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Cutaneous Biology

Epidermal barrier lipids in human vernix caseosa: corresponding ceramide pattern in vernix and fetal skin

P.H.HOEGER, V.SCHREINER,* I.A.KLAASSEN, C.C.ENZMANN, K.FRIEDRICHS† AND O.BLECK

*Departments of Dermatology and †Obstetrics/Gynaecology, University of Hamburg, Martinistr. 48, D-20246 Hamburg, Germany, *Paul-Gerson-Unna Skin Research Center, Beiersdorf Inc., Hamburg, Germany*

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Summary

Background Vernix caseosa is a protective biofilm covering the fetus during the last trimester. Vernix and epidermal barrier lipids (i.e. cholesterol, free fatty acids and ceramides) appear to share protective functions for fetal and neonatal skin.

Objectives To analyse vernix samples for epidermal barrier lipid content, and to compare lipid profiles of vernix with those of fetal and postnatal epidermis.

Methods Vernix samples were collected from 21 healthy term neonates. Skin samples were collected from 10 fetuses aborted between gestational week (GW) 16 and 25, nine infants and 11 older children. Lipids were extracted according to standard protocols and analysed by high-performance thin-layer chromatography.

Results Vernix contained 196·5 ± 70·1 μg barrier lipids mg^{-1} protein (mean ± SD). Cholesterol formed the major barrier lipid fraction (52·8%), followed by free fatty acids (27·7%) and ceramides (20·1%). The ceramide composition of vernix resembled that of mid-gestational (GW 23–25) fetal epidermis both qualitatively and quantitatively, while there were major differences from postnatal epidermis. The total epidermal ceramide concentration increased significantly between prenatal and postnatal samples.

Conclusions The composition pattern of ceramides mirrors that of mid-gestational fetal epidermis. Vernix thus represents a 'homologous' substitute for the immature epidermal barrier in fetal skin. The differential role of individual ceramides in this process remains to be established.

Key words: ceramides, epidermal barrier lipids, fetal skin, vernix caseosa

Skin of premature babies is characterized by increased transepidermal water loss (TEWL), high rates of percutaneous absorption and a propensity for skin trauma,[1,2] which can be explained by an immature epidermal barrier.[3,4] *In utero*, during the last trimester of gestation, the fetus is covered by a protective biofilm called vernix caseosa.[5] It forms a mechanical 'shield' against maceration by amniotic fluid and bacterial infection.[6] Vernix is mainly composed of water (80·5%), proteins and lipids (each 8–10%)[7,8] (Fig. 1). Its high content of squalenes and wax esters originally suggested that vernix lipids were mainly derived from fetal sebaceous glands,[5,9–11] but recent studies using high-performance thin-layer chromatography (HPTLC) demonstrated the presence of all major stratum corneum lipids in vernix.[12]

Stratum corneum lipids are essentially composed of cholesterol, free fatty acids (FFA) and ceramides. They are located in the intercellular space like mortar between the (keratinocyte) bricks.[13] Ceramides represent the major type of lipid in the stratum corneum;[14] precursors of epidermal ceramides are secreted from lamellar bodies.[15] They are formed by amide links between sphingosine and fatty acids. Eight different types of ceramides have been identified.[14,16,17] There is an inverse relationship between the total ceramide

Correspondence: Dr Peter H.Hoeger.
E-mail: hoeger@uke.uni-hamburg.de

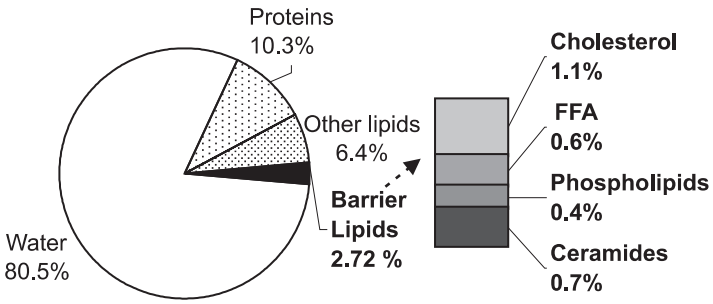


Figure 1. Composition of human vernix caseosa. Protein content and vernix barrier lipids were determined as described in the text. Assays were done with dehydrated samples; data on water content are derived from Pickens *et al.*[8] FFA, free fatty acids.

