The effect of photodamage on the female Caucasian facial stratum corneum corneome using mass spectrometry-based proteomics

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Abstract

BACKGROUND: The effect of photodamage on facial stratum corneum (SC) is still poorly understood.

OBJECTIVE: To describe the SC proteome from tape strippings of Caucasian SC from photoexposed cheek and photoprotected post-auricular (PA) site, a global analysis of photodamage on the skin will be developed leading to a better understanding of keratinocyte signalling pathways and identification of new molecular targets for the treatment of photaged skin.

METHODS: Female Caucasian subjects had nine consecutive tape strippings taken from their cheeks and PA site. Proteins were extracted and the trypsin-digested peptides were analysed by nanochromatography coupled to a high-resolution mass spectrometer. Data-dependent acquisition allowed protein identification that was processed by Paragon algorithm of Protein Pilot software.

RESULTS: Changes in the levels of epidermal differentiation proteins were apparent indicating poor epidermal differentiation and SC maturation (keratins, cornified envelope (CE) proteins) on photoexposed cheeks. Differences in protease-anti-protease balance were observed for corneodesmosylysis (favouring desquamation) and filaggrinolysis (favouring reduced filagrin processing). 12R-LOX, a CE maturation enzyme, was reduced in photodamaged skin but not transglutaminases. Changes in signal keratinocyte transduction pathway markers were demonstrated especially by reduced levels of downstream signalling markers such as calreticulin (unfolded protein response; UPR) and increased level of stratifin (target of rapamycin; mTOR). Evidence for impaired proteostasis was apparent by reduced levels of a key proteasomal subunit (subunit beta type-6). Finally, key antioxidant proteins were upregulated except catalase.

CONCLUSION: Clear examples of poor keratinocyte differentiation and associated metabolic and signalling pathways together with reduced SC maturation were identified in photodamaged facial SC. Corneocyte immaturity was evident with changes in CE proteins. Particularly, the reduction in 12R-LOX is a novel finding in photodamaged skin and supports the lack of SC maturation. Moreover, filaggrinolysis was reduced, whereas corneodesmosylysis was enhanced. From our results, we propose that there is a poor crosstalk between the keratinocyte endoplasmic reticulum UPR, proteasome network and autophagy machinery that possibly leads to impaired keratinocyte proteostasis. Superimposed on these aberrations is an apparently enhanced mTOR pathway that also contributes to reduced SC formation and maturation. Our results clearly indicate a corneocyte scaffold disorder in photodamaged cheek SC.

Résumé

CONTEXTE: Les effets des dommages dus à l’exposition à la lumière sur le stratum corneum (SC) sont encore mal compris.

OBJECTIFS: Décrire le protéome du SC issu de prélèvements par bandes adhésives provenant de la joue, exposée à la lumière, et de la région post-auriculaire, protégée de la lumière, de sujets caucasiens. À cette fin, une analyse globale des dommages cutanés dus à l’exposition à la lumière sera développée pour permettre une meilleure compréhension des voies de signalisation des kératinocytes ainsi qu’une identification de nouvelles cibles moléculaires pour le traitement des peaux ayant subi un photo-vieillissement.

MÉTHODES: 9 prélèvements consécutifs par bandes adhésives ont été réalisés sur des femmes caucasiennes au niveau de la joue et de la zone post-auriculaire. Les protéines ont été extraites des échantillons et les peptides issus de leur digestion trypsique analysés par nano-chromatographie couplée à un spectromètre de masse haute résolution. L’acquisition dépendante des données a permis de mettre en évidence les protéines impliquées par l’algorithme Paragon du logiciel Protein Pilot.

RÉSULTATS: Des changements notables des taux de protéines impliquées dans la différenciation épidermique et la maturation du SC (kératines, protéines de l’enveloppe cornée (EC)) indiquent que celles-ci sont amoindries au niveau des joues photo-exposées. Des différences de l’équilibre protéases-antiprotéases ont été observées pour la corneodesmolysine (favorisant la désquamation) et la filagrinelyse (favorisant la réduction du traitement de la filagrine). 12R-LOX, une enzyme de maturation de l’EC, était réduite dans les peaux photo-dommagées mais pas les transglutaminases. Des changements au niveau des marqueurs de la voie de transduction du signal des kératinocytes ont été observés se caractérisant en particulier par des niveaux réduits de marqueurs de signalisation avuls, tels que la calréticuline (impliquée dans la réponse aux protéines de conformation anormale; alias UPR, unfolded protein response) et un niveau accru de stratifin (voie cible de la rapamycin; mTOR). Des preuves d’une perturbation de l’héméostasie protéique étaient apparentes, avec une diminution des taux d’une
sous-unité clé du protéasome (sous-unité bêta type-6). Enfin, les protéines antioxydantes cruciales, à l’exception de la catalase, étaient régulées positivement.

**CONCLUSIONS:** Des exemples clairs d’un appauvrissement de la différenciation kératinocytaire et des perturbations des voies métaboliques et de signalisations associées ainsi qu’une réduction de la maturation du SC ont été identifiées dans le SC facial photo-endommagé. L’immaturité des cornéocytes a été mise en évidence par des changements de protéines de l’EC. Notamment, la réduction de 12R-LOX est une découverte pour les peaux photo-endommagées et supporte l’idée d’un manque de maturation du SC. De plus, la filaggrinolyse était réduite, tandis que la cornéodémolysé était accrue. D’après nos résultats, nous proposons qu’il existe une altération des interactions entre la réponse aux protéines de conformation anormale du réticulum endoplasmique (UPR), le réseau du protéasome et la machinerie autophagique, cette altération conduisant potentiellement à une déficience de l’homéostasie protéique kératinocytaire. Se superposant à ces aberrations, on trouve un accroissement apparent de la transduction de la voie mTOR, qui contribue également à une diminution de la formation et de la maturation du SC. Nos résultats indiquent clairement une altération de l’échafaudage cornéocytaire dans le SC des joues photo-endommagées.

**Introduction**

Without knowing what consumers want, product development will be meaningless and be like ‘a shot in the dark’. Fortunately, consumer research is continually unravelling the reasoning behind unmet consumer needs. However, without an understanding of the substrate biology behind these unmet consumers’ needs, any innovation will be like a ‘shot in the dark, while firing blanks’. Thus, to identify new molecular targets for visually dry and rough photodamaged facial skin, a better understanding of the cellular biology and biochemistry is required for the substrate in question. Moreover, without this understanding, predictive in vitro models cannot be developed to further probe the relevance of these mechanisms.

Many researchers use surrogate body sites to understand the effects of ingredients on skin, for example, forearm skin, as it is more easily accessible. However, the complexity of facial stratum corneum (SC) barrier and moisturization has recently been demonstrated and highlights the need for studies to be performed directly on facial skin [1]. As a result, this paper is focussed on the effects of photodamage on facial skin, particularly the SC, to better-identify molecular targets for its treatment.

Compared to some other body sites, facial SC has an elevated transepidermal water loss (TEWL), increased corneocyte cohesion but also higher proteolytic activities resulting in premature cornodesmosomal degradation and a thinner tissue [2–4]. This indicates that facial ‘stratum disjunctum’ is diminished or even no longer present anymore and the reason for the increased SC cohesion is the presence of largely a ‘stratum compactum’. The elevated protease activities on the face may be due to a subclinical, micro-inflammatory or pre-inflammatory condition induced, for example, by environmental effects. Thus, increased protease activities might represent key markers for an underlying and sometimes non-observable skin abnormality. Moreover, differences in lipid and natural moisturizing factor (NMF) levels and composition are known compared with other body sites [5,6]. In particular, reduced NMF levels are apparent on the face presumably due to impaired prolaggrin expression and its processing, a reduced SC barrier lipid/protein ratio, increased sphingosine/phytosphingosine ceramide levels and reductions in the chain length of ceramides [7–11].

To determine the effects of photodamage on skin, we have, like other research groups, compared the differences in the physiology, biochemistry and cornocyte morphology of the SC between samples from photoexposed cheek and photoprotected post-auricular (PA) sites [12]. In Caucasian cheek SC, we have found increased plasmin and bleomycin hydrolase, but reduced calpain-1, activities with only marginally increased pyrrolidone carboxylic acid (PCA) levels and greater quantities of immature corneocyte envelopes (CEs) compared with the PA sites. Although this research has contributed to the understanding of the physiology of photoaging of facial SC, more is still to be learned.

Initially, the understanding of SC composition has generally been on an analyte-by-analyte basis, by two-dimensional electrophoretic and chromatographic methods together with multiplex enzymelinked immunosorbent assays. These approaches have been highly successful in helping us to determine the general composition of the SC but each has their limitations. The use of mass spectrometry-based ‘omic’ approaches is on the increase for investigating skin biochemistry especially proteomics [13–19]. Recently, these methods have been used to determine differences in the corneum of dry skin on the leg of post-menopausal women and dandruff on the scalp [20–22]. However, these approaches have only recently been used to study the proteome of facial SC.

