Children with atopic dermatitis show increased activity of β-glucocerebrosidase and stratum corneum levels of glucosylcholesterol that are strongly related to the local cytokine milieu

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Summary

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Background Atopic dermatitis (AD) is characterized by immune dysregulations and an impaired skin barrier, including abnormalities in lipid organization. In the stratum corneum (SC), β-glucocerebrosidase (GBA) mediates transformation of glucosylceramide (GlcCER) into ceramide (CER) and cholesterol into glucosylcholesterol (GlcChol). Alteration in GBA activity might contribute to skin barrier defects in AD.

Objectives To investigate GBA activity in the SC of children with AD before and after topical corticosteroid therapy and to compare it with healthy controls; to determine SC levels of GlcCER- and CER-containing hydroxysphingosine base (GlcCER[H] and CER[H], respectively) and GlcChol; and to relate them to disease severity, skin barrier function and the local cytokine milieu.

Methods Lipid markers and cytokines of innate, T helper 1 and T helper 2 immunity were determined in SC collected from healthy children and from clinically unaffected skin of children with AD, before and after 6 weeks of therapy with topical corticosteroids. AD severity was assessed by Scoring Atopic Dermatitis and skin barrier function by transepidermal water loss (TEWL).

Results Baseline GBA activity and GlcChol levels were increased in children with AD but declined after therapy. CER[H] levels and the CER[H] to GlcCER[H] ratio were increased in AD. GBA activity and GlcChol correlated with TEWL and levels of multiple cytokines, especially interleukin-1α and interleukin-18. GlcChol was strongly associated with disease severity.

Conclusions We show increased GBA activity and levels of GlcChol in AD. Our data suggest an important role of inflammation in disturbed lipid processing. GBA activity or GlcChol might be useful biomarkers in the monitoring of therapeutic responses in AD.

> **What is already known about this topic?**
>
> - Patients with atopic dermatitis (AD) have a reduced skin barrier, mainly caused by altered lipid organization.
> - The mechanisms underlying these lipid anomalies are not fully understood but likely reflect both genetic abnormalities in AD skin and the local cutaneous inflammatory environment.

What does this study add?

- We show increased activity of the ceramide-generating enzyme β-glucocerebrosidase in AD. Activity of this enzyme was correlated with the local cytokine milieu and declined after local corticosteroid therapy.
- We show that glucosylcholesterol levels in the stratum corneum are increased in AD.
- The function of glucosylcholesterol and the physiological consequences of increased levels are not clear yet; however, its levels were strongly correlated with skin barrier function: high transepidermal water loss strongly correlated with high levels of glucosylcholesterol.

What is the translational message?

- Correction of cutaneous inflammation largely restores alterations in lipid metabolism in the stratum corneum of infants with AD.

Atopic dermatitis (AD) is a highly prevalent inflammatory skin disease with a broad range of abnormalities in immune response and skin barrier function, even in nonlesional skin.[1] Skin barrier function resides largely in the uppermost layer of the skin, the stratum corneum (SC), composed of anucleated cells (corneocytes), which are filled with keratin filaments aligned and aggregated by filaggrin protein.[2] Corneocytes are wrapped in lipid envelopes with anchoring sites for lipid lamellae, which fill the intercellular space between corneocytes. Highly ordered lipid lamellae are composed of three main classes of lipids: ceramides (CER), free fatty acids and cholesterol.[3]

Altered composition and structure of lipid lamellae are regarded as the major cause of barrier deficiency in AD. Furthermore, SC lipids, in particular CER, are key molecules in cell signalling that contribute to regulation of vital cellular functions.[4,5] Given the crucial importance of the skin barrier, it is not surprising that skin is highly specialized in the biosynthesis of CER, comprising multiple pathways mediated by numerous enzymes.[6] Several studies have shown alterations in the expression or activity of enzymes involved in elongation of fatty acids in AD, related to reduced skin barrier function.[7,8] In addition, a recent study reported that AD skin has altered expression and/or activity of epidermal β-glucocerebrosidase (in this manuscript referred to as GBA), an acid β-glucosidase that mediates formation of CER from their precursor glucosylceramide (GlcCER).[9,10] In the SC, GBA is the predominant glucosidase.[11] Next to its role in formation of CER from GlcCER, GBA can catalyse transglucosylation reactions between GlcCER and cholesterol, resulting in formation of glucosylcholesterol (GlcChol) (Figure 1).[11,12]

Levels of GlcChol in AD have not been investigated. Reduced expression or activity of GBA is associated with a reduced permeability barrier,[13] while inherited GBA deficiency leads to severe and life-threatening skin abnormalities such as those seen in the lysosomal storage disorder Gaucher disease.[14,15] Additionally, skin barrier disruption leads to an increase in epidermal *GBA* mRNA levels.[16] Skin barrier disruption, a hallmark of AD, might induce expression of GBA in an effort to increase synthesis of

CER and normalize skin barrier function. Recently, Boer *et al* [10] showed an abnormal localization of active GBA, particularly in lesional AD skin, which was related to altered levels of CER and the subclass profile of CER, skin barrier function and local disease severity. The measurement of GBA activity in that study was semiquantitative and no clear insight into the difference in GBA activity between AD and healthy skin could be derived.