content within the corneal layer and the extent of barrier disruption in terms of TEWL.[18–20] Therefore, ceramides are considered to represent the key 'barrier lipids'.[18,21] Few studies so far have addressed the formation of epidermal barrier lipids in the human fetus. In humans, cornification begins at about gestational week (GW) 20. Until GW 16, dermal and epidermal lipids are mainly composed of sterols and phospholipids.[3,4] Following synthesis of their respective key enzymes, ceramides and FFA are increasingly produced thereafter; however, a competent epidermal barrier is not established before GW 30.[3,4,22]

Interestingly, the onset of vernix production coincides with the formation of the corneal layer in the fetus.[22] Vernix and epidermal barrier lipids appear to share protective functions for the fetal and neonatal skin, respectively. The present study was done to analyse the barrier lipid concentration of vernix caseosa and to correlate it with epidermal lipids in the fetal skin at different stages of development.

Materials and methods

Vernix and skin samples

Samples of vernix caseosa (1–2 g) were collected immediately after birth from the shoulder area of 21 healthy term neonates by gentle scraping with a sterile plastic spoon. Vernix samples from pregnancies with a history of premature rupture of the membranes or chorioamnionitis were excluded. Vernix was stored in sterile plastic tubes at − 80 °C until further analysis. Fetal skin samples were collected from 10 fetuses aborted between GW 16 and 25 at the Department of Gynaecology and Obstetrics, University of Hamburg. Indications for abortion were congenital malformations (hydrocephalus, anencephalus, cardiac abnormalities), thalassaemia and chromosomal abnormalities (one case each of trisomy 21 and trisomy 18, respectively); two abortions occurred spontaneously. Samples were frozen immediately in liquid nitrogen and stored at − 80 °C until further analysis. Small skin samples were taken from excessive tissue removed during major abdominal or chest operative procedures at the Department of Paediatric Surgery, University of Hamburg (congenital diaphragmatic hernia, abdominal neuroblastoma) on 20 children aged between 3 weeks and 6 years. The study was approved by the Ethics Committee of the City of Hamburg Medical Council.

Lipid extraction

Lipid content was analysed quantitatively and qualitatively in samples of vernix caseosa and fetal and neonatal skin. After removal of subcutaneous fat tissue, the epidermis was separated by dispase and trypsin digestion as described before.[23] Fetal skin samples were incubated in 10 mmol L^{-1} ethylenediamine tetraacetic acid in calcium- and magnesium-free phosphate-buffered saline for approximately 1 h at 37 °C according to Harris *et al.*[24] The epidermis was gently peeled off the dermis under a dissection microscope. All skin samples were dried on silk and transferred into glass vials for chromatography. Lipids were extracted by ultrasonication (2 × 20 min) in chloroform/methanol (2 : 1 v/v, Merck Inc., Darmstadt, Germany) in a modified procedure according to Bligh and Dyer.[25]

Lipid analysis

Lipids were analysed by HPTLC (horizontal system; Linomat®, Camag Inc., Berlin, Germany) as described before.[23,26] In brief, a standard lipid mixture consisting of Cer(AS), Cer(NS), palmitoleic acid and cholesterol was used as the reference for non-polar barrier lipids. For polar lipids, the reference consisted of sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, glucocerebrosides and Cer(NS). Samples were dissolved in 200 μL of chloroform/methanol (2 : 1 v/v). Samples and standards were run in parallel lanes on HPTLC silica gel plates (Merck)

precleaned with chloroform/methanol. For separation of non-polar lipids, thin-layer chromatography (TLC) plates were developed sequentially with chloroform/methanol/acetic acid (190 : 9 : 1, v/v) and diethylether/hexane/acetic acid (80 : 20 : 1·5 v/v), as described previously.[14,23] For separation of polar skin surface lipids, TLC plates were developed twice with chloroform/methanol/water (65 : 25 : 4 v/v). In preparation for quantitative photodensitometry, the TLC plates were air dried and sequentially fixed in aqueous solutions of 10% $CuSO_3$ in 8% H_3PO_4 (both from Sigma Inc., Deisenhofen, Germany) for 20 s, followed by incubation at 180 °C for 20 min. Lipids were quantified by photodensitometry (Camag TLC Scanner II®). Peaks of ceramides, cholesterol and FFA were numbered according to their position on the TLC plate, and identified according to the comigrating standards. All samples were run in duplicate.

