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# Ichthyosis: Assessing Severity And Genotype-Phenotype Correlations

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Ichthyosis: Assessing Severity and Genotype-Phenotype Correlations

A Thesis Submitted to the  
Yale University School of Medicine  
in Partial Fulfillment of the Requirements of the  
Degree of Doctor of Medicine

by  
Nareh Valerie Marukian  
2017

## ABSTRACT

The ichthyoses, also known as disorders of keratinization (DOK), encompass a group of genetic skin disorders linked by the common finding of abnormal barrier function, which initiates a default compensatory pathway of hyperproliferation, resulting in the characteristic clinical manifestation of localized or generalized scaling.

Ichthyoses are a highly heterogeneous group of disorders, both genetically and clinically. Despite the identification of causative mutations in over 50 genes, clear genotype-phenotype correlations have been difficult to establish due to the rarity of the ichthyoses and because mutations in the same gene can have widely divergent phenotypes, even in individuals with the same disease-causing mutation.

In partnership with the Foundation for Ichthyosis and Related Skin Types (FIRST), we have served as a major national and international referral center and recruited over 800 kindreds with DOK, utilizing whole exome sequencing to identify novel, rare mutations that cause DOK, many of which represent phenotypic expansion.

We systematically evaluated genotypic and phenotypic data for 407 kindreds with ichthyosis, the largest cohort published to date. We identified 156 novel mutations and assessed phenotypic features of DOK with respect to genetic diagnosis to help refine our understanding of this heterogeneous group of disorders (**Chapter 1**). Such a systematic classification of DOK holds promise for the development of customized management plans, generation of targeted therapeutics, and improved understanding of prognostication based on genetic diagnosis.

In the process of performing a large scale genotype-phenotype characterization, we identified kindreds with rare clinical subtypes of ichthyosis, including Bathing Suit Ichthyosis (BSI), a rare disorder caused by mutations in the transglutaminase 1 gene (*TGM1*) and characterized by the restriction of scale to sites of relatively higher temperature such as the trunk, with cooler areas remaining unaffected (**Chapter 2**). We identified novel and recurrent mutations in sixteen subjects with BSI, the largest cohort published to date. Our findings expand the genotypic spectrum of BSI and the understanding of temperature-sensitivity of *TGM1* mutations. Increased awareness of temperature-sensitive *TGM1* genotypes should aid in genetic counseling, and provide insights into the pathophysiology of *TGM1* ichthyoses, and potential therapeutic approaches.

In the course of unifying genetic and clinical data to advance the understanding of the different subtypes of ichthyosis, we realized the critical importance of tools to assess the clinical severity of ichthyosis. We designed a Visual Index for Ichthyosis Severity (VIIS) and tested the instrument for reliability and reproducibility using two different settings: one that utilized scoring of 60 test photographs by 10 dermatologists, and one with in-person evaluations on 85 subjects by 12 dermatologists at the Foundation for Ichthyosis and Related Skin Types (FIRST) conference (**Chapter 3**). The validation process revealed high reliability and reproducibility for both scale and erythema and indicated that the VIIS performs better in person than with photographs, an important consideration in design of clinical trials. This index provides a tool for clinical phenotyping and assessment of therapeutic response for many disorders of keratinization.

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## INTRODUCTION

The ichthyoses are rare genetic skin disorders linked by the common finding of abnormal barrier function, which leads to increased transepidermal water loss and compensatory hyperproliferation. The unifying phenotypic feature of ichthyoses is localized or generalized scaling. Other clinical manifestations can include erythema, palmoplantar keratoderma (PPK), hypohydrosis, anhidrosis, and recurrent infections. DOK can have a range of possible sequelae, including ectropion, or out-turning of the eyelids, alopecia, deforming joint contractures, and failure to thrive **Figure 1**.



### Figure 1 Sequelae of inherited ichthyoses

The range of possible sequelae of DOK includes a. Severe bilateral upper and lower eyelid ectropion b. Scarring alopecia c. Failure to thrive requiring gastric tube placement d. Deforming joint contractures e. Severe plantar keratoderma with resulting limitations in ambulation. Genotype is provided in each panel.

Although ichthyoses are primarily inherited disorders with onset at or shortly after birth, rare acquired forms have been reported in the setting of malignancy, nutritional deficiency, and autoimmune or infectious disease. Mutations in over 50 genes have been reported to cause ichthyoses, and these affect a host of cellular functions including DNA repair, lipid biosynthesis, adhesion, and desquamation as well as other pathways.<sup>1</sup> Despite myriad pathways for pathogenesis, each features disrupted barrier function.

Epidermal barrier function is maintained by a regular pattern of epidermal renewal in which keratinocytes, the primary cell type of the skin, arise from a renewing stem cell pool and undergo a tightly regulated pattern of differentiation as they transit from the innermost stratum basale to the outermost stratum corneum, where they are ultimately sloughed off. This differentiation program is marked by site-specific expression of proteins and, in the suprabasal layers, the production of components necessary for the generation of the lipid barrier.

In the process of differentiation, keratins—intermediate filaments that are responsible for the structural integrity of keratinocytes—are among the first proteins to be expressed in a tightly regulated manner, with keratin 5 and 14 expressed in the basal layer and keratin 1 and 10 expressed in the suprabasal layers. In stratum spinosum, the second innermost layer of the epidermis, components of the lipid barrier (phospholipids, cholesterol, sphingomyelin and glucosylceramides) are packaged into lamellar bodies, which are specialized organelles that house the building blocks of the lipid barrier, as well as enzymes essential to the processing of lipid barrier precursors. At the transition from stratum granulosum—the third layer of the epidermis—to stratum corneum, the contents of lamellar bodies are extruded into the intercellular space to form protective lipid sheets that are responsible for the skin's hydrophobic barrier.<sup>2,3</sup>

This overall process of differentiation results in the formation of a robust barrier in the stratum corneum, composed of keratinocytes (the individual bricks of the barrier) and inter-keratinocyte lipids (the mortar)<sup>4</sup>. Mutation in proteins essential to the formation of this barrier (i.e. keratins, enzymes involved in lipid synthesis) lead to the disruption of barrier integrity, resulting in ichthyosis.

Inherited ichthyoses exhibit marked genetic and phenotypic heterogeneity, and advances in next-generation sequencing technology have allowed for more rapid and cost-effective genetic analysis, leading to the identification of novel, rare mutations that cause DOK. Clear large-scale genotype-phenotype correlations have been difficult to establish, as mutations in the same gene can present with widely divergent phenotypes, even within kindreds bearing the same disease-causing mutation.

In 2009, the Ichthyosis Consensus Conference established a consensus classification for DOK based on pathophysiology, clinical manifestations, and mode of inheritance<sup>1</sup>. This nomenclature system divides DOK into two main groups: 1) nonsyndromic forms, with clinical findings limited to the skin 2) syndromic forms, with involvement of other organ systems.

## **Nonsyndromic ichthyoses**

### Common Ichthyoses

Ichthyosis vulgaris (IV) and X-linked recessive ichthyosis (XLRI) are classified as the “common ichthyoses”, given their high prevalence. IV is the most common form of nonsyndromic inherited ichthyosis, with an estimated incidence of 1 in 250 births.<sup>5</sup>



Typically, IV is a phenotypically mild form of ichthyosis. Clinical findings usually appear around 2 months of age, and include generalized xerosis and fine white to gray scale that is most prominent on the abdomen, chest and extensor surfaces of the extremities. Keratosis pilaris and hyper-linearities of the palms and soles are also frequently associated with IV.

IV is caused by autosomal dominant mutations in the filaggrin gene (*FLG*), which plays an essential role in epidermal differentiation and formation of the skin barrier.<sup>6,7</sup> An autosomal semidominant mode of inheritance has also been described, meaning that while individuals with heterozygous mutations have a mild phenotype, those with homozygous or compound heterozygous mutations can display more severe forms of ichthyosis.<sup>6</sup>

Patients with IV are at increased risk for atopic dermatitis, asthma and allergies.<sup>8,9</sup> This increased risk is likely due to disruption of barrier function, which may allow for greater penetration of the epidermis by potential allergens.<sup>8</sup>

XLRI is the second most common form of inherited ichthyoses, with a prevalence of 1:2000 to 1:6000 males.<sup>10</sup> Clinical findings in XLRI are frequently indistinguishable from IV. Manifestations usually first appear in the neonatal period as generalized desquamation and xerosis, and progress to fine scaling of the trunk and extremities in infancy. Over time, patients develop brownish, polygonal, plate-like scale that is tightly adherent to the skin. XLRI is caused by mutations in the *STS* gene, encoding steroid sulfatase, on the X chromosome.<sup>11</sup>

Autosomal Recessive Congenital Ichthyosis

Autosomal Recessive Congenital Ichthyosis (ARCI) is a genetically and phenotypically heterogeneous group of disorders that includes harlequin ichthyosis (HI), lamellar ichthyosis (LI), and congenital ichthyosiform erythroderma (CIE). The incidence of ARCI has been approximated at 1 in 200,000 births.<sup>12</sup>

HI is caused by loss-of-function mutations in *ABCA12*, which encodes an ATP binding cassette (ABC) transporter. *ABCA12* is necessary for lipid transport into lamellar granules, and central to the process of cornification and lipid barrier formation.<sup>13</sup>

Interestingly, while homozygous loss of function mutations in *ABCA12* lead to HI, missense mutations in *ABCA12* result in milder phenotypes on the LI/CIE spectrum.<sup>14</sup>

Neonates with HI present with thick, armor-like scale with severe ectropion (eversion of the eyelids), eclabium (eversion of the lips), and flattening of the ears. Some patients with HI die during the neonatal period, but survival has been shown to improve with progress in neonatal intensive care and early treatment with systemic retinoids. Rajpopat et al. showed that 83% of HI patients treated with oral retinoids survived compared to 24% of untreated patients.<sup>15</sup>

LI and CIE represent a spectrum of disorders caused by mutations in one of nine genes: *TGM1*, *NIPAL4/ICHTHYIN*, *ALOX12B*, *ALOXE3*, *CYP4F22*, *ABCA12*, *PNPLA1*, *CERS3*, and *LIPN*.<sup>16</sup> Mutations in *TGM1* are the most common, and account for approximately 32% of heritability of ARCI.<sup>17</sup> Fischer et al. found that mutations in the five most common genes (*TGM1*, *NIPAL4*, *ALOX12B*, *CYP4F22*, *ALOXE3*, and *ABCA12*) account for 78% of ARCI cases.<sup>17</sup> Despite this, prior studies of large cohorts of patients with ARCI showed that 22-40% of patients have no mutations in known genes,<sup>17,18</sup> highlighting the heterogeneity of this group of disorders and the importance of continued efforts in gene discovery.

## Keratinopathic Ichthyosis

Keratinopathic ichthyosis is a group of disorders caused by mutations in the keratin family of genes. The major variant of keratinopathic ichthyosis is epidermolytic ichthyosis (EI). Minor variants include superficial epidermolytic ichthyosis (SEI), annular epidermolytic ichthyosis (AEI) and ichthyosis Curth-Macklin.

EI is caused by autosomal dominant mutations in the keratin 1 (*KRT1*) and keratin 10 (*KRT10*) genes, which play an essential role in maintaining structural integrity in suprabasal keratinocytes<sup>19</sup>. EI is characterized by marked skin fragility, leading to generalized blister formation on a background of erythroderma. Neonates present with blistering and erythema at birth, but symptoms improve over time. Blistering becomes less frequent and is usually confined to sites of trauma in adulthood. Palmoplantar keratoderma is often associated with EI, although it is more commonly seen in patients with mutations in *KRT1* than *KRT10*.<sup>19</sup>

SEI, also known as ichthyosis bullosa of Seimens, is caused by mutations in *KRT2*.<sup>20,21</sup> Phenotypic manifestations are milder compared to EI, and include blister formation in response to trauma and hyperkeratosis (thickening of the stratum corneum) over flexural areas.

AEI is a rare phenotypic variant of EI that was shown by Yang et al. to be caused by a unique mutation in *KRT10* that replaces an alanine at residue 12 with a proline.<sup>22</sup> It is characterized by blister formation at birth, which later progresses to the intermittent development of annular polycyclic erythematous plaques on the trunk and extremities.