In this study we aimed to quantitatively measure GBA activity in nonlesional AD skin before and after topical corticosteroid therapy and compare it with activity in healthy skin. Next to GBA activity, we measured in the SC two enzymatic products of GBA – hydroxylated ceramides (CER[H]) and glucosylcholesterol (GlcChol) – and the substrate for GBA, GlcCER[H]. We chose to measure CER[H] as its formation is exclusively catalysed by GBA and not by acid sphingomyelinase, which contributes together with GBA to the formation of CER[S].[17] To explore whether
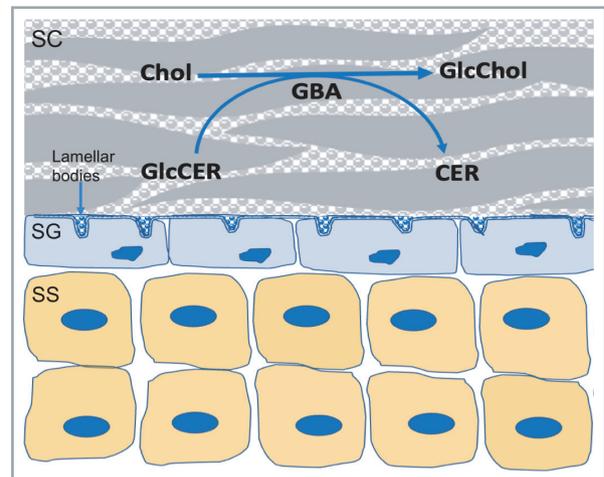

Figure 1 Enzymatic conversion of glucosylceramides (GlcCER) and cholesterol (Chol) mediated by β-glucocerebrosidase (GBA) into, respectively, ceramides (CER) and glucosylcholesterol (GlcChol). SC, stratum corneum; SG, stratum granulosum; SS, stratum spinosum. GBA and GlcCER are extruded from lamellar bodies into the SC at the SC–SG interface.

GBA activity and levels of GlcChol are associated with immune response, inflammation and skin barrier function, we compared levels with disease severity, transepidermal water loss (TEWL) and a range of immune mediators known to play an important role in AD. The study was performed in children on their first presentation when they were treatment naive.

Patients and methods

Study population

Our cohort originates from a larger study in which 100 infants with their first presentation of AD were recruited in a dedicated AD clinic in Children's Health Ireland at Crumlin, Dublin.[18] From the original cohort, three subgroups were randomly selected as follows: 22 children with AD and 17 healthy controls for analysis of GBA activity, 19 children with AD and nine healthy controls for GlcChol, and 17 children with AD and eight healthy controls for analysis of CER. The patients met the Hanifin and Rajka criteria for the diagnosis of AD. Severity of disease was assessed by Scoring Atopic Dermatitis (SCORAD). Objective SCORAD (oSCORAD) is derived from the SCORAD by excluding parental assessment of sleep loss and itch.

TEWL measurements were performed on an area of clinically unaffected skin on the volar forearm using the Tewameter 300 (Courage+Khazaka electronic GmbH, Cologne, Germany). The patients were treatment naive, apart from the use of emollients or over-the-counter hydrocortisone 1% cream or ointment. For controls, children were recruited when attending for elective procedures. They did not have AD, any history suggestive of AD or any other inflammatory skin disease. All patients were asked to refrain from application of any topical agents for 24 h prior to assessment.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the research ethics committee of Children's Health Ireland at Crumlin, Dublin. Written informed consent was obtained from all patients' parents. The demographic and clinical details of the three subgroups are presented in Table 1.

Sampling of the stratum corneum

The SC was collected using circular adhesive tapes (22-mm diameter D-Square Discs; Monaderm, Monaco, France). The tapes were placed on nonlesional forearm skin (2 cm from the nearest lesion) of patients and healthy controls. Adhesive tapes were pressed for 10 s with a pressure of 225 g cm^{-2}, using a D-Square Pressure Instrument D500 (CuDerm Corporation, Dallas, TX, USA). Sequentially, eight consecutive tape strips were collected from the same skin site, placed individually in 2-mL cryovials and immediately stored at −80 °C. For the analysis of GlcChol and GBA activity, the second consecutive tape from the same skin site was used, for CER the third tape, for NMF the fourth tape and for cytokines the fifth tape. In a subgroup of six healthy controls and six patients, GBA activity was determined in the second, sixth and eighth tapes collected at baseline.

Determination of natural moisturizing factor

The analysis of natural moisturizing factor (NMF) was performed by a previously described high-performance liquid chromatography method.[19] NMF was normalized by protein amount, determined by the Pierce Micro BCA Protein Assay Kit (Thermo Fischer Scientific, Rockford, IL, USA).

Determination of cytokines in the stratum corneum

The method for cytokine determination is described in detail elsewhere.[18] Briefly, cytokines were extracted from the tape with phosphate-buffered saline (Merck KGaA, Darmstadt, Germany) containing 0·005% Tween20 (Sigma Aldrich, St Louis, MO, USA). Cytokine concentrations in the extracts were measured on multiplex panels using MESO QuickPlex SQ 120 (MSD, Rockville, MA, USA). We included only cytokines with levels above the limit of detection of the method, including vascular endothelial growth factor, CCL2, CCL4, CCL13, CCL17, CCL22, CXCL8, interleukin (IL)-1a, IL-1b, IL-2, IL-5, IL-13, IL-16 and IL-18.[18] The amount of cytokine in the SC was normalized by protein amount, determined in the extracts using the Pierce Micro BCA Protein Assay Kit.

