 to analyse the red pixels obtained from the Nile red stained cells and the green
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28 pixels from the immunostained cells. The ratio of red/green pixels corresponds to
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30 the extent of corneocyte maturity [45]. Nile red stained images of corneocytes were
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32 used to measure corneocyte envelope (CE) surface area. The percentage of
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34 corneocytes with nuclei in a sample was quantified by the ratio of total number of
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36 cells/CE to the total number of nuclei counted. The Nile red stained cell images
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38 were further processed using IMAGEJ™ to analyse the cell surface area.
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52 **Statistics**

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55 All data were collected in Microsoft Excel 2011 and expressed as mean \pm SEM, with
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57 differences between means analysed with SPSS software version 22 (IBM®, New
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59 York, USA). For all the parameters two body sites were first analysed together and
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3 later analysis was performed for each site separately. The data were also pooled
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5 between ethnic groups (subjects with pigmentation = BA and C) and AA to
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7 investigate structural differences between facial areas. Pair wise group comparisons
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9 were performed using the Student's paired t-test (normally distributed), analysis of
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11 variance (ANOVA) and a non-parametric Mann-Whitney U test with a two-tailed test
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13 of significance. Differences where $p < 0.05$ were considered to be statistically
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15 significant. Differences where $p < 0.05$ were considered to be statistically
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17 significant.
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23 Results

26 **PCA measurements**

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28 AA subjects had a significantly higher amount of PCA compared with the BA and C
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30 subjects (Figure 2). In all subject groups the differences between the
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32 photoprotected post-auricular test sites were not statistically significant. The amount
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34 of SC removed and the SC depth probed was similar in all three groups and no
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36 significant differences were observed for cumulative tape stripping for tapes 1 to 4
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38 and tapes 1 to 6 (data not shown).
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47 **Protease activity measurements**

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49 AA subjects had the highest BH activity in both sites compared with the BA and C
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51 subjects. The BH activity was significantly higher on photoexposed areas for BA
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53 and C subjects (Figure 3). Interestingly, there was no significant difference in BH
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55 activity between BA and C subjects in the photoprotected sites. The AA subjects
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57 had the highest C-1 activity in the cheeks but had significantly lower activity in the
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59 PA site in comparison with the BA and C subjects. In the subject groups with normal
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3 pigmentation, C-1 activity is lower in the BA subjects. Photoprotected sites show
4 elevated activity of the enzyme in all three ethnicities. (Figure 4). The AA and C
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8 subjects had significantly higher plasmin activity on their cheeks, whereas it was
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10 only numerically elevated for the BA subjects (Figure 5).
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12 13 14 15 16 **CE maturity** 17

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19 Differential Nile red and involucrin immunostaining is shown in Figure 6. Differences
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21 in the maturity of corneocytes were observed for the different ethnic groups.
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23 Corneocyte maturity was significantly lower for the AA subjects compared with the
24
25 other two groups. The subjects with normal pigmentations (BA and C) showed no
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27 significant difference in the corneocyte maturity ratio. In addition, the photoprotected
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29 sites generally had more mature CEs than the cheeks in all three groups but this
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31 was only significant for the C cohort (Figure 7). A similar trend was observed
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33 between values for corneocyte surface area and ethnicity. The AA subjects had
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35 corneocytes with the smallest surface areas compared with the other two groups
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37 and there was no significant difference in corneocyte surface area between the two
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39 groups with normal pigmentation (Figure 8). A 10-fold higher value for corneocytes
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41 with nuclei was observed for the AA group compared with the other groups
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43 irrespective of facial site. There was no difference in the percentage of cells with
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45 nuclei observed between the groups with normal pigmentation in both sites (Figure
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Discussion and Conclusion

Ethnic differences in SC properties have been reported previously, particularly for barrier function [6-10, 48-50]. However, there is a paucity of data on the differences in NMF levels, activities of BH, C-1 and plasmin together with corneocyte maturation in different ethnic groups.

We found that the SC of AA subjects contained higher PCA levels irrespective of sun exposure. This was surprising, as previously Voegeli *et al.* had reported that this group of subjects had the lowest skin hydration values and the highest facial dryness and roughness compared to the other groups [6]. Moreover, for other anatomic sites increased skin dryness is usually associated with reduced NMF levels [15, 30]. This suggests that the increased PCA levels are not compensating for the reduced skin hydration and that other SC abnormalities are responsible for this [6]. Nevertheless, an increased NMF level in BA subjects is also associated with increased hydration compared with C subjects [6]. Both of these findings of increased SC NMF levels may be related to the increased SC thickness of the AA and BA subjects relative to a thinner SC of C subjects for the photoexposed site [6]. FLG may simply have a greater length of time to be completely degraded in these subjects. Degradation of FLG has been reported to be compromised on photodamaged facial and forearm skin sites [18, 51]. Nevertheless, the levels of PCA in the BA and C subjects were not different which is consistent with earlier reports [10].