Protein analysis

Epidermal and vernix proteins were quantified as described.[23] In brief, skin samples were completely hydrolysed in 2 mL of 6 mol L^{-1} NaOH for 5 h at 150 °C. After cooling and neutralization with 6 mol L^{-1} HCl, 1 mL of an aqueous buffer containing 20·2% (by weight) propionic acid sodium salt and 9·3% (by volume) free propionic acid was added. Free amino acids were quantified photospectrometrically at 570 nm after derivatization with the ninhydrin reagent. Lipid concentrations were calculated as μg lipid mg^{-1} protein.

Statistical analysis

Data were analysed for statistical significance using Student's *t*-test.

Results

Vernix caseosa contains all barrier lipids

As summarized in Figure 1, vernix is largely composed of water (80·5%), proteins (10·3%) and lipids (9·12%). Among the lipids, about 30% belong to the fraction of barrier lipids. Mean ± SD total barrier lipid concentration in vernix samples from term neonates were 196·5 ± 70·1 μg lipid mg^{-1} protein. Cholesterol formed the major barrier lipid fraction (52·8%), followed by FFA (27·7%) and ceramides (20·1%). Mean ± SD total vernix ceramide concentration was 39·4 ± 13·0 μg mg^{-1} protein. As shown in Table 1, in which ceramide species are designated according to their fatty acid type,[27] Cer(AH) was the most prevalent vernix ceramide (25·83 ± 4·5% of all ceramides), followed by Cer(AS) and Cer(NS). Typical HPTLC scans of vernix caseosa samples are depicted in Figure 2. Of note, the mean vernix ceramide concentration was associated with a relatively small SD, indicating a remarkably consistent composition of vernix lipids. There was no significant difference between vernix samples from male and female neonates (data not shown).

Age-dependent concentrations of epidermal barrier lipids

Unlike in vernix, FFA formed the largest epidermal barrier lipid fraction both in prenatal and postnatal skin. In fetal skin, cholesterol was the second largest fraction, and in postnatal skin, it was ceramides. As shown in Figure 3(a), epidermal concentrations of cholesterol and FFA in early gestation equalled those of older children; they were lowest in the epidermis of GW 23–25 fetuses. Relative and absolute epidermal ceramide concentration rose only slightly between GW

Table 1. Ceramide concentration of human vernix caseosa

	Cer(AH)	Cer(AP)	Cer(AS)	Cer(EOH)	Cer(NP)	Cer(NS)	Cer(EOS)	Total ceramides
Lipid concentration (μg lipid mg^{-1} protein) mean ± SD (range)	9·94 ± 2·62 (5·3–15·1)	3·42 ± 1·57 (1·4–6·5)	6·88 ± 2·61 (3·5–14·0)	4·90 ± 1·42 (3·1–8·5)	3·53 ± 2·33 (0·8–10·1)	6·30 ± 2·77 (3·6–16·3)	4·45 ± 1·63 (2·3–10·1)	39·4 ± 13·0 (25·4–78·9)
Percentage of total ceramides (%) mean ± SD (range)	25·83 ± 4·53 (15·6–34·5)	8·53 ± 2·51 (5·1–12·2)	18·43 ± 4·26 (13·7–35·0)	12·55 ± 1·87 (9·6–18·1)	8·37 ± 3·42 (2·6–14·2)	15·66 ± 2·14 (12·2–20·6)	11·28 ± 1·65 (8·7–13·8)	100

