

## INVESTIGATIVE REPORT

# Expression of Retinoid-regulated Genes in Lamellar Ichthyosis vs. Healthy Control Epidermis: Changes after Oral Treatment with Liarozole\*

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Lamellar ichthyosis is a keratinization disorder caused by *TGMI*, *Ichthyin* and several other gene mutations. A new treatment option is liarozole, which blocks the cytochrome P450 (CYP26)-mediated catabolism of endogenous all-trans retinoic acid. This study focuses on the expression of retinoid-related genes in ichthyotic epidermis before and after treatment with oral liarozole. We first compared the mRNA expression of cellular retinoic acid binding protein II (CRABPII), keratin (KRT) 2 and 4, CYP26A1 and B1, and two markers of inflammation (interleukin-1 $\alpha$  and tumours necrosis factor (TNF)- $\alpha$ ) in shave biopsies from 11 genetically defined, untreated patients and 12 age- and sex-matched healthy controls, finding no overt differences between the groups, besides elevated CRABPII expression. We then studied the biomarkers before and after 4 weeks of treatment with liarozole (75 or 150 mg/day), which produced a better therapeutic response in patients with *Ichthyin* ( $n=3$ ) than in those with *TGMI* ( $n=6$ ) mutations. A significant decrease in the mRNA expression of KRT2 and TNF- $\alpha$ , and trends toward increased expression of KRT4 and CYP26A1 were observed in liarozole-treated patients, consistent with an increased retinoid stimulation of epidermis. However, there were no dose-related responses and the results of the immunostaining did not always parallel the mRNA findings. The results suggest that liarozole exerts a therapeutic effect in lamellar ichthyosis by mildly affecting the expression of retinoid-regulated genes in epidermis. **Key words:** retinoic-acid metabolism blocking agents; keratin; vitamin A metabolism; cytochrome P450, skin disorders.

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Lamellar ichthyosis (LI) is a rare autosomal recessive disorder of keratinization characterized by generalized scaling and a variable degree of skin erythema. The aetiology of LI is heterogeneous: the first discovered gene abnormalities were truncating mutations in *TGMI*, encoding transglutaminase 1, which cross-links the cornified cell envelope proteins in stratum corneum. Subsequently, several other genes involved in the formation of the epidermal lipid barrier have been implicated in the aetiology of LI, including *Ichthyin*, *ALOX12B*, *ALOXE3*, and *ABCA12* (for review see 1, 2).

Treatment of LI comprises topical moisturizers, keratolytic agents and vitamin A analogues (retinoids) (2, 3). Oral therapy with synthetic retinoids is usually effective in ameliorating LI, but may be associated with side-effects (for review see 1, 4). An alternative approach is to try to increase the endogenous level of all-trans retinoic acid (ATRA) in the skin by blocking its catabolism to less active vitamin A metabolites (5). Compounds with this propensity are called retinoic acid metabolism blocking agents (RAMBAs). One such substance, talarozole (also known as Rambazole<sup>TM</sup>, Barrier Therapeutics nv, Geel, Belgium) was recently shown by us to affect the expression of retinoid-regulated genes in healthy human skin (6). This is indirect proof of increased tissue levels of ATRA during talarozole therapy, while ATRA per se cannot be quantified in small skin biopsies due to methodological limitations (Vahlquist, unpublished observation).

Yet another RAMBA, liarozole (Liaza<sup>TM</sup>, Barrier Therapeutics nv, Geel, Belgium), has been shown to be effective as an oral and topical treatment of ichthyosis and psoriasis (for review see 7). Although its effects on ATRA metabolism and expression of retinoid biomarkers have been studied in healthy human skin (8), the corresponding effects in LI epidermis have not previously been studied in any detail.

Vitamin A metabolism in human keratinocytes involves esterification of retinol to fatty acyl esters and oxidation of retinaldehyde to ATRA; processes which are catalysed by lecithin:retinol acyltransferase (LRAT) and retinaldehyde hydrogenase-2 (RALDH2 or ALDH1A2), respectively (for review see 9). The cellular level of ATRA is also regulated by cellular retinoic acid binding protein II (CRABPII), which is highly expressed in human epidermis, especially after ATRA therapy (10). CRABPII

functions both as a "hydrophobic sink" for ATRA (11) and as its shuttle to the cell nucleus where it binds to a family of retinoid receptors all involved in gene regulation (12). Accordingly, ATRA affects the transcriptional activity of epidermal genes, such as those encoding CRABP II, keratin (KRT) 4 and KRT2 (13, 14).

The catabolism of ATRA in epidermis is controlled by several cytochrome P450-dependent enzymes (namely CYP26A1, B1 and C1), which catalyse the formation of less active 4-hydroxy and 18-hydroxy metabolites (15, 16), and are the intended targets for RAMBA therapy. Recent studies have identified a retinoic acid response element in the *CYP26A1* gene (17), supporting the existence of an auto-regulatory loop that controls the cellular level of ATRA (18). Another cytochrome P450 enzyme, CYP2S1, was also shown to catabolize ATRA in epidermis (19), but its importance for ATRA homeostasis is still uncertain.

The main purpose of this study, performed in a subset of patients with LI participating in a randomized multi-centre trial examining the clinical effects of two different doses of oral liarozole<sup>1</sup>, was to investigate whether the epidermal expression of retinoid-related biomarkers and metabolizing enzymes is affected in a manner consistent with the drug's proposed mechanism of action. As a corollary, we compared the gene expression pattern in untreated ichthyotic skin vs. normal skin, and looked for differences in the clinical efficacy of liarozole that could be related to the aetiology of ichthyosis, e.g. *TGMI* or *Ichthyin* mutations.

