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RESEARCH ARTICLE

Early onset of senescence and imbalanced epidermal homeostasis across the decades in photoexposed human skin: Fingerprints of inflammaging

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Abstract

Inflammaging is a theory of ageing which purports that low-level chronic inflammation leads to cellular dysfunction and premature ageing of surrounding tissue. Skin is susceptible to inflammaging because it is the first line of defence from the environment, particularly solar radiation. To better understand the impact of ageing and photoexposure on epidermal biology, we performed a system biology-based analysis of photoexposed face and arm, and photoprotected buttock sites, from women between the ages of 20s to 70s. Biopsies were analysed by histology, transcriptomics, and proteomics and skin surface biomarkers collected from tape strips. We identified morphological changes with age of epidermal thinning, rete ridge pathlength loss and stratum corneum thickening. The SASP biomarkers IL-8 and IL-1RA/IL1-α were consistently elevated in face across age and *cis/trans*-urocanic acid were elevated in arms and face with age. In older arms, the DNA damage response biomarker 53BP1 showed higher puncti numbers in basal layers and epigenetic ageing were accelerated. Genes associated with differentiation and senescence showed increasing expression in the 30s whereas genes associated with hypoxia and glycolysis increased in the 50's. Proteomics comparing 60's vs 20's confirmed elevated levels of differentiation and glycolytic-related proteins. Representative immunostaining for proteins of differentiation, senescence and oxygen sensing/hypoxia showed similar relationships. This system biology-based analysis provides a body of evidence that young photoexposed skin is undergoing inflammaging. We propose the presence of chronic inflammation

Abbreviations: 53BP1, p53-binding protein 1; ALDOA, aldolase A; ALOX12B, arachidonate 12-lipoxygenase,12R; CALML3, calmodulin-like protein 3; CAPN1, calpain 1; CDKN2A, cyclin-dependent kinase inhibitor 2A; CRYAB, alpha-crystallin B chain; CSTB, cystatin B; CXCR2, cytokine receptor type 2/IL8RB; DEJ, dermal-epidermal junction; FLG, filaggrin; HBA, haemoglobin-α; HBB, haemoglobin-β; HIF1α, hypoxia-inducible factor 1, subunit alpha; HMOX1, haem oxygenase 1; IGF1R, insulin-like growth factor 1 receptor; IL-1RA, interleukin-1 receptor antagonist; IL-1α, interleukin-1α; IL-8, interleukin-8; INV, involucrin; KDM3A, lysine demethylase 3A; KDM5A, lysine demethylase 5A; KLF9, Krüppel-like factor 9; KRT14, keratin 14; KRT2, keratin 2; LCE2C, late cornified envelope 2C; LCM, laser capture microdissection; LDHA, lactate dehydrogenase A; LOR, loricrin; mTOR, mammalian target of rapamycin; PGM1, phosphoglucomutase 1; RBL2, retinoblastoma-like protein 2; SASP, senescence-associated secretory phenotype; SIRT1, sirtuin 1; SLC7A11, cystine/glutamate antiporter; SPINK5, serine protease inhibitor Kazai-type 5; SPRY2, Sprouty homologue 2; UEA-1, *Ulex Europaeus*-I Lectin.

in young skin contributes to an imbalance of epidermal homeostasis that leads to a prematurely aged appearance during later life.

KEYWORDS
differentiation, epidermis, epidermal morphology, epigenetics, glycolysis, hypoxia, inflammaging, inflammation, photoexposed, senescence

1 | INTRODUCTION

The skin is one of the largest organs of the human body, providing protection from external insults such as solar radiation, pollution, chemicals and particulate matter. Like all organs of the body, the skin is susceptible to ageing, resulting in structural and functional changes which may be accelerated further by environmental insults.[1] This premature ageing of skin leads to cellular and morphological changes that accumulate over time and ultimately affect the skin's appearance, functionality and homeostatic state. This homeostasis is dependent on an organized and timely renewal process, initiated by basal keratinocytes which proliferate and differentiate to ultimately transform into corneocytes that comprise the stratum corneum. An imbalance in this process has implications on skin's appearance, health and response to stress. Thus, it is essential to understand these changes to identify mechanistic intervention targets that would prevent and repair premature ageing and maintain skin health and appearance.

We previously reported findings from a large base study that evaluated biopsies collected from photoexposed face and dorsal forearms as well as photoprotected buttock sites of Caucasian females across age decades spanning 20's to the 70's, demonstrating that age impacts a wide range of molecular processes in skin.[2] Given that a low-grade chronic inflammatory state is hypothesized to be a significant contributor to premature ageing in the inflammaging theory[3-4], we asked whether this phenomenon could be observed in our previously reported skin biopsy study and investigated its potential impact on epidermal biology and homeostasis. A system biology-based analysis of skin surface biomarkers, transcriptomics, proteomics, metabolomics, histology and immunostaining confirmed that there is underlying chronic inflammation in photoexposed face skin that remains elevated across the decades. Primarily in photoexposed skin, we found an imbalance in epidermal homeostasis beginning in the 20's to 30's and elevation of senescence-related components in the 30's to 40's. A subsequent increase of oxygen sensing/hypoxia and metabolic shift towards glycolysis occurs in the 50's. Additionally, there is a higher epigenetic ageing rate in 60's when comparing to the 30's and is further elevated by photoexposure. Based on these findings, we propose that photoexposed skin undergoes inflammaging which may play a role in the molecular and morphological changes that ultimately lead to a photoaged appearance and less healthy state of skin.

2 | MATERIALS AND METHODS

The detailed protocols and statistical analyses are described in Appendix S1: Supplemental Materials and Methods.

3 | RESULTS

3.1 | Age-associated changes in epidermal morphology

We first performed a histomorphometric analysis of the structural compartments of the epidermis from buttock, arm and face sites across age groups. With age, the overall thickness of the stratum corneum increases (Figure 1A) whereas the epidermal layer becomes thinner (Figure 1B), and the rete ridge path length ratio decreases (Figure 1C). Comparison of the mean data between the 20s and each decade showed that these changes become statistically significant in the older age groups (Table S1). A representative histological stain from a 20- and a 60-year-old face highlights these structural changes (Figure 1D). In an older age sample, we observed relatively lower detection of microcapillary structures using staining against UEA-1, a lectin that binds to endothelial related cells.[3] A representative image shows the differential staining pattern below the basement membrane (Figure 1E, white arrows) as well as staining in the stratum granulosum and corneum. This pattern is similar to what has been previously reported in skin.[5] The structural changes of thickening of the stratum corneum and the thinning of the epidermis suggest an imbalance between proliferation and differentiation that changes with age across all body sites.

3.2 | Proteomics analysis shows elevated presence of proteins associated with differentiation and glycolysis in 60s aged dorsal forearm epidermis over 20s age group

To better understand these measured changes in epidermal structure with age, LCM isolated epidermal sections from 20s and 60s dorsal arms were processed and analysed by label-free quantitative mass spectrometry. Out of 367 proteins identified, 83 showed a significant difference ($p < 0.1$) in levels when comparing between the two age groups (Table S2). Of the 83 proteins, 24 proteins were associated with epidermal differentiation and metabolism/oxygen sensing (Table 1).

