*RXR- and RAR-selective retinoids in psoriasis and (pre)malignant skin disorders*

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# *RXR- and RAR-selective retinoids in psoriasis and (pre)malignant skin disorders*

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

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*Vivo ergo sum,*
*in vivo quaero.*

Voor Jeanette, voor mijn ouders

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# Abbreviation list

| | |
|---|---|
| **ABC** | Avidin-biotin-complex |
| **AEC** | 3-amino-9-ethylcarbazole |
| **AIDS** | Acquired immunodeficiency syndrome |
| **AK** | Actinic keratosis |
| **ALA** | Aminolevulinic acid |
| **ALG** | Antilymphocyte globulin |
| **ALT** | Alanine amino transferase |
| **ANOVA** | Analysis of variance |
| **APC** | Antigen presenting cell |
| **AST** | Aspartate amino transferase |
| **ATG** | Antithymocyte globulin |
| **ATRA** | All-trans retinoic acid |
| **BCC** | Basal cell carcinoma |
| **BD** | Bowen's disease |
| **BSA** | Body surface area |
| **BSA/PBS** | Bovine serum albumin/phosphate buffer saline |
| **CIN** | Cervical intraepithelial neoplasia |
| **CRABP** | Cellular retinoic acid binding protein |
| **CRBP** | Cellular retinoid binding protein |
| **CTCL** | Cutaneous T-cell lymphoma |
| **DAB** | Diaminobenzidine |
| **DBD** | DNA binding domain |
| **DISH** | Diffuse ideopathic skeletal hyperostosis |
| **DNA** | Deoxyribonucleic acid |
| **ECG** | Electrocardiogram |
| **EV** | Epidermodysplasia verruciformis |
| **GRE** | Glucocorticoid response element |
| **HHV** | Human herpesvirus |
| **HIV** | Human inmmunodeficiency virus |
| **HLA** | Human leucocyte antigen |
| **HPV** | Human papillomavirus |
| **HRE** | Hormone response element |
| **ICI** | Immunocompetent individual |
| **INF** | Interferon |
| **IL** | Interleukin |
| **IM** | Intramuscular |
| **ISSCC** | In situ squamous cell carcinoma |
| **IV** | Intravenous |
| **K** | Keratin |
| **KA** | Keratoacanthoma |
| **KIN** | Keratinocytic epidermal neoplasia |
| **KSHV** | Kaposi sarcoma-associated herpesvirus |
| **LBD** | Ligand binding domain |
| **LFA** | Leukocyte function-associated antigen |
| **LOH** | Loss of heterozygosity |
| **LT** | Leukotriene |

# Abbreviation list

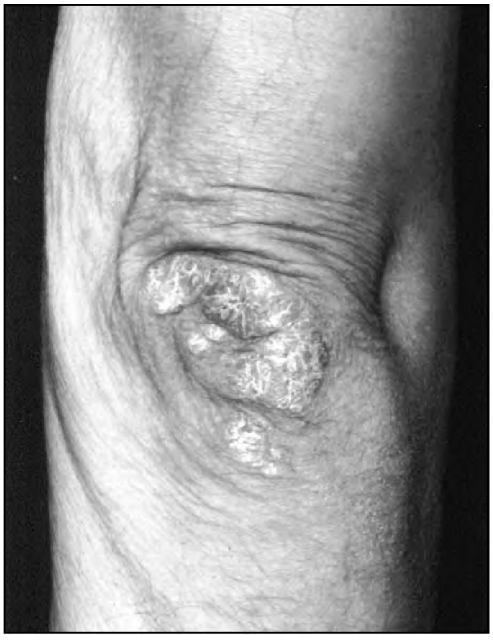
| | |
|---|---|
| **MED** | Minimal erythema dose |
| **mPASI** | Modified psoriasis area and severity index |
| **MTX** | Methotrexate |
| **MW** | Molecular weight |
| **NMSC** | Non-melanoma skin cancer |
| **NS** | Not (statistically) significant |
| **PASI** | Psoriasis area and severity index |
| **PBS** | Phosphate buffer saline |
| **PDT** | Photodynamic therapy |
| **PEL** | Plaque elevation |
| **PGA** | Physicians global assessment |
| **PI** | Proliferation index |
| **PMN** | Polymorphonuclear neutrophils |
| **Pp** | Protoporphyrin |
| **PPAR** | Peroxisome proliferator-activated receptor |
| **PUVA** | Psoralens + UVA |
| **QOL** | Quality of life |
| **RAMBA** | Retinoic acid metabolism blocking agent |
| **RAR** | Retinoic acid receptor |
| **RB** | Retinoblastoma |
| **RTR** | Renal transplant recipient |
| **RXR** | Retinoid X receptor |
| **SC** | Subcutaneous |
| **SCC** | Squamous cell carcinoma |
| **SD** | Standard deviation |
| **SEM** | Standard error of the mean |
| **SPF** | Sunlight protection factor |
| **T4** | Thyroxine |
| **TDI** | Total dermal infiltrate |
| **TNF** | Tumor necrosis factor |
| **TR** | Thyroid receptor |
| **TSH** | Thyroid stimulating hormone |
| **UV** | Ultraviolet |
| **VAS** | Visual analogue score |
| **VDR** | Vitamin D receptor |

# GENERAL INTRODUCTION

*This thesis deals with the clinical and immunohistochemical effects of retinoids on two distinct groups of skin disorders: psoriasis and (pre)malignancies. Retinoids are known to influence proliferation and differentiation, processes that are disturbed in these disorders. In this thesis the effects of a newly developed retinoid and the effects of established retinoids for new indications are described. The introduction comprises the main features of psoriasis, premalignant- and malignant skin disorders, retinoids, and the approaches that were used in the individual studies. Each will be discussed in a separate part.*

# PART I

# PSORIASIS



A psoriatic plaque on the elbow in one of the patients.

# 1.1

## History

The oldest medical records on skin disorders originated in ancient Egypt. The Ebers papyrus is the longest of the medical papyri and is dated to the ninth year of the reign of pharaoh Amenhotep I, about 1534 BC. It comprises 110 pages and contains a few sections on the treatment of skin disorders. In one paragraph topical therapies for *wehau* (skin rash) are described in the form of *gesu* (ointments) and *iner-sepdu* (minerals). Honey, red natron and salt were used to *sepena* (renew) and *senefer* (beautify) the *inem* (skin).[1;2] Unfortunately, there is no clue in the hieroglyphs which enables us to identify these disorders, but it is clear that these ancient Egyptian therapeutic approaches may well have had some effect in an erythematous disease like psoriasis. Many studies have confirmed the beneficial effects of honey in woudhealing of the skin.[3;4] In psoriasis ointments and salt baths are common basic treatments even nowadays.

The first real descriptions that included the typical hallmarks of psoriasis came from Hippocrates (460-377 BC) and Celsius (25 BC-45 AD), but it was Galen (133-200 AD) who introduced the Greek name *psora* (itching eruption) for this disease that often features complaints of itching.[5;6] "Scabbiness" is another meaning for the word *psoriasis*,[7] but this translation is rather misplaced nowadays. Although Robert Willan (1808) described the various forms of psoriasis, he still designated the disease as lepra. In 1841 Hebra eliminated the term lepra for this disease and substituted it psoriasis, however, until 1900 many psoriatics still suffered the same fate as lepers. They were believed to be unclean and infectious and therefore were excluded from society.[5;6]

# 1.2

## Epidemiology

Psoriasis is a chronic disease with a prevalence of 1-3% in the European, North American, East African, and South Asian population.[8;9] However, in certain ethnic groups these indices may differ. In the American Indians, Samoans, Laps, and Eskimos psoriasis is scarcely seen, suggesting the involvement of genetic factors in this disease.[8;10;11] Psoriasis equally affects men and women.[12-14] The age of onset is variable; first manifestations of the disease may occur at any age from birth to even at the age of 108 years.[15;16] However, a study on the age of onset of psoriasis carried out in 2147 patients revealed two peaks of incidence: one occurring at the age of 16 years *(women)*, and another at the age of 60 years *(women)* or 57 years *(men)*.[17] An onset before the age of 5 is rare. In families where psoriasis is commonly seen, there appears to be an earlier onset.[18]

# 1.3

# Clinical aspects

## 1.3.1 Subtypes and cutaneous manifestations

In dermatology many diseases comprise of several different subtypes instead of one single typical clinical picture. With respect to cutaneous manifestations of psoriasis the following common subtypes are seen, that may or may not coexist.[18]

*Plaque-type psoriasis or psoriasis vulgaris*

This is the most common form of psoriasis, accounting for 90 % of all cases. Lesions are symmetrically distributed over the body showing classical psoriatic aspects such as erythema, induration, and silvery scaling. Predilection sites are the elbows, knees, scalp, and the sacral region, but spreading all over the patient's body may occur. The course is mostly chronic with exacerbations and remissions. This thesis deals with this common subtype of psoriasis; in corresponding sections where "psoriasis" is mentioned, the reader should interpret this as "plaque-type psoriasis".

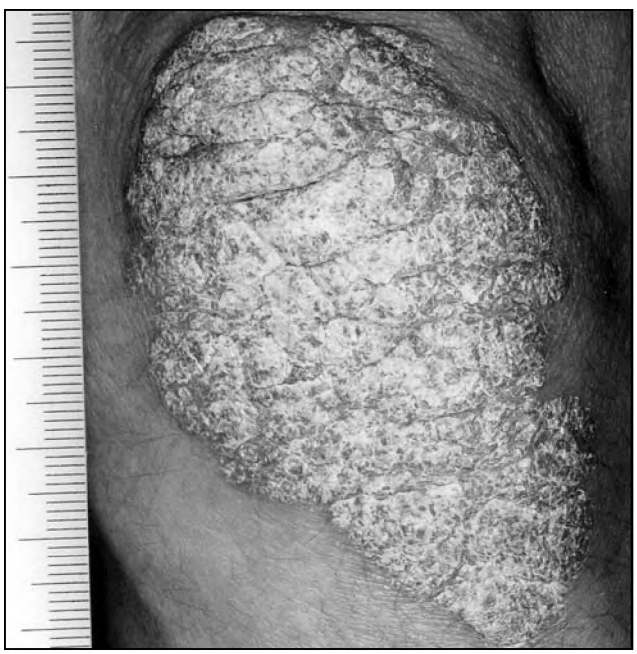


**Figure 1:**

Plaque-type psoriasis: sharply demarcated erythematosquamous plaques are seen.

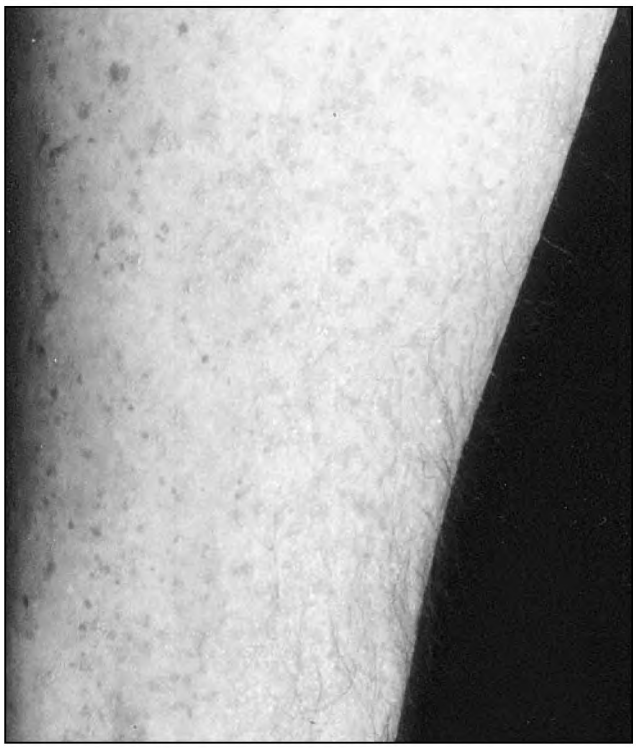


**Figure 2:**

Guttate psoriasis: multiple droplet-like lesions are typical.

*Psoriasis guttata*

This subtype features a pattern of droplet-like (2 millimeters to 1 centimeter) erythematous and scaly lesions disseminated over the body. Compared to plaque-type psoriasis scaling is normally less present, but these droplet-like lesions may confluent to larger and more scaly lesions as seen in plaque-type psoriasis. Psoriasis guttata is often preceded by an upper respiratory tract infection, and is mostly seen in children and adolescents. When a preceding infection is cured, a complete remission of this form of psoriasis can generally be expected after a couple of weeks, although patients are more prone to develop plaque-type psoriasis later on in life.

*Erythrodermic psoriasis*

This is a rare form of psoriasis in which the entire skin becomes erythematous with variable scaling. It may occur in patients with no history of psoriasis, but this form is often seen in patients with plaque-type psoriasis after withdrawal from corticosteroids, after irritation by dithranol or phototherapy, or it may have been triggered by drugs which are known to induce psoriasis. This type of psoriasis may cause severe sickness. Itching is more common and patients are at risk for hypothermia, dehydration, and protein loss. Therefore, admission at the inpatient department is often required.

*Pustular psoriasis*

This is actually a group of psoriasis subtypes that feature macroscopic pustules. Both localized and generalized forms can be distinguished. Localized forms are pustulosis palmoplantaris, acrodermatitis continua of Hallopeau and other non-acral, non-palmoplantar localized forms. Generalized forms comprise pustular psoriasis of Von Zumbusch, impetigo herpetiformis, annular pustular psoriasis, juvenile and infantile pustular psoriasis.[19] These forms are more recalcitrant and often need systemic therapy.

The following subtypes are frequently seen in combination with the above mentioned forms of psoriasis and may therefore be seen as a modification by site; on the other hand these forms are often solitary manifestations of psoriasis that justifies a classification as a separate subtype.

*Flexural psoriasis or psoriasis inversa*

This subtype is mostly localized in the submammary, axillary, and anogenital fold. It is more common in females and in the elderly and often lacks scaling. It is noticed in about 30 % of patients with plaque-type psoriasis.

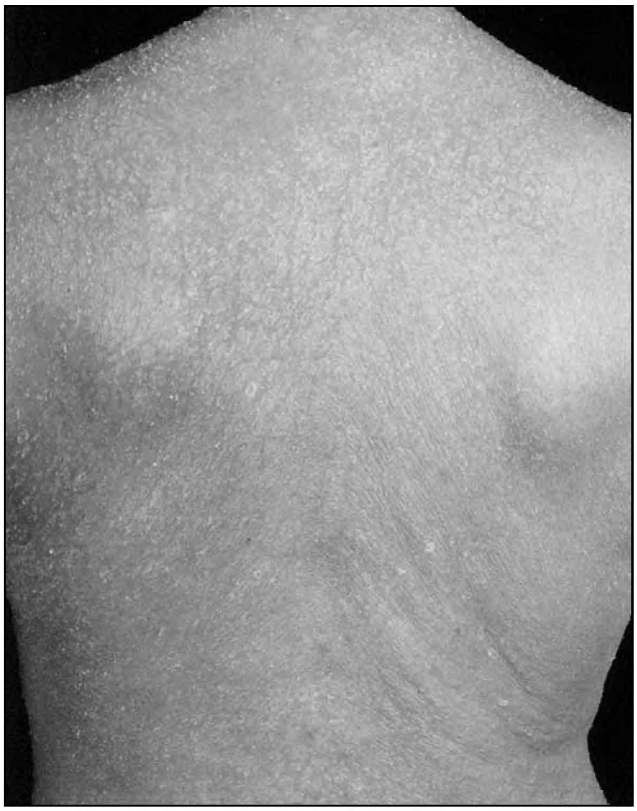


**Figure 3:**

Erythrodermic psoriasis: the entire skin becomes red and scaly.

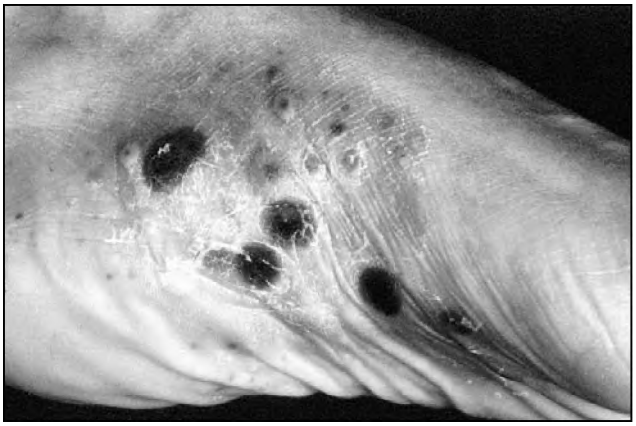


**Figure 4:**

Pustular psoriasis normally represents typical yellow and brown pustules.

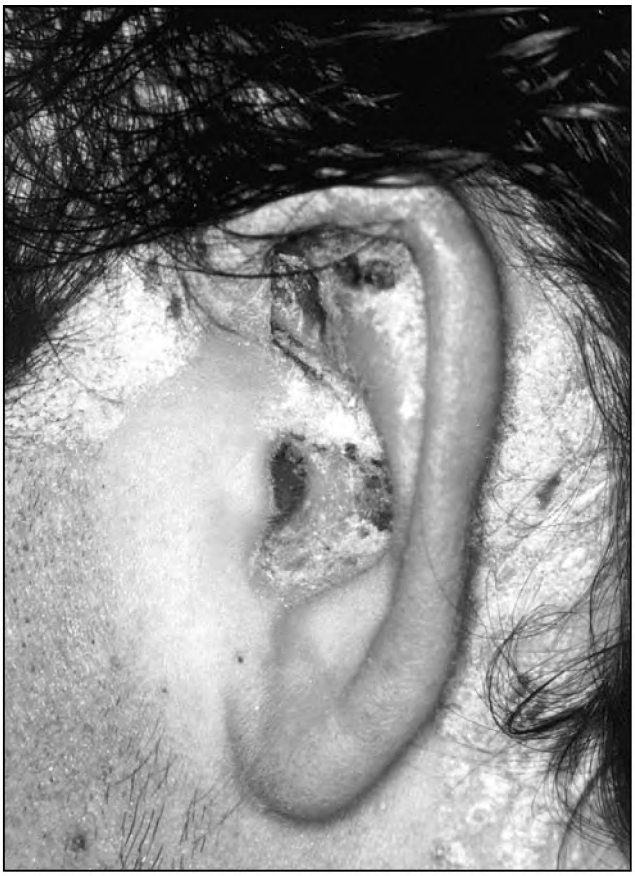


**Figure 5:**

Psoriasis of the scalp often features thickened erythematosquamous plaques.

*Psoriasis of the scalp or psoriasis capitis*

In scalp psoriasis typical scaly and erythematous plaques that are spread over the hairy scalp are seen, but they are often interspersed with normal skin. Frequently scalp psoriasis advances beyond the scalp margin. Significant hair loss is rare.

*Psoriasis of the hands and feet or psoriasis palmoplantaris*

In this form lesions may present as typical scaly patches or as a pustulosis. It may strongly resemble eczema. A relationship with trauma and occupational irritants has been postulated.[18]

*Psoriasis of the nails or psoriasis unguium.*

Involvement of the nails is common in psoriasis and percentages up to 86.5 % have been reported in psoriatic arthritis.[20] Typical signs of nail psoriasis are pitting, discoloration (e.g. 'oil drop' sign: circular areas of discoloration of the nail bed and hyponychium resembling an oil drop below the nail),[21] onycholysis, subungual hyperkeratosis, splinter hemorrhages, as well as crumbling and grooving of the nails.[22-24] This subtype is usually very recalcitrant. Patients may suffer from pain caused by nail changes and are often restricted in their daily activities.[25]

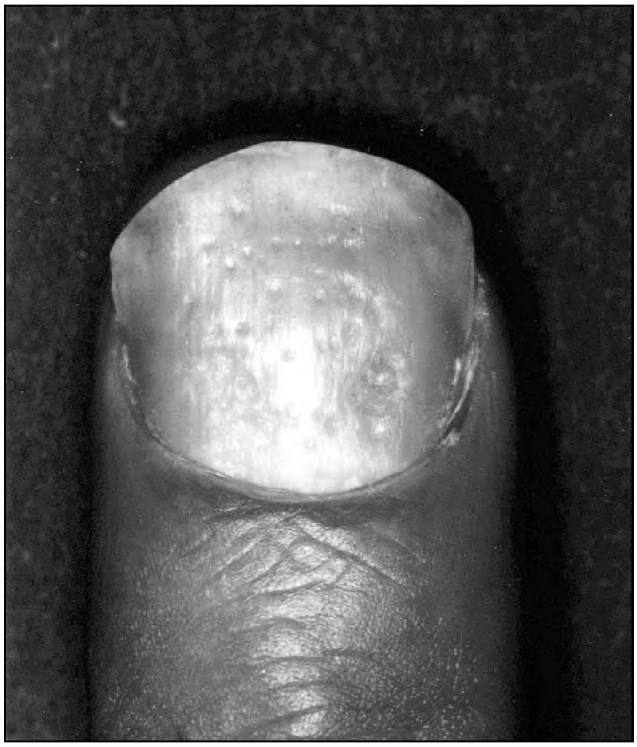


**Figure 6:**

Psoriasis of the nail showing typical signs of pitting.

### 1.3.2 Extracutaneous manifestations

Psoriasis is basically a disease of the skin and appendices (nails). However, *psoriatic arthropathy* or *arthritis psoriatica* is a complication of psoriasis that occurs in 5-10 % of the patients.[26] It equally affects men and women, although differences exist: in men, arthritis of the distal interphalangeal joints of the fingers and spinal disease is predominantly seen, whereas in women symmetrical polyarthritis is more common.[27] Psoriatic arthropathy can be seen in patients without cutaneous lesions. A serological test for rheumatoid factors is negative. In patients with psoriatic arthropathy an increased frequency of HLA B27 and HLA Bw38 is found.[10]

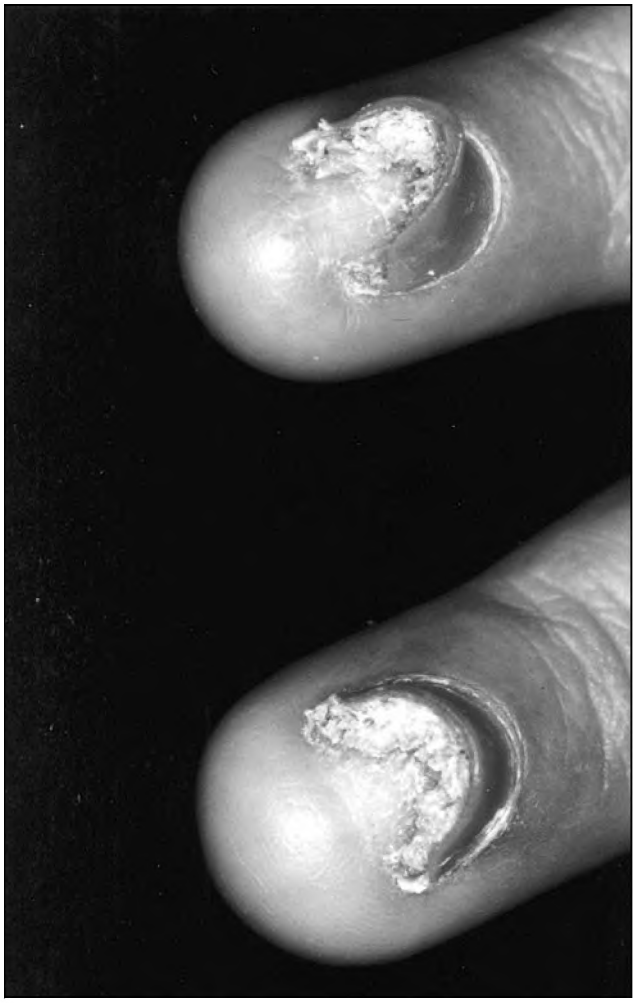


**Figure 7:**

Distal onycholysis and subungual hyperkeratosis in psoriasis of the nails.

### 1.3.3 Clinical hallmarks of plaque-type psoriasis

The proper diagnosis plaque-type psoriasis or psoriasis vulgaris is not difficult, as the clinical appearance of this erythematosquamous disorder is so characteristic that a diagnosis 'a vue' is possible. Typical cutaneous manifestations of plaque-type psoriasis are sharply demarcated-, silvery-, elevated- and non-coherent scales on a homogenous base of erythema. This indicates that both the dermal compartment (erythema), and the epidermal compartment (elevated scales) are involved. The lesions may vary in size between pinpoint lesions and large plaques. Larger lesions have developed out of smaller lesions by centrifugal expansion. Lesions may coalesce and thus form polycyclic, annular or gyratory configurations of plaques. The lesions have a fairly symmetrical distribution pattern.

The clinical picture of psoriasis may depend on the localization of the lesions. Areas of predilection are the elbows, knees, scalp, and sacral region. On the scalp plaques are in general very thick with rather severe scaling, whereas intertriginous psoriasis is milder and generally without scaling. In the sacral area fissures and lichenification can be seen. With respect to erythema slight pink discoloration and very intense redness with almost a livid aspect can be seen, the latter especially on the lower legs. Sometimes a so-called 'ring of Woronoff' can be seen, a clear peripheral zone of blanching around the erythematous center.

A phenomenon characteristic for psoriasis is the 'candle phenomenon', which means that if scales are scratched off from a lesion, the scales cohere similar as the wax flakes scraped from a candle. When all the scales are scratched off from a lesion, characteristic pinpoint bleedings can be observed. This phenomenon is called 'Auspitz sign'.

Psoriatic lesions are normally not very itching and painfulness is rare. As described above, psoriatic arthropathy and involvement of the nails are frequently seen in this group of patients and many suffer from restrictions in daily life activities.

There is no such thing as a typical course of psoriasis. Psoriasis is a chronic disease that may remain stable for years,[28] but exacerbations and remissions are the rule.[18] The following 130-year old quote did not loose strength over the years: "It is impossible to say, in any particular case, how long the disease will last, whether a relapse will occur, or for what period of time the patient will remain free from psoriasis".[29] With respect to exacerbations or the development of new lesions triggering factors are important; these will be discussed in section 1.5.2.

Although the characteristics as described above usually may lead to a correct diagnosis of the disease, difficulty may arise in atypical cases, in particular sites, and when psoriasis is complicated by or alternates with other diseases. The differential diagnosis of psoriasis comprises disorders like seborrhoeic dermatitis, eczema, hyperkeratosis palmoplantaris, lichen simplex, secondary syphilis, candidiasis and parapsoriasis or even cutaneous lymphoma. For an explanation of these disorders the reader is referred to a textbook of dermatology.

# 1.4

## Histopathology

In psoriasis both pathological changes in the dermal and epidermal compartment are seen. Controversy exists as to the location of the initiating events in early psoriasis with respect to both compartments.[30;31] However, data obtained from early pinpoint psoriatic lesions show that the earliest changes consist of a superficial perivascular infiltrate of lymphocytes and histiocytes, with dilatation and tortuousity of the blood vessels in the dermal papillae. Subsequently, with the upward movement of some lymphocytes into the suprabasal compartment of the epidermis, slight intercellular edema develops. Above these spongiotic foci, the granular cell layer disappears, the cornified layer becomes compact, and parakeratosis and epidermal hyperplasia evolve.[32-36] In the psoriatic lesion, mitoses are not limited to the basal cell layer, as seen in normal skin, but are also found in the first two to three rows of cells in the suprabasal compartment.[37] Polymorphonuclear lymphocytes or neutrophils (PMN) may accumulate in the basal and suprabasal layer, and together with remnants of eosinophylic epidermal strands form characteristic spongiform pustules: the micropustules of Kogoj, that are highly diagnostic for psoriasis.[38-40] Sometimes, small sterile abscesses develop, the so-called Munro abscesses. Munro-abscesses represent an accumulation of PMN within the parakeratotic stratum corneum and are easily found in early psoriatic lesions, but may be difficult to find in old lesions.[41;42] In pustular psoriasis, not only microabscesses are formed within the epidermis, but also macroabscesses.[43] The migration of PMN transepidermally from the blood vessels in the dermal papillae to the stratum corneum, is also known as the 'squirting papillae phenomenon'.[44] The inflammatory infiltrate in the upper dermis and papillae mainly consists of T-lymphocytes, but PMN are also present, especially during exacerbation periods of the disease. It has been postulated that also mast cells are increased.[45] On the interface between the dermis and epidermis, the dermal papillae are hypertrophic, and the epidermal rete-ridges are elongated and thickened at the base with suprapapillary thinning of the epidermis. In spontaneously resolving lesions, the disappearance of inflammatory cells is the first change that can be seen, followed by the other features mentioned above, but tortuousity of the blood vessels in the dermal papillae may persist for some time.[32] It is obvious that the histological picture of plaque-type psoriasis may vary with the stage of the lesion. Early lesions and the margin of advancing plaques are the best sites to see most histological signs of psoriasis.[30;31] **Figure 8** depicts a representative histological picture of a psoriatic lesion.

# 1.5

## Etiology

The primary etiological factors that lead to psoriasis are not known. Psoriasis is a disease that still keeps its mysteries. It is well established that a combination of genetic abnormalities and environmental factors are responsible for the heterogeneous picture of the disease. Psoriasis is undoubtedly an inherited disease, but several triggering environmental factors are known.

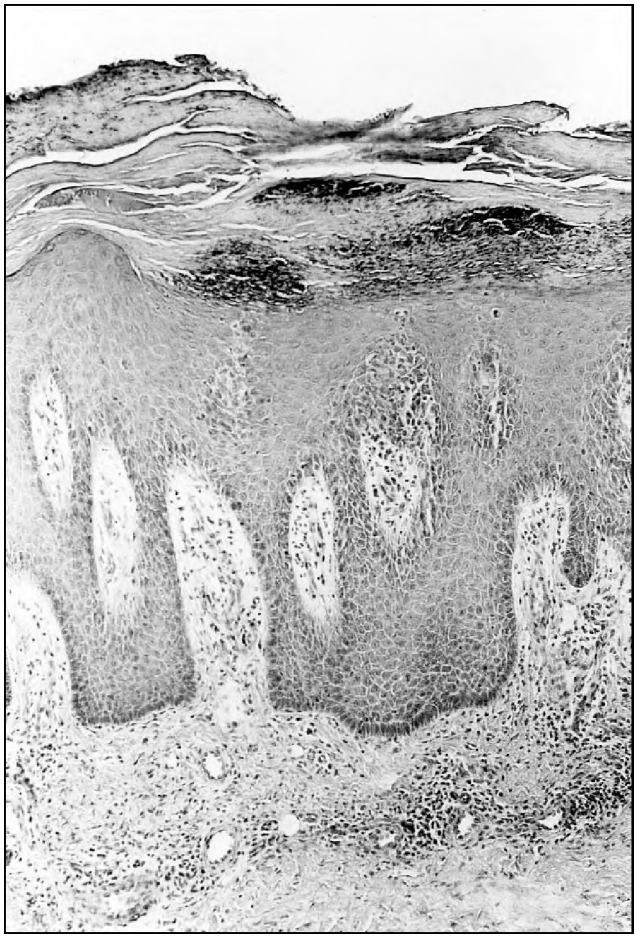


**Figure 8:**

A histological picture of psoriasis showing epidermal rete-ridges that are elongated and thickened at the base, hyperortho- and parakeratosis with characteristic Munroabscessess, and dermal perivascular T-cell infiltrates.

### 1.5.1 Genetics

A genetic base for psoriasis is confirmed by family and twin studies. In twin studies the concordance rate was 73% for monozygotic twins, compared to 20% for dizygotic twins, suggesting a significant heritability of psoriasis.[46] A large population studies in Faroer Island inhabitants indicated that 91% of the psoriatic patients had at least one affected first or second degree relative.[47] Although a genetic component is indisputable, there is still controversy over the mode of inheritance and therefore genetic counseling remains difficult.[48] However, if one parent has psoriasis, and no brother or sister has the disease, the risk to become affected is 10%. If one parent and one brother or sister is affected, the risk is 16%, and if both parents have psoriasis, the risk of getting the disease is even 50%.

HLA subtypes B13, B17, A13, A17, B37, and Cw6 are indicated to be associated with psoriasis.[27;49;50] Cw6 appears to be the HLA that has the strongest association with psoriasis of the subtypes yet known.[18] The risk of those bearing the HLA-Cw6 phenotype to develop psoriasis has been reported as 9 to 15 times normal.[18] This association is unique as no other disease is known to be primarily linked with the HLA-C locus. Other loci associated with psoriasis include the alpha-1-antitrypsin gene locus,[51] and a polymorphism of the IL-1 receptor antagonist gene.[52]

A gene localized to the distal region of human chromosome 17q has been demonstrated to be involved with psoriasis in some families.[53] In these families psoriasis susceptibility is due to variation at a single major genetic locus other than the human lymphocyte antigen locus. Other psoriasis susceptibility loci have been suggested on chromosome 6p,[54] 17q, and 20p.[55]

### 1.5.2 Triggering factors

Several environmental factors may provoke new episodes of psoriasis or cause an exacerbation of the disease.[10;56]

*Traumata*

The 'Köbner phenomenon', the fact that traumata may elicit a lesion in previously uninvolved skin, is a typical phenomenon that is seen in psoriasis. This phenomenon is probably the best evidence that injuries of the skin are triggering factors for psoriasis.[10;57]Any physical injury of the skin may induce a Köbner reaction, such as surgery, shaving, rubbing, pressure, radiation, burning, tattooing, and scratching.[58] Usually the Köbner phenomenon appears 7 to 14 days after the injury.[59] The incidence of this phenomenon ranged from 38 to 76% in psoriatic patients.[28]

*Infections*

Infections have also been noted as triggering factors for psoriasis. The best example may be tonsillitis. Tonsillitis, especially caused by streptococcal infection and in children, may provoke guttate psoriasis.[60;61] In plaque-type psoriasis an association with throat in-

fections is also present; even subclinical streptococcal infections have been reported to be responsible for refractory chronic plaque-type psoriasis.[62] HIV infection is a well-known risk factor causing deterioration of psoriasis.[63-70] This association, however, is somehow paradoxically, as in HIV infection and AIDS the number of CD4+ T-cells is decreased, whereas psoriasis has been postulated to be a T-cell mediated disease.

*Endocrine and metabolic factors*

As the incidence of psoriasis shows a peak at puberty and at the menopause, an endocrine factor may be associated with the disease.[17;71] Hormonal changes during pregnancy have been mentioned to influence psoriasis in individual cases.[28;72;73] From a metabolic point of view, alterations in blood calcium levels and dialysis have been reported to worsen psoriasis.

*Climatological factors*

In colder regions the prevalence of psoriasis is higher than in warmer and humid regions. Patients can experience an aggravation of psoriasis when they move into colder climates, whereas a sunny and warm environment generally improves the disease.[28;72] It is not well understood that in about 5.5% of the psoriatic patients an exacerbation can be induced by sunlight.[74]

*Drugs*

Some 200 drugs have been described that might aggravate or induce the expression of the disease.[75;76] In fact, any drug that has the potential to induce a drug eruption can exacerbate psoriasis as a Köbner reaction.[58] However, several drugs have a marked increased risk to aggravate psoriasis. These include lithium,[76-79] beta-adrenergic blocking drugs,[80] anti-malarials,[81;82] nonsteroidal anti-inflammatory drugs,[83] indomethacin,[84] interferon-alpha,[85-87] and the withdrawal of corticosteroids.[19]

*Psychological stress*

Although the relevance of psychological stress is not fully understood, it has been suggested and documented by several authors to be a triggering factor in psoriasis.[88-93] As psychological stress and immunological alterations are associated with each other, and as psoriasis is a disease where immunological factors play an important role, psychological stress as a causative factor for psoriasis may have its somatic substrate in neuro-immunological mechanisms. There is evidence that reduction of psychological stressors may improve psoriasis and that therefore it could be regarded as part of anti-psoriatic treatment.[90;93]

*Alcohol, smoking, and obesity*

These factors are clearly associated with psoriasis as they have a higher prevalence in psoriatic patients.[94;95] However, it remains to be seen whether these factors are actually etiologically related rather than confounding factors related to psychosociological problems because of the psoriatic disease.[18]

# 1.6

# Pathogenesis

The most striking features of psoriasis are the erythematosquamous plaques. These are the results of three major processes in psoriatic skin: inflammation, hyperproliferation, and disturbed differentiation. Below the simple characteristic clinical picture of the disease lies a world of factors that are more or less involved in the elicitation of these lesions. As mentioned before, both alterations in dermis and epidermis are seen. A difficulty exists in determining whether these factors are causative or secondary to other events. In psoriasis many cell types play a role in the pathological process. In this section I will go through the different cell types that are involved in the psoriatic process.

## 1.6.1 Cellular aspects

*T-lymphocytes*

The mononuclear cell infiltrate in psoriasis is dominated by T-lymphocytes, whereas B-lymphocytes are rarely found in lesional skin.[96] The major part of the T-lymphocytes are T-helper cells (CD4+). T-suppressor cells (CD8+) account for only 25% of all T-cells in lesional skin of chronic plaque psoriasis.[97] The CD4/CD8 ratio is increased compared to normal skin.[98;99] Furthermore, this ratio varies with the different activity stages of the lesion: active psoriasis is associated with CD4+ T-lymphocytes in the epidermis, whereas CD8+ T-lymphocytes are mainly found in regressive plaques.[100-102]

The last decade more evidence has been accumulated for a central role of the T-lymphocyte in the onset of psoriasis. An intriguing case report described a patient without neutrophils and monocytes in the peripheral circulation, who developed psoriasis.[103] Depletion of circulating T-lymphocytes by intravenous administration of anti-CD3/CD4 monoclonal antibodies resulted in improvement of psoriasis.[104] Furthermore, cyclosporin A, an immunomodulating drug with high specificity for inhibition of cytokine production of T-cells (and therefore frequently used in renal transplant recipients; see part II), is very effective in the treatment of psoriasis. And in early psoriatic lesions the involvement of T-lymphocytes is described.[105] T-cells from peripheral blood of psoriatic patients, in the absence of added antigen, show a clear proliferative response in vitro to autologous epidermal cells from lesional as well as uninvolved skin.[106;107] It is unclear whether this response is antigen driven. All these data strengthen the hypothesis that psoriasis is a disease of keratinocyte proliferation induced by T-lymphocytes and T-cell derived pro-inflammatory mediators.

Another recent hypothesis in which the T-cell plays a prominent factor, is the 'super-antigen theory'. This theory has been highlighted by several groups to be of relevance in the pathogenesis of psoriasis.[108-110] Superantigens are a group of bacterial and

viral proteins that are capable of inducing massive T-cell proliferation and cytokine production, without any cellular processing. They bind directly to the major histocompatibility complex class II molecule on the antigen presenting cell (APC) and crosslink the APC with T-cells expressing specific variable segments of the T-cell receptor beta-chain (V-beta), leading to polyclonal T-cell activation and activation of resting keratinocytes.[111] *Staphylococcus aureus* and *streptococci* secrete a large repertoire of exotoxins, which may act as superantigens. In this respect M proteins of group A beta-hemolytic streptococci may be of importance.[112] Studies have demonstrated increased representation of V-beta2 and, to a lesser extent, V-beta5-expressing T-cells in lesional skin of patients with guttate and chronic plaque psoriasis when compared to peripheral blood T-cells from the same patient.[113] Other data that support the superantigen theory are the clinical findings that psoriasis is triggered by infections where these bacteria play a role (e.g. pharyngitis) and (especially in guttate psoriasis) the lesions often disappear after the infection has resolved.

Langerhans cells, dermal macrophages, and keratinocytes are the known antigen presenting cells in the human epidermis and are involved with processing these bacteria. However, psoriasis is not a skin disease 'sensu strictiori', as systemic involvement in the form of psoriatic arthritis is often seen in these patients. Also in rheumatoid arthritis an immune response to such antigens has been proposed as a causative factor.[114]

Another hypothesis forms the 'autoreactive hypothesis'. According to this theory, CD8 + "killer" T-cells generated in the skin or systemically is inappropriately specific to epidermal keratinocytes, producing sublethal damage that triggers regenerative or woundhealing activation.

It is remotely possible that different pathogenic mechanisms could be linked to different genetic loci, especially when considering the genetic heterogenicity and the different theories.

*Polymorphonuclear lymphocytes (PMN)*

Polymorphonuclear lymphocytes or neutrophils are important, especially in early and active psoriatic lesions. Many abnormalities in blood neutrophils from psoriatic patients have been reported, such as enhanced chemotaxis, phagocytosis, increased cytotoxic activity, and adherence.[115-118] As described in Chapter 1.4, in psoriasis PMN are abundantly present in spongiform pustules in the epidermis (pustules of Kogoj) and in microabscesses in the stratum corneum (microabscesses of Munro). Maximal infiltration of PMN in dermis and epidermis is seen in pustular psoriasis.[119;120] Interestingly, accumulation of PMN seems to be associated with parakeratosis in chronic plaque psoriasis, as Munro abscesses were only found within parakeratotic foci.[39] In plaque-type psoriasis 49 % of the patients had a clear infiltrate of PMN.[39] A number of inflammatory mediators can attract and activate PMN. Of these, the most important are leukotriene B4 (LTB4),[121;122] and the cytokine interleukin 8. [123] From a therapeutic point of view, the migration of PMN into the skin seems important, as several anti-psoriatic treatments such as corticosteroids, UVB, PUVA (psoralens + UVA), dithranol, and retinoids have

proven to inhibit intra-epidermal accumulation of PMN in normal skin after epicutaneous application of leukotriene B4, but the exact role of PMN in psoriasis remains to be elucidated.[124-127]

*Keratinocytes*

The thick and scaly lesions are typical for psoriasis and are clinical manifestations of a disturbed differentiation and hyperproliferation of the underlying keratinocytes. So the keratinocyte is an important cell in the whole psoriatic process. Hyperproliferation in psoriasis has first been described by Scott and Van Ekel and can be concluded from the higher number of mitotic figures and the greater number of cells labeled with tritiated thymidine.[128;129] It has been postulated that a shortening of the cell cycle time of the keratinocytes causes epidermal hyperproliferation.[130] However, more recently other studies have conclusively shown that not an intrinsic abnormality in the cell cycle, but an increased recruitment of cycling keratinocytes from the resting G0-compartment is responsible for this phenomenon.[131]

Besides hyperproliferation, alterations in differentiation are seen in psoriatic keratinocytes. In the healthy epidermis, keratinocytes undergo a complex process of terminal differentiation finally leading to formation of the dead stratum corneum. In this process keratinocytes move from the basal compartment, where they are initially fixed to the lamina lucida of the basement membrane by hemidesmosomes and tonofilaments, upwards through the suprabasal compartment until they finally reach the skin surface and are being shed. During this movement inside the keratinocyte a process called 'keratinization' takes place. Hereby, keratin filaments aggregate into bundles through the action of the histidine-rich basic protein filaggrin. During the final differentiation stage the stratum corneum cells have normally lost their nuclei and other recognizable organelles, and basically contain insoluble cysteine-rich, disulphide cross-linked, fibrous proteins or keratins.[132] Intermediate keratin filaments are present in all keratinocytes, however, the type of keratins that are expressed, normally depends on the state (and therefore the location in the epidermis) of the keratinocytes in this differentiation process.[133] At least 20 different epithelial types of keratins have been described.[134;135] Keratins can be subdivided into basic (type II, numbered 1 to 8) and acidic (type I, numbered 9 to 20) subgroups, each the product of a distinct keratin gene.[134;136] Keratins are present in pairs. In normal skin keratin 1 and 10 are abundantly expressed throughout the suprabasal layers of the epidermis, while in the basal layers keratin 5 and 14 are present.[137] In addition, epidermal differentiation involves the synthesis of new envelope proteins, including involucrine, specific enzymes involved in envelope cross-linking, such as transglutaminase, and alterations in membrane glycoproteins.[132]

In psoriasis, however, normal keratinization is incomplete and parakeratosis is seen. With respect to keratin expression a reduction in keratin 1 and 10 has been reported.[138;139] Keratins 1 and 10 are replaced by keratins 6 and 16 in psoriatic lesions and therefore keratinocytes show less expression of keratin 1 and 10 when compared to normal skin.[140-143] Keratins 6 and 16 are associated with hyperproliferative conditions and can

be seen in suprabasal epidermal cell layers.[144;145] Keratin 16 is a parameter that is, apart from the hair follicle, not present in normal skin.[139;145-149] Keratins 17 and 20 are also associated with hyperproliferation, and expression of these keratins in psoriasis has been reported.[148] Other differentiation related parameters, such as involucrin, filaggrin, and transglutaminase, demonstrate alterations in psoriasis as well.[150;151]

*Langerhans cells*

As discussed in the section on lymphocytes, Langerhans cells may play an important role in the onset of psoriasis with respect to their function as antigen presenting cells (APC). Clustering of Langerhans cells has been observed in psoriasis, but it remains controversial whether their numbers are altered in the epidermis.[99;152;153]

*Monocytes*

These cells are also involved in psoriasis and demonstrate increased locomotion,[154] increased phagocytosis,[155] and enhanced cytotoxic activity.[115]

# 1.7

# Therapy

We do not know the exact cause of psoriasis, so there is no single treatment that can cure this disease, but to keep psoriasis under control and suppress its symptoms, a spectrum of treatments is available. Several factors are of relevance to select the optimal treatment for each patient, e.g. type, extent and history of psoriasis, but also personal factors like age, sex, occupation and co-morbidity. In order to enhance the clinical efficacy and to limit side effects, combinations of several anti-psoriatic therapies are often worthwhile.

## 1.7.1. Topical therapies

*Emollients*

Although emollients, such as lanette wax cream and vaseline, do not contain any pharmacologically active metabolites, and are therefore frequently used as a vehicle to investigate the activity of new compounds, they do have a slight therapeutic effect on psoriasis and can therefore not be considered as a placebo treatment.[156] Emollients hydrate the skin and improve the hyperkeratotic and scaly psoriatic plaques.[156;157] The greasier the emollient, the higher its potential effect, but unfortunately the less well accepted it will be from cosmetic point of view.[156] A significant reduction in redness, itching, soreness, and pain has been noticed in about 35 % of the patients when applied twice daily.[158] Therefore, many psoriatic patients use emollients as maintenance therapy to relieve their symptoms.

*Keratolytics*

Salt baths may well be the oldest keratolytic treatment that we know of. As we have seen before, even the ancient Egyptians were aware of the beneficial effects of salt and red natron in skin disorders, and often used them in combination with emollients.[1] However, nowadays more potent keratolytics, like salicylic acid and urea, are used. Keratolytics reduce scaling of the elevated psoriatic plaques and therefore reduce the thickness of the lesions. They also promote absorption and penetration of other psoriatic drugs, which make them a perfect combination therapy with tar, corticosteroids, dithranol, but also photo(chemo)therapy.[156] Nevertheless, high concentrations of salicylic acid should be used carefully, because of its irritating effects on the surrounding uninvolved skin (cave Köbner reaction) and, when applied to large body surface areas, the risk of salicylic intoxication.[159]

*Vitamin D3 analogues*

Vitamin D3 analogues are the first choice of treatment in mild to moderate psoriasis. At this moment there are three vitamin D3 analogues available for topical treatment: calcipotriol (Daivonex®), calcitriol (1α,25-dihydroxy vitamin D3, Silkis®), and tacalcitol (1α,24-dihydroxy vitamin D3, Curatoderm®). The latter has not been registered in the Netherlands so far. These vitamin D3 analogues have in common that they are easy to use from cosmetic point of view and that they are safe and effective in the treatment of psoriasis. A main advantage of these treatments compared to topical corticosteroids is the hold off of dermal atrophy, however, vitamin D3 analogues may alter systemic calcium metabolism when large quantities are applied.

Calcipotriol is an effective treatment that has hardly any side effects when applied twice daily for long-term periods.[160] A dose below 100 gram per week is supposed to have no effect on calcium metabolism.[161] However, calcipotriol can give irritant reactions and therefore facial treatment is less suitable. Calcipotriol can easily be used with several other treatments. A common approach is calcipotriol alternating with a potent topical corticosteroid. Tacalcitol shares similar features with calcipotriol with respect to side effects, although it can be administered once daily.[162] This comparative study has shown that tacalcitol is less effective than calcipotriol.[162] Calcitriol is the natural active vitamin D3 and was recently introduced as a topical treatment in dermatology. It is more effective than a placebo.[163] Double-blind randomized comparative studies of calcipotriol and calcitriol are not available.

Vitamin D3 analogues inhibit epidermal proliferation,[164-167] and modulate differentiation. They can enhance cornified envelope formation and increase transglutaminase activity in vitro.[165;168] Furthermore, vitamin D3 analogues have anti-inflammatory effects, as they inhibit the production of IL-1, IL-2, and IL-6, and the release of arachidonic acid by PMN.[169;170] Vitamin D3 analogues bind to the vitamin D3 receptor (VDR), which is together with the retinoid and other receptors, a member of the nuclear receptor family.[170] See paragraph 1.16 for a more detailed discussion on nuclear receptor mechanisms.

*Corticosteroids*

Topical corticosteroids are the most frequently used treatment for psoriasis nowadays. When applied correctly, they are effective, have no primary irritative potential, do not smell, do not stain clothes and are easy to apply. Topical corticosteroids can be categorized in four increasing classes of potency (I, II, III, and IV). They possess anti-proliferative, anti-inflammatory, and immunosuppressive effects.[171;172] The mode of action is via the glucocorticoid receptor in the cytoplasm, where it forms a complex that is then transported to the nucleus. This is followed by binding to the glucocorticoid response element (GRE) on the DNA and so it can regulate transcription of genes adjacent to this GRE, leading to modulation of inflammation.[173;174] An important target of corticosteroids are the T-cells; these cells are known to play a major role in psoriasis as described in paragraph 1.6.1.

Despite their efficacy and ease of use, topical corticosteroids have disadvantages (especially when more potent corticosteroids are applied for longer periods). The most

prominent side effect is atrophy of the skin. This phenomenon can already be seen microscopically after 3 to 14 days of application. Initially this atrophy is reversible, however, when applied chronically, dermal changes will become evident. A direct antiproliferative effect on fibroblasts is responsible for the dermal atrophy.[175] In a later phase vascular dilatation, telangiectasias, striae, and purpura may be seen. Other side effects are hypertrichosis, hypopigmentation, dermatitis perioralis, and suppression of the pituitary-adrenal axis.[176] Therefore, the use of class IV corticosteroids should be limited to 50 grams per week; less potent corticosteroids may be used to a maximum of 100 grams per week.[177] Topical corticosteroids can be used with all kinds of other anti-psoriatic treatments to increase efficacy.[178] They can also be used under hydrocolloid occlusion.

*Dithranol*

Dithranol is also known as anthralin or cignolin. It is a chemically synthesized analogue of chrysarobin, an extract derived from the araroba tree that has accidentally been found to be of value in the treatment of psoriasis.[179;180] The exact mode of action is still unknown, although it is likely that the anti-psoriatic effect may be caused by its induction of free radicals that can affect the mitochondria and lead to an antiproliferative effect.[181-183] These free radicals are also responsible for the well-known skin irritation that can be seen already at very low concentrations of dithranol. Short-contact application can minimize the skin irritation and is therefore preferable.[184-186] Besides its irritant properties another disadvantage is its irreversible staining of hair and clothes that may limit the use of this treatment outside the clinic.[187;188] In clinical studies dithranol has been proven to be a very effective and safe treatment for psoriasis.[184-186] Dithranol is a promotor of carcinogenesis in mice,[189] however, in humans no carcinogenic effects due to dithranol have been reported in psoriatic patients so far.[18]

*Tar*

Tar can be derived from several sources, but coal tars are the most frequently used in dermatology.[190] Coal tar contains over 1000 ingredients and is a mixture of aromatic hydrocarbons manufactured by primary condensation of coal. Its precise mechanism of action is unknown, but an antimitotic effect has been assumed.[191] Its major beneficial effect is a reduction in pruritus. For over one century tar has remained a common treatment for psoriasis.

It is considered to be a safe and effective therapy, although carcinogenic and teratogenic properties have been reported.[192;193] Therefore, it should not be given to pregnant women and breastfeeding mothers. Tar is generally well tolerated, but disadvantages are its odor, the irreversible staining effect on clothes, the induction of folliculitis, skin irritation, and the enhancement of sunburn. Due to its irritative potential, coal tar is less suitable for erythrodermic and pustular psoriasis. It can be used in combination with photo(chemo)therapy (see paragraph 1.7.1). However, its sunburn enhancing effects may need to take precautions. Coal tar can be applied on psoriatic lesions as pure coal tar (pix lithantracis, 1-10%) or as a tar solution (solutio carbonis detergens, 5-20%). For treatment of psoriasis capitis several tar shampoos and lotions are available.

*Retinoids*

Retinoids are natural or synthetic derivatives of vitamin A. Unlike systemic retinoids (see paragraph 1.7.3) topical retinoids are infrequently used in psoriasis, especially in the Netherlands. Topical retinoids have been proven to be effective in erythematosquamous disorders, such as ichthyosis vulgaris, and also in acne, but their therapeutic potencies in psoriasis are generally less worthwhile. Currently, only all-trans retinoic acid (tretinoin) may be used. In other countries more topical retinoids have been registered, such as isotretinoin, motretinoin, adapalene, and tazarotene.[194] In Part III retinoids, their effects, and their receptor mechanisms will be discussed.

### 1.7.2 Photo(chemo)therapy

It has been known for years that sunlight may have beneficial effects in psoriasis for most patients. Especially when larger body surface areas are involved with psoriasis and when previous therapies with topical treatments were insufficient, photo(chemo)therapy is the treatment of choice above systemic regimens. Photo(chemo)therapy comprises all forms of irradiation therapy that use the ultraviolet spectrum.

*Broad spectrum UVB*

Goeckerman was the first who thoroughly documented the use of UVB irradiation after coal tar applications to treat psoriasis.[195] Much later several studies demonstrated that UVB therapy alone, if given in a slightly erythematogenic dosage, was capable of clearing psoriasis.[196-198] UVB radiation (290-320 nm) induces free radicals and subsequently inflammation. This may be the result of activation of the arachidonic acid pathway.[199] The number of Langerhans cells decreases during UVB treatment and the shape of these cells changes. The mode of action may be through DNA damage, but the precise mechanism is still not fully known.[200] A common and effective irradiation schedule is three times a week, once daily.[196] The duration of remission after UVB therapy is relatively long compared to corticosteroids. A limitation of UVB therapy is its carcinogenicity, although follow-up studies comparing the prevalence of premalignant and malignant skin lesions among extensively UVB-treated psoriatic patients and healthy controls has not shown an increased risk in the psoriatics group.[201-203]

*Small spectrum UVB*

Small spectrum UVB irradiation is a rather new therapeutic approach in psoriasis UV treatment. For this type of irradiation TL01 lamps are used that emit radiation in a narrow band of 311-312 nm. The emission spectrum of small spectrum UVB has an improved ratio between anti-psoriatic and erythematogenic effects. It is the preferred UVB treatment.

*PUVA*

UVA radiation (320-400 nm) causes markedly less erythema compared to UVB radiation and it requires an almost 1000 fold higher intensity than UVB to be therapeutically effective. However, the addition of psoralens as a skin sensitizer to UVA light dramatically improves therapeutic efficacy. PUVA (psoralens + UVA) is a very effective treatment and is even more potent than UVB. Due to the addition of psoralens this treatment is also known as broadspectrum 'photochemotherapy'. Side effects include erythema, pruritus, and sometimes nausea.[200;204] However, its major limitation is the fact that PUVA strongly increases the risk of developing squamous cell carcinomas.[203] The risk increases sharply once a cumulative total dose of 2000J/cm$^2$ is reached. However, in a sun-sensitive population of Celtic subjects an increased risk of developing non-melanoma skin cancer was already found with cumulative doses above only 250J/cm$^2$.[205] Another limitation of UVA-light is the acceleration of photoaging. Therefore, long-term PUVA treatment is not encouraged.

Photo(chemo)therapy is frequently used in combination with topical or systemic retinoids to obtain an enhanced therapeutic effect and/or a reduction in the cumulative UVB or UVA dose necessary to achieve clearance of the disease. However, some experts believe that retinoids may have photosensitizing effects and that patients who are treated with topical or systemic retinoids may need to take precautions with respect to sunlight exposure. In Chapter 4 the presumption of photosensitizing properties of retinoids is challenged.

### 1.7.3 Systemic therapies

*Systemic retinoids*

In this section, I will briefly mention the main features of acitretin (Neotigason®), the retinoid which is most frequently used, and which has become a mainstay in modern psoriasis treatment.[194] Other retinoids like etretinate (Tigason®) and isotretinoin (Roaccutane®) have more or less lost their significance in psoriasis treatment since the introduction of acitretin, due to its shorter half-life. Isotretinoin, however, is still of major importance in severe acne. With respect to efficacy, etretinate and acitretin are equally effective in psoriasis. [206;207] For pustular and erythrodermic psoriasis retinoids are the treatment of first choice.[194;208] In plaque-type psoriasis retinoids are also indicated, often in combination with topical therapy and/or photo(chemo)therapy, as monotherapy for plaque-type psoriasis is less effective.[208] Because both efficacy and side effects can vary substantially among individual patients, proper dosing of acitretin requires a balance between optimizing response and minimizing toxicity for each patient. Optimal dosing for individual patients may be achieved through a dose-escalation strategy involving initiation of therapy at low doses (10 to 25 mg/day) and, if necessary, gradually increasing the dose as tolerated until optimal response is achieved.[209] The mode of action of retinoid therapy, their receptor mechanisms and side-effects will be discussed in Part III.

Apart from direct retinoid treatment, an increased concentration of retinoic acid in the cell can be achieved by retinoic acid metabolism blocking agents (RAMBA's). The imidazol derivative liarozole is such a RAMBA. It is an inhibitor of cytochrome P-450 and causes inhibition of 4-hydroxylation of retinoic acid, leading to increased intracellular retinoic acid levels.[210]

*Methotrexate*

Methotrexate is one of the most effective anti-psoriatic drugs.[211] In 1951 the effect of aminopterin, a folic acid antagonist, on psoriasis was first described by Gubner.[212] Since 1971 another folic acid antagonist, amethopterin (methotrexate, MTX), has been registered for the treatment of severe forms of psoriasis.[213;214] MTX inhibits epidermal DNA synthesis and decreases mitotic activity.[215-217] MTX causes a G1-phase arrest of cycling keratinocytes and MTX kills keratinocytes in the S-phase.[218] However, MTX has also been claimed to inhibit chemotaxis of PMN and thus to decrease inflammation,[219] but some other mechanisms have been postulated as well, including inhibition of intracellular T-cell signaling.[220]

Low dose MTX is a relatively safe therapy, provided that a careful patient selection and regular monitoring for side effects and drug interactions is carried out. Its side effects can cause serious limitations, especially myelosuppression and hepatotoxicity. The latter is related to a high cumulative dose of MTX. The most common side effects comprise nausea, headache, gastric complaints, bone marrow depression, and abnormal serum (liver) transaminases.