UV radiation also causes significant changes in the mechanical properties of the SC [52]. The corneocyte intercellular junctions are the gate-keepers of barrier function and any damage to them causes mechanical challenges to SC function. Rupture of

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2
3 the corneodesmosomes occurs at very low strains and under conditions of low
4 humidity [53-55]. As the SC is thicker and drier in the AA subjects increased levels
5 of NMF may be required to plasticise keratin to ensure that corneodesmosomal
6 rupture does not occur prematurely. Jokura *et al.* highlighted the important role of
7 NMF as a plasticizer of the SC rather than simply being a humectant [13].
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17 To understand better the generation of NMF in the SC in these three ethnic groups
18 we studied the activities of BH and C-1. The active forms of these proteases are
19 mainly localized in the upper layers of the SC and their activity was shown by others
20 to be critical in the final stages of FLG degradation [19-22, 24, 27]. Increased
21 activity of BH was observed in UV-exposed photodamaged sites of all subject
22 groups but the highest activities were observed in the SC of the AA subjects. An
23 increase in activity of BH is associated with higher PCA concentrations in both sites
24 studied. Nevertheless, the differences in the activities of C-1 in SC for the various
25 groups were more complex. For the pigmented subjects higher activities were
26 observed in the post-auricular sites compared to the cheek. In contrast, the activity
27 profile of BH and concentration of PCA was observed highest in cheeks. However,
28 despite having the highest level of NMF on both pre- and post-auricular sites the
29 activity of C-1 was minimal on the post-auricular site in the AA subjects and was
30 comparable on the pre-auricular testing site versus the post-auricular site of C
31 subjects suggesting that the activity of this enzyme is not rate limiting on the face in
32 these subjects. These results contrast with those previously reported that show
33 decreased BH but not C-1 mass levels in forearm dry skin [30].
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4 Equally, although not examined previously for its effects on FLG degradation,
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6 plasmin activities were elevated with increased PCA levels. Photodamage is
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8 expected to cause a feedback mechanism to upregulate the proteases involved in
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10 FLG degradation thereby improving barrier activity by producing more NMF.
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12 Although we did not quantify UCA levels, they are expected to be elevated in the
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14 SC of AA subjects much like that of PCA, and UCA is known to protect against UV
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16 irradiation [56]. Nevertheless, the increased SC thickness and NMF levels in AA
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18 subjects may be a mechanism to protect against UV irradiation when melanin is
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20 absent. These findings are also, in line with reports of hyperkeratosis in patients
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22 with vitiligo offering increased photoprotection [4].
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30 Generally, more mature corneocytes were observed on the post-auricular site but
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32 this only reached statistical significance in the C subjects. Increased quantities of
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34 premature corneocytes observed in the AA group on both testing sites are indicative
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36 of poor barrier function and delayed desquamation [18]. Indications of improper
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38 keratinocyte differentiation and SC maturation were also observed in the AA
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40 subjects where corneocytes retained nuclei (parakeratosis). However, there was no
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42 differences in CE maturity between the BA and C subjects consistent with the facial
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44 study of Hirao *et al.* but inconsistent with the forearm study of Muizzuddin *et al.* [7,
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46 8]. This may reflect the variation in subjects, climatic conditions (adaptation), and
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48 the different methodologies used but is most likely as a result of different body sites
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50 tested. The reduction in CE maturation is probably related to the higher incidence of
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52 dry skin, which is associated with decreased transglutaminase activity, in the AA
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54 subjects and the cheeks of the other two cohorts [6, 35].
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3 Upon comparison between the previously reported skin bio-instrumental data [6] for
4 this study with our biochemical results, the higher water loss and inferior skin
5 capacitance in the AA subjects may be an outcome of poor SC maturation. It
6 appears that the increased SC thickness in the AA subjects does not compensate
7 for elevated TEWL levels. Equally, increased levels of PCA in the AA cohort also do
8 not compensate for the inferior skin hydration. These results highlight the vital
9 importance of corneocyte maturation for a competent skin barrier. Nevertheless, the
10 thickened SC in AA subjects may be a compensation mechanism for the absence of
11 skin pigmentation, providing vital UV protection. It may be considered as an
12 epigenetically-driven alternative adaption to melanogenesis but it has a negative
13 impact on barrier function.
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32 In conclusion, ethnic differences in levels of NMF, activities of BH, C-1 & plasmin
33 and corneocyte maturation occur. Considering the different groups, the AA cohort
34 always had higher activities of BH and plasmin, higher PCA levels and more
35 immature corneocytes compared with the other two ethnic groups. However, the
36 difference in values for C-1 activities is more complex. In addition, this study also
37 demonstrates that photodamage of the cheek causes an increase in the activities of
38 BH and plasmin. Equally, on the sun-exposed site the AA group had the highest C-1
39 activities but demonstrated the lowest C-1 activity on the photoprotected site. These
40 results suggest that C-1 is not the rate-limiting factor for the processing of FLG but
41 BH is highly associated with the final steps in NMF formation. Moreover, it is
42 suggested that both elevated plasmin and BH activities are indicators of epidermal
43 photodamage.
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References

