Importance: Secondary infections and impaired desquamation complicate certain inherited ichthyoses, but their cellular basis remains unknown. In healthy human epidermis, the antimicrobial peptides cathelicidin (LL-37) and human β-defensin 2 (HBD2), as well as the desquamatory protease kallikrein-related peptidase 7 (KLK7), are delivered to the stratum corneum (SC) interstices by lamellar body (LB) exocytosis.

Objective: To assess whether abnormalities in the LB secretory system could account for increased risk of infections and impaired desquamation in inherited ichthyoses with known abnormalities in LB assembly (Harlequin ichthyosis [HI]), secretion (epidermolytic ichthyosis [EI]), or postsecretory proteolysis (Netherton syndrome [NS]).

Design, Setting, and Participants: Samples from library material were taken from patients with HI, EI, NS, and other ichthyoses, but with a normal LB secretory system, and in healthy controls and were evaluated by electron microscopy and immunohistochemical analysis from July 1, 2010, through March 31, 2013.

Main Outcome and Measures: Changes in LB secretion and in the fate of LB-derived enzymes and antimicrobial peptides in ichthyotic patients vs healthy controls.

Results: In healthy controls and patients with X-linked ichthyosis, neutral lipid storage disease with ichthyosis, and Gaucher disease, LB secretion is normal, and delivery of LB-derived proteins and LL-37 immunostaining persists high into the SC. In contrast, proteins loaded into nascent LBs and their delivery to the SC interstices decrease markedly in patients with HI, paralleled by reduced immunostaining for LL-37, HBD2, and KLK7 in the SC. In patients with EI, the cytoskeletal abnormality impairs the exocytosis of LB contents and thus results in decreased LL-37, HBD2, and KLK7 secretion, causing substantial entombment of these proteins within the corneocyte cytosol. Finally, in patients with NS, although abundant enzyme proteins loaded in parallel with accelerated LB production, LL-37 disappears, whereas KLK7 levels increase markedly in the SC.

Conclusions and Relevance: Together, these results suggest that diverse abnormalities in the LB secretory system account for the increased risk of secondary infections and impaired desquamation in patients with HI, EI, and NS.
Impaired desquamation is a hallmark of ichthyoses, but infections also are particularly common in 3 unrelated, inherited forms of ichthyosis: Harlequin ichthyosis (HI), epidermolytic ichthyosis (EI, also known as epidermolytic hyperkeratosis), and Netherton syndrome (NS). The excessive scale in these 3 ichthyoses in part reflects epidermal hyperplasia, secondary to the barrier abnormality. However, the cellular basis for the impaired desquamation and for the increased risk of cutaneous infections in these 3 disorders is unknown. Like all the ichthyoses, these disorders display prominent and occasionally life-threatening abnormalities in permeability barrier function. Because permeability barrier status and antimicrobial defense are closely linked functions, we hypothesized that related cellular mechanisms could account for the increased prevalence of infections and the impaired desquamation in HI, EI, and NS.

Distinctive abnormalities in the lamellar body (LB) secretory system are apparent in HI, EI, and NS, accounting for their prominent permeability barrier abnormalities (eTable 1 in the Supplement). In HI, loss-of-function mutations in the transmembrane lipid transporter ABCA12 (OMIM 607800) result in failure in the secretion of glucosylceramides to nascent LB. As a result, a paucity of this lipid, and perhaps other LB cargo, is delivered to the stratum corneum (SC) interstices. However, the cornified lipid envelope, a structure thought to originate from fusion of LBs with the plasma membrane, is normal in HI, suggesting that formae frustae organelles continue to be formed and secreted in this disorder. Whether the delivery of other LB lipid and/or protein contents also is impaired in HI is not yet known. In contrast, LBs form normally in EI, but cytoskeletal disruption impedes the exocytosis of most LB contents from granular cells, resulting in a paucity of extracellular lamellar bilayers. In contrast, NS epidermis generates abundant LBs, with accelerated en masse secretion of seemingly replete contents into the extracellular spaces of the outer epidermis, likely as a compensatory response to a thin, poor quality SC. This accelerated secretory response likely allows survival of patients with NS in a terrestrial environment.

Netherton syndrome is due to loss-of-function mutations in SPINK5 (OMIM 605010) that encode the serine protease (kallikrein) inhibitor LEKTI. Secreted protein contents, including the 2 ceramide-generating enzymes acidic sphingomyelinase and β-glucocerebrosidase, are rapidly destroyed by unrestricted proteolysis, accounting in large part for the permeability barrier abnormality in NS.