In order to gain a greater insight into the effects of photodamage on the SC and to identify novel protein markers, label-free mass spectrometry-based proteomics was performed on samples from the cheek and PA of Caucasian women in the current study. Defining differences in the expressed proteins will lead to a new understanding of proteomic changes in the photodamaged epidermis/SC and finally to new molecular targets to mitigate the condition.

**Materials and methods**

**Study subjects**

The study was a cross-sectional study and was approved by the School of Health Care Sciences Research Ethics Committee (SREC) together with the Medunsa Campus Research and Ethics Committee (MREC) and was conducted in accordance with the Declaration of Helsinki Principles. Written, informed consent was obtained from all participants before enrolment. Six healthy female Caucasian volunteers (39.0 ± 5.3 years old, Fitzpatrick skin phenotype II and III), living in Pretoria, South Africa, participated in this observational study which took place from the end of November to early December 2013 from a subgroup of the original panel of subjects. The subjects did not apply any dermatological or cosmetic products to their faces for 3 days before expert grading and evaluation of their facial skin. For this 3-day conditioning phase, subjects cleansed the face with tepid water in the morning as well as in the evening. Before tape stripping, the skin was cleaned by gently swabbing with a cotton pad soaked in distilled water at ambient temperature and allowed to dry for 20 min and then acclimatized for 30 min at 21 ± 1°C and 35 ± 10% relative humidity [12].

**Sample collection and SC protein evaluation**

Nine standard D-Squame® (Cuderm Corporation, Dallas, USA) disks with a diameter of 2.2 cm and an area of 3.8 cm² were placed on
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the right cheek (3 cm vertically beneath the outer edge of the eye) and post-auricular area (area opposite to right earlobe). The tapes were applied with 225 g cm\(^{-2}\) of pressure with a pressure device (Cuder Corporation, Dallas, US) for 5 s and then removed by a single stroke movement. All nine stripings were taken sequentially from each site. To minimize variations, the procedure was conducted by the same technician for all volunteers, throughout the study. The interval between the tape stripings was 20 ± 5 s [23,24].

**Protein extraction, digestion and clean up**

Each tape was placed adhesive side up in its own 20-mL borosilicate scintillation vial (Wheaton; Fisher Scientific, Pittsburgh, Pa). Proteins were extracted using PBS buffer containing 0.2% SDS and 0.5% propylene glycol (Sigma-Aldrich, St. Louis, USA) supplemented with 1× HALT protease inhibitors, EDTA-free (Pierce; Thermo Scientific, Rockford, USA). The vials were incubated one hour at room temperature in a sonication bath (Sonorex; BANDELIN electronic, Berlin, DE). Proteins were precipitated in 40 mL ice-cold 90% methanol containing 0.1% formic acid (Sigma-Aldrich, St. Louis, USA). Samples were incubated at −20°C overnight and then centrifuged at 15,000 g at 4°C for 20 min. Protein pellets were resuspended in 0.1 M Tris–HCl – 8 M urea buffer (pH 8.5, Euromedex, Soufflèweseyrheim, FR). The protein content was estimated by BCA assay (Pierce; Thermo Scientific, Rockford, USA). The protein extract was digested according to FASP II procedure as described by Wiśniewski et al. [25], with slight modifications. The protein extracts (150 μg) were loaded onto Amicon Ultra 0.5-ml 10-kDa filters (Millipore, Billerica, USA); filters were washed with 8 M urea in 0.1 M Tris (pH 8.5) to facilitate removal of protein-bound small molecules. Proteins were reduced with 10 mM dithiothreitol (Euromedex, Soufflèweseyrheim, FR) for 60 min and alkylated with 50 mM iodoacetamide (Sigma-Aldrich, St Louis, US) for 30 min. The urea buffer was exchanged by ultrafiltration for ammonium bicarbonate 50 mM (Sigma-Aldrich, St Louis, USA). The cleaned proteins were digested overnight on the filter with trypsin at a 1:50 ratio (Promega, Madison, USA), and the resulting peptides were released from the filter by centrifugation (14,000 g, 20 min), followed by a first wash of the filter in 0.5 M NaCl and second wash of the filter by 50% acetonitrile (both Sigma-Aldrich, St Louis, USA).

The three fractions were pooled, and peptides were diluted at 5% acetonitrile and acidified by 0.1% trifluoroacetic acid 0.1% final concentration. Peptides were desalted using 500 mg SepPak tC18 sample extraction columns (Waters, Milford, USA), eluted with 1.2 mL of 70% acetonitrile, dried and dissolved in 100 μL of water acidified with 0.1% formic acid (Sigma-Aldrich, St Louis, USA). The peptides amount of each digest was estimated by BCA assay (Pierce; Thermo Scientific, Rockford, USA).

**Nano-liquid chromatography and tandem mass spectrometry**

Peptide digests (100 ng per run) were loaded onto a nanoACQUITY UPLC Symmetry C18 Trap Column, 180 μm × 20 mm (particle diameter 5 μm, pore size 100 Å) in trap and elute mode with ACQUITY UPLC Peptide BEH C18 nanoACQUITY Column 75 μm × 250 mm (particle diameter 1.7 μm, pore size 130 Å) (Both Waters, Milford, USA). The run gradient was performed by Eksigent Ultra Plus nano-LC 2D HPLC (AB Sciex, Framingham, USA) system over 90 minutes with a gradient from 3% to 40% buffer B (buffer A: 0.1% formic acid; buffer B: 95% acetonitrile, 0.1% formic acid) at a flowrate of 300 nl min\(^{-1}\). The Eksigent system was coupled to a TripleTOF\(^{3}\) 5600 (AB Sciex, Framingham, USA) mass spectrometer interfaced to a nano-spray III source. The source parameters were set as follows: IS at 2500 V, Curtain gas at 30 psi, gas sprayer 1 at 1 psi and interface heater temperature at 150°C. The acquisition parameters were as follows: for DDA mode one 250 ms MS scan (>30K resolution). Following each survey MS1 scan, MS/MS spectra for the 30 most abundant parent ions (m/z range 350–1250) were acquired (high sensitivity mode, >15 K resolution). For DIA mode one 150 ms MS scan (>30 K resolution), followed by 35 fixed SWATH windows each with a 75 ms accumulation time and a 350–1250 m/z range, MS/MS SWATH scans (high sensitivity mode, >15 K resolution) were set at 26 amu window; Q1 isolation windows were covering entire mass range [26].

**Data analysis and peptide annotation**

DDA spectra processing and database searching were performed with ProteinPilot (v4.5 beta, ABSciex, Framingham, US) using the Paragon algorithm. The search parameters were as follows: sample type: identification; cys alkylation: iodoacetamide; digestion: trypsin; instrument: TripleTOF 5600; special factors: urea denaturation. ID focus: biological modifications. The database was downloaded from Uniprot (June 2014), filtering for reviewed human proteins only (20 194 entries). The resulting group file was loaded into Peakview\(^{3}\) (v2.0, AB Sciex, Framingham, USA), and peaks from SWATH runs were extracted with a peptide confidence threshold of 99% and a false discovery rate <1%. Label-free quantification was performed using Marker View (v1.2.1, AB Sciex, Framingham, USA). The selection of the proper peak was performed using the automated assistance of PeakView. The absolute signal of peptide or protein was calculated by summing the extracted area of all unique fragment ions [27]. These annotated fragment ions are referred to as proteins throughout the text of this article.

**Statistical analysis**

For each protein, a linear mixed model was fit to the log-transformed protein measurements with test site as fixed effect and subject pool as random effect followed by an unadjusted post hoc Tukey test, thus comparing the ethnic groups with each other. Additionally, q-values were calculated (which can be interpreted as adjusted P-values) to take multiplicity issues into account. Each single P-value reports the probability that the observed difference (for this protein and between the two skin sites examined) is due to chance alone. By tradition, a threshold P-value of 0.05 is applied. All comparisons with P-values below that threshold are called ‘significant’ and it is assumed that there is more than noise to it, that is that it is a ‘discovery’. However, as we look at not only one comparison, but a multitude in this analysis (around 1000; 3 group comparisons per protein) a threshold P-value of 0.05 implies in our setting that 5% of all comparisons (i.e. around 50) will be significant due to chance, that is will be ‘false discoveries’. To control the rate of false discoveries (FDR) among all discoveries, we calculated the corresponding q-values, which have also been termed FDR-adjusted P-values. Again, by tradition, a threshold q-value of 0.05 is applied. All comparisons with q-values below that threshold are treated as discoveries, and the theory behind the q-value calculation implies that of these discoveries, only 5% (or x% if another threshold is used) are false discoveries. Unfortunately, it remains impossible to tell which of the discoveries belong to those 5%. For
the resulting P-values of all proteins and comparisons, we again calculated q-values.

