Barrier Dysfunction and Pathogenesis of Neutral Lipid Storage Disease with Ichthyosis (Chanarin–Dorfman Syndrome)

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Neutral lipid storage disease with ichthyosis (NLSDI; Chanarin–Dorfman syndrome) is an ichthyosiform syndrome, often associated with mutations in a lipid hydrolase, CGI-58. The presence of oil red O-positive, neutral lipid droplets in tissue biopsies, and/or in leukocytes on blood smears, coupled with a constellation of multisystem abnormalities and a pruritic ichthyosiform erythroderma, are together diagnostic of NLSDI. We investigated the pathogenesis of the ichthyosiform erythroderma in patients from three unrelated kindreds with a clinical diagnosis of NLSDI. Basal permeability barrier function and stratum corneum (SC) integrity were abnormal, but barrier recovery rates were faster than normal, as in atopic dermatitis. The basal barrier abnormality was linked to the secretion of lipid micro-inclusions, first segregated within lamellar bodies (LB), which then form a non-lamellar phase within the SC interstices, shown by combined ruthenium tetroxide post-fixation and lipid-retaining resin-white embedding. With colloidal lanthanum nitrate perfusion, excess water/solute movement was restricted to the SC interstices, and further localized to non-lamellar domains. Phase separation of excess stored lipid provides a unifying pathogenic mechanism not only for NLSDI, but also in several other inherited ichthyosiform disorders of lipid metabolism, such as recessive X-linked ichthyosis and type 2 Gaucher's disease.

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INTRODUCTION

Neutral lipid storage disease with ichthyosis (NLSDI), or Chanarin–Dorfman syndrome (online Mendelian inheritance in man no. 275630), is a rare, recessive disorder often owing to mutations in a hydrolase, CGI-58 (Lefevre *et al.*, 2001; Akiyama *et al.*, 2003). In NLSDI, triacylglycerols (TAGs) accumulate in cytosolic droplets in multiple tissues, allowing a rapid, unambiguous clinical diagnosis by oil red O staining of frozen tissue sections (Figure 1), or by visual inspection of blood smears (Elias and Williams, 1985; Williams *et al.*, 1985). Although the ichthyosiform phenotype in NLSDI is nonspecific, most closely resembling non-bullous congenital ichthyosiform erythroderma, patients often experience pruritus, with or without atopic features (Williams *et al.*, 1985) (Figure S1). Biopsies of affected skin demonstrate lipid droplets, most prominently in the epidermal basal layer and in appendageal epithelia (Elias and Williams, 1985). However, such storage vacuoles are not likely to account for the ichthyosiform phenotype in NLSDI, because cytosolic inclusions become entombed within corneocytes, where they cannot influence inherently extracellular functions, such as permeability barrier homeostasis or desquamation. Moreover, comparable cytosolic lipid droplets occur as a non-specific response to toxic insults, as well as in many hyperplastic dermatoses (Zaynoun *et al.*, 1983; Johnson *et al.*, 1987; Kanerva, 1990; El-Shoura and Tallab, 1997; Monteiro-Riviere *et al.*, 2004).

More pertinent to disease phenotype instead could be the small, amorphous, lipid micro-inclusions within epidermal lamellar bodies (LBs), which are secreted, along with lamellar membranes, at the stratum granulosum (SG)/stratum corneum (SC) interface (Elias and Williams, 1985). Accordingly, LB encapsulate several types of lipase activity (Menon *et al.*, 1992; Elias *et al.*, 1998; Rassner *et al.*, 1999), including the hydrolase encoded by CGI-58 (Yamaguchi *et al.*, 2004). Therefore, the enzyme mutation and the lipid micro-inclusions in NLSDI could be linked to disease pathogenesis first, through their colocalization within LB; and then, through the persistence of secreted, undegraded TAGs within the SC interstices.