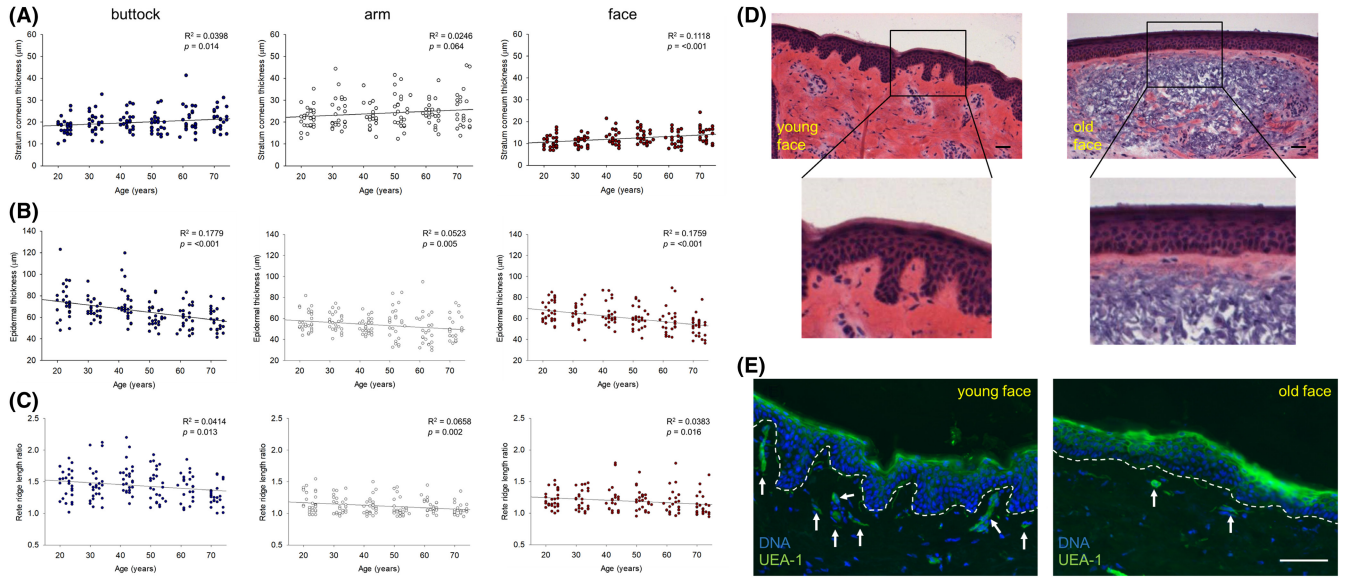

FIGURE 1 Age-associated changes in epidermal morphology. Epidermal structural elements were measured from haematoxylin and eosin-stained histologic sections of skin from buttock, arm and face body sites. Measurements are shown in scatter plots from all subjects and body sites for (A) stratum corneum thickness (μm), (B) epidermal thickness (μm) and (C) rete ridge length ratio (buttock—blue dots; arm—white dots; and face—red dots). Linear regression analysis shows significant changes in structural morphology with age across all body sites. (D) Representative histological section from a 23-year-old and 72-year-old face site is shown along with the region of interest in black box as magnified panel to highlight stratum corneum thickening, epidermal thinning and decrease of rete ridge length ratio in older-aged face. Scale bar (black) is 100 μm. (E) Immunofluorescence staining for microcapillary beds (white arrows) by *Ulex Europaeus*-I Lectin (UVE-1, green) and DNA labelling (blue) show decreased staining of capillary bed structures in a representative section from a 23-year-old and 73-year-old face site. White dotted line denotes bottom of epidermal basal layer. Scale bar (white) is 100 μm.

TABLE 1 Median fold change of detected proteins between 60s and 20s age groups from laser capture microdissection sections of epidermis from photoexposed dorsal forearms and changes in gene expression correlation

Protein	Gene	Median fold change 60s vs. 20s	*p*-value	Gene expression changes with age
Keratin 2	KRT2	1.57	<0.001	Increased
Keratin 10	KRT10	1.50	<0.001	Increased
Cystatin M	CST6	1.86	0.001	Increased
Cystatin A	CSTA	2.37	0.002	Increased
Calpain 1	CAPN1	2.33	0.009	No
Fructose-bisphosphate aldolase A	ALDOA	2.04	0.010	Increased
Arachidonate 12 lipoyxgenase 12R	ALOX12B	2.42	0.011	Increased
Bleomycin hydrolase	BLMH	1.73	0.016	Increased
Annexin A8	ANXA8	1.86	0.020	Increased
Cystatin B	CSTB	2.14	0.022	Increased
Annexin A1	ANXA1	1.41	0.026	Increased
Involucrin	IVL	1.47	0.026	Increased
Transglutaminase 1	TGM1	1.38	0.026	Increased
Annexin A2	ANXA2	1.22	0.026	Decreased
Suprabasin	SBSN	1.37	0.026	Increased
Serine protease inhibitor Kazal-type 5	SPINK5	1.43	0.028	Increased
Calmodulin-like protein	CALML3	1.71	0.030	Increased
Malate dehydrogenase 2	MDH2	0.56	0.044	Decreased
Protein S100-A14	S100A14	1.35	0.046	Increased
Pyruvate kinase M	PKM	1.23	0.049	Increased
Gelsolin	GSN	1.23	0.062	Increased
Transglutaminase 3	TGM3	1.45	0.084	Increased
Haemoglobin alpha	HBA	16.40	0.092	Increased

Twenty-three of these had a similar directional relationship with their representative gene expression pattern with age. The exception is calpain 1 (CAPN1) which showed no significant change in expression levels across the decades (data not shown). Interestingly, we also detected a higher numerical level of haemoglobin-α ($p = 0.092$) and haemoglobin-β ($p = 0.134$, data not shown) present in the older group.

3.3 | Imbalance in epidermal differentiation/proliferation increases with age in photoexposed epidermis

To further understand the age-associated changes in epidermal morphology and corresponding protein level changes, we manually curated transcriptomics data for genes encoding proteins involved in epidermal differentiation and proliferation, including the epidermal differentiation complex, keratins, protease inhibitors, proteases, calcium-binding proteins/AMP (antimicrobial peptides), proliferation and late cornified envelope proteins (Figure 2A).[6,7] Statistical analysis of changes across the decades between 20s and 70s showed a pattern of elevated expression with age of differentiation-associated genes in the photoexposed dorsal arm and face sites in most of these groups (Figure 2A, pink coloration). In contrast, genes associated with proliferation showed a decline in expression with age decades across all three body sites (Figure 2A, blue coloration). Trace profiles of representative probe sets from face of filaggrin (FLG), involucrin (IVL), arachidonate 12-lipoxygenase,12R (ALOX12B), loricrin (LOR), keratin 2 (KRT2), keratin 14 (KRT14), calmodulin-like protein 3 (CALML3), serine protease inhibitor Kazai-type 5 (SPINK5), cystatin B (CSTB), Krüppel-like factor 9 (KLF9), insulin-like growth factor 1 receptor (IGF1R) and late cornified envelope 2C (LCE2C) show the relative expression changes across the decades (Figure 2B). Interestingly, the late cornified envelope proteins did not show as significant of a pattern when comparing across 20s and 70s but exhibits a significant increase up to the 50s and the reversal from 50s to 70s. To further visualize the gene expression profiles, we immunostained for several of these proteins in representative samples from young and old face and arm sites. Immunostaining for filaggrin showed heightened levels in the upper granular/stratum corneum layers in a representative older age face site (Figure 2C) and to a lesser extent for involucrin and loricrin (Figure 2D,E). The basal keratin 14 marker showed a higher overall level of detection in a representative older age arm site (Figure 2F) and a modestly higher level of detection of the suprabasal marker keratin 10 (Figure 2G).