Results
A total of 457 SC proteins were identified 436 on the cheek and 253 on the post-auricular site. 232 common proteins were observed on both sites. 204 and 21 proteins were found to be different on the cheek and the post-auricular site. Comparing between sites, 73 proteins were significantly (P<0.05) upregulated >2.0 fold and 30 proteins downregulated <0.5 fold on the cheek vs the post-auricular site.

Proteases and protease inhibitors
Nineteen proteases were annotated between the cheek and PA sites, a total of 73 were significantly upregulated by factor >2 on the cheek vs the PA site, whereas 30 were downregulated by factor <0.5. A total of 232 common proteins were observed on both sites. However, 204 and 21 proteins were found to be different on the cheek and PA sites, respectively (Fig 1). To relate these protein changes to the known phenotypic changes that occur in photo-damaged SC, the proteins were grouped according to their functions (Table I and Fig 2). All statistical differences, whether significant P-values, q-values or both, are recorded in Table I.

Serum diffusion linked markers
32× increased levels of transitional endoplasmic reticulum–adenosine triphosphatase (ATPase) were observed on the cheek which has been related to impaired barrier function. In contrast, 1.7× reduced levels of serum albumin were seen.

Proteases and protease inhibitors
Four were identified to be involved with desquamation (kalikrein 7; 4.27× and 10; 2.39× together with cathepsin L2; 3.96× and D; 1.43×), whereas four are proposed to be involved with filaggrinolysis (retroviral-like aspartic protease 1 (SASPase 1; 5.73×), calpain-1 (subunits 1; 1.25× and 2; 1.4×), (bleomycin hydrolase; 1.73× and caspase 14; 1.66×). All these were elevated on the cheek. Cathepsin B (1.24×) was slightly elevated and may also be involved in filaggrinolysis.

The levels of serine protease inhibitors serpin B3 (SCCA1; 2.01×), B4 (SCCA2; 1.35×), B5 (3.65×), B7 (13.87×), B8 (1.56×), B12 (1.28×) and B13 (2.72×) levels were increased on the cheek, while those of alpha-2 macroglobulin-like 1 (0.67×) and serpin A12 (0.30×) levels were decreased. The cysteine protease inhibitor, cystatin-A, levels (0.85×) were also decreased.

Proteins related to SC cohesion
Moderate, but statistically significant increases in the levels of desmoglein-1 (1.49×), desmocollin-3 (1.19×) and corneodesmosin (1.20×) were observed on the cheek but the biggest increases were that of epilakin (3.78×), junction plakoglobin (2.63×) and plakophilin-1 (2.59×). Levels of protein POF1B (1.74×) and desmoplakin (1.61×) were intermediary in level.

Proteins related to corneocyte maturation
Cornifelin (0.74×), SPRR 1A (0.20×) and 2E (0.22×), loricrin (0.18×), suprabasin (0.43×) and the late envelope protein LEP7/XP32 (0.16×) were significantly lower on the cheek compared with the PA site, whereas SPRR 1B (1.26×) and 2B (1.45×) were increased. Transglutaminases 1 (1.47×) and 3 (1.64×) were elevated, but 12R-LOX levels were decreased (0.31×). A comparison of epidermal lipoygenase-3 (eLOX3) could not be made as it was only observed on the cheek.

Other enzymes contributing to NMF generation
Arginase-1 (3.05×), histidine ammonia-lyase (2.85×) and gamma-glutamylcyclotransferase (1.10×) were elevated on the cheek. These contribute to urea, urocanic acid (UCA) and PCA, respectively.

Differentiation markers: keratins, annexins
Decreased levels of keratin 1 (0.86×) and 10 (0.79×) were observed on the cheek, whereas increased levels of keratins 5 (1.67×) and 14 (1.40×) were found. Levels of keratin 86 (0.77×) and other keratin species were decreased especially keratins 78 (0.12×), 6B (0.25×), 31 (0.25×), 3B (0.36×), 34 (0.34×) 17 (0.46×), 35 (0.48×), 38 (0.59×), 80 (0.59×), 32 (0.69×), whereas six other keratin species were increased including keratin 39 (4.68×). Annexins A1 (1.79×), A7 (9.18×) and A11 (5.09×) were found to be decreased on the cheek.

Inflammation markers
Increased levels of inflammatory proteins were observed on the cheek, especially interleukin-1 receptor antagonist protein (>107× increase). Immunoglobulin C region proteins were increased (alpha-1 chain (17.1×), lambda-3 chain (1.92×), kappa chain (1.59×)) and decreased (gamma-1; 0.19× and -4; 0.7× chains). Secreted Ly-6/uPAR-related protein 1 (5.64×) and Ly6/PLAUR domain-containing protein 3 (4.01×) were also among the most increased of these markers. Interleukin-16 gamma was moderately increased (1.49×).

Membrane trafficking, microtubule and cytoskeleton markers
Levels of myosin 9 (2.83×) and 14 (2.12×) together with tropomyosin alpha-3 chain (1.19×) were increased, whereas tropomyosin alpha-4 chain (0.52×) and clathrin heavy chain 1 (0.29×) decrease...
Table I  Comparison of proteins between stratum corneum samples of photodamaged cheek and photoprotected post-auricular sites in Caucasian women.

| Gene name         | Protein name                                                                 | Fold change cheek/PA | P value | q value |
|-------------------|------------------------------------------------------------------------------|-----------------------|---------|---------|
| (1) Proteases     |                                                                               |                       |         |         |
| NPEPPS            | Puromycin-sensitive aminopeptidase                                            | 14.62                 | <0.001  | <0.001  |
| ASPRV1            | Retroviral-like aspartic protease 1 (SASPase)                                | 5.73                  | <0.001  | <0.001  |
| PRSS8             | Prostasin                                                                    | 4.86                  | <0.01   | <0.001  |
| KLK7              | Kallikrein-7                                                                  | 4.27                  | <0.001  | <0.001  |
| CT5V              | Cathepsin L2                                                                  | 3.96                  | <0.001  | <0.001  |
| DPP7              | Dipeptidyl peptidase 2                                                       | 3.49                  | <0.05   | <0.01   |
| CPA4              | Carboxypeptidase A4                                                           | 2.51                  | <0.001  | <0.001  |
| APEH              | Acylamino-acid-releasing enzyme                                               | 2.46                  | <0.001  | <0.001  |
| NCSTN             | Nicastin                                                                      | 2.42                  | <0.01   | <0.01   |
| KLK10             | Kallikrein-10                                                                 | 2.39                  | <0.001  | <0.001  |
| BLMH              | Bleomycin hydrolase                                                          | 1.73                  | <0.001  | <0.001  |
| PRCP              | Lysosomal Pro-X carboxypeptidase                                              | 1.66                  | ns      | ns      |
| CASP14            | Caspase-14                                                                    | 1.66                  | <0.001  | <0.001  |
| CTSD              | Cathepsin D                                                                   | 1.43                  | <0.001  | <0.001  |
| CAPNS2            | Calpain small subunit 2                                                      | 1.40                  | ns      | <0.05   |
| SCPEP1            | Retinoid-inducible serine carboxypeptidase                                   | 1.26                  | ns      | <0.05   |
| CAPN1             | Calpain-1 catalytic subunit (calpain-1)                                      | 1.25                  | <0.001  | <0.001  |
| CTSB              | Cathepsin B                                                                   | 1.24                  | <0.05   | <0.01   |
| CTSC              | Dipeptidyl peptidase 1                                                       | 1.18                  | ns      | <0.05   |
| (2) Protease inhibitors |                                                               |                       |         |         |
| SERPINB7          | Serpin B7                                                                     | 13.87                 | <0.001  | <0.001  |
| SERPINB5          | Serpin B5                                                                     | 3.65                  | <0.001  | <0.001  |
| SERPINB13         | Serpin B13                                                                    | 2.72                  | <0.001  | <0.001  |
| SERPINB3          | Serpin B3                                                                     | 2.01                  | <0.001  | <0.001  |
| SERPINB8          | Serpin B8                                                                     | 1.56                  | <0.001  | <0.001  |
| SERPINB4          | Serpin B4                                                                     | 1.35                  | ns      | <0.05   |
| SERPINB12         | Serpin B12                                                                    | 1.28                  | ns      | <0.05   |
| CSTA              | Cystatin-A                                                                    | 0.85                  | <0.05   | <0.01   |
| A2ML1             | Alpha-2-macroglobulin-like protein 1                                          | 0.67                  | <0.01   | <0.001  |
| SERPINA12         | Serpin A12                                                                    | 0.30                  | <0.001  | <0.001  |
| (3) Corneodesmosome constituents |                                                               |                       |         |         |
| EPPK1             | Epiplakin                                                                     | 3.78                  | <0.001  | <0.001  |
| JUP               | Junction plakoglobin                                                         | 2.63                  | <0.001  | <0.001  |
| PKP1              | Plakophilin-1                                                                | 2.59                  | <0.001  | <0.001  |
| POF1B             | Protein POF1B                                                                 | 1.74                  | ns      | <0.05   |
| DSP               | Desmoplakin                                                                  | 1.61                  | <0.001  | <0.001  |
| DSG1              | Desmoglein-1                                                                 | 1.49                  | <0.01   | <0.01   |
| CDSN              | Corneodesmosin                                                               | 1.20                  | <0.001  | <0.001  |
| DSC3              | Desmocollin-3                                                                | 1.19                  | <0.01   | <0.01   |
| (4) Cornified envelope constituents |                                                               |                       |         |         |
| SPRR2B            | Small proline-rich protein 2B                                                | 1.45                  | ns      | ns      |
| SPRR1B            | Cornifin-B (small proline-rich protein IB)                                    | 1.26                  | ns      | ns      |
| CNFN              | Cornifilin                                                                   | 0.74                  | <0.05   | <0.01   |
| SBSN              | Suprabasin                                                                  | 0.43                  | <0.001  | <0.001  |
| SPRR2E            | Small proline-rich protein 2E                                                | 0.22                  | <0.001  | <0.001  |
| SPRR1A            | Cornifin-A (small proline-rich protein IA)                                   | 0.20                  | <0.001  | <0.001  |
| LOR               | Loricrin                                                                     | 0.18                  | <0.001  | <0.001  |
| XP32              | Skin-specific protein 32                                                     | 0.16                  | <0.001  | <0.001  |
| (5) Cornified envelope processing enzymes |                                                               |                       |         |         |
| TGM3              | Protein-glutamine gamma-glutamyltransferase E (transglutaminase-3)           | 1.64                  | <0.001  | <0.001  |
| TGM1              | Protein-glutamine gamma-glutamyltransferase K (transglutaminase-1)           | 1.47                  | <0.01   | <0.001  |
| ALOXE3            | Hydroperoxide isomerase ALOXE3, epidermis-type lipoygenase 3 (eLOX)          | 1.02                  | ns      | ns      |
| ALOX12B           | Arachidonate 12-lipoxygenase, 12R-type (12R-LOX)                              | 0.31                  | <0.001  | <0.001  |
| (6) Filagrin and its processing enzymes |                                                               |                       |         |         |
| FLG               | Filagrin                                                                     | 3.08                  | <0.001  | <0.001  |
| ARG1              | Arginase-1                                                                   | 3.05                  | <0.001  | <0.001  |
| HAL               | Histidine ammonia-lyase                                                      | 2.85                  | <0.01   | <0.001  |
| FLG2              | Filagrin-2                                                                   | 1.12                  | <0.05   | <0.05   |
| GGCT              | Gamma-glutamylcyclotransferase                                                | 1.10                  | ns      | <0.05   |
| (7) Keratins  |                                                                               |                       |         |         |
| KRT39             | Keratin, type I cytoskeletal 39                                               | 4.68                  | <0.001  | <0.001  |
| KRT5              | Keratin, type II cytoskeletal 5                                               | 1.67                  | <0.001  | <0.001  |
| KRT82             | Keratin, type II cuticular Hb2                                                | 1.62                  | <0.05   | <0.01   |
Table I (continued)