1. Hearing VJ. Biogenesis of pigment granules: a sensitive way to regulate melanocyte function. *J. Dermatol. Sci.* **37**, 3-14 (2005).
2. Thomson, M. Relative efficiency of pigment and horny layer thickness in protecting the skin of europeans and africans against solar ultraviolet radiation. *J. Physiol.* **127**, 236-46 (1955).
3. Elias, P.M. Stratum corneum defensive functions: An integrated view. *J. Gen. Int. Med.* **20**,183-200 (2005).
4. Gniadecka, M., Wulf, H., Mortensen, N.N. *et.al.* Photoprotection in vitiligo and normal skin. A quantitative assessment of the role of stratum corneum, viable epidermis and pigmentation. *Acta Derm.Venereol.* **76**, 429-32 (1996).
5. Jung, S.-E., Kang, H.Y., Lee, E.-S. *et.al.* Changes of epidermal thickness in vitiligo. *Am. J. Dermatopathol.* **37**, 289-92 (2015).
6. Voegeli, R., Rawlings, A.V. and Summers, B. Facial skin pigmentation is not related to stratum corneum cohesion, basal transepidermal water loss, barrier integrity and barrier repair. *Int. J. Cosmet Sci.* **37**, 241-252 (2015).
7. Muizzuddin, N., Hellemans, L., Van Overloop, L., Corstjens, H., Declercq, L. and Maes, D. Structural and functional differences in barrier properties of african american, caucasian and east asian skin. *J. Dermatol. Sci.* **59**,123-8 (2010).
8. Hirao, T., Takahashi, M., Kikuchi, K., Terui, T. and Tagami, H. A novel non-invasive evaluation method of cornified envelope maturation in the stratum corneum provides a new insight for skin care cosmetics. *IFSCC Magazine.* **6**, 103-9 (2003).
9. Corcuff, P., Lotte, C., Rougier, A. and Maibach, H. Racial differences in corneocytes. A comparison between black, white and oriental skin. *Acta Dermato. Venereol.* **71**, 146-8 (1990).
10. Hellemans, L., Muizzuddin, N., Declercq, L. and Maes, D. Characterization of stratum corneum properties in human subjects from a different ethnic background. *J. Invest. Dermatol.* **124**, 371 (2005)
11. Palmer, C.N., Irvine, A.D., Terron-Kwiatkowski, A., Zhao, Y., Liao, H., Lee, S.P., et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat. Genet.* **38**, 441-6 (2006).
12. Scott, I.R. and Harding, C.R. Filaggrin breakdown to water binding compounds during development of the rat stratum corneum is controlled by the water activity of the environment. *Dev. Biol.* **115**, 84-92 (1986).
13. Jokura, Y., Ishikawa, S., Tokuda, H. and Imokawa, G. Molecular analysis of elastic properties of the stratum corneum by solid-state ¹³c-nuclear magnetic resonance spectroscopy. *J. Invest. Dermatol.* **104**, 806-12 (1995).
14. Laden, K. Moisturizing agent in skin. *J. Soc. Cosmet. Chem.* **18**, 360 (1967).

15. Rawlings, A.V., Scott, I.R., Harding, C.R. and Bowser, P.A. Stratum corneum moisturization at the molecular level. *J. Invest. Dermatol.* **103**, 731-40 (1994).
16. Chandar, P., Nole, G. and Johnson, A.W. Understanding natural moisturizing mechanisms: Implications for moisturizer technology. *Cutis.* **84**, 2-15 (2009).
17. Harding, C., Aho, S. and Bosko, C. Filaggrin—revisited. *Int. J. Cosmet. Sci.* **35**, 412-23 (2013).
18. Rawlings, A. Molecular basis for stratum corneum maturation and moisturization. *Br. J. Dermatol.* **171**, 19-28 (2014).
19. Takeda, A., Higuchi, D., Yamamoko, T., Nakamura, Y., Masuda, Y., Hirabayashi, T., et al. Purification and characterization of bleomycin hydrolase, which represents a new family of cysteine proteases, from rat skin. *J. Biochem.* **119**, 29-36 (1996).
20. Takeda, A., Nonaka, M., Ishikawa, A. and Higuchi, D. Immunohistochemical localization of the neutral cysteine protease bleomycin hydrolase in human skin. *Arch. Dermatol. Res.* **291**, 238-40 (1999).
21. Schwartz, D.R., Homanics, G.E., Hoyt, D.G., Klein, E., Abernethy, J. and Lazo, J.S. The neutral cysteine protease bleomycin hydrolase is essential for epidermal integrity and bleomycin resistance. *PNAS.* **96**, 4680-5 (1999).
22. Kamata, Y., Taniguchi, A., Yamamoto, M., Nomura, J., Ishihara, K., Takahara, H., et al. Neutral cysteine protease bleomycin hydrolase is essential for the breakdown of deiminated filaggrin into amino acids. *J. Biol. Chem.* **284**, 12829-36 (2009).
23. Ando, Y., Miyachi, Y., Imamura, S., Kannagi, R. and Murachi, T. Purification and characterization of calpains from pig epidermis and their action on epidermal keratin. *J. Invest. Dermatol.* **90**, 26-30 (1988).
24. Yamazaki, M., Ishidoh, K., Suga, Y., Saido, T.C., Kawashima, S., Suzuki, K., et al. Cytoplasmic processing of human profilaggrin by active μ -calpain. *Biochem. Biophys. Res. Comm.* **235**, 652-6 (1997).
25. Miyachi, Y., Yoshimura, N., Suzuki, S., Hamakubo, T., Kannagi, R., Imamura, S., et al. Biochemical demonstration and immunohistochemical localization of calpain in human skin. *J. Invest. Dermatol.* **86**, 346-9 (1986).
26. Ando, Y., Imamura, S., Murachi, T. and Kannagi, R. Calpain activates two transglutaminases from porcine skin. *Arch. Dermatol. Res.* **280**, 380-4 (1988).
27. Hsu, C.-Y., Henry, J., Raymond, A.-A., Méchin, M.-C., Pendaries, V., Nassar, D., et al. Deimination of human filaggrin-2 promotes its proteolysis by calpain 1. *J. Biol. Chem.* **286**, 23222-33 (2011).
28. Kim, S.Y. and Bae, C.D. Calpain inhibitors reduce the cornified cell envelope formation by inhibiting proteolytic processing of transglutaminase 1. *Exp. Mol. Med.* **30**, 257-62 (1998).
29. Kamata, Y., Itoh, Y., Kajiya, A., Karasawa, S., Sakatani, C., Takekoshi, S., et al. Quantification of neutral cysteine protease bleomycin hydrolase and its localization in rat tissues. *J. Biochem.* **141**, 69-76 (2007).