Mean ± SD values from vernix samples of 21 term neonates. The total lipid concentration of vernix was 196·5 ± 70·1 μg mg^{-1} protein. It was mostly composed of cholesterol (103·0 ± 33·5 μg mg^{-1} protein, representing 52·8 ± 5·4% of total lipids), free fatty acids (54·0 ± 29·4 μg mg^{-1} protein, i.e. 27·7 ± 5·7%) and ceramides (39·4 ± 13·0 μg mg^{-1} protein, i.e. 20·1 ± 6·1%). Ceramide species are designated according to their fatty acid (FA) type:[27] E, ester-linked FA; O, ω-hydroxy FA; A, α-hydroxy FA; N, non-hydroxy FA; S, sphingosine; H, 6-hydroxy-4-sphingenine; P, phytosphingosine.

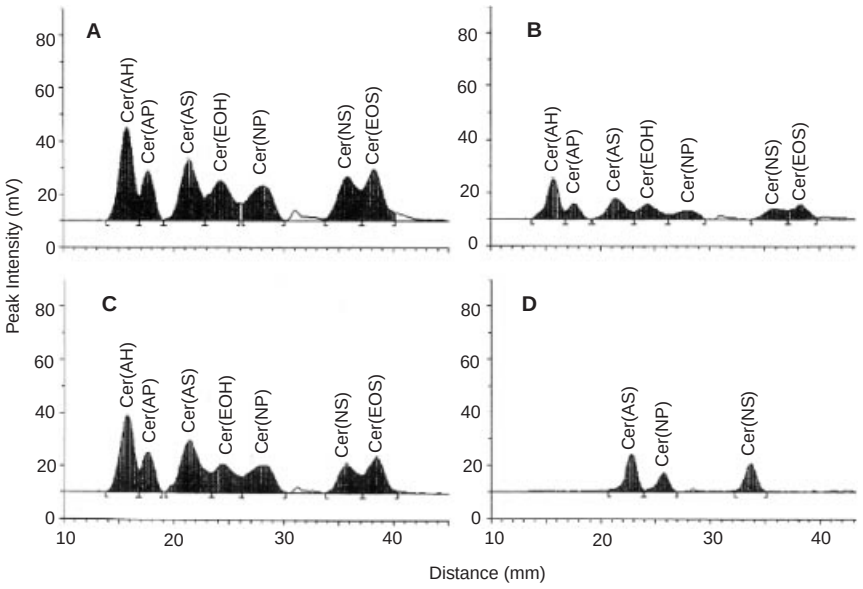
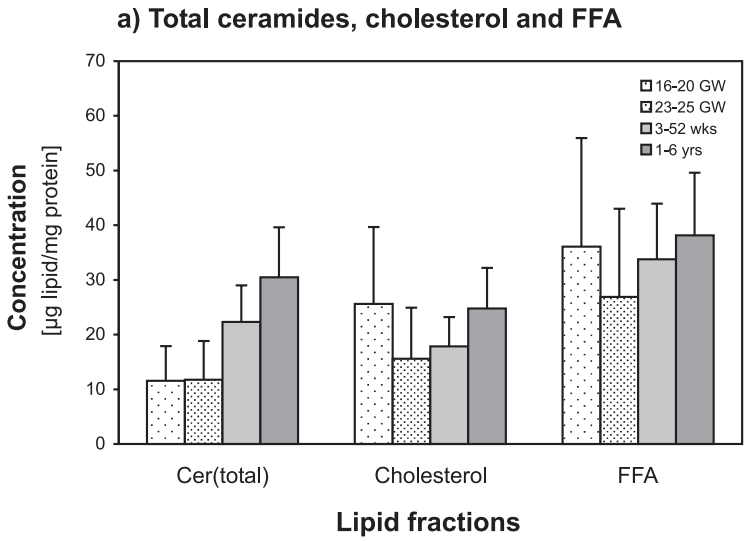


Figure 2. Ceramides in vernix casesosa. (A–C) High-performance thin-layer chromatography scans of vernix caseosa; (D) standard.