We hypothesized previously that the ichthyosiform erythroderma in congenital ichthyosiform erythroderma, and presumably in NLSDI, as well, could be driven by a

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Abbreviations: AD, atopic dermatitis; LB, lamellar body; NLSDI, neutral lipid storage disease with ichthyosis; SC, stratum corneum; SG, stratum granulosum; TAG, triacylglycerol; TEWL, transepidermal water loss

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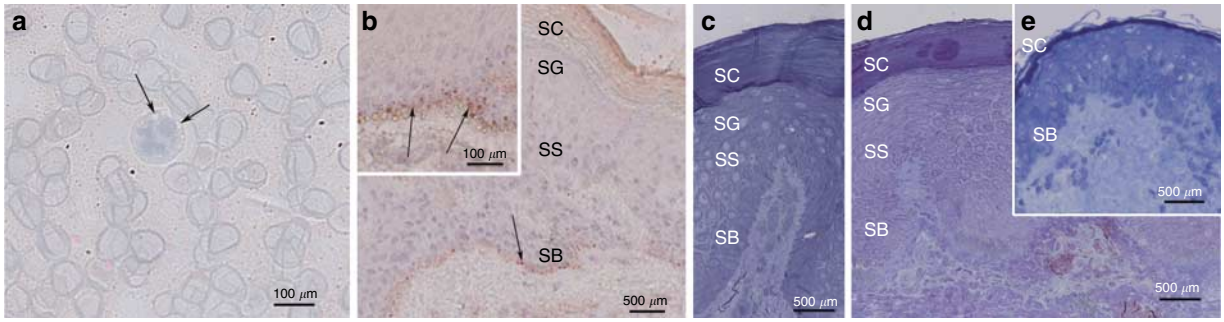

Figure 1. Lipid micro-inclusions in NLSDI. (**a**, arrows) Lipid droplets are visualized in polynuclear cells on a blood smear as empty vacuoles. In the epidermis, after oil red O staining, lipid cytoplasmic micro-inclusions are found in basal keratinocytes (arrows; (**b**) section of the epidermis in frozen sections, with hematoxylin counterstain). Epidermal hyperplasia can be seen in both skin biopsies of patient nos. 1 and 2 on (**c** and **d**) toluidine blue-stained sections in comparison to (**e**) site- and aged-matched control. (SC: stratum corneum; SG: stratum granulosum; SS: stratum spinosum; SB: stratum basal.)

permeability barrier abnormality (Williams, 1992). Normal LBs are replete with lamellar membranes, showing no evidence of non-lamellar lipid micro-inclusions. Following secretion, these membranes normally pack the SG–SC interface, where they transform into the ''mature'' lamellar membrane structures that regulate permeability barrier homeostasis, which again fill the SC interstices (Elias and Menon, 1991). A uniform lamellar phase, which completely fills the SC interstices, therefore equates to permeability barrier competence (Elias and Menon, 1991). In contrast, in NLSDI, we proposed previously that the secreted lipid micro-inclusions transform into electron-lucent, lipid-filled ''clefts'', which could lead to the ichthyosiform dermatosis (Elias and Williams, 1985). But in these earlier studies, the contents of these ''clefts'' could not be visualized owing to prior reliance on osmium fixation; and therefore, they could not be distinguished from the artifact that frequently accompanies tissue processing. If lipids are present within these ''clefts'', they could reflect lamellar/non-lamellar phase separation within the SC interstices, which would form an inherently less-effective permeability barrier than that provided by interstices uniformly replete with lamellar membranes. We asked here first, whether a permeability barrier abnormality exists in NLSDI; second, whether the putative barrier abnormality is linked to lamellar/non-lamellar phase separation of extracellular lipids; and third, whether these structural changes specifically lead to the permeability barrier abnormality.

RESULTS AND COMMENTS

The diagnosis of NLSDI is based upon a characteristic and diagnostic spectrum of clinical and histochemical features (Williams *et al.*, 1985). Although recently attributed to mutations in CGI-58, a lipid hydrolase (Lefevre *et al.*, 2001; Akiyama *et al.*, 2003), mutations in this gene are not invariably found in clinically typical patients (G. Richard, personal communication). The pathway that leads to cytosolic TAG accumulation is not known with certainty. However, labeling studies suggest that the lipolytic activity that utilizes diacylglycerols derived from TAG for phospholipids synthesis is impaired, resulting in TAG accumulation in NLSDI (Williams *et al.*, 1991; Igal and Coleman, 1996, 1998).