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30. Son, E., Kim, Y., Joo, K., Kim, H., Lee, E., Nam, G., et al. Skin dryness in apparently healthy human skin is associated with decreased expression of bleomycin hydrolase in the stratum corneum. *Clin. Exp. Dermatol.* **40**, 247-53 (2015).
31. Kamata, Y., Maejima, H., Watarai, A., Saito, N., Katsuoka, K., Takeda, A., et al. Expression of bleomycin hydrolase in keratinization disorders. *Arch. Dermatol. Res.* **304**, 31-8 (2012).
32. Pellerin, L., Paul, C., Schmitt, A.-M., Serre, G. and Simon, M. Bleomycin hydrolase downregulation in lesional skin of adult atopic dermatitis patients is independent of flg gene mutations. *J. Allergy Clin. Immunol.* **134**,1459-61. e7 (2014).
33. Kamata, Y., Yamamoto, M., Kawakami, F., Tsuboi, R., Takeda, A., Ishihara, K., et al. Bleomycin hydrolase is regulated biphasically in a differentiation-and cytokine-dependent manner relevance to atopic dermatitis. *J. Biol. Chem.* **286**, 8204-12 (2011).
34. Machado, M., Hadgraft, J. and Lane, M. Assessment of the variation of skin barrier function with anatomic site, age, gender and ethnicity. *Int. J. Cosmet. Sci.* **32**, 397-409 (2010).
35. Harding, C., Long, S., Richardson, J., Rogers, J., Zhang, Z., Bush, A., et al. The cornified cell envelope: An important marker of stratum corneum maturation in healthy and dry skin. *Int. J. Cosmet. Sci.* **25**, 157-67 (2003).
36. Kitamura, K. Advances in dry skin care technology extend beyond the category of cosmetic products. *IFSCC Magazine.* **5**, 177-87 (2002).
37. Kitamura, K., Ito, A., Yamada, K. and Fukuda, M. Research on the mechanism by which dry skin occurs and the development of an effective compound for its treatment. *J. Soc. Cosmet. Chem. Jpn.* **29**, 133-45 (1995).
38. Voegeli, R., Rawlings, A., Doppler, S. and Schreier, T. Increased basal transepidermal water loss leads to elevation of some but not all stratum corneum serine proteases. *Int. J. Cosmet. Sci.* **30**, 435-42 (2008)..
39. Contet-Audonneau, J.L., Jeanmaire, C. and Pauly, G. A histological study of human wrinkle structures: comparison between sun-exposed areas of the face, with or without wrinkles, and sun-protected areas. *Br. J. Dermatol.* **140**, 1038-47 (1999).
40. Yan, W., Zhang, L.L., Yan, L., Zhang, F., Yin, N.B., Lin, H.B., et al. Transcriptome analysis of skin photoaging in chinese females reveals the involvement of skin homeostasis and metabolic changes. *PLoS One.* **8**, e61946 (2013).
41. Breternitz, M., Flach, M., Prässler, J., Elsner, P. and Fluhr, J. Acute barrier disruption by adhesive tapes is influenced by pressure, time and anatomical location: Integrity and cohesion assessed by sequential tape stripping; a randomized, controlled study. *Br. J. Dermatol.* **156**, 231-40 (2007).
42. Voegeli, R., Heiland, J., Doppler, S., Rawlings, A.V. and Schreier, T. Efficient and simple quantification of stratum corneum proteins on tape strippings by infrared densitometry. *Skin Res Technol.* **13**(3):242-51 (2007).