## MATERIALS AND METHODS

### Study design

The patients included in this study were part of a larger multi-centre clinical trial designed as a randomized, double-blind study comparing two doses of liarozole (75 mg/day and 150 mg/day) and placebo in 96 patients (at a ratio of 7:7:1) for 3 months followed by a washout period<sup>1</sup>. Both the clinical trial and the biopsy study were approved by the local ethics committee at Uppsala University and by the Swedish authorities, and were performed according to the ethical principles of the Declaration of Helsinki. The patients signed a written informed consent at the beginning of the trial.

Twelve patients with LI, 4 males and 8 females (all Caucasians) aged 18–69 years, were recruited via our genodermatology centre. Seven of the patients carried *TGMI* mutations (20), 4 had *Ichthyin* mutations (21), and one patient was negative for these two types of mutations but showed otherwise typical findings of LI (Table I). One patient (no. 8) did not consent to skin biopsy but in all other aspects completed the clinical trial according to the protocol. Another patient (no. 11) first accepted skin biopsy but was later excluded from the clinical trial because of a heart problem detected at screening. Although patient no. 2 was not available for skin sampling at screening, she accepted biopsy after one month of therapy and again after the washout period at the end of the

clinical trial when all her skin symptoms had relapsed; the latter biopsy was considered analogous to baseline.

All patients had moderate to severe LI, with ichthyosis scores ranging from 20 to 36 (maximal score 44). Seven patients had received acitretin previously (but not within one month prior to the trial), and all were using emollients on a regular basis during the trial (cream application was avoided for > 10 h prior to clinical scoring and skin sampling).

After randomization, the patients received tablets containing liarozole (75 or 150 mg) or vehicle in identical-appearing capsules for 3 months. Vital signs, electrocardiogram (ECG), routine blood chemistry, urinalysis, and severity of skin symptoms were checked before the trial and at monthly intervals during the trial. The results of the laboratory tests were essentially negative, and will be reported separately. At baseline and after 4 weeks of treatment, two punch biopsies (diameter 3 mm) and one superficial shave biopsy, typically containing approximately 80% epidermis (from now on referred to as "epidermis") (22), were obtained from the buttocks after infiltrating the skin with 1 ml of lidocaine-adrenalin (Astra Zeneca, Södertälje, Sweden). The time interval between the samplings (1 month) was chosen so that early rather than late effects of liarozole should be highlighted.

The punch biopsies were first placed in cold Histocon (Histo-lab Products AB, Gothenburg, Sweden) and then subsequently snap frozen and stored at  $-70^{\circ}\text{C}$ . The shave biopsies were immediately frozen on dry ice and kept at  $-70^{\circ}\text{C}$  until isolation of total ribonucleic acid (RNA) was performed. The blinding of the samples (by an independent employee) was performed before the start of the analysis and kept secret until data processing was complete.

Shave and punch biopsies from a group of 12 age- and sex-matched healthy individuals (average age: 41 years, male/female: 5/7), most of whom had participated in a previous study (6), were used to establish the normal mRNA and protein expression profiles for the biomarkers. The control samples were analysed in parallel with the patients' baseline samples.

### RNA isolation and reverse transcription

Isolation of total RNA and reverse transcription has been described in detail previously (23). In brief, RNA was prepared by homogenizing the tissue in 1 ml Trizol (Invitrogen, Carlsbad, CA, USA), and the concentration was determined by spectro-

Table I. Patient's characteristics, doses of liarozole and ichthyosis scores in the clinical trial

No.	Sex	Age (years)	Body weight (kg)	Affected gene	Liarozole dose (mg)	Ichthyosis score	
						Baseline	1 month
1	F	25	55	<i>TGMI</i>	Placebo	35	27
2 <sup>a</sup>	F	26	50	<i>TGMI</i>	150	34	26
3	F	48	75	<i>TGMI</i>	75	36	27
4	M	24	78	<i>TGMI</i>	150	35	34
5	M	28	85	<i>TGMI</i>	75	34	20
6	M	32	64	<i>TGMI</i>	150	35	32
7	M	65	55	<i>TGMI</i>	75	35	34
8 <sup>b</sup>	F	18	56	<i>Ichthyin</i>	150	20	1
9	F	54	65	<i>Ichthyin</i>	75	23	1
10	F	61	71	<i>Ichthyin</i>	75	28	12
11 <sup>c</sup>	F	69	63	<i>Ichthyin</i>	–	21	NA
12	F	66	83	?	75	22	7

<sup>a</sup>The baseline sample in this case was obtained first one month after stopping therapy, i.e. when the ichthyosis score had returned to pre-therapy value.

<sup>b</sup>No biopsies were obtained from this patient.

<sup>c</sup>This patient was excluded from the clinical trial but a baseline biopsy was taken and used in the comparison with healthy controls.

NA: not applicable.

<sup>1</sup>Vahlquist et al., Liarozole effective in treatment of lamellar ichthyosis, poster abstract, IID2008 Meeting, Kyoto, J Invest Dermatol (2008), 128, S60

photometry. First-strand complementary deoxyribonucleic acid (cDNA) synthesis was performed using 1.5 µg of total RNA as template. After synthesis was complete, the cDNA was diluted with dH<sub>2</sub>O to obtain a concentration of 10 ng cDNA/µl.