Ichthyosis Curth-Macklin is another rare disorder and is caused by autosomal dominant mutations in *KRT1*.<sup>23,24</sup> It is characterized by extensive spiky or verrucous hyperkeratosis over the trunk and extensor surfaces of the extremities. It may also be associated with severe palmoplantar keratoderma. In the past five years, 2 novel distinct causative mutations in *KRT1* have been identified, in addition to the two mutations that were initially described.<sup>25,26</sup> While both EI and Ichthyosis Curth-Macklin can be caused by mutations in *KRT1*, EI is caused by amino acid substitutions and in-frame deletions in the gene,<sup>27</sup> while ichthyosis Curth-Macklin is caused by insertions or deletions that lead to a frameshift.<sup>23-26</sup>

### **Syndromic ichthyoses**

In addition to cutaneous involvement, syndromic ichthyoses affect at least one other organ or system. Many causative genes have been identified for syndromic ichthyoses, including *NSDHL* (CHILD syndrome),<sup>28</sup> *EBP* (Conradi-Hunermann-Happle syndrome, CHILD Syndrome),<sup>28,29</sup> and *ALDH3A2* (Sjogren-Larsson syndrome).<sup>30</sup> Depending on the specific gene mutated, a wide range of organ systems can be involved, including the skeletal, nervous, endocrine, and cardiovascular systems. Many of the syndromic ichthyoses may present at birth with isolated cutaneous findings, highlighting the importance of a high degree of clinical suspicion and the usefulness of genetic analysis in early diagnosis of these syndromic cases.<sup>31</sup>

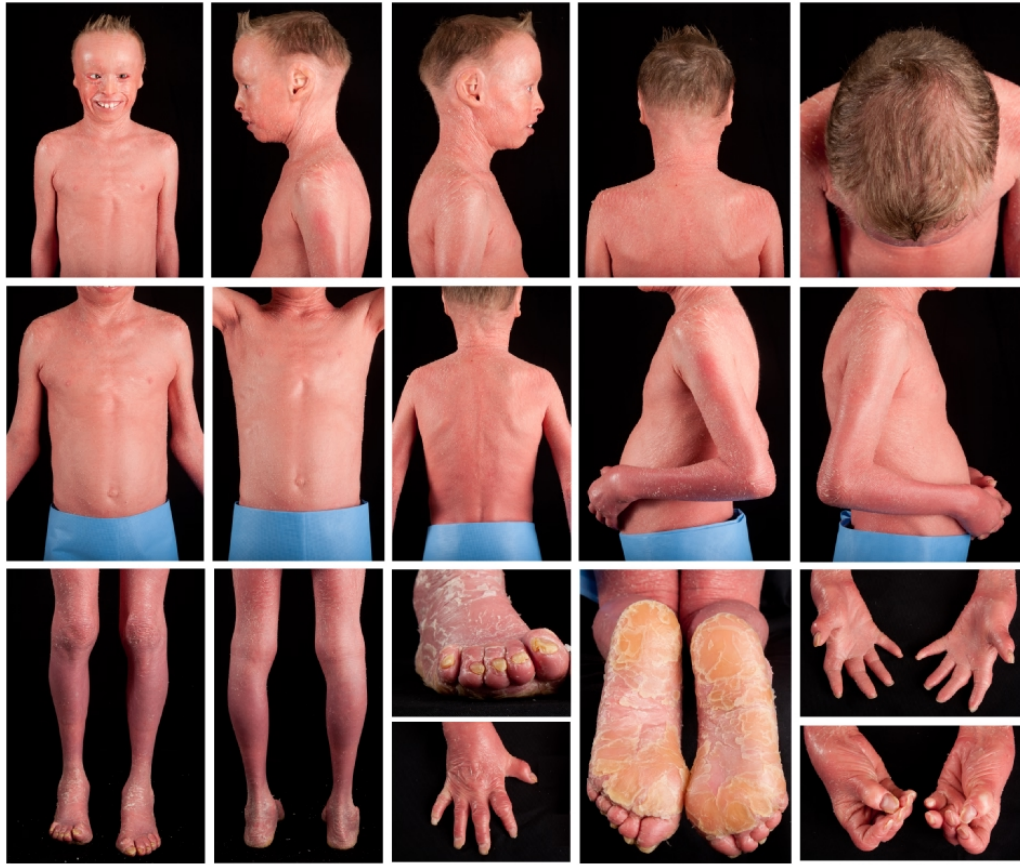
### **Study of ichthyosis in Dr. Choate's lab**

In partnership with the Foundation for Ichthyosis and Related Skin Types (FIRST), we have served as a major national and international referral center and recruited over 800

kindreds with DOK, utilizing whole exome sequencing to identify novel, rare mutations that cause DOK, many of which represent phenotypic expansion. We see patients with ichthyosis at a monthly ichthyosis clinic, as well as the FIRST family conferences, which bring together families from across the country and around the world, and provide access to information, expert advice, and opportunities for families to get involved in research. We had the pleasure of organizing a research program for the FIRST family conference in San Diego in 2016, where we evaluated over 150 families with ichthyosis, providing genetic testing, and enrolling families in the National Registry. To assess phenotypic manifestations of disease and analyze genotype-phenotype characterizations, we took standardized clinical photographs of all enrolled subjects, with the goal of capturing cardinal phenotypic features of disease. Examples of the standardizes poses used for photography are shown in **Figure 2**.

This detailed database of genotypic, phenotypic, and clinical data for patients with ichthyosis has allowed us to conduct a large-scale genotype-phenotype characterization for 407 kindreds with ichthyosis, the largest cohort published to date. We identified 156 novel mutations and assessed phenotypic features of DOK with respect to genetic diagnosis to help refine our understanding of this heterogeneous group of disorders (**Chapter 1**). In the process of performing a large scale genotype-phenotype characterization, we identified kindreds with rare clinical subtypes of ichthyosis, including Bathing Suit Ichthyosis (BSI), a rare disorder caused by mutations in transglutaminase 1 (*TGM1*) and characterized by the restriction of scale to sites of relatively higher temperature such as the trunk, with cooler areas remaining unaffected (**Chapter 2**). We identified novel and recurrent mutations in sixteen subjects with BSI, the largest cohort published to date. Our findings expand the genotypic spectrum of BSI and the understanding of temperature-sensitivity of *TGM1* mutations.

In the course of unifying genetic and clinical data to advance the understanding of the different subtypes of ichthyosis, we realized the critical importance of tools to assess the clinical severity of ichthyosis. We designed a Visual Index for Ichthyosis Severity (VIIS) and tested the instrument for reliability and reproducibility (**Chapter 3**). This index provides a tool for clinical phenotyping and assessment of therapeutic response for many disorders of keratinization.



**Figure 2 Standardized photography for documentation of clinical findings.** Subject with recessive ichthyosis due to homozygous *ABCA12* mutation demonstrating standard poses for high resolution photography.

## **STATEMENT OF PURPOSE**

1. To perform a large scale genotype-phenotype characterization for 407 kindreds with ichthyosis, identifying novel mutations and systematically evaluating phenotypic features of ichthyosis with respect to genetic diagnosis to refine our understanding of this rare group of disorders.
2. Within our cohort, to identify rare subtypes of ichthyosis, including Bathing Suit Ichthyosis (BSI), which is characterized by restriction of scale to sites of relatively higher temperature such as the trunk, with cooler areas remaining unaffected. To identify novel and recurrent mutations for our cohort of subjects with BSI, expanding our understanding of temperature-sensitive mutations.
3. To design and validate a severity index for ichthyosis, a critical tool for evaluating the efficacy of new treatments and advancing research in ichthyosis.



## **Chapter 1: Genotype-Phenotype Correlations and Novel Mutations in 407**

### **Ichthyosis Kindreds**

#### **ABSTRACT**

We identify novel mutations and systematically evaluate phenotypic features of DOK with respect to genetic diagnosis in a cohort of 407 kindreds with ichthyosis, the largest cohort published to date. We recruited over 800 subjects with ichthyoses, and performed gene screening, with exome sequencing employed in the remaining mutation-unknown cases. Clinical photographs were taken to capture phenotypic involvement and questionnaires were utilized to assess additional clinical manifestations. We identified a total of 156 novel mutations, expanding the genotypic spectrum of this group of rare disorders. Our systematic classification of phenotypic manifestations revealed cardinal features that are associated with mutations in each gene, as well as the phenotypic range seen within our cohort for each gene. Our analysis of clinical manifestations revealed that the majority of subjects with ichthyosis are born with a collodion membrane (69%), and identified pruritis (90%), skin pain (70%), anhidrosis (57%), and eye problems (57%) as common clinical features. Such a systematic classification holds promise for the development of customized management plans, generation of targeted therapeutics, and improved understanding of prognostication based on genetic diagnosis.

#### **METHODS**

I verified the Sanger sequencing traces for all of the mutations in the cohort, identifying single nucleotide polymorphism (SNPs), referencing mutations previously reported in the HGMD Professional Database and in the Exome Aggregation Consortium, and

determining which mutations are novel. Working with members of the Choate lab, I organized a research program for the Foundation for Ichthyosis and Related Skin Types (FIRST) Conference, where I recruited and consented patients, took clinical photographs, and obtained saliva samples for genetic analysis. I also recruited and consented patients, taking standardized clinical photographs and obtaining saliva samples, monthly at the ichthyosis clinic. I performed all of the genotype-phenotype correlations reported in the study, evaluating phenotypic features of the clinical photographs taken for our patients, assessing both cardinal features and phenotypic range, and creating the figures of phenotypic manifestations. I also evaluated clinical features by analyzing the in-depth questionnaires filled out by patients at the FIRST conference and at the monthly ichthyosis clinic. Part of accomplishing this task involved curating the paper questionnaires, and I led the effort of creating an online Oncore database and transferring the information into a format that would facilitate analysis. Using the database, I then performed the analysis on the clinical manifestations of ichthyosis, which is reported in this study. The genetic analysis reported in this study was performed by various members of the Choate lab.

### **Subjects and samples**

The study was approved by the Yale Human Investigation Committee, consistent with the Declaration of Helsinki guidelines, and written informed consent was obtained from the subjects or their parents. Subjects with X-linked ichthyosis and ichthyosis vulgaris, as determined by family history and clinical manifestations and confirmed by genetic analysis, were excluded from the study. A total of 427 subjects and 407 kindreds were included in the study.

Each subject (or parent in the case of a child) was asked to complete a detailed questionnaire, including self-reporting of the phenotype at birth, clinical involvement, and associated systemic manifestations. Fully completed questionnaires were obtained from 135 subjects. For subjects seen at the Foundation for Ichthyosis and Related Skin Types (FIRST) family conference, standardized clinical photographs were taken to capture the phenotypic involvement. For figures illustrating the phenotypic range, only subjects who were not on systemic or topical retinoid therapy were included in order to more accurately capture the underlying disease severity. Saliva or blood samples were obtained from all of the subjects for genetic analysis.

### **Genetic Analysis**

Genetic analysis was performed on DNA isolated from the saliva or blood of index cases and both parents, if available. Genomic DNA was extracted using standard procedures. Samples were first screened for mutations in 48 genes (*AAGAB*, *ABCA12*, *ABHD5*, *ALOXE3*, *ALOX12B*, *ALDH3A2*, *ATP2A2*, *ATP2C1*, *AQP5*, *CARD14*, *CDSN*, *CERS3*, *CLDN1*, *CYP4F22*, *DKC1*, *DSC2*, *DSG1*, *DSP*, *EBP*, *EDA*, *FLG*, *GJA1*, *GJB2*, *GJB3*, *GJB4*, *GJB6*, *KANK2*, *KRT1*, *KRT10*, *KRT2*, *KRT9*, *KRT16*, *KRT17*, *KRT6C*, *MBTPS2*, *NIPAL4*, *NSDHL*, *PNPLA1*, *POGLUT1*, *RHBDF2*, *RSPO1*, *SERPINB7*, *SNAP29*, *SLC27A4*, *SPINK5*, *STS*, *TGM1*, *TRPV3*) via multiplex polymerase chain reaction (PCR) and next generation sequencing. Mutations identified by next generation sequencing were verified by Sanger sequencing. If no causative mutation was identified through this screening panel, exome sequencing was employed, allowing for identification of novel, rare causes of DOK.

All novel mutations reported in this manuscript are not referenced as single nucleotide polymorphisms (SNPs) and were not found in the HGMD Professional Database

(<http://www.hgmd.cf.ac.uk>) or in the Exome Aggregation Consortium (<http://exac.broadinstitute.org>).

## RESULTS

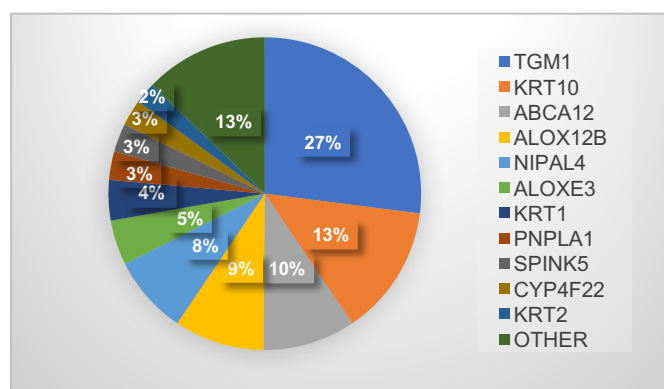
Self-reported demographic characteristics for subjects included in the study (n=427 subjects) are shown in **Table 1** and genotypic information described in **Table 2**. 298 kindreds were found to have autosomal recessive mutations in 21 different genes, and 109 kindreds had autosomal dominant mutations in 17 genes. Clinical characteristics for subset of 135 subjects who completed a detailed questionnaire are shown in **Table 3**.

<b>Gender</b>	Male	196	<b>45.9%</b>
	Female	225	<b>52.7%</b>
	Unclassified	6	<b>1.4%</b>
<b>Ethnicity</b>	Caucasian	181	<b>42.4%</b>
	Hispanic/Latino	65	<b>15.2%</b>
	Asian	36	<b>8.4%</b>
	Middle Eastern	24	<b>5.6%</b>
	Black	18	<b>4.2%</b>
	Mixed	3	<b>0.7%</b>
	Native American	1	<b>0.2%</b>
	Unclassified	99	<b>23.2%</b>
<b>Age</b>	≥ 18 yrs	155	<b>36.3%</b>
	< 18	190	<b>44.5%</b>
	Unclassified	82	<b>19.2%</b>

**Table 1 Demographic characteristics for all subjects included in the study (n=427 subjects)**

a.					b.		
GENE	TOTAL KINDREDS	HET	HOM	NOVEL MUTATIONS	GENE	TOTAL KINDREDS	NOVEL MUTATIONS
TGM1	110	75	35	24	KRT10	55	11
ABCA12	39	34	5	34	KRT1	17	7
ALOX12B	38	30	8	25	KRT2	9	2
NIPAL4	33	7	26	3	CARD14	5	3
ALOXE3	19	8	11	7	GJB2	5	0
PNPLA1	12	3	9	8	DSP	3	0
SPINK5	12	8	4	6	GJA1	3	0
CYP4F22	11	4	7	8	ATP2C1	1	1
ALDH3A2	5	2	3	2	ATP2A2	1	0
ABHD5	4	1	3	0	GJB3	2	1
MBTPS2	3	0	3	1	EBP	1	1
CDSN	2	0	2	2	GJB4	1	0
CERS3	2	1	1	1	DSG1	1	1
CLDN1	1	0	1	1	KRT6B	1	1
CSTA	1	0	1	1	POGLUT1	1	1
KANK2	1	0	1	1	RHBD2	1	0
LIPH	1	0	1	0	TRPV3	1	0
RSPO1	1	0	1	1	ZDHHC4	1	1
SLC27A4	1	1	0	0		<b>109</b>	<b>30</b>
SNAP29	1	0	1	0			
ST14	1	1	0	1			
	<b>298</b>	<b>175</b>	<b>123</b>	<b>126</b>			

c.