In order to minimize the toxicity of MTX, combinations or rotational therapy should be considered. Contraindications for MTX treatment are pregnancy, excessive alcohol intake, abnormalities in renal and liver function, hepatitis, liver cirrhosis, severe anemia, leukopenia, thrombocytopenia, infectious diseases, and non-compliance of the patient.[221]

*Fumarates*

The first report of the beneficial effects of fumarates for psoriasis came in 1959 from the German chemist Walter Schweckendiek, who was a psoriatic patient himself. He hypothesized that psoriasis is caused by a malfunctioning citric acid cycle in the mitochondria, leading to a relative deficiency in the generation of one or more of its important metabolites. [222] Therefore, in an autoexperiment, he orally administered various original and chemically modified citric acid cycle metabolites. Of the tested compounds, only fumarates were able to clear his psoriatic lesions.[222] After a decade of neglecting, fumarates slowly became more accepted in psoriasis treatment and several studies have demonstrated their efficacy.[223] The exact mode of action of fumarates is still unknown, but alterations of the Th1/Th2 lymphocytes balance have been described.

'Fumaric acid' is a frequently used, but incorrect term for therapy with fumarates. Due to its acidity, pure fumaric acid (1,2-ethylenedicarboxylic acid; C4H4O4) is not suitable for oral therapy in psoriasis. Therefore, (m)ethyl ester derivatives of fumaric acid are being used, and these proved to be clinically effective.[223-230]

The most frequently observed adverse events are flushing, gastro-intestinal discomfort, diarrhea, lymphocytopenia, and fatigue, but these are generally well tolerated. [222;223;226-231] Other adverse events are urticaria, osteomalacia, and occasionally nephrotoxicity.[232-235] Gastro-intestinal adverse events tend to diminish during prolonged treatment.

Long-term benefit/risk ratios of orally administered fumarates are such that it permits effective and safe management of psoriasis. However, as this treatment has not yet been approved in the Netherlands, this therapy should only be performed under strictly controlled conditions.

*Cyclosporin A*

It is generally accepted that psoriasis is at least to some extent a T-cell mediated disease, although the precise pathogenesis remains to be elucidated (see paragraph 1.6.1).[105] Therefore, T-cell specific immune-modulating agents have been extensively investigated since the last decade. Cyclosporin A, an immunosuppressive agent used primarily in organ transplant recipients, has also been approved for treatment of severe psoriasis, in whom conventional therapy is either ineffective or inappropriate.[236-239] Besides its application in plaque-type psoriasis, it can also be used in pustular and erythrodermic psoriasis.[240-243]

A well-established treatment regimen is to start with 3 mg/kg/day, in two divided doses, and to increase to a maximum of 5 mg/kg/day, with subsequent tapering to the minimum effective dose. A dose-response relationship has been demonstrated by several studies with respect to both the numbers of patients who responded and the time to response.[236] In a large study in plaque-type psoriasis, cyclosporin A in a dosage of either 2.5 or 5.0 mg/kg/day was effective in respectively 52% and 92% of the patients.[244]

Cyclosporin A binds to cyclophilin, a family of isomerases found in almost all mammalian cells. This newly originated complex inhibits the enzyme calcineurin, which is a key factor in calcium-dependent signaling processes. Calcineurin plays an important role in the signal transduction of the T-cell with respect to cytokine production. By inhibiting calcineurin, the transcription of many cytokines, including T-cell growth factors IL-2 and IL-4 is inhibited.[245]

Adverse events that may be seen during cyclosporin A therapy are nephrotoxicity, hypertension, gastro-intestinal problems, hypertrichosis, gingival hyperplasia, paresthesia, headache, vertigo, muscle cramps, tremor, and (malignant) tumors (e.g. skin cancer and lymphoma; see Part II). A detailed study on nephrotoxic aspects of cyclosporin A indicated that patients who had less than 30% increase in creatinin level did not have nephropathy.[246] However, to reduce the risk of nephropathy, monitoring guidelines have been developed. [247;248] Contra-indications for cyclosporin therapy are impaired renal function, uncontrolled hypertension, past or present malignancy, infection, concomitant immunosuppressive therapy, immunodeficiency, pregnancy and lactation.[236]

## 1.7.4 Potential new therapies

### *1.7.4.1 RXR-selective retinoids*

Current retinoid treatment for psoriasis is limited to retinoids that favor the retinoic-acid-receptor (RAR) pathway. However, besides the retinoic-acid-receptors there is a sub-family of retinoid receptors, the retinoid-X-receptors (RXR), that elicit different transcriptional gene responses, and therefore may provide a completely new approach in psoriasis therapy.

It is known that transcripts of RXRα, representing 90 % of RXR in the epidermis,[249] is 58 % lower in lesional versus non-lesional psoriatic skin, together with a reduced RXRα : RARγ ratio from 3.2 to 1.5, suggesting rexinoid-signaling in psoriasis is abnormal. [250] RXRα has been found in the suprabasal compartment of the epidermis and has been suggested to play a function in the transition from proliferation to differentiation in epidermal keratinocytes.[251] Furthermore, RXRα has been postulated to be an early differentiation marker.[252] In the skin, apart from the RXR-RAR heterodimer, the RXR-PPAR heterodimer may also play a central role, as it modulates keratinocyte proliferation and differentiation, and plays a role in inflammation control.[253] The PPAR (peroxisome proliferator-activated receptor) agonist troglitazone in the treatment of psoriasis indeed showed a clinical and histological response.[254]

Investigations on the potential role of 4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetra-hydro-2-naphthyl)ethenyl]benzoic acid (bexarotene, Targretin®), a new compound with a short half-life of 7 hours,[255] that selectively binds the RXRα, β, and γ subtypes of the nuclear retinoic acid receptor family, in the treatment of plaque-type psoriasis will be presented and discussed in Chapter 2 of this thesis.

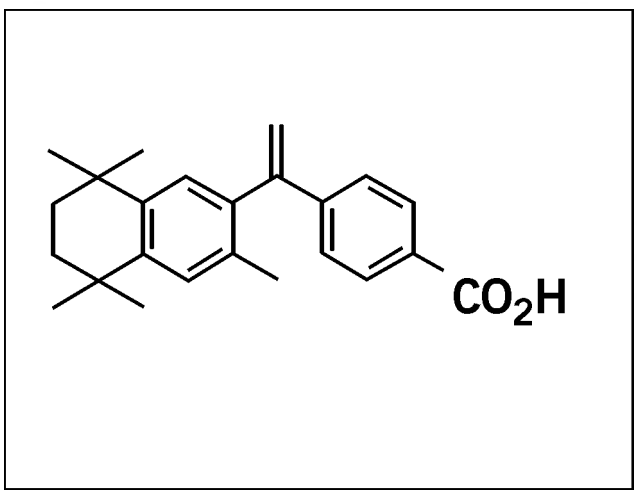



**Figure 9:**

Molecule structure of bexarotene (Targretin®).

#### *1.7.4.2 Cyclic immunosuppressive agents (macrolactams)*

*Ascomycin (ASM 981, Pimecrolimus, Elidel®)*

Ascomycins represent a novel class of anti-inflammatory macrolactams currently under development for the treatment of skin diseases. ASM-981 is at the most advanced stage at this moment. The main biological effect of ascomycin is an inhibition of the synthesis of cytokines of both Th1 (interleukin-2, interferon γ) and Th2 (interleukin-4 and interleukin-10) lymphocytes.[256] In chronic plaque-type psoriasis it is only effective if applied under semi-occlusive dressings with similar effectiveness to clobetasol-17-propionate (0.05%),[257] however the safety profile of ASM 981 is better than that of topical corticosteroids. Only low systemic exposure has been reported even when applied to large areas of skin, and ASM 981 does not induce skin atrophy. Furthermore, ASM 981 holds promise in overcoming the drawbacks of topical corticoids.[258] A topical application of 1% ASM 981 in a cream base is currently available as Elidel®, however, applications in psoriasis are limited, as that formulation is ineffective without occlusion.

*Tacrolimus (FK506, Prograf®, Protopic®)*

Tacrolimus has been shown to be a powerful suppressor of the immune system and is a common treatment for the prevention of allograft rejection in kidney, liver, and heart transplant recipients. Tacrolimus shares many of the immunosuppressive properties of ASM 981. Systemically (Prograf®), it has been demonstrated to be effective in treating psoriasis, probably by inhibiting T-cell infiltration.[259-261] However, another study with topical tacrolimus (Protopic®) proved non-effective in chronic plaque psoriasis.[262] The major side effects of tacrolimus include nephrotoxicity and hypertension.

*Sirolimus (rapamycin, Rapamune®)*

Sirolimus is an immunosuppressive agent that interferes with T-cell activation. It blocks T-cell proliferation in the G1 phase of the cell cycle, but it also inhibits growth of the keratinocytes.[263] Its inhibition appeared more potent than that of cyclosporin.[264] However, severe adverse events of sirolimus include fever, anemia, thrombocytopenia, and capillary leak syndrome.[265;266] The application of sirolimus in combination with a subtherapeutic dose of cyclosporin in severe psoriasis has been opted for permitting a reduction in their respective toxicities, notably cyclosporin-induced nephrotoxicity.[266]

### *1.7.4.3 Monoclonal antibodies and fusion proteins*

In the next couple of years we may see the registration of several new single target compounds for the treatment of psoriasis that act by a completely new and selective approach. These compounds are monoclonal antibodies or fusion proteins that specifically target receptors or other components involved in the psoriatic pathogenesis. As proteins do not penetrate the skin when applied topically, and oral administration will lead to immediate breakdown of the protein by gastric enzymes, intravenous (IV), intramuscular (IM), and subcutaneous (SC) administration is indicated. For detailed information on this subject the reader is referred to a recent review.[267] Here, we will restrict to a few promising new opportunities.

*LFA3tip (Alefacept, Amevive®)*

LFA3tip is a recombinantly engineered LFA-3/IgG1 human fusion protein that modulates immune responses through direct interaction with the T-lymphocytes via the CD2 receptor on the cell surface of the T-lymphocytes.[268] LFA3tip depresses the number of memory effector T-cells and is therefore a relevant new therapy in the T-cell mediated disease psoriasis.[269]

*Infliximab (Remicade®)*

Infliximab is a monoclonal antibody against tumor necrosis factor alpha (TNFα). TNFα is thought to play a role in the pathogenesis of psoriasis, as this proinflammatory cytokine is present in increased concentrations in the skin. A high degree of clinical benefit and rapid time to response in plaque-type psoriasis has recently been demonstrated.[270-272]

*Etanercept (Enbrel®)*

Etanercept is another TNF inhibitor that has shown efficacy in clinical studies in psoriasis.[273]

*Efalizumab (Raptiva®)*

Leukocyte function-associated antigen 1 (LFA-1), consisting of CD11a and CD18 sub-units, plays an important role in T-cell activation and leukocyte extravazation. The humanized anti-CD11a monoclonal antibody efalizumab has recently demonstrated significant clinical and histologic improvement in the T-cell mediated disease psoriasis.[274]

# 1.8

## Research targets

Improved insight in pathogenesis of psoriasis may reveal targets for future drug development. Two major areas of drug development exist:

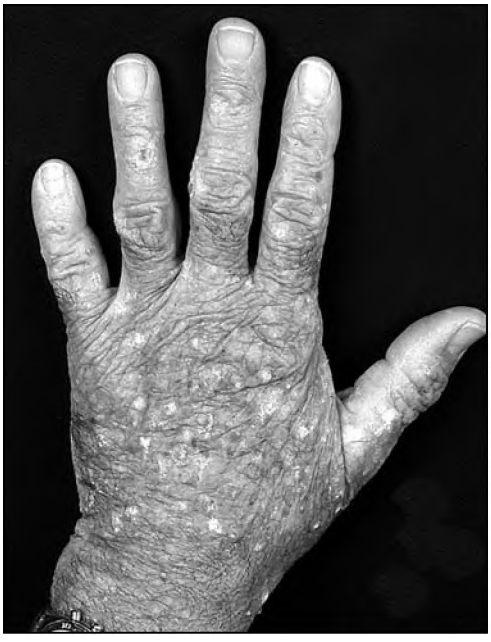
I **Specific single target molecules interfering with immunity.**

II **Modulation of retinoid signaling.**

In the present thesis we will focus on RXR-signaling in psoriasis and study the clinical and immunohistochemical effects of the RXR-selective ligand bexarotene.

# PART II

# (PRE)MALIGNANT SKIN DISORDERS



Multiple actinic keratoses on
the back of the hand in a renal transplant patient.

# 1.9

# Mechanisms of cutaneous carcinogenesis

## 1.9.1 History

Since the 19th century many carcinogens have been identified. Especially the carcinogenic potential of both chemicals and radiation was recognized quite early. The first carcinogen that was recognized was soot, as Percival Potts noted a high incidence of scrotal carcinoma in young chimney sweeps. In 1887 a correlation between arsenic administration and subsequent development of both cutaneous and systemic malignancy has been discovered. After the discovery of X-rays by Wilhelm Conrad Röntgen, scientists became aware of its carcinogenic potential. Hyde was the first to recognize that UV radiation is also carcinogenic. Nowadays many carcinogenic factors are known, including a third group: viruses (see paragraph 1.9.3).[275]

## 1.9.2 Stages of carcinogenicity

### *1.9.2.1 Cellular (functional) stages*

In order to become a malignant cell, a normal cell should surpass several stages. Each stage has specific functional biologic properties that enable a cell to finally become a metastasizing tumor after clonal expansion. The following stages can be determined:[275]

1. Controlled growth to autonomous growth with normal differentiation.
2. Autonomous growth with abnormal differentiation.
3. Both 1 and 2 plus additional nuclear atypia.
4. The ability to pass through the normal natural barrier of the basement membrane.
5. To pass through the dermis to enter the vascular channels.
6. To arrest in nodes (or organs such as the liver and lungs if blood borne).
7. To undergo the clonal expansion of metastases in these sites.

It is important to understand that not all the cells in an early carcinoma in situ (stage 3) have the capacity to complete all these steps. Throughout the stages of tumor development there is also a continuous selection of cells with the functional capacity to proceed to the next stage. In order to facilitate the synthesis of new vascularization in the tumor area for providing nourishment and creating a network for distant dissemination of the tumor cells, some tumors can secrete tumor angiogenic factors.

### *1.9.2.2. 3-stage model of carcinogenesis*

Apart from the functional biologic stages, animal studies have conclusively shown that there are several identifiable steps in the progression from a normal to a malignant cell with metastatic capacity. At several points in this stepwise progression to malignancy, the process can become arrested or can even partially reverse.[276] Three steps have been identified so far: initiation, promotion, and conversion.[277] There is evidence that the 3-stage model of carcinogenesis derived from animal studies is also the mechanism involved in skin cancer. These include (1) the concordance between known carcinogens such as polyaromatic hydrocarbons and UV radiation in mice and humans, (2) evidence for oncogenic activation or tumor suppressor gene inactivation by these agents in the skin of mice and humans, and (3) the long latency between initial exposure to carcinogens and tumor development.[275]

The state of initiation can already be achieved by only one topical or systemic dose of a carcinogen. Initiators have in common that they can bind to nucleotide sites, which interfere with normal basepair reactions in DNA synthesis. This mutation may affect the ras proto-oncogenes and/or p53 tumor suppressor genes among others (see paragraph 1.9.4 and 1.9.5). Initiated cells contain a mutational change in the genome and this remains for the rest of its life; this stage does not seem to be reversible. Initiated cells do not necessarily have to continue with the other steps of malignant progression. In fact, as long as promoting agents are lacking, the cell remains in the initiation phase. Initiated epidermal cells are not malignant, but are insensitive to the normal signals for terminal differentiation in the epidermis. With respect to the skin, however, no clinical signs can be seen at this stage.[275]

The stage of promotion can follow the stage of initiation. A promotor must be applied after an initiator to fulfil its carcinogenic effect. Generally, a promotor stimulus needs to be repeated to be effective in carcinogenesis. Collectively these agents produce a tissue environment that is conductive to the selective clonal outgrowth of the initiated cell population, resulting in a clinically apparent premalignant tumor, with features of hyperkeratosis and scaling in the case of squamous skin tumors, e.i. actinic keratosis, Bowen's disease. However, withdrawal of the promotor may lead to a decrease in carcinogenic capacity, and reversal of the clinical symptoms.

Conversion is the final stage when continued stimulating effects of a promotor lead to progression of the lesion to a malignant carcinoma with metastatic capacities, e.i. squamous cell carcinoma. However, the third stage of carcinogenesis may also occur spontaneously.[275]

Some substances are both initiating and promoting agents, and are therefore called 'complete carcinogens'. A well-known complete carcinogen is UV radiation. Furthermore, in cutaneous carcinogenesis, the concept of co-carcinogenesis may be relevant in humans. This term describes that two substances that may be weak carcinogens when used singly, may have additive or even synergistic effects when used in combination. An example of co-carcinogenesis is the combination of UV irradiation and nitrogen mustard.[275]

Thus, carcinogens can cause DNA defects that can lead to faulty DNA synthesis thereafter, which may eventually lead to carcinomas. In normal epidermis, however, DNA repair is continuously taking place. Several enzyme systems can identify and excise the faulty strands and have a preventative effect in carcinogenesis. In some diseases these mechanism are defective. This is the case in xeroderma pigmentosum, an autosomal recessive disorder, where patients have a 1000 times greater incidence of UV-induced skin cancer.

### 1.9.3 Carcinogens

*Chemicals*

Several chemicals are known to be initiator, promotors or complete carcinogens. In general, cancer induction by chemicals is dose dependent. However, there are interspecies differences with respect to the type of tumor they can induce. Typical initiators are nitrosamines, alkylating agents, and polycyclic aromatic hydrocarbons, such as dimethyl benzanthracene, methylcholanthrene and benzpyrene. Well-known promotors are dithranol, benzyl peroxide, and TPA.[278] However, in humans the promoting potential of dithranol does not seem to play an important role in carcinogenesis, if such a role even exists in humans, as in clinical practice during more than 80 years no increase in carcinogenicity has been observed in dithranoltreated patients so far.[18]

*UV irradiation*

UV irradiation is probably the most important cutaneous carcinogen. As mentioned before, UV radiation is a complete carcinogen with most active wavelengths in the UVB range (290-320 nm). UV light may produce free radicals that cause alterations in DNA. UV irradiation causes immunosuppression, which may play a further role in cutaneous carcinogenesis.[279] UV exposure in human leads to both structural and functional damage to the Langerhans cell, a cell believed to play an important role in antigen presenting in the epidermis. This temporary down-regulation of the immune system may allow genetically altered keratinocytes (that normally are recognized as abnormal) to pass unrecognized.

*Viruses*

The carcinogenic potential of viruses has been discovered the last few decades. To date there are two groups of viruses associated with cutaneous carcinogenesis: the human papillomaviruses (HPV), and retroviruses, such as the human T-cell leukemialymphoma virus group. The latter group is of particular interest in the origin of cutaneous lymphomas. The papillomavirus group comprises over 80 identified genotypes of HPV. Only a few genotypes are associated with carcinogenesis. The E region of the HPV genes code for two particular genes, E6 and E7e, which respectively bind to p53 and retinoblastoma (RB) protein. HPV viruses can increase the risk of malignant transformation by inactivation of wild-type p53 or RB, and this leads to a loss of contact inhibition and immortalization of keratinocytes.[280;281]

The best known HPV genotypes that are associated with cancer are HPV-16 and HPV-18. Both viruses are highly related to genital cancer, in particular cervix carcinoma.[282;283] HPV-5 and HPV-8 are important in the development of squamous cell carcinomas arising on epidermodysplasia verruciformis. HPV-5 and HPV-8 are also hypothesized to play an etiological role in (pre)malignant tumors in immunosuppressed organtransplant recipients.

### 1.9.4 Oncogenes

Oncogenes can simply be defined as virally or mutational activated cellular genes that stimulate proliferation. Oncogenes are frequently the result of an activating mutation in some normal cellular genes. These genes are designated as proto-oncogenes. Such a mutation has a dominant effect, as, from genetic point of view, only one copy of the two alleles needs to be mutated. When proto-oncogenes are transformed to oncogenes by mutations, carcinogenesis is stimulated.

Several other proto-oncogenes have been hypothesized to be associated with non-melanoma skin cancer (NMSC). The ras, fos, and myc proto-oncogene families are probably the best known. Ras mutations are common in murine chemical carcinogenesis of the skin. In human skin the Harvey ras gene, also known as Ha-ras, has been the subject in several studies in NMSC. However, ras mutations show a wide scatter between 0 and 30% for basal cell carcinoma,[284-288] and between 0 and 46% for squamous cell carcinoma.[285;287;289-293] Pelisson *et al* suggested that ras and myc oncogenes are involved in skin carcinogenesis and that these oncogenes may even cooperate with HPV infections in carcinogenesis.[294] They found higher Ha-ras DNA amplification in squamous cell carcinoma than in actinic keratoses. In normal skin myc and ras mutations were not detected. Moreover, no significant differences were observed in the detection of these oncogenes between transplant recipients and non-immunocompromized patients in this study.[294] Pierceall *et al* designated some mutations in Ha-ras to UV-irradiation, as these mutations occurred at high frequency in NMSC found in sun-exposed areas and were located opposite C-C sites.[295]

However, experiments with targeted overexpression of Ha-ras and c-fos only induced hyperkeratosis and papillomas but never malignant progression, while overexpression of the E6 gene of the HPV-18 virus caused the induction of verrucous lesions that sometimes progressed to squamous papillomas. These, upon analysis, were found to harbor an activating Ha-ras gene mutation. It has been proposed that activation of oncogenes is not an early event in the genesis of NMSC, but could be important in the later phase when a tumor progresses from a slow growing carcinoma in situ to a rapidly expanding malignant tumor. However, Ha-ras mutations have also been found in premalignant lesions. The exact role of oncogenes has to be elucidated.

### 1.9.5 Tumor suppressor genes

In the human genome several genes are involved with the regulation of controlled proliferation and differentiation. These genes are of major importance in normal cellular growth and in the prevention of malignancies. Therefore, they are called "tumor suppressor genes". However, if these genes become structurally altered by point mutations, amplification or translocation to a different position on a different chromosome, their normal role of assisting in controlled growth and differentiation may be lost, leading to uncontrolled growth, lack of differentiation, and malignancies.

The most important human tumor suppressor gene is p53. This gene, when unaffected and functioning normally, is the 'wild-type' p53. It controls cell proliferation at critical points in the cell cycle and prevents cells with damaged DNA from entering. Therefore p53 functions as the 'guardian of the genome'. P53 can act both in the G1 and G2 phase of the cell cycle. P53 is believed to be able to shut down the cell cycle when faulty DNA is detected. When the cell cycle is stopped, enzymes have time to repair the faulty DNA, or when such is impossible, to eliminate the cell from the cycling pool by directing the damaged cell along the pathway of apoptosis (see paragraph 1.9.6). By these mechanisms duplication of mutant DNA is prevented. The importance of p53 can also be learnt from the Li Fraumeni syndrome, which is characterized by childhood occurrence of various malignancies. In this syndrome patients often have a germline mutation in one p53 allele, which, after loss of heterozygosity (LOH) in the opposing allele carrying the wild-type p53 gene, may lead to a high incidence of various types of cancers. However, patients with the Li Fraumeni syndrome do not show an excess of NMSC.[296] In transgenic mice where the p53 alleles were knocked out, an increased tumor incidence is found.

Abnormal expression of p53 is common in basal cell carcinomas and squamous cell carcinomas,[297-302] and in premalignant disorders, such as Bowen's disease and actinic keratoses.[303;304] In many instances this increased expression is overexpression of wild-type p53. Wild-type p53 protein expression is increased in human skin following exposure to even doses of ultraviolet radiation too small to induce erythema.[305;306] The largest p53 protein expression has been noted by UVB-light in experiments where equierythemal doses for UVA, UVB, and UVC-light were used.[305] It is assumed that the upregulation of wild-type p53 protein expression in response to UV-radiation is a direct result of DNA damage, although more modest increases are seen following a range of stimuli that are not known to cause DNA damage.[306]

Mutant p53 seems to play an important role in NMSC as p53 mutations have been detected in 20-60 % of basal cell carcinomas,[285;297;307;308] and in 10-90 % of squamous cell carcinomas.[285;288;304;308-312] A specific type of mutation, the CC→TT mutation, is strongly associated with UV exposure. Another mutation, the C→T mutation, is likely related to UV irradiation. Interestingly, these mutations have been found in p53 in actinic keratoses and in cutaneous squamous cell carcinomas,[304;309] but not in p53 from other types of (non-cutaneous) malignancies. Furthermore, inactivated p53 in murine skin reduced the number of sunburn cells following UV-irradiation, implying a reduction in

apoptosis, and a subsequent increase in DNA-damaged cells retaining in the cycling pool.[304] These data provide evidence for an important role of UV-light in p53 associated cutaneous carcinogenesis. A correlation between the presence of p53 mutations, and the morphology and growth patterns of the corresponding squamous cell carcinomas has been demonstrated.[313]

### 1.9.6 Apoptosis

Cell death is not exclusively a pathological mechanism, as cell death plays an important role in homeostasis of living tissues in multicellular organisms. A balance between cell proliferation and cell death maintains homeostasis. Two distinct forms of cell death have been described: necrosis and apoptosis.

Necrosis is a passive and pathological form of cell death resulting from acute cellular injury. Necrotic cells are typified by swelling of cells and organelles, blebbing, vacuolization, and lysis. Cell death by necrosis leads to release of potentially toxic and immunogenic intracellular contents into the surrounding tissue, as necrosis is associated with loss of cell membrane integrity. Next the cytoplasmic contents invoke an inflammatory reaction.

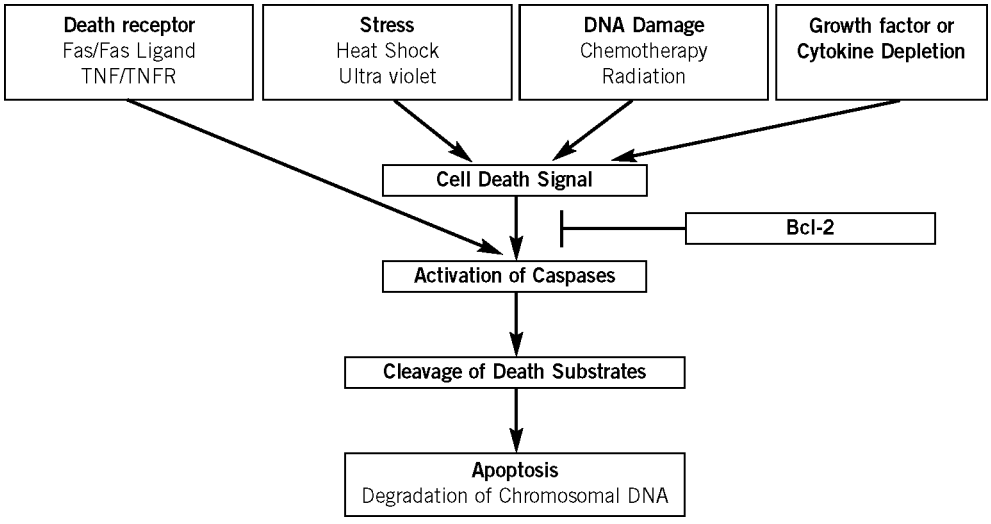
Apoptosis is a functionally different mechanism of cell death. It represents an active and physiological process, by which the nucleus and cytoplasm shrink and often fragment.[314-316] This is followed by phagocytosis by macrophages and other cells. The phenomenon of apoptosis was observed by Wylie *et al*,[317] however, the term apoptosis was introduced by Kerr *et al*.[318] Apoptosis is also known as 'programmed cell death', a term derived from developmental biology. In apoptosis, the nucleus chromatin becomes pyknotic and packed into smooth masses applied against the nuclear membrane. The nucleus breaks up into small pieces (karyorhexis). Finally, the cell itself shrinks and breaks up into membrane-enclosed fragments called 'apoptotic bodies'.[319;320] These apoptotic bodies are recognized and rapidly phagocytosed by neighboring cells including keratinocytes or macrophages. These are effectively degraded and removed within the lysosomes of these cells. Important is the fact that removal occurs before lysis, so the release of intracellular contents from the apoptotic cells into the surrounding tissue is prevented and therefore an inflammatory response, as seen in necrosis, does not occur. The whole apoptotic process takes as long as 1-3 hours in lymphocytes but may last up to 72 hours in keratinocytes.[321] In contrast to necrosis, apoptosis is a process that usually affects individual cells in a more scattered pattern.

Apoptosis is an evolutionary conserved genetic mechanism from insects to mammals where several genes that actively are involved in activating or suppressing apoptosis.[322] The process of apoptosis comprises 3 different phases: initiation, execution, and degradation.[323] During the initiation phase a variety of extrinsic and intrinsic factors can induce or inhibit apoptosis (**Table I**). At some point the apoptosis-inducing factors activate inducers of apoptosis, controlled by several regulators, then the decision to die is defined. This is the execution phase. The execution phase is followed by the degradation

**Table I:**
Factors which induce or inhibit apoptosis. (Modified from Teraki et Shiohara[518]).

| Inducers | Inhibitors |
|---|---|
| TNF-α/FasL | Growth factors |
| Perforin/Granzyme | Cytokines (IL-2, IL-3, IL-4, IL-6) |
| TGF-β | CD40 ligand |
| Glucocorticoids | Zinc |
| Retinoids | Estrogen |
| Ceramid | Androgens |
| Vitamin D3 | Virus genes |
| Zinc deficiency | Calplain inhibitors |
| Calcium | Cystein proteinase inhibitors |
| Growth factor withdrawal | |
| Heat | |
| Cold | |
| Virus | |
| X-ray | |
| UV-radiation | |
| Chemotherapeutic agents | |

**Figure 10:**
Schematic overview of a variety of pro-apoptotic stimuli inducing cell death signals that can trigger the central executioner (e.g. caspases) leading to cleavage of death subtrates and finally resulting in apoptosis. Fas/Fas Ligand and TNF/TNFR can bypass regulation by Bcl-2 and can directly activate caspases. (Adapted from Teraki et Shiohara[518])



phase, in which the activation of the central effector of apoptosis takes place. Here the different private pathways converge into a few common pathways leading to the activation of several caspases, catabolic enzymes (mostly proteases). In the final stage the typical changes of the cell can be seen. In contrast to the initiation phase and the execution phase, the degradation phase is similar in all kinds of cells. The Bcl-2 family of dimerizing proteins controls this pathway. However, members of the TNFR family bypass the regulation of Bcl-2 and can directly activate caspases **(Figure 10)**.

In the regulation of apoptosis many factors are involved. Two factors have been extensively investigated: Fas and Bcl-2. Fas (also known as APO-1 or CD95) is a transmembrane receptor. Binding of its ligand, Fas L, to the Fas receptor leads via a number of caspases and other proteins to apoptosis of the Fas-expressing cell. Fas can be expressed by a variety of cells, including skin, liver, ovary, heart, and lung. Expression of Fas L, however, is more limited to mainly activated T-cells and neutrophils. Fas/Fas L interactions control the peripheral lymphocyte life span and thereby participate in peripheral elimination of no longer needed lymphoid populations. However, certain tumor cells also express Fas L and are capable of killing other cells that express Fas (e.g. cytotoxic T-cells), thus providing malignant cells with resistance to tumor immunity. Several viral infections can lead to increased Fas and/or Fas L expression and increased sensitivity to Fas/Fas L-dependent apoptosis.[324] Other ways of virally infected cells to escape destruction by this Fas/Fas L mechanism comprise of clearing Fas from the cell surface or the encoding of Bcl-2 homologues.

Bcl-2 is a proto-oncogene that has apoptosis inhibiting properties. It prolongs the survival of cells, even in the presence of stimuli such as chemotherapeutical agents, irradiation, TNF and transfection with p53 or c-myc. Thus, genes that inhibit Bcl-2 can stimulate apoptosis. Many tumors act by Bcl-2 involved mechanisms to escape cell death. Many Bcl-2 related Bcl family members are co-expressed in the same cells and can inhibit but also activate apoptosis, so the ratio of anti-apoptotic versus pro-apoptotic proteins may determine whether a cell will undergo apoptosis or not.[325;326]

The best-known histological examples of apoptotic cells in the skin are sunburn cells. These cells are seen in the epidermis as small cells with a very shrunken nucleus and bright pink cytoplasm and can be seen after UV-exposure. In UV-exposed skin p53 is upregulated, and, as shown in paragraph 1.9.5, can push damaged keratinocytes in the direction of apoptosis, thus preventing them from becoming malignancies.[327] UV-irradiation induces both Fas and Fas L. In psoriasis Fas L induction by UV-light has a beneficial effect that may lead to the destruction of fas-bearing T-cells,[328] whereas in NMSC Fas L expression may prevent these tumor cells to be eliminated by the immune system. Apoptosis not only plays a role in the pathogenesis of skin diseases, but possibly also in normal homeostatic terminal differentiation of the keratinocytes in healthy skin. Terminal differentiation has been postulated to be a special form of apoptosis because of the typical apoptosis-associated features in terminal differentiating cells, such as endonuclease activity and DNA fragmentation.[329] Proliferation of keratinocytes may be regulated by apoptotic cell death in order to maintain a constant thickness of the epidermis.

Keratinocytes undergo apoptosis by several mechanisms.[325] Fas-expressing keratinocytes can become apoptotic by binding to Fas L-expressing cytotoxic T-cells. Apoptosis can also be induced via the release of effector cell granules, such as granzyme B and perforin that can activate caspase family members. CD8+ T-cells and NK (natural killer) cells partially use this mechanism for apoptosis. TNF released from mast cells and activated T-cells in the skin may have similar potentials. However, keratinocytes have been mentioned to be able to produce granzyme B, perforin, and Fas L, which can be used to protect the epidermis from pathogens and immune-mediated damage.[330] By expressing Fas L keratinocytes may destruct Fas-expressing keratinocytes (e.g. due to viral infection, drug exposure or malignant transformation) and thus contribute to the elimination of abnormal keratinocytes. In psoriasis (see Part I), however, there is no microscopic evidence for the presence of apoptotic keratinocytes, despite the expression of Fas in lesional skin keratinocytes. Therefore, further studies on the apoptotic mechanisms in psoriasis are indicated.[331]

In non-melanoma skin cancer apoptosis is likely to be of importance. In basal cell carcinomas a high mitotic activity if found, despite a low growth profile. Apoptosis may be the explanation for this discrepancy, as apoptotic cells normally outnumber proliferating cells in BCC.[314] Bcl-2 may play a role in BCC, as has been opted by several studies. BCC cells express Fas L, but not Fas, which may allow tumor growth by destroying Fas-bearing activated T-cells.[332-334] Interferon-α treatment is highly effective in BCC and may work by this Fas/Fas L mechanism, as in interferon-α treated patients the BCC cells also express Fas. Increased proliferation rather than decreased cell death is responsible for the more rapid growth profile in squamous cell carcinoma, which is in contrast to BCC. Spontaneous regression can occasionally be seen in many skin tumors where apoptosis may play an important role. Keratoacanthomas (KA) have been proposed to be a special subgroup of squamous cell carcinomas where the immune system is capable of destructing the tumor and complete regression is the outcome. In regressing KA apoptosis can be observed indeed. In the proliferative stage of KA Bcl-2 is frequently expressed, whereas in the regressive stage such is rarely seen.

# 1.10

## Premalignant skin disorders

The field of premalignant skin disorders comprises many subtypes, such as cutaneous horn, Bowen's disease, erythroplasia of Queyrat, leucoplakia (of mucosal surfaces), actinic porokeratosis, and actinic keratosis. This thesis mainly deals with actinic keratoses, probably the most frequent premalignant skin disorder in dermatology. In the next section actinic keratoses will be discussed more detailed.

### 1.10.1 Actinic keratoses

Actinic keratoses are erythematosquamous and often hyperkeratotic lesions occurring in sun-exposed adult skin, which carry a risk of progression to squamous cell carcinoma. Actinic keratosis (AK) was first identified as 'keratoma senilis' by Freudenthal in 1926. In 1958 Pinkus introduced the term 'actinic keratosis'. Actinic keratoses are also known as solar keratoses or senile keratoses.[275]

Apart from the sunlight-induced actinic keratoses, several other types of keratoses exist that strongly resemble actinic keratoses. These keratoses are associated with specific external factors and comprise postionizing radiation keratoses, tar keratoses, and arsenic keratoses. In this thesis only the sunlight-induced actinic keratoses will be discussed.

#### *1.10.1.1 Epidemiology*

Actinic keratoses are especially seen in elderly and fair-skinned people with a history of significant sun exposure.[277] The global prevalence of these lesions varies strongly. In Western Europe, in Wales, the prevalence for actinic keratosis was 23 %.[335] In Queensland, Australia, actinic keratoses are most common with prevalence up to 60 %.[336] In African blacks, however, actinic keratoses are rarely seen. In immunodeficient and immunosuppressed patients a higher prevalence of actinic keratoses is common.

#### *1.10.1.2 Etiology and pathogenesis*

As mentioned in paragraph 1.9.2.2 the 3-stage model of carcinogenesis is believed to be the mechanism involved in carcinogenesis of the skin. The most important etiological factor for actinic keratosis is UV-light. UV-light is a complete carcinogen, as it has both initiating and promoting capacities. It can damage DNA, including the relevant proto-

oncogenes and tumor suppressor genes, leading to disturbed cell proliferation and carcinogenesis.

Impaired DNA repair mechanism is another factor that is important in the onset of actinic keratoses. Immunosuppressive agents such as azathioprine inhibit DNA synthesis and therefore impede DNA repair.[337] Transplant patients need to take lifelong immunosuppressive drugs in order to prevent from graft rejection, but a side effect of this immunosuppressive state is the formation of a variety of benign and malignant skin tumors. In paragraph 1.12 the skin-related problems in renal transplant recipients will be discussed in detail. Also in some genetically determined disorders, including xeroderma pigmentosa and Bloom's syndrome, DNA repair is impaired.

Genetic factors are important in the development of actinic keratoses. Caucasians with blond hair and blue eyes are more susceptible to solar-induced injury.[277] An association with the skin-type is obvious as pigmented skin prevents from development of actinic keratoses.

Actinic keratoses are not seen in childhood. They appear in midlife and its prevalence rises with age. This is partially due to a higher total sunlight exposure over the years, but other agerelated factors may play a role, such as the decrease in epidermal thickness in elderly persons.

Human papillomavirus (HPV) infections have been of particular interest in carcinogenesis since the last two decades. Especially in grafted patients, but also in immunocompetent patients, it has been suggested HPV DNA is involved in carcinogenesis.[338-343]

Radiotherapy, although therapeutically also effective in actinic keratoses, is another etiological factor in the long-term development of actinic keratoses, due to its DNA-destructive properties.

#### *1.10.1.3 Clinical aspects*

The formation of a typical actinic keratosis is preceded by a local collection of telangiectatic capillaries. This initial sign is often unnoticed by the patient. Once the lesion will demonstrate sandpaper-like scaling with dry, rough, adherent and often yellow- or brown-colored scales (keratosis) on a hyperemic base, one can speak of an actinic keratosis. The scale can be picked off with difficulty, often leading to small bleeding points. Actinic keratoses are mostly well demarcated with sharp edges. They tend to be multiple. Itching, ulceration, and induration may occur.

Actinic keratoses are mostly found on sun exposed areas. Predilection areas are the backs of the hands and forearms, the face, the upper chest, and the lower legs. The cheeks, temples and forehead are more commonly affected than the lower parts of the face. The ears and vermilion of the lip (actinic cheilitis) are also frequently affected, especially in men.

Actinic keratoses can transform into a squamous cell carcinoma (SCC). The like lihood of a fully developed SCC evolving from a given actinic keratosis varies depending on the number of risk factors present. It has been estimated to occur at a rate of 0.075 % to 0.096 % per lesion per year,[344] up to values of even 20 % per lesion per year.[345] Despite

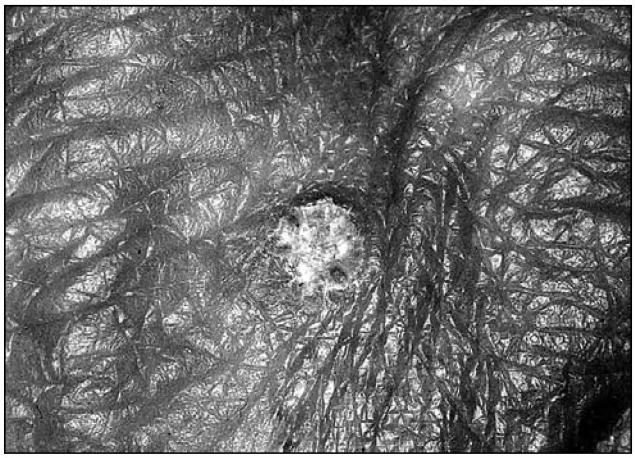


**Figure 11:**

An actinic keratosis between the metacarpophalangeal joints of the hand featuring typical (sharply) demarcated hyperkeratosis at an erythematous base.

its premalignant potential, actinic keratoses may regress spontaneously, especially in the earliest stages. So far there are no parameters available that predict which specific actinic keratosis will progress to squamous cell carcinoma and which actinic keratosis may regress spontaneously, [346] but lesions are at particular risk when they are indurated and/or ulcerated.

#### *1.10.1.4 Histopathology*

Histologically, five types of actinic keratoses can be distinguished: hypertrophic, atrophic, Bowenoid, acantholytic, and pigmented. Multiple types can be found in a single subject and even in a single actinic keratosis combinations of these types can be seen sometimes. In general, actinic keratoses are characterized by hyperkeratosis and parakeratosis, often in columns, with a thin or missing granular layer, some acanthosis, and the presence of actinic elastosis and inflammatory cells in the dermis. The interpapillary ridges may be reduced in number and broader than normal. The affected zone tends to grow under the normal epidermis and around the epithelial ducts, and may separate from it by a cleft or lacuna, resembling Darier's disease. The basement membrane, however, is still intact but basaloid cells may form multiple buds at the junction. In the dermis the papillary vessels are irregularly increased. Variable degeneration of dermal collagen and deposition of material staining like elastin is seen in the upper half of the dermis. A lymphoid infiltrate is seen beneath the lesion.

The transition from AK to SCC is heralded by acanthosis, by the extension of atypical cells into the dermis with ultimate detachment from the surface and by autonomous growth. There is an association with capillary proliferation.

The histological types of actinic keratoses that are summarized above are all characterized by atypical keratinocyte proliferation in the epidermis, but this does not say

anything specific on their malignant potential. Recently, the KIN grading system for actinic keratoses has been proposed that is based on clinical as well as histological data and that is aimed at describing the degree of atypical keratinocyte proliferation.[344] This grading system is analogous to the scoring system used in evolving carcinoma of the uterine cervix (cervical intraepithelial neoplasia or CIN score). It is based upon the vision that actinic keratoses are malignant neoplasms in evolution (keratinocytic intraepidermal neoplasia, KIN) and that they demonstrate histologic and molecular genetic features of malignancy. However, it remains to be seen whether the KIN score may represent a new prognostic parameter for predicting the risk of progression to squamous cell carcinoma.

The KIN score is based upon 3 different levels: **Grade I** lesions are flat, pink macules on solar damaged skin, without roughness or hyperkeratosis. Lesions are often subclinical. Histologically, focal atypia of the keratinocytes is seen, but is not exceeding the lower one third of the epidermis. **Grade II** lesions are pink to red with a hyperkeratotic surface and variable induration. Histologically, focal atypia of the keratinocytes remains limited to the lower two thirds of the epidermis. Common features comprise alternating orthokeratosis and parakeratosis, among others. **Grade III** lesions are red, scaly and indurated plaques. Histologically, atypical keratinocyte proliferation spreads throughout the full thickness of the epidermis. All features of actinic keratoses can be seen. KIN III lesions are currently referred to as squamous cell carcinoma in situ or Bowen's disease.

#### *1.10.1.5 Immunohistochemistry*

There is only a small number of studies available in actinic keratoses where epidermal proliferation, keratinization, differentiation, and apoptosis-associated parameters have been investigated by immunohistochemical analysis. Considering proliferation, a high number of proliferating epidermal cells is found in the lower part of the epidermis, as demonstrated by the proliferation parameter Ki67; and most of them are atypical cells.[347] In a study by Caldwell *et al*, the proliferation index (PI) of actinic keratoses appeared to be nearly 3 times higher than the PI of normal skin, and is intermediary in between the PI for psoriasis and the PI for poorly differentiated squamous cell carcinomas; the latter having the highest PI.

With respect to differentiation parameters keratin 1 and 10 a decreased expression was found in hyperkeratotic areas of actinic keratoses, whereas in the atrophic areas the staining pattern was similar to that in normal epidermis and in well-differentiated squamous cell carcinomas.[348] Hyperproliferation-associated keratin 16 is usually not present in normal skin, but in actinic keratoses, like in psoriasis, it is predominantly expressed in the suprabasal cell layers.[349]

Keratin 13 and keratin 19, retinoid-treatment-associated and embryonic keratins, have previously been found in squamous cell carcinomas, but not in actinic keratoses.[350;351] In Bowen's disease keratin 19 has been found incidentally and mainly in poorly differentiated areas.[348] Therefore, it has been suggested that these simple epithelial keratins are markers for malignant transformation in human epidermis. In mice, keratin 13

expression has already been identified to be a good marker for malignant transformation and skin tumor progression in papillomas.[352;353]

Filaggrin and involucrin expression can be variable in actinic keratoses, but there appears to be no distinct pattern from normal skin and squamous cell carcinoma.[354;355] Involucrin is expressed already in the upper stratum spinosum.[347] There are no data available on transglutaminase staining in actinic keratoses, but in squamous cell carcinoma, depending on the degree of tumor differentiation, transglutaminase expression is disturbed and generally an increase in transglutaminase is seen. The latter is also the case in psoriasis.[356]

P53 protein has been detected in up to 85% of actinic keratoses in several studies.[357;358] It is abundantly expressed throughout the lower regions of the epidermis. Superficial keratinocytes are often spared and the granular cell layer is negative for p53 staining. Nuclear labeling is variable both in intensity and in distribution within a given lesion, but is mostly diffuse.[357] A correlation between the pattern of p53 expression and histological subtype of actinic keratoses (e.g. atrophic, Bowenoid, and hyperkeratotic) has been demonstrated. Diffuse p53 expression is especially seen in hyperplastic and Bowenoid actinic keratoses; focal expression is common in the other types. The adnexa are mostly negative for p53 staining. Atrophic foci often exhibit a stronger and more diffuse p53 labeling.[357] A significant positive correlation with MIB-1 and with the number of atypical cells has been demonstrated.[358;359] Overexpression of p53 in actinic keratoses might be associated with stabilization of wild-type p53 protein as well as p53 gene mutation.[360] Accumulation of p53 could also be due to enhanced synthesis in response to DNA damage.[361] Abnormal p53, either mutant or stabilized p53 protein, probably loses its normal function and might induce or stop prohibiting proliferation or tumorigenesis of the cells. Sim *et al* have shown that p53 expression in actinic keratoses is mainly due to accumulation of mutant-type p53 protein rather than wild-type p53.

#### *1.10.1.6 Treatment*

*Destructive measures*

Destructive measures are the standard of care for treatment of actinic keratoses.[362] Many effective approaches are available depending on factors, such as, the aspect, the number, and the localization of the lesions. Cryotherapy is among the oldest treatment regimens and is considered the standard method of destructive therapy for these lesions.[363] Superficial lesions are easily removed by rapid freezing with liquid nitrogen, but even hyperkeratotic actinic keratoses do often respond well to cryotherapy, especially when applied 2 or 3 times repeatedly.

Other destructive therapies are curettage, desiccation and electrocoagulation. These are all equally effective as cryotherapy, but more likely to leave superficial scaring. Therefore, these approaches should be considered when other therapies are not effective enough.

Biopsy or surgical excision is the therapy of choice for indurated actinic keratoses, so pathological examination of the base can be performed to exclude early invasive squamous cell carcinoma. These approaches are especially indicated for solitary actinic kera-

toses. For multiple actinic keratoses on larger body surface areas other therapies are preferable.

*Topical retinoids*

Topical retinoids are a popular treatment for actinic keratoses. Many articles have been published on topical retinoids in premalignant disorders and photodamaged skin.[345;364-390] In general, topical retinoids improve photodamaged skin clinically and histologically. With respect to the more advanced actinic keratoses most studies report beneficial clinical effects, although several studies do not confirm its therapeutic potential. From histological and especially immunohistochemical point of view little information is available to strengthen the overall clinical beneficial impression. In the subpopulation of renal transplant recipients no histological and immunohistochemical data on topical retinoids are available. A well-known side-effect of topical retinoids is the irritant effect on the skin. This "retinoid dermatitis" is frequently seen and may limit the use of these topical agents. However, many studies report that retinoid dermatitis will subside with continued treatment several weeks after initiation. The exact mode of action of retinoids remains to be elucidated.

All-trans retinoic acid (ATRA) or tretinoin has been used for some decades now and is by far the most frequently used topical retinoid for photodamaged skin and actinic keratoses. Applications with doses varying from 0.01 to 0.3 % have been reported to be effective, although its efficacy is less than with standard destructive procedures.

Partial efficacy has been observed with topical isotretinoin 0.1 % in a cream base. In a placebo controlled study 66 % of the patients had a more than 30 % reduction in the number of actinic keratoses for lesions on the face. However, no significant drug effect was seen for lesions on the scalp or upper extremities.[371]

Tazarotene has been registered recently for acne and psoriasis and has been reported to be effective in basal cell carcinoma.[391] Whether Tazarotene may have some effect in actinic keratoses, remains to be elucidated.

*Topical cytotoxic agents*

Topical cytotoxic agents can be used in the treatment of actinic keratoses and other premalignant skin disorders. Topical 5-fluorouracil (Efudix®) has been extensively studied in actinic keratoses.[392-394] This fluoridated pyrimidin acts as an anti-metabolite. An effective treatment regimen is twice daily application for about 4 weeks. During this treatment period the epidermis becomes erosive, ulcerative and necrotic and patients may complain of worsening of the lesions, which can lead to non-compliance. After the treatment period the skin surface will heal again with renewed epithelialization and, idealically, the actinic keratoses will have disappeared. To improve compliance, 5-fluorouracil can be applied four times daily for shorter periods of time, resulting in clearing of actinic keratoses and patient acceptability.[395] Topical 5-fluorouracil is a highly effective and frequently used approach for actinic keratoses nowadays, however, its use is limited to skin surface areas of about 500 $cm^2$ per treatment. Therefore, especially in renal trans-

plant recipients with often very large areas involved with actinic keratoses, other therapies may be necessary.

*Photodynamic therapy*

Photodynamic therapy (PDT) is a rather new effective method in which actinic keratoses are pretreated with a photosensitizer, 5-aminolevulinic acid (5-ALA), followed by illumination with (broad-spectrum) visible light, leading to destruction of epidermal cells.[396-398] Application of 5-ALA leads to the accumulation of the endogenous photosensitizer protoporphyrin IX (Pp IX) in epidermal cells. Conversion of 5-ALA to Pp IX occurs in skin cells by enzymes in the haem biosynthetic pathway. Rapidly proliferating cells, such as those in actinic keratoses, convert more 5-ALA to Pp IX than do less rapidly proliferating normal epidermal cells.[399] Moreover, since 5-ALA in aqueous solution passes readily through abnormal keratin, but not through normal keratin, the topical application of 5-ALA in aqueous solution to actinic keratoses or superficial basal cell or squamous cell carcinomas induces Pp IX photosensitization that is restricted primarily to the abnormal epithelium. Subsequent exposure to photoactivating light selectively destroys such lesions. Pp IX photosensitization can be induced in cells of the epidermis and its appendages, but not in the dermis. By choosing red (laser) light (630nm) for illumination a deeper penetration can be achieved. But pathologic changes in actinic keratoses are superficially placed in the epidermis and therefore fluorescent (non-laser) blue light is sufficient, especially while Pp IX is more efficiently photo-activated by blue light.[398]

Photodynamic therapy offers the advantages of being non-invasive, well tolerated in slow healing sites, and tissue sparing, leaving the surrounding normal skin intact and functional. Photodynamic therapy is especially effective in superficial actinic keratoses, such as atrophic and Bowenoid actinic keratoses. In general this treatment is less effective in hyperkeratotic actinic keratoses.

*Systemic retinoids*

In patients with widespread actinic keratoses, systemic retinoid therapy may be an option, especially in immunocompromized patients, where multiple actinic keratoses are the rule. Systemic retinoids clinically also improve hyperkeratotic actinic keratoses, probably by enhancing desquamation of the lesions via mechanisms that are not completely understood, leading to a decrease in thickness of the actinic keratoses.

Systemic retinoid treatment has become of interest since the 1980ies, when several clinical studies were undertaken with oral administration of isotretinoin, etretinate, and later on acitretin, in premalignant skin disorders.[400-410] Efficacy has been claimed for all three retinoids in several of these studies with highest efficacy rates for etretinate and acitretin. However, efficacy was often partial and recurrences after discontinuation were frequently seen.

Etretinate, in doses 50 – 100 mg/day, is effective in the clinical setting for actinic keratoses, both in immunocompetent and immunocompromized patients.[406;408] Side ef-

fects of systemic etretinate mainly comprise cheilitis, exfoliation, eye irritation, pruritus, and sweating. Cheilitis and exfoliation were reported in all patients and were often troublesome, but not sufficiently severe to necessitate stopping. These symptoms were most marked in the first month of treatment, and on reducing the dose the symptoms resolved in the majority of patients. After completing therapy all side effects resolved within a month.[408] Other adverse events like hyperlipidemia and elevation of serum transaminases can be seen and, importantly, teratogenicity.

Acitretin shares similar features with etretinate with respect to efficacy, but has a more favorable side-effect profile, especially with respect to teratogenicity, as the half-life time of acitretin is only 2 days, compared to 80-120 days for etretinate.[194]

Isotretinoin is especially used for severe acne. In actinic keratoses a combination of isotretinoin 20 mg daily and 5-fluorouracil is highly effective, but data on monotherapy with oral isotretinoin for actinic keratoses are not available. In Part III retinoids will be discussed in more detail.

*Other approaches*

Radiotherapy is a paradoxical treatment for actinic keratoses, as radiotherapy itself may induce the formation of these lesions after prolonged periods of treatment. However, in elderly persons with multiple actinic keratoses (and suspicion for Bowen's disease or even squamous cell carcinoma) in areas where surgery or other effective destructive treatments are difficult, radiotherapy may be an option.[411;412]

Repeated intralesional injections with interferon alpha or beta have demonstrated some efficacy in actinic keratoses, but topical application of interferon alpha was not

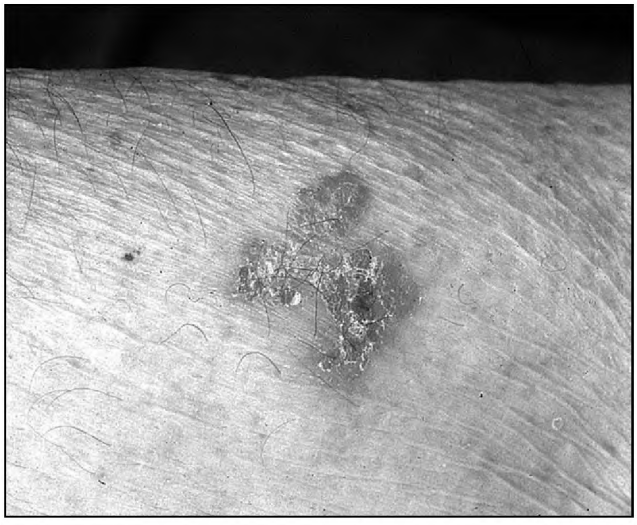


**Figure 12:**

Bowen's disease frequently presents as a progressive erythematosquamous plaque.

effective. [413;414] Although interferons are not a common treatment for actinic keratoses so far, their mode of action may be relevant for the development of new potential effective treatments. An example is imiquimod (Aldara®) that is registered for the treatment of genital and perianal warts, due to its immune response mediating properties. It mainly acts by induction of interferon alpha (IFN-alpha) and other cytokines in the skin, which stimulate several other aspects of the innate and acquired immune response.[415]

Chemical peelings have been used, as monotherapy or in combination with other treatments in actinic keratoses. These peeling agents comprise glycolic acid, lactic acid, trichloroacetic acid, phenol, and salicylic acid among other less frequently used peeling agents.[416;417] Chemical peeling promotes the regeneration of new, healthy skin and therefore improves actinically damaged skin and actinic keratoses.

A promising new therapy may be topical colchicines. Complete healing of actinic keratoses was observed in a pilot-study in 7 out of 10 patients with 1 % colchicine gel applied twice daily for 2 months.[418] No recurrence was observed after 2 months follow-up. First efficacy occurred already in the first week of treatment. Systemic signs of colchicines toxicity did not occur. However, further studies are necessary to define the optimal concentration and the mode of application.

Topical and systemic vitamin D3 analogues have been investigated in several (pre)-cancerous types of lesions and have been claimed to be effective.[419] However, no studies with vitamin D3 derivatives have been performed in actinic keratoses so far, but in this type of lesions efficacy may be expected as well.

*Prevention: sunscreens and clothes*

Last but not least, apart from therapeutic interventions, prevention of actinic keratoses is important to reduce the risk of developing new lesions with time. Especially in high-risk patients, such as immunocompromized patients and fair-skinned individuals who live in areas with a high exposure to sunlight. Encouraging patients to avoid (vivid) sunlight and to cover the skin with appropriated clothes and in some cases to wear a hat, is a first important step for prevention of further actinic damage. The application of sunscreens with a high sunlight-protecting factor (SPF) is a second sensible step.

### 1.10.2 Other premalignant disorders

The previous section dealt with actinic keratoses, the most common premalignant skin disorder. Here, I will briefly describe some other premalignancies that are frequently seen in the normal, but especially in the immunocompromized population.

#### *1.10.2.1 Bowen's disease or Bowenoid actinic keratoses*

Bowen's disease can be defined as a progressive, non-elevated, erythematosquamous or scaly plaque due to an intraepidermal carcinoma (carcinoma in situ). Bowen's disease is mentioned as a separate entity in literature, however, it may be regarded identical to KIN grade III actinic keratoses. Therefore, Bowen's disease may be part of a continuous spectrum that ranges from sun damaged skin to squamous cell carcinoma.[420]

The initial well-demarcated small, red and scaly lesion gradually enlarges, which may take several years, mostly without any subjective symptoms. Although Bowen's disease is usually flat, it may become hyperkeratotic or may ulcerate. The latter is a sign suggestive of development of invasive carcinoma. Lesions are often multiple and may be widespread.

Sunlight is the most important etiological factor and, therefore, these lesions are mostly found on sunlight exposed areas. Arsenic ingestion in the past also plays a role in its etiology.[421]

**Figure 13:**

A cutaneous horn resembles the horn of an animal and often looks quite surrealistic.

The main histopathological feature is proliferation of atypical keratinocytes throughout the whole thickness of the epidermis, but without invasion through the basement membrane. These atypical cells have hyperchromatic nuclei, often larger than normal, giving a freckled appearance to the epidermis. Giant forms of multinucleate cells are seen and mitotic figures may be present. Variable acanthosis is seen with increase in the length and thickness of the interpapillary ridges. The epidermal organization is disturbed and keratinocytes often prematurely keratinize and lose their intercellular connections. The papillary dermis shows an inflammatory infiltrate that is often quite dense. Sometimes the atypical proliferating cells may be surrounded by a relatively normal keratinocyte population, which is called 'Borst-Jadassohn appearance'. The atypical cells often grow down around and along the appendages like a collar and can become invasive. The epidermis above the appendages may even be normal. Parakeratosis is present on the surface.