#### Quantitative real-time polymerase chain reaction

The number of transcripts was studied by quantitative real-time polymerase chain reaction (QRT-PCR) as previously described (23). The primers and probes were obtained from Applied Biosystems; the sequences are described in Table II. Triplicate reaction tubes were set up for each sample and the 25 µl reaction was performed in a MyiQ (BioRad Laboratories, Inc., Hercules, CA, USA). Simultaneous amplification of known amounts of a PCR product generated a standard curve for comparison. The expression of two housekeeping genes (cyclophilin A and β-actin) was used to normalize the expression of the genes of interest, with the help of geNORM software (Center for Medical Genetics, Ghent University, Ghent, Belgium) (24).

#### Analysis of protein expression

Two punch biopsies were obtained at each sampling. One was fixed in Lana's fixative for 30 min and then snap-frozen (for CRABPII analysis), while the other was frozen immediately (for all other histological analysis). Sections (6 µm) were fixed in 100% ice-cold acetone for 5 min, blocked with 10% normal horse or rabbit serum for 15 min (both from Vector Laboratories Inc., Burlingame, Canada), and incubated overnight at 4°C with monoclonal antibodies: KRT4 (clone: 6B10, Gentex, San Antonio, TX, USA)

Table II. Polymerase chain reaction (PCR) primers (F: forward, R: reverse and P: probe). Sequences are given in 5'-3' orientation

CRABPII	F: AGCAGAAGCTCCTGAAGGGA R: CCATCGTTGGTCAGTTCTCT P: AGGGCCCAAGACCTCGTGGAC
KRT4	F: GCAGCTAGATACCTTGGGCAA R: CTTCACTTAGTCTTGAAGTCTCCAC P: CTGCTCGCATGGTCTTCAGCTCAGACT
KRT2	F: TCTTGTGGAGGATTATAAGAAG R: GTAGGCATTGTCCACGTCCTTT P: ACAAAAATCATTCTCAGCAGCTGTGCGCTTA
CYP26A1*	F: TCACTTACCTGGGGCTCTACC R: ACTTGTGTCTTGATTGCTCTTGG P: CCGCTTCCATTACCTCCCGTTC
CYP26B1*	F: CCACCGCCAGCACCTTCAG R: GCAGATGGACGGTTCAGGAAG P: AGGGAGAAGGCTTGTAGGATGG
CYP2S1*	F: CAGACTTGGTGAACGGGACAG R: AGCCAGCCTGCGTAATATCG P: CTGGTCAATCACCTGGACGA
RALDH2*	F: CCGCGCCACCTCCTC R: TCCCTCCAGAAAAAGGCACTGCTCAA
LRAT	F: CGCCAATGACTCAGAGGAAGA R: AGGGCGTCATTCAGGATGAA P: TGGCCAGGCAGTCAGA
IL-1α*	F: TGTTTGTCTACAACATGGGCTACA R: TGCTGGACCCAACACAAATG P: TGCCATCCAACCACTCAGTC
TNF-α*	F: TTCCAGTTTTTCATCTGCACTGCCA R: CTGGCTGCTGACCGAGG P: GAAAGTCTCAAACATGATCTGGGT
Cyclophilin	F: CCTGAACCCCAAGGCCAACCG
β-actin	

\*SYBR Green I was included in the PCR master mix instead of probe. KRT: keratin; LRAT: lecithin:retinol acyltransferase; RALDH2: retinal-dehydrogenase-2; CYP26: cytochrome P450; IL-1α: interleukin 1α; TNF-α: tumour necrosis factor α; CRABPII: cellular retinoic acid binding protein II.

diluted 1/40, KRT2 (clone: Ks2.342.7.1, AbCam plc, Cambridge, UK) diluted 1/800, CYP26B1 (clone: 1H6, AbNova, Taipei City, Taiwan) diluted 1/50, or a polyclonal rabbit anti-human CYP26A1 antibody (Alpha Diagnostics, San Antonio, TX, USA), again diluted 1/50. Biotinylated horse anti-mouse IgG (H+L) (Vector Laboratories Inc.) served as secondary antibody for the KRT4 and CYP26B1. A secondary biotinylated goat anti-rabbit antibody was used for CYP26A1 followed by 30 min incubation with Texas Red Avidin D (Vector Laboratories Inc.). KRT2 was detected by incubating with FITC conjugated rabbit anti-mouse IgG (Dako-Cytomation, Glostrup, Denmark) for 30 min. CRABPII was visualized by using a previously described monoclonal antibody (clone: 5CRA3B3) (12) diluted 1/200, followed by incubation with biotinylated horse anti-mouse and Texas Red Avidin D as previously described (25). The slides were mounted with Vectashield® Hard Mounting Medium containing DAPI (Vector Laboratories Inc.). The staining was analysed and evaluated using a Zeiss Axio Imager Z1 microscope and AxioVision software (Zeiss, Göttingen, Germany) as previously described (6). The microscope was equipped with filters for FITC, Texas Red and DAPI. The staining intensities were scored using a scale from zero (negative) to three (very intense staining) for all examined proteins.

#### Determination of epidermal and stratum corneum thickness

Sections from the punch biopsies were stained with Harris Haematoxylin (Histolab Products AB). The length (L) and area (A) of the epidermis and stratum corneum of each section was measured using a 20× magnification and the microscope and software as described above. Epidermal and stratum corneum thickness (Th) was calculated (according to  $Th=A/L$ ) from these measurements.

#### Statistics

Data obtained for mRNA and epidermal thickness were log-transformed prior to statistical analysis. A paired Student's *t*-test was used to evaluate the statistical significance of differences between data obtained at the baseline visit and data obtained at the visit after 4 weeks of treatment. *p*-values obtained for mRNA gene levels were adjusted for multiplicity using the False Discovery Rate (26) controlling procedure. Two-sided *p*-values less than or equal to 0.05 were considered to indicate statistical significance. For mRNA and epidermal thickness measurements, results were expressed as geometric mean % difference and 95% confidence intervals.