**Table 2 Genotypic characteristics for all kindreds included in the study (n=407 kindreds)**

a. Genes with autosomal recessive pattern of inheritance, b. Genes with autosomal dominant pattern of inheritance, c. Pie chart of the most common genes in our cohort. Abbreviations: HET, kindreds with compound heterozygous mutations; HOM, kindreds with homozygous mutations

	n	Collodion membrane	Skin pain	Pruritis	Skin odor	Skin infections	Anhidrosis	Hearing problems	Eye problems	Hair loss
<i>TGM1</i>	45	87% (39/45)	58% (25/43)	93% (39/42)	43% (19/44)	47% (21/45)	86% (6/42)	62% (28/45)	74% (31/42)	51% (22/43)
<i>ALOX12B</i>	20	100% (18/18)	65% (11/17)	89% (16/18)	30% (6/20)	35% (7/20)	88% (14/16)	32% (6/19)	47% (9/19)	21% (4/19)
<i>NIPAL4</i>	15	50% (7/14)	87% (13/15)	87% (13/15)	47% (7/15)	20% (3/15)	80% (12/15)	40% (6/15)	60% (9/15)	7% (1/15)
<i>ABCA12</i>	13	50% (6/12)	77% (10/13)	100% (13/13)	54% (7/13)	23% (3/13)	69% (9/13)	62% (8/13)	69% (9/13)	54% (7/13)
<i>ALOXE3</i>	8	71% (5/7)	63% (5/8)	75% (6/8)	25% (2/8)	50% (4/8)	71% (5/7)	38% (3/8)	75% (6/8)	43% (3/7)
<i>CYP4F22</i>	8	100% (8/8)	29% (2/7)	86% (6/7)	50% (4/8)	38% (3/8)	88% (7/8)	25% (2/8)	38% (3/8)	25% (2/8)
<i>KRT10</i>	20	14% (2/14)	95% (18/19)	83% (15/18)	89% (17/19)	85% (17/20)	82% (14/17)	30% (6/20)	35% (7/20)	20% (4/20)
<i>KRT1</i>	6	17% (1/6)	100% (4/4)	100% (4/4)	100% (6/6)	33% (2/6)	66% (4/6)	16% (1/6)	16% (1/6)	16% (1/6)
	<b>135</b>	<b>69%</b>	<b>70%</b>	<b>90%</b>	<b>51%</b>	<b>44%</b>	<b>57%</b>	<b>45%</b>	<b>57%</b>	<b>34%</b>

**Table 3 Clinical characteristics for a subset of subjects who completed a questionnaire (n=135 subjects)**

### **Autosomal recessive mutations**

Similar to prior findings,<sup>17, 32</sup> mutations in *TGM1* are the most common in our cohort, accounting for 37% of heritability of the autosomal recessive cases and 27% of heritability of all cases within our cohort. Mutations in the 5 most common genes (*TGM1*, *ABCA12*, *ALOX12B*, *NIPAL4*, *ALOXE3*) account for 80% of the heritability of the autosomal recessive cases. Genotype-phenotype characterizations for the 5 most common autosomal recessive genes are discussed below.

#### *TGM1*

The *TGM1* gene encodes transglutaminase-1, which is an enzyme essential for the cross-linking of proteins during the formation of the cornified envelope.<sup>33, 34</sup> Our cohort includes a total of 110 kindreds with mutations in *TGM1*, 75 with compound heterozygous mutations and 35 with homozygous mutations. We identified 24 mutations in *TGM1* that are novel and have not been described previously.

Phenotypic features of subjects with mutations with *TGM1* are shown in **Figure 3**.

Cardinal features of ichthyosis caused by mutations in *TGM1* include flat armor-like plates of scale, with varying degree of mild to moderate erythema.

Most subjects with mutations in *TGM1* were born with a collodion membrane (87%, n=45). Common clinical features include pruritis (93%, n=42), anhidrosis (86%, n=42), eye problems (74%, n=42), and hearing problems (62%, n=45). The majority of patients reported skin pain (58%, n=43) and alopecia (51%, n=43).





**Figure 3 Phenotypic features of subjects with mutations in *TGM1***

a. Cardinal features include flat armor-like plates of scale, with mild to moderate erythema b. Phenotypic manifestations range from discontinuous smoothing (diminished fine skin markings, shininess) with small scales (left) to confluent, large thick scales (right).

### ABCA12

The *ABCA12* gene encodes an ATP-binding cassette (ABC) transporter that is crucial for the energy-dependent lipid transport into lamellar bodies and the integrity of the lipid barrier.<sup>35</sup> Homozygous loss-of-function mutations in *ABCA12* result in harlequin ichthyosis, a severe form of ichthyosis characterized by neonatal encasement in a thick, armor-like scale with associated findings of eclabium, ectropion and flattened ears. Missense mutations in the gene result in milder phenotypes on the lamellar ichthyosis (LI) and congenital ichthyosiform erythema (CIE) spectrum.

Our cohort includes a total of 39 kindreds with mutations in *ABCA12* (13% of heritability of autosomal recessive cases), 34 with compound heterozygous mutations and 5 with homozygous mutations. Six subjects had truncating mutations in both alleles, resulting in harlequin ichthyosis. We identified 34 mutations in *ABCA12* that are novel and have not been described previously. Cardinal features of ichthyosis caused by mutations in *ABCA12* (**Figure 4**) include significant (often severe) erythema with fine scale and variable PPK, as well as the characteristic findings of tapering digits and hyperconvex nails. Interestingly, all of the subjects in Figure 4 are compound heterozygous with one missense and one nonsense mutation. Despite this, a varying degree of erythema (mild to severe) is observed.

Half of the subjects with mutations in *ABCA12* were born with a collodion membrane (50%, n=12). Common clinical features include pruritis (100%, n=13), skin pain (77%, n=13), anhidrosis (69%, n=13), eye problems (69%, n=13), and hearing problems (62%, n=13). The majority of patients reported skin odor (54%, n=13) and alopecia (54%, n=13).



**Figure 4 Phenotypic features of subjects with mutations in *ABCA12***

a. Cardinal features include significant (often severe) erythema with fine scale and variable PPK, as well as the characteristic findings of tapering digits and hyperconvex nails. b. Phenotypic manifestations range from mild erythema, discontinuous smoothening with small scales (left) to severe erythema with confluent scales (right). Of note, both subjects in panel b are compound heterozygous with one missense and one nonsense mutation.

*ALOX12B and ALOXE3*

The *ALOX12B* and *ALOXE3* genes encode the lipoxygenases 12R-LOX (12R-lipoxygenase) and eLOX-3 (epidermis-type lipoxygenase 3), respectively. The consecutive action of these enzymes leads to the oxygenation of ceramides, which are essential to the formation of the lipid barrier.<sup>36, 37</sup>

Our cohort includes a total of 38 kindreds with mutations in *ALOX12B* (13% of heritability of autosomal recessive cases) and 19 kindreds with mutations in *ALOXE3* (6% of heritability of autosomal recessive cases). We identified 25 novel mutations in *ALOX12B* and 7 novel mutations in *ALOXE3*.

Given that the products of *ALOX12B* and *ALOXE3* function within the same pathway, it is not surprising that the phenotypic manifestations caused by mutations in these 2 genes share common features (**Figures 5 and 6**). Cardinal features of ichthyosis caused by mutations in *ALOX12B* and *ALOXE3* include fine scale, absence of PPK, and mild erythema in some subjects.

Most subjects with mutations in *ALOX12B* and *ALOXE3* were born with a collodion membrane (100% *ALOX12B*, n=18; 71% *ALOXE3*, n=7). Common clinical features include pruritis (89% *ALOX12B*; 75% *ALOXE3*), skin pain (65% *ALOX12B*; 63% *ALOXE3*), anhidrosis (88% *ALOX12B*; 71% *ALOXE3*), and eye problems (47% *ALOX12B*; 75% *ALOXE3*).



**Figure 5 Phenotypic features of subjects with mutations in *ALOX12B***

a. Cardinal features include fine scale, absence of PPK, and mild erythema in some subjects b. Phenotypic manifestations range from discontinuous smoothing and small, fine scale (left) to confluent fine scale and more significant erythema (right).



**Figure 6 Phenotypic features of subjects with mutations in *ALOXE3***

Similar to mutation in *ALOX12B*, cardinal features include fine scale, absence of PPK, and minimal to mild erythema. These features are fairly consistent within our cohort, with minimal variation among subjects.

### *NIPAL4*

The *NIPAL4* gene encodes the NIPA-like domain containing 4 protein, thought to function as an  $Mg^{2+}$  transporter and be involved in lipid processing and lamellar body formation.<sup>38</sup>

Our cohort includes a total of 33 kindreds with mutations in *NIPAL4* (11% of heritability of autosomal recessive cases), 7 with compound heterozygous mutations and 26 with homozygous mutations. We identified 3 mutations in *NIPAL4* that are novel and have not been described previously.

Cardinal features of ichthyosis caused by mutations in *NIPAL4* (**Figure 7**) include fine scale, absence of PPK, and a varying degree of erythema. Similar to prior findings,<sup>39</sup> the A176D missense mutation was common in subjects with mutations in *NIPAL4*, representing 55 of the 66 alleles (83%) examined in our cohort. Interestingly, while 3 of the subjects in Figure 5 are homozygous for the A176D mutation, great variations in the degree of scale and erythema are observed in these subjects.

Half of the subjects with mutations in *NIPAL4* were born with a collodion membrane (50%, n=14). Common clinical features include pruritis (87%, n=15), skin pain (87%, n=15), anhidrosis (80%, n=15), eye problems (60%, n=15).



**Figure 7 Phenotypic features of subjects with mutations in *NIPAL4***

a. Cardinal features include fine scale, absence of PPK, and a varying degree of erythema b. Phenotypic manifestations range from barely perceptible pink erythema and discontinuous smoothing (left) to deep red-purple erythema with confluent smoothing and scale (right). Of note, all subjects depicted in this figure are homozygous for *NIPAL4* A176D.



### **Autosomal dominant mutations**

Keratinopathic ichthyosis, a group of disorder caused by mutations in one of the keratin genes, accounts for 74% of the heritability of the autosomal dominant cases. Keratins are intermediate filaments that are essential to the formation of the cytoskeleton network and maintenance of epidermal integrity. Mutations in *KRT10* and *KRT1* are the most common among the autosomal dominant cases in our cohort, accounting for 50% and 16% of the heritability, respectively. These 2 genes are essential to the structural integrity of the suprabasal epidermis.<sup>40</sup>

Mutations in *KRT10* and *KRT1* most commonly result in epidermolytic ichthyosis (EI), characterized by diffuse erythroderma and skin fragility with blister formation during the neonatal period, evolving into hyperkeratosis and blistering at sites of trauma in adulthood. Ichthyosis Curth-Macklin is a rare phenotypic variant caused by mutations in *KRT1*, characterized by spine-like or verrucous hyperkeratosis. While mutations in *KRT1* can result in both EI and ichthyosis Curth-Macklin, EI is caused by missense mutations and in-frame deletions,<sup>41</sup> while Curth-Macklin is caused by frameshift mutations.<sup>42-45</sup>

Genotype-phenotype characterizations for *KRT10* and *KRT1* are discussed in more detail below.

#### *KRT10*

Our cohort includes a total of 55 kindreds with mutations in *KRT10*, all with heterozygous mutations. We identified 11 mutations in *KRT10* that are novel and have not been described previously.

Phenotypic features of subjects with EI with mutations in *KRT10* are shown in **Figure 8**.

Cardinal features of EI caused by mutations in *KRT10* include columnar hyperkeratosis, scale in which the vertical dimension approaches or exceeds the horizontal dimension (compared to flat scale seen in cases caused by mutations in one of the autosomal recessive gene, such as *TGM1*). Skin fragility is another cardinal feature, especially at sites of trauma. As shown in Figure 8, there may be a variable degree of underlying erythema. PPK is typically present and smooth with occasional focal accentuation.

A minority of subjects with mutations in *KRT10* were born with a collodion membrane (14%, n=14). Common clinical features include skin pain (95%, n=19), skin odor (89%, n=19), skin infections (85%, n=20), pruritis (83%, n=18), and anhidrosis (82%, n=17) Hearing problems (30%, n=20), eye problems (35%, n=20), and alopecia (20%, n=20) were less common.



**Figure 8 Phenotypic features of subjects with mutations in *KRT10***

a. Cardinal features include columnar hyperkeratosis, skin fragility, and PPK b. Skin findings range from discontinuous regions of hyperkeratotic surface accentuation, including organized or geometric exaggeration of coarse skin markings (left) to confluent, primarily columnar hyperkeratosis (right) c. PPK is typically present and smooth with occasional focal accentuation.

### *KRT1*

Our cohort includes a total of 17 kindreds with mutations in *KRT1*, all with heterozygous mutations. We identified 7 mutations in *KRT1* that are novel and have not been described previously.

Phenotypic features of subjects with mutations with *KRT1* are shown in **Figure 9**.

Cardinal features of EI caused by mutations in *KRT1* include columnar hyperkeratosis, with significant involvement of the extensor surfaces of joints in some subjects. PPK is more severe in subjects with EI caused by mutations in *KRT1* than *KRT10*.

A minority of subjects with mutations in *KRT1* were born with a collodion membrane (17%, n=6). Common clinical features include skin pain (100%, n=4), skin odor (100%, n=6), pruritis (100%, n=4), and anhidrosis (66%, n=4).



**Figure 9 Phenotypic features of subjects with mutations in *KRT1***

a. Cardinal features include columnar hyperkeratosis, skin fragility, and severe PPK b. Skin findings range from pink erythema with columnar hyperkeratosis (left) to confluent, organized or geometric exaggeration of coarse skin markings with pink erythema and marked skin fragility (right) c. PPK is severe in most cases.