| Gene name | Protein name | Fold change cheek/PA | P value | q value |
|-----------|-------------|----------------------|---------|---------|
| KRT6C     | Keratin, type II cytoskeletal 6C | 1.57 | ns | <0.05 |
| KRT2      | Keratin, type II cytoskeletal 2 epidermal | 1.50 | <0.001 | <0.001 |
| KRT16     | Keratin, type I cytoskeletal 16 | 1.46 | <0.001 | <0.001 |
| KRT23     | Keratin, type I cytoskeletal 23 | 1.45 | <0.05 | <0.01 |
| KRT14     | Keratin, type I cytoskeletal 14 | 1.40 | <0.001 | <0.001 |
| KRT1      | Keratin, type II cytoskeletal 1 | 0.86 | <0.001 | <0.001 |
| KRT10     | Keratin, type I cytoskeletal 10 | 0.79 | <0.001 | <0.001 |
| KRT86     | Keratin, type II cuticular Ha6 | 0.77 | ns | <0.05 |
| KRT32     | Keratin, type I cuticular Ha2 | 0.69 | <0.01 | <0.001 |
| KRTAP3-1  | Keratin-associated protein 3-1 | 0.68 | ns | <0.05 |
| KRTAP3-2  | Keratin-associated protein 3-2 | 0.65 | ns | <0.05 |
| KRT80     | Keratin, type II cytoskeletal 80 | 0.61 | <0.001 | <0.001 |
| KRT38     | Keratin, type I cuticular Ha8 | 0.59 | <0.05 | <0.01 |
| KRT35     | Keratin, type I cuticular Ha5 | 0.48 | <0.001 | <0.001 |
| KRT17     | Keratin, type I cytoskeletal 17 | 0.46 | <0.001 | <0.001 |
| KRTAP2-1  | Keratin-associated protein 2-1 | 0.42 | <0.01 | <0.001 |
| KRTAP24-1 | Keratin-associated protein 24-1 | 0.41 | ns | <0.05 |
| KRT34     | Keratin, type I cuticular Ha4 | 0.36 | <0.001 | <0.001 |
| KRT33B    | Keratin, type I cuticular Ha3-II | 0.36 | <0.001 | <0.001 |
| KRT31     | Keratin, type I cuticular Ha1 | 0.25 | <0.001 | <0.001 |
| KRT6B     | Keratin, type II cuticular 6B | 0.25 | <0.001 | <0.001 |
| KRT78     | Keratin, type II cytoskeletal 78 | 0.12 | <0.001 | <0.001 |

(8) Calcium binding proteins/AMPs

| Gene name | Protein name | Fold change cheek/PA | P value | q value |
|-----------|-------------|----------------------|---------|---------|
| CALML3    | Calmodulin-like protein 3 | 2.78 | <0.001 | <0.001 |
| S100A16   | Protein S100-A16 | 1.85 | <0.001 | <0.001 |
| CALML5    | Calmodulin-like protein 5 | 1.52 | <0.05 | <0.01 |
| S100A14   | Protein S100-A14 | 0.86 | ns | <0.05 |
| S100A8    | Protein S100-A8 | 0.60 | <0.001 | <0.001 |
| S100A9    | Protein S100-A9 | 0.49 | <0.001 | <0.001 |
| S100A7    | Protein S100-A7 (Psoriasin) | 0.48 | <0.001 | <0.001 |
| S100A3    | Protein S100-A3 | 0.08 | <0.001 | <0.001 |

(9) Glycoprotein folding protein

| Gene name | Protein name | Fold change cheek/PA | P value | q value |
|-----------|-------------|----------------------|---------|---------|
| CALR      | Calreticulin | 0.45 | ns | <0.05 |

(10) Annexins

| Gene name | Protein name | Fold change cheek/PA | P value | q value |
|-----------|-------------|----------------------|---------|---------|
| ANXA7     | Annexin A7 | 9.18 | <0.001 | <0.001 |
| ANXA11    | Annexin A11 | 5.09 | <0.05 | <0.01 |
| ANXA1     | Annexin A1 | 1.79 | <0.05 | <0.01 |

(11) Inflammation-associated proteins

| Gene name | Protein name | Fold change cheek/PA | P value | q value |
|-----------|-------------|----------------------|---------|---------|
| IL1RN     | Interleukin-1 receptor antagonist protein | 107.67 | <0.001 | <0.001 |
| IGHA1     | Ig alpha-1 chain C region | 17.11 | <0.001 | <0.001 |
| SLURP1    | Secreted Ly-6/PLAUR-related protein 1 | 5.64 | <0.001 | <0.001 |
| LYPD3     | Ly6/PLAUR domain-containing protein 3 | 4.01 | <0.001 | <0.001 |
| IGLC3     | Ig lambda-3 chain C regions | 1.92 | <0.05 | <0.01 |
| IGKC      | Ig kappa chain C region | 1.59 | <0.001 | <0.001 |
| IL36G     | Interleukin-36 gamma | 1.49 | <0.001 | <0.001 |
| HLA-DR1    | HLA class II histocompatibility antigen, DR alpha chain | 1.20 | ns | ns |
| IGHI4     | Ig gamma-4 chain C region | 0.70 | ns | <0.05 |
| IGH1G1    | Ig gamma-1 chain C region | 0.19 | <0.001 | <0.001 |

(12) Microtubules/tubulins

| Gene name | Protein name | Fold change cheek/PA | P value | q value |
|-----------|-------------|----------------------|---------|---------|
| TUBA4A    | Tubulin alpha-4A chain | 6.66 | <0.001 | <0.001 |
| TUBA1B    | Tubulin alpha-1B chain | 1.37 | <0.05 | <0.01 |