Because protein delivery to LB is dependent on prior or concurrent lipid deposition in these organelles, we hypothesized that impaired desquamation and infectious complications associated with HI could reflect a failure in the delivery of protein cargo, including antimicrobial peptides (AMPs) and desquamatory proteases into nascent LB. In contrast, impaired desquamation and infections in EI could reflect a concurrent failure in the delivery of AMPs and desquamatory proteases to the SC interstices secondary to cytoskeletal abnormalities. Finally, we hypothesized that the increased risk of infections in NS likely reflects accelerated degradation of the LB-derived AMP cathelicidin (LL-37) because of enhanced kallikrein-related peptidase 7 (KLK7) levels, which likely also accounts for poor SC cohesion in this disorder. Our results confirm that genetic abnormalities that compromise the LB secretory system by unrelated mechanisms produce corresponding deficits in the delivery or persistence of AMPs and KLK7 in the SC extracellular domains. These abnormalities in turn predict an increased prevalence of cutaneous infections and impaired desquamation in these diverse forms of inherited ichthyosis.

### Methods

#### Patient Samples

This study was performed from July 1, 2010, through March 31, 2013. We relied on formaldehyde-fixed, paraffin-embedded library material from previously described and, in most cases, genotyped patients with ichthyosis for all the immunohistochemical studies (eTable 1 in the Supplement). The 3 HI samples were from the pregenotype era, but each had a characteristic, severe neonatal phenotype. Ultrastructural features of these patients have been previously described. Aldehyde-fixed patient samples were stored and refrigerated in 0.1M cacodylate buffer for lipase localization studies (eTable 2 in the Supplement). The paraffin-embedded samples came from the University of California, San Francisco, University of Washington, and Yale University. Controls included samples of healthy human skin (n = 3) (eTable 1 in the Supplement) and samples from genotyped patients with forms of inherited ichthyosis that are not associated with compromised LB secretion, including Gaucher disease (GD) (n = 3), neutral lipid storage disease with ichthyosis (NLSDI) (n = 1), and X-linked ichthyosis (XLI) (n = 3) (eTable 2 in the Supplement).

#### Potential Risks

Under separate institutional review board-approved protocols at each collaborator’s institution, samples from library material of previously obtained skin biopsy specimens were sent to the Elias Lab for study under a separate, University of California, San Francisco–approved protocol. Informed consent was not required because the library material was from routine pathology specimens.

#### LL-37, HBD2, and KLK7 Immunostaining

Sections 10-μm thick of formalin-fixed, paraffin-embedded tissue were deparaffinized and rehydrated, followed by heat-induced epitope retrieval. Sections were rinsed in phosphate-buffered saline (PBS) and incubated for 1 hour in blocking buffer (4% bovine serum albumin and 0.5% cold water fish gelatin in PBS). The primary antibody (monoclonal anti-LL-37) was a gift from Richard Gallo, MD, PhD; the human β-defensin 2 (HBD2) and KLK7 antibodies were from Abcam plc. Primary antibodies were applied overnight at 4°C at 1:500 dilution in blocking buffer. The following morning, sections were washed 3 times in PBS and incubated for 1 hour at room temperature with an Alexa Fluor 488-conjugated secondary antibody, diluted 1:2000 in blocking buffer. Tissue sections then were washed 3 times with PBS, counterstained with propidium iodide, visualized, and photographed at ×40 magnification with a confocal microscope (LSM510; Carl Zeiss AG).
Ultrastructural Cytochemical Analysis
We used the LB content marker acidic lipase to approximate the amount of protein that is assembled into and secreted from LB in stratum granulosum (SG) cells in HI, EI, and NS compared with healthy control samples and samples from 3 unrelated ichthyoses that have normal LB assembly and secretion (ie, XLI, GD, and NLSDI). The rationale for this approach is described in the article by Menon et al, and details of this method can be found in previously published articles.

Electron Microscopy
Biopsy samples were minced to 0.5 mm³ or less, fixed in modified Karnovsky fixative overnight, washed 3 times in 0.1M cacodylate buffer, split into multiple samples that were stored in 0.1M cacodylate buffer until subjected to lipase cytochemical analysis as described above, and then postfixed in 1% osmium tetroxide that contained 1.5% potassium ferrocyanide. After postfixation, all samples were dehydrated in a graded ethanol series and embedded in an EPON-epoxy mixture. Ultrathin sections were examined with and without further lead citrate contrasting with an electron microscope (Zeiss 10A; Carl Zeiss AG) operated at 60 kV.

Results
Controls
Visualization of acidic lipase activity can be deployed as an ultrastructural marker for the extent of protein loading into LB, the efficiency of LB secretion, and/or the fate of secreted LB contents in human epidermis. Although not quantitative, this assay has revealed incomplete protein loading and/or aberrant secretion of LB contents in multiple forms of ichthyosis that correlate with corresponding deficits in permeability barrier function. In healthy human epidermis, abundant lipase activity collected in LB was delivered in toto to the SC interstices, where it persisted into the middle to outer SC (eFigure 1 in the Supplement). Likewise, abundant lipase activity was loaded into and secreted in toto from LB, persisting high into the SC in 3 unrelated, inherited ichthyoses previously found to have a normal LB secretory mechanism (ie, XLI, GD, and NLSDI; eFigure 2 in the Supplement). Abnormalities in LB contents do not impair exocytosis of LB contents in NLSDI.

