# Exploring the Use of Ingenol Mebutate to Prevent Non-Melanoma Skin Cancer

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## LIST OF ORIGINAL PAPERS

- I. Erlendsson AM, Thaysen-Petersen D, Bay C, Hald A, Skak K, Zibert JR, Paasch U, Wulf HC, Haedersdal M. Repeated Treatments with Ingenol Mebutate Prevents Progression of UV-Induced Photodamage in Hairless Mice. *PLoS One.* 2016;11(9):e0162597.<sup>1</sup>
- II. Erlendsson AM, Thaysen-Petersen D, Bay C, Lerche CM, Philipsen PA, Wulf HC, Zibert JR, Hædersdal M. Repeated treatments with ingenol mebutate for prophylaxis of UV-induced squamous cell carcinoma in hairless mice. J Photochem Photobiol B. 2016;163:144-9.<sup>2</sup>
- III. Erlendsson AM, Karmisholt KE, Haak CS, Stender IM, Haedersdal M. Topical corticosteroid has no influence on inflammation or efficacy after ingenol mebutate treatment of grade I to III actinic keratoses (AK): A randomized clinical trial. J Am Acad Dermatol. 2016;74(4):709-15.<sup>3</sup>

## BACKGROUND

## NON-MELANOMA SKIN CANCER

Non-melanoma skin cancer (NMSC) is the most frequently occurring cancer in Caucasians today. Incidence rates in Europe have increased steadily since the 1960s and more than tripled over the last 50 years<sup>4</sup>. NMSCs are keratinocyte-derived carcinomas, primarily composed of basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) that together comprise 99.9% of all NMSC-tumors. While BCCs develop *de novo*, the majority of SCCs are believed to emerge from *in situ* lesions i.e. Bowen's disease, or premalignant lesions, i.e. actinic keratoses (AKs). AKs comprise focal areas of abnormally proliferating and differentiating keratinocytes, clinically manifesting as red scaly patches on the skin<sup>5</sup>. Although spontaneous regression of AKs is common, AKs are biomarkers indicative of underlying photodamage and are associated with increased risk of developing NMSC. While the true rate of malignant progression from AK to SCC is unknown, conversion rates are believed to be between 0.5-3.4% over 1-5 year<sup>6,7</sup>. Recent data suggests that SCC may develop from clinically visible AK, but also from sub-clinical field-cancerization, emphasizing the complex biology of photocarcinogenesis.

# ULTRAVIOLET RADIATION

Solar ultraviolet radiation (UVR) is accountable for ~90% of NMSC, and the cumulative dose of UVR is proportionate to the risk of developing NMSC, especially SCC<sup>8,9</sup>. UVR acts as a complete carcinogen, involved in tumor initiation, promotion, and progression<sup>8</sup>. UVB (290-320nm) constitutes 1-10% of the earth's UVR and is directly absorbed by DNA bases within epidermal cells. The photoreaction results in production of photolesions such as cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts<sup>10</sup>. If not repaired, these photolesions cause signature UV mutations i.e. 'C to T' or 'CC to TT' and play a significant role in photocarcinogenesis<sup>10</sup>. UVA (320-400nm) is nearly unfiltered by the ozone layer and comprises 90-99% of earths UVR; UVA is absorbed by cutaneous chromophores such as melanin, urocanic acid, and riboflavin, resulting in production of reactive oxygen species (ROS)<sup>11</sup>. ROS are believed to cause indirect DNA damage and are proposed to play a role in tumor promotion<sup>12,13</sup>. Through continuous accumulation of mutations, cellular processes like proliferation, differentiation, and apoptosis fail<sup>14</sup>. Unresponsive to normal cell signaling pathways, aberrant keratinocytes may develop into BCC or grow into dysplastic AKs that ultimately undergo malignant progression.

## PREVENTION OF NON-MELANOMA SKIN CANCER

As a result of the field-effect of UVR, NMSC tumors are often multiple and recurring. Treatments include Moh's surgery, surgical excision, and radiotherapy, and for superficial BCCs, photodynamic therapy (PDT), imiquimod, and 5-fluorouracil may provide alternative treatment options<sup>15,16</sup>. Surgical treatments and radiotherapy can be disfiguring and cause functional impairment, especially in patients with multiple tumors, and prevention is thus an important part of NMSC management. For most carcinomas, pathogenesis is complex, depending on numerous genetic and environmental factors. UV-induced NMSC is predominantly dependent on exposure and sensitivity to UVR rendering it largely preventable<sup>17</sup>. Preventive measures include both primary prevention as well as secondary chemoprevention to either prevent progression of UV-damage or reverse it.

Primary prevention of NMSC focuses on minimizing exposure to UVR. Preventative efforts have been made to educate the population of the importance of limiting UVR exposure, including avoiding midday sunlight exposure, use of sunscreen, and restrictive use of tanning beds. Despite such initiatives, incidences of AKs and NMSC continue to rise, and development of effective secondary, chemopreventative strategies is needed <sup>4,18</sup>.

To date, several systemic chemopreventative drugs including, retinoids, non-steroidal anti-inflammatory drugs, and difluoromethylornithine (DFMO) have proven to prevent development of AKs and NMSC in humans<sup>17–20</sup>. These treatments require daily use and extensive monitoring, thus only suited for selected high-risk patient groups<sup>19</sup>. Nicotinomide is another systemic chemopreventative drug with few side effects that holds promise in chemoprevention of NMSC for immune-competent low-risk patients<sup>21</sup>. Topical formulations with retinoids, DFMO, and T4 Endonuclease V have been explored, but as they require daily use, such treatments are cumbersome in practice. Still DNA repair enzymes, such as T4 Endonuclease may prove beneficial as an adjuvant treatment to sunscreen in high risk patients<sup>22</sup>. Recent research has focused on topical treatments requiring less frequent use, and several in-vivo studies have demonstrated that PDT successfully prevents carcinogenesis in mice<sup>23,24</sup>. Although the current clinical evidence is sparse<sup>25,26</sup>, preliminary results from an ongoing clinical trial have demonstrated that continuous biannual PDT treatments in normally appearing skin provides an effective chemopreventative remedy postponing development of AKs in high-risk patients<sup>27</sup>.

For patients presenting with clinical photodamage and AKs, it remains controversial whether or not active treatment is indicated. The most frequently used treatments for AKs includes lesion-directed, physically destructive cryotherapy<sup>28</sup>. However, emerging evidence suggest that AKs should not be regarded as a condition isolated to the individual lesions but rather as a biomarker indicative of significant underlying photodamage<sup>29</sup>. To prevent formation of NMSC, contemporary AK-treatments are thus moving towards field-directed treatments, where the therapeutic target extends to include surrounding subclinical field cencerizaion<sup>30,31</sup>. Currently available field-directed therapies include photodynamic therapy (PDT) and topical pharmacological treatments i.e. 5flourouracil (5-FU), diclofenac, imiquimod, and ingenol mebutate (IngMeb)<sup>29,32-36</sup>.