- 1
2
3 43. Raj, N., Voegeli, R., Rawlings, A.V, Gibbons, S., Munday, M., Summers, B., et
4 al. Variation in stratum corneum protein content as a function of anatomical site and
5 ethnic group. *Int. J. Cosmet. Sci.* (2015) doi: 10.1111/ics.12274.
6
7 44. Kezic, S., Kammeyer, A., Calkoen, F., Fluhr, J. and Bos, J. Natural
8 moisturizing factor components in the stratum corneum as biomarkers of filaggrin
9 genotype: Evaluation of minimally invasive methods. *Br. J. Dermatol.* **161**, 1098-104
10 (2009).
11
12 45. Mohammed, D., Matts, P.J., Hadgraft, J. and Lane, M.E. Variation of stratum
13 corneum biophysical and molecular properties with anatomic site. *AAPS J.* **14**, 806-
14 12 (2012).
15
16 46. Mohammed, D., Matts, P., Hadgraft, J. and Lane, M. Depth profiling of
17 stratum corneum biophysical and molecular properties. *Br. J. Dermatol.* **164**, 957-65
18 (2011).
19
20 47. Voegeli, R., Rawlings, A., Doppler, S., Heiland, J. and Schreier, T. Profiling of
21 serine protease activities in human stratum corneum and detection of a stratum
22 corneum trypsin-like enzyme. *Int. J. Cosmet. Sci.* **29**, 191-200 (2007).
23
24 48. Voegeli, R., Rawlings, A., Seroul, P. and Summers, B. A novel continuous
25 color mapping approach for visualization of facial skin hydration and transepidermal
26 water loss for four ethnic groups. *Int. J. Cosmet. Sci.* **37**, 595-605 (2015).
27
28 49. Jungersted, J., Høgh, J.K., Hellgren, L., Jemec, G. and Agner, T. Ethnicity
29 and stratum corneum ceramides. *Br. J. Dermatol.* **163**, 1169-73 (2010).
30
31 50. Rawlings, A.V. Ethnic skin types: Are there differences in skin structure and
32 function? 1. *Int. J. Cosmet. Sci.* **28**, 79-93 (2006).
33
34 51. Tezuka, T., Qing, J., Saheki, M., Kusuda, S. and Takahashi, M. Terminal
35 differentiation of facial epidermis of the aged: Immunohistochemical studies.
36 *Dermatology.* **188**, 21-4 (1994).
37
38 52. Biniek, K., Levi, K. and Dauskardt, R.H. Solar uv radiation reduces the barrier
39 function of human skin. *PNAS.* **109**, 17111-6 (2012).
40
41 53. Wildnauer, R.H., Bothwell, J.W. and Douglass, A.B. Stratum corneum
42 biomechanical properties i. Influence of relative humidity on normal and extracted
43 human stratum corneum. *J. Invest. Dermatol.* **56**, 72-8 (1971).
44
45 54. Park, A. and Baddiel, C. Rheology of stratum corneum: A molecular
46 interpretation of the stress-strain curve. *J. Soc. Cosmet. Chem.* **23**, 3-12 (1972).
47
48 55. Rawlings, A.V., Watkinson, A., Harding, C.R., Ackerman, C., Banks, J., Hope,
49 J., et al. Changes in stratum corneum lipid and desmosome structure together with
50 water barrier function during mechanical stress. *J. Soc. Cosmet. Chem.* **46**, 41-51
51 (1995).
52
53 56. Barresi, C., Stremnitzer, C., Mlitz, V., Kezic, S., Kammeyer, A., Ghannadan,
54 M., et al. Increased sensitivity of histidinemic mice to uvb radiation suggests a crucial
55 role of endogenous urocanic acid in photoprotection. *J. Invest. Dermatol.* **131**, 188-
56 94 (2011).
57
58
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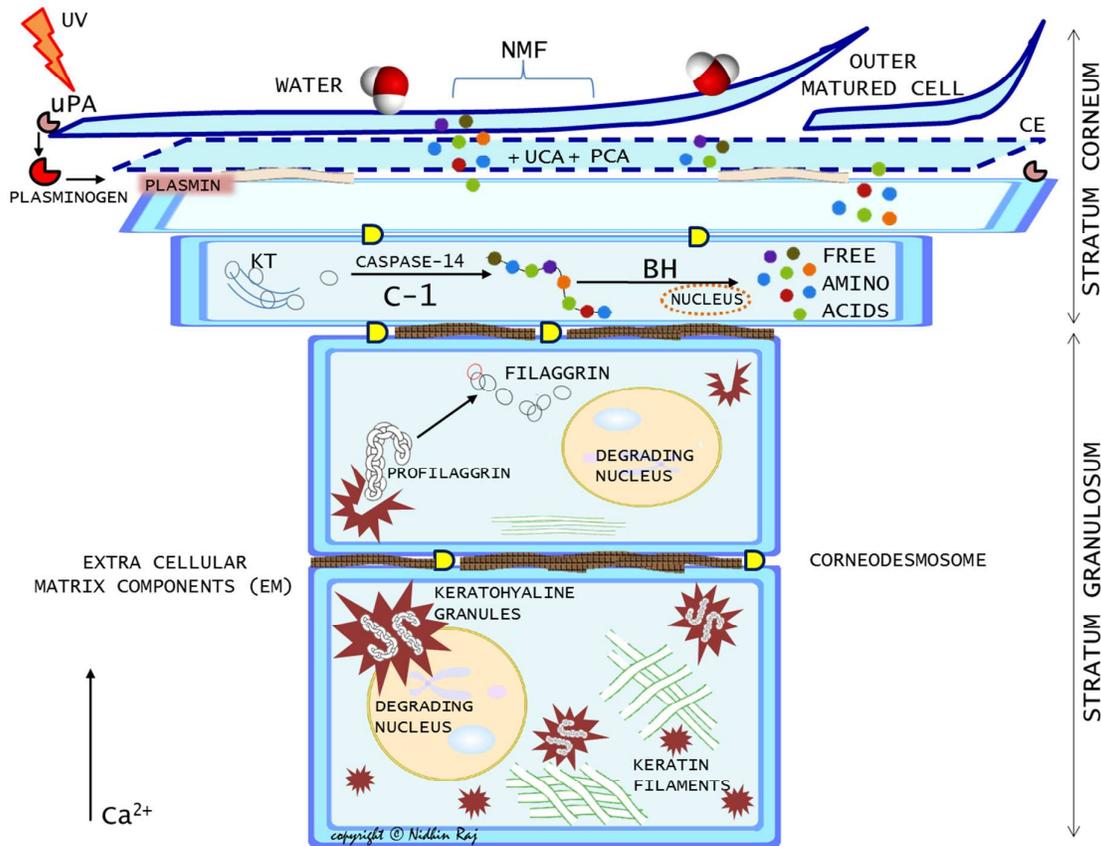