Determination of β-glucocerebrosidase activity

GBA was extracted from the tape by adding 1·2 mL 1% Triton X100 and ultrasonification (Branson 5800; Branson Ultrasonics BV, Ede, the Netherlands) for 15 min in ice water. All chemicals were obtained from Merck KGaA, except glycine (Serva, Heidelberg, Germany). GBA activity in extracts was determined in a reaction mixture (100 μL) containing 5 mmol L^{-1} 4-methylumbelliferone-β-D-glucopyranoside, 1·5% w/v taurocholate and 0·1 mol L^{-1} citrate/0·2 mol L^{-1} phosphate (pH 5·5). The reaction was started by addition of 50 μL skin tape homogenate and incubated at 37 °C for 2 h. The reaction was stopped by adding 2850 μL of 0·2 mol L^{-1} carbonate/glycine (pH 10·5). The reaction product (4-methylumbelliferone) was measured fluorimetrically using a Perkin Elmer fluorescence detector (excitation 360 nm, emission 450 nm; PerkinElmer, Waltham, MA, USA). The GBA activity was normalized by protein amount, determined from optical density measured by SquameScan (CuDerm Corporation). In a pilot experiment, we proved complete inhibition of enzyme activity using CBE (conduritol B epoxide), a known GBA inhibitor.[20]

Determination of ceramides and glucosylcholesterol

CER and sphingoid bases were extracted from the tapes by methanol–chloroform–water, 12 : 6 : 1, v/v/v. The extract was dried (N$_2$, 40 °C) and phase separation was induced by the addition of methanol–chloroform–100 mmol L^{-1} ammonium formate in 20% formic acid, 1 : 1 : 1, v/v/v in order to separate the free sphingoid bases from the CER and GlcCER.[21] The lower phase, containing CER, was dried (N$_2$, 40 °C) and the residue was dissolved in 500 μL of 0·1 mol L^{-1} sodium hydroxide in

Table 1 Demographic and clinical details of the study participants

	GBA		GlcChol		CER	
	Patients with AD	Controls	Patients with AD	Controls	Patients with AD	Controls
Total n	22	17	19	9	17	8
Sex						
Male	14	9	15	7	12	6
Female	8	8	4	2	5	2
Age (months), median (range)	7·8 (4·7–57·4)	11·2 (3·4–72·0)	7·7 (5·3–24·0)	6·8 (3·0–46·0)	8·1 (5·4–21·0)	7·1 (0·0–9·7)

	T0	T6		T0	T6		T0	T6	
SCORAD									
Average	41.8[c3]	13·1	–	48·0[c3]	19·2	–	45.1[c3]	16·6	–
Range	26·5–72·0	4·0–56·6	–	23·4–96·5	0·0–67·2	–	23·4–65·0	0·0–51·0	–
oSCORAD									
Average	31.2[c3]	12·4	–	36.8[c3]	18·5	–	33.4[c3]	14·3	–
Range	14·9–53·0	3·5–42·6	–	17·4–52·5	0·0–48·2	–	17·4–51·0	0·0–37·0	–
TEWL (g m^{-2} h^{-1})									
Average	18.4[c2]	13·4	11.0[a3,b1]	24.5[c2]	17	12.5[a1]	26.4[c2]	14·5	10.6[a2,b1]
Range	10·6–50·9	8·3–48·0	4·0–15·4	8·3–41·6	8·6–29·2	6·3–29·2	8·3–38·5	9·7–29·2	6·3–15·6
NMF (mmol per g protein)									
Average	0·27	0·36	0.81[a3,b3,d1]	0·19	0·26	0.75[a3,b3]	0·34	0·36	1.01[a3,b3]
Range	0·14–0·61	0·04–0·69	0·53–1·12	0·06–0·77	0·07–1·08	0·57–1·02	0·14–0·77	0·07–0·69	0·82–1·32

AD, atopic dermatitis; CER, ceramides; Ctrl, healthy controls; GBA, β-glucocerebrosidase; GlcChol, glucosylcholesterol; NMF, natural moisturizing factor; oSCORAD, objective Scoring Atopic Dermatitis; SCORAD, Scoring Atopic Dermatitis; TEWL, transepidermal water loss. Differences in age, SCORAD, TEWL and NMF between patients within three study cohorts (GBA, GlcChol and CER) were tested by Student's t-test [AD$_{T0}$ vs. Ctrl (a) and AD$_{T6}$ vs. Ctrl (b)] or paired t-test [AD$_{T0}$ vs. AD$_{T6}$ (c)]. Differences between patients with AD and healthy controls within the three study cohorts were tested by ANOVA with Tukey post test (d). Significance is denoted as 1 (P < 0·05), 2 (P < 0·01) or 3 (P < 0·001). Unknown SCORAD for patients with AD: GBA (one at T0, three at T6), GlcChol (one at T6), CER (one at T6). Unknown TEWL for patients with AD: GBA (one at T0, three at T6), GlcChol (one at T0, one at T6), CER (one at T0, one at T6). Unknown NMF for patients with AD: GBA (two at T0, three at T6) and healthy controls: GBA (seven), GlcChol (one).

methanol. Next, samples were deacylated by microwave-assisted deacylation exactly as described previously.[22] After hydrolysis, samples were neutralized by addition of 50 μL of 1 mol L^{-1} hydrochloric acid in methanol, and internal standards were added to each sample [d7-sphingosine(d18:1) and d5-glucosylsphingosine(d18:1)], followed by evaporation of methanol (N$_2$, 40 °C). Thereafter, samples were subjected to extraction with butanol–water (1 : 1, v/v). After drying the butanol phase, samples were dissolved in 120 μL methanol and analysed by liquid chromatography–tandem mass spectroscopy (LC-MS/MS).