## INGENOL MEBUTATE

In 2013, IngMeb was approved in Denmark as a new topical drug for field-directed treatment of  $AKs^{37}$ . IngMeb is available in two concentrations: 150 µg/g and 500 µg/g<sup>38</sup>. The lower concentration is intended to treat AK's in face and scalp and is applied once daily for 3 days. The higher concentration is intended for use on trunk and extremities and is applied once daily for 2 days. Previous studies have demonstrated AK-cure rates between 75%-91.7% in patients with non-hyperkeratotic AKs, but safety and efficacy remains to be established in patients with severe photodamage and hyperkeratotic lesions<sup>36,39,40</sup>. Due to its brief application time and effect on pre-existing actinic damage, early treatments with IngMeb may be used to prevent progression of UV-damage before clinical manifestations emerge<sup>20</sup>.

In vitro and in vivo studies have shown that IngMeb has a dual mechanism of action, causing initial cell death, followed by an immune activation<sup>41</sup>. The drug distributes through the skin, and a concentration gradient over the skin is created. In the superficial layers of the epidermis, a high concentration is found, which induces cell-death by necrosis 42,43. In the deeper epidermal layer IngMeb induces apoptosis, while in deep dermis IngMeb is believed to induce a specific immune activation<sup>44</sup>. This is driven by IngMeb's activation of a broad range of protein kinase c (PKC) isoforms, especially PKC-δ, which in healthy epidermal keratinocytes and fibroblasts upregulates transcription of neutrophil and Tcell mediators, e.g. interleukin-8 (IL-8) and tumor-necrosis factor- $\alpha$  (TNF- $\alpha$ )<sup>45</sup>. Additionally, in response to PKC- $\delta$  activation, endothelial cells increase production of leukocyte adhesion proteins, E-selectin, and intracellular adhesion molecule-1 (ICAM-1), collectively resulting in an acute inflammatory response dominated by neutrophil infiltration<sup>45,46</sup>.

## SIDE EFFECTS ASSOCIATED WITH INGENOL MEBUTATE

The acute inflammation induced by IngMeb manifests as local skin responses (LSR) including erythema, flaking/scaling, crusting, swelling, vesiculation/pustulation, and erosion/ulceration<sup>47</sup>. The typical duration of these responses is 14 days with a peak at day 4, during which the most frequently reported adverse events are pain and pruritus<sup>36</sup>.

The severity of LSR for a given patient are unpredictable, and some individuals may develop insufferable inflammation (*Fig. 1*). As a prophylactic treatment, side effects need to be minimized, but it is currently unknown whether the inflammatory response is essential for achieving optimal treatment response or simply an adverse reaction.



**Figure 1** - Examples of local skin responses following treatment with ingenol mebutate in murine (a, b, c) and human (d, e, f) skin. The responses are unpredictable and vary significantly between individual mice and patients. Subfigure a, b, and c depict responses in mice 48h after a single treatment with 0.015% ingenol mebutate gel. Subfigure d, e, and f depict responses in patients with multiple actinic keratoses on day 4 after finalized label treatment with ingenol mebutate gel (3 x 0.015%).

In a previous murine study, topical administration of a superpotent glucocorticoid, dexamethasone, blocked IngMeb-induced neutrophil invasion. Glucocorticoids may thus potentially be used to reduce IngMeb-induced inflammation and side effects<sup>48,49</sup>. Clobetasol propionate (CP) is a topical glucocorticoid with immunosuppressive, anti-inflammatory, and vasoconstrictive properties<sup>50</sup>. CP downregulates the expression of inflammatory cytokines (IL-2, IL-6, and IL-8) as well as neutrophil and lymphocyte adhesion molecules (E-Selectin and ICAM-1), resulting in reduced leukocyte recruitment to an inflammatory site and<sup>48,50,51</sup>. CP holds promise in alleviating IngMeb-induced LSR, pain, and pruritus, although it is unknown if such alleviation will attenuate the therapeutic effect of IngMeb<sup>45,49</sup>.

# AIM

3.

In this thesis, we sought to investigate if IngMeb can be used to prevent formation of NMSC with minimal side effects.

Specific aims:

- 1. Determine if IngMeb can prevent progression of
  - a. Histologically assessed photodamage (I)
    - b. SCC (II)
- 2. Determine if IngMeb can reverse clinical actinic damage in patients with
  - a. Multiple Grade I-III AKs and field-cancerization (III)
  - Determine if a topical glucocorticoid can reduce IngMeb-induced,
    - a. local skin responses (I, II, III)
    - b. pain (III)
    - c. pruritus (III)

without compromising treatment efficacy.

Methods to achieve aim:

- Standardized histological evaluation of cutaneous UV-damage in mice
- Time to SCC formation in mice
- Treatment efficacy of Grade I-III AKs in field-cancerized skin in patients
- Standardized evaluation of local skin responses in mice and patients

Patient reported pain and pruritus

# MATERIALS AND METHODS

## STUDY OVERVIEW AND CONDUCTION

The presented studies were conducted at the Department of Dermatology, Bispebjerg Hospital, Copenhagen, Denmark, between March 2012 and September 2015.

Study I:	Experimental in vivo study in hairless mice (his-
	tological photodamage)
Study II:	Experimental <i>in vivo</i> study in hairless mice (SCC)
Study III:	Randomized controlled clinical trial in patients with $\ensuremath{AK}$

Studies I and II were conducted under national guidelines for laboratory animal welfare and approved by *The Animal Experimental Inspectorate* (J.nr. 2014-15-0201-00096). Study III was approved by the Danish Health and Medicines Authority (EudraCT#:2013-0022583-80) and the Regional Committee on Health Research Ethics (#H-4-2013-073). The study was conducted according to principles stated in the Declaration of Helsinki and was monitored by Copenhagen University Hospital's Good Clinical Practice Unit (#2013-584).