The issue we addressed here is how a defect in TAG metabolism can provoke an ichthyosifrom erythroderma. Basal transepidermal water loss (TEWL) values for the patient from one of the new NLSDI kindreds (patient no. 2) were compared with similarly obtained, functional data from normal, age- and gender-matched, historical controls. Depending upon body site, basal TEWL levels were up to threefold higher in NLSDI than in age-matched normal controls (Figure 2a). These water loss levels are comparable to those reported for other ichthyoses with a similar phenotype (congenital ichthyosiform erythroderma and transglutaminase 1-negative lamellar ichthyosis) (Lavrijsen *et al.*, 1995; Elias *et al.*, 2004; Moskowitz *et al.*, 2004). Although basal TEWL levels were increased in NLSDI, barrier recovery kinetics after tape stripping were more rapid in NLSDI than in control skin (Figure 2b). Yet, despite accelerated recovery rates, barrier function never declined to normal levels. Thus, NLSDI resembles atopic dermatitis, not only in some of its clinical features but also in the phenomenon of accelerated barrier recovery kinetics, with failure of barrier function to attain normal competence, despite accelerated recovery rates (Seidenari and Giusti, 1995; Gfesser *et al.*, 1997). Moreover, both SC integrity, assessed as the rate that TEWL levels increase with sequential D-squame® tape strippings, and SC cohesion (amount of protein removed per stripping) were impaired in NLSDI in comparison to normal, control subjects (Figure 2c and d). The abnormality in SC integrity and cohesion likely reflects elevated pH of affected SC, which would activate serine proteases, such as kallikrein 5 and 7, which mediate desquamation (Hachem *et al.*, 2003; Caubet *et al.*, 2004). However, this study did not address mechanisms responsible for the desquamation abnormality in NLSDI.

We next assessed the structural basis for the barrier abnormality in NLSDI. Psoriasiform epidermal hyperplasia was observed in skin biopsies from both patients (nos. 1 and 2) (Figure 1c and d), as previously shown for the first kindred (Elias and Williams, 1985). As shown in prior ultrastructural studies of the first kindred (Elias and Williams, 1985), epidermal LB in patient nos. 1 and 2 again contained lipid

