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ORIGINAL ARTICLE

Novel biophysical skin biomarkers discriminate topical anti-inflammatory treatments based on their potential for local adverse effects

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Abstract

Background: Topical corticosteroids (TCS) are efficacious treatments for inflammatory skin conditions, however, there is a risk of adverse effects; understanding how best to use these treatments is an unmet research priority shared by patients and healthcare professionals.

Objectives: To develop non-invasive biomarkers of local adverse effects to facilitate the optimisation of topical therapy.

Methods: An observer-blind randomised within-subject controlled trial in atopic dermatitis patients was undertaken (NCT04194814) comparing betamethasone valerate 0.1% cream (BMV) to a non-steroidal anti-inflammatory treatment, crisaborole 2% ointment (CRB). Participants underwent 4 weeks twice-daily treatment with CRB on one forearm and BMV on the other (left/right randomised). Skin properties were assessed on days 1, 15, 29 of treatment and again on day 57, including imaging of skin microstructure using Optical Coherence Tomography (OCT) and Attenuated Total Reflectance (ATR)-Fourier Transform Infrared (FTIR) spectroscopic assessment of stratum corneum molecular structure. The primary outcome was the difference in the change in epidermal thickness from days 1 to 29.

Results: Thirty-seven participants received the first dose, of which 32 completed the study (all 37 were included in the analysis). Pathologic epidermal thinning at day 29 was significantly greater ($p < 0.0001$) at sites treated with BMV (−31.66; 95% confidence interval: −35.31, −28.01 μm) compared to CRB (−13.76; −17.42, −10.10 μm). From a panel of exploratory biomarkers, superficial plexus depth and stratum corneum carboxyl group levels had the greatest ability to discriminate the effects of the TCS treatment ($p < 0.0001$).

Conclusions: BMV induced 2.3x more pathologic epidermal thinning than CRB after 4 weeks of treatment, suggesting that CRB may be more appropriate for longer-term, proactive-based, treatment strategies where the risks of adverse effects are greatest. By monitoring treatment effects using OCT and ATR-FTIR spectroscopy, two new non-invasive biomarkers of skin health have been identified with the potential to help optimise future safe treatment strategies.

KEYWORDS

atopic dermatitis, optical coherence tomography, PDE4 inhibitor, skin barrier, topical corticosteroid, transepidermal water loss

INTRODUCTION

Atopic dermatitis/eczema (AD) is a common chronic inflammatory skin disease, affecting about 20% of children and 10% of adults worldwide.[1] Topical corticosteroids (TCS) are efficacious treatments for skin inflammation and the first-line option for mild-moderate AD. However, the long-term inappropriate use of TCS carries a risk of adverse effects on the skin, including for example atrophy (skin thinning), striae, telangiectasia and rebound flare.[2] Managing these risks is hindered by uncertainty amongst healthcare professionals over how best to use TCS safely.[3] A barrier to optimising new and existing therapy has been the inability to measure, longitudinally and without the collection of biopsies, the early adverse skin changes that presage the development of clinical adverse effects. Therefore, new tools are needed to rapidly and non-invasively assess the effects of treatments on the skin. These tools should facilitate treatment comparisons based on their local safety to optimise treatment regimens and improve treatment success and long-term control.

Here we showcase the application of two emerging technologies, OCT and ATR-FTIR spectroscopy, for the non-invasive evaluation of the effects of topical AD treatments on the skin. OCT is a non-invasive, high-resolution imaging modality that produces cross-sectional skin images comparable in spacial resolution and contrast to haematoxylin and eosin-stained histological sections of biopsy tissue.[4] ATR-FTIR is a form of molecular spectroscopy ideally suited to determining the chemical composition of surfaces. Applied to the skin, ATR-FTIR reveals marked changes in stratum corneum (SC) between AD patients and healthy controls and can be used to quantify natural moisturising factor (NMF) levels associated with skin health and determine a person's *FLG* phenotype and associated predisposition to AD.[5]

The aim of this study was to develop a new approach to evaluating the effects of AD interventions, utilising OCT and ATR-FTIR. To do this we conducted a clinical trial to directly compare the effects of the potent TCS betamethasone valerate (0.1% cream) to the non-steroidal anti-inflammatory crisaborole (2% ointment) on the properties of the skin.[6]

MATERIALS AND METHODS

Study design and setting

An observer-blind, randomised, within-subject controlled clinical trial in up to 37 AD patients (target to complete 33 allowing for 10% loss-to-follow), wherein each participant underwent 4 weeks of treatment with crisaborole on one forearm and betamethasone on the other (twice daily application in each case and randomised site allocation). At baseline, the skin of the test sites (volar forearms) was clear of the signs of AD so the investigation focused on local adverse effects on the skin as opposed to anti-inflammatory effects. The condition of the skin was assessed before, during and after treatment. A post-treatment washout period of 4 weeks was included to establish how quickly skin changes re-adjust to baseline.

The study (NCT04194814) was conducted at the Sheffield Dermatology Research Skin Barrier Research Facility within the Royal Hallamshire Hospital from Dec 2020 until Sept 2021. The East Midlands—Derby Research Ethics Committee approved the study, under project reference 20/EM/006. It was performed in accordance with the Helsinki Declaration of 1964, and its later amendments and all subjects provided informed consent to participate.

Participants

A single cohort of participants with AD not currently undergoing or requiring active drug treatment at baseline

was recruited. Inclusion criteria included: an AD diagnosis according to the UK working party diagnostic criteria, and being male or female aged 18–65 years old. See supplementary methods for exclusion criteria. Informed consent was obtained from each participant before the collection of demographic information including date of birth, skin type, ethnicity, eczema history and scheduling of the first assessment visit. All participants received compensation appropriate for their involvement.

Treatment

Participants were instructed to apply 1 fingertip unit of product (Table 1) to the appropriate volar forearm (randomised right/left allocation) twice per day (morning and evening, separated by >6 h). All participants received product application training at the start of treatment and undertook the first application under supervision. For details on monitoring treatment compliance, masking and randomisation please refer to the supplementary methods.