# STUDY I & II

## ANIMALS

A total of 210 hairless, immunocompetent C3.Cg/TifBomTac mice of female gender (Taconic, Lille Skensved, Denmark) were randomized to 3 groups of 20 mice in Study I (n=60) and 6 groups of 25 mice in Study II (n=150). Mice were tattooed with consecutive numbers, and each group of 20 or 25 mice was accommodated in

TABLE 1 - STUDY SET-UP, STUDY I (Table 1 was first published in E	Erlendsson et al.1; permission to reuse table has been o	obtained)
Group 1 Control UVR (n= 20)	Group 2 IngMeb (n= 20)	Group 3 CP+IngMeb (n= 20)

Week	UVR	Treatment	Histology	UVR	Treatment	Histology	UVR	Treatment	Histology
1	3x3 SED			3x3 SED			3x3 SED		
2	3x3 SED			3x3 SED			3x3 SED		
3	3x3 SED	-		3x3 SED	Tx1 IngMeb		3x3 SED	Tx1 CP+IngMeb	
4	-		4 mice	-		4 mice	-		4 mice
5	3x3 SED			3x3 SED			3x3 SED		
6	3x3 SED			3x3 SED			3x3 SED		
7	3x3 SED	-		3x3 SED	Tx2 IngMeb		3x3 SED	Tx2 CP+IngMeb	
8	-			-			-		
9	3x3 SED			3x3 SED			3x3 SED		
10	3x3 SED			3x3 SED			3x3 SED		
11	3x3 SED	-		3x3 SED	Tx3 IngMeb		3x3 SED	Tx3 CP+IngMeb	
12	-		4 mice	-		4 mice	-		4 mice
13	3x3 SED			3x3 SED			3x3 SED		
14	3x3 SED			3x3 SED			3x3 SED		
15	3x3 SED	-		3x3 SED	Tx4 IngMeb		3x3 SED	Tx4 CP+IngMeb	
16	-			-			-		
17	3x3 SED			3x3 SED			3x3 SED		
18	3x3 SED			3x3 SED			3x3 SED		
19	3x3 SED	-		3x3 SED	Tx5 IngMeb		3x3 SED	Tx5 CP+IngMeb	
20	-		4 mice	-		4 mice	-		4 mice

separate cages with ad libitum access to water and food. The animal facility was kept at a 12h light-dark cycle and mice were weighed monthly to monitor their wellbeing.

# STUDY SET-UP

Interventions are presented in *Tables 1 and 2*. Mice were irradiated with solar simulated UVR 3 times per week. During the first 20 weeks, 5 single applications with IngMeb were given at four-week intervals (Days 21, 49, 77, 105, 133). Concurrent CP-ointment was applied pre and post IngMeb treatment.

Study I: To follow the development of histological photodamage, biopsies from 4 mice in each group were taken one week after first, third and fifth IngMeb treatment. Primary endpoint was histological UVR-damage score on day 28, 84, and 140. Secondary endpoints included LSR during first, second, third, fourth, and fifth IngMeb treatment.

Study II: To follow formation of SCC, UV-irradiation continued until SCC developed. Primary end-point was time to first tumor. Secondary endpoints included time to second and third tumors, as well as LSR during first, second, third, fourth, and fifth IngMeb treatment.

# ULTRAVIOLET IRRADIATION

UVR was given with a UV6 tube (Waldmann, Wheeling, IL, USA) placed between five Bellarium-S SA-1-12 tubes (Wolff System, Atlanta, Georgia, USA) with a maximum wavelength of 365 nm and 5.9% in the UV-B spectrum<sup>52</sup>. UVR was administered as three standard erythema doses (SEDs) three times weekly (*Fig. 2A*). The UVR-dose was measured using a spectroradiometer (Solatell Sola-Hazard 4D Controls Ltd., Cornwall, UK) and UVR-exposure time adjusted continuously to correspond to 3SED. To allow recovery from IngMeb treatments, each treatment was followed by a one-week pause from UVR.

# INGENOL MEBUTATE & CLOBETASOL PROPIONATE

IngMeb-gel (120 µl; Picato<sup>®</sup> 0.015%, LEO Pharma, Ballerup, Denmark) was applied on the entire dorsal skin of the mice, from neck to tail (*Fig. 2B*). CP-ointment (25µl; Dermovat<sup>®</sup> 0.05%, Glax-oSmithKline Pharma, Brentford, United Kingdom) was correspondingly applied on the entire dorsal skin of the mice once daily for five days prior to IngMeb treatment, 6h after, and 1-day after IngMeb treatment, in total 7 applications.

# OUTCOME MEASUREMENTS

# Histological evaluation of photodamage

UV-damage evaluations were based on UV-changes in stratum corneum, epidermis and dermis. Assessments were conducted by a blinded dermatopathologist on a standardized 0-3 categorical scale evaluating specific parameters, including (i) keratosis grade of stratum corneum ('0' orthokeratotic, '1' focal hyperkeratosis, '2' generalized hyperkeratosis, '3' parakeratosis), (ii) thickness of epidermis ('0' 3-4 cell layers, '1' 5-7 cell layers, '2' 8-10 cell layers, '3' 10+ cell layers), (iii) dysplasia in epidermis ('0' none, '1' present in lower 1/3, '2' present in lower 2/3, '3' present in the entire epidermis), and (iv) chronic dermal UV-damage ('0' none, '1' focal, upper papillary layer, '2' generalized, upper papillary layer, '3' reticular layer). The composite UV-score represented the sum of all subevaluations (0-12), where higher numbers indicate more severe UV-damage.

# Registration of squamous cell carcinoma

Mice were examined weekly for presence of SCC. All SCC that developed on the dorsal skin were registered and mapped onto a template. Time to tumor development was defined as the number of days from study initiation to development of first, second, and third SCC with a diameter  $\geq$  1 mm. Only SCCs growing to a size of 4 mm were included in the analyses. Previous histological examination has demonstrated that all growing tumors developed after UVR are SCC <sup>53</sup>.

	1. Co	ntrol	2. In	gMeb	3. U\	/R	4. UVR+	IngMeb	5. UVR+	-CP	6. UVR+	-CP+IngMeb				
	(n=25)		(n	(n=25)		(n=25)		(n=25)		5)	(n=25)					
Week	Veek UVR Tx		UVR	Tx	Tx	Tx	Tx	Tx	UVR	Тx	UVR	Tx	UVR	Tx	UVR	Tx
1					3x3 SED		3x3 SED		3x3 SED		3x3 SED					
2					3x3 SED		3x3 SED		3x3 SED		3x3 SED					
3				IngMeb	3x3 SED		3x3 SED	IngMeb	3x3 SED	CP	3x3 SED	CP+IngMeb				
4																
5					3x3 SED		3x3 SED		3x3 SED		3x3 SED					
6					3x3 SED		3x3 SED		3x3 SED		3x3 SED					
7				IngMeb	3x3 SED		3x3 SED	IngMeb	3x3 SED	CP	3x3 SED	CP+IngMeb				
8																
9					3x3 SED		3x3 SED		3x3 SED		3x3 SED					
10					3x3 SED		3x3 SED		3x3 SED		3x3 SED					
11				IngMeb	3x3 SED		3x3 SED	IngMeb	3x3 SED	CP	3x3 SED	CP+IngMeb				
12																
13					3x3 SED		3x3 SED		3x3 SED		3x3 SED					
14					3x3 SED		3x3 SED		3x3 SED		3x3 SED					
15				IngMeb	3x3 SED		3x3 SED	IngMeb	3x3 SED	CP	3x3 SED	CP+IngMeb				
16																
17					3x3 SED		3x3 SED		3x3 SED		3x3 SED					
18					3x3 SED		3x3 SED		3x3 SED		3x3 SED					
19				IngMeb	3x3 SED		3x3 SED	IngMeb	3x3 SED	СР	3x3 SED	CP+IngMeb				
20																
21-52					3x3 SED		3x3 SED		3x3 SED		3x3 SED					

## Local skin responses

During all five IngMeb treatments, LSR were registered at baseline and 1h, 6h, 1-, 2- 3-, 4-, 5-, 6-, and 7 days after treatment. The LSR evaluation consisted of separate evaluations of erythema, flaking, crusting, vesiculation, bleeding, and ulceration on a scale from 0-4<sup>47</sup>. The composite LSR-score represented the sum of all sub-evaluations (0-24), where higher numbers indicate more severe skin reactions.