## RESULTS

### Comparison of mRNA expression in ichthyosis vs. normal skin at baseline

To determine whether the patients' mRNA values were abnormal at baseline, a comparison was made with age- and sex-matched healthy controls. As shown in Table III, the mean mRNA values of the genes of interest did not differ markedly between patients and controls, with the exception of CRABPII mRNA, which was six times higher in the patients ( $p < 0.001$ ). There were no obvious correlations between the mRNA expressions and the disease aetiologies (data not shown).

### Effects of liarozole therapy

**Clinical findings.** All patients completed the medication according to the protocol. No overt systemic toxi-

Table III. Epidermal mRNA expression of genes of interest in untreated patients with lamellar ichthyosis and age- and sex-matched healthy controls

Gene product	Mean mRNA expression (Ratio to housekeeping gene $\times 10^3$ )		p-value
	Patients (n=11)	Controls (n=12)	
CRABP II	317000	46800	<b>0.0006<sup>a</sup></b>
KRT2	5700	4700	0.36
KRT4	190	180	0.69
CYP26A1	0.007	0.002	0.17
CYP26B1	73	72	0.76
LRAT	12	3.8	0.09
RALDH2	171	198	0.55
IL-1 $\alpha$	71	62	0.57
TNF- $\alpha$	110	75	0.55

<sup>a</sup>p<0.05 is indicated in bold figures.

KRT: keratin; LRAT: lecithin:retinol acyltransferase; RALDH2: retinaldehydrogenase-2; CYP26: cytochrome P450; IL-1 $\alpha$ : interleukin 1 $\alpha$ ; TNF- $\alpha$ : tumour necrosis factor  $\alpha$ ; CRABP II: cellular retinoic acid binding protein II.

city was noted. The ichthyosis score after one month improved most dramatically in patients with *Ichthyin* mutations (n=3) or unknown aetiology (n=1) who had initially a milder disease; the response in patients with *TGM1* mutations (n=6) was less pronounced, and in some cases not clearly different from that in the placebo-treated patient (see Table I).

Disclosure of the randomization code showed that 6 of the biopsied patients received 75 mg of liarozole per day, 3 received 150 mg/day and one received placebo (see Table I). The unintended skewed distribution of subjects between the liarozole groups was partially due to one patient in the high-dose group declining a repeated biopsy. Because of this distortion, statistical analyses (see below) were performed on all liarozole-treated patients together, irrespective of the dose.

**Analysis of epidermal thickness.** Histological sections from repeated punch biopsies were available in 8 liarozole-treated patients. As shown in Table IV, there

Table IV. Thickness of stratum corneum and viable epidermis after 4 weeks of liarozole treatment (expressed as percentage of baseline value)

	n <sup>a</sup>	Thickness (% of baseline)	95% CI	
Stratum corneum	8	88	(67–117)	NS <sup>b</sup>
Viable epidermis	8	128	(88–188)	NS <sup>b</sup>

<sup>a</sup>Of a total of 10 patients, one did not accept punch biopsies and another received placebo. Therefore only 8 patients were included in this analysis.

<sup>b</sup>Statistically non-significant (p>0.05).

95% CI: 95% confidence interval.

was a tendency towards reduced thickness of stratum corneum and increased thickness of viable epidermis after one month of therapy, but the changes were not statistically significant and no clearly dose-dependent differences were noted (data not shown).

**Changes in mRNA expression.** The mRNA expression of ten genes was studied in paired shave biopsies obtained at baseline and after one month of treatment. Figs 1–3 show the individual results observed in liarozole-treated patients (n=9) in relation to both the underlying genetic cause of LI and the dose of liarozole. The mRNA values of the placebo-treated patient changed only minimally during the trial (data not shown). Table V shows the statistical analysis of liarozole-induced changes in mRNA for all genes described below and for all patients together.

**Effects on well-established biomarkers.** From the combined results presented in Fig. 1 and Table V it is evident that: (i) *CRABP II* expression increased in 7 out of 9 patients on therapy, but the mean change compared with baseline (+37%) was not statistically significant, probably as a result of high pre-therapy levels of *CRABP II* mRNA (see above); (ii) *KRT2* expression decreased in all patients (p<0.003), a result consistent with previous findings in healthy skin exposed to topical ATRA (13) or talarozole (6); (iii) *KRT4* expression increased in 7 patients, but decreased in one patient, explaining