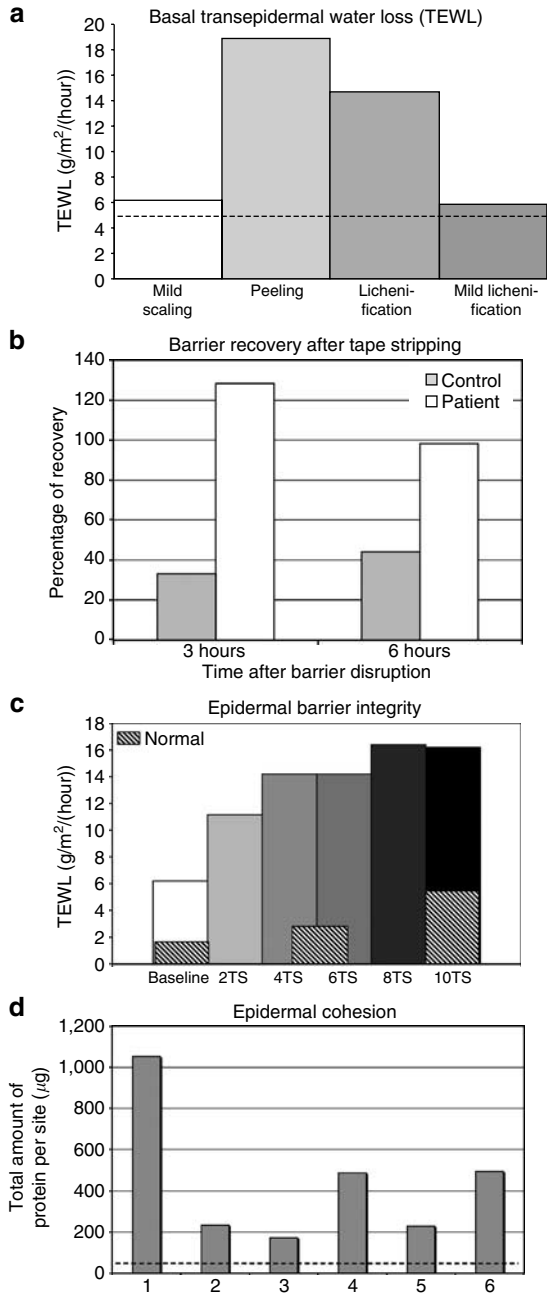

Figure 2. NLSDI displays abnormalities in barrier homeostasis and SC integrity/cohesion. (**a**) TEWL rates from patient no. 2 at baseline, before barrier disruption, were up to three-fold higher than seen in aged-matched historical controls (Seidenari and Giusti, 1995) (horizontal dashed line) (mean age 5±2). (**b**) Barrier recovery after tape stripping in the patient of the new kindred compared to recovery in controls (historical controls in grey boxes). (**c**) Evaluation of epidermal barrier integrity expressed by TEWL after sequential tape-stripping using D-squames®. Normal values are shown in striped boxes (historical controls from adults). (**d**) Cohesion: total amount of protein removed per D-squames® (sum of 10 sequential D-squames® shown) on five different body sites (horizontal line: historical controls) (1: back; 2–6: forearms).

micro-inclusions, which were co-secreted with lamellar contents, at the SG–SC interface (Figure 3). Higher magnification images revealed first that most of these inclusions were membrane bound (Figure 3a and b, black arrows), and that

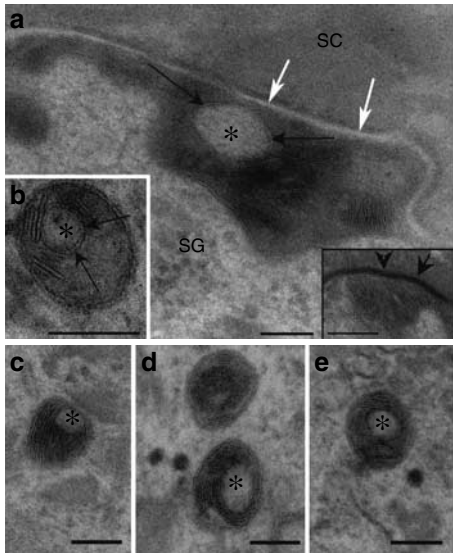

Figure 3. Electron micrographs showing the clefts' at the SC/SG interface. (**a**) White arrows; aged-matched-control is shown in black bordered inset, black arrows. Lipid micro-inclusions (asterisks) appear within the (**a**–**e**) lamellar bodies, which are (**a** and **b**, black arrows) membrane bound. Bars: 100 nm.

these inclusions remained membrane bound following their initial secretion at the SG–SC interface (Figure 3a, black arrows). Despite abnormal LB contents, secretion of organelle content was normal in all of our patients, evidenced by lack of retained LB contents within corneocytes. Thus, NLSDI differs from other types of ichthyoses (e.g., epidermolytic hyperkeratosis), where LB secretion is impaired (Schmuth *et al.*, 2001).

Standard osmium tetroxide post-fixation, followed by Epon–epoxy embedding, demonstrated electron-lucent ''clefts'' initially within the SG–SC interface, and elsewhere within the SC interstices, where these clefts aligned horizontally above parallel arrays of normal-appearing lamellar membranes (Figure 4), as noted previously (Elias and Williams, 1985). Yet, whether these ''clefts'' are artifactual, or whether they contain stored lipid could not be ascertained using this standard protocol. To delineate whether these clefts contain phase-separated (non-lamellar) lipid, we next assessed the parallel samples after ruthenium tetroxide post-fixation, a method that allows visualization of hydrophobic lipid structures, such as the extracellular lamellar membranes (Swartzendruber *et al.*, 1995), coupled with embedding in a lipid-retaining resin-white. Using this method, the ''clefts'' did not appear empty, but rather filled with an amorphous, electron-dense material, adjacent to arrays of lamellar membranes (Figure 5a). These results show that the secreted, LB-derived, lipid micro-inclusions in NLSDI form a non-lamellar phase within the SC interstices. Lamellar/non-lamellar phase separation occurs in polar lipid-based, membrane bilayers, when the amount of nonpolar lipid exceeds the capacity of the membrane to incorporate this lipid (Bangham, 1972; Rehfeld *et al.*, 1986, 1988). Typical,