3.4 | The IL-1RA/IL-1α ratio and IL-8 remain elevated across the decades in photoexposed facial skin, the *cis/trans*-urocanic acid and 53BP1 DNA damage foci are detected in photoexposed sites, and epigenetic age is higher with age in photoexposed arm sites

In addition to proteomics analysis on LCM-derived epidermal sections, we tested for the presence of the senescence-associated secretory phenotype (SASP) inflammatory biomarkers IL-8 and the IL-1RA/IL-1α ratio on the surface of skin.[8] Analysis of tape strip extractions showed the levels of both biomarkers were elevated in photoexposed face compared to dorsal arm and buttock sites (Figure 3A,B). Interestingly, the levels on face remained elevated across the age groups. It was surprising that we did not detect elevated levels of these cytokines in the photoexposed dorsal arm sites. To better understand this difference between the two sites, we analysed for the UV-sensitive metabolite ratio of *cis/trans*-urocanic acid. We showed a significant elevation in both face and arm compared to buttock consistent across the decades (Figure 3C). We also stained arm and buttock sites from both young and old for 53BP1, an indicator of DNA damage response induced by UV-irradiation.[9,10] Quantification showed significantly more foci in the basal layer of aged arm compared to young, while very few foci were detected in buttock (Figure 3D-F). The buttock sites in either young or old did not show any significant increase in DNA damage. Additionally, we quantitated epigenetic age levels, which is based on DNA methylation levels from thousands of ageing-related locis.[11] We showed that both body sites showed elevated epigenetic age levels in the 60s when compared with the 30s, and significantly accelerated ageing in arm sites compared to buttock sites (Figure 3G). The elevated levels of these photosensitive markers with age in arm and face sites support that both sites undergo a certain degree of photodamage. The muted levels of IL-8 and the IL-1RA/IL-1α ratio may be due to unknown physiological differences that merit further investigation.

3.5 | Senescence and inflammation are elevated with age in photoexposed epidermis

The detection from facial skin surface of elevated levels of IL-8 which remains consistently high (Figure 3B) suggests that this site may present a higher senescence/inflammation rate than arm. As shown previously, we report that the senescence-associated gene CDKN2A is elevated with age across all three body sites.[2] To better understand the correlation there may be between senescence, the heightened presence of the SASP-associated inflammatory biomarkers, and the epidermal morphological changes, we manually curated transcriptomics data for a subset of genes encoding for senescence and inflammatory associated proteins. We found an overall pattern of increased expression across the decades between 20s and 70s in both the photoexposed arm and face sites, with more genes being upregulated in face (Figure 4A). In agreement, cyclin-dependent kinase inhibitor 2A (CDKN2A), alpha-crystallin B chain (CRYAB) and cytokine receptor type 2/IL8RB (CXCR2) were upregulated upon ageing (Figure 4B). Similarly, several genes that have been reported to be reduced upon senescence (RBL2, SIRT, LMNB1) showed a general pattern of lowered expression (Figure 4A).[12–14] CDKN2A is known to encode several proteins involved in senescence and linkages to cancer, and ageing, including $p16^{INK4A}$.[15–17] To further visualize the expression patterns, we immunostained biopsy sections from young and old face sites for $p16^{INK4a}$ and observed higher number of p16-positive cells in aged photoexposed facial skin throughout the basal and suprabasal layers (Figure 4C,D, yellow arrows).