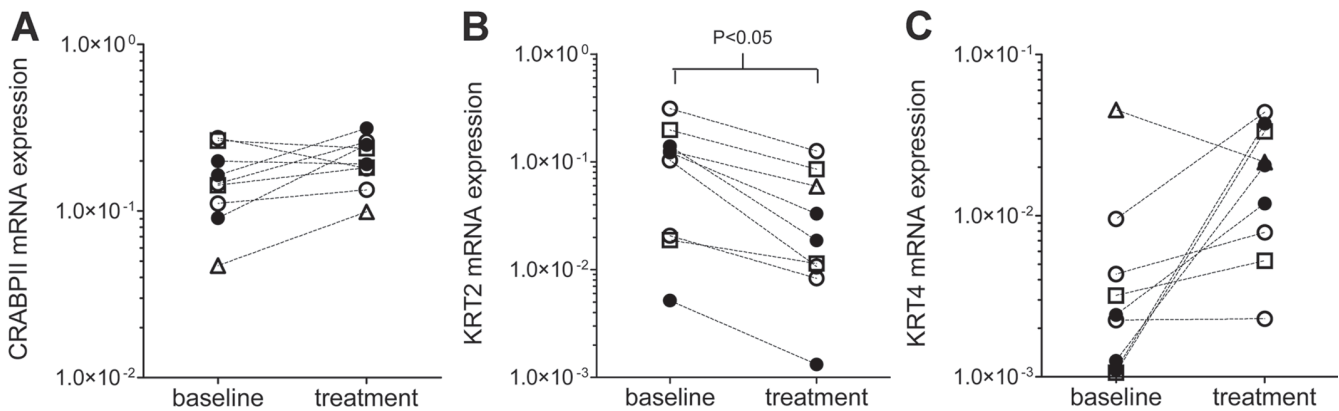


Fig. 1. Individual mRNA expression of three well-known retinoid biomarkers before and after 4 weeks of liarozole treatment. The mRNA expression of: (A) cellular retinoic acid binding protein II (CRABP II); (B) keratin 2 (KRT2); and (C) KRT4 is shown. Circles denote patients with *TGM1* mutation (n=6), squares denote patients with *Ichthyin* mutation (n=2), and triangles denote one patient with unknown mutations. The subjects who received the higher dose (150 mg) are represented by filled symbols and those who received the lower dose (75 mg) by open symbols. All values were normalized to the expression of the two housekeeping genes, cyclophilin and  $\beta$ -actin (using geNORM software). Only KRT2 was significantly affected (p<0.05).

Table V. Epidermal mRNA expression of retinoid biomarkers, retinoid metabolizing enzymes and markers of inflammation after 4 weeks of liorzole treatment expressed as percentage of baseline value (n = 9)

Gene product	mRNA expression (mean % of baseline)	95% CI	False discovery rate adjusted <i>p</i> -value
CRABPII	137	(96–196)	0.151
KRT2	30	(19–47)	<b>0.003<sup>a</sup></b>
KRT4	434	(135–1394)	0.067
CYP26A1	427	(119–1535)	0.077
CYP26B1	142	(81–251)	0.289
CYP2S1	109	(81–146)	0.567
LRAT	76	(22–269)	0.635
RALDH2	117	(74–185)	0.560
IL-1 $\alpha$	45	(12–169)	0.289
TNF- $\alpha$	54	(38–78)	<b>0.022<sup>a</sup></b>

<sup>a</sup>*p* < 0.05 is indicated in bold.

CI: confidence interval; KRT: keratin; LRAT: lecithin:retinol acyltransferase; RALDH2: retinaldehydrogenase-2; CYP26: cytochrome P450; IL-1 $\alpha$ : interleukin 1 $\alpha$ ; TNF- $\alpha$ : tumour necrosis factor  $\alpha$ ; CRABPII: cellular retinoic acid binding protein II.

why a mean change of almost three times above baseline was not statistically significant (*p* < 0.067). KRT4 is not detected in normal skin, but is highly expressed in the upper epidermis during topical treatment with talarozole (6) or ATRA (13). The reason for the high baseline level of KRT4 mRNA in one patient (no. 12) is unclear; her baseline CRABPII and KRT2 values were unremarkable, making any inadvertent or unreported retinoid exposure prior to our study highly unlikely.

**Effects on retinoid metabolizing enzymes.** Despite markedly increased CYP26A1 levels in some patients receiving the higher dose of liorzole (Fig. 2A) and a mean treatment level three times above baseline (Table V), the change was not statistically significant (*p* < 0.077). Interestingly, patients with a strong induction of CYP26A1 by liorzole also showed an increased expression of CRABPII and KRT4 and a decreased expression of KRT2, consistent with the anticipated response to raised epidermal ATRA levels during therapy (results of comparisons not shown).

CYP26B1, LRAT, and RALDH2 mRNA did not change consistently during liorzole therapy (Fig. 2B–D and Table V). CYP2S1 mRNA was barely detectable both before and after therapy (no individual values are shown).

**Effects on pro-inflammatory cytokines.** Although both interleukin 1 $\alpha$  (IL-1 $\alpha$ ) and tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) mRNA were, on average, reduced by 50% during therapy, statistical analysis only showed significant reduction of TNF- $\alpha$  (Table V). In fact, three of the patients showed increased expression of IL-1 $\alpha$  after liorzole (Fig. 3A). In a previous study on healthy skin, topical talarozole was found to significantly down regulate IL-1 $\alpha$ , but not TNF- $\alpha$  expression (6).

**Changes observed in the immunostaining patterns.** The protein expression in skin biopsies obtained before and after liorzole treatment was studied by immunofluorescence analysis using commercially available antibodies against common retinoid biomarkers. Fig. 4