Harlequin Ichthyosis
We next deployed the lipase method to determine whether failure of glucosylceramide loading in HI impedes protein loading into nascent LBs. In contrast to healthy human epidermis, where abundant lipase activity is loaded into LBs and delivered to the SC interstices, reduced amounts of acidic lipase activity decorated the contents of a multitude of smaller vesicular structures in the SG in HI (Figure 1 and eFigure 2 in the Supplement). Although they lack visible lamellar contents, these organelles likely represent forme fruste LBs. A corresponding decrease in enzyme activity was evident at the SG-SC interface (Figure 1).

Epidermolytic Ichthyosis
Protein loading into LBs, indicated by the retention of intense acidic lipase activity, appears normal in EI; however, levels of enzyme activity were reduced at and above the SG-SC interface (Figure 2), consistent with a previous description of a blockade of LB exocytosis in EI. Regardless of levels in the underlying epidermis, LL-37 and HBD2 appeared to largely disappear from the SC in EI. Likewise, immunostaining for KLK7 was markedly reduced in the SC of all EI samples. Of interest, immunostaining for LL-37 (but not HBD2) appeared to increase markedly in 2 of the 4 samples of affected epidermis (eTable 2 in the Supplement), suggesting that these lesions may have been infected at the time of biopsy. Together, these results suggest that LL-37 and KLK7 are produced in normal to increased quantities in EI but that they are incompletely delivered to the SC extracellular spaces.

Netherton Syndrome
Previous studies found that LB production and secretion accelerate in NS, likely as a compensatory response for the often
devastating permeability barrier abnormality in this disease. One study also found that LB-derived, ceramide-generating enzymes, β-glucocerebrosidase and acidic sphingomyelinase, are destroyed because of unrestricted KLK activity in NS. Accordingly, although loading of acidic lipase activity into LBs appeared normal to increased in NS, secreted enzyme activity completely disappeared by one layer above the SG-SC interface (Figure 3).

As expected, KLK7 immunostaining markedly increased in the SC of NS, where enzyme protein prominently localizes to membrane domains. Likewise, although the overall expression of LL-37 and HBD2 appeared normal to increased in the epidermal nucleated layers, LL-37 became undetectable just above the SG-SC interface in NS, paralleling the sudden reduction in acidic lipase activity in the SC (Figure 4 and Figure 5). These results indicate that LL-37 and HBD2, although produced in normal to increased quantities in NS, disappear shortly after secretion in NS, presumably because of unrestricted proteolysis, and that the thinner than normal SC in NS correlates with higher than normal levels of KLK7 in the SC interstices.

Figure 2. Epidermolytic Ichthyosis

A, Levels of extracellular enzyme activity were reduced at and above the stratum granulosum (SG) and stratum corneum (SC) interface (yellow arrows) (original magnification ×50). B, Heavy enzyme labeling of lamellar bodies (red arrows) in the SG but incomplete exocytosis of enzyme contents in the SC extracellular spaces (yellow arrows, A) (original magnification ×50).

A, Rapid disappearance of lipase activity above the transitional cell (TC) area and in the lower stratum corneum (SC) (yellow arrows). B, Enhanced loading of enzyme content into lamellar bodies (LBs) and accelerated secretion of LB contents (red arrows) (original magnification ×50; inset: original magnification ×20). SG indicates stratum granulosum.