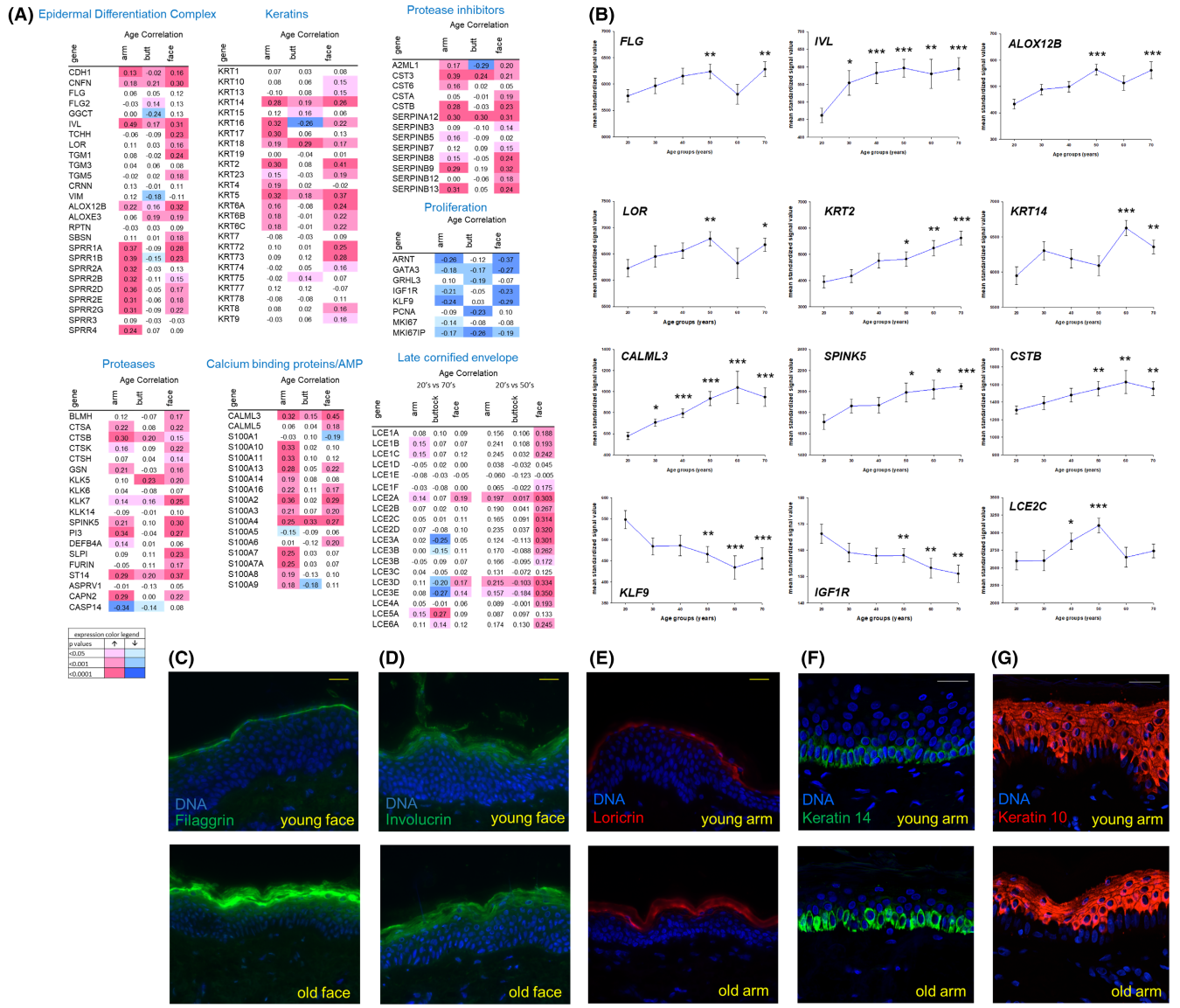

FIGURE 2 Transcriptomics profiling and immunofluorescent staining of epidermal differentiation genes and proteins with age. (A) Transcriptomics statistical heatmap of relative expression changes with age of select genes encoding for proteins associated with regulation of epidermal homeostasis. Genes associated with the epidermal differentiation complex, proteases, keratins, protease inhibitors and calcium-binding proteins/AMP (AMP - antimicrobial peptides) show a pattern of elevated expression across the decades from 20s to 70s (red). Genes regulating proliferation show a pattern of decreased expression across the decades from 20s to 70s (blue). Genes encoding for late cornified envelop proteins show a significant increase from the 20s to 50s (red) but was not significant between 20s to 70s. Colour range reflects average normalized intensity values for each group (colour legend panel: red higher expression and blue lower expression). (B) Trace profiles of probe sets from the face encoding for filaggrin (FLG), involucrin (IVL), arachidonate 12-lipoxygenase, 12R (ALOX12B), loricrin (LOR), keratin 2 (KRT2), keratin 14 (KRT14), calmodulin-like protein 3 (CALML3), serine protease inhibitor Kazai-type 5 (SPINK5), cystatin B (CSTB), Krüppel-like factor 9 (KLF9), insulin-like growth factor 1 receptor (IGF1R) and late cornified envelope 2C (LCE2C). Significance indicates comparisons to 20-year-old cohort mean value (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Representative immunofluorescence images for comparison of filaggrin (C) and involucrin (D) from a 20-year-old (young face) and 64-year-old (old face) face site; loricrin (E), keratin 10 (F), and keratin 14 (G) from a 20-year-old (young face) and 60-year-old (old arm) arm site showing higher detection in older site. Yellow scale bar is 100 μm, and white scale bar is 30 μm.

3.6 | An oxygen sensing/hypoxic fingerprint and metabolic reprograming increases with age in epidermis

The proteomics-based detection of elevated levels of several glycolytic enzymes in 60s aged epidermal arm LCM samples suggests the epidermis was undergoing a metabolic shift. A shift to glycolysis is a hallmark process of cells when exposed to hypoxic conditions. The morphological changes with age of increased stratum corneum thickness, decrease in rete ridge path length ratio, and less vasculature detection could impact oxygen bioavailability in the epidermis. Finally, the increased expression and protein detection

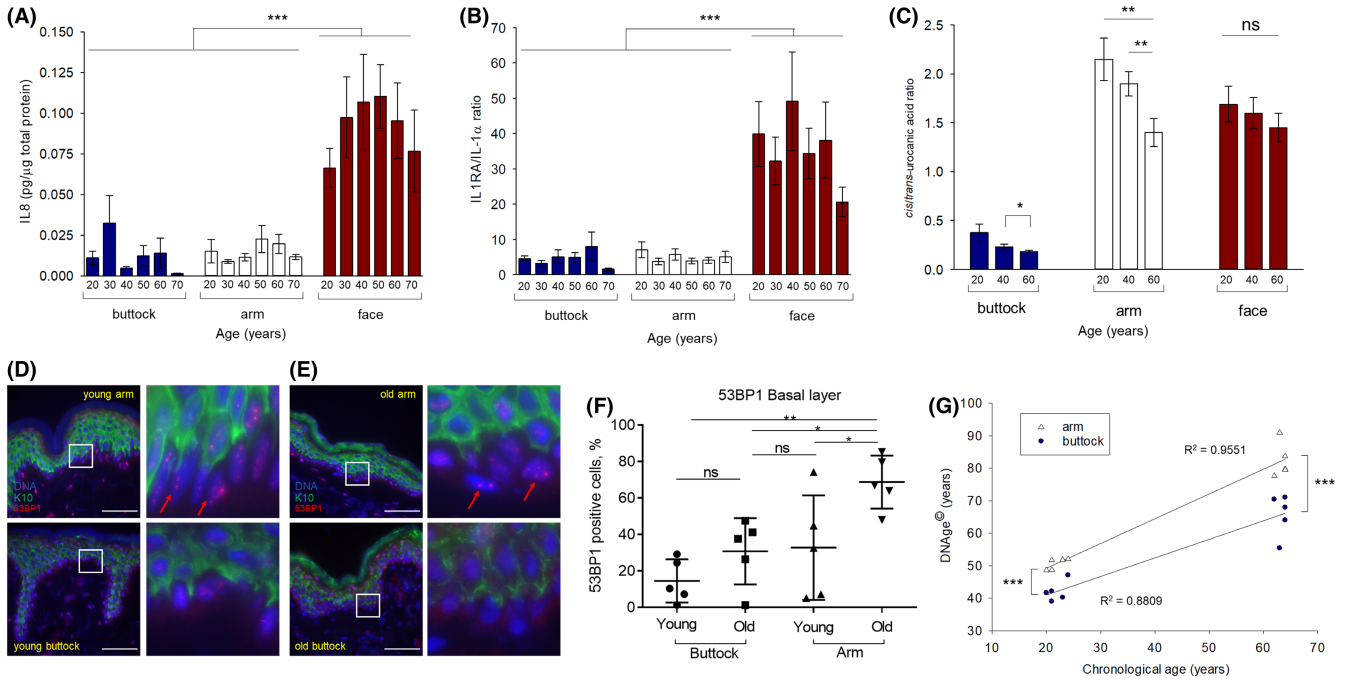

FIGURE 3 Markers of inflammation, photoexposure, and DNA damage and methylation comparisons across body sites and age. Biological material extracted from tape strips collected across all sites and age groups were analysed. (A) IL-1RA/IL-1α and (B) IL-8 are reported from dorsal forearm, cheek and buttock sites across each decade age group showing elevated levels present in face sites across all decades. (C) The *cis/trans*-urocanic acid ratio are reported from dorsal forearm, cheek and buttock sites from 20s, 40s and 60s age groups and show elevated levels across decades in both arm and face sites. Representative immunofluorescence images for detection of foci localization of the DNA damage marker 53BP1 (red) in a 20-year-old young arm and buttock (D) and a 64-year-old arm and buttock (E) are shown. Keratin 10 staining (green) and DNA labelling (blue) are shown. Region of interest outlined in white is shown as magnified panel, and red arrows indicate examples of elevated 53BP1 foci in nuclei of older photoexposed site. Scale bar, 100 µm. (F) Quantification of 53BP1-positive cells in K10-negative basal layer from young and old arm and buttock images ($n = 5$ per site). Data are shown as mean ± SD. Ordinary one-way ANOVA was performed with Sidak's multiple comparison test. (G) Quantitation of epigenetic age levels based on DNA methylation levels from young and old arm and buttock images ($n = 5$ per site). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns non-significant.

of haemoglobin-α further suggest an oxygen sensing response by the epidermis. Thus, we manually curated from transcriptomics data a subset of genes encoding proteins sensitive to oxygen tension or associated with cellular responses to hypoxia. These genes showed an increased expression pattern across the decades between 20s and 70s in arm, buttock and face sites (Figure 5A, pink coloration). Consistent with this, genes encoding proteins known to negatively respond to hypoxia showed decreased expression across the decades, primarily in the photoexposed forearm and face sites (Figure 5A, blue coloration). Genes encoding glycolytic enzymes were also analysed, and several genes were found to have elevated expression patterns across the decades between 20s and 70s in arm and face sites (Figure 5A, red coloration). To further illustrate the statistical findings, representative expression traces are shown for hypoxia-inducible factor 1, subunit alpha (HIF1A, a master regulator of cellular response to hypoxic conditions), haemoglobin-β (HBB), haem oxygenase 1 (HMOX1), cystine/glutamate antiporter (SLC7A11), aldolase A (ALDOA), lysine demethylase 3A (KDM3A), lysine demethylase 5A (KDM5A), Sprouty homologue 2 (SPRY2), lactate dehydrogenase A (LDHA) and phosphoglucomutase 1 (PGM1) (Figure 5B). To further understand HIF1A and haemoglobin gene expression, we immunostained for HIF-1α and haemoglobin-α. A representative image shows staining of HIF-1α in nuclei of young face sites but higher expression was detected in older-aged face sites (Figure 5C,D, red arrows). Representative images of haemoglobin-α staining in both arm and face sites highlight an elevated staining intensity throughout the upper granular/stratum corneum layers in arm (Figure 5E) and face (Figure 5F) from older individuals as compared to younger. Interestingly, there was no observable staining increase in the basal layer and through the dermis, further supporting that the presence of haemoglobin-α was epidermally derived and not erythroid.