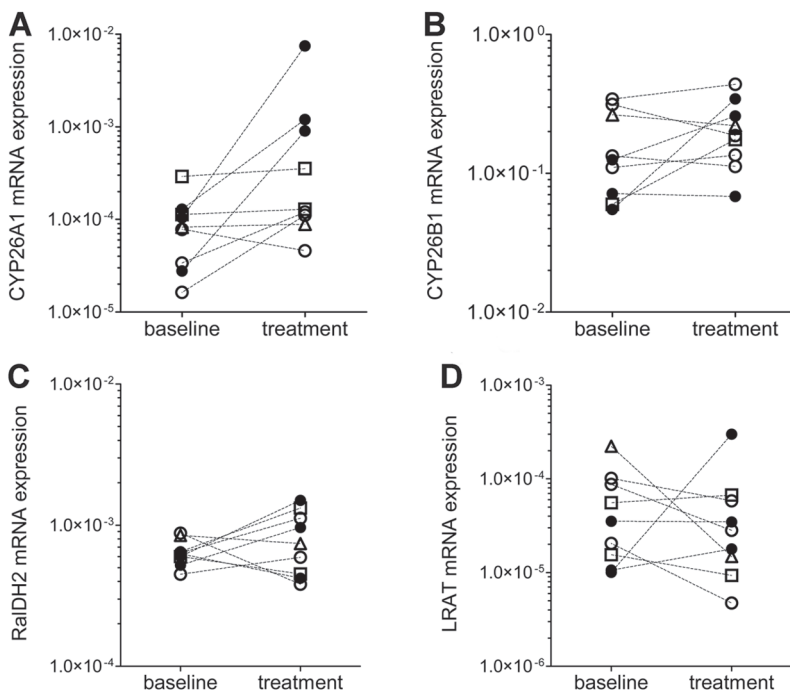


Fig. 2. Individual mRNA expression of four enzymes involved in retinoid metabolism proteins before and after liorzole treatment. The diagrams correspond to: (A) cytochrome P450 (CYP26)A1; (B) CYP26B1; (C) retinaldehydrogenase-2 (RALDH2); and (D) lecithin:retinol acyltransferase (LRAT). (For other explanations see Fig. 1). None of these enzymes of retinoid metabolism were significantly changed as a result of therapy.

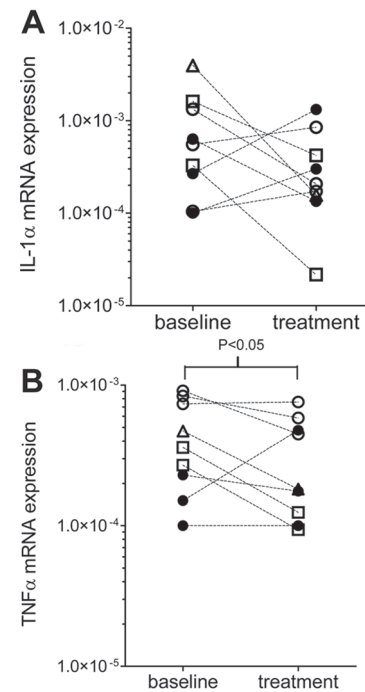
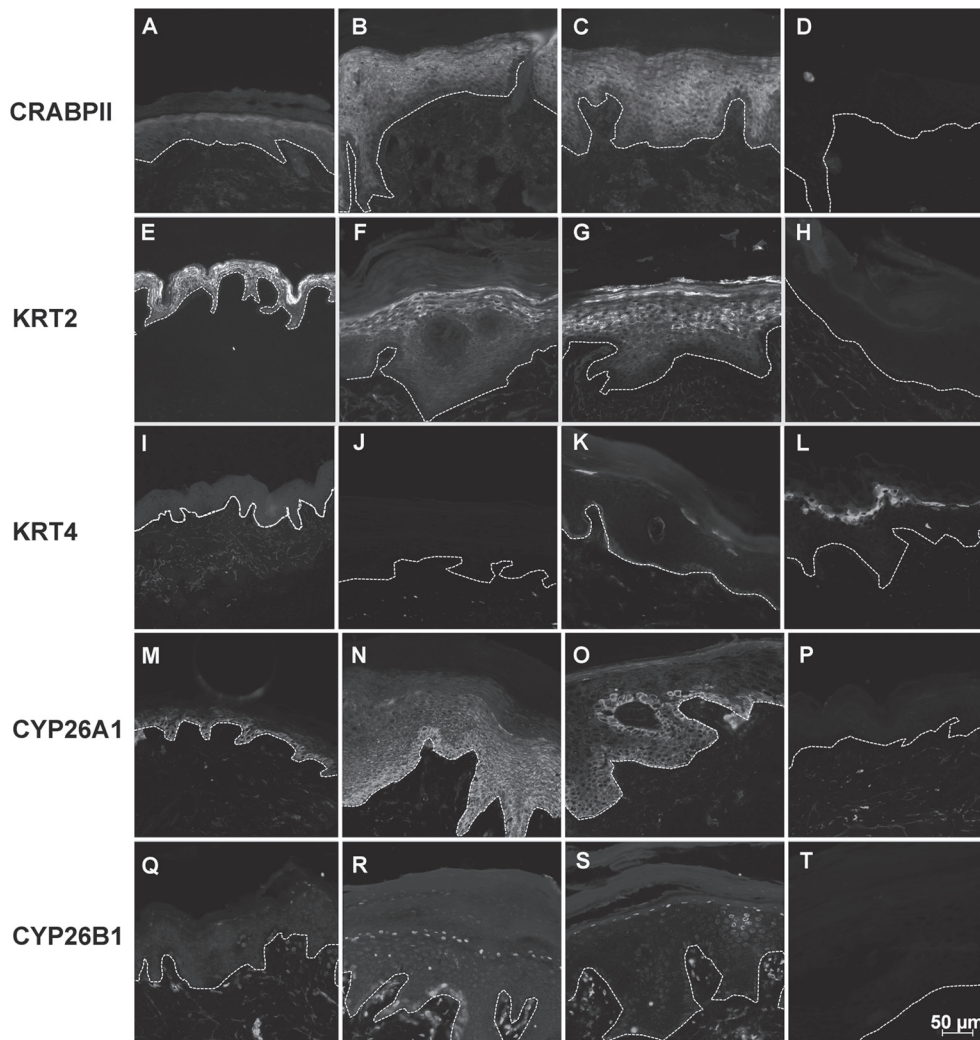