Outcomes

The primary outcome was the difference in the change in epidermal thickness (day 29–day 1), measured from OCT images, between the sites treated with crisaborole and betamethasone. Secondary outcomes included the difference in change in epidermal thickness, visual redness, objective redness, TEWL, and visual dryness during and after 28 days of treatment and the difference in skin barrier (SB) integrity and SC NMF levels after 28 days of treatment. Further analysis of OCT images and ATR-FTIR spectra were exploratory outcomes. Further details on how the outcomes were measured can be found in the supplementary methods.

Sample size determination

The sample size was determined based upon the assumption that the change from baseline in epidermal thickness at day 28 in the betamethasone group will be approximately −4.44 μm with a standard deviation of 5 μm (amounting to 5% epidermal thinning) based upon prior observations.[7] To detect a reduction in this change of 80% (to −0.88 μm) in the crisaborole arm with 80% power, a parallel group study requires 33 participants in each group. We have assumed that this within-participant controlled study will have greater power than a parallel group design.

Statistical analyses

Analyses were done with SAS version 9.4. Graphs were prepared using Prism 9 (GraphPad Software, La Jolla, USA). Due to the exploratory nature of the secondary and exploratory endpoints no multiple testing adjustment was made, therefore, all *p*-values presented for these endpoints should be considered to be nominal. Results are presented as mean ± *SD* unless otherwise indicated (see Supporting Information methods for further details).

RESULTS

A total of 48 adult participants consented to take part in the study and were screened for eligibility. Of these, 37 participants with a confirmed diagnosis of AD were randomised and received the first dose of the study treatments (Figure 1). Thirty-two participants completed the study. There were no important protocol deviations, therefore, no additional per-protocol analyses were carried out (see Table 2 for cohort demographics). The average daily usage of each product was similar (2.13 ± 0.158 g for crisaborole and 2.12 ± 0.204 g for

TABLE 1 Study treatments.

Name	Pack size	Manufacturer	Formulation
Betamethasone (valerate, 0.1% cream)	100 g tube (7 mm nozzle)	Glaxo Wellcome UK Ltd	*Betamethasone Valerate BP 0.122%,* Chlorocresol BP, Cetomacrogol 1000 BP, Cetostearyl alcohol BP, White soft paraffin BP, Liquid paraffin BP, Sodium Acid phosphate BP, Phosphoric acid BP, Sodium hydroxide BP, purified water
Crisaborole (2% ointment)	60 g tube[a] (7 mm nozzle)	Pfizer	*Crisaborole 2%,* White petrolatum, Propylene glycol, Mono- and Di-glycerides, Paraffin, Butylated Hydroxytoluene, Edetate calcium disodium

[a]Two units dispensed to each participant.

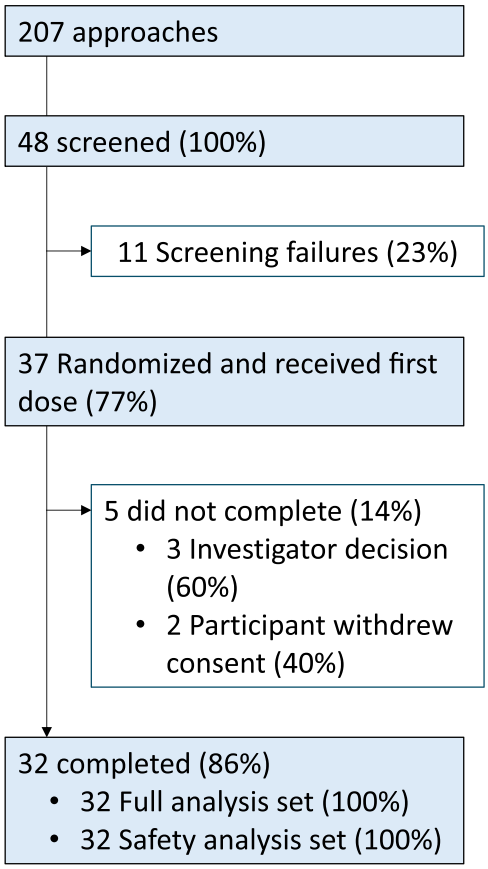

FIGURE 1 Participant flow diagram.

betamethasone) and all participants were fully compliant with the 28-day treatment regimen.

The effect of the treatments on epidermal thickness is presented in Figures 2 and 3a. At baseline, the epidermis at the clinically clear areas of skin on the forearm to be treated with either crisaborole or betamethasone is comparable in thickness (100.8 ± 17.74 vs. $99.5 \pm 15.38\,\mu m$). Over the 28-day treatment period, both sites display decreasing epidermal thickness, however, the reduction is more pronounced at sites treated with betamethasone. After 28 days of treatment, there is a significant difference in the effects of the two treatments on epidermal thickness; with betamethasone inducing 2.3x more thinning than crisaborole (-31.66 vs. $-13.76\,\mu m$, respectively, primary outcome, Table 3). The greatest changes in epidermal thickness were observed during the first 2 weeks of treatment. In the subsequent 2 weeks epidermal thickness remains unchanged at the sites treated with crisaborole but appears to continue on a downward trajectory, albeit with a reduced rate (mean reduction in epidermal thickness by $28.65\,\mu m$ from baseline to day 15 and by $31.66\,\mu m$ from baseline to day 29), at sites treated with betamethasone. Following the cessation of treatment on day 29, epidermal thickness increases towards baseline values at both sites. In contrast to the sites initially treated with crisaborole, the recovery is more rapid at the betamethasone-treated sites, but they fail to fully recover to baseline levels. There was no association between the change in epidermal thickness and the actual number of days of treatment or the average daily dose (data not shown). Stratification by *FLG* genotype revealed no effect of *FLG* status on epidermal thickness (at baseline) or the atrophic response to treatment (Figure S1).

As part of the exploratory analysis vascular changes in the dermis were analysed from the OCT images. The depth of the superficial vascular plexus decreased in response to treatment, with the greatest changes mainly observed at sites treated with betamethasone compared to crisaborole (Figures 2 and 3b). After 28 days of treatment, an ad hoc analysis of the data revealed a significant difference between the treated areas (Table 3). Following the cessation of treatment, the depth of the superficial plexus shifted back towards baseline values consistent with the changes in epidermal thickness. There were no notable changes in vessel diameter (54.3 ± 3.72 vs. $53.7 \pm 2.67\,\mu m$ on day 29) or density (36.0 ± 13.88 vs. 30.0 ± 12.01 on day 29) between the treated areas throughout the study (Figures S2 and S3).