phospholipid-based membrane bilayers display a very limited capacity to incorporate nonpolar species, such as TAGs or cholesterol esters. Our results suggest that ceramide-based membrane bilayers likewise can only incorporate a limited amount of nonpolar lipids, such as the accumulating TAGs in NLSDI.

Finally, we assessed whether lamellar/non-lamellar phase separation accounts for the permeability barrier abnormality in NLSDI. While the interstices of normal human SC completely exclude the water-soluble, electron-dense tracer, lanthanum nitrate (Elias *et al.*, 1977), tracer could be visualized within the extracellular spaces at all levels of the SC in NLSDI, where it further localized to non-lamellar domains (Figure 5b). In contrast, lanthanum was largely excluded from lamellar domains, demonstrating that water and solute movement occurs preferentially through non-lamellar domains in NLSDI. Thus, this structural feature, that is, lamellar/non-lamellar phase separation, underlies the permeability barrier abnormality in NLSDI.

NLSDI represents just one of several lipid metabolic disorders with ichthyosis that could display a common mechanism of lamellar/non-lamellar phase separation as the basis for the barrier abnormality (and phenotype) in these disorders. Pertinently, phase separation of another accumulated lipid, cholesterol sulfate, has been shown to account for the barrier abnormality in recessive X-linked ichthyosis (Zettersten *et al.*, 1998; Elias *et al.*, 2004). Biophysical studies showed that an excess of this polar lipid cannot be completely incorporated into a lamellar lipid phase, consisting of the SC lipids, cholesterol, and free fatty acids (Rehfeld *et al.*, 1986; Table 1). Moreover, the barrier abnormality in type 2 Gaucher disease can also be attributed, at least in part, to accumulation of phase-separated glucosylceramides in the SC interstices (Holleran *et al.*, 1994). Pertinently, phase

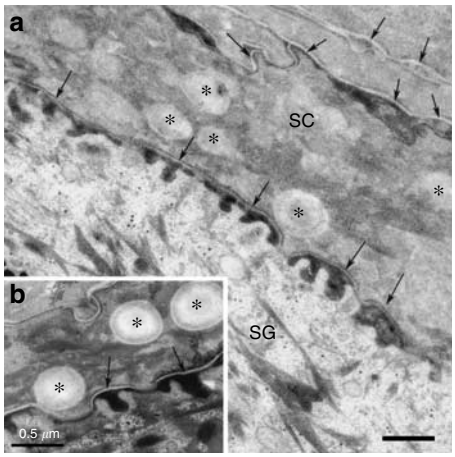

Figure 4. Electron micrograph of epidermis after osmium tetroxide post-fixation and Epon–epoxy embedding. Arrows show empty ''clefts'' at the SC/SG interface and above lamellar material in the SC interstices. Asterisks indicate lipid droplets within the corneocytes. (**a** and **b**) Osmium tetroxide post-fixation. Control, normal epidermis: cf. Figure 3a (inset) and Elias and Williams (1985). Bars: 500 nm.

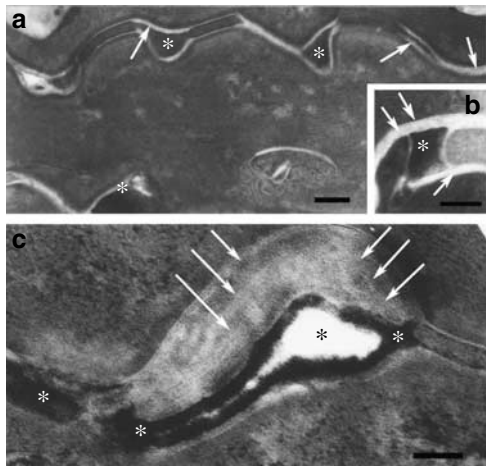

Figure 5. Electron micrographs after ruthenium tetroxide post-fixation coupled with lipid-retaining resin-white embedding. (**a** and **b**) Amorphous electron-dense material forming the non-lamellar phase in the ''clefts'' (asterisks) adjacent to normal-appearing lamellae (white arrows). The electron-dense tracer, lanthanum nitrate, is excluded from lamellar domains, but can be seen within adjacent non-lamellar domains in the SC interstices ((**c**), ruthenium tetroxide post-fixation, Epon embedded). Bars: 100 nm.