Figure 2 – (A) Mice receiving ultraviolet radiation. (B) Mouse receiving prophylactic treatment with ingenol mebutate.

## STUDY III

## PATIENTS

Patients 18 years or older with multiple AKs on face or scalp were recruited for participation. Inclusion required two similar treatment areas of 25 cm<sup>2</sup> with multiple AKs ( $\geq$  7 AKs/25 cm<sup>2</sup>) and signs of field-cancerization (mottled erythema and pigmentation, telangiectasia, sallowness, laxity, and dry skin texture).

Exclusion criteria included: (i) clinical suspicion of non-melanoma skin cancer in the treatment area (ii) previous treatment with

IngMeb (iii) active dermatological condition in the treatment area, (iv) intake of systemic immunosuppressive, cytotoxic, immunemodulating, or retinoid agents within three months of study start, (v) pregnant and breastfeeding women, (vi) patients considered incapable of complying to the trial protocol. Verbal and written consents were obtained from all study patients before inclusion.

# TREATMENT PROCEDURES & OUTCOME ASSESSMENTS

Two contiguous areas (A & B) of 25  $\text{cm}^2$  were identified and AKlesions mapped onto a transparent template for later efficacy evaluation (Fig. 6). Randomization was conducted by consecutively numbered, closed, non-transparent envelopes containing a computer generated allocation of the letter A or B indicating the area assigned to CP treatment.

The first IngMeb application (Picato<sup>®</sup> 0.015%, LEO Pharma, Ballerup, Denmark) was conducted by the treating physician, while applications on day 2 and 3 were patient administered. First application of CP (Dermovat<sup>®</sup> 0.05%, GlaxoSmithKline Pharma, Brentford, United Kingdom) was correspondingly conducted by the treating physician on day 4 when LSR are known to peak<sup>36</sup>. Patients were thereafter instructed to apply a thin layer of CP twice daily (days 4-7) in the CP-allocated treatment area.

Outcome measurements, follow-up visits, scales, and assessment procedures are presented in Table 3. At study initiation, a patient diary was handed out for daily evaluations of pain and pruritus (days 1-15).

At follow-up visits, a blinded dermatologist evaluated AK-clearance (days 15, 57), and cosmetic outcome (day 57; Table 3), while two physicians conducted on-site assessments of LSR (days 1, 4, 8, 15, and 57) and reflectance measurements (days 1, 4, 15, and 57).

TABLE 3 – OUTCOME MEASUREMENTS FOR STUDY III (AK = Actinic keratosis, IngMeb = ingenol mebutate, CP = clobetasol propionate)										
Measurements	Follow-Up (Day)					Scale	Grading System / Assessment procedure			
Efficacy	1	4	8	15	57					
AK Count	x			х	х	n	Individual AKs were evaluated and defined as cleared when not apparent by palpation or sight. In case of non-complete response, the severity Grade (I-III) of the residual AKs was evaluated. New AKs in the treatment area were also registered.			
Inflammation	1	4	8	15	57					
Local skin re- sponses	x	x	x	x	x	0-24	Local skin responses were evaluated on a local skin response (LSR) scale grading the intensity of erythema, flaking, crusting, swelling, vesiculation, and ulceration from 0-4 <sup>47</sup> . The scores from the individual responses were added to give a composite LSR-score ranging from 0-24 with higher numbers indicating more severe response. <sup>47</sup> .			
Pain	1-1	5 (pat	tient d	liary)		0-10	Pain and pruritus was assessed daily by the patient in a patient diary. Pain sensation in skin was			
Pruritus	1-1	5 (pat	tient d	liary)		0-3	evaluated on an analogues scale from 0-10, where "0" indicated no pain and "10" worst imagina- ble pain. Pruritus was evaluated on a scale from 1-3, where "0" represented no pruritus, "1" light pruritus, "2" moderate pruritus, and "3" severe pruritus.			
Reflectance	x	х		x	x	0-100%	Objective quantification of erythema and pigmentation was conducted using a skin reflectance meter (Optimize Scientific 558; Chromo-Light, Espergaerde, Denmark) measuring hemoglobin and melanin content (0-100%) in the skin <sup>54</sup> .			
Cosmetic outcome	1	4	8	15	57					
Hyperpigmentation	x				x	0-3	Hyperpigmentation was graded on a scale where "0" represented no hyperpigmentation, "1" iso- lated hyperpigmentation, "2" mottled/scattered hyperpigmentation exceeding a single spot, "3" generalized hyperpigmentation in the treatment field.			
Hypopigmentation	x				x	0-3	Hypopigmentation was graded on a scale where "0" represented no hypopigmentation, "1" isolated hypopigmentation, "2" mottled/scattered hypopigmentation exceeding a single spot, "3" general- ized hypopigmentation in the treatment area.			
Scarring	х				х	0-3	Scarring was graded on a scale where "0" represented no scarring, "1" isolated scarring, "2" mott- led/scattered scarring exceeding a single spot, "3" generalized scarring in the treatment area.			
Skin texture					х	0-3	"0" represented a rough and rugged skin surface, "1" an even surface without ruggedness, "2" a smooth skin surface, and "3" a silk smooth skin surface. To evaluate changes in skin texture, treated skin was compared to adjacent untreated skin.			
Patient satisfaction	1	4	8	15	57					
Patient Satisfaction					x	0-10	Patients were asked to (i) rate their overall satisfaction with the IngMeb treatment where "0" indi- cated, "could not be more unsatisfied" and "10", "could not be more satisfied", and (ii) state their preferred choice of treatment, IngMeb or IngMeb+CP.			

# 1. HISTOLOGICAL PHOTODAMAGE AND SCC

# PREVIOUS STUDIES

Few experimental and clinical trials have investigated IngMeb for the treatment of NMSC. Four murine studies examined IngMeb treatment of cutaneous SCC (inoculated T7, LK2, and PAM212 tumors), with results ranging from inhibited growth to 100% clearance at follow-up periods between 30 and 150 days<sup>43,45,49,55</sup>.

In patients, two phase II trials investigated IngMeb for treatment of NMSC<sup>56,57</sup>. Ramsay et al. demonstrated cure rates of 57% (16/28) for BCC (superficial n=22, nodular n=6), and 50% (2/4) for SCC at a mean follow-up of 15 months (range 2-15)<sup>56</sup>. Accordingly, Siller at al. reported a histological clearance of 63% (5/8) for superficial BCC<sup>57</sup>, while a case report demonstrated partial clearance of a large (> 4cm) superficial BCC with IngMeb 0.05% after 3 months<sup>58</sup>.