(13) Membrane trafficking/myosin

| Gene name | Protein name | Fold change cheek/PA | P value | q value |
|-----------|-------------|----------------------|---------|---------|
| MYH9      | Myosin-9 | 2.83 | <0.001 | <0.001 |
| MYH14     | Myosin-14 | 2.12 | <0.05 | <0.01 |
| TPM3      | Tropomyosin alpha-3 chain | 1.19 | ns | <0.05 |
| TPM4      | Tropomyosin alpha-4 chain | 0.52 | ns | <0.05 |
| CLTC      | Clathrin heavy chain 1 | 0.29 | <0.05 | <0.01 |

(14) Actin binding proteins/cytoskeleton

| Gene name | Protein name | Fold change cheek/PA | P value | q value |
|-----------|-------------|----------------------|---------|---------|
| GSN       | Gelsolin | 60.67 | <0.001 | <0.001 |
| AHNK      | Neuroblast differentiation-associated protein AHNK | 44.29 | <0.001 | <0.001 |
| PLEC      | Plectin | 31.90 | <0.001 | <0.001 |
| CFL1      | Caspase-11 | 9.07 | <0.001 | <0.001 |
| ACTN4     | Alpha-actin-4 | 5.05 | <0.001 | <0.001 |
| FLNB      | Filamin-B | 3.66 | <0.001 | <0.001 |
| LCP1      | Plastin-2 | 1.32 | <0.05 | <0.01 |
| ACTG1     | Actin, cytoplasmic 2 | 0.36 | <0.001 | <0.001 |
Table I (continued)

| Gene name | Protein name | Fold change cheek/PA | P value | q value |
|-----------|--------------|----------------------|---------|---------|
| *(15) Anti-microbial proteins* | | | | |
| RNH1 | Ribonuclease inhibitor | 13.39 | <0.001 | <0.001 |
| DCD | Dermcidin | 0.76 | ns | <0.05 |
| LYG2 | Lysozyme g-like protein 2 | 0.61 | ns | <0.05 |
| *(16) Proteasome* | | | | |
| PSMB1 | Proteasome subunit beta type-1 | 6.14 | <0.001 | <0.001 |
| PSMB3 | Proteasome subunit beta type-3 | 5.78 | <0.001 | <0.001 |
| PSMA2 | Proteasome subunit alpha type-2 | 5.14 | <0.001 | <0.001 |
| PSMA5 | Proteasome subunit alpha type-5 | 2.47 | <0.001 | <0.001 |
| PSMA1 | Proteasome subunit alpha type-1 | 2.12 | <0.001 | <0.001 |
| PSMA3 | Proteasome subunit alpha type-3 | 1.60 | <0.001 | <0.001 |
| PSMA6 | Proteasome subunit alpha type-6 | 1.42 | <0.001 | <0.001 |
| PSMA7 | Proteasome subunit alpha type-7 | 1.22 | ns | <0.05 |
| RPS27A | Ubiquitin-40S ribosomal protein S27a | 1.22 | ns | <0.05 |
| *(17) Protein folding control* | | | | |
| HSPA5 | 78 kDa glucose-regulated protein | 3.66 | <0.001 | <0.001 |
| *(18) Antioxidant related proteins* | | | | |
| GSTP1 | Glutathione S-transferase P | 7.78 | <0.001 | <0.001 |
| SELENBP1 | Selenium-binding protein 1 | 3.65 | <0.001 | <0.001 |
| GLRX | Glutaredoxin-1 | 3.20 | <0.001 | <0.001 |
| PRDX2 | Peroxiredoxin-2 | 3.14 | <0.001 | <0.001 |
| ME1 | NADP-dependent malic enzyme | 2.37 | <0.001 | <0.001 |
| GGH | Gamma-glutamyl hydrolase | 3.20 | <0.001 | <0.001 |
| ALDH7A1 | Alpha-aminoadipic semialdehyde dehydrogenase | 1.94 | <0.05 | <0.01 |
| PRDX6 | Peroxiredoxin-6 | 1.59 | ns | ns |
| SOD1 | Superoxide dismutase [Cu-Zn] | 1.38 | ns | <0.05 |
| PARK7 | Protein DJ-1 | 1.35 | <0.05 | <0.01 |
| TXN | Thioredoxin | 1.17 | <0.05 | <0.01 |
| PRDX1 | Peroxiredoxin-1 | 1.15 | ns | <0.05 |
| CAT | Catalase | 0.85 | ns | <0.05 |
| *(19) Heat-shock proteins* | | | | |
| HSPA1A | Heat-shock 70-kDa protein 1A/1B | 1.98 | <0.01 | <0.001 |
| HSPB1 | Heat-shock protein beta-1 | 1.23 | <0.05 | <0.01 |
| HSP90AA1 | Heat-shock protein HSP 90-alpha | 0.06 | <0.001 | <0.001 |
| *(20) Signal transduction proteins* | | | | |
| SFN | 14-3-3 protein sigma | 19.83 | <0.001 | <0.001 |
| NPM1 | Nucleophosmin | 4.34 | <0.001 | <0.001 |
| C1QBP | Complement component 1 Q subcomponent-binding protein, mitochondrial | 4.29 | <0.001 | <0.001 |
| YWHAZ | 14-3-3 protein zeta/delta | 2.36 | <0.001 | <0.001 |
| CREG1 | Protein CREG1 | 2.13 | <0.001 | <0.001 |
| BANF1 | Barrier-to-autointegration factor | 1.80 | <0.05 | <0.01 |
| LMNA | Prelamin-A/C | 1.36 | ns | <0.05 |
| CAMK2D | Calcium/calmodulin-dependent protein kinase type II subunit delta | 1.32 | ns | ns |
| ECM1 | Extracellular matrix protein 1 | 1.17 | <0.01 | <0.001 |
| GSDMA | Gasdermin-A | 1.17 | ns | <0.05 |
| KPRP | Keratinocyte proline-rich protein | 0.90 | ns | <0.05 |
| *(21) SC lipid metabolism/transport proteins/enzymes* | | | | |
| PLBD1 | Phospholipase B-like 1 | 4.29 | <0.001 | <0.001 |
| GBA | Glucosylceramidase | 3.75 | <0.001 | <0.001 |
| LCN1 | Lipocalin-1 | 2.00 | <0.001 | <0.001 |
| ASAH1 | Acid ceramidase | 1.50 | <0.01 | <0.001 |
| PSAPL1 | Proactivator polypeptide-like 1 | 1.18 | ns | <0.05 |
| APOD | Apolipoprotein D | 0.59 | <0.001 | <0.001 |
| FABP5 | Fatty acid-binding protein, epidural | 0.57 | <0.001 | <0.001 |
| PSAP | Prosaposin | 0.32 | <0.001 | <0.001 |
| HSD17B4 | Peroxisomal multifunctional enzyme type 2 | 0.26 | <0.001 | <0.001 |
| *(22) Nucleotide metabolism* | | | | |
| TYMP | Thymidine phosphorylase | 2.31 | <0.001 | <0.001 |
| IMPA2 | Inositol monophosphatase 2 | 0.62 | <0.001 | <0.001 |
| CDA | Cytidine deaminase | 0.21 | <0.001 | <0.001 |
| *(23) Secreted proteins* | | | | |
| LGALS7 | Galectin-7 | 1.13 | ns | <0.05 |
| LGALS3 | Galectin-3 | 0.68 | ns | <0.05 |
were decreased on the cheek. A variety of cytoskeletal proteins were increased especially gelsolin (>60×).

**Proteasome markers**

All proteasome subunit proteins alpha and beta types were upregulated on the cheek except proteasome subunit beta type-6 (0.44×) was down regulated.

**Antioxidant markers**

All identified antioxidant markers were upregulated on the cheek except catalase (0.85×). These included glutathione S transferase P (7.78×), selenium-binding protein 1 (3.85×), glutaredoxin (3.30×), thioredoxin (1.17×), peroxiredoxin 1 (1.15×), 2 (3.14×) and 6 (1.59×), Cu-Zn superoxide dismutase (1.38×) and protein DJ-1 (1.35×).