## DISCUSSION

This study, which reports the genotype and phenotype data for 407 kindreds with ichthyosis, represents the largest cohort published to date. We have identified a total of 156 novel mutations, expanding the genotypic spectrum of this group of rare disorders.

While prior studies have reported on the spectrum of phenotypic and genotypic findings in ichthyoses,<sup>32,46-49</sup> most were limited to specific ethnic groups and focused on particular ichthyosis subgroups. We aimed to capture the comprehensive spectrum of genotypes and phenotypes seen in ichthyosis, and reported data on an ethnically heterogeneous cohort with representation of all of the major genes involved in ichthyosis.

The genotype-phenotype correlations in ichthyoses are complex, with significant variations in phenotypic severity and extent of clinical manifestations in subjects with mutations in the same gene. Within our cohort, even subjects bearing the same disease causing mutation (e.g. subjects homozygous for *NIPAL4* A176D) presented with a spectrum of phenotypic severity, with varying degrees of both erythema and scale. This finding highlights the need for further research to elucidate the impact of genetic modifiers and/or environmental factors that may affect disease severity.

Given the intrinsic complexities of genotype-phenotype correlations in ichthyoses, we aimed to capture the cardinal phenotypic features of each of the major genes, while providing the range of disease severities present in our cohort for a given gene. The ability to differentiate potential causative genes based on key phenotypic features may allow for utilization of the more time- and cost-efficient targeted sequencing of the

candidate gene(s), rather than sequencing a panel of ichthyosis genes or pursuing whole-exome sequencing.

To our knowledge, this study is the first to characterize clinical features of ichthyosis based on the specific gene mutated. Hellstrom Pigg *et al.* reported frequencies of clinical features for the different clinical subtypes of Autosomal Recessive Congenital Ichthyosis (Harlequin Ichthyosis, Lamellar Ichthyosis, Congenital Ichthyosiform Erythroderma, and Pleomorphic Ichthyosis);<sup>32</sup> however, there is much phenotypic overlap among these different clinical subtypes and there has been an effort to move away from clinical classification and utilize ichthyosis groupings based on the gene affected.<sup>1</sup> Our analysis showed that the majority of subjects with ichthyosis are born with a collodion membrane (69%, n=135), and identified pruritis (90%), skin pain (70%), anhidrosis (57%), and eye problems (57%) as common clinical manifestations. Certain findings were more common in subjects with specific genotypes, including skin odor in subjects with mutations in *KRT10* and *KRT1* (89% and 100%, respectively), and hearing problems with subjects with mutations in *TGM1* and *ABCA12* (62% for both).

Our findings expand the mutation spectrum of ichthyoses and contribute to the understanding of the relationship between the genotype and the phenotypic and clinical findings. Such a systematic classification holds promise for the development of customized management plans that account for mutation-specific morbidities, and improved understanding of prognostication based on the genetic diagnosis.

## Chapter 2: Expanding the Genotypic Spectrum of Bathing Suit Ichthyosis

### ABSTRACT

Bathing Suit Ichthyosis (BSI) is a rare congenital disorder of keratinization, characterized by restriction of scale to sites of relatively higher temperature such as the trunk, with cooler areas remaining unaffected. Fewer than 40 cases have been reported in the literature. BSI is caused by recessive, temperature-sensitive mutations in transglutaminase 1 (*TGM1*). Clear genotype-phenotype correlations have been difficult to establish, as several *TGM1* mutations have been reported both in BSI and other forms of congenital ichthyosis. We identify novel and recurrent mutations in sixteen subjects with BSI, the largest cohort published to date. We report eight *TGM1* missense mutations that have not been previously found in BSI; five have been previously described in non-temperature sensitive forms of congenital ichthyosis (Arg143Cys, Gly218Ser, Gly278Arg, Arg286Gln, Ser358Arg) and three (Tyr374Cys, Phe495Leu, Ser772Arg) are novel mutations. Three probands are homozygous for Arg264Trp, Arg286Gln, or Arg315Leu, indicating that these mutations are temperature-sensitive. Seven of ten probands with a compound heterozygous *TGM1* genotype have a mutation at either arginine 307 or 315, providing strong evidence that mutations at these sites are temperature-sensitive, and highlighting the importance of these residues in the pathogenesis of BSI. Our findings expand the genotypic spectrum of BSI and the understanding of temperature-sensitivity of *TGM1* mutations. Increased awareness of temperature-sensitive *TGM1* genotypes should aid in genetic counseling, and provide insights into the pathophysiology of *TGM1* ichthyoses, transglutaminase 1 enzymatic activity, and potential therapeutic approaches.



## INTRODUCTION

Autosomal recessive congenital ichthyosis (ARCI) comprises a spectrum of disorders. The major phenotypic subtypes of ARCI include lamellar ichthyosis (LI), congenital ichthyosiform erythroderma (CIE), and harlequin ichthyosis (HI). While ARCI is genetically heterogeneous with at least 9 different genes causative for the most common forms,<sup>16</sup> approximately 30% of the heritability of ARCI is explained by mutations in *TGM1*.<sup>17</sup> *TGM1* encodes transglutaminase 1 (TGase-1), an enzyme involved in the formation of the cornified envelope.<sup>33</sup>

While mutations in *TGM1* most commonly cause a spectrum of LI and CIE phenotypes of varying severity, they also underlie bathing suit ichthyosis (BSI). BSI, a very rare form of ARCI with fewer than 40 reported cases, is characterized by lamellar scaling that is restricted primarily to the trunk, neck and scalp. Affected infants are typically born as collodion babies and develop more localized scaling after shedding of the membrane. BSI is due to the temperature sensitivity of certain *TGM1* mutations.<sup>50</sup> To date clear genotype-phenotype correlations have been difficult to establish due to the rarity of BSI and because many of the BSI mutations have also been reported in subjects with more generalized forms of ARCI.

We identified sixteen subjects with BSI and, employing targeted sequencing, found mutations in *TGM1* in each case. Herein we report phenotypic and genotypic data for this cohort. We identified eight missense mutations newly associated with BSI, including three novel mutations, and employed these genotypes to make inferences about the temperature sensitivity of *TGM1* mutations. Our findings expand the spectrum of *TGM1* mutations known to cause BSI, and the understanding of mutations related to temperature sensitivity.

## **METHODS**

While working on genotype-phenotype characterization, we identified a cohort of individuals with the limited phenotypic manifestations of BSI. I recruited and consented several of these patients at the FIRST conference and the monthly ichthyosis clinics, taking clinical photographs and obtaining saliva samples for genetic analysis. Several of the patients were referred to us by dermatologists across the country and around the world. The genetic analysis was performed by members of the Choate lab and the referring physician in a few cases. I verified the Sanger sequencing traces for all of the mutations in the cohort, identifying single nucleotide polymorphism (SNPs), referencing mutations previously reported in the HGMD Professional Database and in the Exome Aggregation Consortium, and determining which mutations are novel. I performed the genotype-phenotype correlations reported in the study, identifying novel and recurrent and determining which mutations are temperature-sensitive.

### **Subjects and samples**

The study was approved by the Yale Human Investigation Committee, consistent with the Declaration of Helsinki guidelines, and written informed consent was obtained from the subjects or their parents. A detailed clinical history was obtained from each subject, including phenotypic presentation at birth and evolution of disease when available. Self-reporting of ethnicity was obtained to evaluate founder effect. Saliva samples were obtained from all of the subjects for genetic analysis.

### **Genetic analysis**

Genetic analysis was performed on DNA isolated from saliva of index cases and both parents, if available. DNA was extracted using standard procedures. Samples were either screened for mutations in eleven genes including *ABCA12*, *ALOXE3*, *ALOX12B*,

*CYP4F22*, *NIPAL4*, *PNPLA1*, *SPINK5*, *TGM1*, *KRT1*, *KRT2E*, and *KRT10* via multiplex PCR and next generation sequencing, or the coding exons of *TGM1* were amplified using polymerase chain reaction (PCR) and subsequently examined via Sanger sequencing.

## RESULTS

BSI phenotypes and *TGM1* genotypes of each subject are described in **Table 4**.

Representative photos are provided in **Figure 10** (Subjects 8 and 15), and the location of mutations relative to *TGM1* protein domains are shown in **Figure 11**.

Subject #	Presentation at birth	Current presentation					Homozygous or heterozygous	Mutation in coding DNA	Protein Effect
		Scalp	Neck	Trunk	Face	Extremities			
1	Collodion membrane		x	x			HOM	c.857 G>A	Arg286Gln*
2	Normal skin at birth No collodion membrane	x	x	x		x (flexural)	HOM	c.790 C>T	Arg264Trp
3	Normal skin at birth No collodion membrane	x	x	x		x (flexural)	HOM	c.790 C>T	Arg264Trp
4	Collodion membrane	x	x	x			HET	c.790 C>T	Arg264Trp
								c.832G>A	Gly278Arg*
5	Collodion membrane Thickened skin	x		x			HOM	c.944 G>T	Arg315Leu
6	Collodion membrane Thickened skin	x		x			HOM	c.944 G>T	Arg315Leu
7	Collodion membrane Thickened, fragile skin	x	x	x		x (very mild)	HET	c.944 G>T	Arg315Leu
								c.2316 C>A	Ser772Arg <sup>1</sup>
8	Collodion membrane Thickened, fragile skin	x	x	x		x (mild)	HET	c.944 G>T	Arg315Leu
								c.2316 C>A	Ser772Arg <sup>1</sup>
9	Collodion membrane Ectropion as a neonate	x	x	x		x (flexural)	HET	c.944 G>A	Arg315His
								c.832G>A	Gly278Arg*
10	Collodion membrane Thickened skin	x		x			HET	c.944 G>A	Arg315His
								c.876+2 T>C	splice site
11	Collodion membrane	x	x	x			HET	c.919 C>G	Arg307Gly
								c.877-2 A>G	splice site
12	Collodion membrane	x	x	x			HET	c.919 C>G	Arg307Gly
								c.652 G>A	Gly218Ser*
13	Collodion membrane	x	x	x			HET	c.919 C>G	Arg307Gly
								c.1074 C>G	Ser358Arg*
14	Collodion membrane Thickened, fragile skin	x		x			HET	c.919 C>G	Arg307Gly
								c.1121 A>G	Tyr374Cys <sup>1</sup>
15	Collodion membrane	x	x	x		x (flexural)	HET	c.427 C>T	Arg143Cys*
								c.1483 T>C	Phe495Leu <sup>1</sup>
16	Collodion membrane	x	x	x			HET	c. 872 G>A	Gly291Asp
								c. 1407_1416del10insGCTCTGT	I469_C471delinsML <sup>1</sup>

**Table 4. TGM1 mutations causing BSI in sixteen subjects**

Fourteen of 16 subjects presented at birth with a collodion membrane. All eventually developed the characteristic phenotypic pattern seen in BSI, with scaling most prominent on the scalp, neck and trunk and with sparing of the central face and distal extremities. We report eight missense mutations not previously found in BSI. Five have been previously shown to cause ARCI and three are novel mutations.

\* Mutations not previously found in BSI

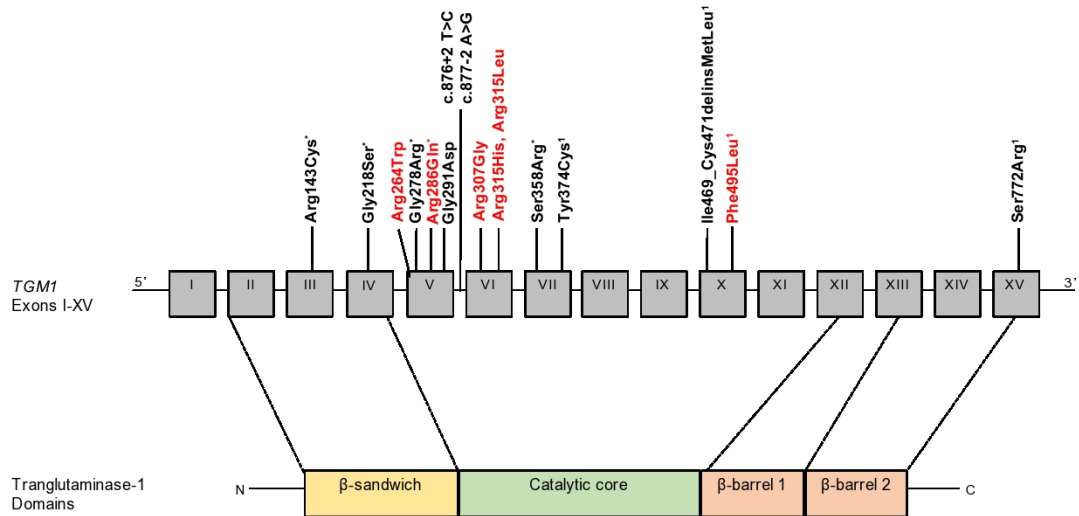
<sup>1</sup> Novel mutations

Temperature-sensitive mutations are shown in red.



**Figure 10. Clinical features of BSI**

Representative photos of the BSI phenotype, notable for plate-like scaling of the trunk and back, with sparing of the extremities and buttocks. (A) Subject 8. (B) Subject 15.



### Figure 11. Protein schematic of TGM1 mutations found in BSI

Thirteen mutations are located in the catalytic core, two in the  $\beta$ -sandwich domain, and one in the  $\beta$ -barrel 2 domain. Nine lead to substitution of charged residues.

\* Mutations not previously described in BSI

<sup>1</sup>Novel mutations

Temperature-sensitive mutations are shown in **red**.

**Homozygous *TGM1* Arg264Trp, Arg286Gln, and Arg315Leu mutations are temperature-sensitive**

Subject 1 is the child of first cousins. He was born with a collodion membrane, and later developed large brown scales on the back, chest and groin, with sparing of the face and extremities. He is homozygous for a *TGM1* Arg286Gln missense mutation. Arg286Gln was previously reported in a compound heterozygous state with Gly278Arg (found in Subject 9 and discussed below) in LI,<sup>51</sup> but has not been previously reported in BSI.