Two case reports have demonstrated complete clearance of Bowen's disease at two months follow-up<sup>59</sup> and six months follow-up<sup>60</sup>, and in a phase II trial a cure rate of 75% (12/16) was found<sup>56</sup>. While no clinical trial has investigated IngMeb for prophylactic purposes, a single murine study examined IngMeb treatments in precancerous UV-damaged skin<sup>61</sup>. Mice were subjected to a carcinogenic UV-dose over a 10-week period, and prior to development of visible tumors, IngMeb was applied once daily for 2 consecutive days. Treatment with IngMeb resulted in eradication of 70% of UV-induced p53 mutated plaques; although the treatments did not postpone tumor formation, the number of tumors that subsequently arose was also reduced by 70%<sup>61</sup>. We sought to investigate if repeated single treatments with IngMeb could prevent formation of UV-induced histological photodamage and SCC.



**Figure 3** - Repeated treatments with ingenol mebutate prevented progression of photodamage compared to mice receiving ultraviolet radiation (UVR) alone; composite UVR-damage score at day 140: 10.0 (UVR) vs. 6.0 (UVR+IngMeb), p = 0.029. Concurrent treatments with clobetasol propionate potentiated the prophylactic effect of ingenol mebutate with a composite UVR damage score at day 140 of 3.0, UVR+IngMeb+CP, p = 0.057. Figure 3 was first published in Erlendsson et al.<sup>1</sup>; permission to reuse figure has been obtained.

# Own investigations

(1) In mice receiving UV-irradiation alone, histological photodamage increased gradually over time. Manifestations included increased keratosis grade, epidermal thickness, dysplasia, and dermally located chronic UV-damaged (*Fig. 3*); composite UVR-score at day 28 was 3.5, which increased to 6.0 at day 84, and 10.0 at day 140. Prophylactic treatments with IngMeb prevented progression of photodamage of all investigated characteristics, including keratosis grade, epidermal hypertrophy, dysplasia, and dermal actinic damage. Composite UV-damage score was 4.0, (UVR vs. UVR+IngMeb, p = 1.000) 4.0 (UVR vs. UVR+IngMeb, p = 0.486) and 6.0 (UVR vs. UVR+IngMeb, p = 0.029) at day 28, 84, and 140, respectively (*Fig. 3*).

(II) All UV-irradiated mice developed SCC (*Fig. 4*). Median time to first tumor was 168 days in mice exposed to UVR alone. Repeated treatments with IngMeb (UVR+IngMeb) resulted in a 3-week delay in tumor formation (UVR 168 days vs. UVR+IngMeb 189 days, p=0.025). Development of second tumor was delayed by 2-weeks (UVR 196 days vs. UVR+IngMeb 210 days, p=0.025), while formation of third tumor was unaffected by IngMeb treatments (UVR 210 days vs. UVR+IngMeb 210 days, p=0.443). No tumors developed in non-UV-irradiated mice receiving IngMeb alone or in the untreated control.



**Figure 4** - Kaplan-Meier plot depicting tumor free survival. Time to first tumor was significantly delayed with ingenol mebutate treatments (day 189) compared to UVR alone (day 168, p = 0.025). Concurrent treatments with clobetasol propionate potentiated the prophylactic effect of ingenol mebutate and further postponed tumor development (day 217 p < 0.001). Figure 4 was first published in Erlendsson et al.<sup>2</sup>; permission to reuse figure has been obtained.

# Discussion

These studies are the first to indicate that IngMeb exerts a prophylactic effect by preventing progression of histological photodamage and postponing formation of UV-induced SCC in hairless mice.

The observed prophylactic properties of IngMeb may depend on various mechanisms. Previous studies have shown that IngMeb induces necrosis and apoptosis in epidermal cells, making it effective in removing subclinical actinic changes, such as clusters of p-53 mutated keratinocytes<sup>61</sup>. Cozzi et al. suggested that epidermal regrowth from bordering hair follicles replaced the mutated keratinocytes with new, non-irradiated epidermis, thus postponing tumor promotion and progression<sup>61</sup>. However, other studies have demonstrated that epidermal renewal alone is not sufficient for SCC postponement, as SCC may originate from hair follicles.

IngMeb has been found to induce immune cell infiltration in hair follicles and may thus be able to treat aberrant cells located in profound hair follicles.

In addition to epidermal clearing, IngMeb also activates PKC- $\delta^{44,62}$ . PKC- $\delta$  is a tumor suppressor gene involved in cell-cycle arrest and apoptotic control of keratinocytes<sup>63</sup>. To evade growth suppressor stimuli, PKC- $\delta$  is commonly downregulated in human SCC<sup>63</sup>. Conversely, IngMeb promotes PKC- $\delta$  activation, inducing apoptosis in keratinocytes displaying critical DNA damage, while increasing innate-immune surveillance by neutrophil recruitment<sup>45,46,64,65</sup>. Through repeated treatments with IngMeb, epidermal renewal and PKC- $\delta$  activation may collectively provide continual clearing of subclinical actinic damage, thus preventing histological photodamage and SCC formation as demonstrated herein.

# 2. MULTIPLE GRADE I-III ACTINIC KERATOSES

# **Previous studies**

IngMeb is approved for the treatment of non-hyperkeratotic AKs<sup>66</sup>. The efficacy of IngMeb has so far been investigated in 7 prospective clinical trials (excluding case series) conducted in patients with non-hyperkeratotic AKs<sup>36,39,40,67–70</sup>. In the study populations, AK burden was specified as 4-8 non-hyperkeratotic AKs per  $25 \text{cm}2^{36,39,40,68,69}$ , or a minimum of 5 AKs per anatomical area<sup>67</sup> and in one study AK-burden was not specified<sup>70</sup>. In 6 studies, overall AK clearance varied from 75%-91.7% at 2-3 months follow-up<sup>39,40,67–69</sup>. In one study, overall clearance was not presented, but a minimum of 75% lesion reduction was observed in 52.6% of patients. More favorable responses were seen in patients treated on the face and scalp (83-91.7%) compared to trunk and extremities (61.8%-86.8%). Complete responders in one study were followed for 12 months, demonstrating an 87.2% mean lesion reduction compared to baseline<sup>71</sup>.

We sought to investigate the therapeutic effect of IngMeb in patients with severe photodamage presenting multiple Grade I-III AKs and signs of field cancerization.

## **Own investigations**

(III) In patients suffering from severe actinic damage (median lesion count: 16 Grade I-III AKs /25cm<sup>2</sup>), IngMeb was found to clear 84% of all AKs at 2-weeks follow up, with clearance rates persisting until two months post treatment (86%; *Fig. 5A, Fig 6*). Complete clearance of all AK-lesions within a treatment area was observed in 29% of the patients.