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**Table 1** (continued)

| Gene name | Protein name | Fold change cheek/PA | P value | q value |
|-----------|--------------|-----------------------|---------|---------|
| (24) Lysosomal proteins | | |
| ACPB | Prostatic acid phosphatase | 14.15 | <0.001 | <0.001 |
| C11orf54 | Ester hydrolase C11orf54 | 9.40 | <0.001 | <0.001 |
| LAMP1 | Lysosome-associated membrane glycoprotein 1 | 6.96 | <0.001 | <0.001 |
| NEU2 | Sialidase-2 | 2.74 | <0.001 | <0.001 |
| GAA | Lysosomal alpha-glucosidase | 2.65 | <0.01 | <0.001 |
| LAMP2 | Lysosome-associated membrane glycoprotein 2 | 1.62 | <0.001 | <0.001 |
| CTSA | Lysosomal protective protein | 1.30 | <0.05 | <0.01 |
| GM2A | Ganglioside GM2 activator | 0.50 | <0.001 | <0.001 |
| (25) Glycolysis/Krebs cycle enzymes | | |
| MDH2 | Malate dehydrogenase, mitochondrial | 16.28 | <0.001 | <0.001 |
| Eno1 | Alpha-enolase | 6.31 | <0.001 | <0.001 |
| TPI | Trisphosphate isomerase | 6.01 | <0.001 | <0.001 |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase | 2.73 | <0.001 | <0.001 |
| PKM | Pyruvate kinase PKM | 1.21 | ns | <0.05 |
| ATP5B | ATP synthase subunit beta, mitochondrial | 1.68 | ns | <0.05 |
| (26) Serum diffusion markers | | |
| VCP | Transitional endoplasmic reticulum ATPase | 31.86 | <0.001 | <0.001 |
| ALB | Serum albumin | 0.60 | <0.001 | <0.001 |
| (27) Protein synthesis | | |
| SSRI1 | Translocon-associated protein subunit alpha | 4.43 | <0.001 | <0.001 |
| EIF6 | Eukaryotic translation initiation factor 6 | 3.00 | <0.001 | <0.001 |
| EEF2 | Elongation factor 2 | 0.44 | <0.01 | <0.001 |
| (28) Aminoacid/purine/pyrimidine metabolism | | |
| GLUL | Glutamine synthetase | 3.78 | <0.001 | <0.001 |
| NUDT5 | ADP-sugar pyrophosphatase | 3.19 | <0.001 | <0.001 |
| GPT | Alanine aminotransferase 1 | 1.95 | ns | <0.05 |
| (29) Retinoid metabolism | | |
| SDR9C7 | Short-chain dehydrogenase/reductase family 9C member 7 | 1.52 | <0.01 | <0.01 |

**Figure 2** Comparison of fold increase of proteins, grouped in protein families, of stratum corneum samples on photodamaged cheek versus photoprotected post-auricular sites in Caucasian women. Values >log 1 (red line) correspond to an increased and values <log 1 to a decreased protein level on cheek. Values above and below the green lines correspond to proteins that are up-regulated >2 fold or down-regulated <0.5 fold on the cheek, respectively.
Heat-shock proteins

Two heat-shock proteins were identified to be upregulated on the cheek (70 kDa protein 1A/1B: 1.98 × and beta-1: 1.23 ×) and down-regulated on the cheek (HSP 90-alpha, 0.06 ×).

Signal transduction markers

Several signal transduction proteins were upregulated on the cheek especially the 14–3–3 proteins (sigma otherwise known as stratifin (19.8 ×) and zeta/delta (2.36 ×)).

SC lipid biochemical markers

SC lipid metabolism enzymes and transport proteins were found to be upregulated on the cheek including phospholipase B-like 1 (4.29 ×), glucosylcerbrosidase (3.75 ×), acid ceramidase (1.50 ×), whereas others were downregulated such as fatty acid-binding protein 5 (0.57 ×) and prosaposin (0.32 ×).

Anti-microbial peptides

Dermcidin (0.76 ×) and lysozyme g-like protein 2 (0.61 ×) were down regulated on the cheek, whereas ribonuclease inhibitor 1 (13.4 ×) was increased.

Lysosomal markers

Several lysosomal markers were increased on the cheek indicative of increased lysosomal activity and possibly lamellar granule biogenesis.

Intermediary metabolism enzymes

Increased levels of glycolytic enzymes were observed on the cheek as well as those involved in amino acid, purine and pyrimidine metabolism, especially malate dehydrogenase (16.3 ×). Equally, protein synthesis markers were increased on the cheek such as translocase-associated protein subunit alpha (4.43 ×) and eukaryotic translation initiation factor 6 (3.00 ×), whereas elongation factor 2 was decreased (0.44 ×) on the cheek.

Protein folding markers

Calreticulin levels (0.45 ×) were decreased on the cheek.

Discussion

We have previously reported on the differences in selected skin physiology measurements, corneocyte morphology and certain SC biochemistry markers between photoexposed cheek and photoprotected PA site [12, 28, 29]. To understand the relevance of these differences with other proteins in female Caucasian skin, and with the aim of identifying new molecular targets for treating photodamaged skin, we chose to use untargeted label-free mass spectrometry proteomics. Here, we discuss the differences in the photodamaged SC corneome to those individual markers that we previously reported.

Previous analysis indicated that reduced keratinocyte differentiation and SC maturation occurred on the photodamaged cheek compared with the PA site, and consistent with this, we observed decreased keratin 1 and 10 levels in our proteomic approach. Cavusoglu et al. [21] also observed decreases in keratins 1, 10 and 17 in subjects with dandruff. In contrast, increased levels of the normally basal cell layer expressed keratins 5 and 14 were observed. Also elevated levels of the hyperproliferation marker keratin 16 were also seen consistent with the results of Proksch et al. [30]. However, unlike the work of Rice et al. [31] on forehead samples, keratin 6A was not observed in the cheek samples and keratin 6B levels were reduced. These and changes in other keratins potentially indicate alterations to the architecture and biomechanical properties of the corneocyte keratin matrix in the photodamaged cheek.

Potentially impaired CE biomechanics were also apparent with the differential expression of certain CE protein markers and associated CE maturation enzymes. Consistent with this, increased levels of SPRR 1B and 2B while decreased levels of SPRR 1A and 2E were found. SPRRs have a specialized role in the CE as they function as cross-bridging agents, which either interconnect or adjoin other CE precursor proteins and affect the biomechanical properties of the CE [31]. However, other CE proteins were also found to be decreased namely cornifilin, suprabasin and especially loricrin together with skin-specific protein 32 or LEF7. Loricrin, also, is cross-linked to itself and other SPRR proteins. These are then cross-linked to the perioplakin–involutin–enoplakin scaffold. Subsequently, filaggrin, S100 family proteins and finally the late CE proteins, are attached [31]. Although we observed increased levels of involucrin on the cheek, we believe this is related to the skin xerosis [30]. The differences in proteome composition in the photodamaged cheek further indicate potential changes to the biomechanical properties of the CE.

Rinnerthaler et al. [32] reported some similar changes with intrinsic ageing to those that we found in our cheek samples: decreased loricrin and SPRR 1A, 2E, S100A7, A8 and A9 levels. However, unlike the study of Trzeciak et al. [33], we observed decreased levels of SPRR1A compared with their recorded increases in subjects with atopic dermatitis. Nevertheless, studying the hand eczema proteome, Molin et al. [16] observed some similar changes to those reported here, that is increased SPRR2B, but also observed the opposite for other proteins, for example, reduced kallikrein 5 and increased S100A7-9 and 11. Like our study, decreased levels of suprabasin were also observed in subjects with dandruff. Any differences in protein levels to our findings probably relate to the differences in (patho)physiology among the different conditions but also possibly differences in extraction methodologies.

Inconsistent with our previous results of reduced CE maturation, levels of peptide sequences for transglutaminases 1 and 3 were found to be increased in the cheek samples [12]. Reduced transglutaminase activities and reduced mass levels are reported in the SC of subjects with post-menopausal dry leg skin, sensitive facial skin conditions, dandruff and xerosis [20, 21, 34, 35]. However, it is possible that the transglutaminases may not be adequately activated or their substrates prepared satisfactorily for their use. Cathespin D and calpain-1 subunits levels, enzymes that activate transglutaminases, were also found to be elevated on the cheek so these differences do not explain the CE phenotype we previously observed. However, ultraviolet radiation (UVR) leads to increased transglutaminase expression [36, 37]. Transglutaminases cross-link CE proteins but they also help to increase the hydrophobicity of the envelope through esterification with ceramides [31]. These ceramides are derived from the very long chain linoleoyl omega-hydroxy ceramides (CER [E05]) [11].
However, the linoleoyl moiety is first oxidized by two lipoxygenases, 12R-LOX and eLOX3, before being hydrolysed and then the free omega-hydroxy ceramides being utilized by transglutaminase 1 [38]. Levels of eLOX3 were found not to change between the cheek and the PA samples, but the levels of 12R-LOX were dramatically lowered. As a result, the attachment of ceramides to the CE by transglutaminase 1 is expected to be decreased and is consistent with the findings of a greater proportion of Nile-red-deficient immature CEs previously reported in these samples [12]. This study shows for the first time the relevance of the reduced levels of 12R-LOX in photodamaged SC.