Figure 1: Schematic representation of NMF generation and the plasminogen pathway. The keratohyalin granules store profilaggrin which is dephosphorylated by phosphatases and the proteolytically cleaved to produce free filaggrin (FLG). FLG monomers bind to keratin filaments causing them to aggregate to form keratin tonofilaments in lower layers of the SC. FLG undergoes further degradation by caspase-14, C-1 and BH to form free amino acids and eventually PCA and UCA. These terminal breakdown products of FLG together with lactate, urea, inorganic ions and sugars are collectively termed NMF. The cornified envelope (CE) is shown as dotted lines.

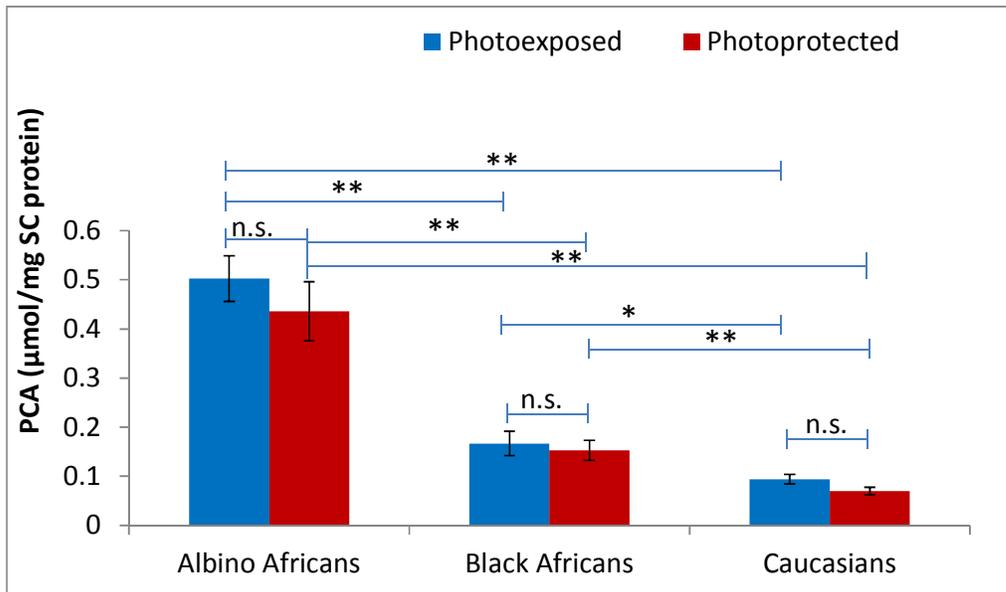


Figure 2: PCA levels of photoexposed cheek and photoprotected post-auricular test sites. Data are mean \pm SEM, n = 20 per group, * p < 0.01, ** p < 0.001, n.s. not significant.

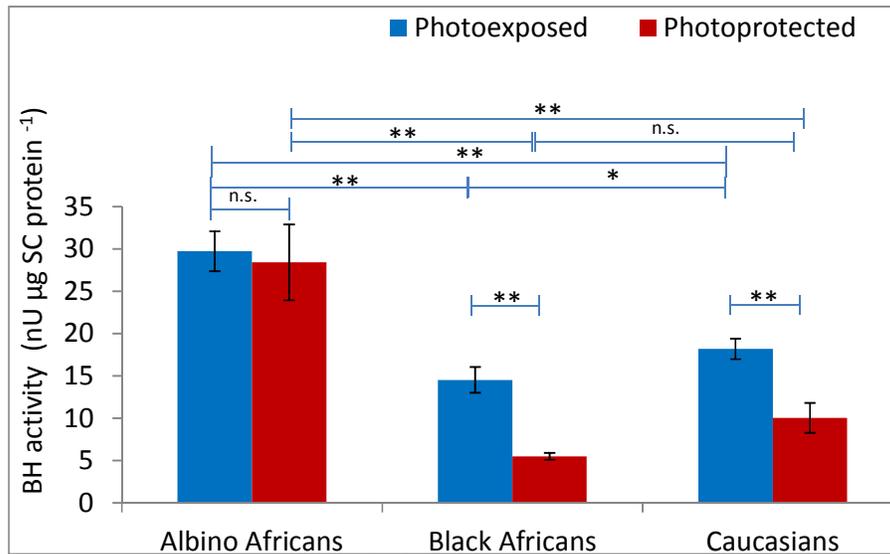


Figure 3: BH activity of photoexposed cheek and photoprotected post-auricular test sites. Data are mean \pm SEM, $n = 20$ per group, * $p < 0.01$, ** $p < 0.001$, n.s. not significant.