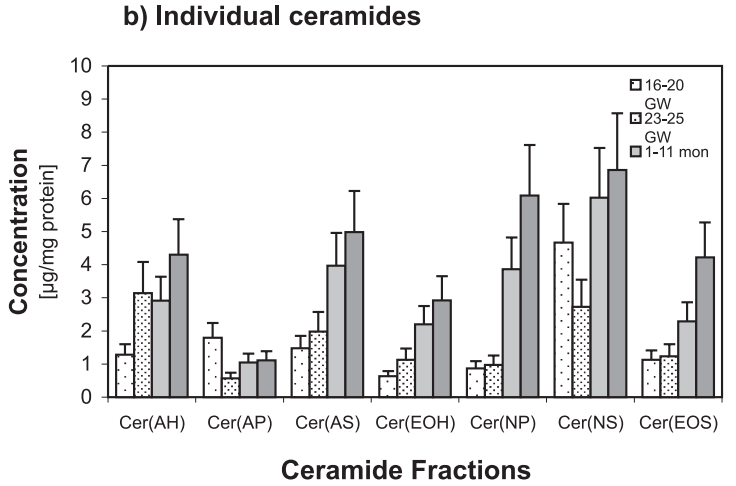


Figure 3. Barrier lipids in fetal and postnatal epidermis. Data are presented as mean ± SD. Number of samples tested: fetal skin, $n = 5$; infantile skin (1–11 months), $n = 9$; children (1–6 years), $n = 11$. (a) Total ceramides, cholesterol and free fatty acids (FFA). Differences between fetal and postnatal lipid concentrations were significant ($P < 0{\cdot}01$) for ceramides only. (b) Individual ceramides. Differences between fetal and postnatal ceramide concentrations were significant ($P < 0{\cdot}01$) except for Cer(AH), Cer(AP) and Cer(NS). GW, gestational week.

16–20 and GW 23–25, but increased significantly after birth. The same holds true for individual ceramides (Fig. 3b), except for the two ceramide fractions Cer(AH) and Cer(AP), which exhibited slightly higher concentrations in fetal than in postnatal skin. An increase in postnatal epidermal ceramide concentrations was likewise observed when ceramide concentrations were not expressed in relation to protein content (μg lipid mg^{-1} protein) but were related to the content of epidermal phosphatidylserine and phosphatidylcholine [(ceramide)/(phosphatidylserine + phosphatidylcholine)], which could be taken as a measure for the amount of living cells (data not shown).

Composition of vernix ceramides reflects that of mid-gestational fetal and neonatal epidermis

The composition of individual ceramide fractions was compared between fetal and postnatal epidermis and vernix (Fig. 4). For six of the seven ceramide fractions investigated [except Cer(AP)], there was no significant difference between the concentration in vernix and in mid-gestational fetal skin (GW 23–25). In both samples, the three most prevalent ceramide fractions were Cer(AH), Cer(NS) and Cer(AS), whereas in postnatal skin Cer(NS), Cer(AS) and Cer(NP) prevailed. Compared with postnatal skin, vernix ceramide concentrations were lower with respect to two ceramide fractions [Cer(NS), Cer(NP); $P < 0{\cdot}05$], and higher with respect to Cer(AH). No significant difference was found for the remaining four ceramide fractions.

Discussion

Our study demonstrates that 30% of total vernix lipids are epidermal barrier lipids, and the composition pattern of ceramides mirrors that of mid-gestational fetal or early postnatal epidermis. Vernix thus represents a 'homologous' substitute for the immature epidermal barrier in fetal skin.

Owing to its high content of squalenes and wax esters, vernix was previously thought to be mostly if not exclusively derived from sebaceous glands.[5,9–11] Fatty acids of wax esters found on the fetal and adult skin surface are derived from sebaceous glands, while fatty acids of sterol esters are also derived from keratinocytes.[5,28] A comparison between vernix from pre-term and term neonates revealed an increasing content of squalenes relative to other lipids,[10] suggesting an increase in fetal sebaceous gland activity proportionate to fetal age. Nevertheless, several earlier reports indicated the presence of epidermal lipids in vernix: as confirmed by our study, cholesterol constitutes the largest fraction of vernix barrier lipids,[10,11,16,29] followed by FFA.[5,7,11,16,29,30]

Androgens have been found to delay the development of epidermal barrier lipid synthesis both *in utero* and *in vitro*.[4] Nazarro-Porro *et al.* reported decreased levels of epidermal lipids in vernix from male as opposed to female neonates.[30] Our results do not corroborate this observation: we found no significant differences in the relative and absolute concentration of vernix ceramides between male and female neonates.