Hirao has demonstrated that the CEs have the potential to mature, increase their hydrophobicity, in the presence of transglutaminase and associated CE protein and lipid components [39]. Nevertheless, immature CEs are also biomechanically weaker [35]. It is also known that CEs are fragile to a sonication stress when there is a loricrin or 12R-LOX deficiency [40,41]. The potential changes in CE biomechanics of the cheek and PA samples are currently under investigation.

Corneodesmolyis is also usually associated with CE maturation and ultimately desquamation [42]. However, in dry skin conditions, non-peripheral corneodesmosomal proteins are not effectively proteolysed which also contributes to the immature CE phenotype and a thicker ‘stratum compactum’. Even though the SC is thinner on the face, a ‘stratum compactum’ phenotype is also apparent indicating the presence of non-peripheral corneodesmosomes [12,43]. Consistent with a lack of CE maturation and changes in desquamation, differences in corneodesmosomal markers were observed between the two body sites. Increased levels of intracorneocyte corneodesmosomes: epilakin, junctional plakoglobin, plakophilin-1 and protein POF1B were seen as well as increased levels of CE membrane spanning proteins such as desmoglein 1, corneodesmin and desmocollin 3. Similarly, Delattre et al. [20] also observed increased levels of desmoglein 1, plakoglobin and corneodesmin, whereas surprisingly desmoglein 1 levels were reported to decline in subjects with dandruff. Increased corneodesmosomal markers can be associated with a thickening of the SC but we had already reported a thinning of the SC in the cheek vs the PA sites [12]. As a result, there must be increased levels of desquamatory enzymes on the cheek leading to an accelerated shedding of ‘stratum disjectum’ while retaining a stratum compactum phenotype.

Indeed, dramatically higher levels of the desquamatory-associated serine protease kallikrein 7 were observed in the cheek samples (4.27 x) as well as kallikrein 10 (2.39 x) which is consistent with our previously reported enzyme activity data [29]. Moreover, increases in levels of calpastatin L2 and D were observed which have also been associated with desquamation [43]. Caspase-14 levels were higher on the cheek and this protease is also known to be localized to the corneodesmosome and may be implicated in this process as well. The largest increase in mass levels of potential desquamatory proteases was observed for Saspase (5.73 x). However, these activities may or may not be counterbalanced by protease inhibitors [43]. With regard to enhancing kallikrein 7 activity, levels of alpha-2 macroglobulin-like protein 1 and serpin A12 (0.30 x) were decreased. Cystatin-A (stefin-1) levels were decreased which may enhance cathepsins B, H and L activities. Increased levels of Serpin B13 were also observed on the cheek and may also inhibit cathepsin L, calpain-1 and some serine proteases. Nevertheless, our previous work indicated increased desquamation on the cheek, and as a result, the protease-anti-protease balance must be in favour of proteolysis on the cheek [12,29]. Increased levels of puromycin-sensitive aminopeptidase may be involved in general proteolysis pathways which was the major protease increased on the cheek samples, but its role in corneodesmolyis is unknown. In addition to their effects on controlling endogenous proteases, the protease inhibitors identified may be part of the immune system to control microflora proteases [43].

Both Serpin B3 (SCCA1) and serpin B4 (SCCA2) were found to be increased in barrier compromised conditions [44,45]. Serpin B3 is known to inhibit cysteine proteases such as cathepsin L2, whereas serpin B4 inhibits serine and cysteine proteases but increases in their levels may be a reflection of SC barrier abrogation [46,47]. Katagiri et al. [45] showed that upregulation of serpin B3 is associated with barrier disruption in psoriasis and following UV irradiation. Yamane et al. [44] also found increased serpin B4 levels in the SC of atopic patients. Moreover, Sivaprasad et al. [48] also reported that increased TEWL was attenuated by an absence of serpin B3. Broccardo et al. [14] also found increased serpin B3 in atopic subjects. Overexpression of serpin B3 also promotes epithelial proliferation [14,49]. In keratinocytes, Serpin B3 binds to c-Jun N-terminal kinase and upon its activation is translocated to the nucleus to play a role in UV-induced apoptotic cell death. In parakeratotic corneocytes, it can be shown to be colocalized with undigested nuclei and it may cause poor barrier function by disrupting the internal structure of the corneocytes [45].

Although facial SC contains significantly lower levels of NMF compared with other body sites, we have shown that there are no differences in PCA levels between the cheek and PA sites [5,6,29]. However, UV irradiation is known to increase the expression of filaggrin and transglutaminase in mouse skin [36,37]. Despite this finding, filaggrin (and transglutaminase 1 together with desmoplakin-1 and desmoplakin-2) levels are reported to be lower at the bottom of a wrinkle in human skin [8]. In contrast to this latter finding, we also observed increased levels of filaggrin with slight increases in the levels of filaggrin-2 in the cheek compared with the PA site. Thus, filaggrinolysis appears to be less effective on the cheek compared with the PA site. In keratinocytes, Serpin B3 binds to c-Jun N-terminal kinase and upon its activation is translocated to the nucleus to play a role in UV-induced apoptotic cell death. In parakeratotic corneocytes, it can be shown to be colocalized with undigested nuclei and it may cause poor barrier function by disrupting the internal structure of the corneocytes [45].
shown by Naeem et al. [55], reduced cathepsin H activities have been observed in conditions where increased mTOR activity (increased regulatory associated protein of mTOR/raptor) was observed, for example, atomic dermatitis resulting in reduced filaggrin processing. Choi et al. [56] have also reported increased mTOR complex 2/raptor-independent companion of mTOR expression following UVR which leads to associated nuclear factor kappa-light-chain enhancer of activated B cells/NF-κB activation and thereby may reduce filaggrin expression. Increased mTOR signalling pathways following UV irradiation has also been documented [57]. Although mTORC1 and mTORC2 regulate epidermal barrier formation their over stimulation appears to be a problem for filaggrinolyis [58]. Although we did not detect specific mTOR-associated proteins, increased mTOR activity results in reduced autophagy, a process known to be essential for keratinocyte differentiation [59,60]. However, certain proteins are increased on the cheek indicative of enhanced mTOR activity, for example, increased levels of the adapter signalling protein, straflin (14-3-3ε) were observed on the cheek [61]. Raaby et al. [62] reported increased straflin expression in psoriasis and contact dermatitis, and its levels are also reported to be increased following UV irradiation leading to decreased proflaggrin expression [61]. Thus, increased mTOR activity is likely in the cheek samples.

Increased filaggrinolyis is also known to occur at the depth in the SC where increased transglutaminase activity occurs [42]. This coincides with changes in the CE phenotype. Presumably increased 12R-LOX activities occur at the same depth. Thus, the reduced 12R-LOX levels we observed on the cheek may also contribute to a reduced proflaggrin processing. Indeed, this has been shown in 12R-LOX knockout mouse models [63]. S100 proteins are small calcium binding proteins, but their roles in skin are largely unknown [64]. Increased levels of S100A16 were observed in the cheek which is normally just found in spinous layers that may be indicative of poor differentiation. Equally, reduced levels of S100A3, A7, A8, A9 and A14 were seen. S100A7 is normally associated with the SC, whereas S100A8, A9 and A14 are normally spinous or granular layer proteins. In contrast, increased levels of S100A8 and A9 were observed in subjects with dandruff and psoriasis [64]. The heterodimeric complex of S100A8 and A9 (calprotectin) functions as anti-microbial peptide and as we observed decreased levels, this may indicate that the cheek might be more susceptible to anti-microbial attack. Likewise, reduced levels of other anti-microbial peptides such as dermcidin and lysozyme g-like protein 2 might contribute to the reduced innate immunity [43]. Similarly, reduced cystatin-A will reduce innate immunity, whereas increases in other serpins will be beneficial. Increases in RNA inhibitor (RNIH1) may also prevent RNAs from functioning [65]. The role of S100A3 and A14 is unknown. S100A7 which also binds epidermal fatty acid protein (E-FABP) suggests it has a role in epidermal lipid barrier formation and its differentiation potential [66].

Epidermal fatty acid-binding protein, E-FABP/FABP5, is actively overexpressed in hyperproliferative diseases such as psoriasis [67]. However, in keratinocytes lacking FABP5 decreased expression of loricrin, involucrin and keratin 1 was observed [68]. This calycin delivers fatty acids intracellularly to peroxisome proliferator receptors [69]. We observed reduced FABP5 levels which may be contributing to the poor differentiation status of the UV exposed cheek SC.

Similar to results of subjects with dandruff, we observed increased calmodulin-like proteins 3 and 5 on photodamaged cheek [21]. These changes have also been observed in subjects with atomic dermatitis and psoriasis [70,71]. Calmodulin-like proteins interact with straflin and control some of the late differentiation genes [72]. These proteins may be increased to try and improve keratinocyte differentiation, but clearly, this is not occurring on the cheek. These proteins also upregulate myosins required for cell motility such as myosin-10 [73]. We observed increases in myosin-9 and myosin-14 possibly being related to a hyperproliferative condition.