The birefringent properties of the skin derived from PS-OCT images (Figure 2) were used to derive a collagen matrix index (CMI); an objective OCT-derived value associated with the structural alignment of collagen fibres in the dermis.[8] CMI was largely unaffected by crisaborole treatment but increased markedly after betamethasone treatment suggestive of structural changes (Figure 3k). An ad-hoc analysis of the data revealed a significant difference between the effects of the treatments (Table 3).

To determine the effect of the treatments on the SB, TEWL measurements were made at each visit (Figure 3c). TEWL values appear to increase marginally at crisaborole-treated areas and remain elevated for the duration of the study. In contrast, TEWL values appear to decrease slightly at sites treated with betamethasone during treatment but subsequently increase after treatment cessation to match the levels at the other site. Accordingly, TEWL change from baseline to days 15 and 29 are significantly different at sites treated with betamethasone compared to those treated with crisaborole (Table 3). Despite a marginally higher baseline TEWL, carriage of *FLG* variant alleles did not noticeably affect TEWL changes in response to treatment (Figure S4). Increases in TEWL can be associated with either decreased SB function or increased skin hydration in normal-appearing skin.[9] Visual assessment revealed no surface dryness in the majority of participants at either of the test sites (Table S1). To help elucidate the effects, SB integrity was determined by measuring TEWL in conjunction with skin tape stripping (STS) to experimentally

TABLE 2 Cohort demographics.

Parameter	Statistic	Total
Age (years)	*n*	37
	Median (min, max)	23 (18, 60)
Ethnicity	White – UK	26 (70%)
	Any other White background	3 (8%)
	Any other background	8 (22%)
Gender	Female	24 (65%)
	Male	13 (35%)
Fitzpatrick skin type	Very fair (1)	5 (14%)
	Fair (2)	12 (32%)
	Medium (3)	14 (38%)
	Olive (4)	4 (11%)
	Brown (5)	2 (5%)
AD?	Yes	37 (100%)
Current severity of eczema (ISGA)	0 - Clear	7 (19%)
	1 - Almost clear	19 (51%)
	2 - Mild	8 (22%)
	3 - Moderate	3 (8%)
Current severity of eczema (EASI)	Median (min, max)	0.6 (0, 9.9)
How long ago was the last flare (months)	Median (min, max)	1 (0, 10)
In the past 12 months, how many times did the eczema relapse/flare up?	0	1 (3%)
	1	2 (5%)
	2	6 (16%)
	3	4 (11%)
	>3	21 (57%)
	Missing	3 (8%)
Allergies (any)?	No	31 (84%)
	Yes	5 (14%)
	Missing	1 (3%)
Number of patients with any known *FLG* variant allele	*n* (%)	9 (24%)
Average daily product use (g ± *SD*)	Crisaborole (mean ± *SD*)	2.13 ± 0.158
	B. valerate (mean ± *SD*)	2.12 ± 0.204

disrupt the SC (Figure 3d). A higher rate of increase in TEWL with each successive round of STS is indicative of reduced SB integrity. TEWL after 20 consecutive STS ($TEWL_{20}$) was significantly higher at sites pre-treated for 28 days with betamethasone compared to crisaborole (mean difference: −11.31, 95% confidence interval [CI]: −17.37, −5.24 $g/m^2/h$) indicative of reduced integrity of the SB (Table 3).

ATR-FTIR-spectra were collected from intact skin sites and during STS (day 29 only) to assess the effects of the treatments on the molecular composition of the skin. ATR-FTIR has a sampling depth of 1.0–1.5 μm, so STS is required to profile the structure of the SC across its depth. Representative spectra of the skin surface and SC (after 5 STS) are presented in Figure 4, and show broad differences between the skin sites after treatment. This

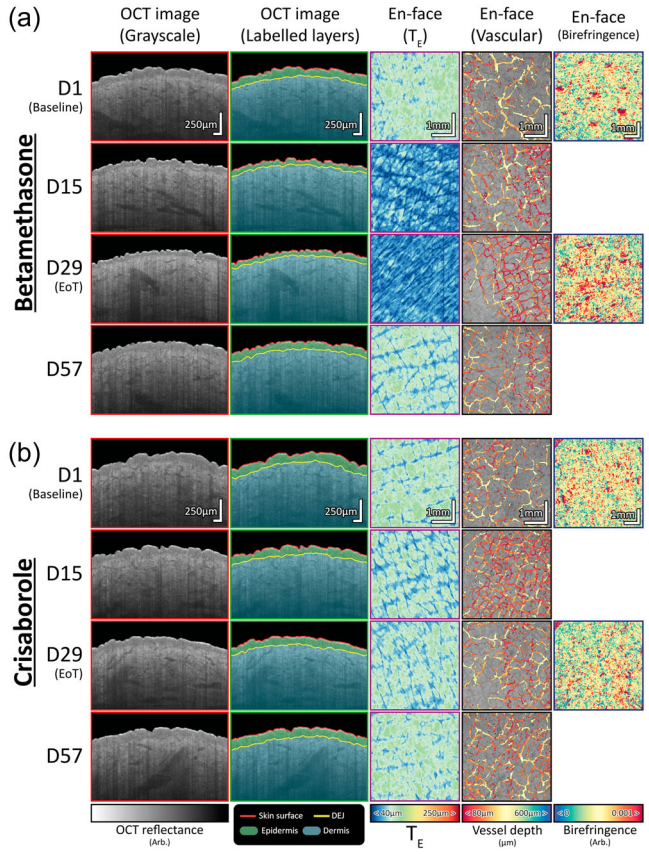

FIGURE 2 Representative OCT and PS-OCT data. Taken from areas treated with betamethasone (a) and crisaborole (b). The first column shows cross-sectional OCT B-scans for each of the study visits. The second column shows these same B-scans with the skin layers labelled for clarity. The third column shows each OCT volume from a top-down (*en-face*) perspective, showing a parametric map of the epidermal thickness (T_E). The fourth column shows the depth encoded vascular network as resolved by OCT-Angiography. The final column shows cross-sectional PS-OCT B-scans of skin tissue birefringence. The final column shows the en-face birefringence computed from the phase-retardance profile of each PS-OCT volume.

includes increased absorbance at 3300 cm^{-1} associated with the O-H bond of water and at 1410 and 1340 cm^{-1} wavenumbers corresponding to the carboxyl functional group of NMF constituents at sites treated with crisaborole compared to betamethasone. Both treatments, delivered from lipid-rich vehicles, were associated with large increases in lipids on the skin surface compared to baseline as expected. Within the SC matrices, there was a higher proportion of lipids following crisaborole treatment compared to betamethasone, which is likely due to the differences in vehicle format (ointment vs. cream).