Differential diagnostically the lesion must be distinguished from lichen simplex, superficial basal cell carcinoma, and erythematosquamous disorders such as psoriasis. Topical steroid treatment may be used for diagnostic purposes; if the lesion does not respond, Bowen's disease is highly suspected. Histological investigation (biopsy) may be necessary to obtain a proper diagnosis.

There are several therapeutic options for Bowen's disease. Recurrences are common, and may be due to extension of the carcinoma in situ around the appendage ducts that were not effected by treatment. Therefore, surgical excision is the best option if the lesion is not too large. Other remedies are cryotherapy, cauterization, topical cytotoxic agents (5-fluor-ouracil; Efudix®), and photodynamic therapy.[422]

#### *1.10.2.2 Cutaneous horn*

Cutaneous horn is strictly not a histological diagnosis, but a diagnosis obtained from clinical examination. Various underlying dermatological conditions, such as epidermal naevus, viral wart, keratoacanthoma, actinic keratosis, and squamous cell carcinoma may cause the typical horny aspect of this lesion. Of these underlying conditions, actinic keratoses were the lesions most commonly found in a large study (37.4%); less than 25% of the underlying disorders was malignant.[423] The lesion resembles the horn of an animal. Its consistency is mostly friable but the lesion can be very hard. It has a yellowish brown color and is often curved with circumferential ridges. The adjacent skin is often normal-looking. The base of a cutaneous horn may be erythematous, which is suggestive of malignant transformation. These lesions are mostly found on sunlight-exposed areas and can be solitary but may also be multiple. Signs of UV-damage of surrounding skin, such as solar keratoses, are often present.

A cutaneous horn is the result of dysplastic epidermal changes similar to those seen in an actinic keratosis. Histologically, the granular cell layer is often deficient or absent. In long-standing lesions there may be budding from the basal layer, indicating early development of a squamous cell carcinoma. Surgical treatment is indicated when an underlying squamous cell carcinoma is suspected.

# 1.11

## Squamous cell carcinoma

The field of skin cancer is very large and comprises non-melanoma skin cancers, melanoma skin cancers, and many dermal skin tumors. This thesis is focussed on actinic keratoses and non-melanoma skin cancers, especially squamous cell carcinomas. Therefore, this subtype will be discussed more detailed in the next paragraph.

### 1.11.1 Definition

Squamous cell carcinoma is a malignant epithelial tumor that may metastasize and arises from keratinocytes.

### 1.11.2 Epidemiology

The incidence of squamous cell carcinoma is related to geographical latitude and skin-type and is about 5-10 times lower than basal cell carcinoma. In sunny areas annual incidences up to 338 per 100,000 for males and 164 per 100,000 for females have been reported in Australia. In temperate climates, however, the incidence is much lower. Its

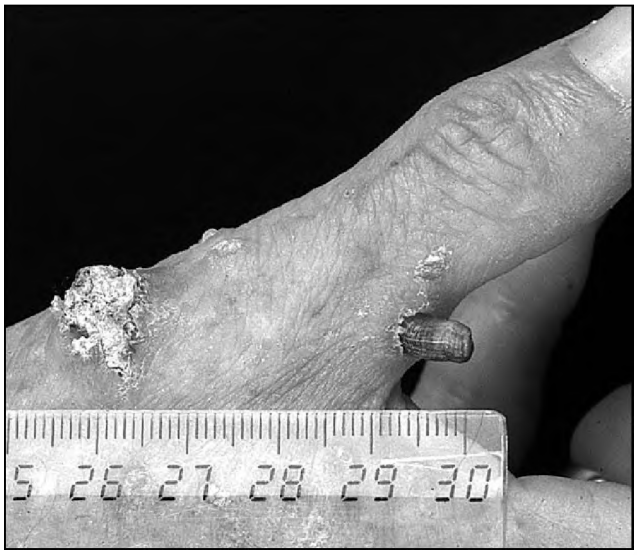



**Figure 14:**

A squamous cell carcinoma on the proximal thumb in a renal transplant recipient. Another squamous cell carcinoma on the distal thumb presented as a cutaneous horn.

incidence is positively related to albinism, but data on its relationship to vitiligo are contradictive.[424;425] Previous injury, burns, and radiant heat have been proposed as risk factors for the arising of squamous cell carcinoma.[426;427] A higher incidence is found in xeroderma pigmentosum patients and in long-standing chronic granulomas.

### 1.11.3 Clinical aspects

Squamous cell carcinoma frequently arises in actinically damaged skin. Predilection areas are the backs of the hands and forearms, the upper part of the face, the upper chest and, especially in males, the lower lip and the ears. The first sign of malignancy is induration. The tumor may present as a small, slightly raised and wart-like hyperkeratotic papule or plaque, sometimes with ulceration. The growth pattern is normally slower than in keratoacanthoma, but faster than in basal cell carcinoma. Initially, pain is uncommon and the tumor may remain indolent, but when growth progresses rapidly, the lesion may become painful. This is especially the case with ulcerating tumors. A hyperkeratotic crust that may shed often caps well-differentiated tumors, whereas less-differentiated tumors frequently have a nodular structure without an overlaying crust. The surface sometimes easily bleeds and the surrounding skin is inflamed. The presentation may be different on mobile structures, comprising a fissure, a small erosion or an ulceration that fails to heal.

The tumor may spread to soft tissues, cartilage or bones. It can spread along the nerves for considerable distances. Metastasis occurs primarily to the local lymph nodes. Metastasis to other organs occurs relatively late. Spread by the bloodstream is uncommon.[428] The frequency of metastasis varies with the grade of differentiation and the subtype; frequencies up to 7% have been reported in transplant recipients.[429] The 3-year cause-specific mortality in a group of 71 patients with metastatic SCC was 54%.[430] In immunocompromized patients squamous cell carcinoma behaves more aggressively and a higher percentage of the tumors metastasizes. Tumors arising in keratoses on the dorsum of the hand are particularly late in metastasizing. This in contrast to anaplastic squamous cell carcinoma, which may resemble a keratoacanthoma. This subtype quite early metastasizes in up to 50% of the tumors. Tumors of 2-3 centimeter generally can be cured in about 90% of cases. However, when squamous cell carcinoma occurs on mucosal tissues, like the vulva or tongue, the prognosis is worse.

### 1.11.4 Pathogenesis

Several etiological factors have been identified for squamous cell carcinoma, such as genetic factors, x-rays, chemical carcinogens, immunosuppression, viral infections (HPV), arsenic, tar, and the most important factor, sunlight or UV-irradiation. The mechanisms of carcinogenesis and the development of premalignant actinic keratoses that may transform to squamous cell carcinomas have been described previously in paragraphs 1.9.2.2 and 1.10.1.3.

### 1.11.5 Histology

When atypical keratinocytes breach the basement membrane, one can no longer speak of a premalignant lesion, such as Bowen's disease or actinic keratosis, but here is where squamous cell carcinoma starts. Thus, the distinction is based on architectural rather than cytological features. With respect to cellular aspects, squamous cell carcinoma cells may vary from large, well-differentiated polygonal cells with vesicular nuclei, prominent nucleoli, numerous tonofibrils in the cytoplasm, and well-developed intercellular bridges, to completely anaplastic cells, which lack a link to their cytological origin. The latter type is often seen in rapidly growing tumor margins.

Well-differentiated tumor cells tend to keratinize, as do normal suprabasal keratinocytes. Tumors that consist of these cells show areas of maturation that form parakeratotic horny pearls, but also dyskeratosis, with lacunae and lumina that contain shed rounded degenerating tumor cells. These tumors may have a pseudoglandular aspect and are called 'acantholytic squamous cell carcinomas'.[431;432]

Anaplastic tumors often show hyperchromatic nuclei, decreased eosinophilia, a decrease in cytoplasmatic tonofibril formation and intercellular adhesions, and an increased number of mitoses with abnormal mitoses.

In the dermis an inflammatory reaction is seen with lymphocytes, plasma cells, histiocytes, and mast cells, together with an increased number of blood vessels.[433] Most tumors penetrate the dermis as coherent strands and columns. In case a squamous cell carcinoma metastasizes, the metastasis normally features the same structure of strands and columns.

To estimate the risk of metastasizing, squamous cell carcinomas can be classified into 4 grades according to Broders. This classification is based on the number of undifferentiated tumor cells in proportion to the number of differentiated tumor cells. However, Broders' classification is somewhat obsolete nowadays, as the risk for metastasizing not only depends on the proportion of undifferentiated cells, but also on the volume and depth of the tumor.

**Table II:**
Population-based standardized incidence ratios of skin cancer in organ transplant recipients. (Adapted from Berg and Otley[430])

| Skin cancer | Increase in incidence |
|---|---|
| SCC | 65-fold |
| SCC of the lip | 20-fold |
| BCC | 10-fold |
| Melanoma | 3.4-fold |
| Kaposi sarcoma | 84-fold |

**Table III:**
Mechanisms of action and newer immunosuppressive medications. (Adapted from Berg and Otley[430])

| Medication | Mechanism of action |
|---|---|
| Cyclosporin A (Neoral®) | Inhibits transcription of interleukin 2 and enzymes essential to cytotoxicity |
| Azathioprine (Imuran®) | Inhibits proliferation of T- and B-effector cells by inhibiting nucleotide synthesis |
| Prednisone | Inhibits proliferation of T cells against antigens |
| Muromonab-CD3 (OKT3) | Acts as anti-T-cell immunoglobulin |
| Mycophenolate mofetil (CellCept®) | Inhibits nucleotide synthesis critical to T- and B-cell proliferation; similar to azathioprine |
| Tacrolimus (Prograf®) | Inhibits transcription; similar to cyclosporin A |
| Rapamycin (Sirolimus®) | Binds to immunophilins and inhibits cytokines; inhibits growth factor and transduction; potentiates cyclosporin A |

### 1.11.6 Treatment

As for many other types of tumors, surgery is the treatment of choice when the tumor can be excised by primary closure. Sometimes by means of additional plastic surgery. It is important to have a margin of at least 3mm from the visible and palpable tumor to minimize the risk of non-radical excision. Surgery is also the treatment of choice when cartilage or bone invasion has occurred or when lymph nodes are involved. Surgery makes an adequate histopathological examination possible for assessment of the radicality of the tumor.

Radiotherapy is an option in poorly-differentiated tumors, particularly when located on the head and neck and not being metastasized. Radiotherapy often leaves fine fragile scars, but radionecrosis may occur. Especially in elderly patients or in patients with contraindication for excision radiotherapy is an option.

For inoperable tumors and metastases of squamous cell carcinoma systemically administered bleomycin can be used. This cytotoxic drug can also be used in combination with other chemotherapeutic agents or in combination with radiotherapy or surgery. In some cases intralesional bleomycin may be tried.

# 1.12

# Skin lesions in renal transplant recipients

## 1.12.1 Introduction

Organ transplant recipients receiving immunosuppressive treatment are at risk for developing a broad spectrum of benign, premalignant and malignant skin disorders, as well as internal malignancies, such as lymphoma, leukemia, and lung tumors. The majority of cancers in this group of patients are derived from the skin. Organ transplant recipients have a 65-fold increased incidence for developing squamous cell carcinoma, which represents the most common malignancy in this population (**Table II**).[430] A 3- to 4-fold increased risk for developing malagnancies in general has been suggested for this population of organ transplant recipients.[434] Typically, this increased risk applies specifically to the high therapeutic doses of immunosuppression after organ transplantation, and not, for example, to immunosuppressive therapy for autoimmune diseases. In the department of dermatology of the University Medical Center Nijmegen a large number of immunocompromized renal transplant recipients visit the outpatient department on a regular base for dermatological follow-up and treatment of the lesions of the skin.

## 1.12.2 Immunosuppressive therapy

Renal transplant recipients use life-long immunosuppressive therapy to prevent rejection of the graft. Since the 1960s several treatment regimens have been used, such as prednisone in a dose of 7.5 to 10 mg per day in combination with azathioprine (Imuran®) 2 to 2.5 mg/kg/day, cyclosporin (Neoral®) in a dose based on the lowest blood levels, and relatively new approaches with mycophenolate mofetyl (Cellcept®) and tacrolimus (FK506, Prograf®) or sirolimus (Rapamune®). Brief courses of antithymocyte globulin (ATG) or antilymphocyte globulin (ALG) are sometimes used as well. **Table III** represents the most commonly used immunosuppressive medications. The development of carcinomas and other skin lesions is directly related to time from transplantation and the intensity of immunosuppression. In heart transplant patients, who generally experience a greater degree of immunosuppression than renal transplant recipients do, the development of skin cancer occurs earlier and lesions progress more rapidly.[435;436] The arising of carcinomas seems to be independent of the immunosuppressive regimen used. However, with respect to the newer approaches, no long-term follow-up data are available at the moment to strengthen this presumed hypothesis. Two mechanisms have been proposed for the accelerated carcinogenesis associated with immunosuppressive agents: decreased immunosurveillance resulting in uncontrolled proliferation of abnormal cells and a direct carcinogenic effect of the drug, including azathioprine and cyclosporin.[437] Immuno-

suppressive agents with powerful antilymphocytic activity, such as cyclosporin, ALT or ALG, may enhance viral oncogenesis because of the alteration of function- or elimination of T-lymphocytes.

A reduction in the level of immunosuppression may lead to a decrease in the number of internal and cutaneous malignancies.[438] Cessation of immunosuppression has been shown to result in a deceleration of cutaneous carcinogenesis within 1 or 2 years and to subjective improvement in skin quality with respect to hyperkeratotic lesions.[437] However, reduction or cessation of immunosuppressive treatment may harbor a greater risk of allograft rejection and is therefore generally not an option.

In the near future the use of immunosuppressive monoclonal antibodies may become more important in organ transplant recipients. The use of immunosuppressive monoclonal antibodies is actively being studied as a means of selectively impairing recipient immune activation against allograft antigens. These blocking antibodies, such as anti-CD40, anti-interleukin 2 receptor antibody, have shown interesting preclinical data with respect to effective immunosuppression without associated pharmacologic toxicities.[439;440]

### 1.12.3 Human papillomaviruses

Apart from the immunosuppressive state and well-known risk factors, such as UV-irradiation, genetic aspects, skin-type, age and radiotherapy, oncogenic viruses may play a role in the etiology of (pre)malignant skin disorders in renal transplant recipients. Several viruses have been identified to play a role in carcinogenesis, including Epstein-Barr virus, herpes simplex virus, herpes zoster virus and polyoma viruses. A special role is reserved for human papillomaviruses (HPV). HPV-16 and HPV-18 have been identified as carcinogenic in cervix carcinoma. In epidermodysplasia verruciformis (EV), a rare genetic disorder in which patients have defective cell-mediated immunity, HPV subtypes 5, 8, 9, 12, 14, 15, 19-25, 36-38, and 47 were found in squamous cell carcinomas and the skin of these patients is usually covered with multiple wart-like lesions.[441] In renal transplant recipients several HPV types, especially those types found in EV, may be involved in the carcinogenic process as well, as HPV-DNA is frequently found in squamous cell carcinoma.[340;442;443] Even normal skin in renal transplant recipients harbors at least one type of (especially EV-associated) HPV in nearly all patients.[444]

First reports on HPV-DNA in renal transplant recipients came from Pfister (1979),[445] and Lutzner (1980),[446] who discovered HPV-3, respectively HPV-5 in carcinomas of renal transplant recipients. Since then a large number of studies has been performed in premalignant and malignant skin disorders in both immunosuppressed and immunocompetent patients, revealing discrepancies in the prevalence and spectrum of HPV types in these lesions, basically due to different techniques used to detect HPV DNA in the skin. The largest study performed so far with the most comprehensive technique comprising 148 samples of both immunosuppressed and immunocompetent patients demonstrated no significant differences between the prevalence and spectrum of squamous cell carcinoma, basal cell carcinoma, and premalignant skin diseases in both groups. However, significant differences were seen as to the prevalence of HPV-DNA in these lesions

between both groups; in immunosuppressed patients this prevalence varies between 75 and 88.2%, whereas in immunocompetent patients much lower prevalences were found, ranging from 27.2 to 54.4.[447]

However, the presence of EV-associated HPV-DNA is not exclusive for (pre)-malignant lesions and normal skin in renal transplant recipients. In the hyperproliferative disease psoriasis EV-associated HPV-DNA is also frequently found and percentages up to 89.4% have been reported.[448]

Furthermore, in contrast to the well-known carcinogenic potencies of UV-irradiation, up to now no relationship between HPV status and p53 mutation could be established.[449]

In conclusion, despite the high number of HPV-DNA found in (pre)malignant lesions in renal transplant recipients, it remains to be established whether HPV plays a primary role in the evolution of non-melanoma skin cancer, or that it is an epiphenomenon.

### 1.12.4 Epidermal disorders

*Common warts and plane warts*

Viral skin lesions in transplant patients consist essentially of common and plane warts. Their prevalence increases with the duration of immunosuppression. One year after transplantation the occurrence of warts is seen in 15-42% of the patients.[450;451] Already after 5 years this percentage is increased to 85%.[452] Viral lesions are particularly localized in uncovered areas, such as the back of the hands and forearms and the face. Clinical features are rather similar to those found in non-immunocompromized patients, however, in RTR especially on severely sun-damaged skin warts may be difficult to distinguish from other keratotic lesions, including actinic keratoses and even SCCs. Warts in RTR are generally multiple and recurrent. HPV-1 and HPV-2 are the viruses particularly related with common warts. Transformation of warts into malignant neoplasms has been described.[337] Apart from common and plane warts, several other wart-like lesions can be seen in renal transplant recipients, such as skin tags (soft warts) and seborrhoeic keratoses (seborrhoeic warts). These lesions, however, have not been associated with malignant transformation in this group of patients, but sometimes may resemble actinic keratoses or non-melanoma skin cancer.

*Actinic keratoses*

The frequency of actinic keratoses in renal transplant recipients is much increased as compared to the general population. Four to 5 years after transplantation incidences of 24-38% have been reported,[453;454] but no data on incidences with longer follow-up periods are available unfortunately. However, with time this percentage seems to increase dramatically and it is likely that eventually (nearly) all renal transplant patients will develop actinic keratoses.

Actinic keratoses in immunosuppressed individuals behave different in several aspects. Their clinical aspect is often less typical and a broad scala of skin lesions characterized by adherent scaling and erythema may be seen in this population. Widespread and multiple actinic keratoses in sunlight-exposed areas are the rule instead of solitary lesions. Histologically, confluent parakeratosis, hyperkeratosis, increased mitotic activity, bacterial colonization, and verrucous changes are moreoften seen.[455] In renal transplant recipients actinic keratoses tend to recur and behave more aggressive. They rapidly evolve into SCC and higher numbers of metastatic spread are reported in this group.[337] Therefore, effective treatments, both for treatment of actinic keratoses and for prevention of squamous cell carcinomas, are urgently needed in these patients. In Chapter 3 several topical and systemic approaches with retinoids will be investigated in actinic keratoses, to obtain further information on this category of potential chemopreventive treatments, especially in renal transplant recipients.

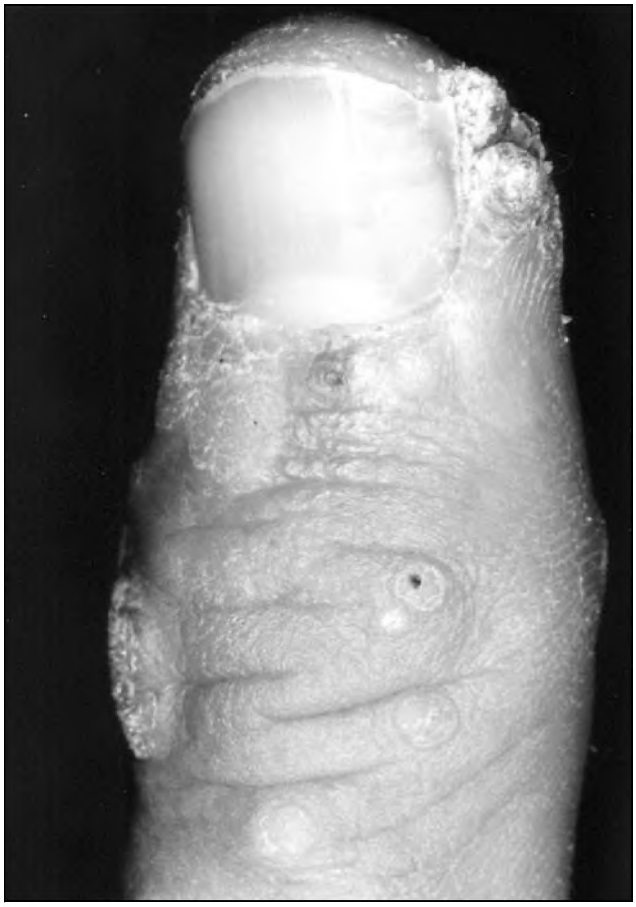


**Figure 15:**

Several common warts located on a finger.

*Porokeratosis*

This is a typical dermatological condition in which an erythematosquamous macula slowly expands superficially due to clonal expansion of abnormal epidermal cells. Clinically, these lesions resemble actinic keratosis or Bowen's disease, but have a raised keratotic edge. They are frequently found on the legs. Histologically, a distinct pattern is seen whose hallmark is a cornoid lamella. Porokeratosis may transform into squamous cell carcinoma in approximately 7% of the cases. In RTR a high incidence of about 10% is seen.[456;457]

*Morbus Bowen*

Bowen's disease or Bowenoid actinic keratoses may already be hard to differentiate from actinic keratosis in the normal population, but in RTR this is even more difficult. In RTR all kinds of warty and hyperkeratotic benign, premalignant and malignant lesions may coexist, making a proper diagnosis sometimes impossible. Data on the incidence of Bowen's disease in renal transplant recipients is limited. A study in 115 renal transplant patients mentions that 2.6% of these subjects had at least one Bowen's disease in an average follow-up period of 12.5 years post transplantation.[458] Another study reports a much higher percentage (36%) of Bowen's disease in 580 renal transplant recipients who were inspected when they were referred for various dermatological conditions.[435]

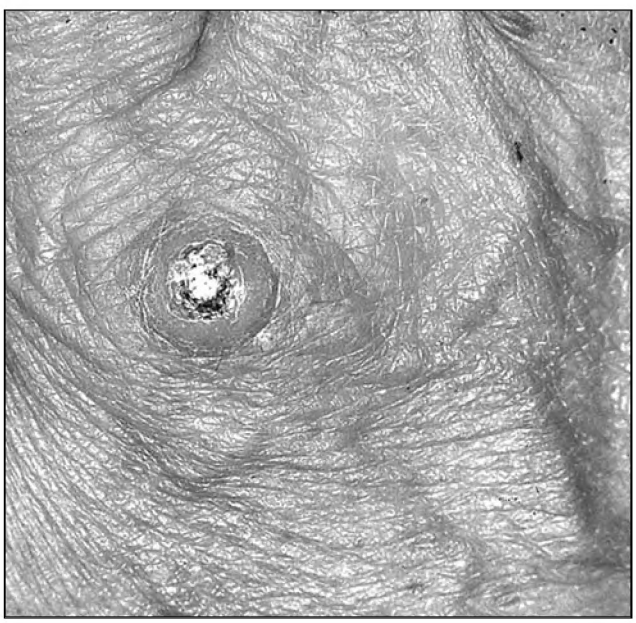


**Figure 16:**

A typical keratoacanthoma showing a central keratin-filled crater. Although this is a benign entity in immunocompetent patients, in immunosuppressed patients it may strongly resemble or transform into a squamous cell carcinoma.

*Squamous cell carcinoma*

The incidence of cutaneous carcinomas in RTR is related to the duration of immunosuppressive treatment. Several studies have reported on the incidence of non-melanoma skin cancer. In the Netherlands 40 % of the patients developed non-melanoma skin cancer 20 years after transplantation,[459] but values up to 70 % after 20 years have been reported.[460] Skin cancers in transplant recipients are found predominantly in sunlight exposed areas, as is the case in non-immunocompromized patients. They occur at a much younger population than is seen at the non-inmmunocompromized group. The average difference is about 30 years.[461;462] In the normal population basal cell carcinomas outnumber squamous cell carcinomas by 5-10 to 1. Interestingly, in RTR this ratio is reversed and squamous cell carcinomas outnumber basal cell carcinomas by 1.8 to 1. The incidence of SCC in RTR is estimated to be 40 to 250 times higher than in the normal population. Squamous cell carcinomas in RTR behave more aggressive.[337] In the majority of patients who have a squamous cell carcinoma multiple tumors will develop. Node metastases are more frequent than in the general population, ranging between 5.7 and 7 % in the different studies.[429;463]

*Keratoacanthoma*

In the normal population keratoacanthoma is a benign tumor with a rapid growth pattern that usually presents on sun-exposed skin. It features a typical central keratin-filled crater. Keratoacanthoma is normally self-limiting and regresses spontaneously. However, in RTR differential diagnosis with SCC is often difficult, both on clinical and on histological grounds. Transformation of a keratoacanthoma into a SCC may be possible, but is hard to prove. The incidence of keratoacanthomas is highly increased compared to the normal population. Euvrard *et al* reported in a total number of 580 renal transplant recipients, who were referred for dermatological investigation, a percentage of 15.7 % keratoacanthomas.

*Basal cell carcinoma*

As mentioned earlier, in contrast to the normal population, in renal transplant recipients basal cell carcinoma is not the most common skin malignancy. Its incidence is about 10 times higher in RTR compared to non-immunosuppressed individuals. The risk of basal cell carcinoma increases in a linear fashion with time, whereas the risk of squamous cell carcinoma increases exponentially.[464] With respect to the clinical course of basal cell carcinoma in this group of patients only limited information is available.

*Kaposi sarcoma*

At present there are 4 different clinical subtypes of kaposi sarcoma. One of them is the subtype that is associated with non-HIV-induced immunosuppression, which is seen in the population of renal transplant recipients. This form of kaposi sarcoma is 400-500 times more frequent in graft patients than in control groups.[465] Its incidence varies from 0.4 to 0.5 % in Western countries to 4.1 % in Saudi Arabia. The difference in incidence is related to the geographical distribution of the major etiological factor of kaposi sarcoma

in transplant patients: kaposi sarcoma-associated herpesvirus (KSHV) or HHV-8. HHV-8-DNA is present in nearly all kaposi sarcoma tissues in grafted patients.[466;467] The role of HHV-8 in kaposi sarcoma has initially been distinguished by Moore *et al.*[468] Kaposi sarcoma appears relatively early after initiation of the immunosuppression with nearly half of the tumors developing already in the first year.[469;470] Most transplant patients who present with kaposi sarcoma are infected with the virus before transplantation,[471] however, a smaller number is infected by an infected allograft or transfusions.[472] The prognosis depends largely on the extent of the lesions and the degree of immunosuppression. On reduction or cessation of these agents a partial or complete regression is usually seen.

*Malignant melanoma*

Malignant melanoma is seen more frequently in renal transplant recipients. A 4 to 5 times higher incidence has been mentioned.[337]

*Merkel-cell carcinoma*

Merkel-cell carcinoma is a rare tumor originating from the neuroendocrien Merkel cells in the skin. In transplant patients this tumor is also frequently seen and is characterized by rapid progression, locoregional recurrences and a high nodal and visceral metastatic power.

Last but not least, one should not forget other side effects of immunosuppressive agents to the skin that may have a serious impact on the quality of life, such as an atrophic friable skin, poor wound healing, skin dryness, hirsutism, acne, hypertrichosis, and opportunistic bacterial and fungal infections.

### 1.12.5 Treatment

Renal transplant recipients often have multiple skin lesions in all sorts that are located preferentially on sunlight-exposed areas. In this population lesions may even develop as coexisting lesions, leading to disorders with mixed histological structures; for example a basal cell carcinoma originating in an actinic keratosis, or a squamous cell carcinoma that arises from a common benign wart. Skin lesions in RTR are sometimes difficult to distinguish from each other both clinically and histologically. Therefore, the different and sometimes difficult presentation of skin disorders in RTR in combination with the fact that (pre)malignant skin disorders in this group of patients generally behave more aggressive, implicates a different and more intensive follow-up in this group of patients.

In clinically atypical verrucous or hyperkeratotic lesions a biopsy may be necessary to exclude an underlying squamous cell carcinoma. Keratoacanthoma, although normally a benign type of lesion, may behave like a squamous cell carcinoma with metastatic spread in RTR. Therefore, these lesions must be met with suspicion and surgery is often indicated.

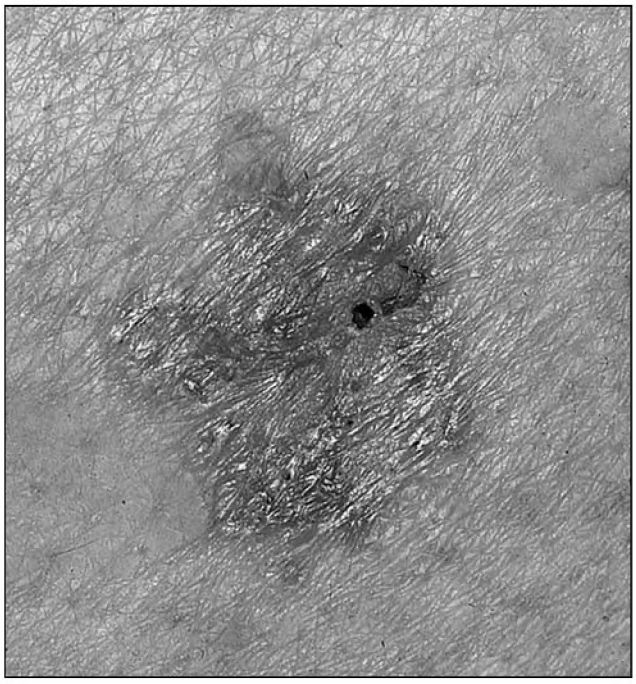


**Figure 17:**

A superficial spreading basal cell carcinoma showing erythema, desquamation and the typical shiny aspect.

Furthermore, patients need to be thoroughly instructed to prevent their skin from UV-damage and to urgently visit the outpatient department in case a rapidly growing lesion arises on the skin.

In general the therapeutic approaches for benign and (pre)malignant skin disorders in RTR are the same as in the normal population. However, with respect to immunostimulating agents, such as immiquimod, caution should be paid as these agents in theory may lead to rejection of the kidney graft. Studies with these agents in immunocompromized patients are carried out at the moment to assess the effect and risk of graft rejection. Furthermore, the atrophic skin that is seen in many renal transplant recipients may be a limitation for several treatments with respect to side effects.

# 1.13

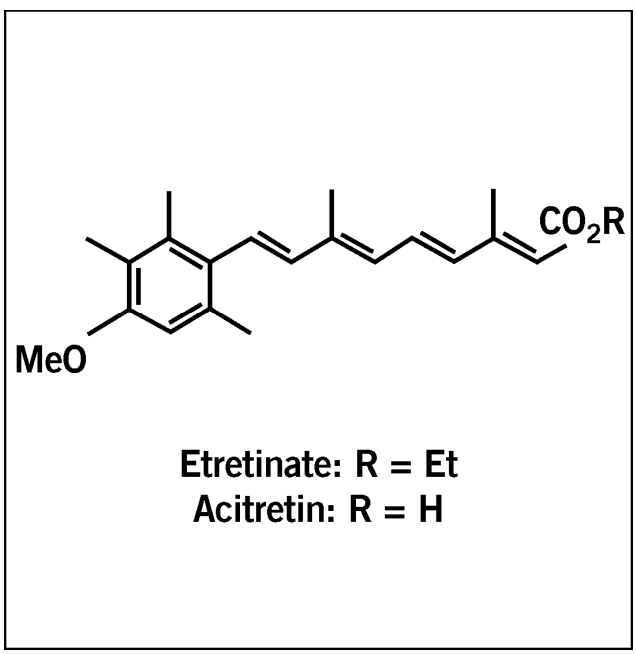
## Research targets

In order to diagnose actinic keratoses and Bowen's disease and to differentiate these conditions from squamous cell carcinoma, the clinical features and histopathological assessment will allow adequate differentiation. In immunocompromized patients the number of non-melanoma skin cancers and of their premalignant conditions can be enormous and the progression to invasive malignancies relatively fast. The occurrence of multiple non-melanoma (pre)canceroses is a major problem that may affect these patients for many years. But also in immunocompetent individuals, which have been exposed extensively to ultraviolet radiation, the number of these lesions can be high and may influence their quality of life for long periods of time.

Treatment approaches that limit the total number of premalignant conditions and/or their malignant behavior, reducing the number of biopsies to be taken in one individual are of major importance. Therapeutic approaches, which are well-accepted, well tolerated and applicable during long-term use, are indicated. In this respect it is attractive to hypothesize that systemic and topical retinoids might serve for this purpose and may provide major opportunities. In Chapter 3 the impact of retinoids on reduction in number and/or alteration in behavior of premalignant conditions will be discussed.

# PART III

# RETINOIDS




The molecule structure of etretinate and its principal metabolite acitretin.

# 1.14

## History

Around 1900 the biological significance of the liposolubile vitamin A was discovered. By that time little information was known on its effects and biological functions on proliferation, differentiation, and inflammation. So therapeutic applications were not available. It took until the 1930s when phrynoderma, a characteristic hyperkeratotic disorder with excessive follicular keratinization, was discovered as a result of vitamin A deficiency.[194]

Since that time vitamin A has been used therapeutically for hyperkeratotic disorders such as psoriasis. Oral application of vitamin A, however, often led to the hypervitaminosis A syndrome, especially when high doses were used for longer periods. Therefore, topical application of vitamin A acid was tried since 1962 for hyperkeratotic disorders, but so far only low efficacy was reported in combination with a high toxicity profile.[473]

In 1968 Bollag *et al* started to chemically alter the vitamin A molecule in order to achieve more effective vitamin A analogues with a lower side-effects profile. With the development of synthetic retinoids a new treatment era started for hyperkeratotic conditions in dermatology.[474]

# 1.15

## Generations

Since the chemical alteration of vitamin A (retinol) began, 3 generations of retinoids have been developed so far that are of importance for dermatological treatment both for topical and systemic application. The clinical application of retinoids was possible after modifications of the structure to minimize vitamin A toxicity.

The first generation is the group of non-aromatic retinoids. The best-known mono-aromatic retinoids are all-trans retinoic acid (ATRA, tretinoin) and 13-cis retinoic acid (isotretinoin, Roaccutane®). ATRA is used topically for disorders such as ichthyosis vulgaris, and has been prescribed since the last couple of decades in the treatment for photodamaged skin and for premalignant hyperkeratotic disorders, such as actinic keratoses. However, it has a similar side-effect profile compared to vitamin A. Isotretinoin was synthesized thereafter. This retinoid is very effective in the treatment of severe acne after oral application and it has been used for other diseases like severe pustular psoriasis. It is also used for prevention of skin cancer.[194]

The second generation is the group of mono-aromatic retinoids. Retinoids such as etretinate and acitretin belong to this generation. Both retinoids significantly improve oral retinoid treatment for a broad scala of skin conditions, including psoriasis, Darier's disease, ichthyosis vulgaris and pityriasis rubra pilaris. Beneficial effects of etretinate and acitretin have been demonstrated in (pre)malignant skin disorders as well.[405-408] Etretinate, however, has a long elimination half-life (80-120 days), which can be a problem in women of childbearing potential with respect to its teratogenicity. Acitretin, the principal metabolite of etretinate, was developed later on. Although acitretin has an elimination half-life of only 2 days due to lower lipid binding properties, it can re-esterificate into etretinate, especially in patients with substantial alcohol-intake, and therefore long-term storage in the subcutaneous adipose tissue account for detectable plasma levels for at least 2 years. Nevertheless, acitretin is the drug of first choice for systemic retinoid treatment of most retinoid sensitive skin disorders nowadays.[194]

The third generation is the group of polyaromatic retinoids or arotinoids. The search for more effective, non-teratogenic retinoids with a shorter elimination half-life and a favorable side-effect profile continued. So far all these retinoids are teratogenic and have similar lipophylic properties as etretinate, leading to long elimination half-life periods. A few polyaromatic retinoids are used in the topical treatment of acne (adapalene, Differin®) and psoriasis (tazarotene, Zorac®) nowadays.[475;476] Bexarotene (Targretin®) is a polyaromatic retinoid that has recently been developed for oral application in the treatment of cutaneous T-cell lymphoma (CTCL or mycosis fungoides).[477] This retinoid is the first RXR-selective retinoid (see the next paragraph) that has become available for human use and will be discussed in Chapters 2 and 4 of this thesis for its potential role in psoriasis and in UV-mediated inflammation.

# 1.16

## Retinoid signaling

Retinoids belong to a superfamily of ligand depending transcription factors that includes receptors for steroid hormones, thyroid hormones, vitamin A, and vitamin D, and peroxisome proliferator-activated receptors. Most members of this receptor family are nuclear in both the absence and presence of the ligand, but some are found in the cytoplasm.[478]

Unlike membrane-receptor second messenger systems, in this pathway the retinoid receptor itself becomes the signal to the nucleus, where it interacts with hormone response elements (HREs) in a target gene and thereby it elicits a transcriptional response. HREs function as transcription enhancers and can be found both upstream and downstream of the transcription start sites of various genes.[479]

Once a retinoid has passed the cellular membrane it is free to interact with intracellular proteins: the Cytoplasmatic Retinoid Binding Proteins (CRBPs). These proteins bind to retinoids and are likely to function as specific transport proteins for retinoids to the nucleus and by steeping up the intracellular concentration gradient of retinoids. CRABP I and II are cellular retinoic acid binding proteins. These molecules do not seem to regulate transcription themselves.[479]

The retinoid acid family as part of the superfamily of ligand depending transcription factors, comprises two different types of subfamilies: the Retinoic-Acid-Receptors (RAR) and the Retinoid-X-Receptors (RXR). Each subfamily contains 3 different receptor subtypes: α, β, and γ. The first retinoid receptor, RARα, has been recognized in 1987, followed by RARβ and RARγ. In 1990 the 3 RXR receptors were found. All retinoid receptor genes are located on different chromosomes. RARα, RARβ, and RARγ are mapped on chromosomes 17, 3, and 12, respectively, whereas RXRα, RXRβ, and RXRγ are located on chromosomes 9, 6, and 1 respectively.[480-484]

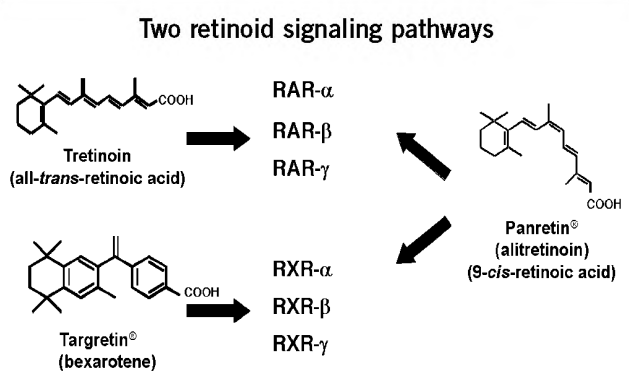



**Figure 18:**

ATRA and bexarotene have different binding properties with respect to binding of the retinoid receptors.

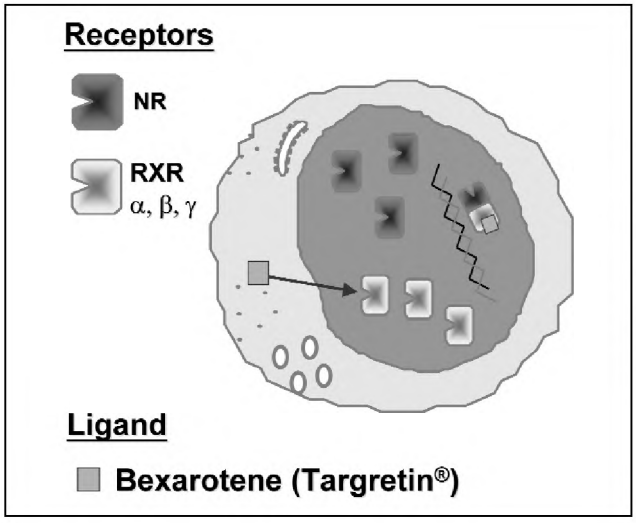



**Figure 19:**

An example of retinoid signaling: bexarotene penetrates the cellular membrane and enters the nucleus, where it can bind to an RXR. The bexarotene-RXR complex then binds to an HRE of a specific target gene and thereby it elicits a transcriptional response. As shown, the RXR can form heterodimers with other nuclear receptors (NR).

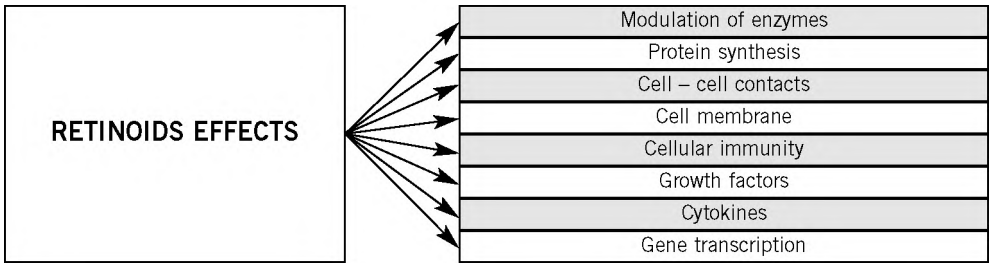
Each retinoid receptor exists of 6 functional domains designated A to F. The most important domains are domain E and domain C. Domain E is the Ligand Binding Domain (LBD), the locus were the retinoid binds to its receptor, and domain C is the DNA Binding Domain (DBD), or the place where the retinoid receptor can bind to the DNA of the target genes (the HREs).[479]

There is a difference in the capacity of the retinoids in stimulating or suppressing the receptor subtypes. So there is receptor specificity. For example, ATRA especially binds to the RAR receptor subtypes and only in high concentrations it can bind to the RXR receptors. The new retinoid bexarotene, on the other hand, especially binds to the RXR receptors and only in high concentrations it may bind to the RAR receptors. But other retinoids, such as 9-cis retinoic acid bind rather equally to both RAR and RXR receptor subtypes. Interestingly, acitretin does not bind to but activates specifically the RAR receptors by an unknown mechanism.[478;479]

The RXR receptor seems to be unique to other nuclear receptors, as it can make, besides the homodimer with itself, also heterodimers by binding to other receptors like the RAR, vitamin D receptor (VDR), and the thyroid receptor (TR). Therefore, in contrast to the RAR receptor, it can act as a master regulator of hormonal signaling. However, vitamin D and thyroid effects do not seem to be RXR dependent, but RXR-selective retinoids may influence these effects. The formation of heterodimers is dependent on the concentration of RXR and is influenced by both the concentration of 9-cis retinoic acid (as this is the major RXR activator) and by the concentration of RAR (mainly retinoic acid). These heterodimers can be activated by either of the two receptor ligands alone or with both ligands simultaneously, yielding in three discrete levels of activation.[479]

Retinoids like retinoic acid can enter the cell directly from the circulation or can be synthesized from retinol and retinal in the target cells. They can be converted intra-

**Figure 20:**
Effects of retinoids. (Adapted from Gollnick and Dümmler[194])




cellularly into other types of retinoids. For example, 9-cis retinoic acid can be converted into ATRA. By the mechanism of conversion the cell may favor specific pathways and so might have control in the different retinoid induced effects.[479]

The concentration of RAR and RXR receptors in human tissue differs. RARα and RXRβ are found in almost all human tissues, but the other receptors are much more restricted to specific tissues. In the skin the total amount of RXR is 5 times higher than the total amount of RAR. RXRα is the predominant RXR subtype. With respect to the RAR receptors the RARγ receptor is the predominant one. Therefore, the RXRα-RARγ is postulated to be the main regulating protein complex of retinoid sensible targetgene expression in human skin.[485;486]

Nuclear receptors bind to specific DNA sequences known as hormone response elements (HREs), but the retinoid receptors differ in affinity for binding these HREs. In other words, there is target-gene specificity. There are two types of HREs for retinoid receptors: RAREs and RXREs. Each of the two has several subtypes and these subtypes, subsequently, require different amounts of retinoid receptors for eliciting a similar response in strength. Furthermore, these HREs can have stimulatory effects as well as inhibitory effects on the transcription of the target genes. The RXREs are predominantly found in the promotors of genes involved in vitamin A, lipid and fatty-acid metabolism, and therefore, RXR-selective retinoids may have an important function in regulating lipid and cholesterol metabolism.[479]

# 1.17

## Cellular effects

Retinoids have a broad spectrum of effects, but most of them still need to be elucidated. A figure derived from Dümmler and Gollnick depicts the most important effects of retinoids in a nutshell **(Figure 20)**.[194] Here, I will briefly mention some retinoid effects that are important for (hyperkeratotic) skin and that are relevant for the studies described in this thesis. Data are derived from RAR-selective retinoids, as these are the retinoids that are commonly used in dermatology nowadays.

Retinoids do have regulatory effects on epidermal keratinization, proliferation, and inflammation.[478] In psoriasis retinoids have a keratolytic effect. They decrease the cohesiveness of the stratum corneum with enhanced fragility of the skin and increased epidermal water loss.[487] Therefore, in combination with other topical treatments retinoids may enhance their permeability. The keratolytic effect is probably caused by a reduction in size and number of desmosomes, especially in the stratum corneum and in the stratum spinosum. However, many other factors have been postulated to play a role, such as widening of the intercellular spaces in the stratum spinosum and stratum corneum associated with deposition of amorphous material. The latter may be a result of rupture and leakage of cells containing the material or might be secondary to serum infiltration into the epidermis.[478]

Retinoids modulate the expression of the cornified envelope protein transglutaminase. They stimulate transglutaminase expression in vivo in normal skin.[488] In hyperkeratotic psoriatic skin, however, retinoids and agents that increase retinoid concentrations reduce transglutaminase activity.[489;490]

With respect to keratinization, retinoids influence expression of cytokeratins. Retinoids alter terminal differentiation towards a non-keratinizing mucosa-like epithelium.[491] They induce expression of retinoids that are normally not present in adult human skin, but that are only seen in embryonic skin and in other adult human tissues, such as the esophagus. Examples of these keratins are keratin 13 and 19 that are induced by retinoids in vitro.[491;492] In vivo in normal skin induction of keratin 13 expression by retinoids has been demonstrated by Rosenthal *et al.*[364] Concerning other keratins, in vitro data have shown that retinoids suppress the expression of keratin 3, 5, 10, 14, and 16. With respect to keratin 6 both a reduction as well an increase due to retinoid treatment have been suggested.[493] In psoriasis and other hyperkeratotic disorders hyperproliferation-associated keratin 6 and 16 are abundantly present, while keratin 10 is less expressed. Retinoids inhibit the expression of keratin 6 and 16 in psoriasis,[490;494] and in contrast to in vitro data, keratin 10 expression tends to normalize.[495]

Epidermal proliferation is influenced by retinoids. In normal skin retinoids promote proliferation, but in psoriatic skin they may act towards normalization of the hyperproliferative state by reducing the number of cycling cells.[496;497] In lesional psoriatic skin the large body of evidence is that RARγ mediated signaling is already upregulated.

CRABP II, a protein which is upregulated by RARγ activation clearly shows such. In contrast RXRγ signaling has been shown to be reduced in lesional psoriatic skin. In view of these features it is attractive to speculate that psoriatic skin is more responsive to RXR stimulation and less to RARγ stimulation.

Retinoids have both immunostimulating and immune-inhibiting effects on humoral and cellular immunity. They can inhibit the migration of neutrophils from the dermal capillaries to the epidermis,[498] decrease inflammation-associated proteins and T-lymphocyte subsets,[497] and increase the number of Langerhans cells in psoriasis, although these effects may be secondary to the clearing of the lesions, as this is a normal feature in healing psoriatic skin. The increase in the number of Langerhans cells in psoriasis due to retinoids is paradoxically, as other effective anti-psoriasis therapies, such as UVB and topical corticosteroids decrease the number of these cells. Retinoids can also inhibit the antigen presenting properties of epidermal cells and of dendritic cells. Retinoids can enhance antibody production and can increase peripheral blood T helper cells, but not natural killer cells.[478] It has been demonstrated in chicken that antigen-specific responses may be directly influenced by ATRA via modulation of RARα expression.[478]

Retinoids have also been reported to have inhibitory effects in skin cancer. The mechanism for this modulation is not known, but may be two-fold: retinoids control cell growth and differentiation, and they also induce apoptosis. Especially when multiple lesions are present, such as in renal transplant recipients, retinoids may be a good alternative. In this group of patients many studies have been performed with topical and systemic retinoids for the treatment of actinic keratoses and non-melanoma skin cancer.[410] However, only one placebo-controlled study by Bouwes-Bavinck *et al* has been conducted so far in this group of patients.[410] In general, these studies reported beneficial effects of retinoids with respect to the number and aspect of the premalignant lesions and beneficial effects in the prevention of carcinomas. However, immunostimulating effects of retinoids, in theory, could lead to an increased risk of transplant rejection. Until now, no signs of an increased rejection incidence have been found in over 60 treated patients.[410;499] In patients with basal cell naevus syndrome, xeroderma pigmentosum, and psoriatic patients overtreated with PUVA, retinoids have been reported to decrease the occurrence of new carcinomas. In keratoacanthomas beneficial results of oral retinoid treatment have also been reported.[500;501] However, so far, no controlled studies are available to substantiate these impressions based on case reports.

# 1.18

## Pharmacokinetics, metabolism and interactions

The absorption of the different retinoids after oral intake is varying. For acitretin, the most used retinoid in dermatology, this is about 60%, but can be increased if combined with a meal rich in fat.[502] Absorption is followed by rapid isomerization of acitretin to 13-cis isoacitretin.[478] The biological effects of isoacitretin are unknown, as rapid re-isomerization to acitretin occurs. Acitretin and isoacitretin are metabolized in the liver via hydroxylation of the aromatic ring and via cytochrome-P450 dependent oxidation of the side chain to biologically inactive metabolites. Clearance of these metabolites occurs via the kidneys and the liver.[503] A small quantity of acitretin is converted into etretinate, especially under influence of alcohol. The teratogenic etretinate is stacked in lipoid tissue.

Acitretin and 13-cis isoacitretin are almost completely bound to albumin; binding to retinol binding protein does not occur. Steady state concentrations are reached after 7-10 days. After three months of treatment, terminal half-life is 50-60 hours,[503;504] and no trans- and 13-cis acitretin is detected anymore after 1 month.[505]

In patients with systemic illnesses pharmacokinetics of retinoids can be different. In patients with renal insufficiency, which is often present in renal transplant recipients, a reduced renal clearance may be seen, as well as a low serum albumin.[506] However, the clinical relevance of such has not been investigated thus far.

Interactions with retinoids and other drugs may occur. Renal transplant recipients usually take a large number of drugs apart from the inevitable immunosuppressive agents (cyclosporin, azathioprine, prednisone, etc) and therefore many interactions are possible. Cyclosporin is metabolized by the cytochrome P450-3A4 system. Retinoids also have an influence on the cytochrome P450 system. Webber has claimed that etretinate is metabolized via a subtype of cytochrome P450 different from the system that metabolizes cyclosporin, and therefore the author suggested that an interaction at this point is unlikely.[507] However, in vitro studies showed that etretinate as well as acitretin do decrease cytochrome P450 activity, and therefore lead to an increase in cyclosporin levels.[508] Retinoids do not seem to influence metabolism of azathioprine and prednisone.[509]

When prescribing drugs with a high protein binding capacity (anticoagulants, antibiotics) in combination with oral retinoids, there is the possibility of competition between these drugs for the available binding sites, eventually leading to an increased free fraction of these drugs, thus leading to more side-effects. However, data are scarce and conflicting.[503;506]

Another possible interaction at the level of cytochrome P450 exists between retinoids and antiepileptic drugs: isotretinoin led to an increase in the dose needed of carbamazepine, and Phenobarbital led to a decreased efficacy of isotretinoin. No data are available regarding possible interactions between acitretin and antiepileptic drugs.

Combination of retinoids and tetracycline is contra-indicated, as both may lead to a cerebral pseudotumor.[503]

# 1.19

## Side-effects

The most common side-effects of retinoids are summarized in **Table IV**. Incidences are related to the retinoid used, its dose, and the duration of treatment. Apart from presumed skeletal abnormalities, all adverse events are fully reversible on cessation.

Mucocutaneous side-effects are seen in nearly all patients and comprise dryness of mucous membranes (mouth, lips, nose, eyes, urethra, vagina), erythema, desquamation, thinning of normal skin and local or generalized loss of hair.[194] Tolerability of these mucocutaneous adverse events largely depends on the dose. In the appropriated doses most mucocutaneous adverse events are generally well-tolerated, but for some patients lowering of the dose is necessary to prevent from cessation of the treatment.

Hyperlipidemia is a frequent complication of the use of retinoids. Particularly an elevation in triglycerides, but also an elevation in total cholesterol and a decrease in high-density lipoprotein cholesterol have been found. Patients with renal insufficiency or after renal transplantation frequently have hyperlipidemia, often aggravated by the use of immunosuppressive drugs. The additional development of retinoid-induced hyperlipidemia could be very deleterious, as cardiovascular morbidity already is very high in these patients.[510]

Shortly after starting retinoids, a rise in alkaline phosphatase, bilirubin and transaminases has been found in some patients. These effects usually disappear on continued use of retinoids. Hepatitis has been described rarely.[511]

Hemeralopia (diminished vision in bright light) or a decrease in night vision may occur, probably due to interference with 11-cis retinaldehyde formation. Cataract has also been described occasionally.[478]

Arthralgia and myalgia may occur in 2 to 5 % of individuals receiving oral retinoids > 0.5 mg/kg/day, especially in adolescents and young adults.[478]

Pseudotumor cerebri has been documented in patients receiving high doses of isotretinoin, particular in combination with tetracyclines.[512] However, in recommended dosages of acitretin no pseudotumor cerebri has been reported, but papilledema should be considered in patients with pre-existing intra-ocular hypertension or glaucoma.

In patients on long-term treatment with high doses of retinoids, notably isotretinoin and etretinate, several effects on the skeleton have been described: osteoporosis,[513] diffuse ideopathic skeletal hyperostosis (DISH),[514] and degenerative changes mainly occurring in the lumbar spine. In patients treated with etretinate and acitretin these changes have been reported. However, no dose-response relationship could be shown and therefore these hyperostoses have been interpreted as idiosyncrasy. No data are available regarding the possible effects of retinoids on the skeleton of patients with renal insufficiency or after renal transplantation. The latter group has several additional risk factors for skeletal problems, like the use of corticosteroids, persistent hyperparathyroidism, and vitamin D deficiency. However, in the few available studies on the use of retinoids in

**Table IV:**
Most common side-effects of retinoids.

| | |
|---|---|
| Mucocutaneous | Cheilitis, dryness of eyes, nasal and oral mucosa (100%)<br>Epistaxis (10%)<br>Xerosis, pruritus (100%)<br>Brittle nails (20%)<br>Hair loss (20–50%)<br>Feeling of burning or sticky skin (20%) |
| Musculoskeletal | Spinal- and extraspinal hyperostoses, ossification of ligaments (spine), degenerative spondylosis, osteoporosis |
| Liver | Elevation of liver enzymes (20%),<br>severe liver damage (<1%) |
| Lipid metabolism | Increase in serum triglycerides and cholesterol;<br>decrease of HDL-cholesterol |
| Teratogenicity | Cranofacial, thymic, cardiac, skeletal, and central nervous system malformations |
| Other | Fatigue, headache, nausea |

these patients, no negative effects of retinoids on the skeleton have been described.[407;410]

Teratogenicity is a limiting factor for women of childbaring potential. Due to the strong teratogenic effect of retinoids and the slow release from the lipid tissue, retinoids should be stopped at least two years before an unadverted pregnancy and retinoids are strictly prohibited during pregnancy and nursing.[504;506;510;515;516] Severe liver dysfunction, severe hyperlipidemia, and excessive alcohol intake are other contra-indications for acitretin therapy.[194]

# 1.20

## Research targets

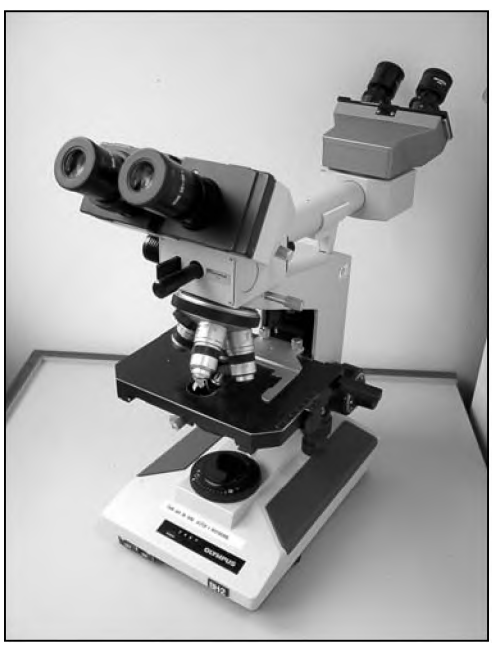
Innovations in retinoid treatment of psoriasis are to be expected from RXR-selective ligands, as RXR signaling is deficient in psoriasis.[250] Therefore, the clinical and cellular responses of psoriasis to the RXR-selective retinoid bexarotene will be evaluated in the present thesis.

In view of circumstantial evidence that treatment with systemic and topical RAR-selective agents results in RAR transactivation and might be effective in non-melanoma (pre)canceroses in immunocompetent and immunosuppressed patients, studies were designed to evaluate the role of RAR-selective retinoids in these conditions.

Available information on photosensitizing properties of retinoids is not conclusive. Therefore, a series of studies was carried out to further clarify this issue.

# PART IV

# INVESTIGATIONAL APPROACHES



Assessment of histological and immunohistochemical parameters was performed by microscopy.

# 1.21

## Aim of the thesis

This thesis focuses on the role of retinoids in the treatment of hyperkeratotic skin disorders. Although retinoids have been used for several decades in psoriasis, current retinoid treatment for this disease is via oral and topical retinoids that favor the retinoic acid receptor (RAR) pathway, such as acitretin, tretinoin and tazarotene. Up to now it is unknown to what extent retinoid X receptors (RXR) may play a role in psoriasis and whether RXR-selective retinoids, or rexinoids, may provide a new therapeutic approach.

Retinoids like acitretin and tretinoin have been claimed to ameliorate actinic keratoses, the most frequently observed premalignant skin disorder. Furthermore, these compounds are used for chemoprevention of non-melanoma skin cancer, in the normal population as well as in immunosuppressed patients, due to their postulated anti-carcinogenic effects. Their clinical effects are well-described, but there are few studies on their immunohistochemical effects in these patients.

Retinoids are frequently used in combination with UV-irradiation therapy to obtain an enhanced therapeutic effect in combination with a reduction in the cumulative UVB or UVA dose necessary to achieve clearance of the disease. Furthermore, it is supposed that retinoids alter the reaction to UV-light and that patients who are treated with topical or systemic retinoids may need to take precautions with respect to sunlight exposure. Effects of a single irradiation with UVB-light, in a dose which is enough to produce a distinct erythema on retinoid pretreated skin, have not been studied so far.