Pooled data for all AK lesions combined showed that 599 out of the 699 (86%) treated AKs were cleared after 2 months. When stratified for AK-grade, IngMeb cleared 88% of Grade I, 70% of Grade II, and 60% of Grade III AKs (*Fig. 5B, Table 4*). In instances where AKlesions were not cleared by initial treatment, 86% of Grade II AKs (18/21) and 100% (6/6) of Grade III AKs were reduced in grade severity (*Table 4*). In addition, only 3 new AK lesions were observed during the study and skin healed without clinical hypopigmentation, hyperpigmentation, or scarring with improved skin texture.

## Discussion

The study presents the first data on IngMeb efficacy in patients with severe photodamage and demonstrates that IngMeb exerts a therapeutic effect on all AK-severity grades, including hyperkeratotic AKs. Despite the high density of AKs in these severely photodamaged patients (16 AKs/25cm<sup>2</sup>), the treatment was well tolerated and efficacious.

Adding to the literature, this study demonstrates that treated AKs are cleared just two weeks after treatment initiation, and cure rates persist until 2 months post treatment. In contrast, application of other patient-administered topical treatments, i.e. imiquimod, diclofenac, and 5-FU, extends beyond three weeks, making IngMeb the most rapidly-acting drug currently available for  $AK^{33,34,72}$ . In the treated areas, few new AKs (n=3) were observed during the

study, which is in accordance with the findings from study I and II and confirms the conception of IngMeb as a field-treatment, targeting not only visible AKs, but also subclinical changes present in the surrounding skin.

## 3. INGENOL MEBUTATE-INDUCED SIDE EFFECTS

### **Previous studies**

In total, 9 prospective clinical trials (excluding case series) have investigated safety, including local skin responses during treatments of AKs<sup>36,39,40,67–70,73,74</sup>. These studies found that IngMeb treatments in 25-100cm<sup>2</sup> skin surface areas are safe, and maximum composite LSR from varied 5.3 to 12.6. Commonly registered adverse events included pain (15%) and pruritus (8%)<sup>36</sup>. In patients treated in face

TABLE 4 - CLEA	ARANCE OF GRAD	E I, II AN		INIC KERATUS	SES (AK = Actinic	keratoses, Ingl	vied = ing	jenoi n	nebutate, CP	= clobetasol pro	opionate)	
	BASELINE			2 WEEKS F	OLLOW-UP		2 MONTHS FOLLOW-UP					
	Total No. of AKs	Cle	ared	Reduced AK-grade	Resistant to treatment	Lost to follow-up	Cle	ared	Reduced AK-grade	Resistant to treatment	Lost to follow-up	
INGMEB												
Grade I AKs	307	254	86%	-	43	10	270	88%	-	37	0	
Grade II AKs	34	22	67%	6	5	1	24	71%	7	3	0	
Grade III AKs	7	5	83%	1	0	1	4	57%	3	0	0	
All AKs	348	281	84%	7	48	12	298	86%	10	40	0	
INGMEB+ CP												
Grade I AKs	308	251	83%	-	50	7	272	88%	-	36	0	
Grade II AKs	35	23	72%	7	2	3	24	69%	11	0	0	
Grade III AKs	8	2	40%	3	0	3	5	63%	3	0	0	
All AKs	351	276	82%	10	52	13	301	86%	14	36	0	
TOTAL												
Grade I AKs	615	505	34%	-	93	17	542	38%	-	73	0	
Grade II AKs	69	45	39%	13	7	4	48	70%	18	3	0	
Grade III AKs	15	7	3%	4	0	4	9	30%	6	0	0	
All AKs	699	557	33%	17	100	25	599	36%	24	76	0	

or scalp, the LSR were more intense, peaking at day 4 and returning to baseline at 2 weeks, compared to trunk and extremities where lower maximum LSR scores were found between day 3 and 8, with a more prolonged downtime of 2-4 weeks. While no clinical trial has studied alleviation of LSR, one study concluded that the best management of the LSR includes thoroughly informing the patients about severe inflammation that may emerge<sup>75</sup>.

We sought to investigate if concurrent or sequential application of CP could minimize IngMeb-induced side effects, including LSR, pain, and pruritus without compromising treatment efficacy.



**Figure 5** - The figure depicts clearance of actinic keratosis (AKs) after ingenol mebutate (ingMeb) treatment. A patient with severe photodamage presented with multiple Grade I-III AKs, field cancerization, and a basal cell carcinoma undergoing radiotherapy (arrow). The white corners mark the intended treatment area, in which 16 AKs (10 Grade I, 4 Grade II, 2 Grade III) were identified at baseline (a) and mapped onto a transparent template (b). After IngMeb treatment, 81% (13/16) of the AKs were cleared including 9/10 Grade I, 3/4 Grade II and 1/2 Grade III AKs (c,d). AKs resistant to treatment, #8, #9 and #14 represent a Grade II, Grade III, and Grade I AKs at baseline, respectively. Parts of figure 6 were first published in Erlendsson et al.<sup>3</sup>; permission to reuse figure has been obtained.

## **Own investigations**

# CP's effect on Local skin responses

**(I,II)** Single treatments with IngMeb in murine skin induced erythema, flaking, crusting, bleeding, vesiculation, and ulceration. Erythema, bleeding, and vesiculation developed rapidly after IngMeb application peaking on day 1. Flaking and crusting emerged on day 2, culminating on day 3 while ulceration had a delayed onset and reached peak intensity on day 5. The skin was normalized by day 10 post-treatment. Contrary to expectations, concurrent CP generated more severe inflammation compared to IngMeb alone. Maximum composite LSR and all individual responses were increased with concurrent CP in both Study I (max LSR treatment 1-5: UVR+IngMeb 2.6-4.3 vs. UVR+CP+IngMeb 3.6-5.5; p < 0.001) and Study II (max LSR, treatment 1-5: UVR+IngMeb 1.3-2.2 vs. UVR+CP+IngMeb 3.2 - 4.9, p < 0.001).



**Figure 6** - The figure illustrates cure rates for actinic keratoses (AKs) after treatment with ingenol mebutate (IngMeb) and IngMeb followed by clobetasolpropionate (IngMeb+CP). IngMeb was found to clear overall 84% (IngMeb) and 85% (IngMeb+CP) of the AKs at 2-weeks, which persisted at 2-months follow-up (86%; A). IngMeb exerted therapeutic effect on all AK severity grades, clearing 88% (542/615) of Grade I, 70% (48/69) of Grade II, and 60% (9/16) of Grade III AKs (B).Figure 5B was first published in Erlendsson et al.<sup>3</sup>; permission to reuse figure has been obtained.