4 | DISCUSSION

The skin is the first line of defence protecting the body from environmental stressors such as solar radiation and pollution. Daily exposure to sunlight is one of the more significant environmental insults that induces DNA damage, oxidative stress and inflammation in skin. Human skin must maintain robust repair capabilities to prevent cumulative damage triggered by these stressors. However, with age this ability is diminished, and the onset of senescence further hinders the skin's capacity to mitigate stress-induced inflammation

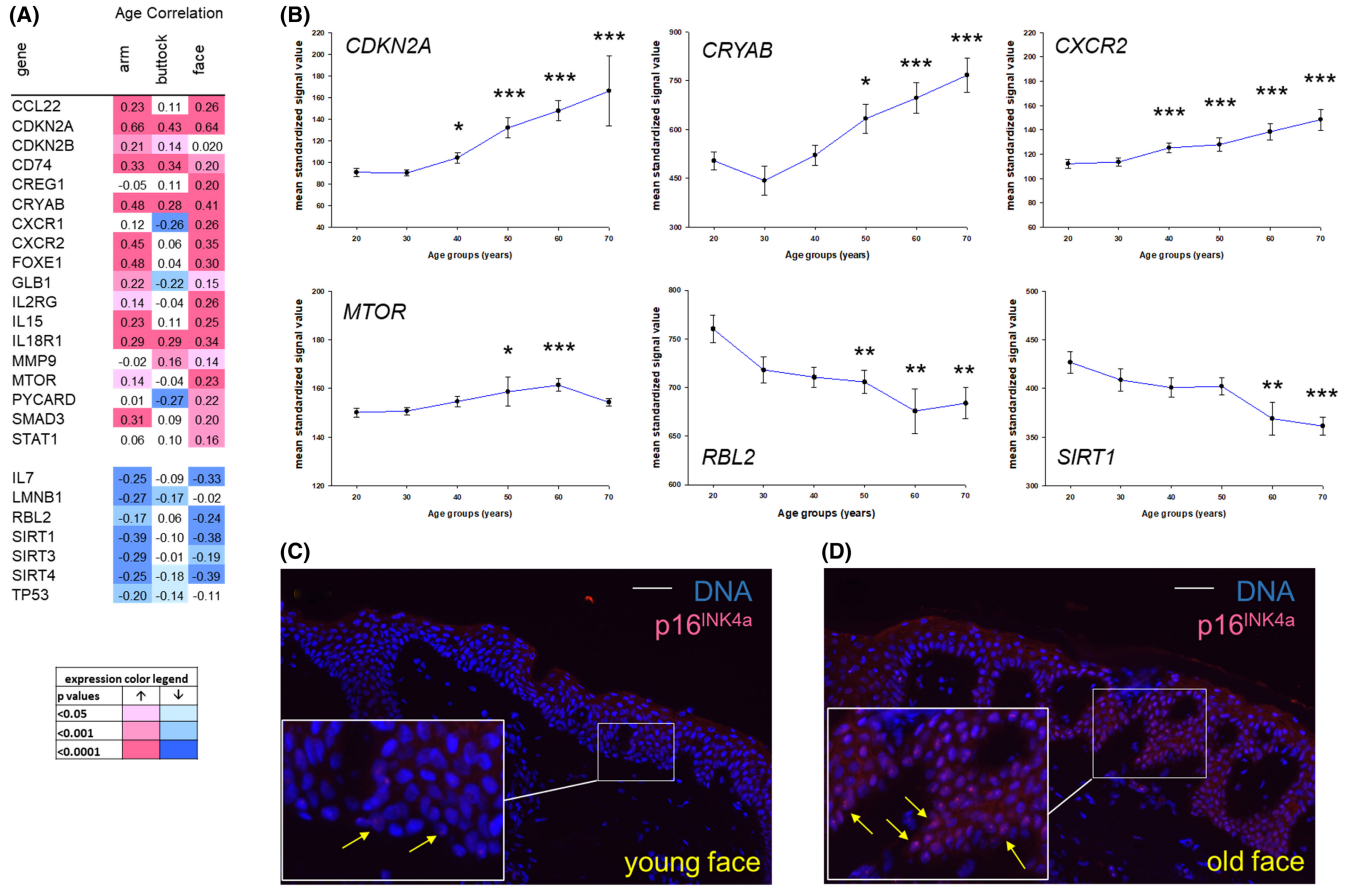

FIGURE 4 Transcriptomics profiling of senescence-associated genes and immunofluorescent staining for p16INK4a. (A) Transcriptomics statistical heatmap of relative expression changes with age of select genes encoding for proteins associated with regulation of senescence. Genes associated with increasing cellular senescence and senescence-associated secretory phenotype show a pattern of elevated expression across the decades from 20s to 70s in the photoexposed sites of dorsal forearm and face (red). Additionally, genes associated with mitigating senescence show a pattern of decreased expression across the decades from 20s to 70s (blue). Colour range reflects average normalized intensity values for each group (colour legend panel: red higher expression and blue lower expression). (B) Trace profiles of probe sets from the face encoding for cyclin-dependent kinase inhibitor 2A (CDKN2A), alpha-crystallin B chain (CRYAB), cytokine receptor type 2/IL8RB (CXCR2), mammalian target of rapamycin (MTOR), retinoblastoma-like protein 2 (RBL2) and sirtuin 1 (SIRT1). Significance indicates comparisons to 20-year-old cohort (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Representative immunofluorescence images for staining of $p16^{INK4a}$ (pink) and DNA labelling (blue) from a 21-year-old (C, young face) and a 63-year-old (D, old face) face site. Region of interest outlined in white is shown as magnified panel, and yellow arrows indicate examples of elevated p16 detection in nuclei of from older face site. Scale bar (white) is 100 μm.

and can lead to the presence of chronic low-level inflammation.[18] This phenotype is a key feature of inflammaging. The evidence for the presence of inflammaging in skin has been previously reviewed, and it was highlighted that while there are clear signs of an inflammaging microenvironment in skin, further work is needed to better understand its role on skin ageing.[19]

To better understand the role of inflammaging on skin ageing, we utilized a system biology-based approach to investigate biological samples collected from photoprotected and exposed female body sites spanning 6 decades of age. A previous report found that patterns of gene expression accelerated with ageing in Caucasian females and differed in a subgroup that appeared exceptionally youthful based on image analysis of facial appearance.[2] The current study focused on the epidermal skin compartment and employed a system biology-based approach to increase our understanding and identify potential intervention strategies to mitigate premature ageing. Our findings provide a body of evidence that photoexposed facial skin appears to be in an inflammaging microenvironment due to the presence of elevated chronic inflammation which, in turn, could be a factor in part that leads to an imbalance in epidermal homeostasis starting in the 30s as measured via histology, transcriptomics and proteomics (Figure 6). This suggests that targeting inflammation in younger-aged skin may be a promising intervention approach to mitigate the molecular and morphological changes that lead to a photoaged appearance of skin and impact on underlying skin health.