In order to achieve further information on these issues, the following aims were formulated:

I. To investigate efficacy and side-effects of oral application of the RXR-selective retinoid bexarotene in the treatment of plaque-type psoriasis, with respect to clinical and immunohistochemical features. *(Chapter 2)*

II. To analyze mechanisms of action of topical and systemic retinoid treatment with RAR-selective retinoids in (pre)malignant skin disorders, in the normal population and in immunosuppressed renal transplant recipients, regarding clinical and immunohistochemical parameters. *(Chapter 3)*

III. To elucidate the modulation of the effect of a single exposure of broadspectrum UVB-light on the skin by RAR-selective retinoids and RXR-selective retinoids. *(Chapter 4)*

In order to study these items, the following approaches were used as explained in the next paragraphs.

# 1.22

## Clinical observations

### 1.22.1 Psoriasis

Clinical scoring of the psoriatic lesions was performed by the previously documented and standardized Physicians Global Assessment (PGA) and the Plaque Elevation (PEL), and by a modified Psoriasis Area and Severity Index (mPASI).

*Modified PASI score (mPASI)*

To determine the mPASI score, the percentage (0-100 %) of body surface area that is involved with psoriasis for each of 4 regions of the body (head, trunk, upper limbs and lower limbs) was evaluated. The percentage of involvement was assigned as an integer value by the following scoring method: 0 = 0 %, 1 = 1-10 %, 2 = 11-30 %, 3 = 31-50 %, 4 = 51-70 %, 5 = 71-90 %, and 6 = 91-100 %. The lesions in each of these body regions were evaluated for erythema, induration, desquamation and pustulation on a 4-point scale as follows: 0 = none, 1 = slight, 2 = moderate, 3 = severe. The regions of the body correspond approximately to the following percentages of total body surface area, which is used to adjust the sum of severity scores from each region: head = 10 %, trunk = 30 %, upper limbs = 20 % and lower limbs = 40 %. The following formula was used for calculating the modified PASI score for each of the 4 body areas: integer of involvement (1-6) X percentage of total body surface area X (sum of erythema, induration, desquamation and pustulation scores). The sum of these 4 values of each body area was the final mPASI score, which could range from 0 to 72.

*Physicians Global Assessment (PGA)*

A physicians global assessment score (PGA), that evaluated the improvement or worsening of the psoriatic plaques as a percentage related to the condition at baseline, was used on a 7-point scale: 0 = completely cleared (100 % improvement), 1 = almost cleared (≥90 to < 100 % improvement), 2 = marked response (≥75 to < 90 % improvement), 3 = moderate response (≥50 % tot < 75 % improvement), 4 = slight response (≥25 to < 50 % improvement), 5 = condition stable (< 25 % change), 6 = condition worsened.

*Plaque elevation (PEL)*

The overall plaque elevation was scored using a 9-point scale: 0 = no plaque elevation (0 mm), 1 = minimal elevation (> 0 to < 0.5 mm), 2 = mild elevation (≥0.5 to < 1 mm), 3 = mild elevation (≥1 to < 1.5 mm), 4 = moderate elevation (≥1.5 to < 2 mm), 5 = moderate to marked elevation (≥2 to < 2.5 mm), 6 = severe elevation (≥2.5 to < 3) mm, 7 = very marked elevation (≥3 to < 3.5 mm), 8 = very severe elevation (≥3.5 mm).

Furthermore, the Psoriasis Disability Index questionnaire was used to get an impression of the psoriatic condition as observed by the patients themselves. Patients had to fill in this questionnaire at specific time points.

### 1.22.2 Actinic keratoses

In contrast to psoriasis, for actinic keratoses no standardized scoring methods are available. Therefore, we had to develop our own methods inspired by scoring methods used in other studies. In the retinoid-treated areas the number of actinic keratoses was counted at specific time-points. Hereby, the total body surface area was divided into several parts based on natural anatomic borders. Actinic keratoses, especially in renal transplant recipients, are sometimes difficult to demarcate, as spectra with verrucae and other types of lesions can be seen, as well as erythematous actinically damaged skin without the well known scaling patterns of actinic keratoses. Therefore, only the typical erythematous, scaling and often hypertrophic lesions were counted.

Erythema, desquamation, and induration of the actinic keratoses in each body surface area was assessed by the following 5-point semi-quantitative score: (0 = none, 1 = slight, 2 = moderate, 3 = moderate-severe, 4 = severe). With respect to adverse event monitoring special attention was paid to mucocutaneous side effects (e.g. cheilitis, conjunctivitis, peeling, hair loss, nail deformations, sweaty and sticky hands). These adverse events were graded as 'absent', 'mild', 'moderate' or 'severe'. Pruritus was scored by a 5-point semi-quantitative method as follows: (0 = none, 1 = slight, 2 = moderate, 3 = moderate-severe, 4 = severe).

To evaluate subjective findings of the patients with respect to "redness of the skin", "thickness of the lesions", "scaling of the lesions" and "general contentment with the skin" (including mucocutaneous side effects), visual analogue scores (VAS) were performed on a 10-point scale.

# 1.23

# Immunohistochemistry

## 1.23.1 Parameters

To assess epidermal proliferation, the monoclonal antibody MIB-1 against the Ki67 antigen was chosen for identification of the number of epidermal cells in the G1, S, G2, and M-phases of the cell cycle.

For determination of epidermal differentiation (keratinization) the following set of antibodies was used: DE-K10 was chosen for assessment of keratin 10 expression, a parameter for normal differentiation of the keratinocytes. The monoclonal antibody LL025 is directed at keratin 16, a parameter for hyperproliferation-associated differentiation, and was used in all actinic keratoses. Ks8.12 was used for detection of keratin 16 in the psoriatic tissues. The monoclonal antibodies 1C7 and 2D7 for keratin 13 expression, respectively, and RCK108 for keratin 19 expression, were chosen to determine retinoid-associated keratinization. The monoclonal antibody BT621, directed to transglutaminase, was used as parameter for terminal differentiation and assessment of the cornified envelope formation.

Dermal and epidermal T-cell mediated inflammation in psoriasis was scored by mononuclear antibodies against CD4 and CD8 positive T-lymphocytes, respectively 1F6 and 4B11. Furthermore, a global inflammation score was used on hematoxylineosin stained sections for assessment of the overall dermal infiltrate. The same global scoring method was used in the studies with actinic keratoses.

The monoclonal antibody DO-7 for p53 protein detection was chosen to provide information on programmed cell-death or apoptosis in the actinic keratoses and psoriatic skin. The cyclindependent kinase inhibitor $p16^{INK4}$, was additionally chosen to assess differences in cell-cycle inhibition in actinic keratoses.

## 1.23.2 Biopsies and staining procedures

For assessment of immunohistochemical parameters using paraffin embedded tissue, punch biopsies of 3, 4 or 6 mm were taken in the different studies under local anesthesia with xylocain/1 % adrenalin. For psoriasis, the center of a representative erythematous and scaly plaque was chosen, preferably on the lower back or the upper legs. After treatment biopsies were taken from the same psoriatic lesion.

In actinic keratoses biopsies were taken from the center of identical lesions that were identified at the baseline, preferably on the lower arms. For determining the actinic keratoses that fulfilled our purpose for biopsy, the lesions had to have a distinct erythematous zone and evident desquamation or hyperkeratosis, but no signs of verrucous origin or clinical features seen in other lesions that may coexist in sunlight exposed skin, especially in renal transplant recipients.

All biopsies were fixed in formalin for 4 hours and then after ethanol washing steps embedded in paraffin cubes. From these paraffin cubes slices of 6µm thickness were cut with a microtome and they were placed on slides that were coated with 3-aminopropyl-triethoxysilane (Sigma Chemicals, St. Louis, USA). Sections were dewaxed in Histosafe, dipped in graded ethanol series, and rehydrated in demineralized water.

To unmask the epitopes that the antibodies are able to recognize, the following procedures were carried out:

- MIB-1, LL025, $P16^{INK4A}$, 1C7, 2D7, 1F6, 4B11, and DO-7 staining required a high temperature microwave antigen retrieval technique. In brief: the slides were placed in 10mM citrate buffer (pH = 6.0) in a microwave oven (760 W) and heated to boil. Subsequently sections were heated twice at 480 W for 5 minutes (boiling was prevented) and slowly cooled down to room temperature (20°C).

- For DE-K10 staining a 15-minute incubation period at 37°C with trypsin 0.1% was necessary.

- For Ks8.12 staining a 45-minute incubation period in 10mM citrate buffer (pH = 6.0) was needed.

- BT621 staining required a 7-minute incubation period with 0.02% protease.

- RCK108 staining required a 7-minute incubation period with 0.1% pronase.

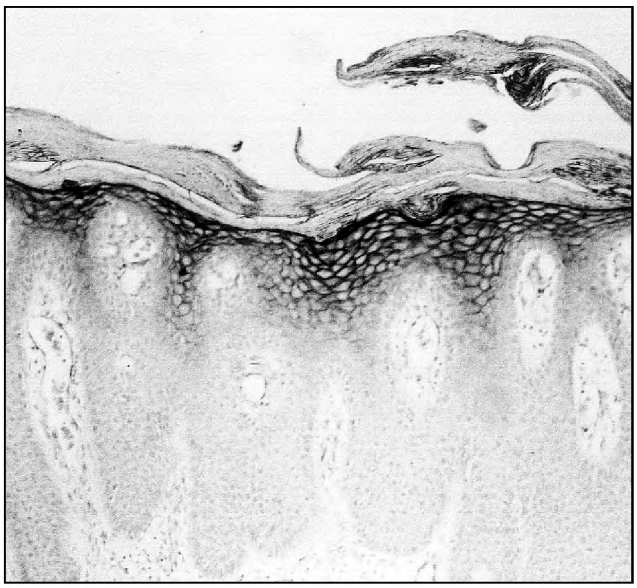


**Figure 21:**

Transglutaminase staining varies between the papillary- and the interpapillary epidermis in psoriasis.

An indirect immunoperoxidase staining technique was used for all antibodies. Slides were incubated with 20% normal horse serum (Vector laboratories, Burlingame, USA) for 15 minutes, with subsequent incubation with the primary antibody (diluted in 1% bovine serum albumin (Organon Technika, Boxtel, the Netherlands)/PBS) for 1 hour. In the cessation study of acitretin (paragraph 3.3) and in the study in warts and Bowen's disease (paragraph 3.6) overnight incubation at 4°C of the primary antibodies was done, instead of this one-hour incubation period. Sections were washed in PBS for 15 minutes. Secondary horse anti-mouse biotinylated IgG antibody (ABC kit-mouse, Vector Laboratories) (dilution 1:200 in 1% BSA/PBS) was added for 30 minutes, and again a 15 minutes wash in PBS was performed. Sections were treated with avidin-biotin complex antibody (ABC kit-mouse, Vector Laboratories)/(avidin/biotin diluted 1:50 in 1% BSA/PBS) for 30 minutes. Slides were washed in PBS for 15 minutes. To visualize staining for MIB-1, DE-K10, 1C7, RCK108, and Ks8.12 3-amino-9-ethylcarbazole (AEC; Calbiochem-Novabiochem Corporation, San Diego, USA) was used. Counterstaining occurred with Mayer's haematoxylin (Sigma Chemicals, St. Louis, USA). Finally, sections were mounted in glycerol gelatin (Sigma Diagnostics, St. Louis, USA). Staining for the other markers was visualized with diaminobenzoyl (Pierce, Rockford, USA). These slides were mounted in permount (Fisher Chemicals, Fair Lawn, USA).

The staining procedures described in paragraphs 3.3 and 3.6 were slightly different from the above mentioned protocol, with a longer incubation time (24 hours) at a lower temperature (4°C), as these were performed in another department.

For global infiltrate scores a heamatoxylin–eosin staining was performed as follows. After dewaxing in Histosafe and dipping in graded ethanol series a haemaluin solution was added to the slides for 10 minutes, followed by a 10-minutes washing step in tapwater. Then an eosin solution was added to the slides for 90 seconds. After washing steps in ethanol and Histosafe, the slides were mounted in permount.

### 1.23.3 Immunohistochemical and histological scoring methods

MIB-1 and DO-7 positive keratinocytes (nuclei) were counted per mm length of section in all studies, with the exception of the cessation study of acitretin (paragraph 3.3), where scoring occurred by using the following 4-point semiquantitative scale:[517] 0 = only basal layer positivity, 1 = positivity confined to basal 1/3 of epidermis, 2 = positivity confined to basal 2/3 of epidermis or 3 = transepidermal positive staining. Transglutaminase scores were assessed as the ratio of BT621 positive epidermal cell layers divided by the total number of epidermal cell layers. Transglutaminase scoring occurred both in the papillary and the interpapillary epidermis. **Figure 21** represents an example of transglutaminase staining in an untreated psoriatic lesion.

For DE-K10, 1C7, RCK108, LL025, Ks8.12, 1F6 and 4B11 staining the following semi-quantitative scale was used: 0 = no staining, 1 = sporadic staining, 2 = minimal staining, 3 = moderate staining, 4 = moderate-pronounced staining, 5 = pronounced staining, 6 = whole epidermis stained. In the cessation study of acitretin (paragraph 3.3) and in the study in warts and Bowen's disease (paragraph 3.6), scoring of 1C7, 2D7, and

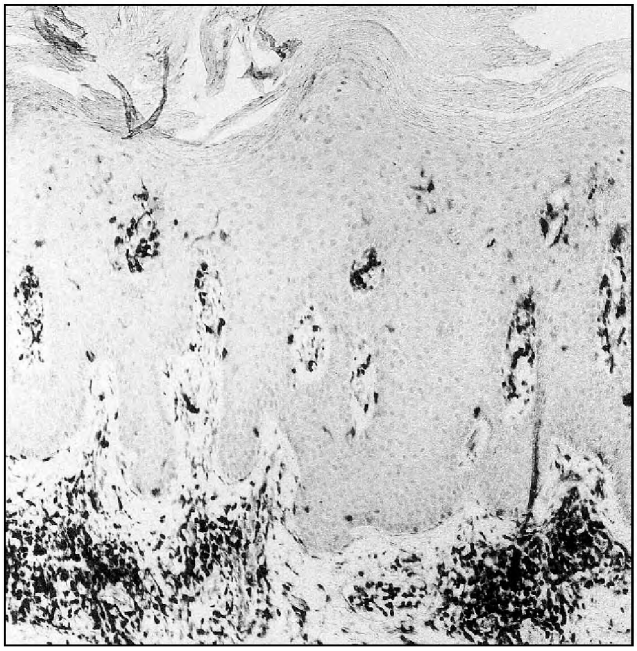


**Figure 22:**

In psoriasis the (epi)dermal infiltrates mainly consist of CD4 positive T-cells.

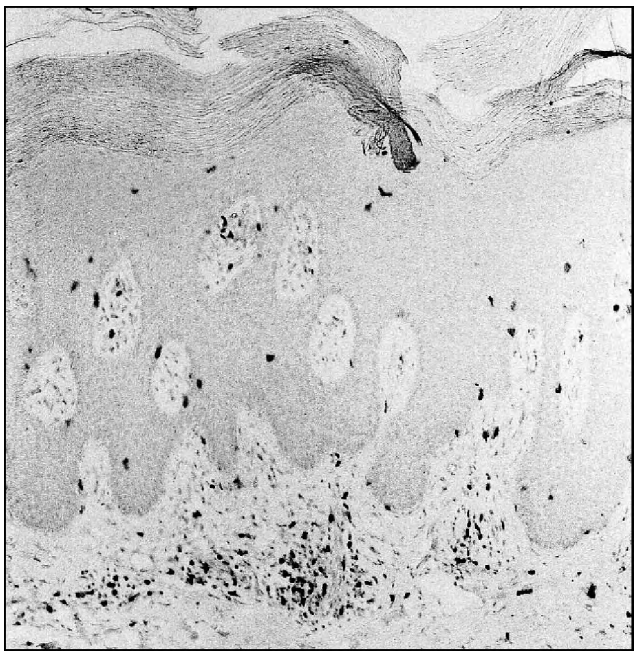


**Figure 23:**

A consecutive slide from the former slide shows that CD8 positive T-cells are the minority in the (epi)dermal infiltrates.

RCK108 occurred by a different semi-quantitative scoring system on a 3-point scale: 0 = negative staining, 1 = suprabasal single cell positivity, 2 = zebroid pattern.

Regarding CD4 and CD8 positive lymphocytes scoring occurred both for their global dermal and epidermal scores as well as for the subset of CD4, respectively CD8 positive T-cells in the dermal infiltrate areas. For the subset of CD4, respectively CD8 positive T-cells in the dermal infiltrate areas the following 6-point score was used: 0 = no staining, 1 = sporadic staining, 2 = 1-25 % staining, 3 = 26-50 % staining, 4 = 51-75 % staining, 5 = 76-99 % staining, 6 = 100 % staining. **Figures 22** and **23** are examples of CD4, respectively CD8 staining in consecutive slides of a biopsy from a representative psoriatic lesion.

Total dermal infiltrate scores were performed by using a semi-quantitative score on a 5-point scale: 0 = no infiltrate, 1 = minimal infiltrate, 2 = moderate infiltrate, 3 = moderate-pronounced infiltrate, 4 = pronounced infiltrate.

Keratinocytic epidermal neoplasia scores (KIN) were assessed according to Cockerell's criteria based on clinical and histological observations.[344] KIN grade I was defined as a flat, pink macula or patch on solar-damaged skin without roughness or hyperkeratosis and focal atypia of basal keratinocytes restricted to the lower one third of the epidermis. A KIN grade II actinic keratosis shows focal atypia of keratinocytes restricted to the lower two thirds of the epidermis in combination with the appearance of other typical hallmarks of actinic keratosis, such as alternating orthokeratosis and parakeratosis; clinically a pink to red papule or plaque with a rough, hyperkeratotic surface and variable induration is seen. KIN grade III is designated to actinic keratoses where diffuse atypical keratinocytic proliferation involves the full thickness of the epidermis in combination with other typical clinical and histological features of actinic keratoses.

All immunohistochemical and histological scoring was performed under blinded conditions.

# 1.24

## Irradiation experiments

Irradiation tests were performed in order to assess the influence of retinoids on the minimal erythema dose and on the degree of erythema after irradiation with broad-spectrum UVB-light.

Patients were pretreated with a topical or systemic retinoid using a certain dose, application schedule and for a distinct period that is known to be sufficient for retinoid-effects to be expressed. In paragraph 4.1 a color matched vehicle cream was used, consisting all ingredients of the comparable cream, but lacking the active metabolite. In the bexarotene study (paragraph 4.2) no placebo-treatment was used, but instead, irradiation tests were also performed before starting with the intake of bexarotene capsules.

After a distinct pre-treatment period with the topical or systemic retinoid, patients were irradiated with a series of 6 (tretinoin study) or 9 (bexarotene study) ascending intensities of UVB-light adjusted for the skin-type of the patient. These different irradiation intensities were performed on circular skin areas of 2.5 cm diameter each.

Irradiation occurred in a Waldman UV7001K cabin, using broad-spectrum UV21 lamps with a spectrum ranging from 285-350 nm and a maximum intensity between 310-315 nm. As sunlight-exposure may bias the study outcome, these tests were performed on the lower back of the subjects, thus using areas that are normally not exposed to high intensities of sunlight. For practical reasons, the 3 series of irradiation tests in the study with tretinoincream were performed on the vertrolateral upper parts of the upper legs. Irradiation always occurred on normal skin (tretinoin study) or uninvolved psoriatic skin (bexarotene study). Patients were instructed not to wash or scrub the test areas during the scoring period, they had to avoid sunlight exposure, and they were not allowed to use emollients or other kinds of topical treatments in the test areas.

Visual scoring occurred 24 hours (bexarotene study), or 24 and 48 hours (tretinoin study) after irradiation, as post-irradiation erythema is highly visible after 24 to 48 hours. The MED was determined by visual scoring and was defined as the lowest UVB-dose that caused a distinct erythema with sharp margins after irradiation. The intensity of the erythema for each irradiated area was measured by visual scoring using a 5-point scale: 0 = no erythema, 1 = weak erythema, 2 = moderate erythema, 3 = dark erythema and 4 = very dark erythema. The total erythema score before and after treatment (bexarotene study) or in the parallel sessions (tretinoin study) was assessed as the sum-score of all individual circular areas in each irradiation series. Visual scoring occurred under standardized conditions of temperature and light. During this scoring period patients continued with the same retinoid application schedule to assure maximum potential of retinoid modulating effects on irradiation-induced inflammation.

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# 2

# RXR-SELECTIVE RETINOIDS IN PLAQUE-TYPE PSORIASIS

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**A phase II multicenter clinical trial of systemic bexarotene in psoriasis.**
**J Am Acad Dermatol 2003:** ***(In press)***

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**Systemic treatment of psoriatic patients with bexarotene decreases epidermal proliferation and parameters for inflammation, and improves differentiation in lesional skin.**
**J Am Acad Dermatol 2003:** ***(In press)***

# 2.1

# Clinical effects of oral bexarotene in plaque-type psoriasis


## Abstract

*Bexarotene, a novel and unique synthetic RXR-selective retinoid, is available as a treatment for cutaneous T-cell lymphoma. In psoriasis, a common retinoid sensitive disease, no data are available on bexarotene-treatment. In this phase II study in psoriasis we investigated the safety, tolerability, and effectiveness of bexarotene at doses from 0.5 to 3 mg/kg/day. Fifty patients with moderate to severe plaque-type psoriasis were treated with bexarotene in 4 sequential dose-defined panels of 12-13 patients at doses of 1, 2, 0.5, and 3 mg/kg/day for 12–24 weeks. Patients were monitored for safety and clinical efficacy. No serious adverse events related to drug occurred. Bexarotene was well tolerated in most patients. Most frequently observed adverse events related to bexarotene were hypertriglyceridaemia (56%) and decrease in free T4 serum levels (54%). Significant improvement of psoriasis after bexarotene at all doses was confirmed by a modified psoriasis area and severity index (mPASI), plaque elevation (PEL), and physician's global assessment (PGA). Overall response rates (≥50% improvement) for mPASI, PEL, and PGA, were 22%, 52%, and 36% respectively. No significant dose-response effect was established for these parameters. The present study indicates an anti-psoriatic effect of bexarotene. Further studies are necessary to assess the optimal dose and the potential for bexarotene as a new therapy for psoriasis.*

## Introduction

Retinoids are frequently used for the treatment of psoriasis. Retinoid receptors belong to a superfamily of ligand-dependent nuclear receptors that includes hormone receptors for glucocorticoids, thyroid, androgens, estrogen, vitamin D and vitamin A among others. Ligand-receptor complexes of this superfamily are transcription factors that bind to the hormone response element of target genes and elicit a transcriptional response. For a detailed review on retinoid signal transduction and general biological aspects of retinoids, the reader is referred to an article by Joseph L. Napoli.[1]

There are 2 subfamilies of retinoid receptors in the superfamily: the retinoid acid receptors (RAR) and the retinoid X receptors (RXR). Each of these subfamilies has three subtypes, designated RARα, RARβ, RARγ, and RXRα, RXRβ and RXRγ. These receptors function as dimers and receptor subtypes may control both overlapping and unique target genes.

Each retinoid may bind and activate retinoid receptor subtypes differentially; there is receptor-ligand specificity. All-trans retinoic acid preferentially binds to the RAR subtypes and in vivo does not significantly bind to the RXR subtypes. 9-cis-Retinoic acid is a natural panagonist that binds with equal affinity to both RAR and RXR receptor subtypes, but so far RXR-selective ligands are synthetic.

Interestingly, the RXR receptors are unique compared to other nuclear receptors, as they function as heterodimers with other nuclear receptors (e.g., RAR, VDR, TR). Furthermore, there are different patterns of expression of retinoid receptors in all tissues of the body. In the skin the total concentration of RXR is 5 times higher than the total concentration of RAR. Of these RXR receptors RXRα is most abundantly present. As RARγ is also abundantly expressed in the epidermis, the RXRα-RARγ heterodimer is postulated to be one of the predominant regulating receptor complexes of retinoid sensitive target-gene expression in normal human skin. Furthermore, the heterodimer of the RXR receptor and the vitamin D3 receptor, RXR-VDR, is of major importance in RXR signaling in human skin.

Recently, a novel synthetic retinoid, bexarotene (Targretin®), has been developed for the treatment of cutaneous T-cell lymphoma (CTCL).[2] Bexarotene selectively binds to the RXRα, RXRβ and RXRγ subtypes.[3] This RXR-selective retinoid or "rexinoid" might also provide a new approach in the treatment of psoriasis. Animal studies demonstrated that bexarotene has antiproliferative effects in a number of tumors of breast, hematopoietic and squamous cell origin.[4-7] It can also induce apoptosis (programmed cell death) in specific tumor cell types.[8] Bexarotene causes thinning of the stratum corneum and in the rhino mouse, possesses antikeratinizing activity when topically applied. Bexarotene has been shown to inhibit T-cell accumulation in cutaneous T-cell lymphoma which is also an important feature of psoriasis.[2,9]

The unique receptor binding profile of bexarotene and the great relevance of RXR to human skin suggested the present dose ranging clinical study. In particular, we investigated the safety profile and tolerability of bexarotene in patients with moderate to severe plaque psoriasis and we addressed the question whether bexarotene can improve chronic plaque-type psoriasis, as measured by mPASI, PEL and a PGA.

## Materials and Methods

### Study design

The study was a phase II multicenter, open-label, dose-ranging study to investigate the safety and tolerability, and the clinical effect of systemic bexarotene in patients with moderate to severe plaque psoriasis.

Patients were enrolled at 4 study centers: the Department of Dermatology of the University Medical Center Nijmegen (The Netherlands), the Department of Dermatology of the University Hospital Antwerp (Belgium), the Department of Dermatology of the University Hospital Brussels (Belgium), and the Department of Dermatology of the University Hospital Ghent (Belgium). Ethics Committee approval prior to this trial was obtained at these 4 study-centers and all patients gave written informed consent prior to study enrollment.

Patients were entered in this study in four sequential dose-defined treatment panels in the following order: 1 mg/kg/day, 2 mg/kg/day, 0.5 mg/kg/day and 3 mg/kg/day (circa 44, 88, 20 and 102 mg/m$^2$/day). The 0.5 mg/kg/day panel was added to the other three panels by protocol amendment after the study had already started. Enrollment of this panel occurred after completion of the 1.0 and 2.0 mg/kg/day panels. This approach of enrollment in sequential dose panels was chosen to provide maximum safety for the patients. Each panel consisted of 12-13 patients. The treatment period was 12 weeks, with the possibility of treatment continuation for another 12 weeks for patients with at least 50 % improvement of psoriasis.

For the 0.5 mg/kg/day panel 10 mg capsules of bexarotene were used and for the 1, 2 and 3 mg/kg/day panels 75 mg capsules of bexarotene were used. Patients took the capsules once daily at the evening meal.

### Patient population

To be eligible for inclusion, patients needed to be 18 years or older with moderate to severe psoriasis involving at least 15 % body surface area at the baseline visit, as well as an overall plaque elevation score of at least moderate (grade 4 on a 9-point scale). Patients could not have a history of pancreatitis or clinical risk factors for it. Organ system functions assessed by blood screening parameters had to be within a protocol-defined range. All patients were required to have normal fasting triglycerides levels before enrollment in this study. The use of emollients and medicated psoriasis shampoos was to be discontinued for 24 hours before each study visit. All drugs that could significantly affect hepatic or renal excretion needed to be on a stable dose throughout the study period.

Exclusion criteria were: erythrodermic, pustular, guttate or inverse psoriasis; spontaneously improving or rapidly deteriorating plaque psoriasis; a history of any dermatological condition that would interfere with protocol evaluations; the use of systemic retinoids within 3 months prior to study entry or etretinate within 1 year prior to study entry; treatment with PUVA, UVB, methotrexate, cyclosporin, azathioprine, mycopheno-

late mofetil, systemic steroids, or fumarates within 4 weeks prior to study entry; the use of vitamin A supplements ≥15,000 IU or any topical anti-psoriatic therapy (except emollients or detergent shampoos) within 1 week prior to study entry; the use of other experimental therapies; the use of medicated or detergent shampoos or cleansers or emollients within 24 hours prior to study entry. Women who were of childbearing potential, pregnant or actively breast-feeding were excluded from participation and male subjects had to use contraceptive devices. Patients had to avoid prolonged exposure to the sun or UV-light sufficient to produce mild erythema.

### Safety and tolerability evaluation

At the pre-study visit the medical history was recorded, an ECG was taken, an ophthalmology examination was made and a physical examination including vital parameters (e.g. blood pressure, pulse, weight, temperature) was completed. Urinalysis and laboratory blood tests for biochemistry and hematology parameters were evaluated.

Patients began treatment with bexarotene within 14 days from the pre-study visit. Clinical evaluations occurred every 2 weeks until Week 12 of treatment. Vital functions were checked at every visit and changes in the physical examination from baseline were recorded. Adverse events reported by patients spontaneously or from inquiry were recorded.

Blood draws were performed every 2 weeks until Week 12 for biochemistry and hematology. If serum triglycerides levels were above the normal laboratory range, antilipidaemic treatment was started or increased. If serum triglycerides rose above 400 mg/dL, blood draws were performed weekly until they were below 400 mg/dL for 2 consecutive lab reports. If fasting triglycerides levels became higher than 800 mg/dL, then a reduction in dose with a possible suspension of treatment was required. Triglycerides levels over 1200 mg/dL required discontinuation from the study medication.

Serial photography of 5 selected psoriatic lesions occurred every 4 weeks. Patient quality of life during treatment was assessed every 4 weeks using the Psoriasis Disability Index[10] and the SF-36™ Health Survey.[11,12]

When patients continued with bexarotene therapy after Week 12, the visits and the blood draws were scheduled every 4 weeks until Week 24. A follow-up visit was performed within 4 weeks after patients stopped bexarotene treatment, both for premature withdrawal and for completion of the study period.

### Clinical efficacy evaluation

Overall psoriasis plaque elevation (PEL) was scored using a 9-point scale in 0.5 mm steps: 0 = no plaque elevation (0 mm), to 8 = very severe elevation (≥3.5 mm).[13,14]

A modified PASI score (mPASI) summing area of disease, erythema, induration, desquamation and pustulation on a 4-point scale (0-3) for 4 body regions was evaluated instead of the regular PASI score, in order to assess potential changes in pustulation. The mPASI score could range from 0 to 72.

A physician's global assessment score (PGA) that evaluated the improvement or worsening of the psoriatic signs and area as a percentage relative to the condition at baseline was evaluated on a 7-point scale: 0 = Clear (100 % improvement); 1 = Almost Clear (≥ 90 % improvement); 2 = Marked Response (≥ 75 % to < 90 % improvement); 3 = Moderate Response (≥ 50 % to < 75 % improvement); 4 = Slight Response (≥ 25 % to < 50 % improvement); 5 = Stable (< 25 % change); 6 = Worse (≥ 25 % worse).[13]

### Statistical analysis

Potential significant differences in baseline values of mPASI, PEL and PGA, were analyzed by Analysis of Variance (ANOVA). The differences in mPASI and PEL between baseline and bexarotene treatment were analyzed by a paired T-test. The onset and duration of response as defined above were analyzed by using the Kaplan-Meier method. Adverse event comparisons between the 4 dose panels were performed using a two-tail Fisher's exact test. For significant differences in laboratory analytes a paired T-test was used. SF-36™ Health and Psoriasis Disability Index questionnaires were summarized using descriptive statistics and analyzed over time using paired T-tests. The data for each questionnaire were summed at each time period by patient. The data at each study time point were also examined by both assigned dose and by all patients who were treated.

# Results

## Patient characteristics

Fifty patients were enrolled and treated with bexarotene capsules (panel 0.5 mg/kg/day 13 patients; panel 1 mg/kg/day 12 patients; panel 2 mg/kg/day 12 patients and panel 3 mg/kg/day 13 patients). The patient population consisted of 44 males and 6 females; with a mean age of 42.9 years ± 13.0 (SD). All patients had prior topical or irradiation therapies and 28 (56%) of the patients had prior systemic therapies for their psoriasis, of which acitretin (38%) and methotrexate (18%) were most frequently used. Forty-two patients (panel 0.5 mg/kg/day 12 patients; panel 1 mg/kg/day 9 patients; panel 2 mg/kg/day 10 patients and panel 3 mg/kg/day 11 patients) completed the 12-weeks treatment period. Eight patients were withdrawn from the study before the week 12 visit: 1 patient due to elevated ALT and AST levels, 3 patients due to hyperlipidaemia, 1 patient due to neck arthralgia, 1 patient for non-compliance, 1 patient for progressive disease, and the remaining 1 patient withdrew consent. Eighteen patients continued therapy after the week 12 visit; 13 of them completed the maximum treatment period of 24 weeks (2 patients in panel 0.5 mg/kg/day; 5 patients in panel 1 mg/kg/day; 4 patients in panel 2 mg/kg/day and 2 patients in panel 3 mg/kg/day).

**Table I:**
Common adverse events at least possibly related to bexarotene treatment with an indidence ≥ 3% for the different dose panels.

| Adverse event | 0.5 mg/kg/day N = 13 N (%) | 1 mg/kg/day N = 12 N (%) | 2 mg/kg/day N = 12 N (%) | 3 mg/kg/day N = 13 N (%) | All panels N = 50 N (%) |
|---|---|---|---|---|---|
| **Hypertriglyceridaemia** | 5 (38.5) | 8 (66.7) | 7 (58.3) | 8 (61.5) | 28 (56.0) |
| **Serum free T4 decrease** | 2 (15.4) | Not Done | Not Done | 12 (92.3) | 14 (53.8)* |
| **Asthenia** | 1 (7.7) | 1 (8.3) | 0 (0.0) | 5 (38.5) | 7 (14.0) |
| **Pruritus** | 1 (7.7) | 0 (0.0) | 2 (16.7) | 4 (30.8) | 7 (14.0) |
| **Cheilitis** | 0 (0.0) | 1 (8.3) | 2 (16.7) | 2 (15.4) | 5 (10.0) |
| **Infections** | 0 (0.0) | 1 (8.3) | 2 (16.7) | 0 (0.0) | 3 (6.0) |
| **Serum ALT increase** | 1 (7.7) | 1 (8.3) | 1 (8.3) | 0 (0.0) | 3 (6.0) |
| **Worsening of psoriasis** | 1 (7.7) | 0 (0.0) | 0 (0.0) | 2 (15.4) | 3 (6.0) |
| **Pain** | 1 (7.7) | 1 (8.3) | 1 (8.3) | 1 (7.7) | 3 (6.0) |
| **Serum AST increase** | 0 (0.0) | 1 (8.3) | 1 (8.3) | 0 (0.0) | 2 (4.0) |
| **Conjunctivitis** | 1 (7.7) | 0 (0.0) | 1 (8.3) | 0 (0.0) | 2 (4.0) |
| **Hair disorder** | 0 (0.0) | 0 (0.0) | 2 (16.7) | 0 (0.0) | 2 (4.0) |
| **Chills** | 1 (7.7) | 0 (0.0) | 0 (0.0) | 1 (7.7) | 2 (4.0) |
| **Vasodilatation** | 1 (7.7) | 0 (0.0) | 1 (8.3) | 0 (0.0) | 2 (4.0) |

* N = 26 subjects.

## Safety and tolerability evaluation

Bexarotene was well tolerated overall in most patients. Four patients were withdrawn from the trial because of laboratory-related adverse events; hyperlipidaemia (3 patients) and mild to moderately elevated ALT/AST levels (1 patient). Hyperlipidaemia was the most frequently observed related adverse event; 56% of the patients had lipid values (mainly triglycerides) above the upper limit of normal during bexarotene treatment. In 4 patients in panels 1, 2, and 3 the increase in triglycerides rose to levels more than 800 mg/dL. One of these patients began atorvastatin therapy and completed 24 weeks of study, but three other patients were discontinued because of elevated triglycerides. A significant increase in patient serum triglycerides was found in each dose panel ($p < 0.01$), however, no significant dose related increase could be observed between the 4 panels. Serum free T4 and TSH values were only monitored in the last dose panels conducted at 0.5 mg/kg/day and 3 mg/kg/day. In both panels a significant decrease in free T4 levels was found ($p < 0.001$). Free T4 levels decreased to levels below normal in 2/13 (15%) of patients in the 0.5 mg/kg/day dose group and 12/13 (92%) of patients in the 3 mg/kg/day dose group. No statistically significant differences were observed in TSH values. Adverse events that were observed during bexarotene treatment and that could possibly be related to bexarotene-induced hypothyroidism were asthenia ($p = 0.049$), a decrease in systolic blood pressure ($p < 0.001$), and hair disorders. However, these symptoms did not require thyroid hormone supplements in any patient.

Typical retinoid-related adverse events like pruritus and cheilitis were found in only 14%, and 10% of the patients, respectively (**Table I**). Other adverse events with an incidence ≥3% that were possibly related to bexarotene were asthenia (14%), infections (6%), increase in serum ALT values (6%), increased psoriasis (6%), pain (6%), increase in serum AST (4%), conjunctivitis (4%), hair disorder (4%), chills (4%), and vasodilatation (4%). Moderately elevated ALT/AST levels (2-3x upper limit of normal) associated with withdrawal of 1 patient appeared to be a function of the patient's prior conditions. Changes in these enzyme levels in other patients were not reported as adverse events at higher doses or longer treatment durations. A statistically significant decrease in ALT values could be demonstrated in panel 2 mg/kg/day ($p = 0.009$) and panel 3 mg/

**Table II:**
mPASI, PEL, and PGA scores (mean ± SEM) for the 4 dose panels at the baseline visit and at the latest visit on bexarotene treatment (endpoint) for all patients who fulfilled at least 6 weeks treatment (N=43).

| | mPASI (0-72) | | | PEL (0-8) | | | PGA (0-6) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Baseline | Endpoint | p-value | Baseline | Endpoint | p-value | Baseline | Endpoint | p-value |
| **0.5 mg/kg/day** | 8.6 ± 0.7 | 5.7 ± 0.8 | 0.003 | 4.2 ± 0.3 | 2.8 ± 0.5 | 0.004 | 5.0 ± 0.0 | 3.8 ± 0.4 | 0.009 |
| **1 mg/kg/day** | 9.1 ± 0.7 | 5.8 ± 0.6 | 0.005 | 4.8 ± 0.3 | 3.3 ± 0.4 | 0.005 | 5.0 ± 0.0 | 3.8 ± 0.4 | 0.022 |
| **2 mg/kg/day** | 9.9 ± 1.1 | 7.1 ± 1.2 | 0.027 | 4.7 ± 0.3 | 2.8 ± 0.4 | 0.001 | 5.0 ± 0.0 | 3.8 ± 0.6 | 0.119 |
| **3 mg/kg/day** | 8.2 ± 0.5 | 4.7 ± 0.5 | < 0.001 | 4.0 ± 0.2 | 2.6 ± 0.3 | < 0.001 | 5.0 ± 0.0 | 3.7 ± 0.3 | 0.001 |

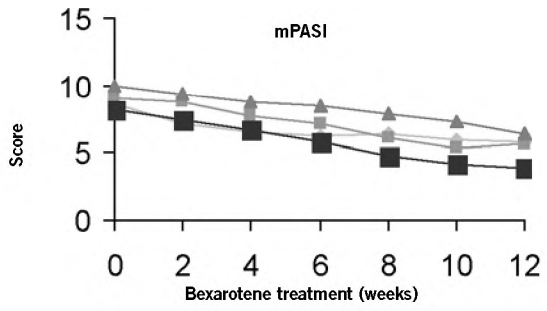


**Figure 1:**

Mean mPASI scores for the 4 different dose panels during 12 weeks bexarotene treatment (N = 42).

0.5 mg/kg/day
1 mg/kg/day
2 mg/kg/day
3 mg/kg/day

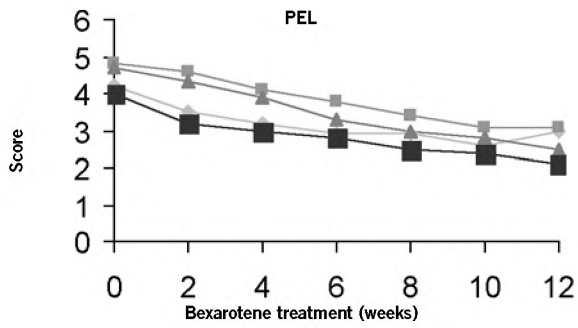


**Figure 2:**

Mean PEL scores for the 4 different dose panels during 12 weeks bexarotene treatment (N = 42).

0.5 mg/kg/day
1 mg/kg/day
2 mg/kg/day
3 mg/kg/day

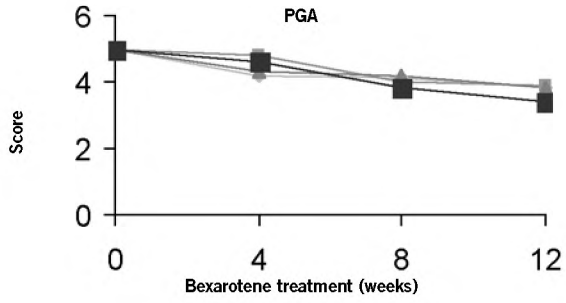


**Figure 3:**

Mean PGA scores for the 4 different dose panels during 12 weeks bexarotene treatment (N = 42).

0.5 mg/kg/day
1 mg/kg/day
2 mg/kg/day
3 mg/kg/day

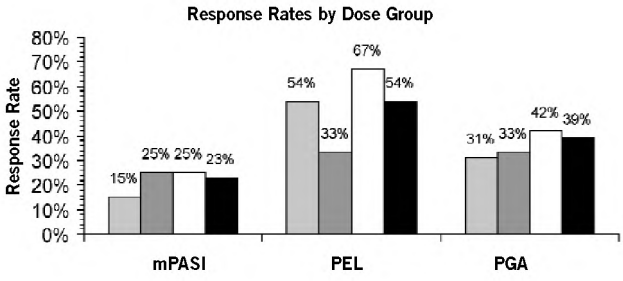


**Figure 4:**

Response rates defined as ≥ 50% improvement in mPASI, respectively PEL, respectively PGA, for the 4 different dose panels.

0.5 mg/kg/day
1 mg/kg/day
2 mg/kg/day
3 mg/kg/day

kg/day ($p = 0.048$) after bexarotene treatment. No significant differences in urinalysis and hematological parameters were seen in this study. Ophthalmologic examinations included 1 worsened cataract and new cataracts in both eyes in 1 patient that were not observed in the baseline examination.

### Clinical efficacy parameters

There were no significant differences in mean scores for body surface area (BSA), mPASI, PEL and PGA among the 4 dose panels at baseline. The BSA at baseline was 25.8 % ± 13.2 % (mean ± SD; N = 50). **Figures 1**, **2**, and **3** demonstrate respectively the mean scores for mPASI, PEL, and PGA during 12 weeks treatment with bexarotene for the 4 different dose groups. According to a paired T-test, significant reductions in mean scores for mPASI ($p < 0.05$) and PEL ($p < 0.01$) were found for all dose panels between baseline and study drug endpoint, which is defined as the last evaluation on treatment for each patient. No pustulation of psoriatic lesions occurred during the study. With respect to PGA scores, significant differences ($p < 0.05$) between baseline and endpoint were observed in all dose panels except for the 2 mg/kg/day dose group. **(Table II)**. No significant dose-response effect could be established for the investigated parameters.

The criteria for response were a 50 % or more decrease in mPASI or in PEL, or a 50 % or more improvement in psoriasis evaluated by PGA, at the week 12 visit. Responses needed to be confirmed over at least 2 consecutive assessments separated in time by at least 4 study weeks. **Figure 4** shows the overall response rates for mPASI, PEL and PGA by dose group. No significant differences in response rates among the 4 dose panels could be demonstrated for mPASI, PEL or PGA. At baseline, no differences between the responding population and the non-responders were observed with respect to clinical parameters, including prior systemic treatments and severity of psoriasis (mPASI, PEL, BSA). Almost complete clearance of ≥90 % according to PGA was observed in 4 patients (8 %); the distribution was comparable in all dose panels. **Figures 5** and **6** represent the same psoriatic plaque in one of the responders before and after 12 weeks treatment.

The onset of response by mPASI, PEL, and PGA for the 4 dose panels was seen after 2 – 20 weeks of bexarotene treatment. The duration of response is the time from the onset of response ( > 50 % improvement) to the time of relapse ( < 50 % improvement) or to the end of the final study visit, in case the response was ongoing at that moment, for each efficacy endpoint (mPASI, PEL, or PGA). Duration of response varied from 8 – 16 weeks.

**Figure 7** represents the mean scores for mPASI, PEL, and PGA for all responders who completed bexarotene treatment until week 24 (N = 13). These figures suggest no incremental benefit from bexarotene after the initial 12-week treatment period.

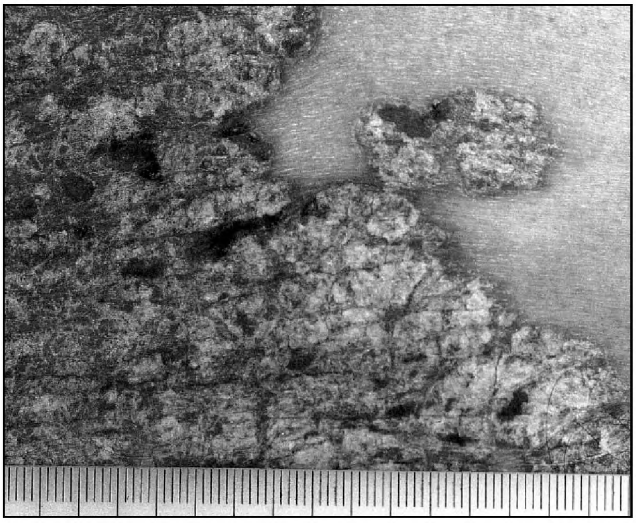

**Figure 5:**

A psoriatic plaque on the back in one of the patients from the 2 mg/kg/day panel before bexarotene treatment.

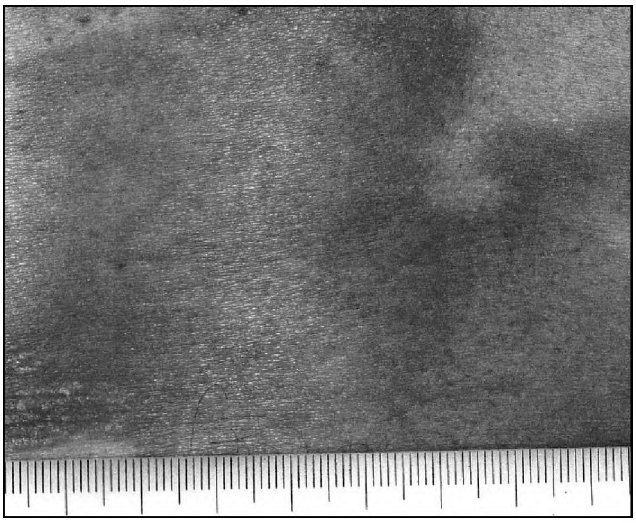

**Figure 6:**

The same psoriatic plaque as depicted in figure 5 after 12 weeks treatment with bexarotene.

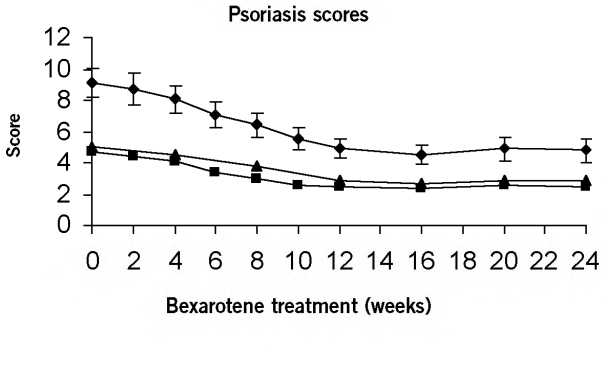


**Figure 7:**

mPASI, PEL, and PGA scores (mean ± SEM) during the study from all 13 responders who completed the maximum bexarotene treatment period of 24 weeks.

### SF-36™ Health and Psoriasis Disability Index questionnaires

No statistically significant differences were observed in the SF-36™ General Health questionnaires. The mean QOL response scores did not improve or worsen substantially in any dose panel. The Psoriasis Disability Index demonstrated a significant improvement in the 2 mg/kg/day panel after 24 weeks treatment ($p = 0.027$) and the 3 mg/kg/day panel after 12 weeks treatment ($p = 0.002$). However, the small number of patients, especially the remaining patients who continued bexarotene until week 24, may limit the significance of this outcome. No significant differences were observed in the lower dose panels of 0.5 and 1 mg/kg/day.

## Discussion

Bexarotene was studied at various doses in 50 patients with moderate to severe plaque psoriasis. Overall, this RXR-selective ligand demonstrated a statistically significant improvement of psoriasis (≥50%) with mean response rates of 22% for mPASI, 52% for PEL and 36% for PGA. No significant dose response relationship was demonstrated at the doses studied. It's intriguing that during the evaluation of the large dose finding study of acitretin in psoriasis no dose response relationship could be shown either.[15] Studies performed in psoriasis with acitretin showed response rates (≥50% improvement) for PASI scores up to 53.8%,[15] so data obtained at doses in the present study suggest the RXR-selective retinoid bexarotene is not superior to the currently used RAR-selective retinoids. However, as increases in dose up to the occurrence of mild cheilitis is relevant to reach maximal clinical efficacy in individual patients, and as in this study cheilitis has been observed in only 10% of the patients, it is tempting to speculate that bexarotene has not yet expressed maximal clinical efficacy at a dose of 3 mg/kg/day. On the other hand it is possible that the dose of maximal efficacy for bexarotene does not coincide with cheilitis due to its unique RXR binding profile. The lack of a dose response relationship in the present study and during acitretin treatment suggests that the optimal therapeutic dose in individual patients might be highly variable.

Clinical experience suggests that a large variation occurs in the bioavailability of oral acitretin among patients and that the dose has to be individualized in order to reach optimal efficacy. It should be stressed that no placebo group was included in the present study design, which aimed to find the dose that is tolerated well in patients with moderate to severe psoriasis.

Side effects such as a decrease in free T4 and hyperlipidaemia may restrict the use of higher doses of bexarotene in psoriatic patients. The decrease in free T4 levels that occurred at 3 mg/kg/day can lead to the clinical symptoms of hypothyroidism and may be related to heterodimer formation of the RXR with the thyroid receptor.[16] Although a dose-related reduction in free T4 levels was observed in this study, the clinical symptoms of hypothyroidism were absent or mild at doses up to 3 mg/kg/day and did not require thyroid supplements. Hyperlipidaemia is a well-established limitation of systemic retinoid treatment.[17,18] During acitretin treatment in a dose of 30-60 mg/day a 35% increase in serum triglycerides was observed.[18] Indeed 56% of the patients treated with bexarotene developed hyperlipidaemia as reported as an adverse event in the present study, but this effect was satisfactorily controlled with atorvastatin anti-lipaemic therapy in almost all patients.

In a study of bexarotene in CTCL in 48% (27 of 56) of the patients who received a dose of 300 mg/m$^2$/day (approximately 6.8 mg/kg/day) a partial or complete response (> 50% improvement) was found in PGA.[2] In the present study in psoriasis, where lower doses of bexarotene were given, we found in 36% (18 of 50) of the patients a partial or complete response (> 50% improvement) with respect to PGA. Concerning the adverse events, in the CTCL study similar adverse events were seen as in the present study, al-

though the frequency and severity was mostly higher in the CTCL study. The main adverse event in both studies is hyperlipemia (CTCL study: 82 %; psoriasis study: 56 %).

The present study suggests an anti-psoriatic effect of bexarotene. Based on clinical data, doses higher than 3 mg/kg/day and lower than 0.5 mg/kg/day should be investigated by using a placebo-controlled design that includes physician blinding with respect to laboratory findings in order to find the maximum effective dose of bexarotene. However, side effects, especially hypertriglyceridaemia and hypothyroidism, may be important factors requiring intervention. Bexarotene should also be evaluated in combination with other anti-psoriatic therapies.

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# 2.2

# Immunohistochemical effects of oral bexarotene in plaque-type psoriasis


## Abstract

*Bexarotene, a novel synthetic RXR-selective retinoid, has been reported to have anti-proliferative and apoptotic stimulating effects in cutaneous T-cell lymphoma. In benign, hyperproliferative and retinoid sensitive disorders, such as psoriasis, bexarotene has not been evaluated so far and no information on these parameters is available. In the present study immunohistochemical parameters for proliferation, differentiation, inflammation, and apoptosis were investigated in a group of bexarotene-treated psoriatic patients. Twenty-nine patients with plaque-type psoriasis were treated for 12 weeks with oral bexarotene in four dose-defined treatment panels. Treatment was initiated in the following consecutive order: 1 mg/kg/day, 2 mg/kg/day, 0.5 mg/kg/day, and 3 mg/kg/day. Biopsies for immunohistochemical analysis were taken at baseline and after 12 weeks treatment. Significant reductions in Ki-67, keratin 16, transglutaminase, dermal CD4, epidermal CD8 and inflammation scores were seen after bexarotene treatment, in combination with a significant increase in keratin 10 score. No induction of keratin 13 and 19 and no alterations in apoptosis-associated p53 expression were observed. Apart from a weak significant dose-response effect for Ki-67, no other significant dose-response effects were seen. We have demonstrated efficacy of oral bexarotene in psoriasis in doses up to 3 mg/kg/day during 12 weeks treatment for proliferation, differentiation, and inflammation parameters. Studies investigating higher doses of bexarotene in a larger number of patients are necessary to reveal potentially dose-related immunohistochemical effects of this new rexinoid and to elucidate the role of RXR-signaling in retinoid-associated keratin expression.*

## Introduction

Epidermal hyperproliferation, disturbed differentiation and cutaneous inflammation are the most important hallmarks of psoriasis. Retinoids have been demonstrated to modulate these processes and, therefore, they are frequently used in the treatment for this disease. Oral acitretin in a dose of 25 mg/day has been shown to reduce epidermal proliferation (Ki-67), hyperproliferation associated keratin 16, terminal differentiation (transglutaminase) and dermal inflammation.[1] However, retinoids currently used, such as acitretin and isotretinoin, only activate the retinoic acid receptor (RAR) pathway, even though they do not bind the RARs directly.[2] Agents that selectively bind to the retinoid X receptor subtypes or selectively activate this pathway, have not been investigated in psoriasis so far. As each retinoid receptor subtype controls a series of overlapping as well as unique target genes, and as RXR is abundantly expressed in human skin,[3] RXR-selective retinoids, like RAR-selective retinoids, may have beneficial effects in this retinoid sensitive disease.

Recently, a novel synthetic RXR-selective retinoid, bexarotene (Targretin®, LGD1069), has been developed for the treatment of cutaneous T-cell lymphoma (CTCL).[4] Bexarotene has been shown to cause thinning of the stratum corneum. Furthermore, bexarotene inhibits the growth of tumor cell lines of breast, hematopoietic and squamous cell origin[5-7] and induces apoptosis (programmed cell death) in a number of tumor cell lines.[8] In the rhino mouse topical application of bexarotene demonstrated antikeratinizing activity.

The present study was undertaken to analyze the impact of bexarotene on epidermal proliferation, keratinization, inflammation, and p53 protein expression in plaque-type psoriasis.

## Materials and Methods

### Study design

The study was conducted as a satellite laboratory study of the phase II multicenter, open-label, dose-response study designed to investigate the safety and tolerability, and the clinical efficacy of oral bexarotene in patients with moderate to severe plaque-type psoriasis. In the present study the immunohistochemical effects of bexarotene with respect to proliferation, differentiation, inflammation, and p53 protein-expression in psoriasis were investigated. Ethics Committee approval for this trial was obtained and all patients gave written informed consent prior to study enrollment.

Patients were entered in this study in four sequential dose-defined treatment panels in the following consecutive order: 1 mg/kg/day, 2 mg/kg/day, 0.5 mg/kg/day, and 3 mg/kg/day. The treatment period was 12 weeks. For the 0.5 mg/kg/day panel Targretin® capsules of 10 mg were used; for the 1, 2 and 3 mg/kg/day panels Targretin® capsules of 75 mg were used. Patients had to take the capsules daily at the evening meal.

### Patient population

Only patients entered at the University Medical Center Nijmegen were included in the present study. To be eligible for inclusion, patients had to meet the criteria of the multicenter clinical study, including: age older than 18 years; moderate to severe psoriasis involving at least 15 % body surface area at the baseline visit; an overall plaque elevation score of at least moderate (grade 4 on a 9-point scale); discontinuation of emollients and medicated psoriasis shampoos 24 hours before each study visit. In case of itching, patients were allowed to use an emollient (cremor lanette I) on the target lesion that had been chosen for biopsy purposes, but not in the 24 hours before each biopsy.

Protocol exclusion criteria were defined: erythrodermic, guttate or inverse psoriasis; spontaneously improving or rapidly deteriorating plaque psoriasis; a history of any dermatological condition that would interfere with protocol evaluations; the use of systemic retinoids within 3 months prior to study entry or etretinate within 1 year prior to study entry; treatment with PUVA, UVB, methotrexate, or cyclosporin within 4 weeks prior to study entry; the use of vitamin A supplements ≥15,000 IU or any topical anti-psoriatic therapy (except emollients or detergent shampoos) within 1 week prior to study entry; the use of medicated or detergent shampoos or cleansers or emollients within 24 hours prior to study entry. Patients had to avoid prolonged exposure to the sun or UV-light sufficient to produce mild erythema.

**Table I:**
Antibodies used in the study.

| Antibody | Specifity | Marker for | Concentration | Source |
|---|---|---|---|---|
| **MIB-1** | Ki-67 | Epidermal proliferation | 1:50 | Immunotech, Marseilles, France |
| **DE-K10** | Keratin 10 | Normal keratinization | 1:100 | Monosan, Uden, Netherlands |
| **1C7** | Keratin 13 | Retinoid-induced keratinization | 1:10 | Monosan, Uden, Netherlands |
| **Ks8.12** | Keratin 13,15,16 | Hyperproliferation | 1:100 | Sigma Chemicals, St. Louis, USA |
| **RCK108** | Keratin 19 | Retinoid-induced keratinization | 1:50 | Monosan, Uden, Netherlands |
| **BT621** | Transglutaminase | Terminal differentation | 1:10 | Biomedical Technologies Inc., Stoughton, MA, USA |
| **DO-7** | p53 | Apoptosis | 1:200 | DAKO, Glostrup, Denmark |
| **1F6** | CD4 | T-helper lymphocytes | 1:100 | Novocastra laboratories Ltd, Newcastle Upon Tyne, UK |
| **4B11** | CD8 | Cytotoxic T-lymphocytes | 1:100 | Novocastra laboratories Ltd, Newcastle Upon Tyne, UK |

## Biopsies and staining procedures

For assessment of immunohistochemical parameters punch biopsies of 3 mm were taken from the same, representative psoriatic lesion, under local anesthesia with xylocaine/1% adrenaline: one biopsy at the baseline and one after 12 weeks of treatment with bexarotene. All biopsies were fixed in formalin for 4 hours and then embedded in paraffin cubes after ethanol dehydration. From these paraffin cubes slices of 6 mm thickness were cut with a microtome and were placed on slides that were coated with 3-aminopropyl-triethoxysilane (Sigma Chemicals, St. Louis, USA). Sections were dewaxed in Histosafe, dipped in graded ethanol series, and rehydrated in demineralized water.