(III) In patients with severe photodamage, daily applications with IngMeb for three consecutive days induced erythema (100%), flaking (100%), crusting (91%), swelling (91%), vesiculation (69%), and erosion (29%). Application of CP was initiated at day 4 when LSR were most severe (IngMeb 9.95, IngMeb+CP 9.52, p = 0.285). No reduction in LSR was observed in areas receiving CP (day 8, IngMeb 6.81 and IngMeb+CP 6.81; p = 0.939; Fig. 7, Fig. 8). Two weeks after treatment initiation, LSR returned to baseline in both IngMeb (0.67) and IngMeb+CP treated areas (0.38; p=0.250; Fig. 7, Fig. 8). Reflectance measurements supported the clinical findings; no difference in erythema was found between IngMeb and IngMeb+CP treated areas. Peak values were observed on day 4 (IngMeb, 57%; IngMeb+CP 57%; p = 0.976), and while minimal subclinical redness was present on day 15 (IngMeb, 50%; IngMeb+CP 54%; p = 0.543), reflectance-evaluated erythema returned to baseline at two months follow-up (IngMeb 45%; ingMeb+CP 48%; p = 0.076).







**Figure 8** - The figure illustrates progressive development of local skin responses (LSR) in a patient treated with ingenol mebutate (IngMeb; a,c,e,g,i) and IngMeb followed by clobetasolpropionat (IngMeb+CP; b,d,f,h,j). In this patient, IngMeb induced erythema, flaking, crusting, swelling, and vesiculation. Application of CP started at day 4 when LSR peaked (d), but no alleviating effect on LSR was observed. Identical LSR were found at day 8 (e,f). On day 15 responses were back to baseline in both treatment areas (g,h). Two months after treatment (day 57) no LSR were observed (i,j). Figure 8 was first published in Erlendsson et al.<sup>3</sup>; permission to reuse figure has been obtained.

# CP's effect on pain

**(III)** A majority of patients experienced pain during and after IngMeb treatment (71%). Pain scores were of mild to moderate intensities and started on the day of first IngMeb application. Pain intensity peaked on day 3 (IngMeb 2.55 vs. IngMeb+CP 2.85; p = 0.500) and declined gradually thereafter (*Fig. 9A*). CP application (day 4-7) had no impact on pain, and similar scores were reported in areas receiving IngMeb and IngMeb+CP (*Fig. 9A*; p > 0.500). On day 6, less than half of the patients experienced pain (IngMeb 1.15 vs. IngMeb+CP 1.10; p = 1.00), and on day 15 pain scores were back to baseline (IngMeb 0.0 vs. IngMeb+CP 0.0; p = 1.00).

## CP's effect on pruritus

(III) Pruritus was experienced by 67% of the patients and had a delayed onset compared to pain (*Fig. 9B*). Pruritus was low during the first two days of IngMeb application ( $\leq 0.30$  vs. IngMeb+CP  $\leq 0.30$ ; p = 1.00), but exacerbated on day 3 before reaching peak intensities of 1.0 vs. 1.21 on day 7 in IngMeb and IngMeb+CP treated areas, respectively (p = 0.312). Pruritus decreased gradually after day 7, and returned to baseline on day 14 in IngMeb treated areas and day 15 in IngMeb+CP treated areas. CP application had no alleviating effect on pruritus during application (day4-7), and following CP treatment (day 8-10), pruritus was even greater in IngMeb+CPtreated areas (day 9; IngMeb 0.84 vs. IngMeb+CP 1.11; p = 0.042) compared to IngMeb alone.

# CP's effect on treatment efficacy

(*I,II*) Interestingly, concurrent CP applications potentiated the prophylactic effect of IngMeb on both photodamage and SCC. Composite UV-damage score was 3.0 on day 28 (UVR vs. UVR+IngMeb+CP, p = 0.067), 4.0 on day 84 (UVR vs. UVR+IngMeb+CP, p = 0.086), on 3.0 on day 140 (UVR vs. UVR+IngMeb+CP, p = 0.057; *Fig. 3*). The prophylactic effect of IngMeb+CP applications compared with IngMeb alone was improved on first, second, and third tumor formation (UVR+IngMeb vs UVR+CP+IngMeb: 1<sup>st</sup> tumor 189 vs. 217 days, p < 0.001; 2<sup>nd</sup> tumor 210 vs. 224 days, p = 0.012; 3<sup>rd</sup> tumor 210 vs. 231 days, p = 0.002; *Fig. 4*).

(III) CP application had no effect on AK cure rates in patients with severe photodamage; at 2-weeks follow-up, overall 84% (IngMeb) and 85% (IngMeb+CP) of AKs were cleared (p = 0.585), and clear-ance rates were identical at two months follow-up (IngMeb 86%; IngMeb+CP 86%; p = 0.991; *Fig. 5A*).

#### Discussion

Topical glucocorticoids are widely used in dermatology and provide effective treatments of many inflammatory skin diseases<sup>76</sup>. Glucocorticoids have several proposed mechanisms of action, including a down-regulatory effect on pro-inflammatory cytokines (IL-2, IL-6, and IL-8) and leucocyte adhesion proteins (E-selectin, ICAM-1), which prevent leucocyte invasion and thus inflammatory response<sup>48,76–78</sup>. These immunosuppressive effects directly interfere with the immune regulatory pathways stimulated by IngMeb, and glucocorticoids were thus assumed to reduce IngMeb-induced LSR<sup>46</sup>. However, as indicated herein, CP failed to alleviate IngMeb-induced LSR, pain, and pruritus in patients and <u>exacerbated</u> the responses in murine skin.

In mice treated with CP, intracutaneous hemorrhage was observed with a higher frequency than in mice treated with IngMeb alone. Bleeding emerged only hours after IngMeb application, which was followed by crusting, and ulceration. Previous murine studies have shown that if IngMeb penetrates into dermis and the capillary plexus, intra-cutaneous hemorrhage and subsequent eschar formation develop<sup>45,49,61</sup>. The increased LSR observed in CP-treated mice may thus be accounted by a deeper penetration of IngMeb. Since the murine skin is only three cell-layers thick, the five pretreatments with CP-ointment may have weakened the skin barrier and enabled IngMeb to reach dermal capillaries, resulting in bleeding and severe LSR. In addition, CP-ointment was applied 6h and 24 h after IngMeb treatment and may have caused temporary occlusion of IngMeb, which has been demonstrated to increase penetration of IngMeb. followed by PKC- $\delta$  activation and rapid neutrophil recruitment to treated skin<sup>45,48,50,51,65</sup>. Histological analyses have revealed that the majority of keratinocytes undergo apoptosis or necrosis, which may leave them unresponsive to glucocorticoid stimuli<sup>61</sup>. In addi-



#### A - Pain in areas treated with ingenol mebutate vs. ingenol mebutate + clobetasol propionate

B - Pruritus in areas treated with ingenol mebutate vs. ingenol mebutate + clobetasol propionate



Figure 9 - The figure illustrates the evolution of pain (A) and pruritus (B) in patients treated with ingenol mebutate (IngMeb) and IngMeb followed by clobetasolpropionate (IngMeb+CP). Columns indicate the percentage of patients experiencing pain (A) and pruritus (B), while the line diagrams represent mean scores in population.