Quantitative analysis of the carboxyl bands reveals reduced surface levels of carboxyl groups (relative to the amide II group of protein) with treatment, but the reduction is greater with betamethasone compared to crisaborole (Figure 3e, Figure S5 and Table 3). Following cessation of treatment, the levels of carboxyl groups recover at both treatment areas to baseline levels. Profiling carboxyl groups across the SC depth confirmed that the surface measurements reflect changes occurring within the SC (Figure 3f and Table 3). Quantification of SC NMF metabolites on day 29 by laboratory assay suggests that a broad reduction in NMF constituents may contribute to this change in carboxyl groups, with a significant difference in urocanic acid levels noted between treatments (Figure 3g–i). Lipid chain conformational order was assessed by quantifying the mean frequency of the lipid peak at 2850 cm^{-1} (corresponding to the symmetric stretching of the CH_2 group of lipids) across the 5 depths sampled during STS (Figure 3k). The frequency of the lipid peak was slightly higher at sites treated with betamethasone indicative of less ordered SC lipid packing (not significant, Table 3).

With regard to tolerability, a small proportion of participants in both groups displayed a small increase in visual redness on day 29 which improved slightly following the cessation of treatment by day 57. There were no clear differences in visual redness between the treatment groups (Table S2). Objectively measured skin redness suggested a slight decrease in response to both treatments, which normalised by 28 days post-cessation of treatment. The effect was significantly more pronounced at sites treated with betamethasone (Table 3). Thirty (81%) participants experienced at least 1 treatment-emergent AE, of which only 1 participant experienced a serious AE which was unrelated to treatment. Table S3 lists the AEs that could be related to a single treatment, all of which were either known AEs associated with the treatments or expected in this population.

Conditional logistic regression analyses were employed to investigate which of the exploratory biomarkers measured could discriminate between the baseline and day 29 observations of skin sites treated with betamethasone. Sites treated with crisaborole were not used, as the expectation was that crisaborole would produce less epidermal atrophy. In the first step, 6 variables were identified for distinguishing between treated and untreated skin (Table S4). Next, a stepwise approach was used to investigate subsets of variables which could discriminate between treated and untreated skin. A shallow superficial plexus and low level of carboxyl groups in the SC (Figure 5 or CMI, see Figure S6) were most strongly associated with betamethasone treatment and provided the greatest ability to differentiate treated from untreated skin [superficial plexus depth: odds ratio 0.02424 (95% CI: 0.01291, 0.04552), carboxyl groups: odds ratio 0.00022 (95% CI: 0.00005, 0.00105)]. Overlaying the

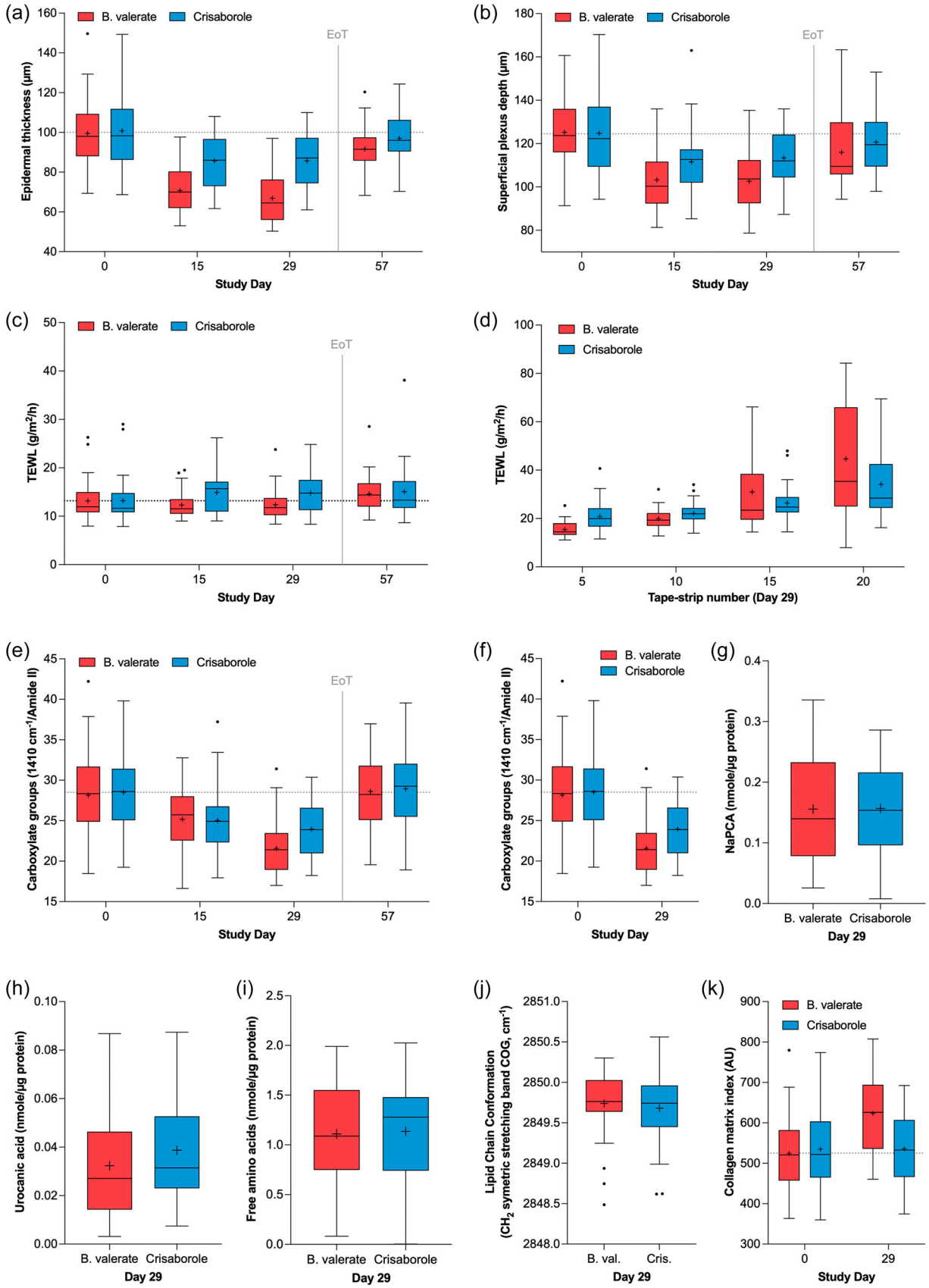

FIGURE 3 (See caption on next page).