Recent studies using HPTLC have demonstrated the presence of all major stratum corneum lipids in vernix,[12] including ceramides,[31] which are not synthesized by sebaceous glands. Vernix is therefore composed of two types of lipid: wax esters formed in sebaceous glands and epidermal barrier lipids derived from keratinocytes. Similar to postnatal skin, sebum and epidermal lipids apparently mix within vernix in order to provide on the fetal skin surface what has been referred to as the 'skin surface lipid film'.[32] Ceramides are of crucial importance for the prevention of TEWL

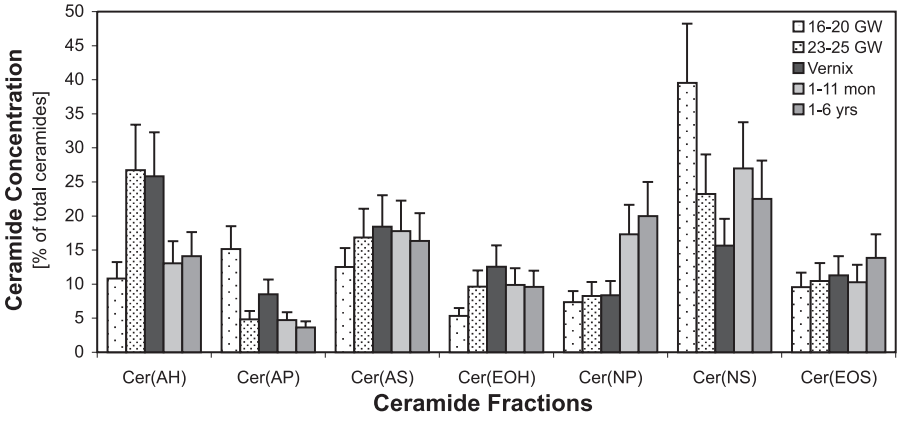


Figure 4. Ceramide composition of human vernix: comparison with fetal and postnatal epidermis. Data are presented as mean ± SD. Number of samples tested: vernix, $n = 21$, fetal skin, $n = 5$; infantile skin (1–11 months), $n = 9$; children (1–6 years), $n = 11$. Differences between vernix and late fetal skin (gestational week, GW 23–25) were significant only for Cer(AP) ($P < 0{\cdot}05$). Differences between vernix and postnatal skin (1–11 months) were significant only for Cer(AH) ($P < 0{\cdot}001$) and Cer(NP) ($P < 0{\cdot}05$).

both prenatally and postnatally.[3,18–20] We were therefore interested to compare the ceramide composition of vernix with that of fetal and postnatal skin. The idea of a correlation between vernix lipids and fetal skin was originally suggested by Nicolaides *et al.*[28] who found similar saturated fatty acids of wax esters in vernix and adult skin; however, fetal or neonatal skin was not assessed, and the authors did not differentiate between individual barrier lipids. As shown in Table 1 and Figure 3, ceramides represented the third largest fraction (20·1–23·6%) of all barrier lipids both in vernix and in fetal skin, while in postnatal skin ceramides were the second largest fraction of barrier lipids (24·1–29·7%). In absolute terms, vernix had the highest concentration of ceramides (39·4 μg mg^{-1} protein) as compared with fetal skin (11·8 μg mg^{-1} protein) and postnatal skin (22·3–30·5 μg mg^{-1} protein); as lipid concentration is expressed in relation to protein, the higher protein content of epidermis limits its direct comparison with vernix.