The histomorphologic analysis in this study found that the epidermis undergoes significant changes with age, including stratum corneum thickening, implying that there may be a stronger barrier in older-aged skin. While counter-intuitive, several reported studies have shown that trans-epidermal water loss values decrease in

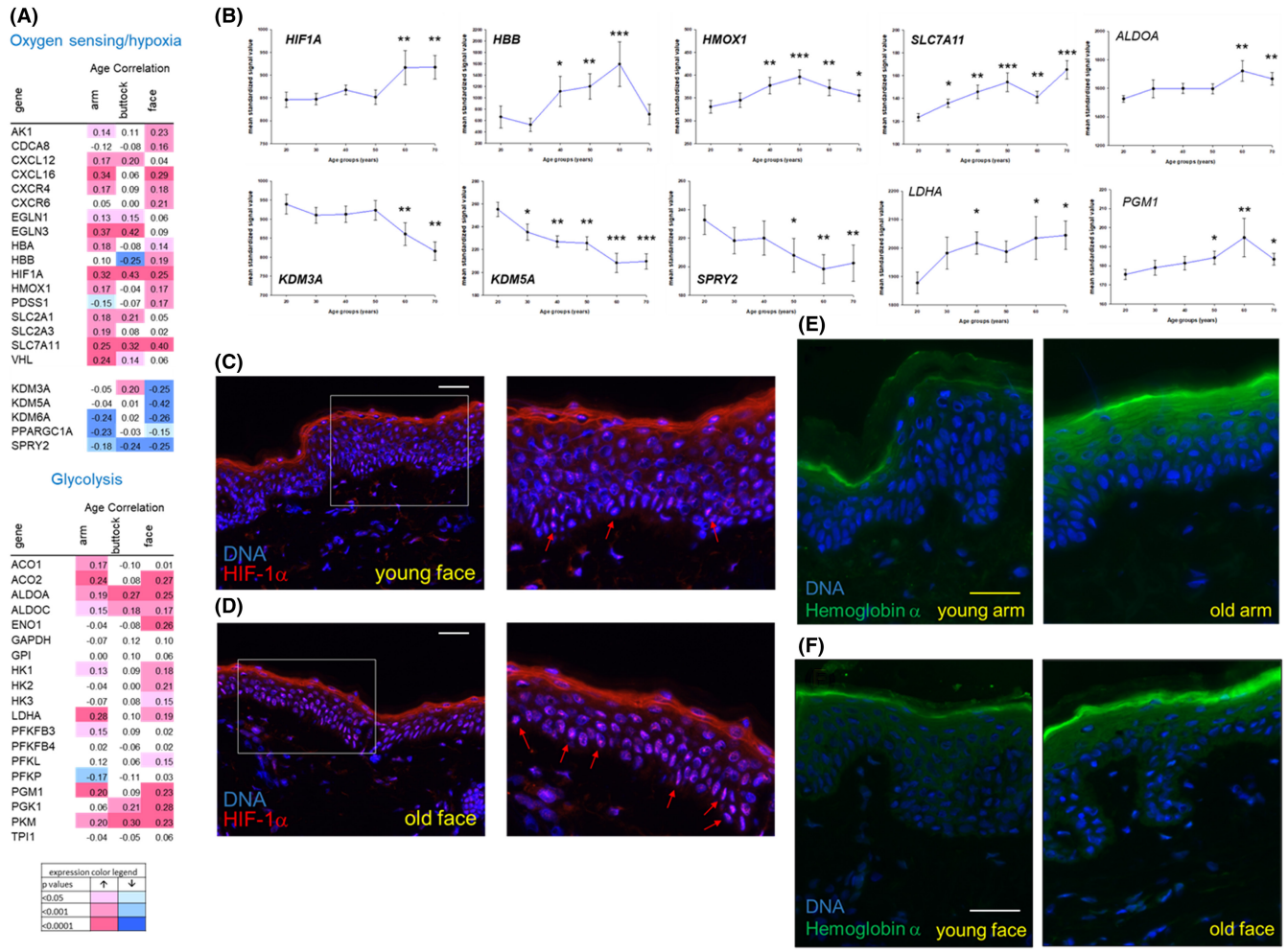

FIGURE 5 Transcriptomics profiling of oxygen sensing and hypoxia associated genes and immunofluorescent staining for HIF-1α and haemoglobin α. (A) Transcriptomics statistical heatmap of relative expression changes with age of select genes encoding for proteins associated with oxygen sensing and hypoxia. These genes show a pattern of elevated expression across the decades from 20s to 70s in the photoexposed sites of dorsal forearm and face (red). Additionally, genes associated with being downregulated under hypoxic conditions show a pattern of decreased expression across the decades from 20s to 70s (blue). Colour range reflects average normalized intensity values for each group (colour legend panel: red higher expression and blue lower expression). (B) Trace profiles of probe sets from the face encoding for hypoxia inducible factor 1, subunit alpha (HIF1A), haemoglobin-β (HBB), heme oxygenase 1 (HMOX1), cystine/glutamate antiporter (SLC7A11), aldolase A (ALDOA), lysine demethylase 3A (KDM3A), lysine demethylase 5A (KDM5A), Sprouty homologue 2 (SPRY2), lactate dehydrogenase A (LDHA) and phosphoglucomutase 1 (PGM1). Significance indicates comparisons to 20-year-old cohort (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Representative immunofluorescence images for comparison of HIF-1α between a 22-year-old (C, young face) and a 60-year-old (D, old face) face sites. Region of interest outlined in white is shown as magnified panel, and red arrows indicate examples of elevated HIF-1α staining in nuclei of older site. Scale bar (white) is 100 μm. Representative immunofluorescence images for comparison of haemoglobin-α between a 22-year-old (E, young arm) and a 63-year-old (E, old arm) arm sites and between a 21-year-old (F, young face) and a 63-year-old (F, old face) face sites showing elevated detection throughout the granular layer stratum corneum in the older sites. White scale bar is 25 μm, and yellow scale bar is 50 μm.

older-aged subjects, suggesting that the barrier integrity improves with age.[20] However, the underlying health of the skin plays a role to ensure optimal repair response kinetics to damaging agents. Older-aged skin has been shown to have a slower and weaker response profile to damage such as wounding and tape strip removal.[21,22] We also show that with age the epidermis becomes thinner, the rete ridge path length flattens, and these changes correlate with changes in gene expression and protein levels associated with differentiation and proliferation, similar to in vitro data previously published.[23] Expression changes occur in a large proportion of genes encoding proteins associated with the epidermal complex, keratins, proteases, protease inhibitors, calcium bindings proteins/AMP and late cornified envelope proteins. Additionally, these changes are more apparent in the photoexposed arm and face sites than the buttock site, confirming previous in vitro data where UVB irradiation led to increased levels of late differentiation markers.[24] This imbalance in differentiation and proliferation processes appears to shift in the 30s and could be a factor in the observed morphological changes detected starting in the 40s. For example, the representative expression traces for FLG, LOR, ALOX12B, KRT2, CALML3, SPINK5 and CSTB all show a similar