The following parameters were investigated: Ki-67 for proliferation, keratin 10 for keratinization, keratin 13 and keratin 19 for retinoid-induced keratinization, keratin 16 for hyperproliferation-associated differentiation, transglutaminase for terminal differentiation, p53 for apoptosis, CD4 and CD8 for inflammation and a hematoxylineosin staining for global infiltrate scores (Table I).

To unmask the epitopes that the antibodies are able to recognize, the following pretreatments were necessary:

- MIB-1, 1C7, 1F6, 4B11 and DO-7 staining required a high temperature microwave antigen retrieval technique. In brief: the slides were placed in 10 mM citrate buffer (pH = 6.0) in a microwave oven (760 W) and heated to boil. Subsequently sections were heated twice at 480 W for 5 minutes (boiling was prevented) and slowly cooled down to room temperature (20°C).

- For DE-K10 staining a 15-minute incubation period at 37°C with trypsin 0.1% was necessary.
- For Ks8.12 staining a 45-minute incubation period in 10 mM citrate buffer (pH = 6.0) was needed.
- RCK108 staining required a 7-minute incubation period with 0.1% pronase.
- BT621 staining required a 7-minute incubation period with 0.02% protease.

An indirect immunoperoxidase staining technique was used for all antibodies. Slides were incubated with 20% normal horse serum (Vector laboratories, Burlingame, USA) for 15 minutes, with subsequent incubation with the primary antibody (diluted in 1% bovine serum albumin (Organon Technika, Boxtel, the Netherlands)/PBS) for 1 hour. Sections were washed in PBS for 15 minutes. Secondary horse anti-mouse biotinylated IgG antibody (ABC kit-mouse, Vector Laboratories) (dilution 1:200 in 1% BSA/PBS) was added for 30 minutes, and again a 15 minutes wash in PBS was performed. Sections were treated with avidin-biotin complex antibody (ABC kit-mouse, Vector Laboratories)/(avidin/biotin diluted 1:50 in 1% BSA/PBS) for 30 minutes. Slides were washed in PBS for 15 minutes. To visualize staining for MIB-1, DE-K10, 1C7, and Ks8.12, RCK108 3-amino-9-ethylcarbazole (AEC; Calbiochem-Novabiochem Corporation, San Diego, USA) was used. Counterstaining occurred with Mayer's haematoxylin (Sigma Chemicals, St. Louis, USA). Finally, sections were mounted in glycerol gelatin (Sigma Diagnostics, St. Louis, USA). Staining for the other markers was visualized with diaminobenzoyl (Pierce, Rockford, USA). These slides were mounted in permount (Fisher Chemical, Fair Lawn, USA).

For global infiltrate scores a hematoxylin–eosin staining was performed as follows. After dewaxing in Histosafe and dipping in graded ethanol series a hematoxylin solution was added to the slides for 10 minutes, followed by a 10-minute washing step in tap water. Then an eosin solution was added to the slides for 90 seconds. After washing steps in ethanol and Histosafe, the slides were mounted in permount.

### Immunohistochemical scoring methods

MIB-1 and DO-7 positive keratinocytes (nuclei) were counted per mm length of tissue section. Transglutaminase scores were assessed as the ratio of BT621 positive epidermal cell layers divided by the total number of epidermal cell layers. Transglutaminase scoring occurred both in the papillary and the interpapillary epidermis. For DE-K10, 1C7, Ks8.12, RCK108, 1F6 and 4B11 staining the following semi-quantitative scale was used: 0 = no staining, 1 = sporadic staining, 2 = minimal staining, 3 = moderate staining, 4 = moderate-pronounced staining, 5 = pronounced staining, 6 = whole epidermis stained. With respect to CD4 and CD8 positive lymphocytes, scoring occurred both for global dermal and epidermal scores as well as for the subset of CD4, respectively CD8

positive T-cells in the dermal infiltrate areas. For the subset of CD4, respectively CD8 positive T-cells in the dermal infiltrate areas the following 6-point score was used: 0 = no staining, 1 = sporadic staining, 2 = 1-25 % staining, 3 = 26-50 % staining, 4 = 51-75 % staining, 5 = 76-99 % staining, 6 = 100 % staining.

Finally, total dermal infiltrate scores were performed by using a rather similar semi-quantitative score on a 5-point scale: 0 = no infiltrate, 1 = minimal infiltrate, 2 = moderate infiltrate, 3 = moderate-pronounced infiltrate, 5 = pronounced infiltrate. All immunohistochemical scoring was performed blinded by one evaluator.

### Statistical analysis

In order to substantiate potential dose-related differences and differences with respect to the immunohistochemical parameters between the baseline evaluation and the evaluation after 12 weeks treatment, a two way Analysis of Variance (ANOVA) test was used. In case this test revealed significance, a post hoc comparison using the Duncan multiple range test was performed.

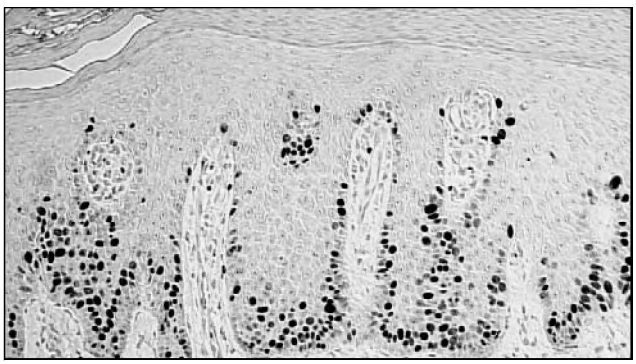


**Figure 1:**

MIB-1 staining in a tissue sample of a psoriatic plaque from one of the responders at the baseline visit.

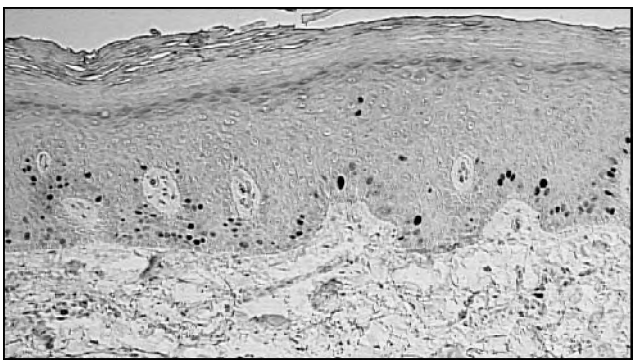


**Figure 2:**

MIB-1 staining in a tissue sample of the same psoriatic plaque as depicted in figure 1 after 12 weeks treatment with bexarotene. A significant decrease in the number of MIB-1 positive keratinocytes compared to the baseline is clearly seen.

## Results

In a total of 29 patients biopsies were taken before and after 12 weeks treatment with bexarotene. Patients received the following doses: 0.5 mg/kg/day, 8 patients; 1 mg/kg/day, 5 patients; 2 mg/kg/day, 8 patients; 3 mg/kg/day, 8 patients.

Evaluation of the parameter Ki-67 (MIB-1) showed a decrease in the number of proliferating keratinocytes in 24 patients. In 5 patients an increase was observed; 4 of these patients were from the 0.5 mg/kg/day panel and one from the 3 mg/kg/day panel. In **Figures 1** and **2** examples of MIB-1 staining are shown in tissue samples of lesional skin from one of the responders before and after 12 weeks treatment with bexarotene. **Figure 3** shows the mean MIB-1 scores for the 4 different dose panels. Statistical analysis demonstrated a significant decrease in MIB-1 scores over all panels ($p < 0.001$). A trend in dose-response was observed ($p = 0.080$). The 1.0, 2.0, and 3.0 dose panels demonstrated a statistically significant ($p < 0.05$) higher reduction in Ki-67 scores compared to the 0.5 mg/kg/day panel; no significant differences between the 1.0, 2.0, and 3.0 mg/kg/day dose panels were seen **(Figure 4)**.

**Figure 3:**
Ki-67 count (mean ± SEM) for the 4 different dose panels before and after 12 weeks treatment with bexarotene.

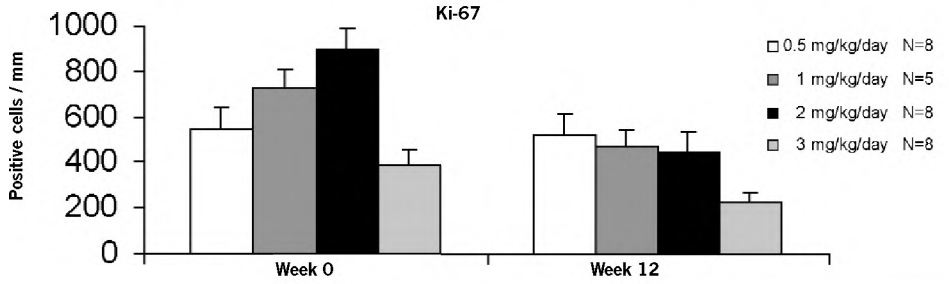
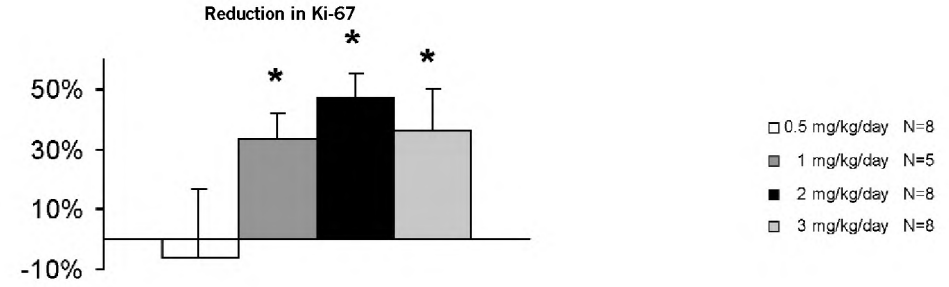


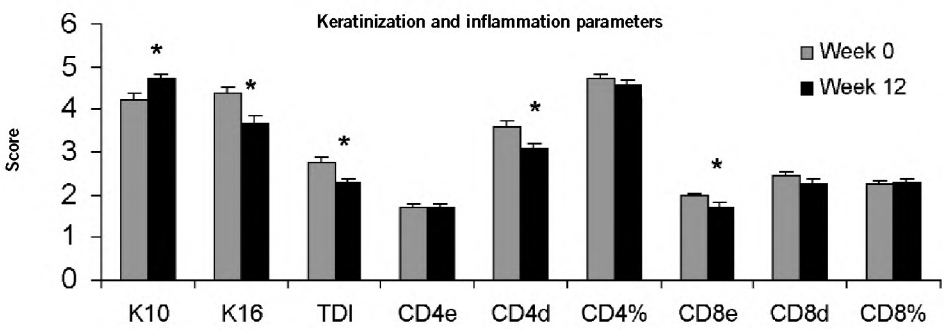

**Figure 4:**
Reductions in Ki-67 count (mean ± SEM) after 12 weeks bexarotene treatment for the 4 dose panels as percentage from baseline. Significant ($p<0.05$) stronger MIB-1 reductions for the higher dose panels compared to the 0.5 mg/kg/day panel are marked with an asterisk.



Keratinization and inflammation parameters before and after 12 weeks treatment with bexarotene are depicted in **Figure 5**. Keratin 13 (1C7) and keratin 19 (RCK108), markers for RAR retinoid-induced keratinization, were absent in all slides both before and after 12 weeks treatment (data not shown in Figure 5). Eccrine ducts and sweat glands in the tissue sections and esophageal tissue served as positive controls for these markers. Comparing baseline values with values after bexarotene treatment for all dose panels combined, a significant increase in keratin 10 expression (DE-K10), a marker for normal keratinization, was found ($p = 0.005$), as well as a significant decrease in hyperproliferation-associated keratin 16 (Ks8.12) ($p = 0.007$). Ks8.12 could be used as a marker for keratin 16, as keratin 13 was absent in all slides and as keratin 15 is only present in minor amounts in the basal layer of the epidermis. In **Figures 6** and **7** examples of keratin 10 staining are shown in tissue samples of lesional skin from one of the responders before and after 12 weeks treatment with bexarotene.

Total dermal infiltrate scores showed a significant reduction after 12 weeks treatment with bexarotene ($p < 0.001$). Specification for CD4 and CD8 positive lymphocytes showed a significant reduction in dermal CD4 positive T-cells ($p = 0.001$). No significant differences in epidermal CD4 scores were seen. For CD8 positive T-cells a significant decrease was found in epidermal scores ($p = 0.011$), but no changes were seen in dermal CD8 scores. No significant shift in T-cell populations was found when looked at the subset of CD4, respectively CD8 positive T-cells, in the dermal infiltrate areas. No significant dose-response relationships as to these inflammation parameters could be demonstrated.

**Figure 5:**
Keratinization and inflammation parameters (mean ± SEM) before and after 12 weeks treatment with bexarotene for all patients (N=29). Significant changes ($p<0.05$) are marked with an asterisk.




K10 = keratin 10; K16 = keratin 16; TDI = total dermal infiltrate; CD4e =epidermal CD4+ T-cells; CD4d = dermal CD4+ T-cells; CD4% = subset of CD4+ T-cells: CD8e =epidermal CD8+ T-cells; CD8d = dermal CD8+ T-cells; CD8% = subset of CD8+ T-cells

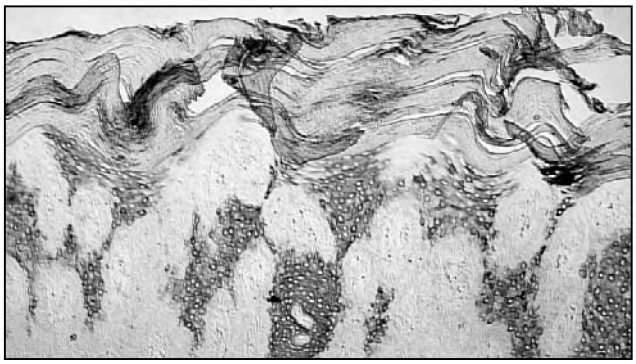


**Figure 6:**

Keratin 10 staining in a tissue sample of a psoriatic plaque from one of the responders at the baseline visit.

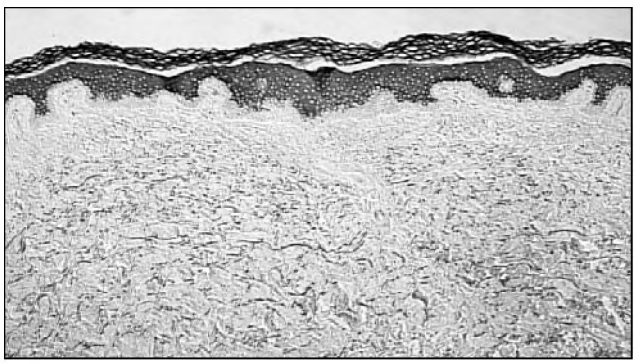


**Figure 7:**

Keratin 10 staining in a tissue sample of the same psoriatic plaque as depicted in figure 6 after 12 weeks treatment with bexarotene. A significant improvement in keratin 10 staining is clearly seen.

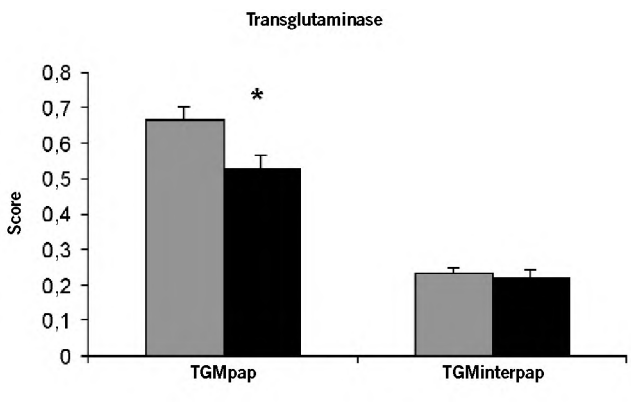



**Figure 8:**

Transglutaminase scores (mean ± SEM) before and after 12 weeks treatment with bexarotene for all patients (N=29). Significant changes ($p<0.05$) are marked with an asterisk.

TGMpap = transglutaminase score in papillary areas of epidermis; TGMinterpap = transglutaminase score in the interpapillary areas of epidermis.

Overexpression of terminal differentiation parameter transglutaminase (BT621) significantly decreased in the large interpapillary areas ($p = 0.012$) after bexarotene treatment (**Figure 8**). However, no significant differences were found in the smaller papillary areas. No significant dose response effects were observed with respect to transglutaminase. P53 protein was abundantly present in the psoriasis lesions. However, p53 expression remained unchanged during 12 weeks treatment with bexarotene for all dose panels (**Figure 9**).

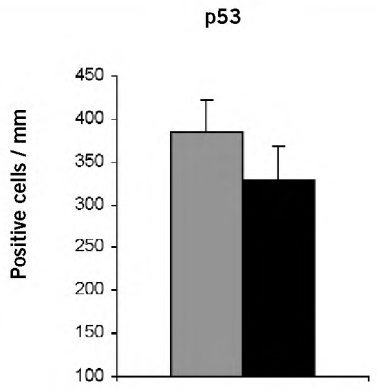



**Figure 9:**

p53 protein expression (mean ± SEM) before and after 12 weeks treatment with bexarotene for all patients (N=29).

## Discussion

In a non-randomized study where patients are enrolled in dose panels in consecutive order, differences in baseline values may be seen between the dose groups with respect to clinical and immunohistochemical parameters. In general, patients with severe psoriatic symptoms who easily fulfilled the inclusion criteria (e.g., at least 15 % body surface area involvement) are expected to be included in the first two dose panels, while the later two dose panels are expected to contain patients with milder symptoms who just fulfilled the inclusion criteria. Therefore, in this study we expected that patients enrolled in the first dose groups, respectively the 1.0 and 2.0 mg/kg/day panels, would have more severe values of clinical and immunohistochemical psoriatic parameters at the baseline than the 0.5 and 3.0 mg/kg/day panels.

The present study clearly shows that differences in baseline values for several parameters are present between some of the 4 dose groups. These differences in baseline values are in accordance with our hypothesis, as the 1.0 and 2.0 dose group show more severe values for several parameters than the 0.5 and 3.0 mg/kg/day panels and as this pattern is analogous for most parameters, including Ki-67, keratin 10, keratin 16, dermal CD4, modified PASI score and plaque elevation score. (The latter two parameters are reported in the corresponding multicenter clinical article).

Nevertheless, markers for Ki-67, keratin 10, keratin 16, transglutaminase, dermal CD4 count, and total dermal infiltrate scores clearly demonstrate improvement of psoriasis after bexarotene treatment with respect to proliferation, differentiation and inflammation. Except for a weakly significant dose response effect for MIB-1, in just 7-8 patients per dose panel, no other significant dose-response effects were found.

No significant differences were observed in p53 protein expression after bexarotene treatment in this group of patients. In some other studies bexarotene has been demonstrated to induce apoptosis. However, these studies were performed in malignant conditions.[7,8] Therefore, in psoriasis, bexarotene may not be able to influence apoptosis-associated p53 protein expression, or the number of patients and/or the doses used in the present study may be too low to detect such an effect.

Interestingly, no keratin 13 and/or keratin 19 expression, markers for conventional retinoid keratinization,[9,10] was observed after bexarotene treatment. It is known that RAR-selective retinoids can induce keratin 13 expression in doses sufficient for clinical and immunohistochemical improvement.[11-13] In this study efficacy has been demonstrated for several immunohistochemical parameters of proliferation, differentiation, and inflammation, as well as for clinical parameters, including a modified PASI score and the plaque elevation score. Typical retinoid associated adverse events, such as cheilitis and hyperlipidaemia, which were observed in some patients, strengthen the conclusion that bexarotene at the doses tested has activity in psoriasis. As bexarotene may elicit different transcriptional responses compared to RAR-selective retinoids, it is possible that RXR-selective retinoids, in contrast to RAR-selective retinoids, do not induce expression of retinoid-associated keratins. On the other hand it is possible they may do this only in higher doses via heterodimer formation with the RAR subtypes.

When comparing immunohistochemical results on epidermal proliferation, epidermal differentiation, and dermal inflammation in psoriasis after 12 weeks oral bexarotene treatment (the present study) with 12 weeks acitretin treatment (Kuijpers *et al*[1]), the following reduction percentages (bexarotene versus acitretin) are seen. Ki-67 35% versus 60%; Keratin 16 16% versus 77%; transglutaminase interpapillary 21% versus 29%; transglutaminase papillary 4% versus 36%; dermal infiltrate 18% (total dermal infiltrate) versus 65% (elastase). As demonstrated, the RXR-selective retinoid bexarotene has similar qualitative effects as the RAR-pathway favoring retinoid acitretin, although these data suggest that for the treatment of psoriasis the investigated concentrations of bexarotene in the present study are less effective than acitretin in a dose of 25-50 mg/day. Since bexarotene at the doses tested does not bind to the RARs, the observed effects are likely to be RXR-related or may be the result of binding of bexarotene to the RXR-RAR heterodimer.

In conclusion, we have demonstrated efficacy of oral bexarotene in psoriasis in doses up to 3.0 mg/kg/day during 12 weeks treatment for proliferation, differentiation, and inflammation parameters. However, no placebo was used in the present study and efficacy of the tested doses of this new compound seemed to be less when compared to regular doses of acitretin. Apart from a trend in dose-response for MIB-1 expression, no significant dose-response effects were found. Data obtained from clinical practice demonstrate a large interindividual variation in the bioavailability of oral retinoids, suggesting that the optimal dose in individual patients might be highly variable, and thus a dose-response effect may not be observed in this relatively small number of patients. Therefore, further investigation of these immunohistochemical parameters in a higher number of bexarotene-treated patients, by using a placebo-controlled approach that includes higher dose levels and serum level measurements, is indicated to reveal potential dose-response effects of bexarotene. Furthermore, pharmacogenetic studies focussing on identification of the responding population may be worthwhile. The role of RXR-signaling in retinoid associated keratin expression remains to be elucidated.

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# 3

# RAR-SELECTIVE RETINOIDS IN (PRE)MALIGNANT SKIN DISEASES

*This chapter was based on the following publications:*

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Departments of Nephrology[1] and Dermatology,[2] University Medical Center, Nijmegen, The Netherlands. **Acitretin treatment of (pre)malignant skin disorders in renal transplant recipients: clinical effects of a randomized trial comparing two doses of acitretin.**
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Departments of Dermatology[1] and Pathology,[2] University Medical Center, Nijmegen, The Netherlands. **Effects of temporary cessation of long term treatment with acitretin on keratinocytic intraepidermal neoplasia (KIN) in renal transplant recipients.**
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Departments of Dermatology[1] and Pathology,[2] University Medical Center, Nijmegen, The Netherlands. **Short-term treatment with topical all-trans retinoic acid for actinic keratoses: a randomised double-blind placebo-controlled clinical and immunohistochemical study. (Submitted).**

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Departments of Pathology[1] and Dermatology,[2] University Medical Center, Nijmegen, The Netherlands. **Retinoids strongly and selectively correlate with keratin 13 and not keratin 19 in cutaneous warts of renal transplant recipients.**
**Arch Dermatol 2002;138(1):61-5.**

# 3.1

# Clinical effects of oral acitretin in the treatment of actinic keratoses in renal transplant recipients


## Abstract

*After renal transplantation, the incidence of (pre)malignant skin lesions is high. Treatment with acitretin improves the number and aspect of actinic keratoses and appears to reduce the incidence of squamous cell carcinomas, but treatment is hampered by frequent side effects. No optimal long-term dosing advice is available. 26 long-term renal transplant recipients were randomized to 1 year treatment with acitretin, either 0.4 mg/kg/day throughout the whole year, or 0.4 mg/kg/day during the first three months, followed by 0.2 mg/kg/day for the remaining 9 months. On 9 different time points, the number of actinic keratoses and tumors was counted, and erythema and thickness of the lesions and the severity of side effects were scored. Patient's judgement was recorded using visual analog scores. In both groups, the number of actinic keratoses decreased by nearly 50%, but the number of new malignant tumors during the study year was similar to the number of tumors in the year before the study. Thickness of the keratoses decreased significantly in both groups. Acitretin dose had to be reduced in most patients because of the frequent occurrence of (mainly mucocutaneous) side effects. Patients' contentment about the aspect of their skin increased significantly, with no differences between groups. Acitretin therapy decreased the number of actinic keratoses in renal transplant recipients, also at a low maintenance dose of 0.2 mg/kg/day, and significantly decreased the degree of thickness of the lesions. However, the incidence of new skin malignancies remained unchanged. Despite the high incidence of mucocutaneous side effects, patient's contentment with the aspect of their skin increased significantly.*

## Introduction

After successful renal transplantation, the incidence of premalignant and malignant skin lesions is high. Especially actinic keratoses, keratoacanthomas and squamous cell carcinomas (SCC) are very common.[1] The most important risk factors for the development of these lesions are the long-term use of immunosuppressive drugs, sun exposure, and infection with human papillomavirus.[2,3] As no causal therapy is available, therapeutic options are limited to local excision, cryotherapy, topical chemotherapeutic agents, and radiotherapy. Usually many lesions are present in one patient, making it difficult to treat all these lesions at the same time. Therefore the risk of transition from a premalignant lesion to a malignant lesion is high. Recent studies have suggested that retinoids may be effective for the treatment of actinic keratoses and the prevention of squamous cell carcinomas (SCC) in renal transplant recipients.[4,5,6] However, long-term use of retinoids is often hampered by side effects,[7] whereas lesions recur rapidly after the treatment is stopped.[4] No data are available regarding the optimal long-term dosing advice.

We have performed a randomized study in renal transplant recipients with histologically proven actinic keratoses to compare maintenance doses of 0.2 and 0.4 mg/kg/day of acitretin respectively. Both treatment regimens were equally effective and decreased the number of actinic keratoses and improved patient well being. However, no effect on the development of SCCs was observed.

## Materials and Methods

### Patients

Adult renal transplant recipients with a stable graft function, who were on a stable dose of immunosuppressive therapy, were included. Patients should have a history of at least one squamous cell carcinoma of the skin and multiple ( > 10) actinic keratoses, of which at least one was histologically proven. Excluded were patients with a nephrotic syndrome, with hypercholesterolaemia ( > 9 mmol/l), hypertriglyceridaemia ( > 10 mmol/l), or elevated transaminase levels (ALT and/or AST more than twice the upper limit of normal) and patients with pregnancy (-wish), with excessive alcohol intake and patients using anti-epileptic drugs. The washout period for systemic retinoids was 3 months. Topical retinoid treatment had to be discontinued at least 4 weeks prior to study enrollment.

### Study design

This study was conducted as an open label clinical study to investigate the effect of long-term treatment with two different doses of oral acitretin on the number and aspect of actinic keratoses and the incidence of new tumors in renal transplant recipients. Patients were randomized to treatment with acitretin 0.4 mg/kg/day during the first 3 months, followed by either treatment with 0.2 mg/kg/day or treatment with 0.4 mg/kg/day during the next 9 months.

### Assessments

Clinical parameter assessment occurred at the following visits: baseline, week 2, month 1, month 2, month 3, month 4.5, month 6, month 9, and month 12. At these visits the number of all actinic keratoses was counted in separate body areas, laboratory parameters were monitored according to the protocol developed by Dutch dermatologists,[8] and suspicious lesions were biopsied or, if necessary, surgically removed. At baseline and again at 12 months, a spinal X-ray was performed.

The clinical picture of actinic keratoses in renal transplant recipients is often difficult to demarcate, as spectra with verrucae vulgares and verrucae planae can be seen as well as erythematous actinically damaged skin. Therefore, only the typical erythematous, scaling and often thickened lesions were counted.

Furthermore, erythema and thickness of actinic keratoses in each body surface area was assessed. For these parameters we used a semi-quantitative score on a 5-point scale (0 = none, 1 = slight, 2 = moderate, 3 = moderate-severe, 4 = severe).

With respect to adverse event monitoring special attention was paid to muco-cutaneous adverse events *(See appendix)*. These mucocutaneous adverse events were graded on a semi-quantitative scale also (0 = absent, 1 = mild, 2 = moderate, 3 = severe).

To evaluate subjective findings of the patients with respect to "redness of the skin", "roughness of the lesions", "scaling of the lesions" and "general contentment with the skin" (including mucocutaneous side effects), visual analog scores (VAS) were performed on a 10 point scale.

### Randomization procedure

At baseline, patients were randomly assigned to one of the treatment groups, with stratification for age ( $>$ 50 versus $\leq$ 50 years), skin-type (according to Fitzpatrick;[9] type 1 and 2 versus type 3 and 4), and number of previous carcinomas (1 versus $>$ 1). Randomization was carried out by opening a sealed envelope with the lowest available study number. Medical Ethics Committee approval was obtained and all patients gave written informed consent prior to study enrollment.

### Statistical analysis

Analysis was performed following the intention to treat principle. Wilcoxon's rank sum test, Wilcoxon's signed ranks test, Fisher's exact test and ANOVA for repeated measurements with post hoc Dunn's test were used as appropriate. Calculations were performed using the SAS system, version 6.12 (SAS institute, Cary, NC, USA). Results are expressed as means $\pm$ standard deviation unless stated otherwise. A p-value $<$ 0.05 was considered statistically significant.

## Results

### Demographics

Twenty-six patients were enrolled in the study. Demographic data of these patients are summarized in **Table I.** All patients were of Caucasian origin. No significant differences existed between the groups at baseline. The majority of patients also participated in a study focussing on the immunohistochemical effects of acitretin treatment on actinic keratoses (J.V. Smit *et al.*, in press).

### Dosage

Initially this study was planned as a randomized study to assess the effect of long-term acitretin treatment at a dose of 0.4 mg/kg/day or 0.2 mg/kg/day, after an initial treatment with 0.4 mg/kg/day for a period of 3 months. However, most patients could not tolerate the starting dose of 0.4 mg/kg/day due to mucocutaneous side effects (see below). We had to lower the dose in most patients already during the first 3 months. Fortuitously, the dose was lowered more in the low-dose group compared to the high-dose group, resulting in a significant difference in acitretin dose between the groups already after one month. The average actual dose of acitretin at 3 months was 0.35 ± 0.13 mg/kg/day in patients randomized to continuation of the 0.4 mg/kg/day dose and 0.18 ± 0.11 mg/kg/day in patients randomized to the 0.2 mg/kg/day group ($p = 0.004$; **Figure 1**). Beyond 3 months, acitretin dose decreased further in the high dose group but doses remained significantly different between the groups at all time points **(Figure 1)**.

One patient in each group stopped acitretin treatment, one because of severe headache, one because of mucocutaneous side effects. In five other patients (1 in the high-dose group, 4 in the low-dose group) treatment with acitretin was interrupted for a few weeks, in four of them because of mucocutaneous side effects, and was restarted later in a low dose. The duration of interruption of treatment was on average 4 weeks, and occurred beyond three months of treatment in all five patients. Nineteen patients continued to take acitretin over the full study period, although in reduced doses in most cases. Only 3 patients in each group used the planned dose of acitretin throughout the study period.

### Clinical efficacy parameters

In the 24 renal transplant recipients that completed the one-year study period, the number of newly formed tumors was counted and compared with the number of tumors in the 12 months before acitretin treatment. Thirty new SCCs developed during treatment, versus 28 in the year before treatment (NS). Only 1 basal cell carcinoma (BCC)

was diagnosed during the study, compared to 3 BCCs in the year before the study (NS). A non-significant reduction in the number of Bowen's disease (9 during treatment versus 20 in the year before treatment) and keratoacanthomas (7 during treatment versus 16 in the year before treatment) occurred during acitretin treatment. There were no significant differences in the incidence of SCCs, BCCs, keratoacanthomas, or Bowen's disease between the two dose groups during the study period.

Three out of 7 patients who did not have a SCC during the year before the study, remained free of SSC during the study; the other 4 patients had at least 1 SSC during the study. Seven out of 17 patients with at least 1 SCC during the year before the study remained free of SCCs; 10/17 had at least 1 new SCC.

At the start of the study, the number of actinic keratoses was 251 ± 190 in the high-dose group and 280 ± 165 in the low-dose group (NS). The lesions were located mainly on the arms (high-dose group 58 ± 26%, low-dose group 66 ± 14%; NS) and the legs (high-dose group 25 ± 25%, low-dose group 20 ± 15%; NS). A rapid decrease in the number of keratoses occurred in both groups **(Figure 2)**, which was significant beyond two months in both groups (ANOVA: both $p < 0.0001$). No differences between the groups occurred at any time point, nor were there differences in effect between different body regions.

**Table I:**
Demographic data.

| | High dose (N=14) | Low dose (N=12) | P |
|---|---|---|---|
| **Age (years)** | 54 ± 11 | 57 ± 8 | NS |
| **Sex (male/female)** | 8 / 6 | 5 / 7 | NS |
| **Years of immunosuppression** | 15 ± 7 | 17 ± 7 | NS |
| **Maintenance immunosuppression** | | | |
| Azathioprine/prednisone | 12 | 7 | NS |
| Cyclosporin/prednisone | 1 | 4 | |
| Other | 1 | 1 | |
| **Creatinine (μmol/l)** | 121 ± 27 | 119 ± 51 | NS |
| **Skin-type 1 + 2*** | 3 | 5 | NS |
| **Skin-type 3 + 4*** | 11 | 7 | |
| **Number of previous SCCs**** | | | |
| During year before study entry (No/patient) | 1.5 ± 1.6 | 0.8 ± 0.8 | NS |
| All previous SCCs** (No/patient) | 2.8 ± 2.6 | 3.5 ± 3.6 | NS |
| **Previous systemic retinoid use** | | | |
| Within 1 year before study | 0 | 0 | |
| More then 1 year before study | 1 | 2 | |
| **Previous local retinoid treatment** | 1 | 4 | |

* Skin-type (according to Fitzpatrick[9]): On sun exposure, the skin 1 = always burns, never bronzes; 2 = always burns, rarely bronzes; 3 = always bronzes, rarely burns; 4 = always bronzes, never burns

** SCC: squamous cell carcinoma. Values are given as means ± SD.

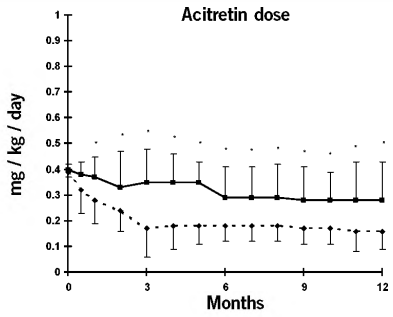



**Figure 1:**

Acitretin dose (means ± SEM; * = doses significantly different between groups).

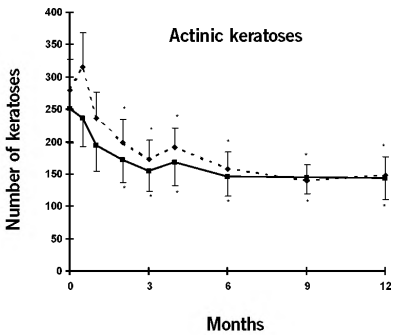
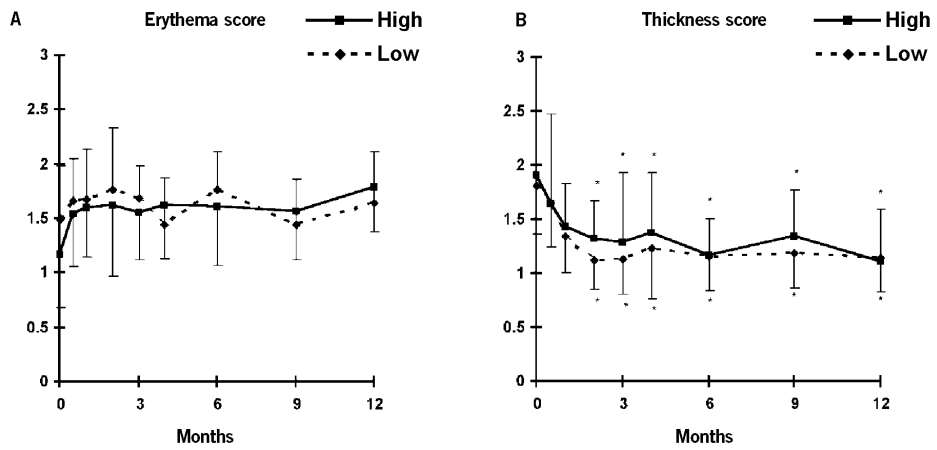



**Figure 2:**

Number of actinic keratoses per treatment group (means ± SD; * = numbers significantly different compared to baseline).

**Figure 3:**
Erythema (**3A**) and thickness (**3B**) scores of actinic keratoses per treatment group (means ± SD; * = numbers significantly different compared to baseline).



The degree of erythema and the thickness of the actinic keratoses throughout the study as observed by the investigators are depicted in **Figure 3**. No significant changes in erythema scores of the actinic keratoses were found **(Figure 3a)**. Thickness of the lesions decreased significantly in both groups (both $p < 0.01$ for comparison with baseline at all time points beyond 1 month of treatment; **Figure 3b**). No differences in response to acitretin treatment were observed with different immunosuppressive regimens; however, the number of patients not using azathioprine plus prednisone is small. **Figure 4** shows numerous hyperkeratotic actinic keratoses on the back of a hand in an average responder to acitretin at baseline. **Figure 5** shows the same hand after 12 weeks treatment with acitretin. A significant improvement in aspect and number of the lesions is obviously.

Although this study was focussed on actinic keratoses, our clinical impression was that the extent of warty lesions, such as common-, plane-, and sessile warts, which are generally present in large numbers in renal transplant recipients with multiple skin lesions, did also improve on oral acitretin.

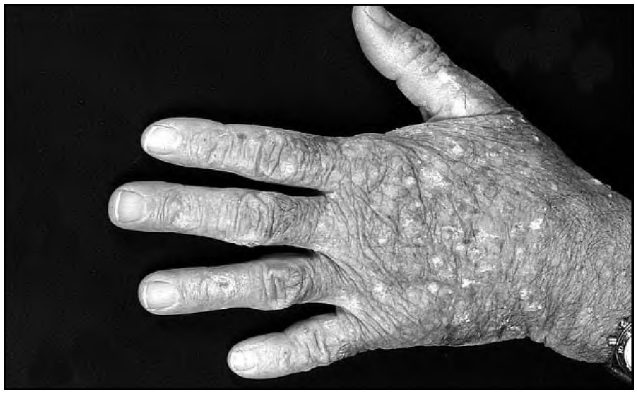


**Figure 4:**

Numerous hyperkeratotic actinic keratoses on the back of the hand in one of the subjects at baseline.

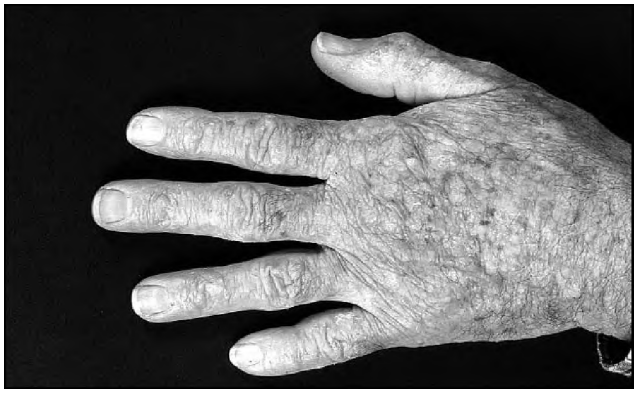


**Figure 5:**

The same hand as depicted in figure 4 after 12 weeks' treatment with acitretin.

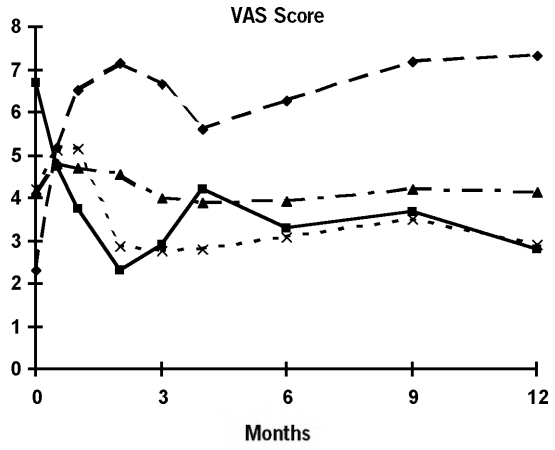



**Figure 6:**

Visual Analog Scores (all patients). Significant changes occurred in the roughness (p=0.002) and contentment (p=0.001) scores.

## Visual Analog Scores

Visual analog scores were available in 15/26 subjects (7/14 in the high dose group and 8/12 in the low dose group) at the scheduled follow up visits. Data are presented on all patients together, as there were no significant differences between the subgroups at any time point.

Roughness of the lesions decreased significantly during treatment with maximum efficacy after 2 months treatment ($p = 0.002$). The decrease in roughness slightly fluctuated, but remained till the end of the study period **(Figure 6)**.

Small changes in redness and scaling of the lesions were noticed during the study. In the first months of treatment scaling seemed to increase; thereafter scaling decreased to values even below the baseline value. These changes remained however non-significant.

A global skin score for evaluation of the contentment of the patients with respect to effect and adverse events, showed a significant increase with maximum contentment after 2 months treatment ($p = 0.001$).

## Side effects

Scoring of the severity of mucocutaneous side effects by the patients revealed within 2 weeks in both groups a significant increase of dryness of the lips, from "absent" to "mild" ($p < 0.001$). The dryness of the lips remained "mild" over the whole study period, although the acitretin dose decreased over time. A significant increase in scaling of the skin was observed during the first two months in both groups ($p < 0.05$). Dryness of the skin decreased significantly in both groups during the first two months ($p < 0.05$), but

increased to the baseline level thereafter. No significant changes in the other items occurred. Analyzing the number of patients developing a specific side effect within the first three months revealed significant rises in the number of patients complaining of dry lips, nasal crusts, and scaling of the hands (all $p < 0.01$). Beyond 3 months, nearly half of the patients started complaining of mild hair loss and/or brittle nails (both $p < 0.01$).

Renal function was stable in all patients and no significant change in the amount of proteinuria occurred. There were no liver function disturbances, nor a significant change in cholesterol or triglyceride concentration. No clinical or radiographic signs of skeletal damage were observed. In 2 patients, a significant decrease in hemoglobin level occurred; additional investigations revealed no explanation for the anemia. After completion of the study and interruption of the study medication, hemoglobin levels normalized spontaneously, suggesting a hitherto unknown side effect of acitretin.

Three female patients, who rarely had urinary tract infections in the years before the study, had recurrent symptomatic urinary tract infections during the study period, suggesting that the use of acitretin played a role (e.g. by thinning of vulvar and urethral mucosa). In one patient acitretin was interrupted temporarily because of an otherwise unexplained tendinitis of an Achilles' tendon.

## Discussion

In this study, an important reduction was found by treatment with acitretin in the number of actinic keratoses in renal transplant recipients (RTRs) receiving long-term immunosuppressive therapy. This effect occurred within two months after starting treatment, and could be maintained over the next 10 months by a low dose of 0.2 mg/kg/day acitretin. This reduction in the number of actinic keratoses by 50 % is even larger than described by Bouwes Bavinck,[4] who found a reduction in the number of keratoses of approximately 30 % in patients with a previous SCC. In two smaller studies that evaluated the effect of systemic acitretin treatment in RTRs, the number of keratoses also appeared to be reduced by retinoid treatment; however, quantitative data are lacking.[6,10] Apart from the reduction in the number of actinic keratoses, also a reduction in the degree of thickness was found.

In contrast with other studies,[4,6,10] no reduction in the incidence of SCC was found when comparing the number of SCCs in the year before the study with the incidence in the year of the study. There was a slight reduction in the number of keratoacanthomas and Bowen's disease as well as BCCs; this was however not significant.

Several hypotheses can be put forward to explain why a reduction in tumor incidence was not observed. First, the dose of acitretin could have been too low. However, the actual dose proved to induce mucocutaneous side effects in the majority of patients, indicating substantial acitretin availability. Also, the dose of acitretin in the patients in our high dose group was similar to the doses used by Gibson[10] and McKenna[6] (0.3 mg/kg/day of etretinate and acitretin, respectively), and only slightly lower than the dose used by Bouwes Bavinck[4] (30 mg/day). In addition, in our study no relation between the occurrence of new tumors and the use of a high or a low dose of acitretin could be found.

Second, it could be that in both dose groups tumor incidence remained unchanged during treatment with acitretin, where it would have increased if patients had been left untreated. This idea is supported by the data from the control group of Bouwes Bavinck, where the incidence of new tumors is much higher than in our treated groups.[4] However, as no control group was included in our study, this option cannot be proven.

Other possible explanations like differences in demographic characteristics seem unlikely: all studies have been performed in neighboring countries, with comparable sun exposure. Time after transplantation and the immunosuppressive regimen are similar, with the majority of patients on azathioprine/prednisone. Therefore, we have no definite explanation for the unchanged tumor incidence in this study.

The use of acitretin appeared to be safe. As retinoids have several effects on the immune system, the theoretical risk of inducing transplant rejection exists. In our study, the only patient in whom a decrease in graft function occurred already had chronic transplant dysfunction, and stopped treatment after 4 weeks due to mucocutaneous side effects. Thus it seems very unlikely that acitretin caused the decline in graft function in this case.

With the doses used, we did not find any evidence of well-known side effects of retinoids like liver function disturbances, hyperlipoproteinemia, nor skeletal abnormalities. This is an important finding, as RTRs are already at high risk for developing these side effects as a complication of their continuous use of immunosuppressive agents. On the other hand, some of the apparent side effects in our patients have been reported only rarely or even not at all, like anemia, Achilles' tendinitis and urinary tract infections.[11]

The great majority of side effects consisted of the well known mucocutaneous side effects of systemic retinoid use, as observed in patients with psoriasis and disorders of keratinization. However, in our study RTRs experienced these side effects at lower dosages than used for the treatment of psoriasis, where the advised dosage is 25-50 mg/day. A possible explanation could be that the skin of RTRs is already altered by the long-term use of systemic corticosteroids, leading to easy bruisability and atrophy of the skin. Alternatively, the occurrence of side effects at relatively low doses could be caused by a different pharmacokinetical profile of retinoids in RTRs. However, no literature data are available regarding this point.

Although many mucocutaneous side effects occurred that necessitated a dose reduction, patients' contentment with the aspect of their skin was good. Especially the softening of the skin, the thinning of the lesions and the alterations in macroscopic aspect were important for patients' contentment.

Taking together the incidence of mucocutaneous side effects, the efficacy in reducing the number of keratoses and the contentment of the patients, our dosing advice would be to start treatment with 0.25-0.30 mg/kg/day, and lower this dose if mucocutaneous side effects are severe or frequent. If only a modest therapeutic effect is seen, the dose should be increased to the maximal tolerated dose.

In conclusion, acitretin reduces the number of actinic keratoses in renal transplant recipients. This effect can be maintained at a low dose of acitretin (0.2 mg/kg/day). Although the incidence of mucocutaneous side effects is high, patients' contentment with their skin appearance improved. In contrast to earlier studies, no reduction in the number of new tumors was observed. Additional studies, including immunohistochemical studies, are needed to clarify the question whether retinoids merely have a cosmetic effect or really change the process of malignant degeneration of actinic keratoses in RTRs.

## Appendix

*Items in the semi-quantitative score of mucocutaneous adverse events:*

Dryness of lips
Dry mouth
Dry nose / crusts
Conjunctivitis
Dryness of skin
Scaling of hands
Scaling of other skin
Loss of hair
Brittleness of nails

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# 3.2

# Immunohistochemical effects of oral acitretin in the treatment of actinic keratoses in renal transplant recipients


## Abstract

*After renal transplantation, the incidence of (pre)malignant skin lesions is high. Treatment with acitretin improves the number and aspect of actinic keratoses, and appears to reduce the incidence of squamous cell carcinomas. However, no histological and immunohistochemical studies have been performed to further substantiate these observations. In 33 renal transplant recipients, biopsies were taken before and after 3 months of treatment with acitretin in doses up to 0.4 mg/kg/day. Histological and immunohistochemical parameters for dysplasia, epidermal thickness, proliferation, differentiation, apoptosis and dermal inflammation were analyzed. After acitretin treatment, a significant reduction in epidermal thickness ($p=0.002$) and a significant increase in normal differentiation parameter K10 ($p=0.02$) was observed. Epidermal proliferation did not change, nor did apoptosis, inflammation, keratinocytic epidermal neoplasia score, or transglutaminase staining. Remarkably, at baseline in 11 actinic keratoses a single cell expression pattern of K13 and/or K19 was found. This pattern was associated with high levels of parameters indicative of high-risk lesions ($p<0.05$). After acitretin treatment an increase in K13 ($p=0.006$) and K19 ($p=0.05$) was found, together with a change in expression towards a focal or band-like staining pattern normally seen after retinoid treatment. The latter expression pattern was not associated with high-risk parameters. These data suggest we deal with two epiphenomena: malignant transformation-associated dedifferentiation (single cell expression) and retinoid-associated differentiation (focal or band-like expression). In conclusion, acitretin improves the aspect of actinic keratoses via alteration of keratinization, resulting in peeling of the stratum corneum. No significant change in proliferation was found, which may explain for the rapid recurrence of actinic keratoses seen in other studies after cessation of acitretin treatment.*

## Introduction

In renal transplant recipients (RTRs) receiving long-term immunosuppressive treatment, an increased incidence of several benign wart-like lesions and (pre)malignant conditions has been found. The most frequently occurring (pre)malignant lesions are actinic keratosis (AK), Bowen's disease, squamous cell carcinoma (SCC), keratoacanthoma, basal cell carcinoma, and porokeratosis.[1,2] Major etiological factors include previous sun exposure, the duration of immunosuppressive therapy, and human papillomavirus (HPV) infections.[1,3] AKs are the lesions most frequently seen in this population and have been reported in up to 38 % of these patients already after 5 years follow-up, and even increase thereafter.[4] The percentage of AKs that will convert into a SCC within one year varies between 0.25 % and 16 % depending on the number, sort and duration of risk factors present, especially the use of immunosuppressive drugs.[5]

AKs in RTRs usually are multiple and behave more aggressive when compared to identical lesions in non-immunocompromized individuals.[6] Treatment modalities like surgical excision, cryotherapy, or topical fluorouracil, are often difficult to perform in view of the multiple localizations and involvement of large body areas. Therefore, a systemic treatment preventing malignant transformation is urgently needed.

Data obtained from 3 studies in RTRs where AKs were treated with systemic etretinate or its biologically active metabolite, acitretin, showed a significant decrease in the number and an improved aspect of the AKs.[7,8,9] In 5 studies it was mentioned that these systemic retinoids also decreased the number of SCCs.[7,8,9,10,11] However, only one of these studies was randomized and placebo-controlled.[7] After cessation of systemic retinoid treatment a recurrence in the number and aspect of AKs[7,9] and in SCCs[9,10,11,8] was frequently seen.

Ideally, the criteria for improvement of AKs comprise both changes in clinical appearance and changes in relevant histopathological and immunohistochemical features of the preneoplastic process. Clinical improvement without improvement of dysplastic and proliferative characteristics may mask these premalignant keratoses and may mislead both patient and physician. Apart from data on Langerhans cells,[12,13] no histological and immunohistochemical studies investigating the effect of systemic retinoids on AKs in RTRs have been performed to strengthen or contradict the clinical impression.

It is known that malignant transformation is represented by dedifferentiation, which is associated with a switch in synthesis of high molecular weight proteins, such as keratin 1 (K1) and keratin 10 (K10), towards low molecular weight proteins, such as keratin 13 (K13) and keratin 19 (K19).[14] In normal human epidermis these keratins are absent,[15] but they can be expressed in SCCs.[16] No information is available on the expression of these low-molecular weight molecules in AKs.

Therefore, in the present study we evaluated both parameters for dysplasia, epidermal thickness, dermal infiltrate, epidermal proliferation, epidermal differentiation, and apoptosis, as well as parameters for dedifferentiation and retinoid-induced keratinization, the low molecular weight proteins K13 and K19 in AKs in RTRs.

## Materials and Methods

### Study design

This study was conducted in adult RTRs with a stable graft function and a stable dose of immunosuppressive therapy. Patients initiated treatment with acitretin 0.4 mg/kg/day, unless it was known from previous acitretin treatment that this dose was intolerable because of side effects. In that case the highest dose still tolerable by the patients was applied. The duration of treatment was 12 weeks. After this 12-week study period patients remained on acitretin therapy for another 9 months for further clinical evaluation.

Patients should have a history of at least one SCC of the skin and ≥10 AKs; or ≥20 AKs if no previous SCC had occurred. Exclusion criteria were: excessive alcohol intake, the use of anti-epileptic drugs, nephrotic syndrome, hypercholesterolemia ( > 9 mmol/l), hypertriglyceridemia ( > 10 mmol/l), elevated transaminase levels (ALT and/or AST more than twice upper limit of normal) and pregnancy (–wish). The washout period for systemic retinoids was 3 months. Topical retinoid treatment had to be discontinued at least 4 weeks prior to study enrollment to ensure an adequate epidermal turnover (in normal skin 2-3 weeks; in hyperproliferative disorders < 1 week[17]) with respect to potential previous retinoid-associated effects by these agents. Medical Ethics Committee approval was obtained and all patients gave written informed consent prior to study enrollment.

### Biopsies and staining procedures

Punch biopsies of 4mm were taken under local anesthesia with xylocain/1 % adrenaline at baseline and after 3 months treatment. Biopsies were taken from AKs that were clinically identical at baseline and had the typical erythematous, scaling and mostly elevated aspect and from the same body region. Suspicious lesions that were present at baseline or that newly formed during the study, were biopsied for diagnosis or, if necessary, surgically removed.

Biopsies were analyzed using standard histological techniques. The following histological parameters were assessed: thickness of the lesions, total dermal infiltrate score (TDI), and keratinocytic epidermal neoplasia score (KIN).[18]

The following immunohistochemical stainings were performed, using the avidin-biotin-complex (ABC) staining method and indirect immunoperoxidase techniques: Ki-67 (MIB-1, epidermal proliferation), p53 (DO-7, apoptosis association), K10 (DE-K10, normal keratinization), K13 and K19 (1C7 and RCK108 respectively, retinoid-associated keratinization), K16 (LL025, hyperproliferation-associated keratinization) and transglutaminase (BT621, terminal differentiation). Esophageal tissue, eccrine ducts, and sweat glands served as positive controls for K13 and 19 staining. Further details on the immunohistochemical markers are provided in **Table I.**

**Table I:**
Antibodies used in the study.

| Antibody | Specifity | Marker for | Concentration | Source |
|---|---|---|---|---|
| **MIB-1** | Ki-67 | Epidermal proliferation | 1:50 | Immunotech, Marseilles, France |
| **DE-K10** | Keratin 10 | Normal keratinization | 1:100 | Monosan, Uden, Netherlands |
| **1C7** | Keratin 13 | Retinoid-induced keratinization | 1:10 | Monosan, Uden, Netherlands |
| **LL-025** | Keratin 16 | Hyperproliferation-associated keratinization | 1:10 | Novocastra laboratories Ltd, Newcastle Upon Tyne, UK |
| **RCK108** | Keratin 19 | Retinoid-induced keratinization | 1:50 | Monosan, Uden, Netherlands |
| **BT621** | Transglutaminase | Terminal differentation | 1:10 | Biomedical Technologies Inc., Stoughton, MA, USA |
| **DO-7** | p53 | Apoptosis | 1:200 | Dako, Glostrup, Denmark |

## Histological and immunohistochemical scoring

With respect to histological analysis, epidermal thickness (mm) was measured in the center of the AKs, and additionally assessed by counting the number of cell layers. TDI scores were performed by using a similar semi-quantitative score on a 5-point scale: 0 = no infiltrate, 1 = minimal infiltrate, 2 = moderate infiltrate, 3 = moderate-pronounced infiltrate, 4 = pronounced infiltrate. KIN scores were assessed according to Cockerell's criteria for keratinocytic epidermal neoplasia.[18] In brief, lesions were graded based on the degree and extent of dysplasia on a 4-point scale.

Immunohistochemical scoring was based on methods previously published[19] and occurred along the complete length of the slides, but only in the sections that showed typical histological signs of AKs. Transglutaminase scores were assessed in the center of the AKs as the ratio of BT621 positive epidermal cell layers divided by the total number of epidermal cell layers. Ki-67 and p53 positive keratinocytes (nuclei) were counted per mm length of section. For scoring of keratinization parameters K10, K13 and K16, the following semi-quantitative scale was used: 0 = no staining, 1 = sporadic staining (= single cell expression), 2 = minimal staining, 3 = moderate staining, 4 = moderate-pronounced staining, 5 = pronounced staining, 6 = whole epidermis stained.

All histological and immunohistochemical scoring occurred under blinded conditions. Demographic and dosage-related results are expressed as means ± standard deviation (SD); histological and immunohistochemical results are expressed as means ± standard error of the mean (SEM) unless stated otherwise.

## Statistical analysis

For assessment of statistical significant differences before and after 12 weeks treatment with respect to the histological and immunohistochemical parameters, a Wilcoxon matched pairs test was used. Assessment of statistical significant correlations between parameters was performed by calculating Spearman's rank correlation. Differences between K13 and/or K19 responders versus non-responders were analyzed by using the Mann-Whitney U-test. A p-value $< 0.05$ was considered statistically significant. Calculations were performed using Statistica version 9.0 (StatSoft Inc, 2300 East 14th Street, Tulsa, OK 74104, USA).

**Table II:**
Demographic data.

| | |
|---|---|
| **Age (years)*** | 53.6 ± 9.7 |
| **Sex (male/female)** | 15 : 18 |
| **Years of immunosuppression*** | 16.4 ± 6.7 |
| **Maintenance immunosuppression** | |
| Azathioprine/prednisone | 25 |
| Cyclosporin/prednisone | 6 |
| Mycophenolate mofetil/prednisone | 1 |
| Cyclosporin/azathioprine | 1 |
| **Number of previous SCCs (N/patient)** | |
| All previous SCCs | 2.6 ± 2.8 |
| During year before study | 1.0 ± 1.3 |
| **Previous systemic retinoid use** | |
| Within 1 year before study | 0 |
| More then 1 year before study | 3 |
| **Previous local retinoid treatment** | 7 |

* Values are given as means ± SD.

## Results

### Demographics

Demographic data are summarized in **Table II**. Thirty-three patients (15 men and 18 women) were included in this study. Most patients also participated in a randomized study focussing on the clinical effects of acitretin on AKs (R. de Sévaux *et al*, in press). The mean age was 53.6 ± 9.7 years. The follow-up period after renal transplantation was 16.4 ± 6.7 years. Twenty-nine patients had a history of one or more SCCs, with a mean of 2.6 ± 2.8 SCCs per patient (excluding keratoacanthomas). Maintenance immunosuppressive treatment consisted of: prednisone plus azathioprine (25 patients), cyclosporin plus prednisone (6 patients), mycophenolate mofetil plus prednisone (1 patient), and cyclosporin plus azathioprine (1 patient).