Fig. 3. Individual mRNA expression of two pro-inflammatory cytokines before and after liorzole therapy. The diagrams correspond to: (A) interleukin (IL)-1 $\alpha$ ; and (B) tumour necrosis factor (TNF)- $\alpha$ . (For other explanations see Fig. 1). Only TNF- $\alpha$  was significantly affected by the treatment with liorzole (*p* < 0.05).

shows examples of CRABP<sub>II</sub>, KRT2, KRT4, CYP26A1 and CYP26B1 staining in normal skin, in ichthyotic skin at baseline and after liarozole therapy, and in appropriate controls. In accordance with the mRNA results (see Table III) and previous protein analysis (27), CRABP<sub>II</sub> was over-expressed in untreated ichthyotic epidermis compared with normal epidermis, which was stained only in the stratum granulosum. No clear-cut increase in CRABP<sub>II</sub> staining was observed in liarozole-treated skin. KRT2, which was strongly expressed in the suprabasal layers of normal epidermis, occurred in reduced amounts in ichthyotic skin at baseline. However, in contrast to the strongly reduced mRNA levels after

therapy (see above), KRT2 protein expression was slightly *increased* by liarozole. KRT4 was negative both in normal skin and in ichthyotic skin at baseline, but a faint staining of the granular layer appeared after liarozole therapy, and this co-localized with the staining in healthy skin exposed to ATRA.

Figure 4 further shows that CYP26A1 expression, which was confined to the basal layer in normal skin, was increased in both intensity and extension in ichthyotic epidermis before therapy, and *decreased* somewhat after therapy. This was in sharp contrast to the increased CYP26A1 mRNA expression seen after therapy (see above). Finally, the weak and scattered CYP26B1



**Fig. 4.** Immunofluorescence staining of cellular retinoic acid binding protein II (CRABP<sub>II</sub>), keratin (KRT)2, KRT4, cytochrome P450 (CYP26)A1 and CYP26B1 in normal skin and in ichthyotic skin before and after liarozole treatment (patient number 6, Table I). The first row shows the expression of CRABP<sub>II</sub> protein in (A) normal and (B) ichthyotic skin before therapy showing staining of suprabasal layers, which did not change after therapy (C). A negative control omitting primary antibody is shown in (D). The second row shows examples of KRT2 expression in (E) normal human skin, (F) ichthyotic skin at baseline, and (G) ichthyotic skin after 4 weeks of liarozole (150 mg per day). A negative control is shown in (H). The third row shows negative expression of KRT4 in (I) normal skin and (J) the baseline skin biopsy; this is in contrast to (K) a faint positive staining of the stratum granulosum in liarozole-treated skin and (L) a strong staining of this layer in normal skin treated with all-trans retinoic acid for 4 days under occlusion (positive control). The fourth row represents CYP26A1, showing (M) staining of the basal layer in normal skin (which could be blocked by specific peptide), (N) a more diffuse staining of ichthyotic skin at baseline, (O) attenuation after liarozole therapy and (P) a negative control. The last row shows CYP26B1 staining which was (Q) essentially negative in normal skin, and patchily positive in the epidermis and papillary dermis of ichthyotic skin both (R) at baseline and (S) after liarozole therapy. (T) is a negative control omitting the CYP26B1-specific antibody. Bar: 50 µm. Dotted line: basal membrane zone.

Table VI. Scoring (0–3) of the protein expression in skin biopsies obtained before and after liorzole treatment. Data are presented as change in relation to pre-treatment value ( $n = 8^a$ )

Protein	Mean change	<i>p</i> -value
CRABP II	0.5	0.124
KRT2	0.7	<b>0.037<sup>b</sup></b>
KRT4	0.5	0.051
CYP26A1	–0.9	<b>0.032<sup>b</sup></b>
CYP26B1 Dermis	0.0	0.844
CYP26B1 Epidermis	–0.2	0.629

<sup>a</sup>Of a total of 10 patients, one did not accept punch biopsies and another received placebo. Therefore only 8 patients were included in this analysis.

<sup>b</sup> $p < 0.05$  is indicated in bold.

KRT: keratin; CYP26: cytochrome P450; CRABP II: cellular retinoic acid binding protein II.

staining in normal skin and at baseline did not increase as a result of therapy.

Table VI shows the mean changes in the immunofluorescence scores observed in paired biopsy specimens before and after therapy. Epidermal CRABP II protein expression was not significantly altered by therapy, whereas staining of KRT2 and KRT4 was up-regulated ( $p = 0.037$  and  $0.051$ , respectively), and that of CYP26A1 was down-regulated ( $p = 0.032$ ).

## DISCUSSION

This is the first study exploring the mechanism of action of RAMBA therapy in LI and attempting to correlate the therapeutic outcome to the underlying genetic abnormalities in this complex skin disease. A limitation of this study is the scarcity of patients with LI and the fact that for ethical and practical reasons we could only include Swedish patients who were enrolled in a multicentre clinical trial of two fixed doses of liorzole and who were also willing to accept repeated skin sampling. Two types of skin samples were obtained: punch biopsies and shave biopsies. The latter type of biopsy is, in our experience, the gentlest way of producing an epidermis-enriched sample that can be directly extracted for RNA.