Subjects 2 and 3 are siblings and the children of second cousins. Both were born with normal skin at birth and no collodion membrane, and went on to develop plate-like scale restricted to the neck, scalp, trunk, and flexural areas of upper extremities. Non-scarring alopecia is present in both subjects. Both siblings are homozygous for a *TGM1* Arg264Trp missense mutation, which has previously been found in a compound heterozygous state in a patient with BSI.<sup>50</sup> It was also found in a heterozygous state in Patient 4, a female with *TGM1* mutations Arg264Trp and Gly278Arg. The Gly278Arg mutation has been previously described in both LI<sup>51</sup> and in self-healing ichthyosis, a rare form of ARCI characterized by the presence of a collodion membrane at birth with spontaneous healing of the phenotype within the first few weeks.<sup>52, 53</sup>

Subjects 5 and 6 are African-American siblings, with no known consanguinity in the family. Both were born with a collodion membrane and later developed plate-like scale restricted to the scalp and trunk. They are both homozygous for a *TGM1* Arg315Leu mutation, which has been reported by Arita *et al.* in a cohort of 8 South African BSI subjects.<sup>54, 55</sup>

The observation of homozygous mutations in these five subjects with BSI provides evidence that these *TGM1* mutations (Arg264Trp, Arg286Gln, and Arg315Leu) result in temperature sensitivity. All fall within the catalytic core of the transglutaminase 1 enzyme (**Figure 9**).

**Temperature-sensitive substitutions at R315 *TGM1* are common compound heterozygote mutations in BSI**

Subjects 7 and 8 are siblings. Both were born with a collodion membrane and were noted to have thickened, fragile skin at birth. They developed dark plate-like scaling, most prominent on the back, chest, and neck. Both are compound heterozygous for *TGM1* Arg315Leu, a temperature-sensitive mutation described above, and Ser772Arg, a novel mutation falling within the  $\beta$ -barrel 2 domain (**Figure 11**).

Subject 9 is male born with a collodion membrane and ectropion. He now has brown plate-like scales that are most prominent on the neck, scalp and trunk, with sparing of the face and the extremities. He also exhibits attention deficit hyperactivity disorder and developmental delay. He is compound heterozygous for *TGM1* (Arg315His and Gly278Arg). While both mutations fall within the catalytic core, Arg315His affects the same residue as Arg315Leu (found in subjects 5-8 and described above), and has been commonly reported in subjects with BSI.<sup>50, 56</sup> The Gly278Arg mutation was also found in Subject 4.

Subject 10 is a male born with a collodion membrane, and later developing scaling restricted to the scalp and trunk. He has *TGM1* mutations Arg315His and c.876+2 T>C, a mutation within the donor splice site of exon 5 previously described in generalized ARCI.<sup>57</sup>



The observation of a missense mutation at R315 in six of our 16 BSI subjects highlights the prevalence of substitutions at this site in BSI, and contributes to evidence that such mutations are temperature-sensitive.

***TGM1* Arg307Gly is a common BSI mutation and is temperature-sensitive**

Subject 11 is a female born with a collodion membrane that peeled at a few weeks of age, and developed thick dark scale on the scalp, neck, axillae, and groin by 1 year of age. She is compound heterozygous for *TGM1* Arg307Gly, which has been commonly described in BSI,<sup>50,56</sup> and c.877-2 A>G, a mutation within the acceptor splice site of exon 6, which has previously been found in BSI in conjunction with Arg307Gly (as in Subject 11) as well as with Arg264Trp and Arg315His in our cohort.<sup>50,56</sup>

Subject 12 is a male born with a collodion membrane. He now has brown plate-like scales that are most prominent on the neck, scalp, axillae, and trunk. He is compound heterozygous for *TGM1* Gly218Ser and Arg307Gly. The Gly218Ser mutation has been previously reported in an LI subject with a collodion membrane at birth and later development of thick scales and ectropion.<sup>58</sup>

Subject 13 is a female born with a collodion membrane. She now has thick dark scale restricted to the neck, scalp and trunk. She has *TGM1* mutations Arg307Gly and Ser358Arg. The Ser358Arg mutation has been previously reported in two siblings with LI, who were born with collodion membranes and later developed generalized scaling with facial and palmoplantar involvement.<sup>59, 60</sup>

Subject 14 is a female born with a collodion membrane, later developing scaling restricted to the trunk and scalp. She has *TGM1* mutations Arg307Gly and Tyr374Cys. The Tyr374Cys mutation is within the catalytic domain and has not been described previously.

The observation of the Arg307Gly mutation in four out of our sixteen BSI subjects contributes to evidence that Arg307Gly is relatively common in BSI, and is a temperature-sensitive mutation.

***TGM1* Phe495Leu is a temperature-sensitive mutation**

Subject 15 is a male born with a collodion membrane. He now has thick scale restricted to the neck, scalp and trunk, as well as flexural involvement of the extremities. He has *TGM1* mutations Arg143Cys and Phe495Leu. Homozygosity for the Arg143Cys mutation has been previously described in two patients with LI.<sup>58</sup> We therefore presume that Phe495Leu, which is novel, is the temperature-sensitive mutation in this subject.

*TGM1* Ile469\_Cys471delinsMetLeu is a novel mutation in the catalytic core

Subject 16 is a female born with a collodion membrane. She now has scale restricted to the neck, scalp and trunk. She has *TGM1* mutations Gly291Asp and Ile469\_Cys471delinsMetLeu. The Gly291Asp mutation was previously described in a compound heterozygous state in a patient with BSI,<sup>61</sup> as well as a patient with generalized ARCI.<sup>62</sup> The *TGM1* Ile469\_Cys471delinsMetLeu is a novel in-frame indel mutation that affects the catalytic core.

## DISCUSSION

BSI is a rare ARCI phenotype characterized by presentation at birth with a collodion membrane, followed by clinical improvement of ichthyosis on the face and extremities during the first few weeks of life. The resulting phenotype of scaling restricted to the trunk, neck, and scalp is a distinguishing feature of BSI, and can be differentiated from somatic mosaicism by the lack of a distribution pattern along Blaschko's lines.

Prior to this report, 21 missense mutations had been reported in BSI subjects. Of these, 9 had been reported only in subjects with BSI, while 12 had been observed in both BSI and generalized ARCI.<sup>62</sup> Both truncating mutations (nonsense, splice site, and frameshift) and missense mutations in *TGM1* have been found in subjects with BSI.<sup>50, 54, 56, 61, 63, 64</sup> However, while homozygosity or compound heterozygosity for truncating mutations has been observed in generalized forms of ARCI,<sup>59, 65, 66</sup> such a genotype has never been observed in BSI. This is consistent with the hypothesis that near or total loss of TGase-1 function causes generalized forms of ARCI, while genotypes that include a missense mutation resulting in a partially active, temperature-sensitive TGase-1 result in the more limited BSI phenotype.

In 2006, Oji *et al.* investigated TGase-1 enzymatic activity in BSI tissue, assessing uptake of biotinylated cadaverine into cornified envelopes, finding that areas of healthy skin in patients with BSI show nearly normal TGase-1 activity, while affected areas display clearly reduced and abnormal activity. Furthermore, digital thermal imaging showed close association between skin temperature and the degree of scaling in patients with BSI, with warmer body sites exhibiting greater scaling. Functional TGase-1 testing of normal-appearing skin of a BSI subject homozygous for the missense mutation Tyr276Asn showed clear temperature-sensitivity, with reduction in enzyme activity at

37°C compared to 25°C. This may explain the increased degree of scaling at sites of relatively higher temperature, such as the trunk.<sup>50</sup>

In addition to the *TGM1* mutation Tyr276Asn, several other mutations have been previously presumed to be temperature-sensitive based on their presence in a homozygous state in subjects with BSI, including *TGM1* mutations Ile304Phe, Arg307Gly, Arg315Leu, Arg315His, Val383Met, Arg687His.<sup>50, 54-56, 61, 63, 67</sup>

Here we report phenotypic and genotypic data from 16 subjects with BSI, the largest cohort published to date. Aside from a pair of siblings who had normal skin at birth with no collodion membrane (subjects 2 and 3), the phenotypes were consistent with prior descriptions of BSI. We identified a total of 16 unique mutations in our cohort, including 13 missense, two splice site, and one indel mutation. Eight of the missense mutations have not been previously reported for BSI; of these, five have been previously described in generalized ARCI (Arg143Cys, Gly218Ser, Gly278Arg, Arg286Gln, Ser358Arg), while three (Tyr374Cys, Phe495Leu, Ser772Arg) are novel mutations. The indel mutation *TGM1* I469\_C471delinsML is also novel.

Transglutaminase 1 consists of three domains: an N-terminal  $\beta$ -sandwich domain, a catalytic core domain, and two C-terminal  $\beta$ -barrel domains.<sup>68</sup> Most BSI mutations have been located in exons 5 and 6 of *TGM1*, encoding the N-terminal portion of the catalytic core domain.<sup>50</sup> Of the 13 unique missense mutations reported in this study, only two are within the  $\beta$ -sandwich domain (Arg143Cys, Gly218Ser) and one is within the  $\beta$ -barrel 2 domain (Ser772Arg). In stark contrast, ten are within the catalytic core (Arg264Trp, Gly278Arg, Arg286Gln, Gly291Asp, Arg307Gly, Arg315His, Arg315Leu, Ser358Arg, Tyr374Cys, Phe495Leu), including all three of the mutations homozygous in our BSI

subjects. **(Figure 9)**. All of our BSI subjects have at least one mutation within the catalytic core, and catalytic core mutations represent 88% of the mutations in our unrelated probands (23 of 26 alleles). Given that the catalytic core is only 38% of the total protein, our findings underscore a striking clustering of BSI mutations in this domain.

Based on our observation of BSI subjects homozygous for *TGM1* mutations Arg264Trp, Arg286Gln, and Arg315Leu, we conclude that these mutations are temperature-sensitive. Furthermore, the recurrence of mutations affecting R307 and R315 in our cohort, which among unrelated probands are present in 1/3 of homozygotes and 7/10 of compound heterozygotes, comprising 35% of the mutations (9 out of 26 total alleles), bolsters prior evidence that these mutations are common in BSI (also reported by Bourrat *et al.*),<sup>56</sup> and temperature-sensitive. Finally, we hypothesize that the novel mutation Phe495Leu is temperature-sensitive, given that the *TGM1* genotype of subject 15 includes this mutation and Arg143Cys; the latter mutation is presumably not temperature-sensitive given that subjects homozygous for Arg143Cys are described as exhibiting generalized LI.<sup>58</sup>

Though our findings provide evidence for temperature sensitivity of *TGM1* mutations, clear genotype-phenotype correlations have been difficult to establish, as several *TGM1* mutations have been reported in both BSI and generalized ARCI. For example, homozygosity for Arg315Leu has been found in a pair of twins who were described as having lamellar ichthyosis, whose phenotype at two months of age included thick plate-like scaling affecting the trunk and extremities, with sparing of the face.<sup>69</sup> Another patient described as having characteristic phenotypic findings of lamellar ichthyosis was found

to be compound heterozygous for *TGM1* mutations, including Arg286Gln, which we describe here as temperature-sensitive.<sup>51</sup>

The reporting of these mutations in both BSI and generalized ARCI may represent evolution of the phenotype, as patients with BSI can present with more generalized scaling earlier in life, with appearance of a bathing-suit distribution later in childhood. Thus, phenotypic characterization within the first few months of life may lead to misclassification. This dynamic nature of BSI highlights the importance of continued follow-up of subjects with presumed temperature-sensitive mutations, with phenotypic re-evaluation at multiple ages. Additional environmental or genetic factors that may determine the level of enzyme activity and response to temperature in subjects with *TGM1* mutations remain unclear.

Patients with BSI typically respond well to agents that improve barrier function and promote desquamation, including keratolytics and topical or systemic retinoids. Topical tazarotene led to substantial improvement in two subjects within our cohort.

Our findings expand the genotypic spectrum of BSI, and provide evidence supporting the temperature sensitivity of specific *TGM1* mutations (Arg264Trp, Arg286Gln, Arg307Gly, Arg315Leu, Arg315His, Phe495Leu), which are clustered in the catalytic core. Although patients respond well to topical and systemic therapies, further research into the pathogenesis of BSI could lead to the development of novel therapeutic approaches targeting enzymatic stability and consideration of environmental modifications that might modify disease severity.

### Chapter 3: Establishing and Validating an Ichthyosis Severity Index

#### ABSTRACT

We designed and validated a Visual Index for Ichthyosis Severity (VIIS) for scale and erythema that provides 1) written descriptions of the features characteristic of each level of severity 2) visual standards for 4 body sites, and 3) two distinct standards to account for different types of scale. We tested the VIIS for reliability and reproducibility using two different settings: one that utilized scoring of 60 test photographs by 10 dermatologists, and one with in-person evaluations on 85 subjects by 12 dermatologists at the Foundation for Ichthyosis and Related Skin Types (FIRST) conference. The validation process revealed high reliability and reproducibility for both scale and erythema. The inter-rater and intra-rater ICCs for scale were consistently near or greater than 0.7 in both settings. By contrast, the inter-rater reliability for erythema was higher during in-person validation compared to validation on test photographs. Our analysis indicates that the VIIS performs better in person than with photographs, an important consideration in design of clinical trials. Power analysis predicts that a 1-step difference on this 5-step scale would be detectable with 12 subjects in each of two defined groups. This index provides a tool for clinical phenotyping and assessment of therapeutic response for many disorders of keratinization.

## INTRODUCTION

Careful examination of patients who have ichthyosis reveals that the amount of scale on specific sites is quite variable, and that there are subtle differences in the quality of scale between different genotypes. While textbooks often suggest a monomorphous presentation for different types of ichthyosis, our experience from carefully photographing and genotyping over 500 individuals with ichthyosis shows significant patient to patient variability in amount of scale at any given body site. Previous estimates of scale severity have been limited by focus on a single body site,<sup>71</sup> by examples of severity that utilize different body sites as comparators,<sup>72</sup> or by concentration on a single phenotype.<sup>73</sup>

Erythema is often a clinically significant component of ichthyosis even though the relative roles of vasodilation and inflammation are not well understood. Erythema is an important component of many skin diseases, but there is no agreement as to how its severity should be measured. Strong arguments have been made for the clinical validity of visual analog scales,<sup>74, 75</sup> or the superiority and sensitivity of reflectance spectroscopy.<sup>76-78</sup> To our knowledge, all measurements of erythema in ichthyosis have used a visual analog scale without critical validation.