Table 1. Ichthyoses that have or could have lamellar phase separation as basis for barrier abnormality

Disease	Enzymatic defect	Barrier dysfunction	Lamellar/non-lamellar phase separation	Phase-separated lipid
Neutral lipid storage disease	Hydrolase (CGI-58)	Demonstrated[1]	Demonstrated[1]	Triglycerides
Recessive X-linked ichthyosis	Steroid sulfatase	Demonstrated[2]	Demonstrated[2]	Cholesterol sulfate
Refsum's disease	Phytanoyl-CoA hydroxylase (PhyH)	Not assessed	Not assessed	Phytanic acid in all glycerolipids[3]
Sjogren–Larsson	Fatty aldehyde deshydrogenase	Not assessed	Demonstrated[4]	Not assessed
Gaucher disease	β-Glucocerebrosidase	Demonstrated[5]	Demonstrated[5]	Glucosylceramides
CHILD syndrome	NAD(P)H 3β-hydroxysteroid deshydrogenase (NSDHL)	Not assessed	Not assessed	Not assessed
Niemann-Pick disease	Acid sphyngomyelinase	Demonstrated[6,7]	Demonstrated[7]	Not assessed

[1]These studies; [2]Elias *et al.* (2004); [3]Van den Brink *et al.* (2004); [4]Shibaki *et al.* (2004); [5]Holleran *et al.* (1994); [6]Jensen *et al.* (1999); [7]Schmuth *et al.* (2000).

separation has been utilized as a strategy to enhance transdermal drug delivery. For example, topical neutral lipid-based penetration enhancers, such as oleic acid, 3-α-hydroxysterols, and *trans*-vaccenic acid, form non-lamellar lipid phases within the SC interstices (Mao-Qiang *et al*., 1993; Jiang *et al*., 2000), producing enhanced transcutaneous permeability, presumably by a mechanism analogous to the development of a non-lamellar phase in NLSDI. It is also likely, but not yet demonstrated, that in several other inherited, lipid-storage diseases associated with ichthyosis, such as Refsum disease, Sjögren–Larsson syndrome, and Congenital Hemidysplasia with Ichthyosiform erythroderma and Limb Defects (CHILD) syndrome, a similar pathomechanism could be operative. An additional pathomechanism could also be operative in recessive X-linked ichthyosis and CHILD syndrome; that is, accumulation of lipid precursors (cholesterol sulfate and desmosterol/zymosterol, respectively) could result in lamellar membranes that are deficient in cholesterol. Cholesterol is one of the key lipids (with ceramides and free fatty acids) that are required to form mature lamellar membranes, and such cholesterol-deficient membranes provide a sub-optimal barrier (Feingold, 1991).

In conclusion, the presumed basis for the permeability barrier abnormality in NLSDI relates to the limited capacity of lamellar membranes to incorporate excess nonpolar lipids into lamellar membranes, which already are fully ''loaded'' with nonpolar species. Yet, it should also be noted that in most of the inherited disorders where we are positing this mechanism, neither barrier function nor membrane structure has been assessed (Table 1).

MATERIALS AND METHODS (see figure legends for further details)

Patients

The Committee for Human Research, University of California, San Francisco, approved all the described studies, which were conducted according to the Declaration of Helsinki Principles. All participants provided formal written, informed consent before their participation in this study.

We had access to one or more family members from three unrelated kindreds with NLSDI. The clinical, pathological, and biochemical abnormalities of the four affected members from the first kindred (Palestinian) have been described in detail (Elias and Williams, 1985; Williams *et al*., 1985; Figure S1).