GlcChol was extracted from the tapes by methanol–chloroform–water, 12 : 6 : 1, v/v/v and the extract was dried (N$_2$, 40 °C). After addition of an internal standard (stigmasterol) separation of phases was induced by methanol–chloroform–water, 1 : 1 : 0·9, v/v/v according to the Bligh and Dyer method.[23] The lower phase was transferred to a 2-mL tube and the upper phase was washed with chloroform. The combined lower phases were dried (N$_2$, 40 °C) and the sample residues were dissolved in 150 μL of 10 mmol L^{-1} ammonium formate in methanol prior to analysis by LC-MS/MS, the method for which has been described previously[24,25] and is detailed in Table S1 (see Supporting Information).

A schematic overview of the methods used to analyse GlcChol, CER and GlcCER is provided in Figure S1 (see Supporting Information).

Statistical analysis

Calculations were performed using Prism 8 software (GraphPad, La Jolla, CA, USA). Distribution of data was tested by the Shapiro–Wilk normality test. P values were corrected for multiple testing using a Benjamini–Hochberg procedure. The applied statistical test is indicated within the figures or legends.

Results

Patients with AD had moderate-to-severe disease, which significantly improved after topical corticosteroid therapy (Table 1). At baseline, patients with AD had reduced skin barrier function, as assessed by TEWL, and lower NMF levels than healthy controls (Table 1). After therapy, TEWL in children with AD was still higher than in healthy controls, while NMF levels were lower. At baseline, GBA activity was significantly higher in patients with AD than in healthy controls, but it decreased after 6 weeks of therapy (Figure 2). The same pattern was observed for GlcChol; baseline values were higher in patients with AD

than in healthy controls and decreased after therapy. We calculated the relative ratio of GlcChol before and after therapy, and the median value amounted to 0·5 (range 0·04–4·8), an average decrease of 50%. After therapy, GlcChol and GBA activity were still higher in patients with AD than in healthy controls.

To investigate a possible relationship between SC depth and activity we determined the GBA activity at three different depths (tape numbers 2, 6 and 8, collected from the same skin site) in six patients with AD at baseline and six healthy controls. As shown in Figure 3, there was a gradual increase in activity of GBA with SC depth, and this pattern was observed in both patients with AD and healthy controls. Consistently, patients with AD showed higher GBA activity than healthy controls at all investigated depths. Levels of a subclass of CER – CER[H] – the corresponding glucosylceramide GlcCER[H] and their ratio are presented in Figure 4. CER[H] levels were higher in patients with AD than in controls, while no difference between these two groups was observed for GlcCER[H]. The CER[H]-to-GlcCER[H] ratio was higher in patients with AD than in controls (Figure 4). As described in the methods section, the values of CER[H] are approximate, as for the calibration the standard of sphingosine was used due to the lack of a hydroxylated standard.

Next, we investigated whether lipid parameters were associated with disease severity, skin barrier parameters and immune response. For this purpose, we performed a Spearman's rank correlation analysis for GBA activity and GlcChol with clinical parameters (SCORAD and oSCORAD), markers of the skin barrier (TEWL and NMF) and immunological markers (cytokines). The results of the Spearman correlation analysis including correlation coefficients and corresponding significance levels are presented as a heat map in Figure 5. The results revealed a

strong association of GlcChol with oSCORAD, TEWL and NMF and most immunological markers that we had previously shown to be elevated in this cohort of children with AD.[18] CER [H] was associated with several cytokines, including IL-18, CCL22, IL-5 and CCL4, and furthermore with NMF and TEWL (Figure 5). GlcChol was positively correlated with vascular endothelial growth factor, CCL2, CCL22, CCL17, IL-18 and CXCL8, while an inverse association was seen for IL-1α, CCL13, CCL4, IL-5, IL-2 and NMF. In contrast to GlcChol, GBA activity was not significantly correlated with disease severity, but showed a strong association with the skin barrier parameters TEWL and NMF and several immunological markers. GBA activity was positively associated with CCL2, CCL22, CCL17, IL-18 and CXCL8, while significant negative correlation was observed with NMF, CCL13 and IL-1α. In general, GBA and GlcChol showed the strongest associations with the cytokines of innate immunity: IL-1α and IL-18 (Figure 6).