data gathered from Crisaborole-treated skin (not part of the model) reveals a pattern that is more closely aligned to untreated skin than betamethasone-treated skin (Figure 5).

DISCUSSION

In patients with AD, the application of the corticosteroid betamethasone twice daily for 2 weeks to areas of skin without visible signs of inflammation led to substantial thinning of the epidermis (by approximately 30%). After 4 weeks of treatment, the level of thinning seen was 2.3x greater than observed with crisaborole following the same regimen. Thinning of the epidermis was accompanied by concordant changes in vascular structure. Compared to areas of skin treated with betamethasone, those treated with crisaborole displayed greater SB integrity, which was supported by subtle improvements in SC molecular structure. The effects of both treatments on the skin were transient. Whilst baseline values were almost fully restored 28 days following the last dose of crisaborole, evidence of epidermal thinning persisted in the areas treated with betamethasone.

The findings confirm previous studies showing the atrophic effects of TCS on the skin and validate this model for studying the local adverse effects of this class of treatment.[7] We show that OCT is a highly sensitive method for quantifying the atrophic effects of TCS on the skin and further characterise the changes by revealing the associated change in the position of the superficial vascular plexus that sits directly below the rete pegs of the papillary dermis. The reduced depth of the superficial vascular plexus confirms the significant thinning of the epidermis, including the papillary dermis. Remarkably we observed high levels of thinning, representing a mean reduction of 30% in the epidermal layer, even after just 2 weeks of treatment. This is higher than previously reported,[7], which may be due to the robust training and regular compliance monitoring undertaken here to fully appreciate the effects of compliance with this regimen. It is widely accepted that compliance with topical therapy is usually low. The transient nature of TCS-induced epidermal and dermal thinning has been reported, however, we show that recovery can take more than twice as long as the 2 weeks previously suggested.[7]

Inflammation brings about significant thickening of the epidermis (hyperplasia), and so thinning that brings the epidermis back down to the normal physiologic range is desirable.[10] At baseline epidermal thickness is 'close' to the level expected for healthy skin at the volar forearm, however, the lack of a robust reference dataset in a large population makes this determination challenging.[11,12] We note that the SD is slightly higher at baseline compared to the measurements made 28 days after the cessation of treatment and markedly higher compared to data from other healthy cohorts.[4] All participants either have current signs of AD at other anatomical locations or have a recent history of AD. We have previously shown that unaffected sites in AD patients with active AD display epidermal thickening in the region of 5%–10%.[4] Therefore, sub-clinical inflammation may be present in at least some of the participants at baseline, and could contribute to the high variance. Whilst the reduction of pre-existing sub-clinical inflammation may explain a small degree of thinning in response to treatment, it is very unlikely to explain the marked thinning at betamethasone-treated sites that takes epidermal thickness outside of the expected range for untreated skin.[4,7,11] Establishing a robust reference dataset for epidermal thickness is essential for future interpretation of the nature of epidermal thinning.

Skin atrophy is caused by the suppression of cell proliferation in the dermal and epidermal layers.[2] In the dermis, the reduced growth of fibroblasts is accompanied by reduced synthesis of collagen. PS-OCT captures information about collagen fibre alignment.[13] Striae for example are visible scars that form along mechanical stress lines where collagen is deposited. The development of striae is a recognised adverse effect of chronic TCS use.[2] Using PS-OCT the alteration in collagen density and structure of striae can be visualised due to the changes in birefringence.[14] During the relatively short 28-day treatment regimen undertaken in this study, betamethasone, but not crisaborole, significantly increased the birefringent properties of the dermis, suggesting an alteration in collagen density or arrangement. The banding pattern of birefringence, evident at striae lesions,

FIGURE 3 Quantitative study outcomes by study day. (a) OCT-derived epidermal thickness; (b) superficial plexus depth derived from OCT-angiography; (c) TEWL; (d) SB integrity on day 29; (e) ATR-FTIR-determined skin surface carboxyl groups; (f) SC mean carboxyl groups relative to amide II groups (determined in conjunction with STS on day 29); (g) HPLC-derived SC pyrrolidone carboxylic acid level; (h) HPLC-derived SC urocanic acid levels; (i) SC free amino acid levels; (j) ATR-FTIR-determined mean SC lipid structure; (k) PS-OCT-derived collagen matrix index (CMI). Boxes indicate the median, 25th and 75th percentiles, with '+' for the mean and whiskers showing 1.5x interquartile range (IQR). Treatment ended on day 29 (EoT).

TABLE 3 Study outcomes.