### Dosage

Except for two patients who started with a lower dose (0.2 mg/kg/day) due to previous intolerability of higher acitretin doses, the remaining 31 patients initiated treatment with acitretin 0.4 mg/kg/day. Most of these patients could not tolerate the starting dose of 0.4 mg/kg/day due to mucocutaneous side effects (mostly cheilitis, intensive peeling, and eye irritation/conjunctivitis). Therefore, all patients received the maximum dose of acitretin that was still tolerated. The starting dose was 39.8 ± 8.6 mg/day (0.38 ± 0.07 mg/kg/day). At the end of the 12 weeks treatment period the mean dose had been reduced to 20.2 ± 9.6 mg/day (0.26 ± 0.13 mg/kg/day).

### Histological results

Biopsies taken at baseline and after 12 weeks of treatment with acitretin were available in 32 out of 33 patients. One patient withdrew from the study because of severe mucocutaneous side effects after 8 weeks of treatment. In 28 baseline samples a typical actinic keratosis was found, in 2 samples only hyperkeratosis without dysplasia was seen, one actinic keratosis had already progressed to a SCC, and one hyperkeratotic lesion appeared to be a common wart. One biopsy was not intact and was therefore excluded from evaluation. Therefore, 27 pairs of biopsies taken before and after acitretin treatment could be used for histological and immunohistochemical analysis.

On histological examination, a mean reduction in epidermal thickness of 44.0 % ($p < 0.01$) was found after acitretin treatment. This reduction was completely caused by a decrease in stratum corneum thickness; no significant reduction could be seen in the other strata **(Figure 1)**.

At baseline, minimal to moderate TDI scores were found in 85 % of the AKs, whereas the remaining 15 % demonstrated a moderate-pronounced TDI. The infiltrates were

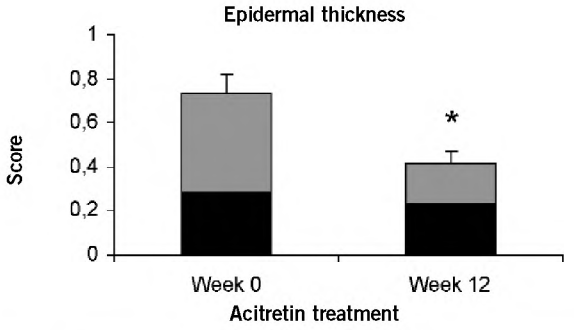
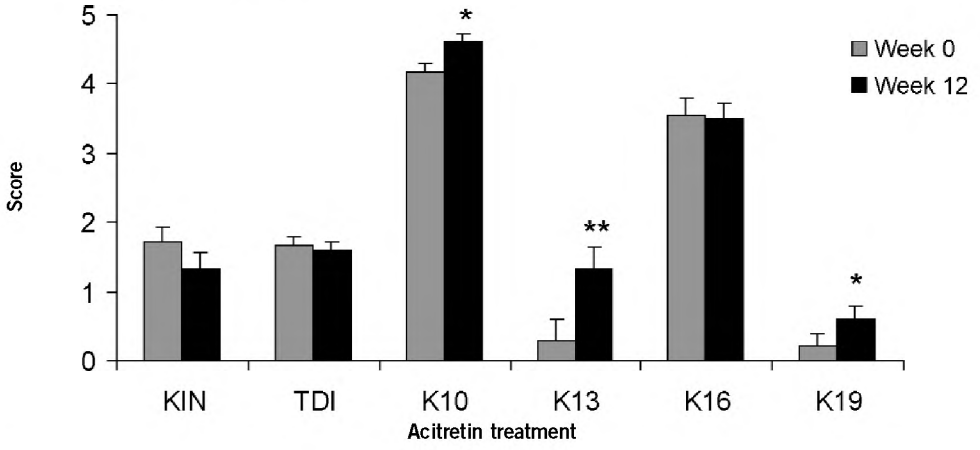



**Figure 1:**

Epidermal thickness as the sum score of stratum corneum thickness and thickness of the other strata in millimeter at baseline and after 12 weeks treatment with acitretin (mean ± SEM) (* $p < 0.01$; N=27).

Stratum corneum
Other strata

**Figure 2:**
Semi-quantitative scores for keratinocytic intraepidermal neoplasia (KIN), total dermal infiltrate (TDI), and keratins K10, K13, K16, and K19 at baseline and after 12 weeks treatment with acitretin (mean ± SEM) (* $p < 0.01$; **$p \leq 0.05$; N=27).



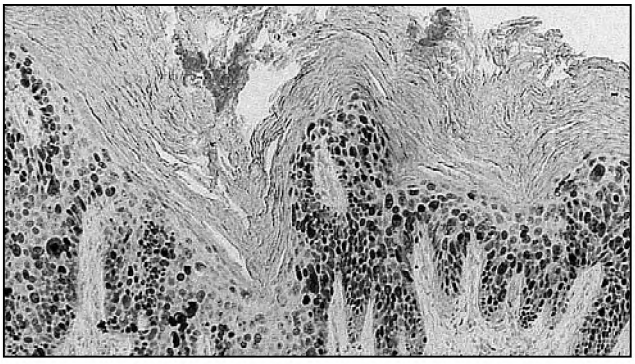



**Figure 3:**

Numerous proliferating keratinocytes are often seen in actinic keratoses.

localized perivascular and in the upper dermis. After 12 weeks of treatment, no significant alterations were seen in TDI scores (Figure 2).

The KIN score, a scoring system for dysplasia, did not change significantly during the study period. Before treatment the KIN score was 1.7 ± 0.2 versus 1.3 ± 0.2 after treatment (Figure 2).

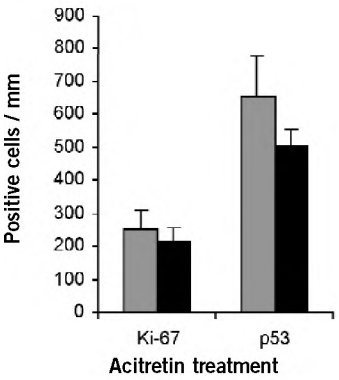



**Figure 4:**

Ki-67 and p53 scores as the number of positive stained cells per millimeter length of section of the slides at baseline and after 12 weeks treatment with acitretin (mean ± SEM) (N=27).

Week 0
Week 12

## Immunohistochemical results

At baseline, in most AKs the epidermal proliferation parameter Ki-67 revealed numerous proliferating cells mostly in the basal cell layer, but also to a variable extent depending on KIN score in the suprabasal regions of the epidermis. Ki-67 expression was restricted to the nuclei and featured a rather homogenous staining pattern in the horizontal plane of the epidermis. An example of MIB-1 staining for Ki-67 expression is seen in Figure 3. Treatment with acitretin did not lead to a significant alteration in the number of Ki-67 positive keratinocytes (Figure 4). In 13 patients an increase in Ki-67 positive cells could be demonstrated, whereas in 14 patients a decrease in this number was found after acitretin treatment.

Apoptosis associated p53 protein at baseline was mostly observed in the basal and lower suprabasal cell layers of the epidermis, but in some AKs p53 expression throughout all epidermal cell layers was seen. P53 expression basically followed the pattern of Ki-67. However, incomplete staining areas for p53 were often observed, whereas the corresponding Ki-67 staining in a consecutive slide was continuous along the complete length of the slide. Figure 5 clearly shows this focal staining of p53 in an actinic keratosis. Overall, the number of p53 positive cells was also much higher than the number of Ki-67 positive cells. Statistical analysis revealed no significant change in p53 staining after acitretin treatment (Figure 4).

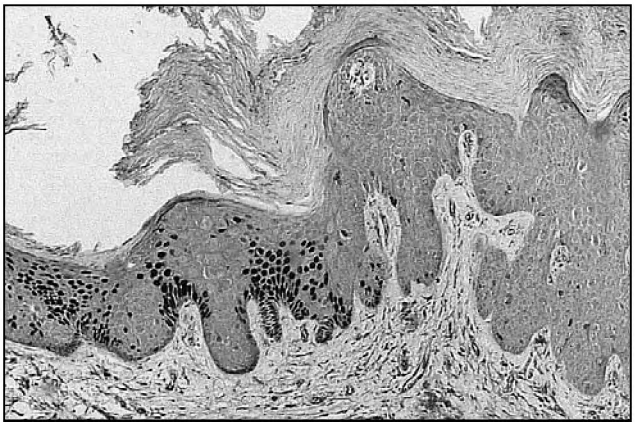


**Figure 5:**

Focal expression of p53 is frequently observed in actinic keratoses.

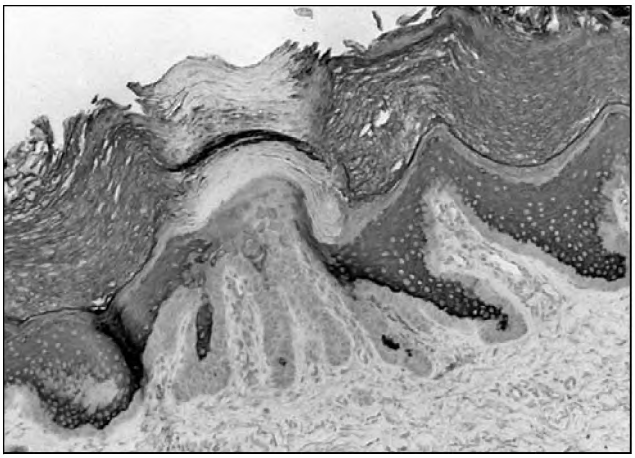


**Figure 6:**

In actinic keratoses rather complete suprabasal staining of K10 is seen. In this slide minimal staining in the center of the stratum corneum is seen, which may well be the beginning of the formation of a cutaneous horn.

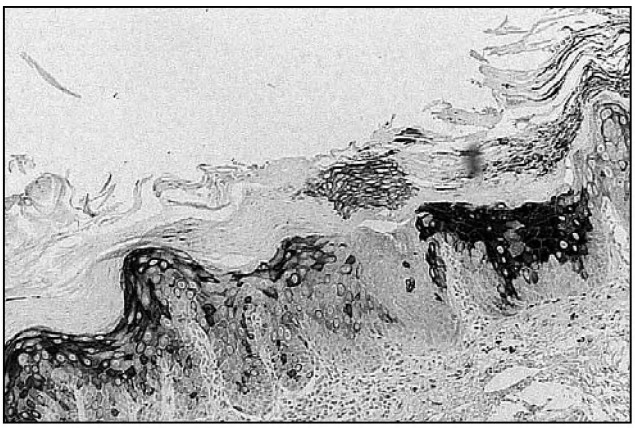


**Figure 7:**

Numerous K16 positive keratinocytes are the rule in actinic keratoses.

Changes in the different parameters of keratinization are depicted in **Figure 2.** Epidermal differentiation parameters at baseline showed the following patterns. Normal differentiation-associated K10 expression was only seen in the suprabasal compartment. Its staining pattern was less when compared to normal skin and often showed unstained foci. An example of K10 expression is shown in **Figure 6.** After acitretin treatment, a significant increase in K10 expression was found with a more complete staining pattern ($p = 0.02$).

Hyperproliferation-associated K16 was expressed in the suprabasal compartment of all AKs. Expression was moderate to pronounced, with staining of all suprabasal cell layers. Most intense staining for K16 was observed suprabasally in the hypertrophic center of AKs directly below the stratum corneum. In **Figure 7** an example of K16 expression is depicted. As **Figure 8** demonstrates, staining can be seen in the basal cells. The degree of K16 expression before and after acitretin treatment was similar.

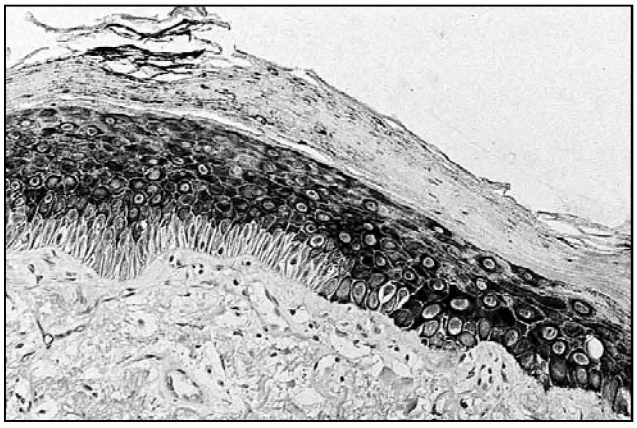


**Figure 8:**

Basal cell staining of K16 can be seen in actinic keratoses.

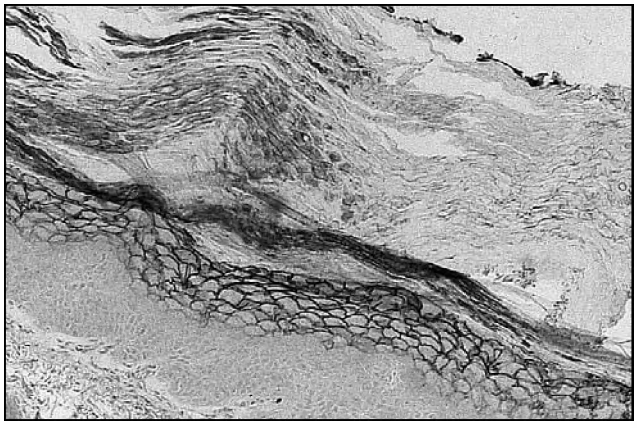
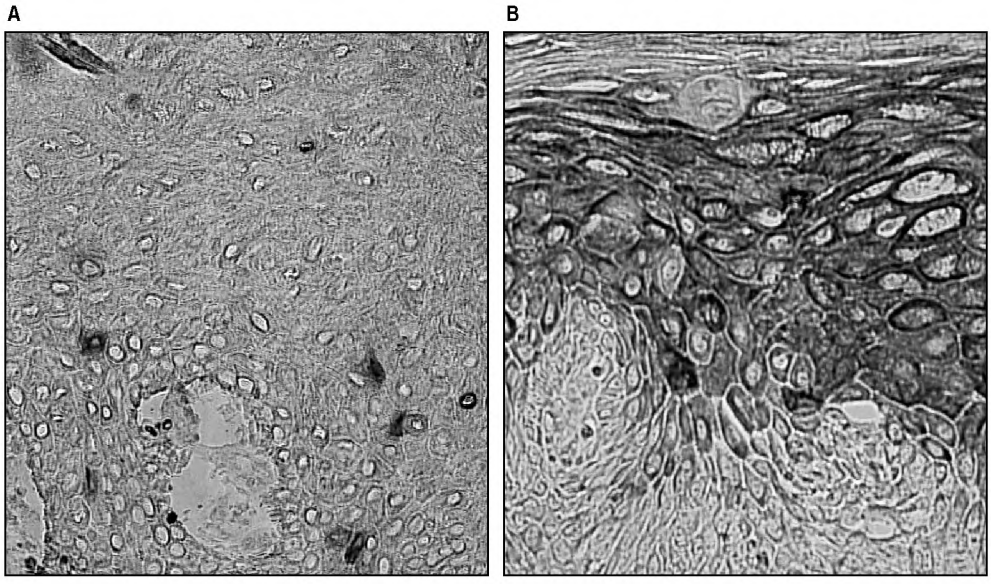


**Figure 9:**

In actinic keratoses multiple transglutaminase positive cell-layers are normally seen.

In the untreated AKs, terminal differentiation-associated transglutaminase staining was seen in the stratum granulosum and stratum spinosum. The number of positive cell layers varied among the AKs from 1 to 15. **Figure 9** represents a typical example of transglutaminase expression. In addition, also in individual AKs differences could be seen in the number of positive cell layers. In general, in the interpapillary areas the number of positive cell layers was the highest. No significant changes in transglutaminase scores could be detected after acitretin treatment.

**Figure 10:**
K13 expression (1C7) in tissue samples of a non-retinoid treated actinic keratosis (single cell expression; Figure **10A**, 200x) and of an acitretin treated actinic keratosis (increased expression; Figure **10B**, 200x).



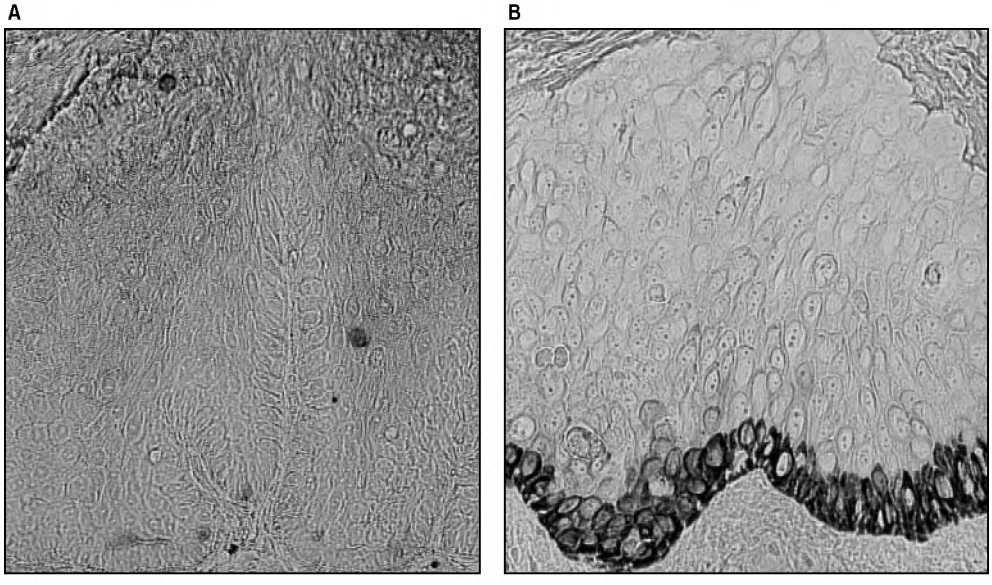

## Keratin 13 and keratin 19

At baseline, K13 expression was found in 8 patients and this was localized in the suprabasal cell layers of the AKs. The staining pattern in these sections was always sporadic with single cell expression mostly scattered in the epidermis. After acitretin treatment K13 expression was found in 14 patients featuring increased expression in the suprabasal regions of the epidermis. No K13 staining was observed in the basal cell compartment. This increase in K13 expression (in number and score) after acitretin treatment was significant ($p < 0.01$). In **Figure 10**, K13 expression is shown in a non-retinoid treated actinic keratosis and in an acitretin treated actinic keratosis.

At baseline, in 4 patients K19 expression was found. Its grade of expression varied from sporadic to minimal, with typical single cell expression mainly located in the suprabasal compartment. After acitretin treatment K19 could be detected in 10 AKs. After treatment K19 was primarily located in the basal cell layers with minimal expression in the suprabasal compartment. The expression of K19 increased ($p = 0.05$) from single cell to continuous staining **(Figure 11)**.

With respect to the histological and immunohistochemical parameters no differences in response to acitretin treatment were observed with different immunosuppressive treatment regimens; however, the number of patients not using azathioprine plus prednisone is small.

**Figure 11:**
K19 expression (RCK108) in tissue samples of a non-retinoid treated actinic keratosis (single cell expression; Figure **11A**, 200x) and in an acitretin treated actinic keratosis increased expression; (Figure **11B**, 200x).



## Correlations

In the baseline samples, a significant positive correlation was found between Ki-67 and p53 ($R = 0.61$; $p = 0.001$), between Ki-67 and KIN score ($R = 0.50$; $p = 0.013$) and between KIN and p53 score ($R = 0.48$; $p = 0.02$).

With respect to K13 expression, significant positive correlations were found with Ki-67 ($R = 0.41$; $p = 0.036$) and with the number of SCCs in each patient that had developed in the 12 months immediately before treatment ($R = 0.43$; $p = 0.024$) in the baseline samples.

For K19 a similar positive correlation with Ki-67 ($R = 0.56$; $p = 0.003$) was found in the baseline samples as well as significant positive correlations with p53 ($R = 0.56$; $p = 0.002$) and KIN score ($R = 0.53$; $p = 0.007$). No statistical significant correlation between baseline K13 and K19 expression was found.

In order to assess whether induction of K13 and/or K19 in AKs by acitretin is correlated with a better response concerning a decrease in epidermal thickness, an increase in K10 expression, and/or the number of newly formed SCCs in this study, we had to compare K13 and/or K19 responders with non-responders. This was done by comparing patients who had at least grade 2 expressions in either K13 or K19 ($N = 10$) with patients who were both K13 and K19 negative ($N = 8$) after acitretin treatment. Grade I (single cell) expression was excluded because this was also seen in untreated biopsies.

When comparing K13 and/or K19 responders with K13 plus K19 non-responders, no significant differences with respect to a decrease in epidermal thickness, an increase in K10 expression, and/or the number of newly formed SCCs were found in this study. After the above described correction for single cell expression in acitretintreated AKs a statistical significant correlation ($R = 0.49$; $p = 0.039$) between K13 and K19 could be demonstrated in these 18 patients.

No statistical significant correlations between the remaining histological and immunohistochemical parameters could be established.

## Discussion

Histological data obtained from this study demonstrated a 44% reduction in epidermal thickness of the AKs after acitretin treatment, but other histological parameters (TDI, KIN) remained unchanged. With respect to immunohistochemical data, we have demonstrated changes in keratinization parameters K10, K13, and K19. No effect on K16 and transglutaminase was seen after 12 weeks treatment. Moreover, acitretin treatment did not lead to significant changes in epidermal proliferation and p53 protein expression, two important hallmarks of AKs that are associated with the degree of dysplasia and the malignant transformation process. These results are in contrast to psoriasis, where a 12-week treatment period with systemic acitretin in a similar dose led to significant reduction in epidermal proliferation, transglutaminase, K16 and dermal inflammation in a group of 10 patients.[19]

From previous studies it is known that retinoids can improve the aspect of AKs by reducing its roughness.[7,8,9] Our data indicate that this clinical improvement is caused by a decrease in thickness of the stratum corneum, whereas the other strata remain unchanged with respect to thickness or the number of cell layers. In general, retinoids are known to alter normal differentiation of the cornifying epidermis towards an esophagealtype of differentiation by stimulating the synthesis of low molecular weight keratins, such as K13, that are normally present in internal squamous epithelia.[20,21]

Concerning K13 and K19, these keratins are embryonic keratins that are normally not present in adult human epidermis.[14,15] In studies with cultured keratinocytes, coupled induction of K13 and K19 by retinoids has been described.[20,22] With respect to in vivo studies, K13 expression was induced by topical application of all-trans retinoic acid (ATRA) on photo-aged human skin[23] and by systemic treatment in warts in RTRs.[24] To our knowledge, no in vivo studies have been performed that report an induction of K19 by retinoids. The present study in AKs in RTRs also demonstrates that induction of K13 by acitretin is correlated with K19 induction, although, in contrast to fetal skin, expression of both parameters was also seen uncoupled. Uncoupled expression of K13 and K19 has been reported previously in a human SCC cell line[25] and in warts in RTRs.[24] Our data also suggest that induction of K13 in AKs by acitretin is more frequently seen than induction of K19. Earlier findings in human epidermal cultures indeed showed stronger induction of K13 than of K19 by retinoids with already a marked increase of K13 in response to very low levels of retinoids, while for K19 induction a higher concentration was necessary.[20]

Apart from the induction of K13 and K19 by acitretin, striking is the single cell expression of these keratins already in several AKs at baseline. None of the patients who showed K19 expression at baseline, and only 2 out of 8 patients who showed K13 expression at baseline, had previously used retinoids. It has been suggested that the presence of these low molecular weight proteins in human skin can be a sign of malignant transformation.[20,16] To assess whether K13 and/or K19 expression in non-retinoid treated AKs is positively correlated with malignant transformation, correlations between K13/K19

and epidermal proliferation (Ki-67),[26,27] (mutant) p53 expression,[26,27] and KIN score[18] were analyzed. In addition, the frequency of newly formed SCCs in a patient with multiple AKs may also be indicative of an actinic keratosis to become malignant. In the present study, we found evidence that K13 expression in non-retinoid treated AKs is correlated with higher epidermal proliferation and a higher incidence of SCCs before treatment. Furthermore, K19 expression in non-retinoid treated AKs is correlated with higher epidermal proliferation, higher (mutant) p53 protein expression, and higher degree of dysplasia. Therefore, these data indicate that assessment of K13 and K19 in non-retinoid treated AKs may provide further information on the risk of malignant transformation and thus may be of interest for the identification of high-risk lesions.

It is intriguing that retinoid treatment induces keratins that are correlated with malignant transformation and on the other hand cause a reduction in skin cancer incidence. [7,8,9,11,10,12] Therefore, it is likely that, with respect to K13 and K19 expression, we deal with epiphenomena, related to two distinct pathways: malignant transformation-associated dedifferentiation and retinoid-associated keratinization.

KIN scores based on histological criteria did not always correlate well with the clinical picture in this group of RTRs. While all 27 baseline biopsies were taken from typical AKs with evident erythematosquamous hallmarks, suggesting a grade II-III KIN score, in 2 biopsies no dysplasia (KIN 0), and in 10 biopsies a KIN grade I was found. Therefore, in RTRs, who often have numerous erythematosquamous and hypertrophic AKs, the directly visible clinical hallmarks of these lesions may not always reflect the grade of dysplasia in the epidermis of these lesions.

In some sections we found areas featuring numerous Ki-67 positive keratinocytes with sparse p53 positive keratinocytes, thus areas with a high level of epidermal proliferation and no sign of apoptosis or staining of mutant p53. Histologically, these areas consisted of KIN III dysplasia and may therefore be at increased risk for malignant transformation.

Combining clinical-, histological-, and immunohistochemical data, it can be concluded that treatment with systemic acitretin improves AKs probably via alteration of the keratinization process of the keratinocytes, leading to peeling of the hypertrophic stratum corneum of these lesions and clinically resulting in softening of the skin. No significant decrease in proliferation and dysplasia was found, which might be the explanation why recurrence of AKs is normally seen after cessation of acitretin treatment. Expression of low molecular weight keratins K13 and/or K19 in non-retinoid treated AKs is correlated with parameters that are indicative for high-risk AKs. Therefore, K13 and K19 may provide two new diagnostic parameters for assessment of AKs at particular risk for transformation into a SCC.

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# 3.3

# Effects of cessation of oral acitretin on actinic keratoses and verrucae vulgares in renal transplant recipients


## Abstract

*The aim of this study was to investigate effects of temporary cessation of acitretin treatment in renal transplant recipients (RTRs) on skin (pre)cancer development and expression of keratin 13 and cell-cycle-associated proteins (MIB-1, p53 and p16$^{INK4A}$). A prospective, non-randomized clinical and immunohistochemical design was used. Nine RTRs on acitretin treatment (mean duration 2.3 ± 0.6 years; mean dosage 18.3 ± 9.9 mg) were asked to interrupt their treatment for three months. Patients gave written informed consent. Patients were seen at intervals of 6 weeks, starting at the moment of acitretin withdrawal. Each visit epidermal lesions were counted; erythema and desquamation of perilesional skin and actinic keratoses (AKs) with induration of AKs were recorded, a visual analogue score (VAS) for patient's contentment was performed, and clinically comparable AKs were biopsied. The main outcome measurements were assessment of a change in the numbers of skin lesions and skin (pre)cancers after acitretin cessation and to compare expression of keratin 13 (K13), MIB-1, p53 and p16$^{INK4A}$ in keratinocytic intraepidermal neoplasia (KIN) of RTRs, with and without acitretin. After 3 months, the number of warts had increased significantly. VAS score had reduced significantly. No significant increase in number of AKs or SCCs was found. K13 expression decreased significantly; for zebroid K13 expression only a trend towards reduction was present. No significant alterations in expression of MIB-1, p53 or p16$^{INK4A}$ were found. It was concluded that acitretin withdrawal in RTRs leads to 1. clinical deterioration within 3 months, without significant increase in skin (pre)cancer, and 2. significant reduction of aberrant K13 expression, without alteration in expression of cell-cycle-associated markers.*

## Introduction

Retinoids, synthetic vitamin A analogues, interfere with epidermal growth and differentiation. These effects of retinoids on proliferation and differentiation make them of interest in the treatment of hyperproliferative skin diseases, such as psoriasis, disorders of keratinization and as an antitumor drug.[1]

Renal transplant recipients (RTRs) are known to develop multiple warts, actinic keratoses (AKs) and non-melanoma skin cancers (NMSCs), especially on sun-exposed parts of the body,[2-4] which lead to a major co-morbidity. In the past two decades, systemic treatment with retinoids has shown to be promising in chemoprevention of AKs and squamous cell carcinoma (SCC),[5] also in RTRs.[6-10]

Several studies suggested a beneficial effect of systemic retinoid treatment (either etretinate or acitretin), with prevention of SSCs and reduction in the number of hyperkeratotic lesions in RTRs, especially in those with NMSC or severe lesions in their history.[5, 6, 8-10]

However, after stopping retinoid treatment the beneficial effect disappeared, with report of relapses varying from 4-12 months after discontinuation of retinoid therapy.[5, 6, 9, 11] Therefore, some have recommended continuous treatment.[7]

So far it is unknown how retinoids exert their chemopreventive effect. Recently, we demonstrated that retinoids alter differentiation in almost 90 % of warts of RTRs, with induction of keratin 13 (K13) expression,[12] a keratin normally not present in the adult epidermis.[13] A specific zebroid K13 expression pattern was associated with retinoid bioactivity in warts of these patients. Whether comparable K13 expression patterns are present in retinoid-treated keratinocytic intraepidermal neoplasia (KIN) lesions of RTRs, remains to be established.

In view of the clinical efficacy of retinoids in NMSCs on RTRs, it is feasible that retinoids not only influence differentiation, but also interfere with cell-cycle-associated proteins. Thus far, effects of retinoids on expression of cell-cycle-associated markers in KIN lesions of RTRs have not been published. The most current and so far best documented cell-cycle-associated marker for intraepithelial neoplasia (especially in neoplasia of the uterine cervix) is the proliferation marker, MIB-1.[14-16] Other cell-cycle-associated markers of interest with respect to KIN are the tumor suppressor p53 and $p16^{INK4A}$. In AKs of both immunocompetent individuals and RTRs, frequent overexpression of the p53 tumor suppressor is reported.[17, 18] Recent data have shown that besides the p53 pathway, also the pathway involving the tumor suppressor $p16^{INK4A}$ is involved in skin carcinogenesis;[19] in high-grade KIN lesions of both RTRs and immunocompetent individuals we found frequent $p16^{INK4A}$ overexpression *(own unpublished data)*.

The present prospective study was designed to study the effects of 3 months withdrawal of long term acitretin treatment on clinical aspect of the skin, the development of (pre) cancerous lesions in RTRs, and to assess its effects on histology and immunohistochemical expression of the three biomarkers MIB-1, p53 and $p16^{INK4A}$ in KIN lesions of RTRs. Furthermore, we examined whether K13 expression in acitretin-treated KIN le-

sions showed a zebroid K13 pattern as previously found in warts during retinoid treatment, and whether K13 expression reduced after acitretin withdrawal.

## Materials and Methods

### Patients

RTRs on systemic acitretin (mean duration of acitretin treatment 2.3 ± 0.6 year; mean dosage 18.3 ± 9.9 mg/day) were asked to interrupt their treatment for 3 months.

Initially, 11 out of 16 approached patients agreed to participate and in all cases written informed consent was obtained. However, one female withdrew after the first visit due to interfering breast cancer and 1 patient withdrew because of progression of AKs and SCCs, 6 weeks after the start of the study. Both patients were excluded from further evaluation because only baseline biopsies were obtained.

The characteristics of the remaining 9 patients (3 males, 6 females) that finally completed the 3-month study period, including data on previous retinoid therapy and type and duration of immunosuppression are listed in **Table I.**

**Table I:**
Characteristics of the 9 participating patients; M=male; F=female; yrs=years; A=azathioprine; P=prednisolone; S=sirolimus; AK=actinic keratoses.

| Patient | Age (years) | Sex | Duration imm. suppression (years) | Type imm. suppression | Dosage acitretin at T0 (mg/day) | Dosage acitretin at T0 (mg/kg) | Duration of acitretin treatment (years) |
|---|---|---|---|---|---|---|---|
| **1** | 65 | M | 33 | A+P | 20 | 0.24 | 2.9 |
| **2** | 60 | F | 6 | A+P | 35 | 0.30 | 2.9 |
| **3** | 53 | F | 27 | P+S | 20 | 0.29 | 2.8 |
| **4** | 64 | M | 8 | A+P | 10 | 0.20 | 1.5 |
| **5** | 64 | F | 24 | A+P | 15 | 0.16 | 2.6 |
| **6** | 48 | F | 22 | A+P | 15 | 0.15 | 2.6 |
| **7** | 60 | M | 21 | A+P | 15 | 0.19 | 2.3 |
| **8** | 51 | F | 26 | C | 25 | 0.26 | 1.9 |
| **9** | 60 | F | 13 | A+P | 10 | 0.13 | 1.5 |
| **Mean (±SD)** | 58.3 | | 20 (± 9.1) | | 18.3 (± 9.9) | 0.21 (± 0.06) | 2.3 (± 0.6) |

## Design of the study

During the 3-month study period the patients were seen at three defined moments, with intervals of 6 weeks in between each visit. At time T0 the study started and on this day systemic acitretin was stopped. Control visits were respectively 6 (T1), and 12 (T2) weeks after acitretin stop.

At each visit all warts and AKs in each patient were counted and scored by the same dermatological investigator, familiar with this type of patients and their skin lesions. Erythema and desquamation of lesional and perilesional normal skin and induration of lesional skin were judged, using a scale of 0-3 (0 = absent, 1 = minimal, 2 = moderate, 3 = severe).

Patients were asked to give a subjective judgment of their skin condition using a visual analogue score (VAS), with a scale from 0-10 (0 = very discontented, 10 = very satisfied). At the end of each visit a 6 mm skin biopsy was taken; it was attempted to biopsy clinically identical (at baseline defined) AKs from the same location in all three sessions, preferentially from the forearm.

Of each participating RTR, the number of biopsied Bowen's diseases (BDs) and NMSCs during the 3-month study period was compared with the number of lesions, which had occurred in the last 3 months with retinoid treatment.

## Histopathology and immunohistochemistry

A KIN-classification was assigned to all skin biopsies.[20,21] Immunohistochemical analysis was performed on all lesions using standard avidin-biotin-peroxidase complex system with diaminobenzidine (DAB) as the chromogen. In brief, 4 µm thick paraffin sections were deparaffinized, hydrated, and washed in buffered saline phosphate. The monoclonal antibodies used, the antigen they recognize, and pretreatment and dilutions are listed in Table II. After incubation with primary antibodies, sections were incubated for 30 minutes with (a 1:200 dilution of) biotinylated horse anti-mouse (Vector laboratories, Burlingham, CA), followed by a 45-minute incubation period with (a 1:50 dilution

**Table II:**
Antibodies with the recognized antigens, used dilution, pretreatment, incubation time and temperature.

| Antibody | Antigen | Source | Dilution | Pretreatment | Incubation time and temperature |
|---|---|---|---|---|---|
| **DO-7** | P53 | Neomarkers | 1:400 | microwave | 4°C overnight |
| **P16$^{INK4A}$/MTS1 Ab-4** | P16$^{INK4A}$ | Neomarkers | 1:100 | microwave | 4°C overnight |
| **MIB-1** | Ki-67 | Progen | 1:100 | microwave | 4°C overnight |
| **2D7 (IgG2b)** | Keratin 13 | G. van Muyen | 1:1 | microwave | 4°C overnight |

of) avidin-biotin complex (Vector laboratories, Burlingham, CA). Sections were counterstained with Mayer's haematoxylin.

Immunoreactivity was scored semi-quantitatively for all applied immunohistochemical markers, in the following manner:

*MIB-1* and *p53*: for both staining was nuclear. Staining was scored as previously published by Keating *et al.* in cervical neoplasia[22]: 0 = only basal layer positivity, 1 = positivity confined to basal 1/3 of epidermis, 2 = positivity confined to basal 2/3 of epidermis or 3 = transepidermal positive staining.

*K13*: staining was cytoplasmic. Scoring was performed as described previously[12]; 0 = negative staining, 1 = suprabasal single cell positivity, 2 = zebroid pattern.

*P16$^{INK4}$*: staining was both nuclear and cytoplasmic. Staining was scored as for MIB-1 and p53. Scoring of immunostaining was performed without knowledge of patient history.

## Statistics

The Lilliefors test for normality disclosed that the data were not gaussian distributed. Therefore, non-parametric statistical procedures were used to analyze the data. Since the small series of patients included in this study the exact significance level is given or an unbiased estimate of exact significance level, calculated by repeatedly sampling (= Monte Carlo method). The relations between the grade of KIN and expression of K13, MIB-1, p53, and p16$^{INK4A}$ were assessed with both rank correlation analysis and the Jonckheere-Terpstra test, a distribution-free test for ordered alternatives.[23]

**Table III:**
T0=start of the study; T1=6 weeks after acitretin withdrawal; T2=12 weeks after acitretin withdrawal; p1=p value T0-T1(week0-6); p2=p value T0-T2(week 0-12); SEM=standard error of the mean; AKs= actinic keratoses; VAS=visual analogue score. Figures in bold indicating statistically significant findings.

| Parameter | T0 mean±SEM | T0 mean±SEM | T0 mean±SEM | P1 | P2 |
|---|---|---|---|---|---|
| Number of warts | **1.6 ± 0.8** | 1.9 ± 1.0 | **2.7 ± 1.1** | 0.5 | **0.02** |
| Number of AKs | 129.6 ± 36.4 | 108.1 ± 26.7 | 109.6 ± 25.4 | 0.1 | 0.2 |
| Erythema perilesional normal skin | **0.9 ± 0.3** | **0.2 ± 0.2** | **0.2 ± 0.2** | **0.03** | **0.03** |
| Desquamation perilesional normal skin | 0.6 ± 0.2 | 0.3 ± 0.2 | 0.3 ± 0.2 | 0.3 | 0.3 |
| Erythema AK | 1.9 ± 0.4 | 1.7 ± 0.3 | 1.7 ± 0.3 | 0.4 | 0.4 |
| Desquamation AK | 1.1 ± 0.3 | 2.00 ± 0.3 | 1.6 ± 0.4 | 0.05 | 0.3 |
| Induration AK | **0.9 ± 0.2** | **2.0 ± 0.2** | 1.9 ± 0.3 | **0.004** | 0.05 |
| VAS | **5.6 ± 0.7** | **4.4 ± 0.7** | **3.1 ± 0.6** | **0.04** | **0.004** |

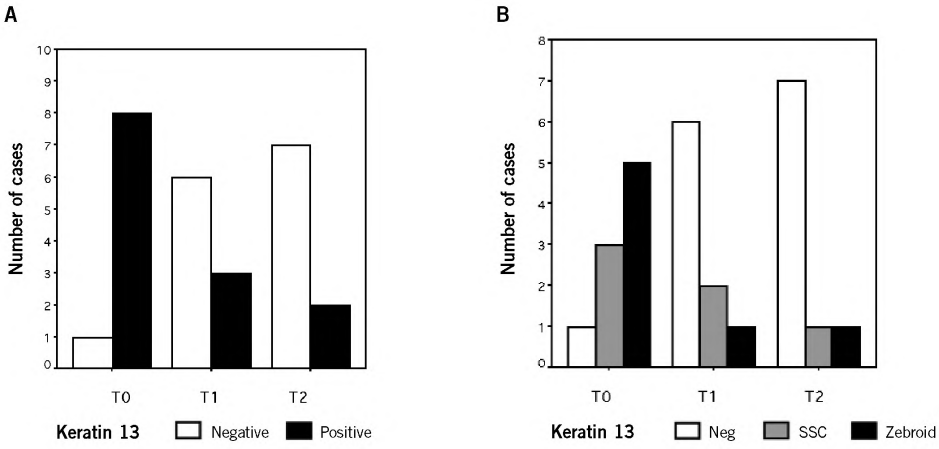
In this study a before-after design[23] was used to study the effect of cessation of retinoid treatment on clinical and immunohistochemical parameters. Significance of effects on short term (after 6 weeks, p1) and longer term (after 12 weeks, p2) were calculated. A partial rank correlation analysis controlled for KIN grade was used to assess the effect of retinoid treatment cessation on the KIN-grade dependent immunohistochemical features.

For the KIN-grade, independent immunohistochemical features, and clinical parameters, the following statistical procedures were used to analyze the treatment cessation effect: two planned pair wise comparisons (T1 versus T0 and T2 versus T0) were used to study the short and longer term effects (with significance levels p1 and p2 respectively as outlined above). The Wilcoxon matched-pairs signed-rank test and McNemar test were used for these pair wise comparisons. The latter test was used for the dichotomous dependent variables. With a limited number of planned pair wise comparisons the Bonferroni adjustment of the Type I error rate can be omitted.[23] Because there is a definite expectation about the direction of the therapy cessation effect, a directional (one-tailed) alternative hypothesis was used. All statistical analyses were performed with SPSS 10.0 for Windows. Results are expressed as means ± standard error of the mean (SEM), unless stated otherwise.

**Figure 1:**
Expression (patterns) of keratin 13 (K13) in keratinocytic intraepidermal neoplasia (KIN) lesions of renal transplant recipients at the start of the study (T0) and 6 (T1) and 12 (T2) weeks after acitretin withdrawal.

**A.** K13 positive versus negative cases. The number of K13 expressing KIN lesions significantly reduced 12 weeks after acitretin withdrawal (p=0.02).

**B.** K13 positive cases subdivided in cases having suprabasal single cell (SSC) expression or zebroid K13 expression. The latter pattern was previously found correlated with retinoid treatment in warts of renal transplant recipients[12]. For zebroid K13 expression a decreasing trend was present with 5/9 KIN having zebroid K13 expression at the start of the study and only 1/9 KIN at T1 and T2. This reduction in zebroid K13 expression was not significant, probably due to the small number of cases (p=0.10).



## Results

### Clinical effects of interruption of systemic acitretin

The numbers of counted warts and AKs at each control visit are listed in **Table III**. Only for the number of warts a significant increase was seen after 3 months when compared to the start of the study ($p = 0.02$); for AKs no significant change in the number of lesions was found.

Desquamation of clinically normal and lesional skin did not alter significantly during the 3-month study period **(Table III)**. Erythema of normal skin reduced significantly already after 6 weeks ($p1$ and $p2 = 0.03$): induration of AKs increased significantly only in the first 6 weeks after acitretin withdrawal ($p1 = 0.004$): this trend sustained also after 3 months, although no longer significant ($p2 = 0.05$).

The mean VAS-score had reduced significantly after 6 weeks of acitretin withdrawal ($p1 = 0.04$), which persisted after 12 weeks ($p2 = 0.004$) **(Table III)**.

During the 3-month study period, the patients developed 2 BDs, 5 SCCs and 1 basal cell carcinoma (BCC), compared to 2 SCCs in the 3 months pre-study period while still on acitretin therapy. Differences for the number of SCCs ($p = 0.3$) were not significant. However, numbers are small and follow-up is relatively short.

### Histopathological and immunohistochemical effects of interruption of systemic acitretin

Since immunohistochemical parameters can be dependent of KIN grade, we had to take this into account while studying the effects of cessation of acitretin. Of the 9 patients three biopsies at respectively T0, T1 and T2 were taken from clinically comparable AKs. Despite clinical resemblance of the biopsied AKs within one patient at each control visit, histological examination revealed sometimes variation in KIN grade. In 8 patients in all visits either KIN 1 or 2 was biopsied, in 1 patient each control visit a KIN 3 lesion was biopsied.

A correlation analysis showed that MIB-1 score strongly correlated with KIN grade ($R = 1$). All KIN 1 lesions showed a 1+ MIB-1 score, all KIN 2 lesions a 2+ and all KIN 3 lesions a 3+ MIB-1 score. Also p53 expression was positively correlated with KIN grade ($R = 0.5$, $p = 0.01$). K13 and $p16^{INK4A}$ expression were not significantly correlated with KIN grade ($R = 0.33$, $p = 0.10$ and $R = -0.03$, $p = 0.90$ respectively).

When taken the KIN grade into account, we found no significant correlation between the biopsy number and the expression of MIB-1, p53, and $p16^{INK4A}$. Therefore, in this small patient group no influence of acitretin withdrawal on expression of these three biomarkers could be demonstrated. However, a negative correlation between K13 expression and the time of biopsy ($R = -0.50$, $p = 0.01$) was present.

K13 expression decreased significantly from $1.44 \pm 0.24$ to $0.33 \pm 0.24$ after 12 weeks ($p = 0.02$). At the start of the study 90% of KIN lesions were K13 positive, after 6

**Figure 2A:**

KIN lesion of RTR, biopsied at the start of the study (first day of acitretin withdrawal); an abrupt transition from normal to lesional skin with hyperorthokeratosis and acanthosis is seen; a slightly disordered epidermal structure with atypia in the basal one third of the epithelium is present (HE, 100x).

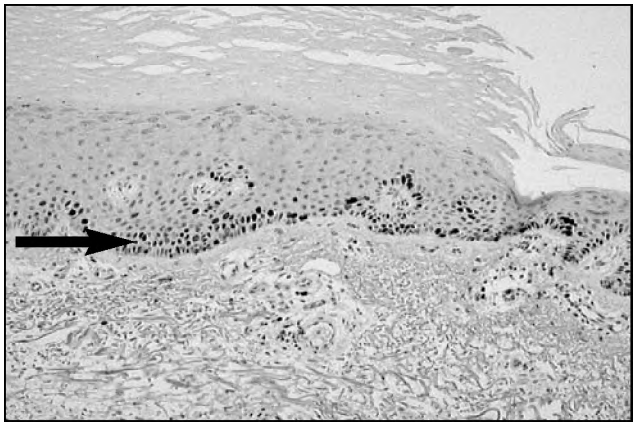


**Figure 2B:**

MIB-1 shows positive nuclear staining in the basal one-third layer of the epidermis (arrows). A and B, are consistent with KIN 1(MIB-1, 100x).

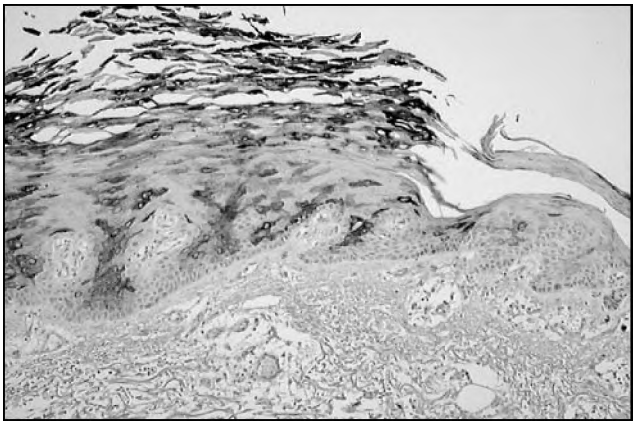


**Figure 2C:**

In lesional skin, a zebroid K13 expression pattern is present with alternating columns of K13 positive and negative cells (K13, 100x).

weeks 33% and after 12 weeks 22% (**Figure 1A**). If we only considered KIN lesions showing zebroid K13 expression, there was clearly a trend in decrease of the zebroid K13 expression pattern after acitretin withdrawal ($p = 0.10$): at the start of the study zebroid K13 expression was found in 5/9 patients (56%), while after 6 weeks and 12 weeks of acitretin withdrawal zebroid K13 expression was only present in 1/9 patients (11%, **Figure 1B** and **2**).

Histopathology of the skin biopsies with a zebroid K13 pattern at the start of the study, revealed in the areas of immunohistochemical K13 expression often compact hyperorthokeratosis, hypergranulosis, acanthosis and swelling with focally cytoplasmic vacuolization of the keratinocytes when compared to the K13 negative adjacent epithelium (**Figure 2** and **3**). These are known histological changes following topical and systemic retinoid treatment.[24] The transition with the K13 negative epithelium was mostly sharp and abrupt.

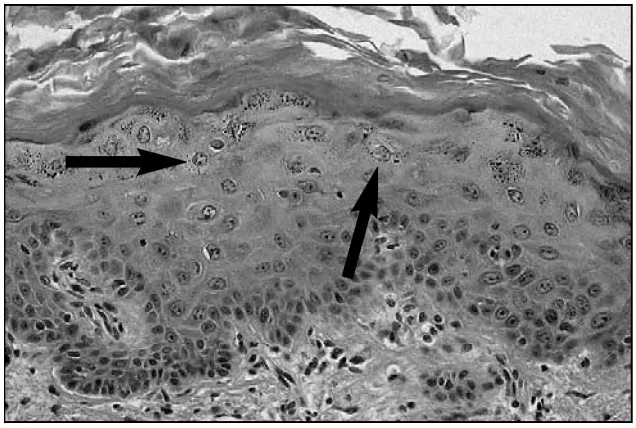


**Figure 3A:**

KIN lesion of another RTR than in fig.1, also at the start of the study, showing dysplasia in the basal one third epidermal layer consistent with KIN1. Hyperorthokeratosis is evident at the surface (HE, 200x) with hypergranulosis. Keratinocytes show swelling with a bluish cytoplasmic hue (mucine-like material), focally with cytoplasmic vacuolization (arrows).

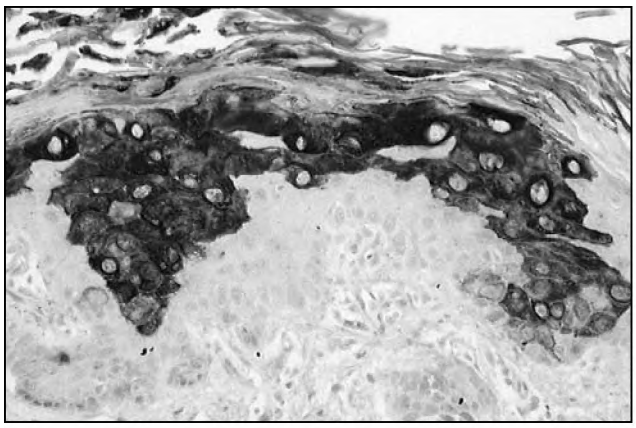


**Figure 3B:**

Parallel slide, showing strong suprabasal K13 in this area (K13, 200x).

## Discussion

The present study shows that 3 months cessation of systemic acitretin treatment is followed by reduction of aberrant differentiation in KIN lesions of RTRs with significant decrease of K13 expression. Expression of the proliferation marker MIB-1, and of cell-cycle-associated proteins p53 and $p16^{INK4A}$, all often found overexpressed in KIN[17,25] ($p16^{INK4A}$, *own unpublished data*), were not altered on acitretin withdrawal. These data suggest that retinoids in RTRs exert their effect by inducing aberrant differentiation (induction of K13, normally present in the suprabasal layers of internal stratified squamous epithelia, like the esophagus[26]) and not by decreasing proliferation or influencing important tumor suppressors in cutaneous carcinogenesis. Previously we found a particular zebroid K13 expression pattern related to retinoid treatment in warts of RTRs which we postulated to be indicative of retinoid bioactivity in warts of these RTRs;[12] almost 90% of warts in RTRs showed this typical zebroid K13 pattern in case of retinoid treatment. In the present study, concerned with KIN lesions, we found a much lower percentage of zebroid K13 pattern at the start of the study, than would be expected from the previous findings in warts; only 5/9 patients (56%) on acitretin showed zebroid K13 expression. This zebroid K13 expression markedly reduced on acitretin withdrawal, with only 1/9 patients (11%) displaying this zebroid K13 pattern after 6 and 12 weeks, as we had already anticipated based on the previously found correlation between zebroid K13 expression and retinoid treatment in warts. This marked reduction, however, proved not significant ($p = 0.1$), probably due to the small number of patients. Combining the two patterns of K13 expression, the decrease in K13 expression reached significance after 6 and 12 weeks of acitretin withdrawal ($p = 0.03$ and $p = 0.02$ respectively).

The lower frequency of zebroid K13 expression presently found in KIN lesions when compared to warts, might be attributable to the dysplastic changes in KIN due to genetic alterations that might interfere with the retinoid receptor or interactions with the retinoid receptor or alterations in keratin gene regulation (e.g. K13) that would otherwise have been transcriptionally induced. Indeed, recently Xu *et al.* found progressive decrease in nuclear retinoid receptors during skin squamous carcinogenesis.[27] On the other hand in warts, (transforming) types of human papillomavirus might increase the sensitivity of keratinocytes to retinoid effects.[28] Future research on for instance warts and actinic keratoses within one patient during systemic acitretin treatment might elucidate whether there is an interlesional (warts versus AKs) variation in retinoid susceptibility, maybe related to retinoid receptor differences.

In parallel to the immunohistochemical decrease in K13 expression, clinical progression was seen with significant increase in warts. We could not demonstrate a significant increase in number of AKs and SCCs when acitretin was interrupted for 3 months. The relatively small number of patients and the short study period could cause this. This possibility is being supported by the previous studies by Bouwes Bavinck, where a relapse of new skin cancers occurred between 3 and 6 months after acitretin withdrawal, and by Shuttleworth,[10] who described the development of further lesions 6 months after etretinate cessation.

VAS score also significantly decreased in the 3 months study period, reflecting a reduction in contentment of the patients with their skin condition.

Already after 6 weeks of acitretin withdrawal, erythema of normal perilesional skin diminished significantly, as could be expected since erythema is a well-known side effect of topical and oral retinoid treatment.[5,29] After 3 months a trend towards increased induration of AKs was present; since we found no significant concomitant increase in SCCs, induration seems more likely due to increased hyperkeratosis (in the absence of retinoids) instead of infiltrative growth. Retinoids could indeed have an antihyperkeratotic effect, by induction of aberrant suprabasal K13 expression causing terminal differentiation towards non-keratinizing stratified squamous epithelia.[26] This diminished hyperkeratosis with retinoids leads to better cosmetic appearance of the skin, important for patient's contentment. Increased induration in the absence of retinoids could make it more difficult for the clinician to differentiate AKs from invasive lesions (masking effect), necessitating more frequent biopsies in these RTRs, which are already subject to frequent skin biopsies.

So far, effects of systemic retinoids on epidermal proliferation in RTRs were only studied by means of $^{3}H$ thymidine labeling index; Shuttleworth *et al*[10] found a significant increase in the labeling indices after 6 months of systemic etretinate treatment in normal sun-exposed skin, but not in sun protected skin in 6 RTRs; effects on dysplastic skin were not assessed. Besides Gibson *et al.*, who found no significant differences in histological features of SCCs,[8] no other studies on chemoprevention by systemic retinoids in RTRs[6,9,30] included histological or immunohistochemical evaluation of retinoid effects on skin lesions, to study for instance effects on proliferation or differentiation.

In the present study, clinically comparable actinic keratoses were biopsied, which on histological examination varied in KIN grade. When taken the KIN grade into account, we found no significant correlation between the time of biopsy and the expression of MIB-1, p53, and $p16^{INK4A}$. Therefore in this small patient group no influence of acitretin withdrawal on expression of these three biomarkers could be demonstrated.

In conclusion, this study demonstrates that 3 months cessation of acitretin therapy in RTRs, leads to a significant reduction of aberrant K13 expression, without alteration in expression of proliferation and cell-cycle-associated markers (MIB-1, p53, $p16^{INK4A}$), in KIN lesions of RTRs. Therefore, our data imply that if retinoids in RTRs exert an effect, it is by modifying differentiation (K13 expression), without altering proliferation.

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# 3.4

# Topical all-trans retinoic acid for actinic keratoses in immunocompetent patients


## Abstract

*All-trans retinoic acid (ATRA) has been claimed to ameliorate actinic keratoses clinically. However, little information is available on histological and immunohistochemical parameters for proliferation, differentiation, inflammation and apoptosis. A randomized double-blind placebo-controlled study was undertaken to determine clinical, histological and immunohistochemical effects of short-term treatment of actinic keratoses with topical ATRA. In 9 patients, two symmetrical and comparable skin areas with multiple actinic keratoses were randomly assigned for treatment with ATRA cream 0.05% versus vehicle cream, once daily for 6 weeks. Biopsies were taken from clinically comparable actinic keratoses at baseline and after treatment. After 6 weeks treatment neither ATRA nor its vehicle significantly reduced the number of actinic keratoses. ATRA significantly increased pruritus, erythema, and desquamation. Keratin 10 and keratin 13 expression were significantly higher in the ATRA-treated actinic keratoses. No differences were observed between the two creams with respect to immunohistochemical parameters for epidermal proliferation, apoptosis, and dermal inflammation, and no changes compared to the baseline sections were observed for these parameters. The present study suggests that topical ATRA may have beneficial effects in the treatment of actinic keratoses via alterations in keratinization rather than via effects on epidermal proliferation, inflammation, and apoptosis. Apart from an association with retinoid treatment, keratin 13, when it is expressed in non-retinoid treated actinic keratoses, may also be associated with malignant transformation in actinic keratoses.*

## Introduction

Actinic keratoses (AKs) are erythemato-squamous and often hyperkeratotic, pre-malignant lesions that are mainly found on sunlight-exposed skin surfaces. They can transform into a squamous cell carcinoma (SCC). Between 0.25 % and 16 % of AKs will convert into a SCC within one year, depending on the number of risk factors present.[1]

Many studies report beneficial effects of topical all-trans retinoic acid (ATRA) in the treatment of AKs,[2-4] although some studies do not confirm this.[5;6] The study periods varied from several months to a few years. Little information is available on immunohistochemical effects of ATRA in the treatment of AKs, especially where early effects are concerned. Treatment with topical ATRA frequently causes skin irritation already after several days of treatment, which may reduce patient compliance to treatment in an early stage.[7-9]

Therefore, in the present study we focussed on clinical and histological parameters, and immunohistochemical parameters for epidermal proliferation, keratinization, terminal differentiation, and apoptosis of short-term therapy of AKs with topical ATRA.

**Table I:**
Monoclonal antibodies used in the study.

| Antibody | Specifity | Concentration | Source |
|---|---|---|---|
| **MIB-1** | Ki-67 | 1:50 | Immunotech, Marseilles, France |
| **DE-K10** | Keratin 10 | 1:100 | Monosan, Uden, Netherlands |
| **1C7** | Keratin 13 | 1:10 | Monosan, Uden, Netherlands |
| **LL-025** | Keratin 16 | 1:10 | Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK |
| **BT621** | Transglutaminase | 1:10 | Biomedical Technologies Inc., Stoughton, MA, USA |
| **DO-7** | p53 | 1:200 | DAKO, Glostrup, Denmark |

## Materials and methods

### Study design

A randomized double-blind placebo-controlled approach was used in this trial. Patients with multiple AKs in a symmetrical pattern on the dorsal area of their forearms and/or hands were randomized for treatment with topical ATRA cream 0.05 % on the left forearm/hand or on the right forearm/hand. The color-matched vehicle cream of ATRA, which was prepared by the pharmacist, was used as placebo on the contra-lateral site. Application of both creams occurred once daily for 6 weeks. An independent research nurse carried out randomization by opening a sealed envelope that contained the application procedure, and prepared the labels for the creams (left versus right), so patient and investigator were unaware of the contents of the creams. Ethics Committee approval was obtained and all patients gave written informed consent prior to study enrollment.