Discussion

Impaired skin barrier is an important aetiological factor in AD; this study contributes to our knowledge about the interplay between the skin barrier and the local immune milieu in this highly prevalent inflammatory skin disease. We measured the activity of GBA and its enzymatic product GlcChol in the SC of children with AD before and after topical corticosteroid therapy, and related these levels to the local cytokine milieu, disease severity and skin barrier function. The results convincingly demonstrate that nonlesional skin of children with AD shows increased GBA activity and higher levels of the GBA enzymatic product, GlcChol. After 6 weeks of therapy, activity
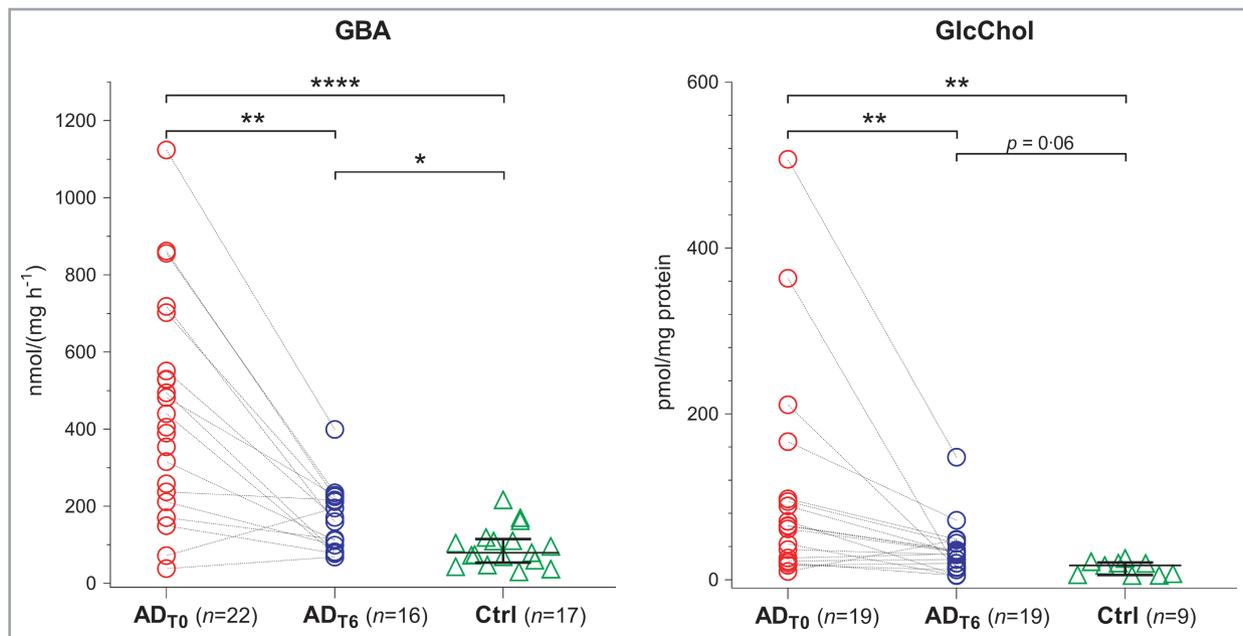

Figure 2 Activity of β-glucocerebrosidase (GBA) and stratum corneum levels of glucosylcholesterol (GlcChol) in patients with atopic dermatitis at baseline (AD$_{T0}$) and after therapy (AD$_{T6}$), and in healthy controls (Ctrl) (mean and SD). Differences between values before and after therapy were tested by Wilcoxon matched-pairs signed-rank test and between patients with AD and healthy controls by two-tailed Mann–Whitney test. Benjamini–Hochberg corrected P values: *P < 0·05, **P < 0·01, ****P < 0·0001.
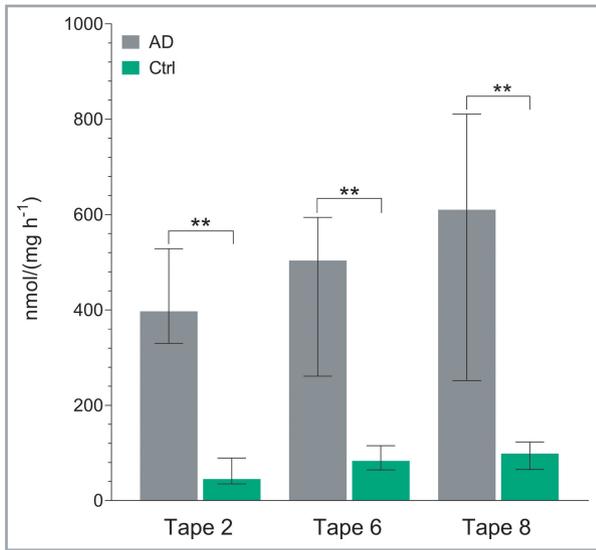
Figure 3 Activity of β-glucocerebrosidase (GBA) determined in the stratum corneum of patients with (AD) (n = 6) and healthy controls (Ctrl) (n = 6) at different depths (consecutive tape numbers 2, 6 and 8). Differences between patients with AD and healthy controls were tested by two-tailed Mann–Whitney test. Data are presented as the median and interquartile range. Benjamini–Hochberg corrected *P* values: **P < 0·01.

of GBA and GlcChol decreased significantly from baseline; however, their levels were still increased compared with whose of healthy control children. In line with increased activity of GBA and GlcChol levels, levels of a specific ceramide subclass, CER[H], and the CER[H]-to-GlcCER[H] ratio were higher in AD. Formation of CER[H], a ceramide class with 6-hydroxysphingosine as a sphingoid base, is exclusively

catalysed by GBA, while conversion into the sphingosine-related subclass CER[S] is also mediated by acid sphingomyelinase.[10] Previously, it has been shown in a human skin equivalent model that inhibition of GBA activity leads to a dose-dependent increase in the ratio of GlcCER[EOH] to CER[EOH], supporting our findings.[26]

GBA plays an important role in biosynthesis of CER and is essential for formation and functioning of the skin barrier.[9] A decrease in GBA activity is associated with reduced skin barrier function,[13] while inherited deficiency of GBA with complete lack of expression or enzyme activity leads to severe and life-threatening skin abnormalities as demonstrated by Gaucher disease.[14] Along with precursors of CER – sphingomyelins and GlcCER – and various other biotransformation enzymes, GBA is stored in lamellar bodies and secreted into the extracellular space at the interphase of the stratum granulosum and SC shortly before keratinocytes transform into corneocytes.[27] GBA activity is present throughout the outer parts of the epidermis, with predominant localization of active GBA in the extracellular space of the SC lipid matrix.[26]