	Treatment mean (95% confidence limit)		Treatment comparison (Crisaborole – Betamethasone)		
	Crisaborole	**B. valerate**	**Mean**	**95% CI**	***p*-value[1]**
Epidermal thickness (μm)					
Baseline to day 29[a]	−13.76 (−17.42, −10.10)	−31.66 (−35.31, −28.01)	17.90	(14.93, 20.86)	<0.0001[b]
Baseline to day 15[c]	−14.41 (−17.35, −11.48)	−28.65 (−31.58, −25.73)	14.24	(12.13, 16.35)	<0.0001[b]
Days 29–57[c]	11.63 (8.07, 15.19)	24.94 (21.39, 28.50)	−13.31	(−16.67, −9.96)	<0.0001[b]
Superficial plexus depth (μm)[d]					
Baseline to day 29	−9.51 (−16.99, −2.02)	−20.34 (−26.63, −14.06)	10.84	(3.24, 18.44)	0.0067[e]
Collagen matrix index (AU)[d]					
Baseline to day 29	−3.99 (−32.32, 24.33)	100.02 (63.21, 136.84)	−104.02	(−137.95, −70.08)	<0.0001[e]
TEWL ($g/m^2/h$)[c]					
Baseline to day 29	2.06 (0.96, 3.16)	−0.52 (−1.62, 0.59)	2.58	(1.49, 3.66)	<0.0001[b]
Baseline to day 15	1.66 (0.85, 2.48)	−0.92 (−1.74, −0.11)	2.59	(1.90, 3.27)	<0.0001[b]
Days 29–57	0.21 (−1.26,1.67)	2.25 (0.79, 3.72)	−2.04	(−3.71, −0.38)	0.0172[b]
Skin barrier integrity ($TEWL_{20}$, $g/m^2/h$)[c]					
Day 29	34.13 (28.84, 39.42)	45.44 (37.14, 53.74)	−11.31	(−17.37, −5.24)	0.0006[e]
ATR-FTIR determined skin surface carboxyl group levels (AU)[d]					
Baseline to day 29 ($1410\,cm^{-1}$ band)	−4.88 (−6.84, −2.91)	−6.75 (−8.84, −4.65)	1.87	(0.25, 3.49)	0.0252[e]
Baseline to day 29 ($1340\,cm^{-1}$ band)	1.31 (−2.40, 5.01)	−2.45 (−4.22, −0.69)	3.76	(−0.26, 7.77)	0.0654[e]
Lipid chain conformational order (symmetric stretching of CH_2 band centre of gravity, cm^{-1})[d]					
Day 29	2849.68 (2849.52, 2849.84)	2849.74 (2849.59, 2849.89)	−0.058	(−0.154, 0.037)	0.2219[e]
SC NMF levels, day 29 (nmol/μg protein)					
Total NMF[c]	1.331 (1.119, 1.543)	1.299 (1.080, 1.518)	0.032	(−0.161, 0.224)	0.7395[e]
Pyrrolidone carboxylic acid[f]	0.157 (0.131, 0.182)	0.156 (0.125, 0.186)	0.001	(−0.023, 0.025)	0.9228[e]
Urocanic acid[f]	0.039 (0.031, 0.046)	0.032 (0.024, 0.040)	0.006	(0.001, 0.012)	0.0215[e]
Free amino acids[f]	1.135 (0.947, 1.323)	1.111 (0.925, 1.297)	0.024	(−0.142, 0.190)	0.7693[e]
Objective redness (AU)[c]					
Baseline to day 29	−1.16 (−17.04, 14.72)	−27.61 (−43.48, −11.73)	26.44	(12.40, 40.48)	0.0005[b]
Baseline to day 15	−23.61 (−37.65, −9.57)	−45.04 (−59.08, −31.01)	21.43	(9.70, 33.16)	0.0006[b]
Days 29–57	2.84 (−12.02, 17.69)	37.66 (22.80, 52.51)	−34.82	(−50.16, −19.48)	<0.0001[b]

[a]Primary outcome.

[b1]*p*-value from repeated measures mixed model with change as the outcome, treatment, time point as factors together with a treatment by time point interaction, subject as a random effect and baseline as a covariate. Means and confidence intervals (CI) estimated from the same model.

[c]Secondary outcome.

[d]exploratory outcome.

[e]*p*-value from ad hoc paired *t*-test comparing crisaborole and betamethasone; AU, Arbitrary units. NB *p*-values, other than for the primary objective, should be considered nominal as no multiple testing adjustment has been made.

[f]additional ad hoc analyses.