Other dermatological conditions or the use of other topical agents or immunosuppressive drugs that might interfere with this study were contra-indicated. The washout period for topical treatments and emollients on the test areas was at least 2 weeks, with the exception of topical retinoids, where a washout period of 4 weeks was chosen to assure an adequate epidermal turnover (normal skin 2-3 weeks; hyperproliferative disorders < 1 week).[10]

Punch biopsies for immunohistochemical and histological scoring were taken at baseline and after 6-weeks treatment from clinically identical AKs located at the two predefined areas. Clinical parameters (pruritus, erythema, desquamation and thickness of the AKs) in these areas were monitored biweekly during the application period and at the follow-up visit, which was performed 2 weeks after the final application. The number of AKs in the two test areas was counted each visit.

### Biopsies and staining procedures

Punch biopsies were processed according to standard methods by using the avidin-biotin-complex (ABC) staining method and indirect immunoperoxidase techniques on paraffin-embedded sections. The following immunohistochemical parameters were assessed: Ki-67 (MIB-1, epidermal proliferation), p53 (DO-7, apoptosis associated), Keratin 10 (K10) (DE-K10, normal keratinization), K13 (1C7, retinoid-associated keratinization[11-13]), K16 (LL025, hyperproliferation-associated keratinization), and transglutaminase (BT621, terminal differentiation) **(Table I)**. Furthermore, total dermal infiltrate (TDI) scores and epidermal thickness were assessed. Sunlight-exposed normal skin was used as a control for all immunohistochemical parameters. Esophageal tissue was used as a positive control for K13 staining.

## Scoring methods

Pruritus, erythema, desquamation and thickness of the AKs were assessed by using a semi-quantitative score on a 5-point scale: 0 = none, 1 = slight, 2 = moderate, 3 = moderate-severe, 4 = severe.

MIB-1 and p53 were counted as the number of positive cells per millimeter length of section. Keratins were scored by semi-quantitative analysis on a 7-point scale: 0 = no staining, 1 = sporadic staining, 2 = minimal staining, 3 = moderate staining, 4 = moderate-pronounced staining, 5 = pronounced staining, 6 = whole epidermis stained. Transglutaminase scores were assessed as the ratio of BT621 positive epidermal cell layers divided by the total number of epidermal cell layers. Epidermal thickness was measured in millimeters. TDI was scored by using the following 5-point scale: 0 = no infiltrate, 1 = minimal infiltrate, 2 = moderate infiltrate, 3 = moderate-pronounced infiltrate, 4 = pronounced infiltrate. Immunohistochemical scoring occurred along the complete length of the slides. All histological and immunohistochemical scoring occurred under blinded conditions by two independent investigators.

## Statistics

In order to substantiate differences between baseline biopsies and biopsies taken after 6 weeks treatment, as well as differences between ATRA-treated biopsies and vehicle-treated biopsies, the Wilcoxon matched pairs test was used. For statistical analysis of the clinical parameters a paired T-test was used. Assessment of a statistical significant correlation between Ki-67 and p53 was performed by calculating Spearman's rank correlation. A p-value $< 0.05$ was considered statistically significant. Calculations were performed using Statistica version 9.0 (StatSoft Inc, 2300 East 14th Street, Tulsa, OK 74104, USA).

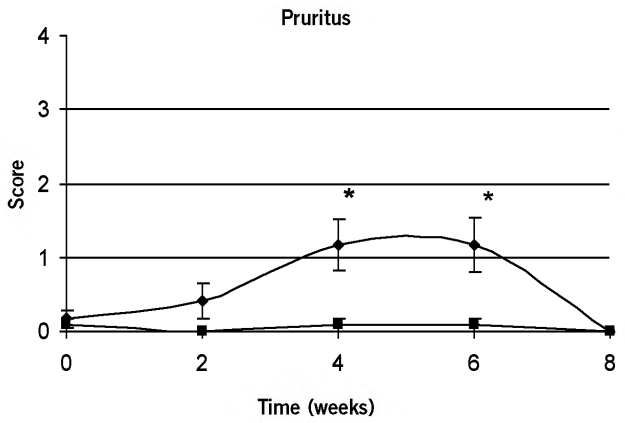


**Figure 1:**

Pruritus scores on a 5 point semi-quantitative scale as experienced by the subjects during the 6-week treatment period and 2 weeks follow-up with topical ATRA, respectively vehicle (* significant difference between ATRA and vehicle; $p < 0.05$).

## Results

### Demographics

In total 9 patients were included: 3 women and 6 men. The average age was 63 ± 11 (mean ± SD) years. Patients have had AKs since 6.8 ± 3.7 (mean ± SD) years. In 3 out of these patients one or more SCCs had developed before study inclusion and 5 out of these patients had a history of one or more basal cell carcinomas. In general, patients responded well to previous treatments, comprising cryotherapy and topical 5-fluorouracil. Before the washout period most patients used indifferent creams for smoothening of the lesions and sunscreens for prevention of new AKs. Only one patient had previously used topical ATRA.

### Clinical results

No significant differences in baseline values were seen between the two test areas. After 6 weeks treatment, the ATRA-treated AKs showed significant more pruritus ($p = 0.04$; **Figure 1**), more erythema ($p < 0.001$; **Figure 2**), and more desquamation ($p < 0.01$; **Figure 3**) compared to the vehicle-treated lesions. In two patients application of the creams had to be reduced to every other day after 4 weeks of treatment because of skin irritation. Due to the irritation properties of ATRA, the area that was randomized for application of topical ATRA could be distinguished during the final stage of the study in some patients, somehow reducing the success of blinding. Both creams demonstrated significantly less thickness after 6 weeks treatment compared to the baseline ($p = 0.03$), but no differences with respect to thickness were observed between the two creams.

Two weeks after cessation of the applications the differences between the two creams had disappeared and values had returned to baseline levels. None of the two creams was able to significantly reduce the number of AKs during this study period **(Figure 4)**.

### Histological and immunohistochemical results

Histological and immunohistochemical data are summarized in **Table II**. Compared to normal skin, keratin 10 is less expressed in AKs, where areas without K10 expression can be seen in the suprabasal compartment. A statistically significant higher expression of K10 ($p = 0.05$) was found in ATRA-treated compared to untreated AKs.

In normal skin K13 is absent, but in AKs K13 expression was seen. Six out of 9 patients (67%) showed mild to moderate focal expression of K13 in the ATRA treated AKs. Remarkably, also 2 untreated AKs and 1 vehicle treated AK showed minimal K13 expression **(Figure 5)**. One of these patients had used tretinoincream 4 weeks prior to the study. The other two patients had not used topical retinoids, creams containing vita-

min A, or oral intake of high doses of vitamin A during the previous year. K13 expression was significantly higher ($p = 0.04$) in ATRA-treated compared to vehicle-treated AKs.

All other histological and immunohistochemical parameters did not reveal any significant alterations when comparing the two creams or when comparing baseline and post-treatment situation. Ki-67 and p53 expression is increased in AKs compared to normal skin. In general, the expression pattern of p53 largely followed the distribution of Ki-67 positive nuclei. A statistically significant positive correlation between the number of Ki-67 positive nuclei and the number of p53 positive nuclei was found in this study ($p = 0.02$; $R = 0.79$). Hyperproliferation-associated K16 expression was, in contrast to normal skin, abundantly expressed both in the basal and suprabasal layers. Transglutaminase was not only found in the granular layer, as seen in normal skin, but also in the spinal layer of the epidermis in the AKs. Furthermore, dermal infiltrate and thickness of the epidermis were higher in AKs than in normal skin.

**Table II:**
Histological and immunohistochemical parameters (Mean ± SEM) for untreated AKs and AKs that have been treated for 6 weeks with topical ATRA, respectively vehicle.

| Parameter | Scoring method | Untreated | ATRA | Vehicle | Normal skin (reference number) |
|---|---|---|---|---|---|
| Ki67 | positive cells/mm | 165 ± 47*** | 175 ± 26 | 185 ± 50 | 28[24] |
| Keratin 10 | 7-point scale | 4.1 ± 0.2* | 4.9 ± 0.1* | 4.1 ± 0.3 | 5.0[25] |
| Keratin 13 | 7-point scale | 0.6 ± 0.4 | 1.0 ± 0.4** | 0.1 ± 0.1** | 0[26] |
| Keratin 16 | 7-point scale | 3.8 ± 0.3 | 3.8 ± 0.2 | 3.6 ± 0.2 | 0[24] |
| Transglutaminase | positive-/total cell layers | 0.18 ± 0.04 | 0.18 ± 0.03 | 0.21 ± 0.04 | 0.20[27] |
| p53 | positive cells/mm | 418 ± 115*** | 626 ± 163 | 588 ± 195 | 20 (own unpubl. data) |
| Epidermal thickness | mm | 0.44 ± 0.09 | 0.33 ± 0.08 | 0.37 ± 0.08 | 0.30 (own unpubl. data) |
| Dermal infiltrate | 5-point scale | 2.0 ± 0.4 | 2.0 ± 0.4 | 2.2 ± 0.5 | 1.0 (own unpubl. data) |

* Significant difference between ATRA-treated AKs and untreated AKs ($p < 0.05$);
** Significant difference between ATRA-treated AKs and vehicle-treated AKs ($p < 0.05$);
*** Significant correlation between Ki67 and p53 in untreated AKs ($p < 0.01$).

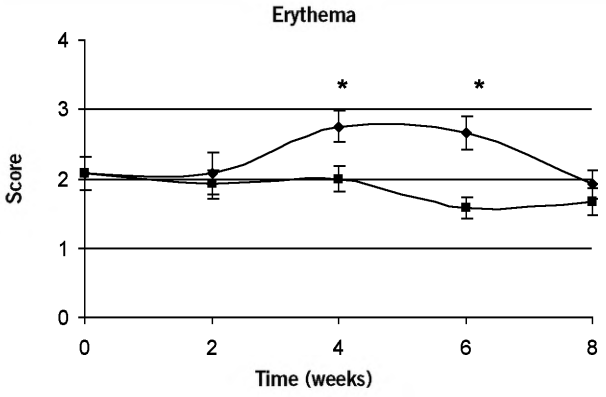


**Figure 2:**

Erythema scores on a 5 point semi-quantitative scale for the ATRA- and vehicle treated test areas during the 6-week treatment period and 2 weeks follow-up (* significant difference between ATRA and vehicle; $p < 0.05$).

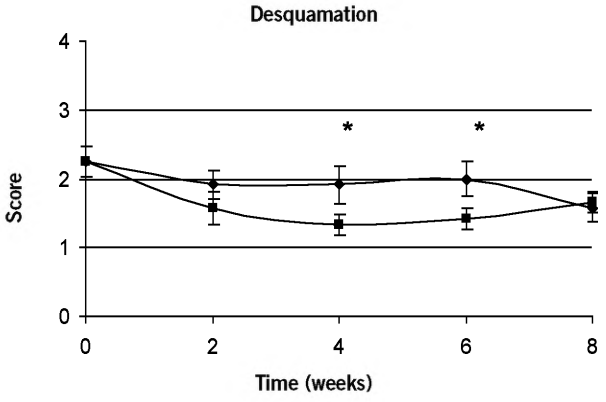


**Figure 3:**

Desquamation scores on a 5 point semi-quantitative scale for the ATRA- and vehicle treated test areas during the 6-week treatment period and 2 weeks follow-up (* significant difference between ATRA and vehicle; $p < 0.05$).

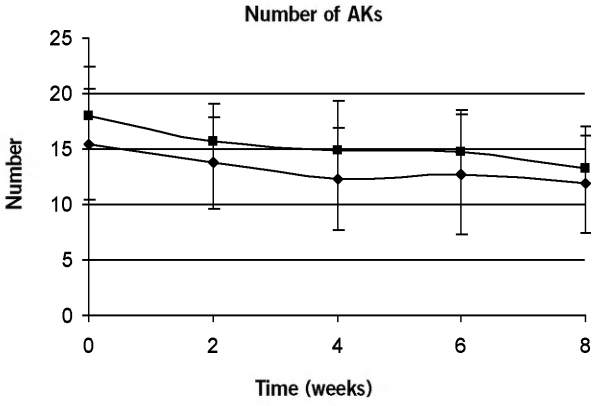


**Figure 4:**

The number of AKs at the ATRA- and vehicle treated test areas during the 6-week treatment period and 2 week's follow-up.

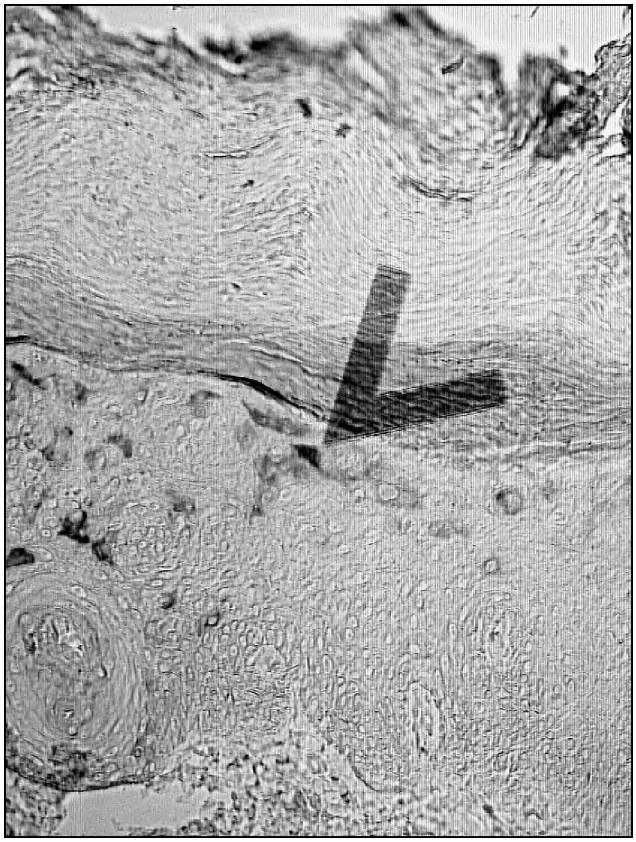

**Figure 5:**

Scattered expression of K13 in an untreated AK without previous use of retinoids. In the suprabasal compartment of the epidermis several dark-stained K13 positive keratinocytes can be seen (arrow) (100x).

## Discussion

In this study, that is focussed on short-term effects of topical ATRA, a significant higher expression of K13 and significant skin irritation was seen in ATRA-treated AKs compared to vehicle–treated AKs, suggesting the study design was adequate to reveal early retinoid effects. Nevertheless, although important hallmarks of AKs are a hyperproliferative state, an increased expression of (especially mutant-type) p53,[14] and disturbed differentiation, only an alteration in differentiation parameters was seen in this study, as represented by an increase in K10 and in K13 expression. No decrease in epidermal proliferation was found after 6 weeks treatment with ATRA, even though retinoids are known to suppress proliferation in psoriasis and other hyperproliferative skin condi-

tions[15] and as AKs are hyperproliferative conditions that feature high levels of Ki-67 positive keratinocytes.[16] Furthermore, no alterations in apoptosis associated p53 protein expression were seen, despite the fact that retinoids can induce apoptosis and may act as a chemopreventive treatment.[17;18] A positive relationship between epidermal proliferation and p53 protein in AKs has been reported in several studies.[19;20] The present study reconfirms this relationship. Other parameters, including hyperproliferation-associated K16, transglutaminase, dermal infiltrate, and epidermal thickness, did not change either. In contrast to other clinical studies, no reduction in the number of AKs was found, but this may be explained by the relatively short study period.

With respect to keratinization, retinoids are known to alter normal differentiation of the cornifying epidermis towards an esophageal-type of differentiation. Retinoids stimulate synthesis of low molecular weight keratins, such as K13, which are normally present in internal squamous epithelia, and inhibit the production of higher molecular weight proteins, normally present in adult human skin.[12;21]

Remarkably, as this study demonstrates, untreated AKs and vehicle treated AKs can also incidentally express K13 in the absence of retinoid therapy. In human skin K13 expression has been found in poorly differentiated SCCs but not in well-differentiated SCCs.[22] Dedifferentiation can be a feature of malignant transformation, which is associated with a switch in synthesis of high molecular weight proteins towards low molecular weight proteins.[23] Bearing this in mind, expression of the low molecular weight K13 in a non-retinoid treated AK may well be sign of a more dedifferentiated state of this AK, and thus a lesion with an increased risk for transformation towards a SCC. It is somehow contradictory that ATRA in one way may have chemopreventive capacities in the treatment of AKs and SCCs, and on the other hand may induce a keratin that is postulated to be associated with malignant transformation. However, an explanation for this phenomenon could be that we deal with two independent epiphenomena, related to two distinct pathways: malignant transformation associated dedifferentiation and (benign) retinoid-associated keratinization. In that situation, it is attractive to speculate whether K13 expression in non-retinoid-treated AKs may provide additional information on the risk of malignant transformation of AKs for use as a diagnostic parameter. It is obvious that further studies are necessary to resolve this issue.

Integrating the clinical, histological and immunohistochemical findings of this study in AKs, we observed differences in keratinization between ATRA and its vehicle. We could not demonstrate reductions or alterations in the number of AKs, nor in important hallmarks of AKs, such as epidermal proliferation and p53 expression, during 6-week treatment with topical ATRA. This implicates that topical ATRA does not alter epidermal proliferation and p53 expression, but only changes the process of keratinization in AKs during a 6-week treatment period. Studies with longer treatment duration or higher concentrations of ATRA are needed to further elucidate these findings. However, the relevance of this treatment regimen may be limited, while ATRA causes substantial skin irritation.

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# 3.5

# Combination of topical calcipotriol and all-trans retinoic acid in actinic keratoses in renal transplant recipients


## Abstract

*A double bind placebo-controlled study was undertaken to determine clinical, histological and immunohistochemical effects of topical calcipotriol and all-trans retinoic acid (ATRA) in the treatment of actinic keratoses in renal transplant recipients. In 13 renal transplant recipients 4 comparable skin areas with multiple actinic keratoses were treated twice daily for 6 weeks by using the following double-blind approach: 1. Calcipotriol cream. 2. ATRA 0.02%. 3. Calcipotriol cream (mornings) and ATRA cream 0.02% (evenings). 4. Vehicle cream. Examination of the test areas was carried out biweekly and punchbiopsies were taken before and after treatment. No single treatment resulted in a reduction of the number of actinic keratoses. Both clinically and immunohistochemically (epidermal proliferation, keratinization, inflammation and apoptosis) no significant differences were found between the 4 treatment regimens. The present study indicates that short-term topical treatment with calcipotriol cream and/or ATRA cream 0.02% is not effective in actinic keratoses in renal transplant recipients.*

## Introduction

In patients receiving immunosuppression following renal transplantation, actinic keratoses have been reported in 38% after 5 years follow-up.[1] However, it has been speculated that with time nearly all these patients will develop actinic keratoses. The major etiological factors for the origin of these lesions are previous sunlight exposure, the duration of immunosuppression and HPV infections.[2,3] In general, the percentage of actinic keratoses that will convert into a squamous cell carcinoma (SCC) within one year varies between 0.25% and 20%, depending on the number of risk factors present.[4,5] Since actinic keratoses in renal transplant recipients are, as a rule, multiple and behave more aggressive when compared to identical lesions in non-immunocompromized individuals, these patients are in need of a chemopreventive treatment that is quickly effective (within weeks) and well tolerated.[5]

It has been suggested that actinic keratoses might be potential targets for vitamin D and retinoids.[6] Several clinical studies have shown efficacy of topical retinoids in the treatment of actinic keratoses.[7,8] But other studies did not confirm this clinical impression.[9,10] No histological and immunohistochemical studies with topical retinoids on actinic keratoses in the population of renal transplant recipients have been performed so far to strengthen or contradict the presumed efficacy. The criteria for improvement of actinic keratoses have to comprise both the clinical appearance and relevant histopathological and immunohistochemical features of the preneoplastic process. Clinical improvement alone without improvement of proliferative characteristics may mask the keratoses and can result in a recurrence of lesions after cessation of treatment.[11,12] No studies have been performed on vitamin D derivatives in the treatment of actinic keratoses. The aim of the present study was to find out whether monotherapy or a combination of topical ATRA and the vitamin D3 derivative calcipotriol provides an approach for actinic keratoses which is effective from clinical, histological and immunohistochemical perspective in a treatment period of 6 weeks and that is well tolerated by the patients.

## Materials and methods

### Study design

Thirteen renal transplant recipients with multiple actinic keratoses were included in this double-blind, placebo-controlled study. All patients applied each of the following treatment regimens for 6 weeks to 4 comparable and distinct areas of actinic keratoses on the extremities: (I) calcipotriol cream 50μg/g twice daily, (II) ATRA cream 0.02% twice daily, (III) the combination of calcipotriol 50μg/g cream once daily and ATRA cream 0.02% once daily, and (IV) cremor cetomacrogolis twice daily. With respect to the combination therapy, calcipotriol cream was used in the morning and all-trans retinoic acid cream was used in the evening. ATRA in a concentration of 0.2% was selected, since the skin of renal transplant recipients often is atrophic and subsequently irritant reactions can be severe. The washout period for any topical treatment on the test areas was 2 weeks. A follow-up visit was performed 2 weeks after treatment discontinuation. Medical Ethics Committee approval was obtained and all patients gave informed consent prior to study enrollment.

### Formulations

Calcipotriol 50μg/g cream (Daivonex®, cream) was obtained from LEO Pharma, Weesp, The Netherlands. The pharmacist formulated ATRA cream 0.02%. The cream base consisted of cremor cetomacrogolis, alcohol ketonatus and butylhydroxytolueen. As placebo cream, cremor cetomacrogolis was used, which approached the physical-chemical characteristics of the other two creams. The pharmacist manufactured the creams color-matched with a randomization scheme.

### Clinical efficacy and adverse events

Pruritus, erythema, induration and desquamation of the actinic keratoses in the test areas were assessed biweekly during the application period and at the follow-up visit, which was scheduled 2 weeks after the final application. For these parameters we used a semi-quantitative score on a 5-point scale (0 = none, 1 = slight, 2 = moderate, 3 = moderate-severe, 4 = severe). Furthermore, the number of actinic keratoses in the test areas was counted each visit.

### Immunohistochemical techniques and scoring methods

Three millimeter biopsies taken at the baseline and after 6-weeks treatment from clinically identical actinic keratoses of the 4 test areas were processed by using indirect immunoperoxidase techniques on paraffin-embedded sections, and were analyzed based on previously published scoring methods by two blinded investigators.[13] Esophageal tissue was used as a positive control for keratin 13 staining. With respect to proliferation (MIB-1 for Ki67), and apoptosis (DO-7 for p53 protein) the number of positive cells per millimeter length of section was counted. Keratinization (DE-K10 for keratin 10, 1C7 for keratin 13, and LL-025 for keratin 16) and dermal infiltrate scores were assessed by semiquantitative scoring techniques. Terminal differentiation (BT621 for transglutaminase) was assessed as the number of stained epidermal cell layers divided by the total number of epidermal cell layers. Epidermal thickness was measured in millimeters.

### Statistical analysis

In order to substantiate the differences between the baseline situation and the situation after 6 weeks treatment, with respect to the clinical and immunohistochemical parameters, an Analysis of Variance (ANOVA) test was used.

**Table I:**
Monoclonal antibodies used in the study.

| Antibody | Specifity | Concentration | Source |
|---|---|---|---|
| **MIB-1** | Ki-67 | 1:50 | Immunotech, Marseilles, France |
| **DE-K10** | Keratin 10 | 1:100 | Monosan, Uden, Netherlands |
| **1C7** | Keratin 13 | 1:10 | Monosan, Uden, Netherlands |
| **LL-025** | Keratin 16 | 1:10 | Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK |
| **BT621** | Transglutaminase | 1:10 | Biomedical Technologies Inc., Stoughton, MA, USA |
| **DO-7** | p53 | 1:200 | DAKO, Glostrup, Denmark |

## Results

The mean age of the 13 subjects was 47.5 (37 – 68 years). The mean number of years after transplantation was 19.7 (6 – 29 years). Patients had actinic keratoses since 9.0 ± 4.6 years (mean ± SEM) after transplantation. In 7 out of these patients invasive squamous carcinomas had occurred before study inclusion. In 1 patient a squamous cell carcinoma developed from an actinic keratosis in the calcipotriol-treated area during the application period of the study.

Clinical parameters showed no significant differences between the 4 treatments, although, overall significantly more erythema ($p = 0.04$; **Figure 1**) and less induration ($p = 0.007$; **Figure 2**) were observed after 6-weeks treatment compared to the baseline. No differences in desquamation and in the number of actinic keratoses could be found **(Figure 3)**. Mild pruritus was seen in all test areas during treatment, except for the sites that were only treated with the vehicle, and had disappeared 2 weeks after treatment discontinuation **(Figure 4)**.

Histological and immunohistochemical data-analysis revealed no significant differences between each of the 4 treatments or between baseline and post-treatment situation, for the following parameters: MIB-1, DE-K10, LL-025, BT621, DO-7, epidermal thickness, and dermal infiltrate **(Table II)**. In some actinic keratoses very typical and different staining patterns for DO-7 **(Figure 5)** and MIB-1 **(Figure 6)** were observed in consecutive slides, suggesting that the correlation between p53 and Ki-67, which has been found in several studies, may vary in individual areas of actinic keratoses in renal transplant recipients. Keratin 13 expression (1C7) was observed in 3 ATRA-treated actinic keratoses, in 3 ATRA-calcipotriol-treated actinic keratoses, and in 2 calcipotriol-treated actinic keratoses. No keratin 13 expression was found in the untreated actinic keratoses and in the vehicle treated actinic keratoses.

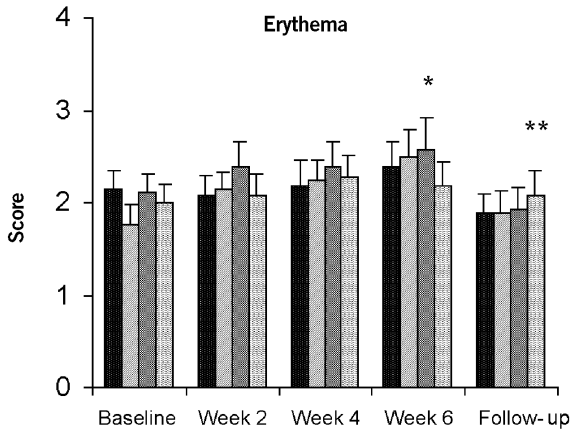


**Figure 1:**

Erythema scores of the actinic keratoses for the 4 treatments at the test areas during the study. (* Indicates a significant increase in erythema after 6 weeks treatment compared to the baseline ($p < 0.05$); ** indicates a significant reduction in erythema after cessation of the cream applications compared to the week 6 visit ($p < 0.05$)).

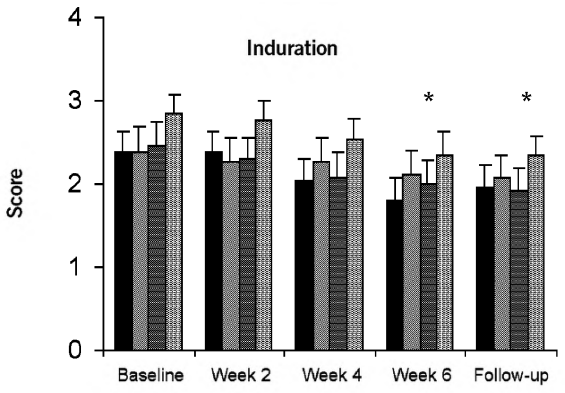


**Figure 2:**

Induration scores of the actinic keratoses for the 4 treatments at the test areas during the study. (* Indicates a significant reduction in induration of the actinic keratoses compared to baseline ($p < 0.01$)).

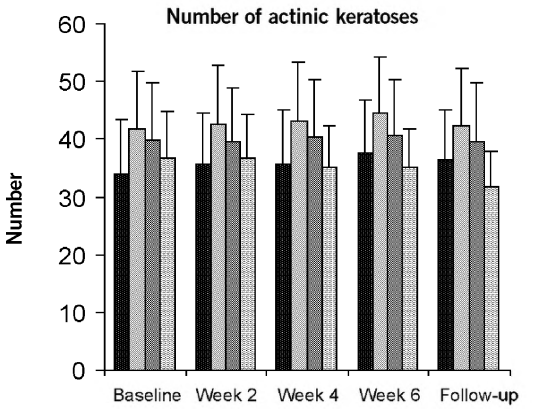


**Figure 3:**

The number of actinic keratoses for the 4 treatments at the test areas during the study.

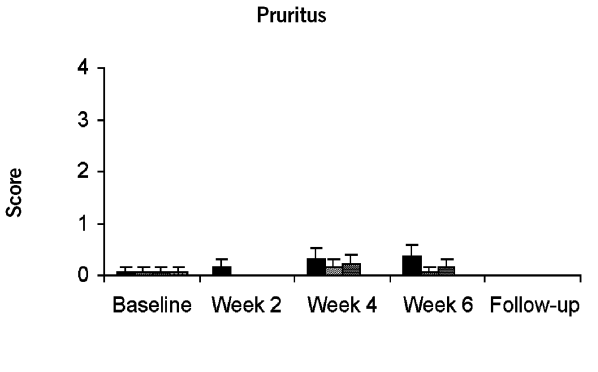


**Figure 4:**

Pruritus scores for the 4 treatments at the test areas during the study.

**Table II:**
Histological and immunohistochemical parameters (Mean ± SEM) for the untreated actinic keratoses and after 6 weeks treatment with ATRA cream, calcipotriol cream, ATRA-calcipotriol combination and vehicle cream.

| Parameter | Scoring method | Untreated | ATRA | Calcipotriol | Combination | Vehicle |
|---|---|---|---|---|---|---|
| Dermal infiltrate | 5-point scale | 2.2 ± 0.2 | 1.9 ± 0.3 | 1.8 ± 0.3 | 1.8 ± 0.2 | 1.6 ± 0.2 |
| Keratin 10 | 7-point scale | 4.6 ± 0.2 | 4.6 ± 0.2 | 4.5 ± 0.2 | 4.5 ± 0.2 | 4.3 ± 0.2 |
| Keratin 13 | 7-point scale | 0 | 0.3 ± 0.2 | 0.3 ± 0.2 | 0.4 ± 0.2 | 0 |
| Keratin 16 | 7-point scale | 3.8 ± 0.3 | 3.4 ± 0.4 | 3.7 ± 0.4 | 3.8 ± 0.3 | 3.9 ± 0.2 |
| Transglutaminase | positive-/total cell layers | 0.37 ± 0.03 | 0.37 ± 0.03 | 0.37 ± 0.02 | 0.38 ± 0.03 | 0.36 ± 0.03 |
| Ki–67 | positive cells/mm | 218 ± 29 | 272 ± 64 | 189 ± 27 | 270 ± 45 | 227 ± 32 |
| p53 | positive cells/mm | 388 ± 40 | 252 ± 32 | 247 ± 43 | 364 ± 58 | 313 ± 53 |
| Epidermal thickness | mm | 0.57 ± 0.08 | 0.38 ± 0.07 | 0.45 ± 0.09 | 0.35 ± 0.06 | 0.51 ± 0.10 |

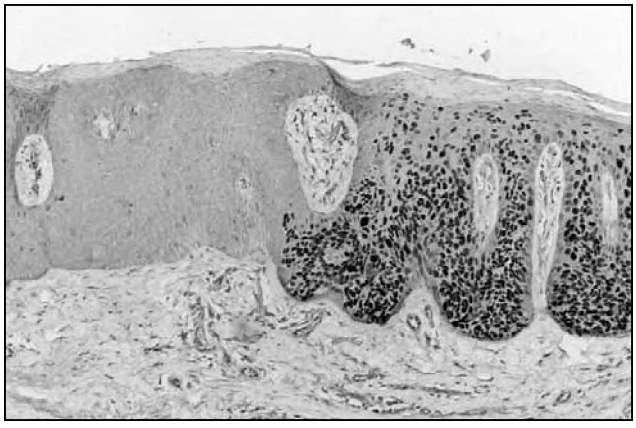

**Figure 5:**

P53 protein expression (DO-7) in an untreated actinic keratosis: the focal expression pattern is clearly visible.

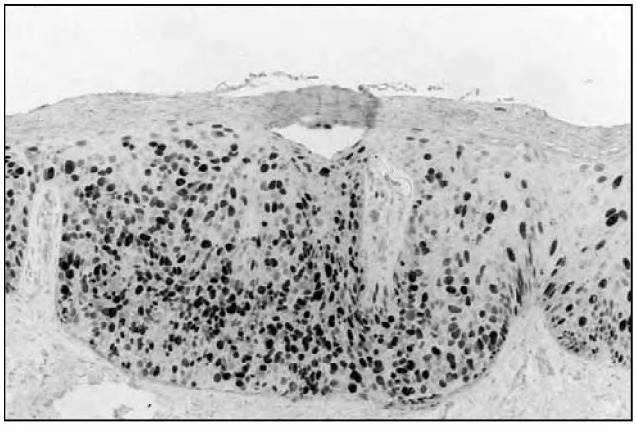

**Figure 6:**

Ki-67 expression (MIB-1) in a consecutive slide of the same untreated actinic keratosis as shown in previous figure: the Ki-67 expression pattern spreads throughout the epidermis and does not correlate with p53 protein expression.

## Discussion

The present study demonstrated no significant differences in clinical, histological and immunohistochemical parameters between the 4 different therapies during a 6-week treatment period. Post-treatment values for these parameters did not reveal a significant improvement, apart from a minimal reduction in induration, that was not represented in general histological features such as epidermal thickness, and that was probably caused by a softening effect of the creams. Integrating these findings, it can be concluded that a 6-week treatment with calcipotriol 50μg/g cream, ATRA 0.02% cream or the combination of both, is not effective in the treatment of actinic keratoses in renal transplant recipients. In contrast, a 6-week treatment of topical retinoic acid in ichthyoses, and calcipotriol in psoriasis, has been reported to result in marked clinical improvement, reduction of epidermal hyperproliferation, and a decrease of several abnormalities with respect to inflammation and keratinization. First clinical and histological efficacy has already been found after 2-4 weeks treatment.[13,14]

It is remotely possible that a prolonged treatment schedule might show an effect for some parameters. However, the dynamics of actinic keratoses rapidly progressing to squamous cell carcinomas in renal transplant recipients makes such an option not adequate. Treatment with formulations containing higher concentrations of ATRA might be more effective, however treatment with such a cream causing irritation, obviously reduces compliance to treatment. In conclusion, the common believe that topical retinoids and vitamin D3 analogues might be of value in actinic keratoses of renal transplant recipients appears to be unsubstantiated. Topical 5-fluorouracil (Efudix®) and cryo-therapy remain the first therapeutic approaches in actinic keratoses in these patients.

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# 3.6

# Keratin 13 and keratin 19 expression in verrucae vulgares and morbus Bowen in renal transplant recipients and immunocompetent patients


## Abstract

*The aim of the present study was to compare frequency and expression patterns of keratins K13 and K19 in cutaneous warts of renal transplant recipients (RTRs) and immunocompetent individuals (ICIs). In RTRs the effect of retinoids on both keratins was assessed. For this retrospective, non-randomized immunohistochemical study, excisions of cutaneous warts from RTRs and ICIs were retrieved from the archives of the department of Pathology, University Medical Center Nijmegen, the Netherlands. Twenty-one warts from both RTRs and ICIs were examined. In 10 specimens from RTRs, patients received either systemic acitretin or topical all-trans retinoic acid. A significant higher percentage of RTR-related warts expressed K13 when compared to warts of ICIs, 86% versus 14% ($p<0.001$). In RTR-related warts, retinoid treatment correlated significantly with a particular strong strikingly segmental ("zebroid") K13-expression pattern. Without retinoids, K13 was mostly restricted to suprabasal single cells. K19 was absent in all warts of both patient groups. It was concluded that: 1.Retinoids strongly correlate with K13 in a characteristic "zebroid" pattern in RTR-related warts, making K13 a sensitive marker for retinoid bioactivity in skin(lesions) of RTRs. 2. In non-retinoid treated renal transplant patients, K13 is also frequently found in warts, but without the dramatic "zebroid" pattern noted with retinoid treated warts.*

## Introduction

Epithelial keratins comprise a heterogenous group of acidic (type I) and neutral-to-basic (type II) proteins. As a general rule, they are coexpressed in specific pairings, each pair consisting of a type I and type II keratin. For instance, in the normal adult skin, keratin pairs K5/K14 predominate in the basal layer and K1/K10 in the suprabasal compartment.[1]

The type I keratins K13 and K19, are usually expressed separately in adult epithelia. Combined expression occurs only in fetal skin. Both keratins are thought to be absent in normal skin of adults except for certain body sites such as penile foreskin that still contains K13. Furthermore, K13 in adults is abundantly present in internal stratified epithelia and associated (with terminal differentiation) with suprabasal expression.[1-4] K19 expression in adults is found in simple epithelia, such as most glandular epithelia.

In the past years several murine and in vitro studies have demonstrated that vitamin A and its derivatives (retinoids) are important regulators of epidermal differentiation and influence keratin gene expression. In cultured keratinocytes, the induction of an embryonic type of differentiation by retinoids with reinduction of keratin 13 and 19 expression has been well documented.[5,6] In vivo topical application of retinoids on photoaged human skin also showed induction of K13.[7]

Although retinoids are used as chemopreventive agents for inhibiting skin cancer in renal transplant recipients,[8,9] to the best of our knowledge there are no studies regarding the effects of retinoids on keratin expression in skin or skin lesions of these patients.

Renal transplant recipients (RTRs) are known to develop multiple warts and skin neoplasms. Immunosuppressive treatment, sun exposition and viral infection with human papillomavirus are all implicated in the etiology of cutaneous tumorigenesis in RTRs.[10-14] The frequent simultaneous occurrence of warts and skin cancers in renal transplant recipients led us to the assumption that the verrucae or warts in renal transplant recipients might be more prone to become malignant than equivalent lesions in normal immunocompetent individuals (ICIs). The existence of high and low risk papillomas was previously shown in mice models on cutaneous carcinogenesis.[15] Interestingly, these high-risk papillomas showed expression of K13 which was absent in low risk papillomas.

The present study shows that RTR-associated warts in contrast to warts in normal ICIs indeed show pronounced K13 expression. This suggests that altered keratin expression may reflect an important molecular event inherent in malignant degeneration of warts in RTRs. Furthermore, this K13 expression in warts of RTRs strongly correlates with retinoid therapy but in contrast to findings in animal studies and in cultured human keratinocytes,[5] we could not demonstrate influence of retinoids on K19 expression in these patients. Retinoid related K13 expression in epithelial skin lesion of RTRs displays a highly characteristic (zebroid) pattern, making K13 a useful marker for evaluating the effect of retinoid treatment in these patients.

## Materials and methods

### Tissues

For this retrospective immunohistochemical study we retrieved formalin fixed and paraffin embedded skin excisions of warts from renal transplant recipients (21 excisions, 18 patients, average age 50.8 years, mean duration of immunosuppression 16.4 years) and from normal immunocompetent individuals (21 excisions, 19 patients, average age 33.3 years) out of our archival material at the department of Pathology, University Medical Center Nijmegen St. Radboud, the Netherlands.

### Retinoid treatment

Retinoids were only used by renal transplant recipients. Nine RTRs (10 excisions) received retinoid treatment. In 3 patients (4 excisions) topical all-transretinoic acid (concentration 0.025-0.05%) was used and 6 patients received systemic acitretin (dose at time of biopsy varying from 10-35 mg). Patients on systemic acitretin participated in a clinical trial, not related to the present study, studying the effects of systemic retinoid on cutaneous carcinogenesis in RTRs. Inclusion criteria for this trial were either the presence of at least one SCC in the patients' history or the presence of 10 or more actinic keratoses, at least one confirmed histologically. Initially most patients on acitretin started with a dose of 30-35 mg/day, a dosage that in an earlier study proved to prevent squamous cell carcinomas in renal transplant recipients.[8] However, in a relative large number of patients doses of acitretin had to be lowered because of mucocutaneous side-effects (peeling of palms and soles and/or cheilitis). Retinoid and non-retinoid-treated patients showed no obvious differences with respect to duration, dosage and type of immunosuppression, all factors implied in the etiology of skin cancers in immunosuppressed patients (data not shown).

### Histopathology

Histology of all studied lesions was revised according to World Health Organization (WHO) definitions of verrucae.[16] The verrucae consisted predominantly of common warts or verrucae vulgares with a smaller group of verrucae plana especially in the ICIs, usually located on hands and feet. Condylomata acuminata or anogenital warts were not included.

### Immunohistochemistry

Immunohistochemical analysis was performed on all excisions by using standard avidin-biotin-peroxidase complex system with either diaminobenzidine (DAB) and/or 3-amino-9-ethylcarbazole (AEC) as the chromogens. In brief, 4-micron thick paraffin sections were deparaffinized, hydrated and washed in buffered phosphate. For K13 staining sections were cooked in buffered citrate (10mM, pH 6.0) in the microwave oven for twice 5 minutes (600 Watt). After a cooling down period of (at least) 20 minutes and preincubation with 20% normal horse serum for 15 minutes, the sections were incubated with undiluted primary antibody, overnight at 4°C. We used two mouse monoclonal primary antibodies, 1C7 (IgG2a) and 2D7 (IgG2b), both recognizing K13.[17] As positive control normal esophageal tissue was used. After incubation with primary antibodies sections were incubated for 30 minutes with (a 1: 200 dilution of) biotinylated horse anti-mouse (Vector laboratories, Burlingham, CA), followed by a 45-minute incubation with (a 1:50 dilution of) avidin-biotin complex (Vector laboratories, Burlingham, CA). For development with AEC, ABC concentrations were doubled.

For K19 immunohistochemistry (mouse) monoclonal antibody, RCK108 (DAKO) was used. Besides different pretreatment (0.1% pronase for 10 min.), the same procedure as for K13 was followed. Eccrine ducts and sweat glands served as positive internal controls. Sections were counterstained with Mayer's hematoxylin for 2 minutes.

### Scoring of immunohistochemical results

Immunoreactivity was scored as negative, slightly positive in a suprabasal single cell pattern ( < 10% lesional keratinocytes positive) or strongly positive in a suprabasal segmental columnar pattern (zebroid pattern). Scoring was performed without knowledge of patient history and use of retinoid therapy.

### Statistics

The Pearson Chi-square ($X^2$) test was used in all statistical analysis and significance was set at $p < 0.05$.

## Results

### General aspects of immunostaining for K13 and K19

In all cases, immunostaining for K13 was restricted to the cytoplasm. Slight variations in staining intensity were observed in lesional skin when comparing antibodies 1C7 and 2D7, with overall stronger staining with the 2D7 antibody. Principally, however the staining pattern of lesional skin with both antibodies was identical. Normal esophagus, which was used as positive control, showed strong diffuse suprabasal staining.

K19 immunostaining was also localized in the cytoplasm. Eccrine ducts and sweat glands, serving as internal controls, showed marked positivity.

### Expression of K13 and K19 in warts of RTRs versus ICIs and effects of retinoid treatment

There was a statistically significant difference in K13 expression between warts of RTRs when compared to warts of ICIs ($p < 0.001$). A high percentage (86 %) of the warts from RTRs showed K13 expression, whilst in benign warts of ICIs almost all lesions were negative except for 3 out of 21 cases with suprabasal single cell positivity (14 %) (**Table I** and **Figure 1**). This statistical difference in K13 positivity stayed present when we left retinoid treated patients out; 82 % K13 positivity in non-retinoid-treated RTRs versus 14 % in the controls ($p < 0.001$). Besides number of positive excisions, also the proportion of positive lesional cells differed and was far more pronounced in the warts from transplant patients: the 3 positive warts of ICIs showed only suprabasal single cell positivity (**Figure 2**); in RTRs, half of the 18 positive warts also showed this single suprabasal cell positivity, while the other half showed strong positive staining in a remarkable pattern of segmental positive suprabasal full-epithelium thickness columns, a pattern we termed zebroid (**Figure 1B, D** and **Figure 2**). This particular pattern was not linked to eccrine ducts or hair follicle structures; the latter actually seemed to be spared. This zebroid pattern (statistically) correlated ($p < 0.001$) with retinoid treatment (topical and systemic) when comparing retinoid treated RTRs (warts and ISSCCs) with non-retinoid treated RTRs (**Table II**). Only 1 patient without (anamnestically traceable) retinoid treatment exhibited the same K13 expression pattern.

Most warts were superficially excised with no perilesional skin available. In 1 retinoid treated RTR the perilesional skin showed K13 positivity comparable with the above described zebroid pattern.

All warts in both groups were negative for K19 with retinoids having no demonstrable effect on K19 expression (**Figure 1C, D**).

**Table I:**
K13 positive and negative warts in RTRs and ICIs.

| | Warts of RTRs (N=21) | Warts of ICIs (N=21) |
|---|---|---|
| **K 13 positive** | 18 (86%) | 3 (14%) |
| **K 13 negative** | 3 (14%) | 18 (86%) |

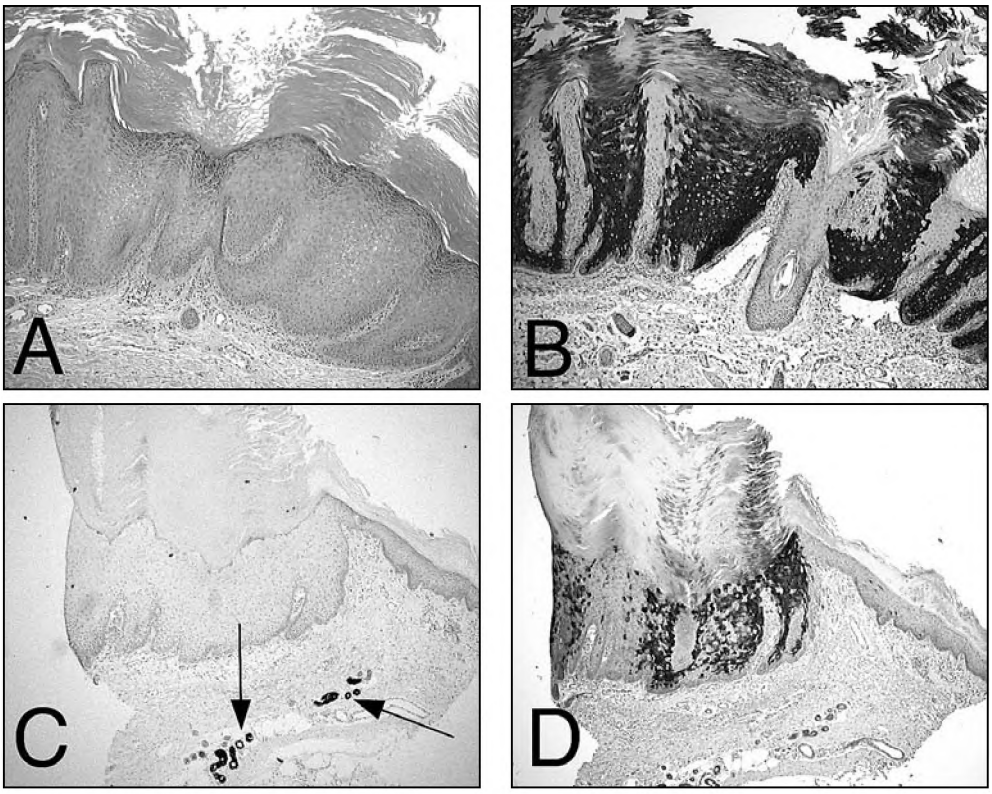


**Figure 1:**

**A.** Hematoxylin-eosin staining of a wart from a retinoid-treated renal transplant recipient (RTR) (original magnification X40).

**B.** Immunohistochemical analysis of a wart from a retinoid-treated RTR for K13 (monoclonal antibody 2D7, DAB as the chromogen) showing the particular strong 3+ positive zebroid pattern of alternating suprabasal columns of K13-positive and K13-negative keratinocytes. This zebroid pattern proved significantly correlated to retinoid treatment in RTRs (original magnification X40).

**C-D.** Wart of retinoid treated RTR showing uncoupled regulation of K13 and K19 expression by retinoids with strong zebroid pattern K13 positivity (D), while K19 expression is absent (C). The arrow (C) point to K19-positive sweat glands, which serve as internal control (original magnification X40).

**Table II:**
Warts in RTRs. Strong K13 positivity (zebroid pattern) and not zebroid K13 positivity vs. retinoid therapy.

| | Retinoid treated warts (N=10) | Non-retinoid treated warts (N=11) |
|---|---|---|
| **K 13 zebroid pattern** | 9 (90%) | 1 (10%) |
| **K 13 not zebroid pattern** | 1 (9%) | 10 (91%) |

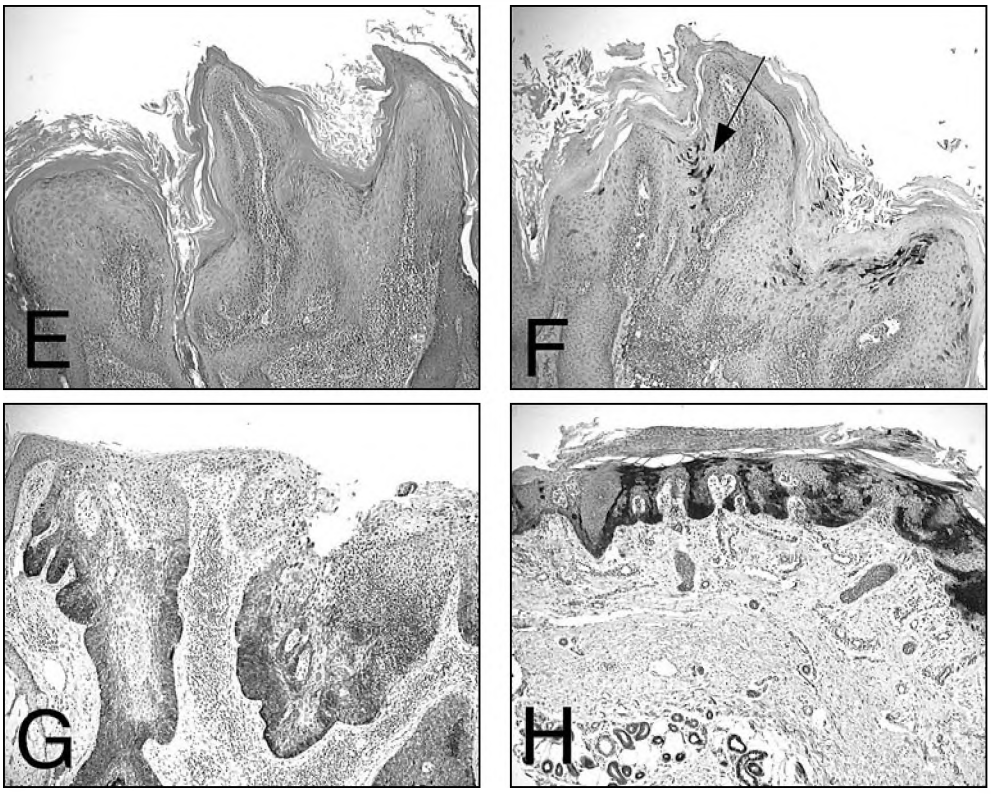


**Figure 1** *(continued)*:

**E.** Hematoxylin-eosin staining of a wart from an immunocompetent individual (ICI) (original magnification X40).
**F.** Immunohistochemical analysis of a wart from an ICI for K13 (2D7, DAB) showing suprabasal single cell positivity (arrow). This pattern of K13 expression was also typical of warts from non-retinoid-treated RTRs (original magnification X40).
**G.** In situ squamous cell carcinoma (ISSCC) of an RTR showing K13 (2D7) expression partially centered around hair follicle structures (original magnification X40). This staining pattern differs considerably from the retinoid therapy-related zebroid K13 pattern.
**H.** Perilesional skin specimen from an ISSCC of an RTR receiving retinoid treatment. Immunohistochemical analysis of K13 (2D7) shows the same zebroid pattern as in lesional skin of most warts of retinoid-treated RTRs (original magnification X40).

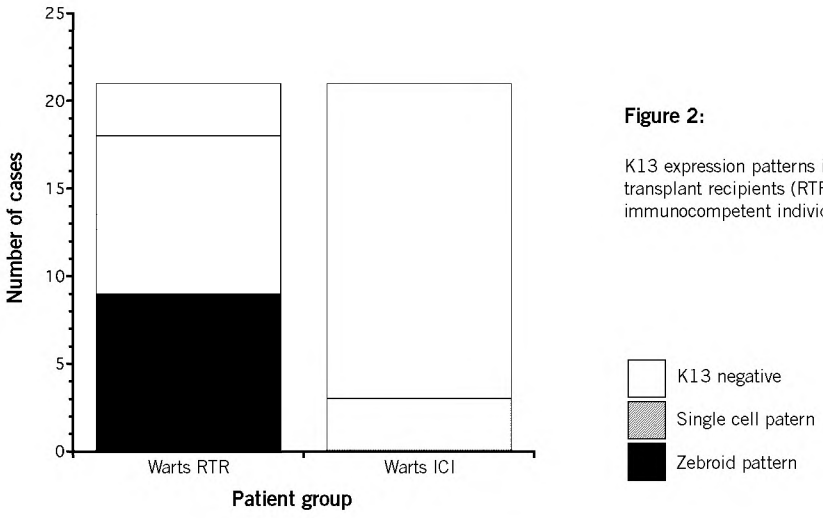


**Figure 2:**

K13 expression patterns in warts of renal transplant recipients (RTRs) versus immunocompetent individuals (ICIs).

## Discussion

Data from numerous animal and in vitro studies with cultured human keratinocytes have indicated that retinoids influence epidermal proliferation and differentiation.[5,6,18-20] Retinoids repressed expression of differentiation-specific keratins (K1/K10) and strikingly reinduced K13 and K19 expression, two keratins that are only coexpressed in fetal skin but not normally present in epidermis of adults.[2,4] In contrast to these findings of coupled K13 and K19 induction by retinoids, Agarwal was the first to report uncoupled regulation of K13 and K19 expression in a human squamous cell carcinoma cell line.[21]

Interestingly, our in vivo data with immunohistochemical evaluation of K13 and K19 expression in warts of RTRs versus ICIs, showed that retinoids used as a chemoprotective agent in the transplant recipients for preventing skin cancer, also only strongly relate to K13 and not K19 expression. Our finding of retinoid-related uncoupled K13 and K19 expression in these patients could be threefold. First, the retinoid concentration in our patients could be sufficient to enhance K13 expression but not K19 expression. Earlier findings in human epidermal cultures indeed showed stronger induction of K13 than of K19 by retinoids with already a marked increase of K13 in response to very low levels of retinoids, while for K19 induction a higher threshold retinoid concentration was necessary.[6] Although in a considerable number of our patients on systemic retinoids dosages had to be lowered in the course of treatment because of severe mucocutaneous side-effects, also in patients still receiving the higher dosages of acitretin no induction of

K19 was found. Second, response of keratinocytes to retinoids in vitro might not be representative for the response in vivo and retinoids in humans in vivo might not induce an embryonic type of differentiation and only selectively induce K13. In the only previously performed in vivo study on effects of retinoids, in photo-aged skin, only K13 and not K19 expression was studied.[7] Finally, this differentiation towards "esophageal-type" of epithelium in contrast to so-called "embryonic-type" of differentiation found in animals and in vitro, could be specific for skin and skin lesions in RTRs: earlier studies already showed that effects of retinoids differed in normal keratinocytes when compared to diseased keratinocytes.[19]

Retinoid treatment significantly correlated with a specific pattern of K13 expression in skin lesions of transplant recipients. This pattern, which we termed zebroid, because of alternating suprabasal columns of K13 positive and negative keratinocytes, is suggestive of a genetic mosaicism, reflecting a clonal expansion of genetically altered stem cells. In warts and slightly dysplastic skin of RTRs, segments of epidermis may contain keratinocytes with a genetic abnormality making them more susceptible to inductive actions of retinoids. Future studies might reveal the underlying genes that are involved in this process and if for instance (transforming types of) human papillomavirus (HPV) might play a role.[22]

The interpretation of the biological impact of K13 expression in retinoid and non-retinoid treated warts of RTRs could be twofold. The first interpretation relates presence of K13 in skin to differentiation, in parallel to internal squamous epithelia in which K13 is restricted to the suprabasal epithelial compartment and associated with differentiation. Retinoids, by inducing K13 expression c.q. directing keratinocytes towards (esophageal) differentiation, might act chemopreventive by "freezing" of cells in this differentiated state and preventing them to (further) dedifferentiate. Indeed previous studies on retinoid effects on epidermal keratinocytes have shown that in response to retinoid treatment higher molecular weight keratins, typically encountered in squamous epithelia disappear, and synthesis of two new low molecular weight keratins, a 40 – and 52-kD keratin, corresponding to K19 and K13 respectively, is enhanced.[6,23,24] In the absence of vitamin A, the opposite occurs, with enhanced terminal *epidermal type* of differentiation.[25] Retinoid induced *"esophageal-type"* of differentiation, could provide an explanation for the cosmetically skin improvement of lesional skin in these transplant recipients who often had multiple hyperkeratoses before treatment: esophageal epithelium is, in contrast to the keratinizing epidermis, a non-keratinizing squamous epithelium. By inducing a non-keratinizing differentiation, retinoids could lower the number of hyperkeratotic skin lesions. As a side-effect, in normal skin the diminished cutaneous keratinization caused by retinoids leads to desquamation of palms and soles which normally show the most prominent keratinization. On the lips, the outer cutaneous side becomes more vulnerable because of differentiation towards wet epithelium leading to cheilitis, another known side-effect of acitretin also present in our patients.[8,9,26]

The second interpretation relates presence of K13 to a more dedifferentiated and potentially malignant phenotype. Regarding cutaneous carcinogenesis, malignant transformation is indeed heralded by a switch from production of high molecular weight

(MW) keratins normally present in adult skin (K1/K10) to low MW keratins also characteristic of fetal skin and simple epithelia (e.g. K8/K18 and K19).[1] Presence of K13, a low MW embryonic keratin, would fit within this concept. It is tempting to attribute a relevance to the high frequency of K13 in warts of RTRs and speculate it may be related to the higher susceptibility of warts in these patients to become malignant. This would be in analogue to mouse models on skin carcinogenesis in which aberrant K13 expression is a consistent finding in chemically and v-Ha-ras-induced papillomas and squamous cell carcinomas.[15,27,28] In in situ squamous cell carcinomas (ISSCC) of RTRs and ICIs we indeed found frequent K13 expression in respectively 75% and 45% of lesions (20 ISSCC tested within each group, data not shown), which is in concert with this second hypothesis. The pattern of K13 expression in ISSCCs of both groups was strikingly different from the retinoid therapy related K13 expression (zebroid pattern, compare **Figure 1B** and **G**). Only 4 RTRs with ISSCCs used retinoids, and in these patients the retinoid related zebroid K13 pattern was most pronounced or only present in perilesional only slightly dysplastic skin **(Figure 1H)**.