The affected family member from the second, Greek/Lebanese kindred (patient no. 1) is a 29-year-old female, who presented with generalized, fine scaling and hyperkeratosis. She is the only affected member of a non-consanguineous kindred. Physical examination showed generalized erythroderma with more severe involvement of flexural and extensor surfaces, along with prominent hyperkeratosis. No keratoderma was noted on her palms. Of note, she was somewhat overweight, with an unusual pattern of fat deposition over the uppertrunk, face, and neck. Her clinical history began with a dry and flaky, scaly rash in early infancy when a skin biopsy was performed that was inconclusive. In addition to clinical finding of an enlarged liver, associated with repeatedly elevated liver function tests, a liver biopsy was performed at that time which demonstrated moderate steatosis with mildly active steatohepatitis. Moreover, peripheral blood smears showed neutrophils, basophils, and monocytes with multiple, small-to-medium-sized, spherical cytoplasmic vacuoles. An audiology examination demonstrated sensorineural hearing loss, while an ophthalmologic evaluation revealed a small cataract. Her medical history included primary ovarian failure, as well as type IIa hyperlipoproteinemia. Neither cutaneous nor respiratory allergies were noted. No other family members were affected, except for a sister with a history of mild xerosis of the skin (see pedigree Figure S2b).

When first seen at University of California San Francisco, the patient's blood smear demonstrated pale, oil red O-positive vacuoles within polynuclear leukocytes (Figure 1a), and frozen sections of a skin biopsy also clearly showed neutral lipid-enriched, cytoplasmic vacuoles by oil red O staining (Figure 1b). These laboratory findings, in conjunction with evidence of multisystem involvement, coupled with an ichthyosiform erythroderma, were considered diagnostic of NLSDI.

The affected patient from the third, Indian kindred (patient no. 2) is a 12-year-old girl, who also presented initially with generalized erythroderma, scaling and xerosis. Physical examination showed erythroderma with generalized scaling, including the scalp, severe lichenification of the neck and flexural areas (Figure S2a), and hyperkeratosis on the extensor aspects of both knees and wrists. The patient also reported generalized, but moderate pruritus. Her parents were first cousins (see pedigree: Figure S2c). Family history revealed no other affected members, but asthma was present in her mother and grandfathers, and her father had a history of atopic dermatitis and hay fever. Laboratory testing at the age of 2 years revealed hypertriglyceridemia, with abnormal liver functions, and a liver biopsy showed moderate hepatic fibrosis. A blood smear demonstrated vacuoles in polynucleated leukocytes consistent with stored neutral lipids, while a skin biopsy again showed lipid inclusions in epithelial and appendageal keratinocytes. The diagnosis of NLSDI was therefore confirmed by this characteristic constellation of clinical and histolopathologic findings.

Methods

We assessed basal permeability barrier function as mean TEWL, with a Tewameter (Courage and Khazaka, Cologne, FRG) over four separate sites, which displayed varied clinical features (e.g., lichenification), on one patient (patient no. 2), who desisted from application of topical emollients for 1 week before study. Barrier function, SC integrity, and cohesion were assessed after sequential tape-stripping (i.e., repeated application/removal of D-squame® adhesive discs (Cuderm Corp., Dallas, TX, USA)). After aldehyde pre-fixation of punch biopsies from patient nos. 1 and 2, samples were split and post-fixed in osmium tetroxide and ruthenium tetroxide, followed by dehydration and embedding either in an Epon–epoxy mixture or in lipid-retaining resin-white, a lipid-retaining epoxy resin (Newman *et al*., 1983). To further assess and localize permeability barrier dysfunction by an alternate method, portions of skin biopsy samples from patient nos. 1 and 2 were immersed in the water-soluble, electron-dense tracer, 4% colloidal lanthanum, for 4 hours, followed by osmium tetroxide and ruthenium tetroxide post-fixation and Epon/epoxy embedding, as we have described (Schmuth *et al*., 2001, 2004; Elias *et al*., 2004). Ultrathin sections were examined and photographed in a Zeiss 10A electron microscope, operated at 60 kV.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. Clinical presentation of NLSDI.

Figure S2. Clinical features and pedigrees.

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