Although there is redundancy in their function, we observed increases in the levels of annexin 1, 7 and 11 indicating reduced keratinocyte differentiation potential [74]. Annexin A2 is increased in dry skin, but together with annexin A3, it was reported to be decreased in dandruff, whereas A1 was increased [20,21]. Annexin 7 is reported to mobilize calcium from inositol-1,4, 5-triphosphate-sensitive endoplasmic reticulum stores [75]. Gelsolin, an actin binding protein, alpha-actinin and filamin are usually less abundant in suprabasal cells compared with basal keratinocytes [76]. Thus, our finding of greatly increased levels of gelsolin on the cheek indicated a hyperproliferative epidermis. Interestingly, increased plasma gelsolin levels are known in atopic dermatitis [77]. This hyperproliferation is also indicated by the increases levels of glycolytic enzymes on the cheek. Broccardo et al. [14] also found increased x-enolase and triosephosphate isomerase in atopic dermatitis indicative of increased epidermal hyperproliferation.

Secreted Ly-6/uPAR-related protein 1 is a selective antagonist of the nicotinic acetylcholine receptor that reduces keratinocyte proliferation [78]. However, it is also reported to be pro-apoptotic increasing caspase 3 and transglutaminase 3 levels [79]. We observed increased levels so the skin may be trying to mitigate the excessive proliferation on the cheek and could account for the increases in levels of transglutaminase 3 we observed. However, transglutaminases may be increased to protect against photodamage [80].

Clearly, the cheek is undergoing UV-induced oxidative stress. Although IL-1 receptor antagonist protein can be associated with keratinocyte differentiation, we believe the increased levels together with increases in other inflammatory proteins were indicative of UV-induced inflammation on the cheek [81, 82].

To counteract this, the skin will attempt to mitigate any inflammation and upregulate its antioxidant systems and we observed increases in many antioxidant proteins on the cheek. However, we observed reduced catalase levels. Similar to our cheek samples, Ryu et al. [83] reported increased expression of glutathione S-transferase 1 and peroxiredoxin 2 in psoriatic skin as was also shown for dandruff SC. Declercq et al. [84] have previously reported decreased catalase in the SC in the summer months of the year clearly as a result of UV stress similar to our samples. Protein deglycase DJ-1 was also increased on the cheek which is known to be elevated in UV-induced skin damage [85]. Increased levels of malate dehydrogenase were also observed on the cheek which reportedly increases the levels of the cellular antioxidant reduced nicotinamide adenine dinucleotide whereas gamma-glutamyl hydrolyase levels were also increased that is involved in folate transport [86,87]. An increased level of thioredoxin was also observed presumably to control the redox state of the cells [88]. HSP27 is increased in dry skin, but we observed HSP90α to be decreased in our samples indicating that there may be reduced cell mobility [89]. HSP70/1A levels are higher in darker skin phenotypes and our increases may be related to an increased tanning response on the cheeks [90]. Overall, the skin is trying to defend itself against further oxidative stress. mTOR activity may also be influencing the expression of these proteins as Song et al. [91] have shown that autophagy inhibition using
keratinocytes with inactivated autophagy-related 7 gene underwent more oxidative stress to an external challenge. Thus, over activation of the mTOR pathway, which inhibits autophagy, will likely have the same effects and may result in some of the changes we have observed in other proteins.

Calreticulin levels were decreased on the cheek. Calreticulin is an intracellular chaperone that is critical for the proper folding and transport of proteins through the endoplasmic reticulum and recruits cells during wound healing [92]. The lack of this chaperone leads to increased endoplasmic reticulum (ER) stress which can activate the unfolded protein response (UPR). However, ER signalling leading to an elevated UPR in response to UVB has been reported but clearly increased levels of unfolded proteins can over activate this mechanism [93]. A mild UPR has been reported to increase the innate immunity β-defensin anti-microbial peptides through terminal differentiation [94]. However, in the cheek samples, we observed decreased levels of anti-microbial peptides and increased ribonuclease inhibitor levels which again indicate poor keratinocyte differentiation possibly due to a deficiency in the UPR.

Usually, proteasomes degrade unfolded proteins. However, we observed a proteasome insufficiency in the cheek samples because of reduced levels of one of the essential 20S proteasome subunits, namely PSMB6 [95]. Impairment of proteasome function on UV irradiation of keratinocytes has been reported [96]. Also, proteasome inhibitor treatments lead to reductions in calreticulin [97]. The likely impaired proteasome functioning in the cheek samples may be the cause of the reduced calreticulin and thereby reduced UPR.

Our analysis of cheek samples indicates poor cross-talk between the keratinocyte ER UPR-proteasome network–autophagy mechanisms and mTOR. UV irradiation clearly leads to oxidation of cellular proteins, and as a result, control of proteostasis is important. Keratinocytes in particular need to control protein overload in the ER; otherwise, this will lead to ER stress due to hydrophobic residues exposed from unfolded or misfolded proteins interacting with cellular membranes. This normally leads to an UPR to mitigate these problems and damaged proteins are translocated to the cytosol to be degraded by proteasomes. Autophagy is another route to disposing of damaged proteins as well as organelle degradation. mTOR is also important in these processes as it negatively and positively influences autophagy and the proteasome mechanisms. Nevertheless, these processes are important for terminal differentiation. However, when these mechanisms are under- or overactivated as appears on female Caucasian cheeks, disturbed or poor keratinocyte differentiation events occur [98]. The UPR process can be reduced in diseases such as psoriasis which may lead to reduced filaggrin/NMF levels, whereas it can be over activated in disorders such as keratosis linearis with ichthyosis congenital and keratoderma syndrome (KLICK) that results from a deficiency of the proteasome maturation protein (POMP) leading to proteasome insufficiency which in turn leads to a problem with profilaggrin processing and the later differentiation events [99]. Activation of mTOR also occurs in many dermatological disorders, and the resulting blockage of autophagy leads to poor differentiation events [100]. The UPR response is also believed to activate autophagy, but in our clinical samples, poor differentiation was still apparent, and as a result, the mTOR response may be over riding any UPR signalling [101]. Thus, the UPR and proteasome mechanisms appear to be reduced on the cheek, whereas mTOR activity is increased.

It should be borne in mind, however, that although a tremendous amount of information has been gathered on the SC corneome, proteomics has its limitations. In this study, we have not evaluated post-translational processing protein events, and this approach does not provide information on the activity and location of the proteins identified. New target proteins need to be validated with activity and localization measurements.

In conclusion, we have observed a complex SC corneome in photodamaged skin of the face that shows some differences and similarities to that reported for dandruff, dry skin and skin diseases (atopic dermatitis, psoriasis). These similarities or differences may

![Figure 3](https://i.imgur.com/3GJG7.png)

**Figure 3** Schematic of relevant stratum corneum maturation protein changes between stratum corneum samples of photodamaged cheek and photoprotected post-auricular sites in photodamaged Caucasian women.
relate to the underlying diversity in the (patho)physiology of these disparate conditions and/or differences in methodology or selection of body site. Nevertheless, this study broadens the catalogue of the proteins found within the SC and especially in photodamaged sites. Relating to our previously published findings on SC of cheek compared with that of the PA site, clear differences in corneocyte and CE protein components together with CE maturations enzymes were observed and diametrically opposed changes in the protease–anti-protease balance were measured for corneodesmolyis and flaggrinolysis as summarized in Fig 3. From these findings, we expect proteolysis to be favoured for corneodesmosis causing premature desquamation on the face but protease inhibition for flaggrinolysis on the cheek compared with the PA site. Moreover, 12R-LOX, an upstream CE maturation enzyme was identified as a key new target for photodamaged skin as its levels were lowered. This enzyme would normally provide the substrate for transglutaminases to hydrophobe the CE. The levels of transglutaminases were unchanged in this study. These enzyme and structural CE protein changes indicate a corneocyte scaffold disorder in photodamaged facial skin. Furthermore, this study provides insights into the potential molecular mechanisms involved in SC maturation/keratinocyte differentiation and their aberration in photodamaged skin. In this respect, analysis of proteins indicated a perturbed cross-talk of keratinocyte ER UPR-proteasome network–autophagy mechanisms with an enhancement of mTOR activity in keratinocytes. Changes in the levels and activities of these proteins need further validation as only peptide fragments have been annotated. Nevertheless, the biochemical diversity of the cheek and PA SC corneome identified in this study has highlighted new molecular pathways/targets that need to be influenced to better treat photodamaged skin.

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Conflicts of interests and disclosures

RV and RS are employees of DSM. AVR is a consultant to DSM. BS and JMM report no conflicts of interest.

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Supporting Information

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

Table SI. Documentation of all proteins observed between stratum corneum samples of photodamaged cheek and photoprotected post-auricular sites in Caucasian women.

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