It is well-known that topical retinoids and RAMBAs affect the expression of ATRA biomarkers such as CRABP II, KRT4 and KRT2 in normal human epidermis (6, 10, 13). In contrast, little is known about the corresponding effects in LI skin, and after oral administration of RAMBAs. At the outset of this study it was not even known if the expression of retinoid biomarkers is intrinsically abnormal in ichthyotic epidermis, thus potentially affecting the response to liorzole therapy. In fact, we found increased CRABP II mRNA levels in many LI patients (Table III) and the protein was expressed in all suprabasal cells instead of only stratum granulosum (Fig. 4). Previously, increased CRABP protein levels have been found by binding assay in LI epidermis (27). A similar abnormal CRABP II protein

expression pattern has been noted in psoriasis lesions (25) also characterized by keratinocyte hyperproliferation. Therefore, increased CRABP II expression may be secondary to epidermal hyperplasia in LI and not a reliable indicator of ATRA stimulation during liorzole therapy. Indeed, neither CRABP II mRNA nor the protein levels changed significantly in our study.

Interestingly, despite only marginal effects on CRABP II expression, good clinical results were obtained after only 4 weeks of liorzole therapy, particularly in the patients with *Ichthyin* or other non-*TGMI* mutations who had initially a somewhat milder ichthyosis than these with *TGMI* mutations. Since none of these patients had previously been given oral retinoids or RAMBAs, we do not know whether their superior therapeutic response is specific for liorzole. *Ichthyin* is a putative transmembrane receptor that transports lipids to the lamellar bodies (LB) essential for the formation of a skin water barrier (28). Interestingly, functional deficiency of *ichthyin* results in characteristic ultra-structural abnormalities of LBs that are not seen in patients with *TGMI* mutations (21). In a study of retinoic acid receptor  $\gamma$  null mice (29), similar LB abnormalities were observed in murine skin, implying a possible relationship between retinoid signalling and *ichthyin* shuttling of lipids in the maintenance of skin barrier function. In theory, it might be possible partially to compensate for the negative impact of *ichthyin* deficiency on LB function by increasing the retinoid activity in the skin. Further studies on a larger group of patients with *Ichthyin* mutations are needed to test this hypothesis, although this may be problematic due to the rarity of such patients (only 7 cases are known in Sweden).

Keratin protein expression has been studied previously in ichthyosis patients treated with oral liorzole (30), but no corresponding mRNA data were available at the onset of this study. Our results show that the transcription KRT2 and KRT4 changed relatively little after 4 weeks of oral liorzole treatment, especially when comparing the effects with those of topical treatment with ATRA (13) or talarozole (6) in healthy control skin. KRT2 is negatively regulated by retinoid stimulation of normal skin (6, 13). The reason for the reduced KRT2 protein expression in LI epidermis at baseline is unknown, but this intrinsic abnormality may explain why no further reduction occurred after oral liorzole treatment.

Belonging to the group of putative retinoid biomarkers are the enzymes that regulate epidermal vitamin A metabolism. For example, exposure of normal skin to topical ATRA results in increased retinol esterification (31), which may serve as a feedback loop by which epidermal retinol is diverted from oxidation to ATRA. In the present study, we examined the mRNA expression of LRAT and RALDH2, two enzymes which are rate-limiting in retinol esterification and retinal oxidation, respectively (9). No significant changes were seen in the expression of these enzymes in LI skin, which suggests that the ATRA levels

attained during liarozole therapy usually remain below the threshold value needed to activate any auto-regulatory mechanisms in the epidermal retinoid metabolism. However, slightly increased ATRA levels must have occurred during liarozole therapy, since a few other retinoid biomarkers in the epidermis reacted in accordance with the RAMBA concept. For instance, CYP26A1 mRNA tended to increase in a dose-dependent manner during liarozole therapy (see Fig. 2). Of course the expression of this and other CYP26 enzymes is of special interest, because they are the actual targets for liarozole therapy, namely inhibition of CYP26-mediated 4-hydroxylation of ATRA (15, 32–34). Somewhat surprisingly, several LI patients displayed increased protein staining of CYP26A1 before therapy and *reduced* staining after therapy (see Fig. 4N and O), despite increased CYP26A1 mRNA mean levels during therapy (see Table V). The poor correlation between CYP26A1 mRNA and protein expressions in epidermis probably illustrates the methodological problems implicit in comparing the expression of two widely different molecules (RNA and protein) in different types of samples (shave and punch biopsies) obtained from a heterogeneous tissue such as ichthyotic skin. However, we cannot rule out the possibility that the translation of CYP26A1 mRNA is fundamentally altered in LI skin under the influence of liarozole or its metabolites. Nevertheless, the fact that CYP26A1 reacted most strongly in patients receiving high-dose liarozole therapy (Fig. 3A) suggests that this gene retains its ability to respond to excessive ATRA accumulation in epidermis, which is potentially toxic to the cells.

When transcripts of two pro-inflammatory cytokines (TNF- $\alpha$  and IL-1 $\alpha$ ) were examined in skin specimens from liarozole-treated patients, a significant down-regulation of TNF- $\alpha$  was noted, whereas IL-1 $\alpha$  was more variably affected. Although a decreased IL-1 $\alpha$  expression occurred in most patients on therapy, those with a poor clinical response or a high dose level of liarozole (150 mg per day) occasionally showed *increased* expression. It remains to be seen whether this is related to a mild retinoid-like dermatitis sometimes observed as a side-effect in liarozole-treated patients. In contrast, topical application of talarozole in normal skin has previously been shown to result in a down-regulation of IL-1 $\alpha$  (6).

In conclusion, our study of a rare congenital form of ichthyosis, indicates that: (i) with the exception of CRABP2, the expression of retinoid biomarkers in LI epidermis is essentially normal at baseline; (ii) the expression pattern of retinoid biomarkers slightly changes during liarozole therapy in a way that is mostly consistent with the drug's suggested mode of action; and (iii) the clinical response to liarozole therapy appears to be related to the aetiology of the disease (there is a better response in patients with *Ichthyin* mutations than in those with *TGMI* mutations).

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