A reliable method of assessing the clinical severity of subjects with ichthyosis is critical to evaluating the efficacy of new treatments and to grading the severity of ichthyosis due to mutations in a single gene and between different genes. Although prior studies have used visual scales to measure the clinical severity of this rare group of disorders,<sup>71-73, 79, 80</sup> to date, no widely-accepted index exists.



Our goal was to develop a severity index that could be used across many types of ichthyosis and many body sites without requiring separate scales for each body site, genotype or phenotype.

We aimed to create severity index using photographs and detailed written descriptions for the degree of scale and erythema in ichthyosis.

We designed a prospective study to evaluate the reliability and reproducibility our instrument, the Visual Index for Ichthyosis Severity (VIIS), using two different settings: one that utilized scoring of test photographs and one with in-person evaluations.

## **METHODS**

While working on the genotype-phenotype characterization, we identified the need for a reliable method to assess ichthyosis severity. Working with Dr. Choate and Dr. Milstone, we identified photographs that were representative of each level of severity, and created detailed written descriptions for each level of severity. I recruited and consented patients at the FIRST conference and the monthly ichthyosis clinics, taking standardized clinical photographs. Working with Dr. Choate and Dr. Milstone, I led both stages of validation. Statistical analysis was performed by Yanhong Deng and Geliang Gan from the Yale Center for Analytical Sciences.

### **Study Design**

To design a user-friendly tool, we developed photographic standards for two characteristics of ichthyosis: scale and erythema. For scale, photographic standards for four representative body sites that are typically less aggressively groomed (upper arm, upper back, lower leg and dorsal foot) were created. Each of the four representative body sites was carefully defined using anatomical landmarks. Two different sets of scale

standards were developed: the lamellar (“L”) set of standards for the typical flat scales that are seen in most forms of ichthyosis and the keratoderma (“K”) set for the more columnar scales that are typical of epidermolytic ichthyosis and erythrokeratodermas. We employed five-point Likert scales (0-4) with increasing clinical severity. The combined scores, therefore, range from 0 to 16 (score of 4 for all 4 body sites) for each category (erythema and scale). The final score accounts for both erythema and scale, and ranges from 0 to 32. Each photographic standard includes representative clinical photographs for each severity score, along with detailed written descriptions of features characteristic of the severity score represented. All of the clinical photographs were obtained at Foundation for Ichthyosis and Related Skin Types (FIRST) family conferences. The photographs included in the standards were limited to subjects with Fitzpatrick 1-3 skin type. Photographs were chosen with the aim of demonstrating severity alone. All of the photographic standards can be found in the **Appendix**.

Validation of our instrument was performed in two stages the first utilized scoring of test photographs, and the second involved in-person evaluations. During the first stage, ten dermatologists were provided with a series of 60 test photographs (15 photographs for each of the 4 body sites) and asked to independently use our index to score for scale and erythema (Stage 1, Round 1). The re-shuffled photographs were sent to the same dermatologists four weeks later (Stage 1, Round 2) to determine intra-rater reliability. This four-week interval was chosen to reduce rater recall of prior scoring.<sup>71</sup>

The second stage of validation was performed at the FIRST family conference in San Diego in June 2016. Eligible participants included all subjects enrolled in the National Registry for Ichthyosis and Related Skin Types who participated in a clinical screening appointment at the conference. None refused to participate. Subjects were seen in one

of three clinical evaluation rooms with four dermatologists assigned to each room. A total of twelve dermatologists participated, and they were asked to independently rate the clinical severity of the subjects seen in their room. The rating process was limited to the first 5 minutes of each twenty minute appointment. Discussion of ratings was not permitted and all raters attested that they adhered to this requirement. Four (two in room 2 and two in room 3) out of the twelve dermatologists had previously participated as raters during the validation on test photographs.

### **Participants**

The study was approved by the Yale Human Investigation Committee, consistent with the Declaration of Helsinki guidelines, and written informed consent was obtained from the participants or their parents. Each participant was asked to fill out a questionnaire, including self-reporting of gender, age, ethnicity, and ichthyosis type.

### **Statistical analysis**

Intra-rater Intraclass Correlation Coefficients (ICCs) for agreement were estimated using the one-way random model. The estimated ICCs are the variances contributed by the different subjects divided by the summation of variances contributed by subjects and the variances of random error. Combined intra-rater ICCs were estimated using the mixed model controlling for raters. Inter-rater ICCs for agreement were estimated using the two-way random model. The estimated ICC is the variance contributed by the different subjects divided by the summation of the variance contributed by subjects, variance introduced by the raters and the variance of random error. Inter-rater ICCs for consistency were estimated using the two-way random model as well. The estimated

ICC is the variance contributed by the different subjects divided by the summation of the variance contributed by the subjects and the variance of random error. ICCs less than 0.7 were considered unacceptable, 0.7 – 0.79 fair, 0.8 – 0.89 good, and greater than or equal to 0.9, excellent.<sup>11</sup> The reliability of choosing the “L” or “K” standard by the same rater was estimated by the Cohen’s Kappa, which is a measure of intra-rater reliability for a nominal variable. The reliability of choosing the “L” or “K” standard on same subject across all raters was estimated by the Kuder-Richardson Formula 20, which is a measure of inter-rater reliability for a nominal variable. All confidence intervals were calculated at 95% confidence level. For the power analysis, a two-sided two-sample t-test was performed with a significance level (alpha) of 0.05. All analyses were carried out using SAS 9.4.3 (SAS Institute, Cary, NC).

## **RESULTS**

### **Validation of VIIS on test photographs (Stage 1)**

A total of 60 photographs (15 for each body site) were sent to ten dermatologists for baseline scoring (Stage 1, Round 1). All raters were experts in ichthyosis, and members of the FIRST Medical & Scientific Advisory Board (MSAB). The re-shuffled photographs were re-sent in four weeks to test reproducibility (Stage 1, Round 2). Demographic and clinical details for the subjects whose photographs were used as the test images can be found in **Table 5**.

		Upper Back	Upper Arm	Lower Leg	Dorsal Foot	Total	
						n	%
<b>Gender</b>	Male	5	6	5	5	21	35.0%
	Female	10	9	10	10	39	65.0%
<b>Ethnicity</b>	Caucasian	12	11	11	11	45	75.0%
	Hispanic/Latino	1	1	0	1	3	5.0%
	Black	0	1	1	0	2	3.3%
	Unknown	2	2	3	3	10	16.7%
<b>Age</b>	≥ 18 yrs	9	8	6	7	30	50.0%
	< 18	6	7	9	8	30	50.0%
<b>Ichthyosis Type</b>	EI	5	4	6	6	21	35.0%
	ARCI	6	7	6	7	26	43.3%
	Netherton's	1	0	0	1	2	3.3%
	Ichthyosis vulgaris	0	1	1	0	2	3.3%
	Normal control	1	1	0	0	2	3.3%
	Unclassified	2	2	2	1	7	11.7%

**Table 5. Demographic and clinical characteristics for patients whose photographs were used for the first stage of validation (n=60)**

Abbreviations: EI, Epidermolytic Ichthyosis; LI, Lamellar Ichthyosis; CIE, Congenital Ichthyosiform Erythroderma.

### Inter-rater reliability was higher for scale than for erythema

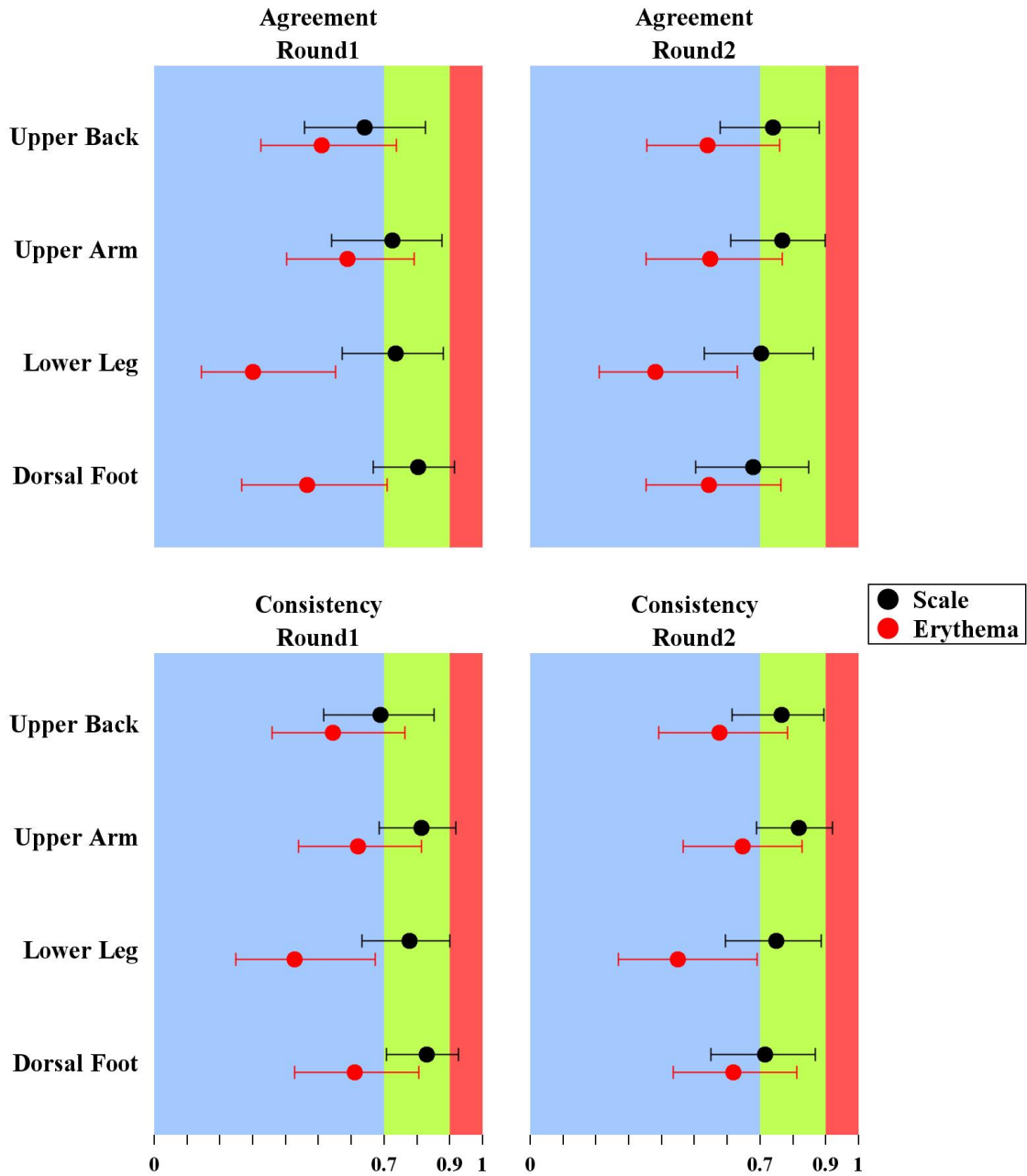
Descriptive statistics for inter-rater reliability (IRR) for scale and erythema can be found in **Figure 12**. Two measures of IRR were evaluated: ICC for agreement, which is the more rigorous metric and requires absolute agreement among the raters, and ICC for consistency, which measures whether the raters provide scores that have the same rank order rather than requiring agreement on the absolute values. ICCs for consistency are especially relevant when evaluating a change in severity from baseline, such as assessing response to treatment. As expected, ICCs for consistency are higher than ICCs for agreement for the same data points. For both rounds 1 and 2, the ratings were found to be significantly correlated for scale (ICCs near 0.7 or greater). However, the inter-rater reliability for erythema was poor (ICCs < 0.7).

### Raters were consistent in choosing the specific set of scale standards for a given subject

Raters were given two separate sets of scale standards (lamellar or “L” set of standards and keratoderma or “K” set of standards) to account for the two different types of scales most commonly seen in subjects with ichthyosis. Raters were consistent in choosing a set of standards (“L” or “K”) for a given patient, and there was excellent inter-rater reliability for all body sites during both rounds of testing (Kuder-Richardson Formula 20 > 0.9) **Table 6**. There was also high intra-rater reliability between Round 1 and Round 2 for the same rater, with Kappa near or greater than 0.7 for all body sites.

### Intra-rater reliability was higher for scale compared to erythema

To determine intra-rater reliability, we employed a test-retest approach with a 4-week interval between Rounds 1 and 2. Across all ten raters, the intra-rater ICC was 0.75 (CI 0.60, 0.90) for scale and 0.59 (CI 0.39, 0.79) for erythema.



**Figure 12. IntraClass Correlation Coefficients (ICCs) for agreement and consistency for the two rounds of photographic testing (Stage 1, Rounds 1 and 2)** For both rounds of testing, the ratings were significantly correlated for scale (ICCs near 0.7 or greater). The inter-rater reliability for erythema was lower (ICCs less than 0.7).

	<b>ROUND 1</b>	<b>ROUND 2</b>
<b>Dorsal Foot</b>	0.95 (0.89, 0.98)	0.95 (0.89, 0.98)
<b>Lower Leg</b>	0.94 (0.88, 0.98)	0.91 (0.82, 0.96)
<b>Upper Arm</b>	0.90 (0.81, 0.96)	0.92 (0.83, 0.97)
<b>Upper Back</b>	0.92 (0.85, 0.97)	0.94 (0.89, 0.98)

**Table 6. Kuder-Richardson Formula 20, KR20, values for the two rounds of photographic testing (Stage 1, Rounds 1 and 2)**

Raters were consistent in choosing the specific set of standards for scale (“L” or “K”) for a given patient, with excellent inter-rater reliability for all body sites (KR20 > 0.9).