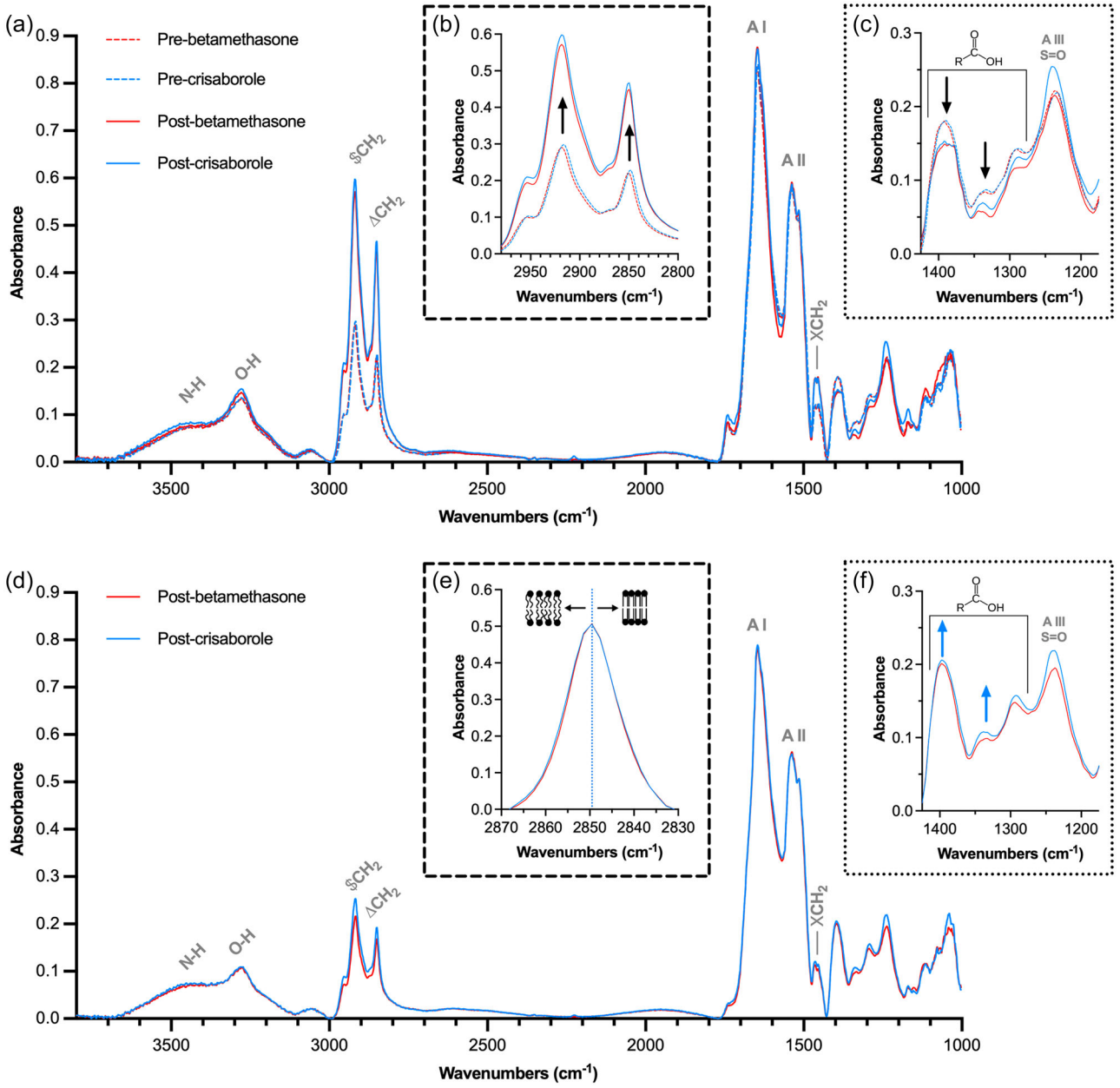

FIGURE 4 Average ATR-FTIR spectra. Spectra were collected from (a) the skin surface and (d) within the SC (collected after 5 STS) before (dashed line) and after (solid line) treatment with crisaborole (blue) and betamethasone (red). All spectra were baseline corrected and normalised (to the amide II band at 1550 cm^2) before averaging. (b) magnified spectra showing the asymmetric and symmetric stretching of CH2 (of lipids) at ~2950 and ~2850 cm^{-1}, respectively; treatment is associated with significantly increased levels of lipids (black lines). (e) Magnified spectra showing the symmetric stretching of the CH_2 functional group of lipids at ~2850 cm^{-1} (hatched box). When SC lipids are tightly packed in the orthorhombic formation critical for water permeability barrier function the absorbance of this band appears at a low frequency (no difference between treatment observed). (c, f) Magnified spectra showing the bands in the region from 1180 to 1420 cm^{-1} associated with the carboxyl group, common to natural moisturising factor (NMF) components in the skin (dashed box). Spectral bands are labelled by functional group: $\$CH_2$, asymmetric stretching vibration of CH_2; ΔCH_2, symmetric stretching vibration of CH_2; XCH_2, scissoring vibration of CH_2; AI, amide I functional group; AII, amide II; AIII, amide III; O-H of water and N-H, NH stretch of primary amine.

was not clearly observed in this study, consistent with the absence of visible scar tissue after the short treatment duration. However, this change may be an early biomarker of pathophysiologic changes that lead to striae development.

Skin blanching is a known consequence of TCS exposure that was only observed following betamethasone treatment.[2] There was no evidence of vasoconstriction or an increase in vessel density associated with either treatment. Several factors may have limited our ability to

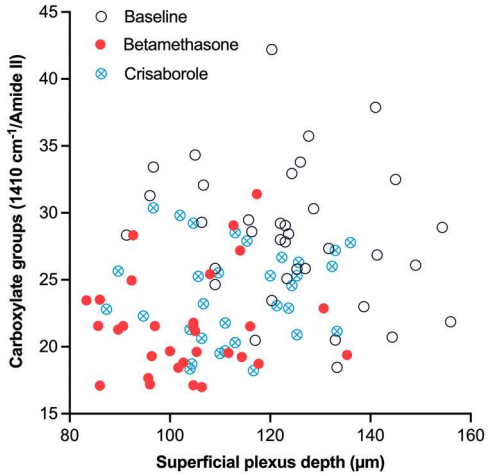

FIGURE 5 Conditional logistic regression modelling. Superficial plexus depth (μm, x axis) and ATR-FTIR-determined skin surface carboxyl groups (1410 cm^{-1}) relative to amide II (AU, y axis) are identified as candidate biomarkers for discriminating skin treated with TCS (betamethasone, day 29 of treatment, red circles) from untreated (baseline measurements, clear circles) skin. Betamethasone treatment is associated with a shift in skin properties towards the low left-hand corner. Overlaying the measurements for crisaborole-treated skin (day 29, blue crossed circles—not part of this statistical analysis) reveals a distribution that lies between treated and untreated skin.

measure such changes including the level of precision obtained for these fine measurements and the effect of temperature on the blood vessels (thermoregulation). It is also possible that skin blanching is driven more by changes in the deeper skin vessels. The considerable shift in superficial vascular plexus position, bringing it closer to the surface of the skin is consistent with epidermal atrophy and may mark early changes leading to telangiectasia, another adverse effect of TCS use.[2] Further work is required to establish whether the observed changes in collagen matrix index and superficial plexus depth are important early skin changes associated with striae and telangiectasia, and at what point these changes become irreversible.

A significant difference in the effects of the treatments on TEWL was observed; with betamethasone-treated sites displaying a lower level than crisaborole-treated sites.[15] In normally hydrated skin (as in this case) increases in TEWL are seen with increased levels of hydration.[9] Supporting this we also see a comparative increase in the levels of SC carboxyl groups that are required to bind water. Laboratory analysis of SC samples suggests that this change is attributed to variations in the levels of specific components of NMF, including urocanic acid.[16] Supporting the reduction in SB function at sites treated with betamethasone compared to crisaborole is the lipid structure data, which suggests a small shift towards a less ordered arrangement throughout the upper half of the SC. Changes in lipid structure, indicated by a shift in the 2850 cm^{-1} ATR-FTIR band frequency of skin have previously been associated with SB integrity, and AD severity.[17–19] Taken together these lines of evidence point towards increased skin hydration and improved SB condition at sites treated with crisaborole compared to betamethasone. Stratification by *FLG* genotype, which has been shown to affect the effect of SB-targeted therapies,[20] revealed no effect on the response to either anti-inflammatory treatment.