The significant rise in total ceramide concentration in postnatal skin (Fig. 3) most probably represents an adaptive response of the epidermis to environmental 'stress', i.e. transition from humid to dry ambient atmosphere. It is reminiscent of the rapid normalization of TEWL in neonates following premature delivery.[2,33] Even in adults, regional adaptation of stratum corneum ceramide levels to dry environmental conditions has been reported.[34] Pre-school children's skin still revealed increasing ceramide levels (Fig. 3a,b), although (environmental or genetic) factors responsible for this 'late' increase remain to be elucidated. With increasing age, i.e. beyond the age of about 20 years, however, epidermal ceramide concentrations tend to decrease.[20] Looking at the composition of individual ceramides, we found similar patterns of ceramide fractions in vernix caseosa and mid-gestational (GW 23–25) fetal epidermis (Fig. 4): in both, Cer(AH), Cer(NS) and Cer(AS) prevailed, and there was no significant difference in the relative amount of all ceramides except Cer(AP). Most strikingly, in both samples Cer(AH) constituted the biggest fraction. By contrast, less Cer(AH) and Cer(EOH) was present in early as compared with later fetal skin and vernix. This difference may be due to the different rates of synthesis of individual ceramides during embryogenesis; it may likewise point to different functional requirements of individual ceramides at different developmental stages. The increase in epidermal Cer(EOS) during mid-gestation and postnatally indicates barrier maturation, as Cer(EOS) plays an important role in the formation of a functional epidermal barrier.[30] Fetal lipid synthesis correlates with cutaneous development.[3,4] Recent studies have shown that barrier formation in the human fetus is patterned,[22] and it is likely that synthesis of ceramide fractions is likewise a patterned process. Cer(NS) reaches high intracellular concentrations within living cells, and can act as a second messenger triggering cellular differentiation and apoptosis.[35] This could partly explain its preponderance in early fetal skin. Oku *et al.*[31] recently reported low concentrations of two novel branched-chain ceramides [Cer(EAS) and Cer(NH)] in human vernix, but these were not assessed in our study.

Synthesis of epidermal barrier lipids is influenced by hormones (glucocorticoids, thyroid hormones, oestrogen, androgen), nicotinamide and nuclear receptors (peroxisome proliferator activated receptor, farnesol receptor),[4] but the molecular regulation of ceramide synthesis during human embryogenesis is incompletely understood so far. As shown in our study as well as in previous studies in fetal rat, pig and human skin,[3,4,36,37] increasing ceramide concentrations within the epidermis correlate with maturation of the transepidermal barrier. While the overall pattern of barrier lipids reported in these studies was similar to our observations, individual ceramides were not analysed.[3,4] Interestingly, in patients with atopic eczema, the epidermal concentration of Cer(NP) was found to correlate inversely with increased TEWL.[38]

Unlike postnatal skin, sebum and keratinocytes are not shed in the fetal period but adhere to the skin. Barrier lipids are thus retained, and accumulate on the skin surface. The synthesis of epidermal barrier lipids increases during the second trimester, preceding the formation of vernix. Vernix ceramides are derived from the fetal stratum corneum. Thus, fetal corneocytes detach along with their barrier lipids to form vernix. This may be viewed as a protective mechanism compensating for the immaturity of the transepidermal barrier. Conversely, shedding of the vernix membrane towards the end of gestation—a phenomenon that is utilized to estimate fetal maturity[39]—suggests that at that time the transepidermal lipid barrier is sufficiently developed to maintain transepidermal homeostasis by itself. As vernix lipids are to a significant part derived from fetal epidermis, we propose that their composition reflects fetal epidermal lipid synthesis. We suggest that the process of lipid accumulation might compensate for the relative deficiency of barrier lipids during the second and third trimester of gestation. In order to clarify this issue further, studies are thus needed to

analyse fetal epidermal lipids during the third trimester, and to compare vernix from premature and mature babies. Preliminary studies indicate that the application of vernix to normal adult skin results in increased surface hydration.[40] It proved different from standard water-in-oil emulsions or petrolatum, which have previously been used to protect against TEWL in premature babies. Vernix or vernix-like substances have so far not been evaluated in this setting. We suggest that synthetic emollients resembling vernix should be assessed for their use in protecting premature infants against TEWL and transcutaneous infections.

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