## **Live validation of VIIS at the FIRST conference (Stage 2)**

A total of 85 subjects (27 in Room 1, 27 in Room 2, 31 in Room 3) were enrolled in the study and evaluated by three or four dermatologists. Demographic and clinical details for all subjects can be found in **Table 7**.

### Inter-rater reliability was high for both scale and erythema

Descriptive statistics for inter-rater reliability (IRR) for scale and erythema can be found in **Figure 13**. Across all rooms, the scores were found to be highly correlated for both scale and erythema, with ICCs near or greater than 0.7. The inter-rater reliability for scale was generally greater than that for erythema. The scores in Room 1 showed the highest degree of inter-rater reliability, with ICCs greater than 0.7 for both scale and erythema for all 4 body sites.

### Raters were consistent in choosing the specific set of scale standards for a given subject

Similar to the data from validation on test photographs discussed above, the raters were consistent in choosing the specific set of scale standards (“L” or “K”) for a given subject. The inter-rater reliability was high with Kuder-Richardson Formula 20 greater than 0.77 for all body sites **Table 8**. In room 2, all of the raters unanimously agreed on “L” or “K” for all of the subjects seen (Kuder-Richardson Formula 20 of 1).

### Inter-rater reliability was high for the combined scores for scale and erythema, as well as the final total scores

An important feature of the design of our index is the ability to allow for a comprehensive assessment of ichthyosis severity that accounts for the severities at the 4 representative sites. We determined IRR for the combined scores that range from 0 to 16 (score of 0-4

for each of 4 body sites) for each category (erythema and scale). We also determined IRR for the final score (sum of combined scores for scale and erythema) that ranges from 0 to 32. As shown in **Figure 14**, the inter-rater reliability was high, with ICCs consistently higher than 0.7.

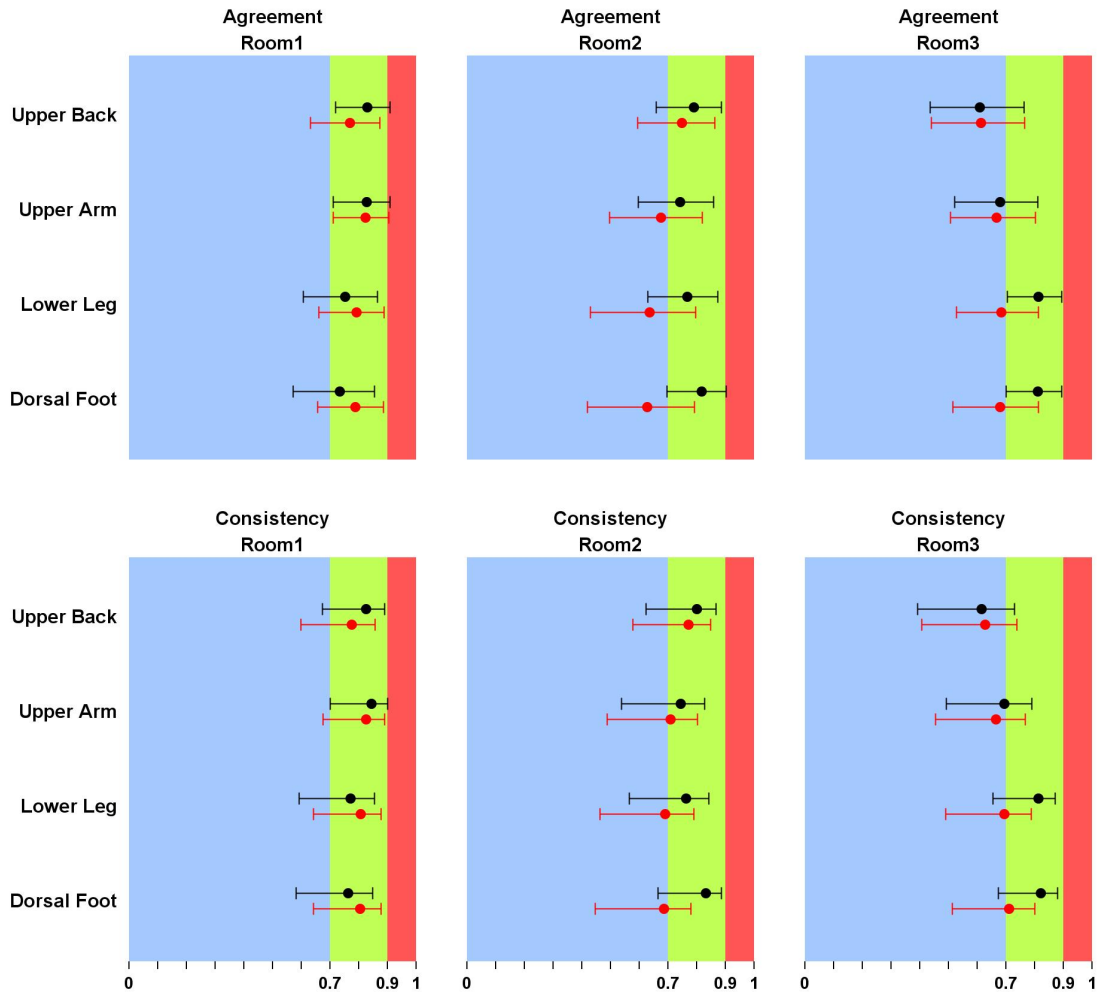
#### Power analysis to predict sample size for 1-step improvement

To determine the feasibility of using VIIS in future clinical trials, we performed a power analysis to determine the number of subjects that would need to be enrolled to detect a single step improvement or decrement on our 5-step scale. For our cohort of 85 subjects, the mean scores for erythema and scale are 1.78 and 2.35, respectively, across all body sites. In many clinical trials, inclusion criteria require subjects to start with a severity of at least 2 to measure a 1-2 step improvement. Approximately 50% and 70% of subjects had scores greater than or equal to 2 for erythema and scale, respectively. For subjects with average scores greater than or equal to 2, the mean score was 2.8 with standard deviation of 0.7. Given the null hypothesis that the control and treatment groups have equal means of 2.8, the alternative hypothesis that the mean of the treatment group is 1.8 (1 unit difference), and an estimated group standard deviations of 0.7, a sample sizes of 12 in the control group and 12 in the treatment group will achieve 92% power to detect a one unit difference.

		Room 1	Room 2	Room 3	Total	
					n	%
<b>Gender</b>	Male	12	16	11	39	45.9%
	Female	15	11	20	46	54.1%
<b>Ethnicity</b>	Caucasian	21	19	26	66	77.6%
	Hispanic/Latino	2	1	2	5	5.9%
	Black	1	1	0	2	2.4%
	Asian	2	4	1	7	8.2%
	Unknown	1	2	2	5	5.9%
<b>Age</b>	≥ 18 yrs	19	14	9	42	49.4%
	< 18	6	8	21	35	50.6%
<b>Ichthyosis Type</b>	EI	6	6	9	21	24.7%
	ARCI	9	8	13	30	35.3%
	Netherton's	0	2	4	6	7.1%
	X-linked ichthyosis	1	1	0	2	2.4%
	Ichthyosis vulgaris	1	1	1	3	3.5%
	HI	0	2	1	3	3.5%
	IWC	2	0	0	2	2.4%
	EKV	1	0	0	1	1.2%
	KID syndrome	1	0	0	1	1.2%
Unclassified	6	7	3	16	18.8%	

**Table 7. Demographic and clinical characteristics for patients enrolled in the second stage of validation at the FIRST conference (n=85)**

Abbreviations: EI, Epidermolytic Ichthyosis; LI, Lamellar Ichthyosis; CIE, Congenital Ichthyosiform Erythroderma; HI, Harlequin Ichthyosis; IWC, Ichthyosis With Confetti; EKV, Erythrokeratoderma Variabilis.

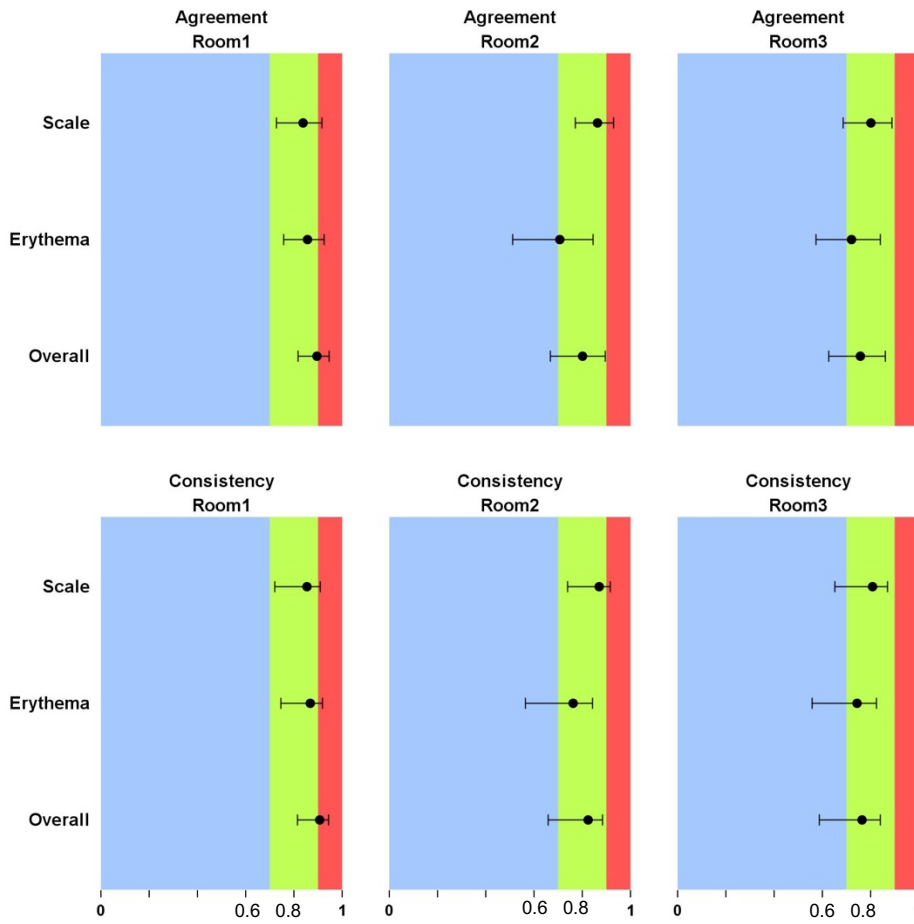


**Figure 13. IntraClass Correlation Coefficients (ICCs) for agreement and consistency for the three rooms during in-person evaluations at the FIRST conference (Stage 2)**  
 For all rooms, the ratings were found to be highly correlated for scale and erythema (ICCs near 0.7 or greater).

	<b>ROOM 1</b>	<b>ROOM 2</b>	<b>ROOM 3</b>
<b>Dorsal Foot</b>	0.85 (0.72, 0.92)	1.00 (1.00, 1.00)	0.94 (0.89, 0.97)
<b>Lower Leg</b>	0.85 (0.73, 0.92)	1.00 (0.99, 1.00)	0.92 (0.87, 0.96)
<b>Upper Arm</b>	0.77 (0.59, 0.88)	1.00 (0.99, 1.00)	0.92 (0.87, 0.96)
<b>Upper Back</b>	0.83 (0.69, 0.91)	1.00 (1.00, 1.00)	0.92 (0.86, 0.96)

**Table 8. Kuder-Richardson Formula 20, KR20, values for the three rooms during in-person evaluations at the FIRST conference (Stage 2)**

Raters were consistent in choosing the specific set of standards for scale (“L” or “K”) for a given patient, with KR20 greater than 0.77 for all body sites.



**Figure 14. IntraClass Correlation Coefficients (ICCs) for agreement and consistency for the combined scores for scale and erythema, as well as the final total scores during in-person evaluations at the FIRST conference (Stage 2)**  
 For all rooms, the ratings were found to be highly correlated for hyperkeratosis and erythema (ICCs greater than 0.7).

## DISCUSSION

We have designed and validated a visual severity index for ichthyosis, a critical tool for research and clinical evaluation in this rare group of disorders. The Congenital Ichthyosis Severity Index (CISI) is the only other ichthyosis severity index to have undergone validation. Similar to our instrument, the CISI employs visual Likert scales for scale and erythema.<sup>72</sup> We aimed to improve the convenience and applicability of the index by providing 1) clear written descriptions of the features characteristic of each level of scale 2) visual standards for four different body sites, and 3) two different sets of scale standards to account for two different types of scale seen in subjects with ichthyosis.

To create a user-friendly index, we focused on two different types of scale (lamellar and keratoderma), instead of creating a separate standard for each subtype of ichthyosis as was done for the CISI.<sup>80</sup> We found high inter-rater and intra-rater ICCs for scale (near 0.7 or greater for all rounds of testing). Raters were highly consistent in deciding which set of scale standards to choose for a given subject (Kuder-Richardson Formula 20 greater than 0.77 for all rounds of testing), even though our subject population was heterogeneous with respect to clinical diagnosis (Table 5 and Table 7). Subgroup analysis revealed that high ICCs for scale were maintained even in the rare cases where raters disagreed on which set of standards to use for a given subject; that is, ICCs for subjects for whom all the raters unanimously chose the same set of standards did not differ significantly from ICCs for subjects for whom raters chose differently. This indicates that our two different sets of scale standards capture increments of severity in a similar fashion, possibly due to the detailed written descriptions that standardize the scoring regardless of which set of standards is used. This suggests that the standardized written descriptions may allow or even be critical for scoring of the many different clinical subtypes of ichthyosis—even those not represented in the photographs.

While the inter-rater reliability for erythema was high (ICCs near 0.7 or greater) during in-person validation at the FIRST conference, it was poor (ICCs less than 0.6) during validation on test photographs. This finding emphasizes the difficulty of capturing and reproducing erythema on photographic images. Many factors, including room lighting and color saturation settings, can impede consistent evaluation of erythema on photographs.