One prior study quantitatively measured GBA activity in the SC of Japanese adult patients with AD.[17] In contrast to the present study, there was no difference in GBA activity between healthy and AD skin; however, that study did not report details on disease severity, skin barrier function or sampling distance from lesional skin.[17] In another study, Boer *et al.* reported differences in the localization, expression and activity of GBA in the epidermis between healthy and AD skin.[10] Altered localization of active GBA in AD skin was mainly detected in lesional skin, while the difference between healthy and nonlesional atopic skin was inconsistent. In that study, *in situ* expression of active GBA was visualized across the epidermal layers; however,
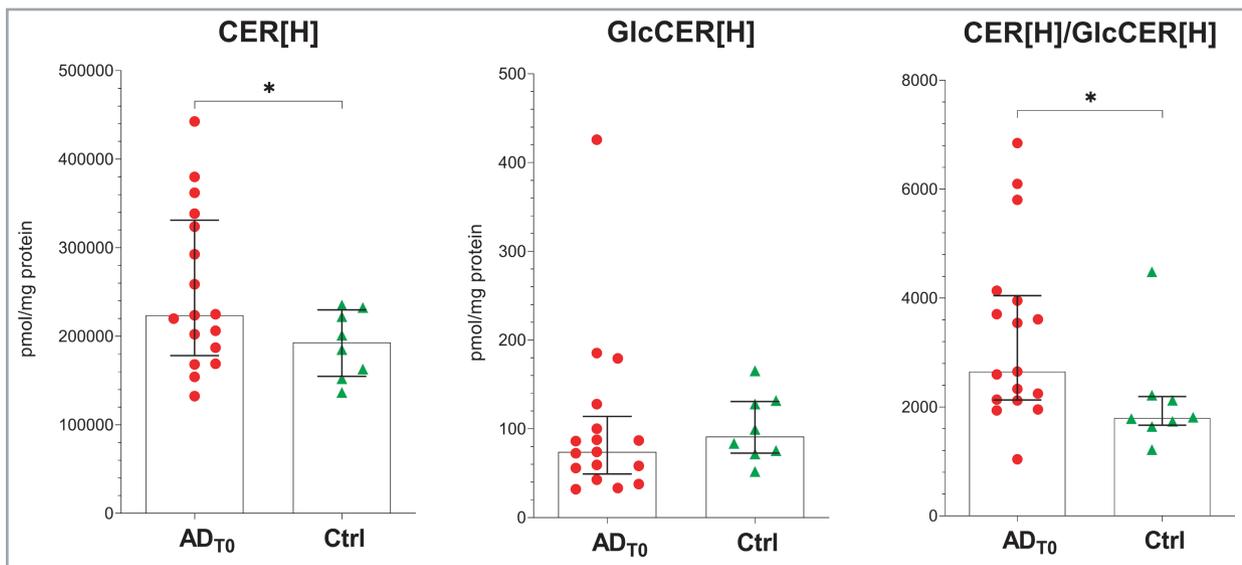

Figure 4 Baseline levels of hydroxylated ceramides {CER[H]}, hydroxylated glucosylceramide (GlcCER[H]) and their ratio in patients with atopic dermatitis (AD) and healthy controls (Ctrl). The difference in lipid markers between the patients with AD and controls was tested by two-tailed Mann–Whitney test. Data are presented as the median and interquartile range. Benjamini–Hochberg corrected *P* values: *P < 0·05. Note that the values of CER[H] and GlcCER[H] are not true values, as due to the lack of their standards for the calibration sphingosine and glucosylsphingosine were used.
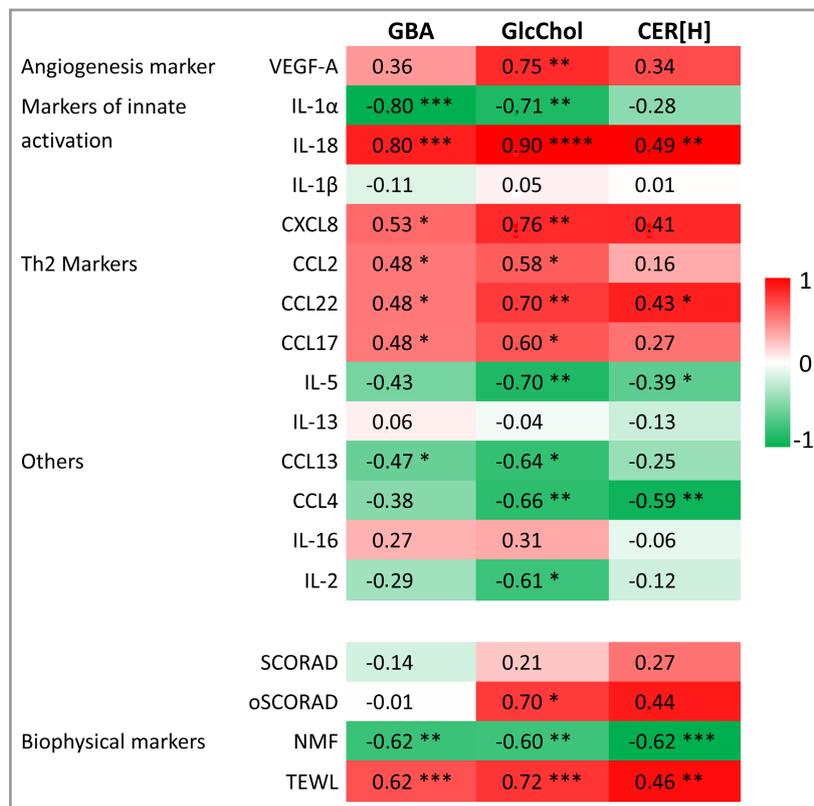
		GBA	GlcChol	CER[H]
Angiogenesis marker	VEGF-A	0.36	0.75 **	0.34
Markers of innate activation	IL-1α	-0.80 ***	-0.71 **	-0.28
	IL-18	0.80 ***	0.90 ****	0.49 **
	IL-1β	-0.11	0.05	0.01
	CXCL8	0.53 *	0.76 **	0.41
Th2 Markers	CCL2	0.48 *	0.58 *	0.16
	CCL22	0.48 *	0.70 **	0.43 *
	CCL17	0.48 *	0.60 *	0.27
	IL-5	-0.43	-0.70 **	-0.39 *
	IL-13	0.06	-0.04	-0.13
Others	CCL13	-0.47 *	-0.64 *	-0.25
	CCL4	-0.38	-0.66 **	-0.59 **
	IL-16	0.27	0.31	-0.06
	IL-2	-0.29	-0.61 *	-0.12
	SCORAD	-0.14	0.21	0.27
	oSCORAD	-0.01	0.70 *	0.44
Biophysical markers	NMF	-0.62 **	-0.60 **	-0.62 ***
	TEWL	0.62 ***	0.72 ***	0.46 **