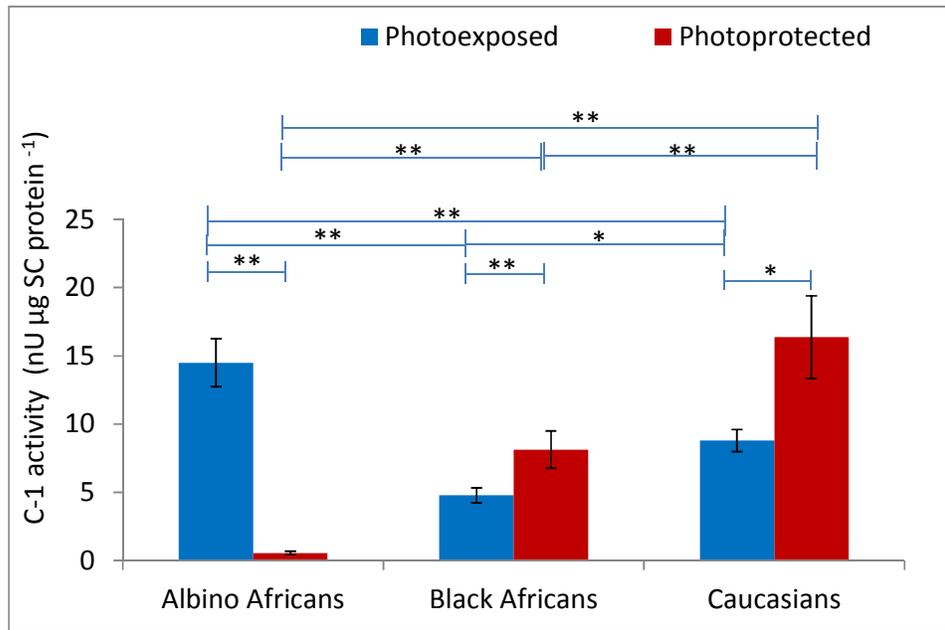


Figure 4: C-1 activity of photoexposed cheek and photoprotected post-auricular test sites. Data are mean \pm SEM, n = 20 per group, * p < 0.01, ** p < 0.001, n.s. not significant.

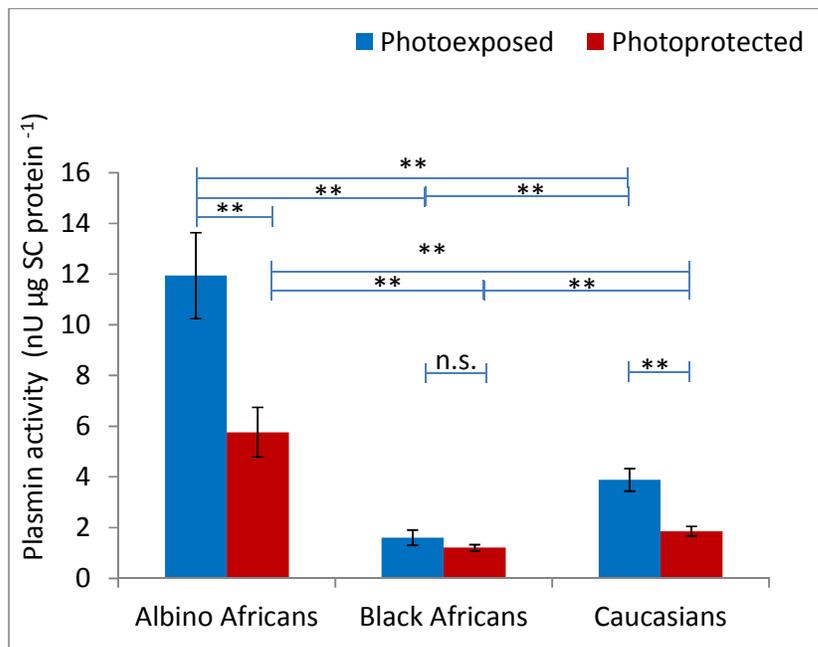


Figure 5: Plasmin activity of photoexposed cheek and photoprotected post-auricular test sites. Data are mean \pm SEM, n = 20 per group, ** p < 0.001, n.s. not significant.

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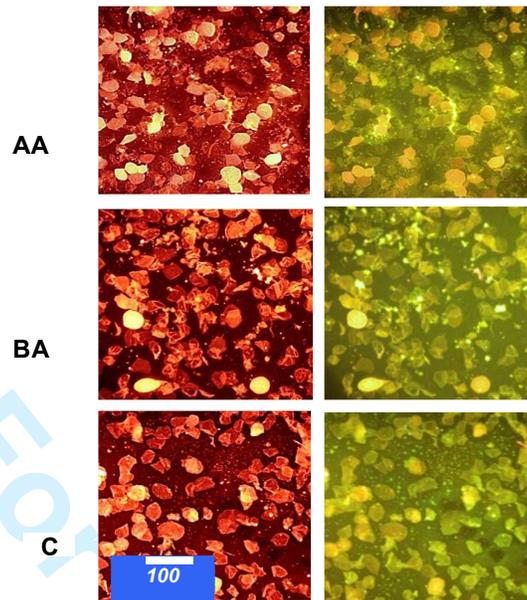


Figure 6: Differential immunostaining of corneocytes from cheek (tape stripping 1), Nile red staining (left column) and involucrin antibody immunostaining (right column). The nuclei inside the corneocytes (dark stained) and CE as loosely bound aggregates (green) in involucrin immunostaining, (2.85 pixels/ μm), scale, bar = 100 μm .