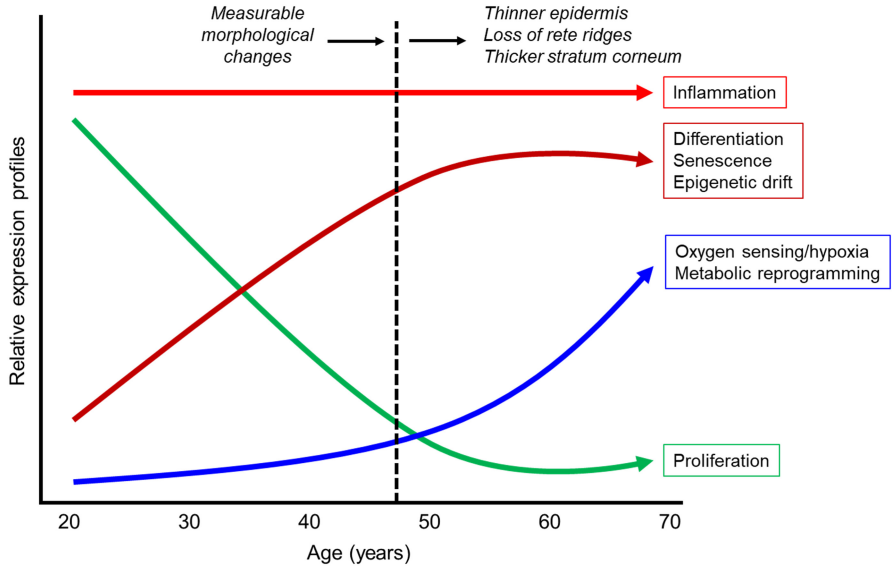

FIGURE 6 Schematic representation of key biological processes changing with age in epidermis of photoexposed facial skin. A heightened inflamed status remains constant across the decades in the epidermis of photoexposed facial skin and underlying processes such as differentiation, senescence, oxygen sensing/hypoxia, epigenetic drift and metabolic reprogramming steadily increase. In contrast, epidermal proliferation declines with age. These suggest that inflammaging leads to an imbalance in epidermal homeostasis with photoaging.

pattern of increased expression beginning in the 20s to 30s and continuing to increase across the decades. It is worth noting that some of these markers show alterations of this trend in the 50s, presumably due in part to hormonal changes as recorded in the previous study.[2] This is particularly highlighted in the respective traces presented as well as the overall expression patterns for the late cornified envelope proteins which showed significant changes in expression between the 20s and 50s but lost significance when comparing between the 20s and 70s. Several of the proteins expressed by these genes were also detected via proteomics profiling between the photoexposed arm of young and old subjects. A similar proteomics profiling has been reported in which the authors used tape strip collection to quantitate the levels of surface proteins associated with differentation.[25] Their findings are similar to the ones presented here with the exception that several proteins showed contrasting reduced levels in photoexposed skin compared to the elevated levels of those same proteins in our study. It should be pointed out that the age comparison between the 20s and 60s in this work was selected due to reversal of expression levels in the older 70s cohort. Future work will include additional analyses across all the age groups. Overall, there is an apparent correlation between the differentiation-associated gene expression changes that begins in the 20s and correlates with the morphological changes that become significantly measurable starting in the 40s. This suggests an imbalance in epidermal homeostasis which could impact its response profile to environmental insults and maintenance of normal cellular function.

To better understand the inflammatory and photoexposure status of the subjects in this study, we evaluated for the presence of inflammatory and photosensitive biomarkers isolated from the skin's surface. Detection of elevated levels of IL-8 has been shown to be elevated in eczema, atopic dermatitis, and psoriasis skin and in 3D skin models after UVB exposure.[26–28] We found elevated levels of IL-8 on photoexposed facial skin surface sites that remain elevated across age groups. The ratio of IL-1RA/IL-1α present on the skin's surface is known to be an indicator of underlying inflammation associated with skin dermatitis conditions and UV exposure.[29–31] Relative to impact of age and photoexposure on this inflammatory biomarker, it was reported that the IL-1RA/IL-1α ratio was elevated in photoexposed face compared to non-exposed upper inner arm and remained constant across age groups.[29] Relatedly, we show similar patterns when comparing between photoexposed face where the IL-RA/IL-lα ratio was consistently high and consistently low in photoprotected buttock sites across the decades. Surprisingly, we did not see an increase in these cytokines in photoexposed dorsal arm samples since we had previously reported there are significant histological indications of photoaging.[2] We show that several biomarkers associated with photoexposure are increased in arm sites, including the *cis/trans*-urocanic acid ratio, foci of the DNA damage response marker 53BP1 that is sensitive to UV exposure, and epigenetic age derived from methylation levels of DNA, an indicator of epigenetic ageing.[11,32–34] These methylation patterns are similar to what has been previously reported where the biopsies were enzymatically separated into epidermis and dermis fractions in contrast to LCM in our study.[35] Overall, this supports that the photoexposed arms undergo photodamage. We do not believe the lower levels of IL-8 or the IL-1RA/IL-1α ratio on photoexposed arm or buttock sites are an artefact since we performed the analysis in two independent experiments from duplicate tapes. The difference could reflect a dose–response or a level of chronic exposure or, alternatively, facial skin is among the thinnest in the body and may be more susceptible to injury. While overall our results support the hypothesis that photoexposed skin is in a heightened state of inflammation, and that inflammation is present early in the 20s and remains persistent across the decades, future work is needed to understand the physiological relevance in photodamaged arms. Overall, the implications of this constant inflammatory pressure could be an indicator of skin inflammaging that leads to the changes in gene expression patterns and correlating protein levels in photoexposed skin.

We previously reported CDKN2A, a gene that encodes for proteins associated with senescence induction, to be elevated

with age.[2] CDKN2A is known to encode for $p14^{ARF}$, $p15^{INK4B}$ and $p16^{INK4A}$, all of which are involved in senescence and play significant roles in cancer and ageing, including in skin.[15–17] In the current study, we wished to better understand this correlation beyond CDKN2A and performed a focused transcriptomics profiling of select genes encoding for proteins associated with regulation or induction of senescence in skin.[36] It has been established that photoexposure can cause keratinocytes to prematurely enter senescence and these cells can be characterized by secretion of an altered secretome called the senescence-associated secretory phenotype (SASP), enriched with pro-inflammatory cytokines such as IL-6, IL-8 and IL-1β.[8]

The elevated skin surface levels of IL-8 early in the 20s age cohort on photoexposed face sites support there may be an early onset of a SASP-associated phenotype in photodamaged facial skin. We see significant elevated levels of expression of genes encoding proteins associated with senescence in the photoexposed sites. For example, GLB1 encodes SA-β-gal (beta-galactosidase), a well-known biomarker of senescence in numerous tissues, including skin.[36] Several chemokine receptors were observed to increase in expression levels with age in the photoexposed arm and face sites. CXCR1 and CXCR2 encode receptor proteins that bind with IL-8 and showed elevated expression in both arm and face.[37] Interestingly, this provides a potential correlation of inflammatory response with the elevated levels of IL-8 present on the skin's surface. A survey of candidate SASP components from a comparison between in vitro senescence models and in vivo tissue and fluid samples showed the elevated presence of CCL22, IL15 and MMP9 under senescent-impacted conditions.[38] The mammalian target of rapamycin (mTOR) is suggested to be a master regulator of metabolite sensing that impacts senescence induction and overall cellular ageing.[39,40] We show in both photoexposed epidermal sites an increase in mTOR expression levels with age (Figure 4A) that becomes significant in the 50s compared to the 20s for face (Figure 4B). CREG (cellular repressor of E1A-stimulated genes 1) co-expression with $p16^{INK4a}$ can further enhance senescence than either expressed alone.[41] Recently, CRYAB and HMOX1 have been proposed to be senolytic targets in humans cell models.[42] Interestingly, it was also demonstrated that HMOX1 expression levels were increased during differentiation, which supports a similar correlation as measured in our study.[43] As reported here, we observe a significant increase in the expression patterns of these genes starting in the 30s and continuing into the 70s in photoexposed facial epidermis sites. In addition, we evaluated genes that encode proteins that mitigate senescence, including RBL2, SIRT1, SIRT3, SIR4 and TP53.[12,44–46] These show varying patterns of decreased expression in the epidermis of photoexposed sites with significance starting in the 50s and 60s. Finally, we immunostained for $p16^{INK4A}$ and detected nuclear localized puncti in both basal and spinous layers. Interestingly, it has been reported that $p16^{INK4a}$ is primarily detected in epidermal melanocytes by immunohistochemistry methods.[47] However, this may not be an exclusive scenario since it has also been reported that $p16^{INK4a}$ can be detected in keratinocytes in both basal and suprabasal layers, findings that are similar to ours.[48] These findings suggest that future work is needed to better define the role of this important senescence marker in the skin individual cell types.