Figure 5 Spearman correlation coefficients for the relationships between lipid markers and disease severity [Scoring Atopic Dermatitis (SCORAD) and objective SCORAD (oSCORAD)], transepidermal water loss (TEWL), stratum corneum natural moisturizing factor (NMF) and cytokine levels. Benjamini–Hochberg corrected P values: *P < 0·05, **P < 0·01, ***P < 0·001, ****P < 0·0001. CER[H], hydroxylated ceramides; GBA, β-glucocerebrosidase; GlcChol, glucosylcholesterol; IL, interleukin; Th2, T helper cell 2; VEGF, vascular endothelial growth factor.

in contrast to the present study only semiquantitative measures of GBA activity were ascertained. In accordance with the study of Boer *et al.*,[10] GBA activity also varied with SC depth in this work. We found a gradual increase of GBA activity with SC depth in nonlesional AD skin, as well as in healthy skin, and AD skin consistently showed significantly higher GBA activity than healthy skin at all investigated depths.

As GBA activity and the levels of GlcChol decreased after therapy we investigated their association with disease severity (SCORAD or oSCORAD), skin barrier function (TEWL) and levels of NMF and immunological mediators. Interestingly, activity of GlcChol, but not GBA, showed a significant correlation with disease severity (oSCORAD). Both GBA and GlcChol were positively associated with TEWL and inversely correlated with SC levels of NMF, a mixture of hygroscopic substances known to contribute to hydration and acidification of the SC.[28]

The strong correlation of GBA and GlcChol with expression of the IL-1 family cytokines IL-1α and IL-18 is striking. IL-1α is abundant in the SC and is constitutively expressed by keratinocytes.[29] IL-1α is often used as a measure of skin barrier damage, and in the SC, IL-1α levels decrease after challenges such as skin barrier damage with skin irritants.[30,31] In our previous study in the same cohort of children, we showed, as would be expected, a significantly impaired skin barrier (e.g. increased TEWL) and lower IL-1α values in children with AD

compared with healthy controls.[32] It might be speculated that IL-1α acts as an alarmin, triggering repair mechanisms. Thus, increased production of CER through elevated activity of GBA might represent one repair mechanism aimed to restore skin barrier homeostasis. This view is further supported by the finding that skin barrier disruption significantly increased epidermal *GBA* mRNA levels, with a 2·8-fold increase over untreated control levels.[16] Furthermore, a single topical application of a GBA inhibitor, which almost completely inhibited enzyme activity, significantly delayed permeability barrier recovery.