Discussion

In this study, we asked whether the well-documented increase in prevalence and severity of cutaneous infections, as well as the impaired desquamation in HI, EI, and NS, could be attributed to previously documented defects in the LB secretory system in these disorders (Table 1 in the Supplement). A limitation of this study was our reliance on aldehyde-fixed library material, which made it impossible to quantitate assays for AMP or KLK7 content. Nonetheless, in all 3 diseases, we found striking differences in the assembly, secretion, or postsecretory fate of LB contents, including reduced to absent levels of 4 LB-derived proteins (acidic lipase, LL-37, HBD2, and KLK7) in the SC. In contrast, healthy controls and 3 cases of other inherited ichthyoses that exhibit an intact LB secretory system (eTable 2 in the Supplement) had a normal distribution of lipase activity and a normal pattern of AMP and KLK7 immunostaining. Thus, reduced extracellular LL-37 (and likely HBD2 in the case of HI and EI) levels in the SC could explain, at least in part, the increased prevalence and severity of cutaneous infections in patients with HI, EI, and NS. Likewise, the prominent desquamation abnormality in HI and EI levels in the SC could explain, at least in part, the increased prevalence and severity of cutaneous infections in patients with HI, EI, and NS. Likewise, the prominent desquamation abnormality in HI and EI, although in part due to barrier-driven epidermal hyperplasia, probably can be attributed at least in part to a failure to deliver the LB-derived serine protease KLK7 and other LB desquamatory proteases to the SC interstices. Thomas et al described reduced levels of 2 other desquamatory proteases, KLK5 and cathepsin D, in the SC of patients with HI. Moreover, a preliminary study by the Roop Laboratory (University of Colorado, Denver) found that the thicker SC in a transgenic mouse model of HI can be attributed to persistence of corneodesmosomes. These investigators conversely found that topical applications of recombinant KLK5 and KLK7 facilitated desquamation in explants.
from these mice. However, delivery of LB-derived proteins is normal in NS, but unopposed proteolytic activity, reflected by excessive KLK7 activity, likely accounts for the poor cohesion of the SC and the accelerated degradation of the protease-sensitive AMP LL-37 (and likely also HBD2) in NS.

Both LL-37 and HBD2 are packaged within LB and secreted at relatively low, constitutive levels into the SC interstices in normal epidermis. However, delivery of these AMP accelerates in response to (1) acute barrier disruption, (2) pathogen challenges, (3) suberythrogenic UV-B exposure, (4) topical applications of the
of importance, the cellular mechanisms that lead to decreased levels of AMP and KLK7 in the SC differ in these 3 disorders. In HI, few of these proteins appear to be packaged into nascent LB, and as a result, reduced amounts of LB-derived proteins are delivered to the SC interstices. In contrast, production of LL-37 and HBD2 appears to accelerate in NS, but these AMPs then appeared to be destroyed as rapidly as they are secreted, consistent with the known susceptibility of LL-37 to proteolytic

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degradation. Accordignly, we found that levels of one key desquamatory protease (eg, KLK7) appear to increase markedly in NS.

Increased cutaneous infections and a prominent desquamatory abnormality are also hallmarks of EI, in which LB secretion is impaired due to a cytoskeletal abnormality, a finding that correlated with an apparent failure to deliver LL-37 and KLK7 to the SC interstices. In summary, abnormalities in the assembly, secretion, or postsecretory processing, due to excessive proteolysis of LB-derived proteins, predict abnormalities in cutaneous antimicrobial defense and desquamation in 3 genetically diverse forms of ichthyosis.

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Author Affiliations: Dermatology Service, Department of Veterans Affairs Medical Center, University of California, San Francisco (Chan, Godoy-Gijon, Nuno-Gonzalez, Crumrine, Hupe, Elias); Department of Dermatology, University of California, San Francisco (Chan, Godoy-Gijon, Nuno-Gonzalez, Crumrine, Hupe, Williams, Elias); Department of Dermatology, Yonsei University, Wonju College of Medicine, Wonju, South Korea (Cho); Department of Dermatology and Venereology, Medical University of Innsbruck, Innsbruck, Austria (Gruber); Department of Pediatrics, University of California, San Francisco (Williams); Department of Dermatology, Yale University, New Haven, Connecticut (Choate); Department of Pathology, Yale University, New Haven, Connecticut (Choate); Division of Dermatology, University of Washington, Seattle (Fleckman).

Author Contributions: Dr Elias had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Drs Chan, Godoy-Gijon, and Nuno-Gonzalez are all considered first authors.

Study concept and design: Chan, Godoy-Gijon, Williams, Elias.

Acquisition, analysis, or interpretation of data: Chan, Godoy-Gijon, Nuno-Gonzalez, Crumrine, Hupe, Choi, Gruber, Choate, Fleckman, Elias.

Drafting of the manuscript: Chan, Nuno-Gonzalez, Gruber, Choate, Elias.

Critical review of the manuscript for intellectual content: Chan, Godoy-Gijon, Crumrine, Hupe, Choi, Gruber, Choate, Fleckman, Elias.

Statistical analysis: Crumrine, Hupe.

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REFERENCES

Conclusions
We have determined that HI is due to decreased protein loading into LBs, leading to decreased LL-37 levels within the SC interstices. Netherton syndrome is due to increased loading and increased secretion of protein into LBs, which cannot compensate for accelerated proteolysis of enzyme proteins and LL-37 within the SC interstices. Epidermolytic ichthyosis is due to decreased exocytosis of protein from LBs, leading to decreased levels of LL-37 within the SC interstices. Increased prevalence of secondary cutaneous infections in HI, NS, and EI can be attributed to distinctive abnormalities in the LB secretory system that collectively decrease the bioavailability of LL-37.