In total, the data presented here support that photoexposed skin is undergoing an accumulation of senescent cells with age. The chronic presence of the SASP factor IL-8 could be a causative indicator of senescence but further work is needed to establish cause and effect linked to the imbalance in differentiation−/proliferation and morphological changes.[49] The implications of skin undergoing these changes in inflammation and senescence due to photoexposure also have potential implications on overall body health. A recent review suggests there is a correlation between the accumulation of senescent cells in the skin and a negative impact on overall systemic health and longevity that occurs via the hypothalamic–pituitary–adrenal axis.[50] Future work is planned to further correlate the gene expression and protein detection across individuals and body sites in this data set and from a recent clinical study.

Oxygenation of the epidermis occurs via passive diffusion from direct contact with atmospheric oxygen and from microcapillary beds intertwined underneath the basement membrane.[51] This may explain why the epidermis is considered to have a relatively low oxygen tension estimated to range between 0.3% and 8%, and why the epidermis could be considered hypoxic in contrast to the highly vascularized dermis where oxygen levels are estimated to be >7%.[51,52] The morphological changes measured in the epidermis with age suggested to us a further limitation of oxygen supply due to the longer diffusion path length through the thickened stratum corneum, as well as the reduced surface area interface with microcapillary beds from reduction of rete ridge undulation pattern. It has been previously reported that ageing can lead to a measured increase in hypoxic-related response profiles.[53] That work utilized suction fluid blisters from young and older aged upper arms for transcriptomics profiling. In our study, we utilized the sensitivity of LCM dissection to localize the epidermis in both photoprotected and photoexposed skin sites for further investigation and an overall system biology body of evidence. The impact of a lowered oxygen tension in the epidermis is controlled in large part by hypoxia-inducible factor-1α (HIF-1α), a transcription factor and master regulator of cellular response to oxygen tension condition.[54] In addition to HIF-1α, an expanded transcriptomics profiling of select genes encoding proteins associated with regulation or responsiveness to oxygen tension changes or hypoxia supports our hypothesis that photoaged skin is transitioning into a more hypoxic microenvironment. For example, hypoxic conditions have been shown to induce HMOX1 gene expression at 1% O_2 in vitro and 7% O_2 in vivo and this was mediated by HIF-1α activity.[55] Gene expression of the CXCL16-CXCR6 axis, CXCR4 and CXCL12 has been reported to be elevated under chronic hypoxic conditions.[56] PDSS1 encodes for decaprenyl diphosphate synthase subunit 1 and was recently identified as a member of a hypoxia signature in hepatocellular carcinoma cells.[57] We identified several genes whose expression patterns are negatively regulated under hypoxic conditions. Lysine demethylase 3A (KDM3A) has been reported to regulate PGC1α (PPARGC1A) and is inhibited under hypoxic

conditions.[58] Silencing of SPRY2 gene expression was shown to correlate with elevated levels of HIF-1α.[59] Prolonged exposure to hypoxic conditions is known to shift cellular metabolism to a greater reliance on glycolysis due to the more anaerobic conditions.[60] We observed a similar shift based on elevated expression of genes encoding enzymes involved in glycolysis such as ALDOA, ENO1, LDHA, PGM1 and PKM. This was further supported by the detection of higher protein levels for ADLOA and PKM in older-aged arm samples compared to younger-aged samples. Expression for the glucose transporters SLC2A1, SLC2A3 and SLC7A11 was also elevated with age, which have been reported to be stimulated in response to hypoxia.[56,61] Interestingly, we detected elevated expression of haemoglobin-α and haemoglobin-β (HBA and HBB) and a numerically greater level of haemoglobin-α protein in older-aged photoexposed arms. Of note, we did not see any significant staining for haemoglobin-α through the dermis and neither did we identify haemoglobin differences from proteomics of dermal sections (data not shown). While haemoglobin is well known for its role in O_2 and CO_2 gas exchange in red blood cells, an increasing number of non-erythroid tissues have been reported to endogenously express haemoglobin.[62] The exact function of haemoglobin in non-erythroid tissue is not clear but it has been speculated it could include regulation of haem, iron and oxygen levels.[63] It has also been proposed that haemoglobin plays a role in response to oxidative stress by helping protect against ROS damage.[64] Overall, the significant increase in expression of genes associated with hypoxia and glycolytic enzymes suggests a phenotype reflecting a hypoxic microenvironment in photoexposed skin and, to a weaker extent, in non-exposed skin. The reported range of O_2 tension in the epidermis has a broad range between 0.3%–8%,[51,52] and we would propose that in this study cohort the tension was significantly lower in the older age group compared with the younger group. Further work is needed to validate these findings with quantitation of differences in oxygen content in the epidermal compartment as a function of age and photoexposure.

Limitations exist in this study since it is not clear on the causal relationship between the molecular changes ascribed and the cascade across the decades to the morphological changes.

5 | CONCLUSIONS

In summary, this system biology-based approach to analyse inflammatory and photosensitive biomarkers, proteomics, transcriptomics and immunostaining strongly suggests that photoexposed facial skin is undergoing inflammaging that begins as early as in the 20s and that multiple biologic pathways are affected in this process. We propose that the chronic presence of inflammation and SASP early in age may contribute to the molecular reprogramming, imbalance of epidermal homeostasis and morphological changes. The presence of heightened senescence, oxygen sensing/hypoxic response, epigenetic drift and metabolic shift may also play roles leading to this imbalance. While this work provides further evidence on the role of senescence and inflammation in impacting ageing in photoexposed skin, further evidence is still required.,[65,66] Finally, the detection of non-erythroid-derived haemoglobin in the epidermis is a novel finding that merits further evaluation on its function and role in skin biology and ageing.

AUTHOR CONTRIBUTIONS

BBJ, CYRT, CYH, TTL, XY, LC, SP, SB, OD and JEO conceived the experiments. BBJ, CYRT, CYH, ALS, TTL, XY, CN, WG, YCC, YMD, LC and PSG performed experiments and analysed the data. JEO wrote the manuscript. All authors reviewed/edited the manuscript.

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CONFLICT OF INTEREST

The authors state no conflict of interest. XY, CN, WG and YCC are full-time employees of Zymo Research Corporation. BBJ, YMD and JEO are full-time employees of The Procter & Gamble Company.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Supplemental Materials and Methods.

Table S1. Quantification of histological measurements of epidermal morphology across age groups and face, arm, and buttock sites (mean ± SEM).

Table S2. Complete list of detected peptides and corresponding protein associations.

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