Another reason for altered GBA activity might be increased pH in AD skin.[9] One of the regulators of skin pH is urocanic acid, a degradation product of the abundant epidermal protein filaggrin and a constituent of NMF.[28] Indeed, we found reduced NMF in AD skin, and NMF levels were inversely associated with GBA activity. The optimal pH for GBA activity is 4·5, and some but not all studies have shown increased pH in AD skin.[33] As discussed by Boer *et al.*,[9] GBA activity might also be affected by the presence of cofactors such as saposin C, which facilitates GlcCER degradation by GBA. Next to the markers of innate immune activation, GBA activity and GlcChol were positively associated with SC levels of the cytokines of T helper 2 immunity CCL17 and CCL22. However, the degree of correlation was not as strong as that found with IL-1α and IL-18. Another cytokine that showed association with GlcChol was IL-5, a cytokine that is mainly
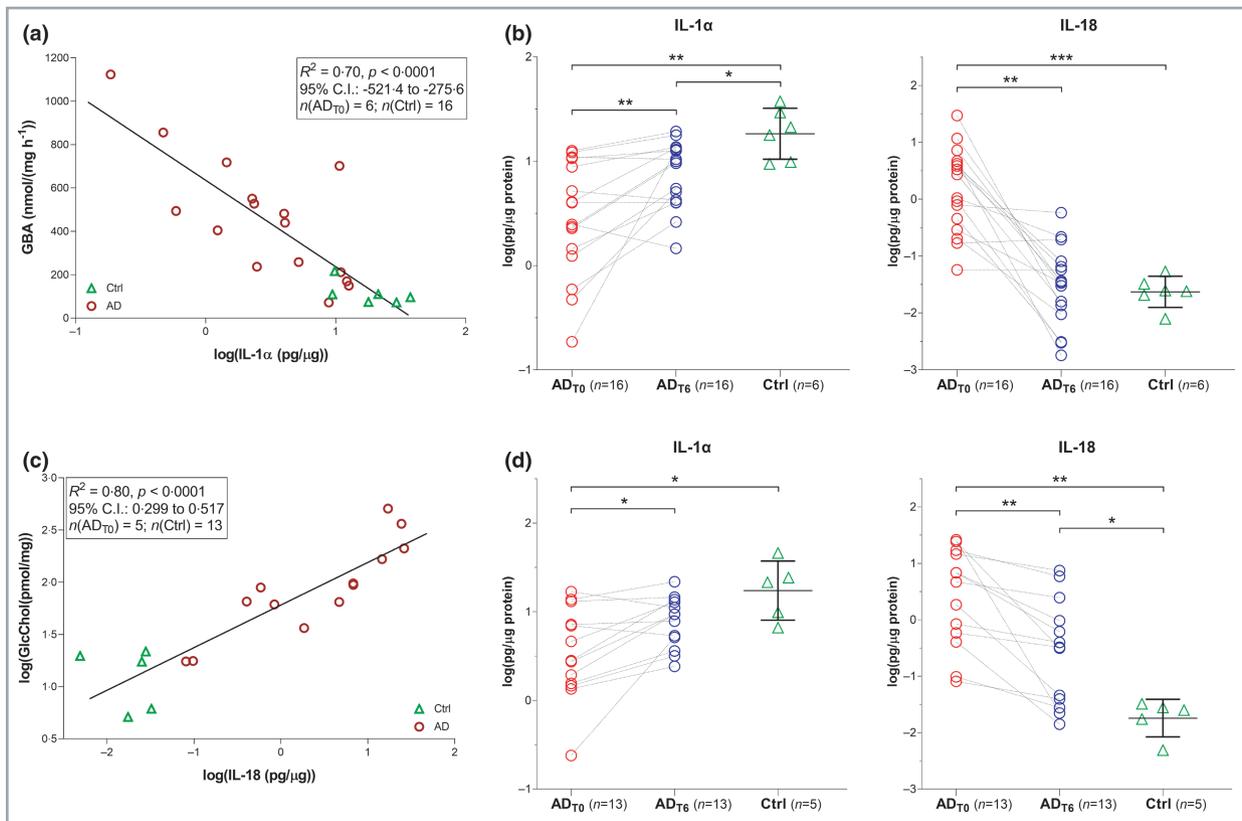
Figure 6 (a, c) Linear regression analysis between baseline values of interleukin (IL)-1α and IL-18 and activity of β-glucocerebrosidase (GBA) and glucosylcholesterol (GlcChol). (b, d) IL-1α and IL-18 values (mean and SD) before and after therapy in the respective GBA and GlcChol subgroups. AD_{T0}, baseline in patients with AD; AD_{T6}, values 6 weeks after therapy; CI, confidence interval; Ctrl, healthy controls. R^2 = regression coefficient. Differences between values before and after therapy were tested by two-tailed paired t-test, and between patients with AD and healthy controls by two-tailed t-test. Benjamini–Hochberg corrected P values: *P < 0·05, **P < 0·01, ***P < 0·001.

produced by T helper 2 and mast cells.[34] In our previous study we found lower IL-5 levels in the SC of children with AD than in healthy controls.[18] Consistently with this, in the present study inverse correlation of GlcChol with IL-5 was found.

Here we show that GlcChol is increased in AD and that its levels parallel GBA activity in the SC. Previously, it was shown *in vitro* that GBA is able to form GlcChol by transglucosylation of cholesterol, by a process that is reversible and depends on the local concentrations of cholesterol, CER and GlcCER.[25] The role of GlcChol in skin barrier function the and pathophysiology of AD is unknown, but increased water solubility due to glucosylation of cholesterol might affect the organization of lipid lamellae. Similarly to GlcChol, another water-soluble cholesterol derivative, cholesterol sulfate, is increased in AD.[35] Cholesterol sulfate is important in regulation of differentiation and desquamation; however, its generation is mediated by another enzyme, cholesterolsulfotransferase. Future research will be needed to reveal whether elevated levels of GlcChol contribute to skin barrier defects in AD, like cholesterol sulfate does.

To conclude, this study shows that SC in nonlesional skin of patients with AD has increased activity of GBA, an important enzyme for biosynthesis of CER in the SC. Consistently, increased activity of GBA is paralleled by elevated levels of its enzymatic product GlcChol. Local anti-inflammatory therapy

corrects this increased GBA activity and SC levels of GlcChol, and both markers show strong association with the local immune milieu, and GlcChol also with disease severity. These findings, taken together, suggest that increased GBA activity in AD is both acquired and modifiable. Therefore, GBA activity and GlcChol might be useful biomarkers in monitoring of therapeutic responses in AD. Further work should explore these markers before and after other AD therapies including topical calcineurin inhibitors and systemic therapies, including broad immune suppressants and targeted biologic therapies. This would help to distinguish potential pathomechanisms underlying biochemical changes in AD.

The strength of our study is that we analysed not only GBA activity, but also its substrate (GlcCER) and the enzymatic products GlcChol and CER. Moreover, to the best of our knowledge we are the first to directly compare these lipid components before and after local therapy and to relate their levels to disease severity, skin barrier function and local cytokine profile. The limitation of our study is the lack of insight into the mechanisms underlying the increase of GBA activity (e.g. due to increased expression of GBA) and the effect of other lipid enzymes and cofactors.[27] Furthermore, we did not investigate the ratio of CER[H] to GlcCER[H] before and after therapy. The activity of GBA and the levels of CER, GlcCER and GlcChol were

determined in different subgroups of the cohort, and therefore the association between these markers could not be investigated. This is the first time that GlcChol was measured in atopic skin, but its function still must be clarified.

Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1 Schematic overview of glucosylcholesterol and sphingolipid analysis.

Table S1 Liquid chromatography–tandem mass spectroscopy settings for sphingolipids and glucosylcholesterol.