In the clinical study, IngMeb was well tolerated in patients with severe photodamage and maximum composite LSR scores were of similar intensity as scores presented in previous studies on 4-8 non-hyperkeratotic AKs/ 25 cm<sup>2 36,39,67-70</sup>. CP had no impact on LSR or cure rates, which may have several explanations. IngMeb induces a complex inflammatory process, initially causing cell death

tion, neutrophil invasion, which is prevented by CP, is most pronounced in the early phase of IngMeb-induced inflammation; accordingly, initiating CP on day 4 may be too late to impact LSR<sup>45</sup>. Previous studies attempting to alleviate externally induced inflammation, such as acute sunburn, have failed to do so using subsequent topical glucocorticoids<sup>62,79</sup>. In contrast, when applied prior to the inflammatory stress, both acute sunburn and photodynamic therapy-induce inflammation have been successfully reduced<sup>62,80</sup>.

Concurrent CP application in murine skin increased LSR, likely due to an enhanced penetration of IngMeb that resulted in a greater therapeutic effect compared to IngMeb alone. In patients with AKs, sequential application of IngMeb and CP did not reduce local skin responses, pain, or pruritus nor did it affect treatment efficacy. CP can thus not be used to alleviate IngMeb-induced LSR, pain, or pruritus.

# CONCLUSIONS

- Early repeated treatments with IngMeb prevent progression of histological photodamage and postpone formation of SCC in hairless mice.
- IngMeb reverses clinical actinic damage in patients with multiple AKs and field-cancerized skin, showing a therapeutic effect on all AK severity grades: Grade I 88%, Grade II 70%, Grade III 60%.
- Concurrent or sequential application of CP does not alleviate IngMeb-induced LSR, pain, or pruritus compared to IngMeb treatment alone.

# **FUTURE PERSPECTIVES**

In this thesis, we demonstrate that repeated field-directed treatments with IngMeb are able to prevent progression of histological photodamage as well as SCC formation. In a clinical setting, patients amenable to photodamage e.g. inhabitants of tropical and subtropical latitudes, patients receiving immunosuppressive treatments, fair-skinned, elderly, and people with outdoor occupations may be identified and offered prophylactic therapy before clinical signs of actinic damage emerge<sup>81,821,283</sup>. Chemoprevention is however complicated by a lack of knowledge of the amount of UV-damage required before for such treatments are indicated. Future clinical trials are needed to address issues as to when prophylactic treatments are mandated and how frequently such remedies should be administered.

In NMSC chemoprevention, preventative treatments are equally important as treatment of pre-existing actinic damage. In a recent study, Erlendsson et al. surveyed the treatment of AKs performed by Danish dermatologists. Cryotherapy was overall the most frequently used treatment modality (58%), even in areas with multiple AKs (47%). Such lesion-directed treatment does not exert any therapeutic remedy to neighboring skin, and in areas with multiple lesions and field cancerization, field-directed treatments are recommended. Until 2013, topical field-directed treatments were limited to 5-FU, diclofenac, and imiquimod where the application time alone is in excess of 3 weeks. IngMeb offers a rapid treatment, which may nurture a greater use of field-directed therapies and provide a better prevention of NMSC for patients with pre-existing photodamage.

In order for IngMeb to gain clinical impact as a prophylactic remedy, it is essential to minimize the side effects generated by the treatment. IngMeb-induced LSR, pain, and pruritus were not alleviated by concurrent or sequential application of CP. Future studies investigating different applications of glucocorticoids as well as the exploration of other remedies, such as non-steroidal anti-inflammatory drugs in alleviating IngMeb-induced LSR, are thus needed.

In the future, prophylactic treatments with IngMeb may support primary preventative efforts in reducing NMSC incidence.

# SUMMARY

Non-melanoma skin cancer is the most frequently occurring cancer in Caucasians today. Incidence rates in Europe have increased steadily since the 1960s and more than tripled over the last 50 years. Despite primary preventative efforts, incidences of non-melanoma skin cancer continue to rise and development of effective chemopreventative strategies is needed. In 2013, ingenol mebutate was approved in Denmark as a new topical drug for field-directed treatment for actinic keratoses. Ingenol mebutate has a dual mechanism of action, causing initial cell death, followed by an immune activation. The treatment induces an acute inflammation, manifesting as local skin responses, often accompanied by pain and pruritus. The severity of local skin responses for a given patient is unpredictable, and some individuals may develop insufferable inflammation. The overall aim of the thesis was to investigate if ingenol mebutate could be used as a chemopreventive agent to prevent development of non-melanoma skin cancer with minimal side effects. Specific aims included:

- Determine if ingenol mebutate can prevent progression of histological photodamage and squamous cell carcinoma (murine)
- Determine if ingenol mebutate can reverse clinical actinic damage in patients with multiple actinic keratoses and fieldcancerized skin (clinical)
- Determine if a topical glucocorticoid (clobetasol propionate) can reduce ingenol mebutate-induced local skin responses, pain, and pruritus without compromising the treatment efficacy (murine clinical)

In two *in vivo* murine studies, ingenol mebutate's effect on photodamage and squamous cell carcinoma formation was investigated. Mice were irradiated with solar simulated ultraviolet radiation. During the first 20 weeks, 5 single applications with ingenol mebutate were given at four-week intervals with and without concurrent application of clobetasol propionate. Prophylactic treatments with ingenol mebutate prevented progression of histological photodamage of all investigated characteristics, including keratosis grade, epidermal hypertrophy, dysplasia, and dermal actinic damage. In addition, tumor formation was postponed by 3 weeks.

In the clinical trial, patients with multiple actinic keratoses and field-cancerized skin were treated with ingenol mebutate, according to label, with and without sequential application of clobetasol propionate. Ingenol mebutate treatments were found to clear overall 86% of all actinic keratoses, exerting a therapeutic effect on all severity grades; cure rates were 88%, 70%, and 60% for Grade I, II, and III actinic keratoses, respectively.

Ingenol mebutate treatments generated erythema, flaking, crusting, vesiculation, swelling/bleeding, and ulceration. Concurrent application of clobetasol propionate increased local skin responses in murine skin, likely due to an enhanced penetration of ingenol mebutate that resulted in a greater therapeutic effect compared to ingenol mebutate alone. In patients with actinic keratoses, sequential application of ingenol mebutate and clobetasol propionate did not reduce local skin responses, pain, or pruritus, nor did it affect treatment efficacy compared to ingenol mebutate alone.

In conclusion, the thesis highlights ingenol mebutate's potential as a prophylactic remedy for non-melanoma skin cancer with promise to support primary preventative efforts in reducing non-melanoma skin cancer incidence.

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