Limitations and generalisability

The results of this study clearly show that compared to crisaborole treatment, the prolonged use of betamethasone on clinically clear-appearing skin can induce marked epidermal atrophy and SB damage. The extent of the damage will most likely depend on age, anatomical site, ethnicity and the level of sub-clinical inflammation because all of these factors are associated with TCS uptake.[21] It is well established that different types of corticosteroids, different potencies and differences in vehicle formulation can affect the level of atrophy observed. Subsequent trials will need to explore the effects of lower potency TCS and reduced frequencies of dosing. Given the rapid onset of atrophy just 2 weeks into the regimen and the slower, but continuing, progression of atrophy in the subsequent 2 weeks it will be important to establish whether a saturation point has been reached.

Clinical implications

In the 2013 eczema priority setting exercise, the best and safest way of using TCS was identified as the most important uncertainty shared by patients and healthcare professionals.[3] Addressing this priority has been impeded by a lack of tools to assess the effects of TCS over short-treatment durations for which the current marketing authorisation restricts their use. Here we present a panel of new non-invasive biomarkers that can quantify the early, and transient, sub-clinical changes associated with local adverse effects of TCS. Of these biomarkers, superficial plexus depth and the level of SC carboxyl groups, or superficial plexus depth and CMI had the greatest ability to discriminate between TCS-treated and untreated skin. This will enable broader testing of the effects of different TCS regimens, varied by dose, frequency, potency and population to establish the parameters for safe use. Not only is the aim to prevent harm but also to provide data to reassure patients concerned about

the adverse effects of TCS (in the context of steroid phobia) of the safe ways in which TCS can be effectively used to encourage greater compliance. In this preliminary trial, we establish that 28 days of treatment with betamethasone induces significant pathologic epidermal thinning when applied to normal-appearing skin. Whilst this was transient, it took longer than 28 days for the skin to recover, which should inform how this treatment is used.

In addition, we demonstrate that crisaborole does not induce the same level of damage as betamethasone. The reduced risk of adverse effects means treatment can continue for longer to ensure sub-clinical inflammation is inhibited. Proactive treatment strategies to maintain inflammation-free skin have been shown to increase the period of remission between flares.[22–24] We propose that local safety is an important consideration alongside efficacy when developing a long-term treatment strategy to control AD. The effect of treatments on epidermal and dermal atrophy, could therefore be an important outcome on which future reimbursement is based.

CONCLUSIONS

Betamethasone induced 2.3x more thinning of skin than crisaborole following 4 weeks of treatment with differences seen as early as 2 weeks from starting treatment. This suggests that crisaborole may be more suitable for longer-term and proactive-based treatment strategies where the risks of TCS-induced atrophy are greatest. By monitoring the effects of these therapies on the skin using OCT and ATR-FTIR spectroscopy, two new non-invasive biomarkers of skin health have been identified. These 'at-the-bedside' tests have the potential to help optimise future safe topical anti-inflammatory treatment regimens.

AUTHOR CONTRIBUTIONS

Conceptualisation: Simon G. Danby, Stephen Matcher, Amy Cha, Roni Adiri, John Werth, and Michael J. Cork. *Methodology*: Simon G. Danby, Stephen Matcher, Rosie Taylor, Amy Cha, Roni Adiri, Chuanbo Zang, John Werth, and Michael J. Cork. *Investigation*: Simon G. Danby, Robert Byers, Sura Sahib, Paul Andrew, Kirsty Brown, Linda Kay, Carl Wright, Abi Pinnock, John Chittock, and Mengqiu Duan. *Visualisation*: Rosie Taylor. *Funding acquisition*: Simon G. Danby, Stephen Matcher, and Michael J. Cork. *Project administration*: Simon G. Danby. *Supervision*: Simon G. Danby, Stephen Matcher, and Michael J. Cork. *Writing – original draft*: Simon G. Danby and Rosie Taylor. *Writing – review & editing*: Simon G. Danby, Stephen Matcher, Robert Byers, Rosie Taylor, Sura Sahib, Paul Andrew, Kirsty Brown, Linda Kay, Carl Wright, Abi Pinnock, John Chittock, Mengqiu Duan, Amy Cha, Roni Adiri, Chuanbo Zang, John Werth, and Michael J. Cork.

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CONFLICT OF INTEREST STATEMENT

SGD, has received fees for giving lectures and/or attending advisory boards and unrelated research funding from Almirall, Astellas Pharma, Bayer Dermatology, Leo Pharma, MSD, Pfizer, and Stiefel-GSK who manufacture topical anti-inflammatory treatments for eczema. MJC, has been/is a Clinical Trial Investigator for the following organisations: Atopix, Galapagos, Hyphens, Johnson & Johnson, Kymab, Leo, L'Oreal/La Roche Possay, Novartis, Pfizer, Regeneron, and Sanofi-Genzyme. He is an Advisory Board member, Consultant &/or invited lecturer for the following organisations: Abbvie, Amlar, Astellas, Atopix, Boots, Dermavant, Galapagos, Galderma, Hyphens, Johnson & Johnson, Kymab, Leo, L'Oreal/La Roche Possay, Menlo, Novartis, Oxagen, Pfizer, Procter & Gamble, Reckitt Benckiser, Regeneron, Sanofi-Genzyme. SJM has previously received funding from Michelson Diagnostics. AC, RA, CZ and JW are full-time employees of and shareholders in Pfizer. RB, RT, SS, PA, KB, LK, CW, AP, JC, MD none to declare.

DATA AVAILABILITY STATEMENT

Upon request, and subject to review, the study Sponsor, Sheffield Teaching Hospitals NHS Foundation Trust, will provide data that support the findings of this study. Subject to certain criteria, conditions and exceptions, Sheffield Teaching Hospitals NHS Foundation Trust may also provide access to the related individual de-identified

participant data. Contact Sheffield Teaching Hospitals NHS Foundation Trust for more information.

ETHICS STATEMENT

The study (NCT04194814) was conducted at the Sheffield Dermatology Research Skin Barrier Research Facility within the Royal Hallamshire Hospital from Dec 2020 until Sept 2021. The East Midlands - Derby Research Ethics Committee approved the study, under project reference 20/EM/006. It was performed in accordance with the Helsinki Declaration of 1964, and its later amendments and all subjects provided informed consent to participate. No major amendments to the original protocol were made, and this manuscript has been prepared according to CONSORT guidelines.

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SUPPORTING INFORMATION

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

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