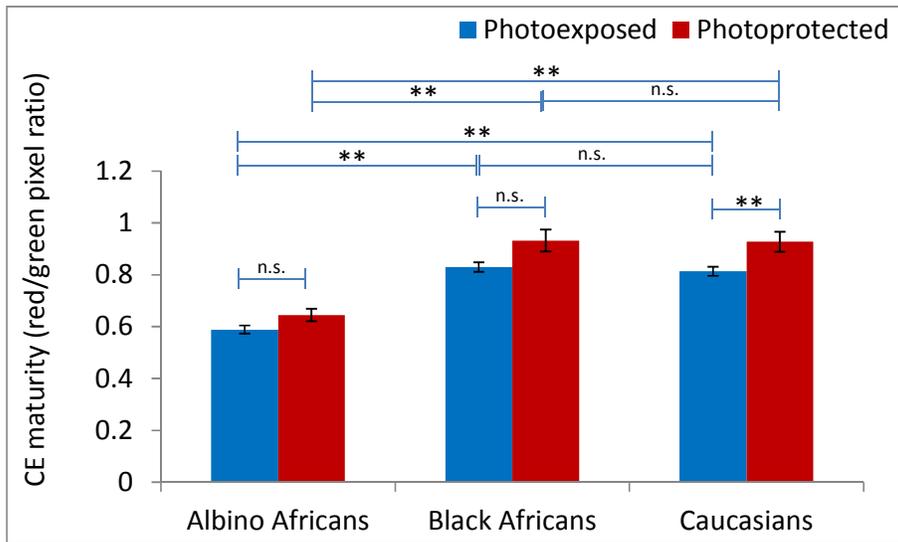


Figure 7: Maturity of corneocytes originating from photoexposed cheek and photoprotected post-auricular test sites. Data are mean \pm SEM, $n = 20$ per group, ** $p < 0.001$, n.s. not significant.

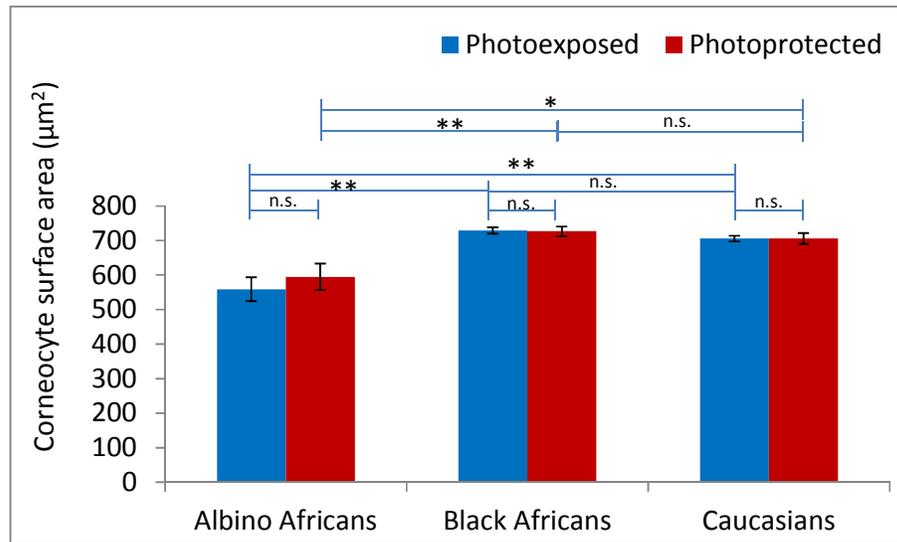


Figure 8: Corneocyte surface area between photoexposed and photoprotected sites. Data are mean \pm SEM, ** p < 0.001, * p < 0.01, n.s. not significant.

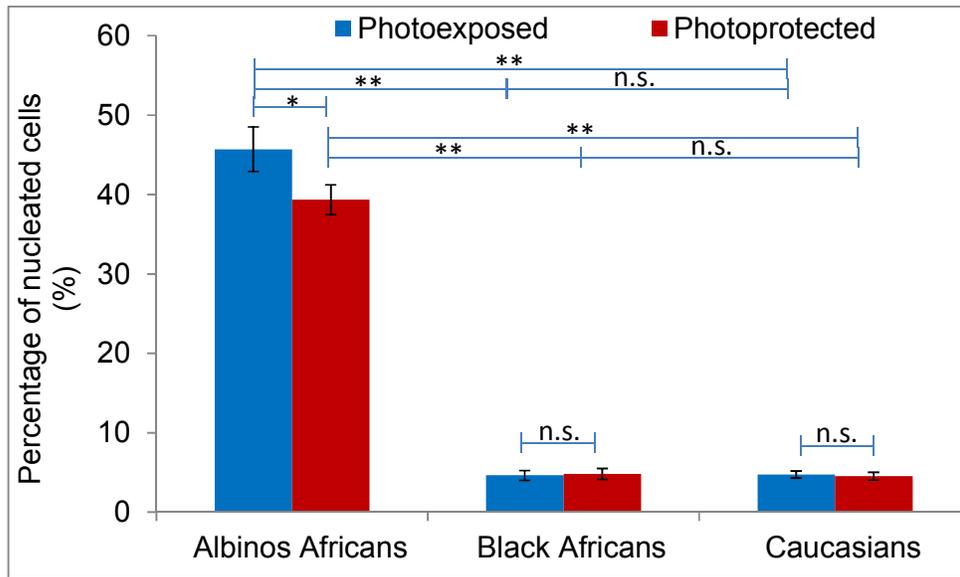


Figure 9: Percentage of nucleated cells between photoexposed and photoprotected sites in three different ethnicities. Data are mean \pm SEM, ** $p < 0.001$, * $p < 0.01$, n.s. not significant.