Much literature has been published on evaluation of erythema, with compelling evidence for the use of both visual analog scales, and more objective methods, such as reflectance spectrophotometry. Reflectance spectrophotometry calculates an erythema index by comparing the intensity of light absorbed by hemoglobin (green light) to that of light absorbed by melanin (red light). Several studies have provided evidence for the reliability of reflectance spectrophotometry;<sup>77,78</sup> however, others have shown lack of superiority of readings from a reflectance spectrophotometry compared to visual analog scales.<sup>75, 82</sup> Various operator factors can influence the reading obtained from reflectance spectrophotometry, including the pressure with which the instrument is held and the angle used.<sup>83</sup> Furthermore, several individual factors can impact reflectance spectrophotometry readings, including blood pressure, smoking, caffeine intake, and skin phototype.<sup>83,84</sup> Latreille *et al.* showed that erythema induced by methyl nicotinate was not detected by reflectance spectrophotometry in the darker phototypes, possibly because the emitted light is predominantly absorbed by the melanin in the epidermis and does not reach the hemoglobin in the dermis.<sup>84</sup> Given the time- and cost-effectiveness of visual observation, and lack of strong evidence for the superiority of reflectance spectrophotometry, we utilized a visual analog scale for the evaluation of erythema in our index.



During in-person evaluation, our index showed high inter-rater reliability for both erythema and scale with ICCs near 0.7 and greater. An important feature the VIIS is its flexibility in either evaluating the severity at a specific individual body site, as may be done in a clinical trial, or utilizing ratings of all 4 body sites to arrive at a comprehensive assessment of ichthyosis severity. Our analysis showed high inter-rater reliability for both the individual ratings for each body site, as well as the combined scores.

A limitation of our index is that the photographs included in the standards were from subjects with Fitzpatrick I-III skin type. As shown in Table 5 and Table 7, the majority of the subjects during the validation process were Caucasian. Ideally, visual standards would have been created for a range of skin phototypes. We aimed to increase the generalizability of our instrument while maintaining convenience by including written descriptions for each level for severity that are applicable to all skin types. Further studies will be necessary to determine whether our standards can be reliably used for Fitzpatrick IV-VI skin types, especially for erythema since evaluation of erythema can be more challenging in individuals with darker phototypes.

We have designed a Visual Index for Ichthyosis Severity (VIIS), and performed several rounds of rigorous validation to show high reliability and reproducibility. Such a standardized method of assessing disease severity has the potential to advance ichthyosis research. This user-friendly index was easily applied by dermatologists during a busy schedule of clinical evaluations at the FIRST conference, and with high ICCs for evaluation of scale and erythema. Our analysis also indicates that to reliably capture all aspects of the index, it is essential that the evaluations be performed in person. This finding is critical to the study design of clinical trials and emphasizes the importance of

in-person evaluations rather than assessments of photographs, especially when erythema is one of the outcome measures. In addition, power analysis revealed that a sample size of 24 (12 in control group, 12 in treatment group) will achieve 92% power in detecting a unit difference, which is a feasible sample size. We expect this index to facilitate clinical phenotyping and to provide a measure of response for therapeutic trials which assess the cardinal features of this class of disorders.

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APPENDIX

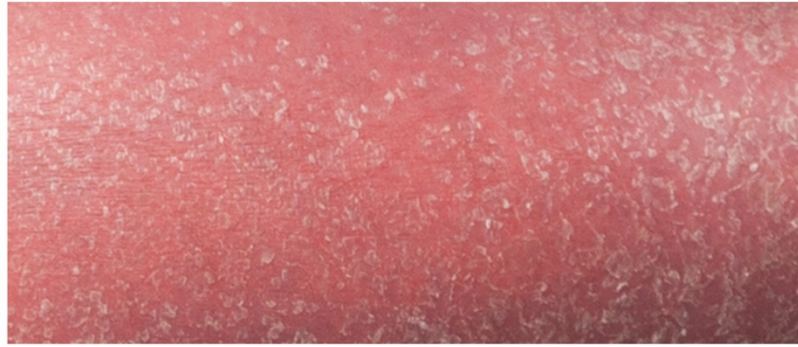
**ERYTHEMA SEVERITY STANDARDS**  
(Severity score 0: no erythema)



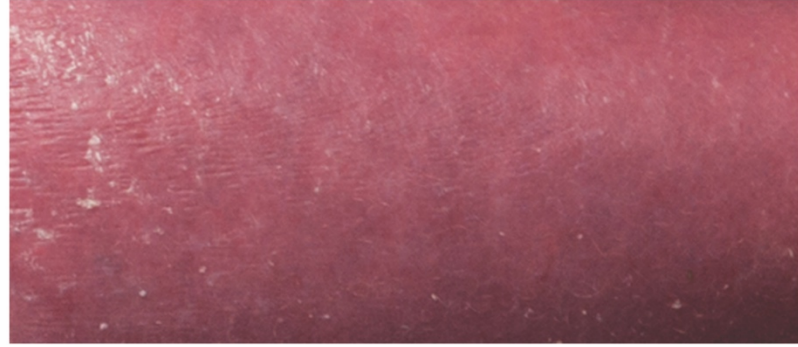
**Severity score 1:**  
barely perceptible pink



**Severity score 2:**  
pink



**Severity score 3:**  
red



**Severity score 4:**  
deep red-purple

## LAMELLAR SCALE SEVERITY STANDARDS - ARM

(Severity score 0: normal skin; no perceptible scale or smoothening)



**Severity score 1:** areas of normal skin intermixed with areas showing smoothening (diminished fine skin markings; shininess; waxiness) or small scales (visibly separated/fractured stratum corneum).



**Severity score 2:** confluent smoothening (diminished fine skin markings; shininess; waxiness) or small scales (visibly separated/fractured stratum corneum).



**Severity score 3:** confluent scales (visibly separated/fractured stratum corneum) including some large (>1cm), thick scales.



**Severity score 4:** confluent, primarily large (>1 cm), thick scales.

## KERATODERMA SCALE SEVERITY STANDARDS - ARM

(Severity score 0: normal skin; no perceptible scale or surface accentuation)



**Severity Score 1:** areas of normal skin intermixed with areas showing hyperkeratotic surface accentuation (organized or geometric exaggeration of coarse skin markings, such as corrugation or follicular prominence).



**Severity Score 2:** confluent surface accentuation (organized or geometric exaggeration of coarse skin markings, such as corrugation or follicular prominence) and/or flat scale.



**Severity Score 3:** confluent surface accentuation (organized or geometric exaggeration of coarse skin markings, such as corrugation or follicular prominence), scales and some columnar hyperkeratosis (piles of scale with vertical fracturing).



**Severity Score 4:** confluent, primarily columnar hyperkeratosis (piles of scale with vertical fracturing).

**LAMELLAR SCALE SEVERITY STANDARDS - LEG**

(Severity score 0: normal skin; no perceptible scale or smoothening)



**Severity score 1:** areas of normal skin intermixed with areas showing smoothening (diminished fine skin markings; shininess; waxiness) or small scales (visibly separated/fractured stratum corneum).



**Severity score 2:** confluent smoothening (diminished fine skin markings; shininess; waxiness) or small scales (visibly separated/fractured stratum corneum).



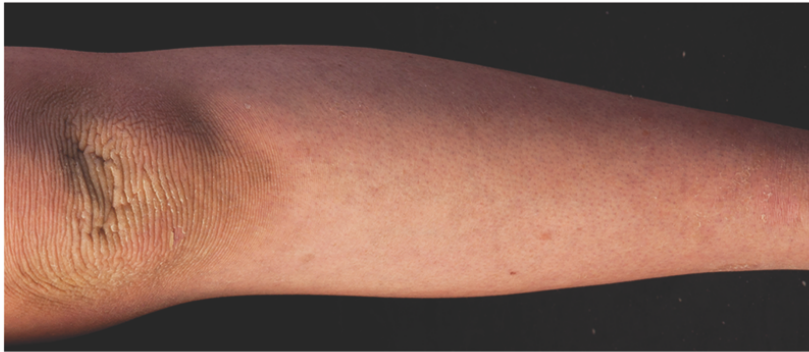
**Severity score 3:** confluent scales (visibly separated/fractured stratum corneum) including some large (>1cm), thick scales.



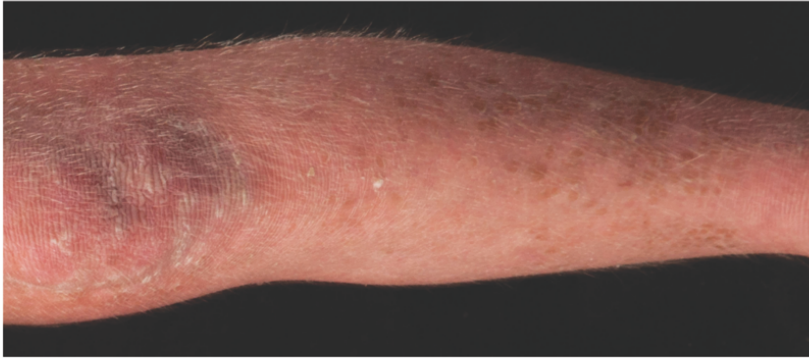
**Severity score 4:** confluent, primarily large (>1cm), thick scales.

## KERATODERMA SCALE SEVERITY STANDARDS - LEG

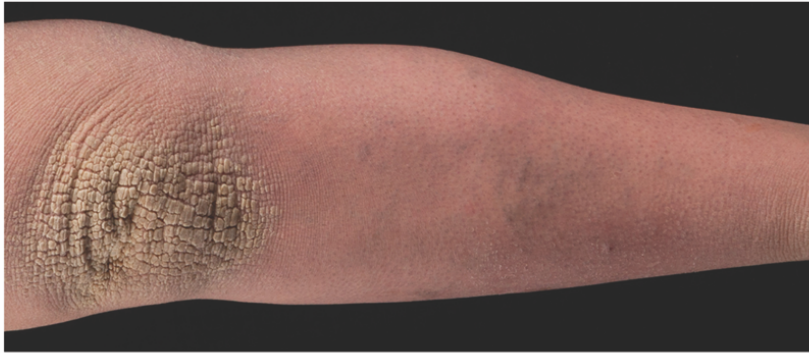
(Severity score 0: normal skin; no perceptible scale or surface accentuation)



**Severity score 1:** areas of normal skin intermixed with areas showing hyperkeratotic surface accentuation (organized or geometric exaggeration of coarse skin markings, such as corrugation or follicular prominence).



**Severity score 2:** confluent surface accentuation (organized or geometric exaggeration of coarse skin markings, such as corrugation or follicular prominence) and/or flat scale.



**Severity score 3:** confluent surface accentuation (organized or geometric exaggeration of coarse skin markings, such as corrugation or follicular prominence), scales and some columnar hyperkeratosis (piles of scale with vertical fracturing).



**Severity score 4:** confluent, primarily columnar hyperkeratosis (piles of scale with vertical fracturing).

## LAMELLAR SCALE SEVERITY STANDARDS - FOOT

(Severity score 0: normal skin; no perceptible scale or smoothening)



**Severity score 1:** areas of normal skin intermixed with areas showing smoothening (diminished fine skin markings; shininess; waxiness) or small scales (visibly separated/fractured stratum corneum).



**Severity score 2:** confluent smoothening (diminished fine skin markings; shininess; waxiness) or small scales (visibly separated/fractured stratum corneum).



**Severity score 3:** confluent scales (visibly separated/fractured stratum corneum) including some large (>1cm), thick scales.



**Severity score 4:** confluent, primarily large (>1 cm), thick scales.

## KERATODERMA SCALE SEVERITY STANDARDS - FOOT

(Severity score 0: normal skin; no perceptible scale or surface accentuation)



**Severity score 1:** areas of normal skin intermixed with areas showing hyperkeratotic surface accentuation (organized or geometric exaggeration of coarse skin markings, such as corrugation or follicular prominence).



**Severity score 2:** confluent surface accentuation (organized or geometric exaggeration of coarse skin markings, such as corrugation or follicular prominence) and/or flat scale.



**Severity score 3:** confluent surface accentuation (organized or geometric exaggeration of coarse skin markings, such as corrugation or follicular prominence), scales and some columnar hyperkeratosis (piles of scale with vertical fracturing).



**Severity score 4:** confluent, primarily columnar hyperkeratosis (piles of scale with vertical fracturing).

## LAMELLAR SCALE SEVERITY STANDARDS - BACK 1 & 2

(Severity score 0: normal skin; no perceptible scale or smoothing)



**Severity score 1:** areas of normal skin intermixed with areas showing smoothing (diminished fine skin markings; shininess; waxiness) or small scales (visibly separated/fractured stratum corneum).



**Severity score 2:** confluent smoothing (diminished fine skin markings; shininess; waxiness) or small scales (visibly separated/fractured stratum corneum).



## LAMELLAR SCALE SEVERITY STANDARDS - BACK 3 & 4



**Severity score 3:** confluent scales (visibly separated/fractured stratum corneum) including some large (>1cm), thick scales.



**Severity score 4:** confluent, primarily large (>1 cm), thick scales.

## KERATODERMA SCALE SEVERITY STANDARDS - BACK 1 & 2

(Severity score 0: normal skin; no perceptible scale or surface accentuation)



**Severity score 1:** areas of normal skin intermixed with areas showing hyperkeratotic surface accentuation (organized or geometric exaggeration of coarse skin markings, such as corrugation or follicular prominence).



**Severity score 2:** confluent surface accentuation (organized or geometric exaggeration of coarse skin markings, such as corrugation or follicular prominence) and/or flat scale.

## KERATODERMA SCALE SEVERITY STANDARDS - BACK 3 & 4



**Severity score 3:** confluent surface accentuation (organized or geometric exaggeration of coarse skin markings, such as corrugation or follicular prominence), scales and some columnar hyperkeratosis (piles of scale with vertical fracturing).



**Severity score 4:** confluent, primarily columnar hyperkeratosis (piles of scale with vertical fracturing).