When this latter interpretation would be applicable to retinoid related K13 expression, retinoids might actually be dangerous for these patients. This is contradicted by studies on long term safety of retinoid therapy, since no increased incidence of skin cancer is reported.[29] Studies on skin cancer chemoprophylaxis in RTRs with retinoid actually showed reduction in the skin cancer incidence.[8]

In conclusion, this retrospective in vivo study on embryonic keratin expression in warts of renal transplant recipients shows that retinoids strongly relate to K13- but not K19 expression. By keeping keratinocytes in this "esophageal-type" of differentiation retinoids might act chemopreventive. Retinoids correlate with a highly distinctive and strong K13 expression, which we termed zebroid, making K13 a useful marker for evaluating retinoid treatment in these patients. The alternating zebroid K13 pattern is suggestive of an underlying genetic mosaicism. Even in the absence of retinoids, a significant higher percentage of K13 positivity is found in warts of RTRs when compared to warts of ICIs. Future prospective, randomized and well-controlled studies need to establish the relevance of this finding and whether K13, in analogue to mouse models on skin carcinogenesis, might become a predictive marker for malignant progression.

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# 4

# UVB-IRRADIATION EXPERIMENTS WITH RAR- AND RXR-SELECTIVE RETINOIDS

*This chapter was based on the following publications:*

*Jürgen V. Smit, Elke M.G.J. de Jong, Gijs, J. de Jongh, Peter C.M. van de Kerkhof.*
**Topical all-trans retinoic acid does not influence minimal erythema doses for UVB light in normal skin.**
**Acta Derm Venereol 2000 Jan-Feb;80(1):66-7.**

*Jürgen V. Smit, Elke M.G.J. de Jong, Peter C.M. van de Kerkhof*
**Effects of oral bexarotene (Targretin®) on the minimal erythema dose for broad-spectrum UVB-light.**
**Skin Pharmacol Appl Skin Physiol 2003: (In press)**

# 4.1

# UVB-irradiation in topical all-trans retinoic acid treated normal skin


## Abstract

*The study was a double-blind, placebo-controlled, within subject comparison, of 15 healthy volunteers, that focussed on clinical effects of topical all-trans retinoic acid (ATRA) on UVB-light-induced inflammation. Three distinct skin areas on the ventrolateral parts of the upper legs were treated for 10 days as follows: 1. ATRA (tretinoincream 0.05 %) twice daily; 2. vehicle cream twice daily; 3. no treatment. On day 8 of the application period irradition occurred with a series of 6 ascending intensities of UVB-light adjusted for the skin-type of the patient on each of the 3 test areas. Clinical scoring for erythema was performed on day 8 (before irradiation), day 9 and day 10; assessment of the minimal erythema dose (MED) occurred on day 9 and day 10. Before irradiation (day 8) the grade of erythema in ATRA-treated skin was significantly higher compared to its vehicle ($p < 0.01$). However, after UVB-irradiation no significant differences were found between ATRA-treated skin, vehicle-treated skin and untreated skin with respect to minimal erythema doses, both 24 and 48 hours after irradiation. No significant changes in the grade of post-irradiation erythema between the three different treatment regimens were found. Furthermore, a typical perifollicular erythema pattern was seen in ATRA-treated skin compared to a diffuse erythema pattern that was visible after UVB-irradiation. The present study suggests that ATRA 0.05% has no effect on UVB-induced erythema.*

## Introduction

Retinoids are used in several skin disorders, such as acne, psoriasis and ichthyoses. It is well-established that retinoids interfere with epidermal proliferation,[1,2] keratinization[3] and inflammation control.[4] Retinoids influence UV-induced skin changes.[5] Topical ATRA improves photodamaged skin.[5,6]

Parallel with the therapeutic effect, topical retinoids often cause irritation with erythema and some scaling.[7] Although the irritant effect is probably not involved in the therapeutic action of retinoids, it is remotely possible that the irritation property of ATRA could be, in part, accountable for some therapeutic effects.[1,7]

In this study we would like to challenge the common belief that topical retinoids enhance UV-induced inflammation. We therefore evaluated the minimal erythema dose (MED) for UVB-irradiation on topical ATRA (tretinoin cream) pre-treated skin compared with vehicle cream pre-treated skin and untreated skin. The degree of erythema at different times before and after UVB-irradiation was scored in the three different skin areas.

The following questions were addressed: 1) To what extent does a 7 days treatment of normal skin with ATRA cream or its vehicle alone induce erythema? 2) To what extent do these creams influence the minimal erythema dose (MED) for UVB-irradiation? 3) What are the dynamics of erythema 24 and 48 hours after UVB-irradiation in ATRA-treated skin, vehicle-treated skin and untreated skin?

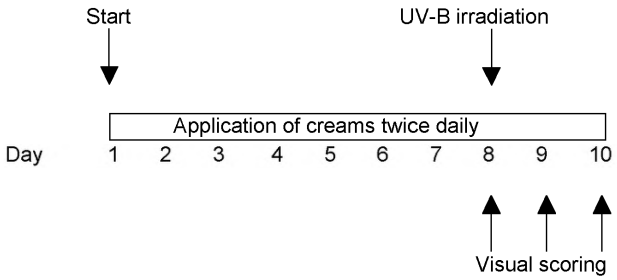


**Figure 1:**

Schematic view of the study procedures

## Materials and methods

The study was a double-blind, placebo-controlled study. Approval of the Ethics Committee was obtained. A total of 15 healthy volunteers (8 men and 7 women) participated in this trial. The mean age was 25 years (range 21 - 30 years). Skin-types of the patients varied from type I to type III according to Fitzpatrick's classification.[8]

An area of 8 x 20 cm on the ventral upper part of one leg, was treated twice daily with ATRA (tretinoin cream 0,05%), which is a frequently used clinical concentration, for 10 days. An area of the same size on the upper part of the other leg was treated with the vehicle of tretinoin cream (per 100 gram: cremor cetomacrogolis 88 gram, alcohol ketonatus 12 gram and butylhydroxytolueen 40 mg) also twice daily and for 10 days. The application of both creams on the legs was selected at random and was applied in a double-blind manner. From the beginning of the application, until 4 days after the last application of the creams, the volunteers were instructed to avoid sunlight exposure and not to wash the applied skin for at least one hour after each application.

On day 8 of treatment, irradiation of the skin was performed in 12 evaluable subjects to determine the minimal erythema dose (MED) with 3 series of 6 increasing intensities UVB-light related to the skin-type of the subjects: one series on ATRA-treated skin, one series on vehicle-treated skin and one series on non-treated skin on the lateral side of the upper part of one leg. Each dose was given to a piece of skin of 4 $cm^2$. For UVB-irradiation, broad-spectrum UV21 lamps (Waldmann UV7001K cabin, 285-350 nm) were used.

Before irradiation on day 8, on day 9 and on day 10 the skin was examined for erythema by visual scoring. The MED was defined as the lowest UVB dose that caused a distinct erythema with sharp margins over the irradiated area 24, respectively 48 hours, after irradiation. For erythema the following scale was used: 0 = no erythema, 1 = weak erythema, 2 = moderate erythema and 3 = strong erythema. Visual scoring occurred under similar conditions of light and temperature. In **Figure 1** a schematic view of the study procedures is depicted.

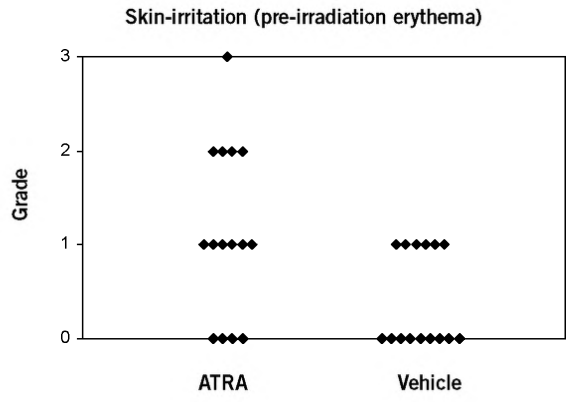


**Figure 2:**

Pre-irradiation erythema scores for test areas treated with ATRA and its vehicle for the 15 individuals.

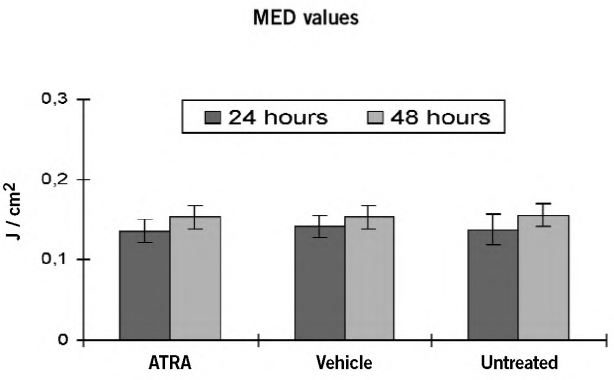


**Figure 3:**

Minimal erythema doses (mean ± SEM) for UVB-light at test areas treated with ATRA, vehicle and untreated skin 24 and 48 hours after irradiation.

## Results and discussion

Previous studies showed that erythema induced by topically applied retinoids is frequently seen during the first weeks of treatment and usually subsides with continued treatment. This erythematous reaction is clinically similar to a mild irritant dermatitis[9] and therefore it is also designated as *"retinoid dermatitis"*.[10] In this study the Wilcoxon matched-pairs signed-ranks test showed a significant difference ($p < 0.01$) between ATRA and its vehicle with respect to the induction of erythema (skin-irritation) before UVB-irradiation **(Figure 2)**. Thus, this study reconfirms the irritative effect of ATRA following twice daily application during 7 days.

Dermatologists tend to avoid ATRA applications in conjunction with UV exposure in order to avoid additive irritation. Some studies investigated the relationship between pre-treatment with ATRA and UVB-irradiation, regarding this expected additive irritation or intensity of erythema.[11,12] However, no data are known about the question whether ATRA can influence the value of the minimal erythema dose for UVB-light itself.

The present study showed no significant difference ($p > 0.05$) as to MED values both 24 and 48 hours after UVB-irradiation between ATRA-treated skin, vehicle-treated skin and untreated skin **(Figure 3)** using the ANOVA test. When looked at the relationship between skin-type and MED value, a significant higher MED value was found in skin-type 3 versus skin-type 1 and 2 ($p < 0.01$).

As both ATRA in itself can induce erythema of the skin, it might be difficult to grade post UVB-irradiation erythema in retinoid treated areas. However, a marked difference in erythema pattern between ATRA-induced erythema and UVB-induced erythema was found. UVB-induced erythema showed a diffuse erythema, while ATRA-induced erythema showed a marked erythema restricted to the perifollicular areas. This perifollicular pattern may be related to the actions of ATRA on the pilosebacious duct and can be seen in other irritants as well, e.g. glycolic acid.[13] These two distinct patterns of erythema permitted to score both parameters independently and separately.

The present study shows that topical treatment with ATRA does not influence the response to a single exposure to UVB-light and does not change the minimal erythema dose.

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# 4.2

# UVB-irradiation in oral bexarotene-treated uninvolved psoriatic skin


## Abstract

*Photo(chemo)therapy and oral retinoid therapy for psoriasis or cutaneous T-cell lymphoma (CTCL) are frequently combined to obtain an enhanced therapeutic effect with lower safety risks. Bexarotene, a new RXR-selective retinoid (rexinoid), has been developed for the treatment of CTCL and has recently been investigated in the treatment of psoriasis. In the present study the UV-modulating properties of bexarotene were evaluated by assessment of the minimal erythema dose (MED) for UVB-light. In 11 patients participating in a phase II study of oral bexarotene 0.5 mg/kg/day (7 patients) or 3.0 mg/kg/day (4 patients) for plaque-type psoriasis, MED-tests were performed on uninvolved psoriatic skin on the lower back of the subjects before and after 12 weeks treatment. Clinical scores of erythema and determination of the MED 24 hours after irradiation did not show statistically significant changes between the exposed areas before and after bexarotene treatment or between the two doses tested. No photosensitising reactions were observed. This study demonstrates that a single exposure to UVB-irradiation is well-tolerated in patients treated with bexarotene 0.5 – 3.0 mg/kg/day and suggests it is not necessary to take precautions with respect to short term effects of sun exposure during bexarotene treatment. Further study of bexarotene-photo-(chemo)therapy in CTCL and psoriasis is warranted.*

## Introduction

Bexarotene (Targretin®, LGD1069) is a novel retinoid that selectively binds and activates the RXR receptors.[1] RXR signalling is fundamentally different from retinoid signalling via the RAR receptors.[2] RXR retinoids can form heterodimers with several other nuclear receptors (e.g., RAR, PPAR, VDR, TR) and these heterodimers have DNA binding domains that act different from the RAR binding domains and different genes may be activated. RAR activity can be seen at high doses of bexarotene around 6 mg/kg/day.

Bexarotene was approved for the treatment of refractory cutaneous T-cell lymphoma.[3] In plaque-type psoriasis we have demonstrated some efficacy[4]. In patients with cutaneous T-cell lymphoma and psoriasis retinoid treatment is often combined with UV-irradiation and may reduce the cumulative UVB or UVA dose necessary to achieve disease clearance. The combination treatment can reduce the short-term side effects of retinoids and the longer-term carcinogenic and skin-ageing effect of phototherapy.[5-8] UV absorption of bexarotene begins at 310 nm, slowly rising to 280 nm, with a distinct peak at 202 nm.

Despite the benefits of combination therapy, some studies suggest that retinoids might induce photosensitivity. Ferguson *et al* showed a phototoxic potential of acitretin.[9] On the other hand Diffey *et al* suggested that photosensitivity due to isotretinoin did not occur.[10] Retinoids may thin the stratum corneum of the skin and lead to an enhanced cutaneous reaction to UV-light. In anticipation of a possible highly effective combination of bexarotene in combination with phototherapy, we evaluated the potential of bexarotene to modulate UV-induced erythema by measuring MED before and after bexarotene treatment.

**Table I:**
UVB-irradiation series according to skin-type (Fitzpatrick).

| Skin-type | UVB-irradiation series J/cm$^2$ | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **1** | 0.02 | 0.04 | 0.06 | 0.08 | 0.10 | 0.12 | 0.14 | 0.16 | 0.18 |
| **2** | 0.03 | 0.06 | 0.09 | 0.12 | 0.15 | 0.18 | 0.21 | 0.24 | 0.27 |
| **3** | 0.05 | 0.10 | 0.15 | 0.20 | 0.25 | 0.30 | 0.35 | 0.40 | 0.45 |
| **4** | 0.07 | 0.14 | 0.21 | 0.28 | 0.35 | 0.42 | 0.49 | 0.56 | 0.63 |

## Materials and methods

The evaluation of the MED was performed on a subset of patients in a phase II dose-response study to investigate bexarotene capsules as a monotherapy in the treatment of adult patients with moderate to severe plaque-type psoriasis. In this evaluation, the influence of bexarotene on the minimal erythema dose (MED) for broad-spectrum UVB-light and the intensity of erythema after irradiation in non-lesional psoriatic skin were investigated. Ethics Committee approval for this trial was obtained and all patients gave informed consent for the MED evaluation prior to study enrollment.

Patients of two sequential dose-defined treatment panels: 0.5 mg/kg/day, the lowest dose and 3.0 mg/kg/day, the highest dose were evaluated. The treatment period was 12 weeks. The following washout periods were defined: systemic retinoids: 3 months (etretinate: 1 year); PUVA, UVB, methotrexate or cyclosporin: 4 weeks; emollients (on the test sites) and topical anti-psoriatic therapies (on lesional psoriatic skin): 1 week. Patients had to avoid prolonged exposure to the sun or UV-light during the study.

Irradiation tests were performed on the day before treatment and after 12 weeks of treatment with bexarotene. Bexarotene treatment was initiated once the baseline MED had been determinded. Bexarotene capsules were taken at dinner, as this is the normal intake schedule for systemic retinoids. Post-treatment irradiation tests where performed 18 hours after the final bexarotene intake (during midday), to approach the timepoint when exposure to sunlight occurs normally. Irradiation tests were performed as follows: 2.5 cm diameter circles of uninvolved skin were irradiated using broad-spectrum UV21 lamps (Waldmann UV7001K cabin, 285-350 nm). This was carried out on the lower back / upper buttocks on non-lesional psoriatic skin by using a series of 9 ascending intensities of UVB-light adjusted for the skin-type of the patient **(Table I)**.

Scoring occurred as follows: twenty-four hours after each UVB-irradiation the intensity of the erythema for each irradiated area was measured by visual scoring using a 5-point scale: 0 = no erythema, 1 = weak erythema, 2 = moderate erythema, 3 = dark erythema and 4 = very dark erythema. The total erythema score before and after treatment was assessed as the sum-score of all individual irradiated areas. The MED was defined as the lowest UVB dose that caused a distinct border to the area of erythema 24 hours after irradiation (erythema grade 1). Visual scoring was performed under standardized conditions of light and temperature.

A paired T-test was used to analyse the differences in the minimal erythema doses and the intensities of erythema between the baseline and after 12 weeks of treatment.

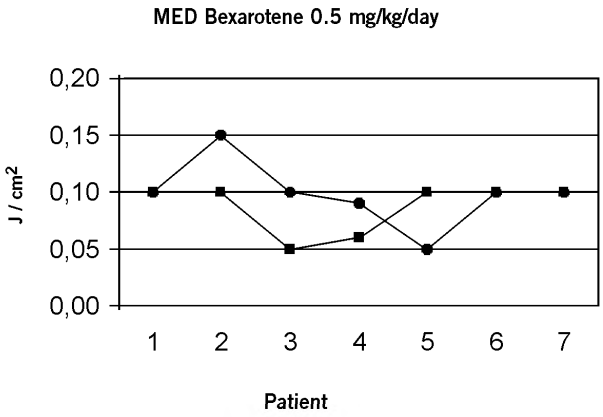


**Figure 1:**

MED values for broadspectrum UVB-light before and after 12 weeks treatment in the 0.5 mg/kg/day bexarotene panel.

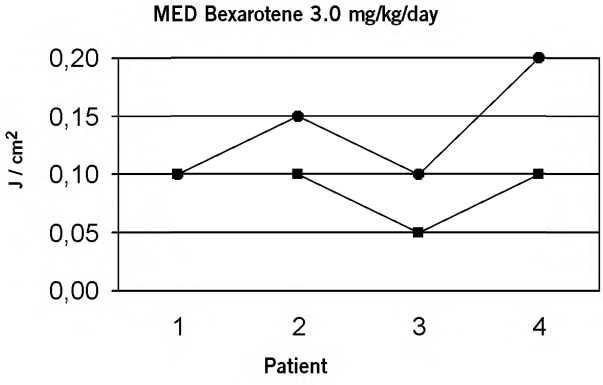


**Figure 2:**

MED values for broadspectrum UVB-light before and after 12 weeks treatment in the 3.0 mg/kg/day bexarotene panel.

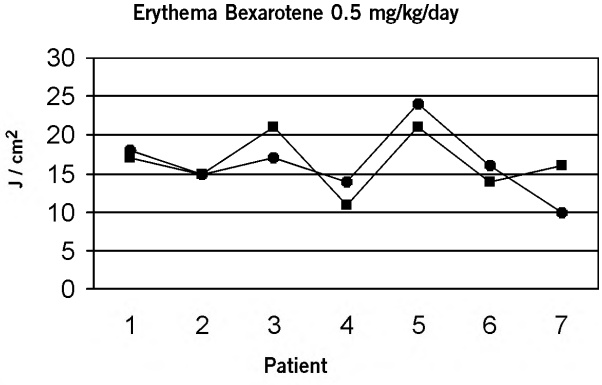


**Figure 3:**

Erythema values for broadspectrum UVB-light before and after 12 weeks treatment in the 0.5 mg/kg/day bexarotene panel.

## Results

A total number of 11 male-patients with moderate to severe plaque psoriasis participated in the study. The mean age was 42 years (range 25 – 65 years). Seven patients received 0.5 mg/kg/day bexarotene and 4 patients received 3.0 mg/kg/day bexarotene. Their skin-types were varying from type II to type III according to Fitzpatrick's classification.[11]

The irradiation was well tolerated in all patients, although, 4 patients reported some itching 24 hours after irradiation in particular within the areas that received the highest intensities UVB-light. No significant differences in the incidence of itching were experienced between the UVB-irradiated areas before and after bexarotene treatment or between the two doses tested.

**Figure 1** shows the MED before and after treatment with 0.5 mg bexarotene for all individual patients. In 3 patients the MED was slightly lower after treatment, in 1 patient it was higher and in the remaining 3 patients there were no differences in MED between baseline and 12 weeks of treatment with bexarotene. The mean MED before treatment was 0.10 ± 0.01 J/cm$^2$ (mean ± SEM); after treatment it was 0.09 ± 0.01 J/cm$^2$ (mean ± SEM).

**Figure 2** shows the MED before and after treatment of 3.0 mg bexarotene for all individual patients. In all but one patient the MED was lower after treatment. Before treatment the mean MED was 0.14 ± 0.02 J/cm$^2$ (mean ± SEM). The mean MED after 12 weeks treatment with bexarotene was 0.09 ± 0.01 J/cm$^2$ (mean ± SEM). However, there was no statistical difference in the limited sample sizes between baseline MED's and MED's after 12 weeks of treatment with bexarotene. No significant dose-response effect could be shown.

Following 12 weeks treatment with bexarotene, 4 out of 7 patients of the 0.5 mg panel had a decrease in erythema scores, 2 patients had an increase in erythema and 1 patient had no differences in erythema between baseline and week 12 **(Figure 3)**. The mean erythema score before treatment was 16.3 ± 1.6 (mean ± SEM) for these 7 patients and after treatment it was 16.4 ± 1.4 (mean ± SEM).

At the 3.0 mg/kg dose, all 4 patients showed an increase in erythema scores following bexarotene treatment **(Figure 4)**. The mean erythema score in this group before treatment was 12.3 ± 1.7 (mean ± SEM) and after treatment it was 17.8 ± 1.9 (mean ± SEM). Statistical analysis, however, did not show any significant differences in erythema scores between baseline and week 12 and a dose-response relationship was not found either.

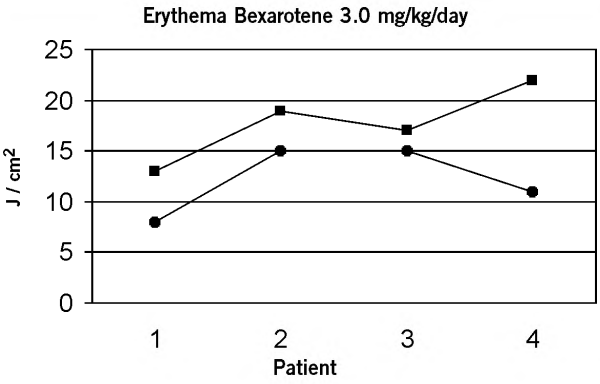


**Figure 4:**

Erythema values for broadspectrum UVB-light before and after 12 weeks treatment in the 3.0 mg/kg/day bexarotene panel.

## Discussion

The present study provides evidence that bexarotene in the dose-range 0.5 – 3.0 mg/kg/day is not interfering with the erythematous response to a single irradiation of UVB.

Some clinical data suggested that retinoids induce cutaneous photosensitivity,[12,13] although other reports are contradictory and do not demonstrate a photosensitization.[10] Phototesting a group of subjects taking oral isotretinoin proved negative.[10] Furthermore, retinoid effects like erythema of the skin and dryness and increased epidermal fragility may bias or enhance the clinical picture of retinoid induced photosensitivity.

In a previous study, where we applied all-trans retinoic acid to the skin of healthy volunteers, we found no significant effect of all-trans retinoic acid on the minimal erythema dose for broad-spectrum UVB light.[14] With respect to the intensity of erythema, Juhlin and Shroot found a significant decrease 12 hours after 0.2 J of UVB-irradiation in an area previously treated with 0.01 % ATRA under occlusion for 4 days.[15] However, twenty-four and 48 hours after UVB-irradiation no significant modulation of erythema could be observed. Meunier, Voorhees and Cooper concluded that after UVB-irradiation with 1 MED no significant differences in erythema were found between areas tested with ATRA 0.1 %, vehicle and untreated skin.[16]

Although a tendency for an increase in erythema ($p = 0.066$) and a decrease in MED ($p = 0.091$) in the bexarotene 3.0 mg/kg/day dose panel was observed in 4 patients, this decrease was not statistically significant. No dose response relationship between MED and the dose of bexarotene was found and no abnormal responses to UVB-irradiation were seen.

Therefore, the present study suggests that patients treated with bexarotene 0.5 – 3.0 mg/kg/day do not need to take precautions with respect to short term effects of sun exposure. Further studies are warranted to investigate the efficacy of bexarotene-photo-(chemo)therapy in cutaneous T-cell lymphoma and psoriasis, especially at higher dose levels of bexarotene.

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# 5

# SUMMARY AND GENERAL DISCUSSION

*The effect of retinoids on clinical and immunohistochemical parameters for proliferation, differentiation, and inflammation, is the central theme of this thesis. In Paragraph 1.21 three aims were defined.*

*The first aim focused on clinical and immunohistochemical effects of the RXR-selective retinoid bexarotene in the treatment of plaque-type psoriasis. In Chapter 2 this issue has been discussed in detail. In Paragraph 5.1 a brief summary of the primary outcome is given, including suggestions for future studies.*

*The second aim was to investigate the mode of action of RAR-selective retinoid treatment in actinic keratoses in immunocompetent and immunosuppressed patients. In Chapter 3 the effects of topical and systemic retinoids on these disorders were discussed. In Paragraph 5.2 the results are summarized and will be discussed further.*

*The third aim dealt with the UV-modulating properties of RAR-selective retinoids and RXR-selective retinoids after a single exposure to broadband UVB. These issues were discussed in detail in Chapter 4; in Paragraph 5.3 the major conclusions have been summarized.*

# 5.1

# RXR-selective retinoids in the treatment of plaque-type psoriasis

## Clinical efficacy, adverse events and immunohistochemical effects of oral bexarotene in plaque-type psoriasis

It is known from clinical and immunohistochemical studies that psoriasis is a retinoid sensitive disease and, therefore, retinoids are a major treatment strategy for this disorder. So far, in contrast to RAR-selective retinoids, RXR-selective retinoids have not been investigated in the treatment of psoriasis. As mentioned in **Chapter 1**, the total concentration of RXR in human skin is 5 times higher than the total concentration of RAR and is decreased in psoriatic lesions. RXR-signaling is of major importance and occurs in concerted action with vitamin D3 signaling. These observations, in combination with reported efficacy of RXR-selective retinoids in cutaneous T-cell lymphoma and in animal studies, justified an investigation on the efficacy and side effects of the RXR-selective retinoid bexarotene in plaque-type psoriasis.

In **Chapter 2** clinical efficacy of oral bexarotene has been demonstrated in doses ranging from 0.5 to 3 mg/kg/day as shown by significant improvement in a modified psoriasis area and severity index (mPASI), plaque elevation (PEL), and physicians global assessment (PGA) during the 12 to 24 weeks treatment period. The onset of response varied for these parameters between 2 to 8 weeks and the clinical improvement was maintained for another 8 to 16 weeks. A significant decrease in the psoriasis disability index, visualizing the subjective improvement experienced by the patients themselves, strengthened these data. However, no significant dose-response effect for these clinical parameters could be found in this group of patients, which might be explained by inter-individual variation with respect to the bioavailability of oral bexarotene, as is the case with acitretin, the retinoid inducing RAR transactivation. Therefore, the dose has to be individualized in order to reach optimal efficacy. It is possible that studying a larger group of subjects, adjusting the dose to blood levels, is necessary to reveal a potential dose-response effect for these parameters.

On the other hand, it is also possible that the optimal dose of bexarotene for plaque-type psoriasis, is higher than the doses that were evaluated in this study. Typical retinoid-associated adverse events like pruritus and cheilitis were found in only 14 %, respectively 10 % of the patients. Both percentages are much lower than the percentages seen in treatment with oral RAR-selective retinoids, suggesting that these patients might have received a dose of bexarotene that is below the optimum, or that RXR-activation does not coincide with these adverse events to the same level as RAR-activation does.

No serious adverse events were seen and bexarotene was well tolerated overall. On the other hand, 56% of the patients had an increase in triglycerides during the study and had to be treated with atorvastatin. Furthermore, 92% of the patients in the 3 mg dose panel demonstrated a decrease in FT4 values, which is an indication that bexarotene-induced hypothyroidism, due to heterodimer formation with the thyroid receptors, may be a serious limitation for oral application of this drug. The fact that a decrease in FT4 values was seen especially in the highest dose panel and in only 15% of the subjects in the lowest dose group, indicates that bexarotene-induced hypothyroidism is a dose depended phenomenon, which was already shown in previous studies for other indications. With respect to the other adverse events no significant differences between the 4 different dose panels were observed.

When these data are combined with the data obtained from the corresponding immunohistochemical study of bexarotene in plaque-type psoriasis that focused on epidermal proliferation, differentiation, and dermal inflammation, the clinical efficacy of bexarotene for this disease is strengthened by the immunohistochemical findings. Significant reductions in Ki-67, keratin 16, transglutaminase, dermal CD4, epidermal CD8 and global inflammation scores were seen after bexarotene treatment, in combination with a significant increase in keratin 10. A borderline significant dose-response effect for Ki-67 was observed. However, the other immunohistochemical markers did not show a dose response effect. No alterations in apoptosis associated p53 expression were observed, suggesting bexarotene does not improve psoriasis via an increase in apoptosis of the keratinocytes.

Interestingly, no induction of keratin 13 and keratin 19 was seen in our samples. The present study suggests that RXR-activation does not induce these keratins which are induced by RAR-activation. On the other hand it cannot be ruled out that higher doses of bexarotene than used in our study are necessary to express induction of keratin 13 and keratin 19. However, acitretin, at doses sufficient to demonstrate clinical and immunohistochemical efficacy in psoriasis already induces these keratins.

### Future studies with RXR-selective retinoid treatment in psoriasis and other hyperkeratotic disorders

In order to further clarify the potential of oral bexarotene as a new treatment for psoriasis and to reveal a potential dose-response effect, clinical and immunohistochemical studies are indicated that focus on higher doses of bexarotene in a larger group of patients and by using a placebo-controlled approach. A study reconciling blood levels of bexarotene might be indicated. As mentioned before, hypertriglyceridaemia and hypothyroidism may be a serious limitation of oral bexarotene treatment.

In order to circumvent the systemic side effects, topical treatment with bexarotene represents a promising treatment of psoriasis. Future trials investigating the role of topical bexarotene or other RXR-selective retinoids for psoriasis are warranted. In a later phase, if such studies prove to be positive, one might think of a combination of either oral or topical RXR-selective retinoids and other commonly used treatments for psoriasis, as

described in **Chapter 1**. Especially the combination of RXR-selective retinoids and calcipotriol or other vitamin D derivatives are likely to have a synergistic effect as these ligand-activated receptors form heterodimers.

In addition, RXR-selective retinoids may be of importance for a broad spectrum of retinoid sensitive diseases, such as ichthyosis vulgaris, acne, and Darier's disease. It is worthwhile to investigate the potential of topical or systemic bexarotene in these skin conditions.

Finally, an intriguing field is the development of RXR subtype-selective retinoids. The RXR family comprises three receptor subtypes: α, β, and γ. Each receptor subtype has a different chemical structure that controls unique as well as overlapping target genes. Therefore, efficacy and side-effects may vary among these RXR subtypes. By creating RXR subtype-selective retinoids, it may well be possible to bypass or minimize side-effects like hypothyroidism and hyperlipidaemia. Especially RXRα-selective retinoids may represent an innovation in treatment of skin disorders, as RXRα is abundantly present in adult human skin.

# 5.2

# RAR-selective retinoids in the treatment of (pre)malignant skin disorders

## The relevance of topical all-trans retinoic acid and calcipotriol in actinic keratoses in immunocompetent and immunosuppressed patients

Topical retinoids have been reported to be beneficial for photodamaged skin; clinical appearance and histological features proved to be improved by topical retinoids. With respect to actinic keratoses, resulting from long-term exposure to UV-light, less information is available on the effect of topical retinoids. Most of the studies that have been performed on these lesions focussed on clinical parameters only. Immunohistochemical data on the effects of topical retinoids on actinic keratoses, regarding parameters for epidermal proliferation, differentiation, dermal inflammation and apoptosis, were not available so far. Preferably, the criteria for improvement of actinic keratoses have to comprise both the clinical appearance and relevant histopathological and immunohistochemical features of the preneoplastic process, as clinical improvement without improvement of dysplastic and proliferative characteristics may mask these premalignant keratoses and may mislead both patient and physician, who are satisfied with the cosmetic improvement. Furthermore, retinoids frequently cause skin irritation, which might reduce patient compliance and may restrict its applicability in the treatment of these lesions.

In **Chapter 3** we assessed clinical and immunohistochemical parameters in topical all-trans retinoic acid-treated actinic keratoses, both in (immunocompromized) renal transplant recipients and in non-immunocompromized patients. In non-immunocompromized patients we concluded that short-term (6 weeks) treatment with 0.05% all-trans retinoic acid (ATRA) once daily in a cream base did not reduce the number of actinic keratoses located on the forearms and hands, while causing skin irritation in some patients. A small but statistically significant increase in differentiation parameter K10 was seen ($p = 0.049$) after ATRA treatment and a significantly higher staining for K13, when compared to vehicle-treated actinic keratoses ($p = 0.043$). No alteration was found in epidermal proliferation (Ki-67), apoptosis (p53), hyperproliferation associated keratinization (K16), terminal differentiation (transglutaminase), epidermal thickness or dermal inflammation.

In a group of renal transplant recipients, who were treated with topical ATRA 0.02% twice daily for actinic keratoses located on the forearms and hands during 6 weeks, after treatment no significant improvement in the same clinical and immunohistochemical parameters as mentioned above was found, especially no significant induction of K10 and/or K13. In this group of renal transplant patients we also evaluated the

efficacy of topical calcipotriol 0.05 mg/gram twice daily as monotherapy, or once daily in the morning in combination with topical ATRA 0.02 % in the evening. Again, no significant change in clinical or immunohistochemical parameters was seen between baseline and post-treatment lesions for both calcipotriol monotherapy and the combination therapy.

Combining these studies, at baseline no significant differences were observed between non-immunocompromized patients and renal transplant recipients with respect to the investigated parameters. With respect to efficacy, a change towards normal keratinization (K10) as well as induction of retinoid-associated keratinization (K13) was observed after ATRA-treatment in actinic keratoses of immunocompetent patients. We could not demonstrate a significant effect on epidermal proliferation (Ki-67) and apoptosis (p53), or on the number of lesions, after treatment with commonly used doses of topical ATRA. Therefore, the reported beneficial clinical effects of topical ATRA in the treatment of actinic keratoses in several studies may be explained by alterations in keratinization rather than by alterations in epidermal proliferation, inflammation, and/or apoptosis. Topical calcipotriol as monotherapy, or in combination with topical ATRA, did not show any significant improvement in the investigated parameters in these actinic keratoses. This is in contrast to other hyperkeratotic disorders, such as psoriasis, where calcipotriol has been reported to improve epidermal proliferation and differentiation even in a 6-week treatment period. We cannot rule out that long-term treatment with these agents, or application of higher doses, may have some effect on these parameters, especially when investigated in a large number of patients. However, as significant irritation has been observed in some patients using ATRA, we question the relevance of higher doses and longer treatment durations with ATRA with respect to compliance to treatment. Therefore, we do not recommend these applications for actinic keratoses, but favor other treatments, such as cryotherapy and topical 5-fluorouracil (Efudix®).

### The relevance of systemic acitretin in the treatment of actinic keratoses and common warts in renal transplant recipients

It has been reported that systemic retinoids, such as acitretin and isotretinoin, are chemopreventive treatment modalities that clinically reduce the number of actinic keratoses and may inhibit the development of non-melanoma skin cancers. Especially in renal transplant recipients, systemic retinoids may be relevant due to the high number of actinic keratoses generally present in these patients and due to the high incidence of malignancies in this population. However, little information is available on histological and immunohistochemical effects of systemic retinoid treatment for actinic keratoses. As explained before, clinical improvement without improvement in dysplastic and proliferative characteristics may mask these premalignant keratoses and may mislead both patient and physician. Therefore, it is important to assess clinical as well as histological and immunohistochemical parameters in patients with actinic keratoses that were treated with systemic retinoids.

In **Chapter 3** the results are described of 26 renal transplant recipients that were treated with oral acitretin in a randomized setting. All patients started with 0.4 mg/kg/day during the first 12 weeks, an effective dose by data obtained from previous studies. After the initial 12 weeks treatment half of the patients were planned to continue with a dose of 0.2 mg/kg/day, whereas the remaining half continued with the initial dose of 0.4 mg/kg/day. However, in contrast to other studies, in most patients we had to reduce this dose already within 12 weeks of treatment due to mucocutaneous side effects. We concluded that 0.25-0.30 mg/kg/day is likely to be a better starting dose that is well tolerated. Acitretin was safely used in this group of patients, without the occurrence of severe adverse events related to the drug. No deterioration of renal graft function was seen. Clinical parameters revealed a significant improvement in thickness of the actinic keratoses ($p < 0.01$) and in the number of lesions that featured the typical erythemato-squamous hallmarks ($p < 0.0001$) in both groups. Although not scored, warts also seemed to improve during the study. No significant decrease in the number of non-melanoma skin cancers was found during the study period of 12 months, compared to the year before treatment. Visual analogue scores showed that the patients in general experienced benefit from acitretin treatment already in the first 2-3 months with respect to roughness ($p = 0.002$) and general skin contentment ($p = 0.001$). After the first months of treatment no additional benefit was noticed for the remaining study period. No differences with respect to clinical efficacy parameters and side effects were seen between the two dose groups, suggesting a low maintenance dose of 0.2 mg/kg/day after 3 months initiation with higher doses is adequate for maintaining clinical improvement. Treatment was safe.

The corresponding immunohistochemical study in actinic keratoses, as described in paragraph 3.2, which was performed in 33 renal transplant recipients, demonstrated that the clinical improvement of the actinic keratoses during acitretin treatment originated from a reduction in stratum corneum thickness, probably due to changes in the keratinization process. Significant improvement in K10 ($p = 0.02$) and induction of K13 ($p < 0.01$) and K19 ($p = 0.05$) indicate that a direct effect on keratinization is the underlying mechanism. However, no significant reduction or alteration in epidermal proliferation (Ki-67), apoptosis (p53), hyperproliferation associated keratinization (K16), terminal differentiation (transglutaminase), and dermal inflammation was seen.

Data obtained from the cessation study of systemic acitretin in the treatment of actinic keratoses (paragraph 3.3) that was performed in 9 renal transplant recipients, demonstrated no significant increase in the number of actinic keratoses or squamous cell carcinomas 3 months after acitretin treatment had been stopped. This in contrast to the number of warts that was significantly increased after this period ($p = 0.02$). Induration of actinic keratoses increased significantly only in the first 6 weeks after acitretin withdrawal ($p = 0.004$). A VAS score, representing patient contentment, reduced significantly 12 weeks after cessation ($p = 0.004$). With respect to immunohistochemical parameters, significant reduction of K13 expression was seen 6 weeks after cessation of acitretin, without alteration in expression of MIB-1, p53 or $p16^{INK4A}$. Combining these data, acitretin withdrawal in RTRs leads to clinical deterioration within 3 months, without significant increase in skin (pre)cancer, and from immunohistochemical point of view, it leads

to a significant reduction of K13 expression, without alteration in expression of cell-cycle-associated markers.

In conclusion, systemic treatment with acitretin improves the aspect of actinic keratoses and warts in RTRs by alterations in keratinization, comprising an improvement towards normal differentiation as well as induction of retinoid-associated differentiation. Acitretin does not influence epidermal proliferation, apoptosis, and dermal inflammation in our studies. Clinically, a reduction in the number of typical actinic keratoses, featuring erythema, desquamation, and thickened horny scales, is seen during acitretin treatment, although erythematous maculae often persist. These maculae still represent features of actinic keratoses, such as dysplasia and high proliferation levels, assessed by histological and immunohistochemical investigations. Thus, actinic keratoses are clinically masked by acitretin. The reduction of hyperkeratosis, in theory, might also mask squamous cell carcinomas and other non-melanoma skin cancers. However, it is our impression that these lesions are still easily detected clinically, as they rapidly progress despite retinoid treatment. But in slower progressing SCCs and especially in actinic keratoses with micro-invasion, a masking effect may indeed exist. The masking effect could also explain the sudden increase in the number of (typical) actinic keratoses and squamous cell carcinomas that has been reported after cessation of acitretin treatment in some other studies.

Therefore, long-term treatment seems necessary, with the lowest effective dose possible.

## The relevance of retinoid-associated keratin expression in (pre)malignant skin disorders

K13 and K19 are not expressed in adult human epidermis, but can be found in embryonic epidermis and are induced by retinoids. From **Chapter 3** it can be learned that non-retinoid treated actinic keratoses may also express K13 and/or K19. In the acitretin study on actinic keratoses in RTRs, expression of K13 and K19 in non-retinoid treated actinic keratoses was seen in 30%, respectively 15%, of the biopsies. In this study only patients were included with at least 10 AKs and one or more SCCs, or more than 20 AKs without an SCC in their medical history. In the study in RTRs where actinic keratoses were treated with topical ATRA and/or calcipotriol, no K13 expression was observed in the non-retinoid treated actinic keratoses. In that study patients with a relatively low number of actinic keratoses and squamous cell carcinomas were included. K13 expression, unlike K19 expression was also observed in non-retinoid treated common warts and with a significant higher incidence in renal transplant recipients (82%) than in non-immunocompromized patients (14%), as described in paragraph 3.6. Data from the cessation study of acitretin in actinic keratoses showed a significant reduction in K13 expression 6 weeks after acitretin has been stopped ($p = 0.02$). However, in one patient at this time point and in another patient 12 weeks after cessation a focal expression pattern of K13 was still visible.

Most patients with K13 and/or K19 positive actinic keratoses or common warts at baseline have not used topical or systemic retinoids prior to the study, however, a minority of the patients were on retinoid treatment and needed a washout period (4 weeks for topical retinoids; 3 months for systemic retinoinds) in order to participate. Therefore, there must be another explanation for the expression of these keratins in baseline biopsies than a retinoid effect. Interestingly, expression of these keratins in baseline (non-retinoid treated) actinic keratoses and common warts featured low expression levels with single cell positivity, whereas expression of these keratins after retinoid treatment showed a more extensive focal or band-like expression pattern.

Other (animal) studies pointed out that expression of low-molecular weight keratins, such as K13 and K19, could be found in skin malignancies as a sign of a more dedifferentiated state. Therefore, expression of K13 and/or K19 in a non-retinoid treated premalignant lesion is possibly a sign of a more dedifferentiated lesion and thus a lesion with an increased risk for malignant progression. Therefore, we looked at other parameters that have been positively identified to be associated with high-risk actinic keratoses. Indeed, we found significant associations between K13 and/or K19 expression and several high-risk parameters, including Ki-67, p53, KIN, and the number of recently developed squamous cell carcinomas. The significant higher expression of K13 in common warts from renal transplant recipients compared to common warts in the normal population, fits to this hypothesis, as certain types of HPV are postulated to be associated with malignant transformation, especially in renal transplant recipients.

Therefore, it can be concluded that K13 and K19 are two new parameters that can be relevant in the identification of high-risk actinic keratoses. It is likely that K13 and K19 expression are epiphenomena related to two distinct pathways: malignant transformation and retinoid-associated keratinization. The pattern of K13 and/or K19 expression may be helpful to differentiate between these two pathways.

Future studies may focus on the behavior of actinic keratoses and squamous cell carcinomas during retinoid treatment. To what extent do retinoids in the clinical management of the patients indeed mask these lesions? It is also relevant to investigate the efficacy of RXR-selective retinoids, such as bexarotene, in the treatment of actinic keratoses. Furthermore, even though topical calcipotriol was not effective in the treatment of actinic keratoses, the combination of a vitamin D3 derivative and an RXR-selective retinoid may be worthwhile to investigate, because of the heterodimer formation of their nuclear receptors.

# 5.3

# Modulation of UV-induced inflammation by retinoids

## Modulation of UVB-induced erythema and the minimal erythema dose (MED) for UVB-light by topical all-trans retinoic acid

Despite the fact that the role of RAR-selective retinoids in combination with photo-(chemo)therapy has been extensively studied in many dermatological conditions and that these treatment regimens are commonly used nowadays, up to now little information is available on the UV-modulating capacities of retinoids. It is known that retinoids influence inflammation and may induce a phenomenon called 'retinoid dermatitis', that comprises features like increased desquamation and erythema of the skin. Furthermore, retinoid treatment may lead to a decrease in epidermal thickness, and thus, may increase efficacy of photo(chemo)therapy in especially hyperkeratotic dermatological conditions by increasing penetration of UV-light through the skin.

In this thesis we found a significant increase in erythema after one week treatment of normal skin with twice daily topical applications of all-trans retinoic acid 0.05%, as compared to its vehicle. A typical perifollicular erythema pattern was seen, which can be interpreted as retinoid-induced dermatitis. After UVB-irradiation no statistically significant differences could be found in MED between ATRA pre-treated skin, vehicle pre-treated skin and untreated skin both 24 and 48 hours after irradiation. With respect to the intensity of erythema following these three different treatment regimens, no significant differences were found either. Thus, apart from an increase in (perifollicular) erythema due to retinoid treatment, no evidence exists that all-trans retinoic acid, after topical application, enhances erythema after a single exposure to UVB-light.

## Modulation of UVB-induced erythema and the minimal erythema dose (MED) for UVB-light by oral bexarotene

It is clear that RXR-selective retinoids may be an innovation in retinoid treatment for a broad spectrum of dermatological conditions. As RAR-selective retinoids are frequently combined with photo(chemo)therapy in order to potentiate the therapeutic effect of PUVA or UVB therapy, it is interesting to speculate to what extent RXR-selective retinoids also may potentiate photo(chemo)therapy. Therefore, it is important to know whether RXR-selective retinoids modulate UV-induced erythema.

In this thesis we described the effect of the RXR-selective retinoid bexarotene on UVB-induced inflammation by assessment of the MED and the degree of erythema. The

single exposure of a series of 9 increasing intensities of broadspectrum UVB-light on the lower part of the back was well tolerated and no photosensitizing reactions were observed.

After 3 months of treatment with either 0.5 or 3 mg bexarotene per kg/day a tendency for an increase in erythema and a decrease in MED in the bexarotene 3 mg/kg/day dose panel was observed in a total of 4 patients, but these alterations were not statistically significant. No dose response relationship between MED and the dose of bexarotene was found and no abnormal responses to UVB-irradiation were seen. However, we cannot rule out that, especially higher concentrations of bexarotene, studied in a larger number of patients may reveal some alterations of the MED and the degree of erythema. On the other hand, our data suggest that patients treated with bexarotene 0.5 – 3 mg/kg/day do not need to take precautions with respect to short-term effects of sun exposure and suggest that combination therapy of RXR-selective retinoids and UV-therapy is possible.

Further studies are warranted to investigate the efficacy of bexarotene-photo(chemo)-therapy in cutaneous T-cell lymphoma and psoriasis especially at higher dose levels of bexarotene. Apart from the combination of oral treatment with bexarotene with UV-treatment, topical application of bexarotene may represent another attractive option. Future trials may focus on RXR subtype-selective retinoids in combination with photo-(chemo)therapy.

# Samenvatting en conclusies

Het effect van retinoïden op klinische en immunohistochemische parameters voor proliferatie, differentiatie en inflammatie, vormt het centrale thema van dit proefschrift. In paragraaf 1.21 werden 3 doelstellingen geformuleerd.

De eerste doelstelling had betrekking op klinische en immunohistochemische effecten van het RXR-selectieve retinoïd bexaroteen in de behandeling van psoriasis vulgaris. In **hoofdstuk 2** werd hier dieper op in gegaan. In paragraaf 5.1 is een korte samenvatting van deze resultaten beschreven, inclusief suggesties voor toekomstige studies.

Het tweede doel richtte zich op onderzoek naar de onderliggende mechanismen van RAR-selectieve retinoïden in de behandeling van actinische keratosen in immuuncompetente en immuungecompromitteerde patiënten. In **hoofdstuk 3** werden de effecten van topicale en systemische retinoïden op deze aandoeningen uiteengezet. In paragraaf 5.2 worden deze resultaten opgesomd en nader bediscussieerd.

Bij de derde doelstelling stonden de UV-modulerende eigenschappen van RAR en RXR-selectieve retinoïden centraal na een eenmalige blootstelling aan breedspectrum UVB licht. Deze items werden belicht in **hoofdstuk 4**; in paragraaf 5.3 worden de resultaten van deze studies besproken.

# RXR-selectieve retinoïden in de behandeling van psoriasis vulgaris

## Klinische effectiviteit, bijwerkingen en immunohistochemische effecten van systemisch bexaroteen in psoriasis vulgaris

Het is bekend van klinische en immunohistochemische studies dat psoriasis een aandoening is die doorgaans een goede respons laat zien op retinoïden en dat daarom retinoïden een belangrijke plaats innemen in de behandeling van deze ziekte. In tegenstelling tot RAR-selectieve retinoïden zijn RXR-selectieve retinoïden tot op heden nog niet onderzocht in de behandeling van psoriasis vulgaris. Zoals beschreven in **hoofdstuk 1**, heeft de retinoïd X receptor unieke kenmerken en is bovendien de totale concentratie RXR in humane huid 5 maal hoger dan de totale concentratie van RAR, terwijl de expressie in de psoriasis laesie verlaagd is. Verder heeft RXR-activatie een centrale regulerende rol in de epidermis waarbij de interactie met vitamine D3 signaaltransductie relevant is. Deze observaties, in combinatie met de gerapporteerde effectiviteit van RXR-selectieve retinoïden in andere dermatologische aandoeningen en in dierexperimenteel onderzoek, maken het onderzoek naar de effectiviteit en bijwerkingen van het RXR-selectieve retinoïd bexaroteen in psoriasis vulgaris betekenisvol.

In **hoofdstuk 2** hebben we de effectiviteit van systemisch bexaroteen gedemonstreerd in doseringen variërend van 0,5 tot 3 mg/kg/dag gedurende een behandelperiode van 12 tot 24 weken, zoals de significante verbeteringen in de gemodificeerde psoriasis area and severity index (mPASI), plaque elevation (PEL), en physicians global assessment (PGA), laten zien.

Het begin van de respons varieerde voor deze parameters van 2 tot 8 weken en klinische verbetering hield gemiddeld genomen aan gedurende 8 tot 16 weken (einde van de evaluatieperiode). Een significante afname in de psoriasis disability index, welke een maat is voor de subjectieve verbetering van de patiënten zelf, versterkt deze data, hoewel geen significant dosisrespons effect voor deze klinische parameters kon worden aangetoond. Dit laatste kan mogelijk verklaard worden door interindividuele variatie in biologische beschikbaarheid van bexaroteen per os, zoals eveneens gezien wordt bij oraal acitretine, een retinoïd dat RAR-transactivatie stimuleert. Daarom is het mogelijk dat de dosis per patiënt afzonderlijk dient te worden bepaald om optimale effectiviteit te bereiken. Onderzoek in een grotere patiëntenpopulatie met bepaling van bexaroteen bloedspiegels is wellicht noodzakelijk om eventuele dosisrespons effecten aan te kunnen tonen met betrekking tot bovengenoemde parameters.

Aan de andere kant is het ook mogelijk dat de optimale dosis van bexaroteen voor psoriasis vulgaris hoger ligt dan de doseringen welke in deze studie werden geëvalueerd. Typische retinoïdgeassocieerde bijwerkingen, zoals jeuk en schrale lippen, werden gevonden in slechts 14%, respectievelijk 10%, van de patiënten. Beide percentages zijn veel lager dan de percentages welke gezien worden in de behandeling met orale RAR-

selectieve retinoïden. Dit suggereert dat deze patiënten een suboptimale dosering kregen, dan wel dat RXR-activatie niet gepaard gaat met dezelfde mate van mucocutane bijwerkingen als RAR-activatie.

Wanneer gekeken wordt naar bijwerkingen in zijn algemeenheid, werden geen ernstige bijwerkingen gezien en werd bexaroteen over het algemeen goed verdragen. Aan de andere kant liet 56 % van de patiënten een stijging zien van de triglyceriden in het serum gedurende de behandeling, en diende dit (protocollair) behandeld te worden met atorvastatine. Verder had 92 % van de patiënten uit de 3 mg groep een verlaging van het vrije T4 gehalte in het serum, hetgeen er op kan duiden dat bexaroteen geïnduceerde hypothyreoïdie door heterodimeerformatie van de RXR met de schildklierhormoonreceptor, een serieuze beperkende factor kan vormen voor toepassing van dit medicijn in psoriasis. Het feit dat een afname in vrij T4 met name gezien werd in de groep patiënten die de hoogste concentratie bexaroteen ontvingen en slechts in 15 % van de patiënten uit de laagste doseringsgroep, duidt er op dat bexaroteen geïnduceerd hypothyreoïdisme mogelijk een dosisafhankelijk fenomeen is. Studies waarin bexaroteen gebruikt werd voor andere aandoeningen, hebben dit inderdaad al eerder aangetoond. Met betrekking tot de andere bijwerkingen, werden geen significante verschillen gezien tussen de 4 verschillende doseringsgroepen.

Wanneer deze data gecombineerd worden met de resultaten verkregen uit de corresponderende immunohistochemische studie van bexaroteen in psoriasis vulgaris, waarbij epidermale proliferatie, differentiatie en dermale inflammatie centraal stonden, ondersteunt dit de gevonden klinische effectiviteit van bexaroteen voor deze aandoening. Significante reductie in Ki-67, keratine 16, transglutaminase, dermaal CD4, epidermaal CD8 en inflammatie werd gezien na behandeling met bexaroteen, in combinatie met een significante toename in keratine 10. In deze immunohistochemische studie werd een significant dosisrespons effect voor Ki-67 waargenomen. Echter, de overige markers toonden geen dosisrespons relatie. Interindividuele variatie in de biologische beschikbaarheid van oraal bexaroteen zou ook hier een verklaring kunnen vormen. Geen veranderingen in apoptosegeassocieerde expressie van p53 werden gezien, hetgeen suggereert dat het therapeutische effect van bexaroteen in psoriasis vulgaris niet voortvloeit uit een toename van apoptotische keratinocyten.

Een interessant gegeven is het feit dat geen inductie van keratine 13 en keratine 19 werd gezien in de epidermis van onze biopten. De huidige studie suggereert dat RXR activatie niet leidt tot inductie van deze keratinen, in tegenstelling tot RAR activatie. Aan de andere kant kan het niet uitgesloten worden dat hogere doseringen bexaroteen dan werden gebruikt in deze studie nodig zijn voor inductie van keratine 13 en keratine 19. Daar staat echter weer tegenover dat acitretine, in doseringen welke juist hoog genoeg zijn om klinische en immunohistochemische effecten te bewerkstelligen in psoriasis, deze keratinen wel reeds kan induceren.

## Toekomstige onderzoeksbenaderingen met RXR-selectieve retinoïden in de behandeling van psoriasis en andere hyperkeratotische aandoeningen

Om het maximale effect van systemisch bexaroteen voor psoriasis te verhelderen en ter bepaling van een eventueel dosisrespons effect, zijn klinische en immunohistochemische studies geïndiceerd die focussen op hogere concentraties bexaroteen in grotere studiepopulaties middels een gerandomiseerde en placebogecontroleerde opzet. Hierbij is het tevens van belang om de resultaten te relateren aan de feitelijke bloedspiegels van bexaroteen. Zoals reeds eerder genoemd, vormen hypertriglyceridaemie en hypothyreoïdie mogelijk een beperkende factor in de behandeling van psoriasis met bexaroteen.

Ten einde deze systemische bijwerkingen te omzeilen, vormen topicale toepassingen van bexaroteen mogelijk een veelbelovende therapie. Toekomstige studies gericht op topicale applicatie van bexaroteen of andere RXR-selectieve retinoïden zijn daarom relevant. In later fase, wanneer dergelijke studies een positief resultaat laten zien, zou men kunnen denken aan combinatie van systemisch of topicaal bexaroteen met andere beproefde behandelingen voor psoriasis, zoals beschreven in **hoofdstuk 1**. Met name de combinatie van een RXR-selectief retinoïd met calcipotriol of een ander vitamine D derivaat is interessant; deze stoffen kunnen immers heterodimeren vormen en derhalve mogelijk synergistisch werken.

Naast een eventuele toepassing van bexaroteen in psoriasis, zijn RXR-selectieve retinoïden mogelijk ook relevant in de behandeling van een breed scala aan retinoïdgevoelige aandoeningen, zoals ichthyosis vulgaris, acne, en morbus Darier. Het is interessant om de effectiviteit van bexaroteen, systemisch of topicaal, te onderzoeken in deze huidziekten.

Tenslotte vormt de ontwikkeling van RXR subtype selectieve retinoïden een belangrijk onderzoeksterrein. De RXR familie omvat drie receptor subtypen: $\alpha$, $\beta$ en $\gamma$. Elk receptorsubtype heeft een aparte chemische structuur welke unieke als ook overlappende genen controleert. Daarom kunnen de effectiviteit en bijwerkingen variëren tussen deze RXR subtypen. Door de ontwikkeling van RXR subtype selectieve retinoïden is het wellicht mogelijk om bijwerkingen als hypothyreoïdie en hyperlipidaemie te omzeilen of te beperken. Met name RXR$\alpha$-selectieve retinoïden representeren mogelijk een innovatie in de behandeling van huidziekten, aangezien RXR$\alpha$ veelvuldig voorkomt in volwassen humane huid.

# RAR-selectieve retinoïden in de behandeling van (pre)maligne huidaandoeningen

## De relevantie van topicaal all-trans retinoic acid en calcipotriol in actinische keratosen in immuuncompetente en in immuungecompromitteerde patiënten

Diverse klinische en histologische onderzoeken laten zien dat topicale retinoïden een positief effect hebben op door UV-licht beschadigde huid. Met betrekking tot actinische keratosen, lesies die ontstaan door UV-schade, is er echter minder informatie bekend over het effect van topicale retinoïden. De meeste studies welke zijn verricht naar de effecten van topicale retinoïden op actinische keratosen hadden enkel betrekking op klinische parameters. Immunohistochemische gegevens over effecten van topicale retinoïden op actinische keratosen, met betrekking tot parameters voor epidermale proliferatie, differentiatie, dermale inflammatie en apoptose, waren niet voor handen.

Idealiter zouden criteria voor een therapeutisch effect op actinische keratosen zowel klinische als relevante immunohistochemische kenmerken van het preneoplastische proces moeten omvatten, aangezien klinische verbetering zonder verbetering van dysplastische en proliferatieve karakteristieken deze premaligne keratosen zou kunnen maskeren en derhalve zowel arts als patiënt kan misleiden. Een relevant aspect van retinoïden vormen de mucocutane bijwerkingen, welke de compliance van de patiënt kunnen verminderen en zodoende een beperkende factor zouden kunnen vormen voor het gebruik van retinoïden bij deze huidaandoening.

In **hoofdstuk 3** werden klinische en immunohistochemische parameters onderzocht in actinische keratosen die behandeld werden met topicaal ATRA, zowel in (immuungecompromitteerde) nierrecipiënten als in niet-immuungecompromitteerde patiënten. In niet immuungecompromitteerde patiënten leidde 6 weken behandeling met 0,05% ATRA in een crèmebasis éénmaal daags niet tot een statistisch significante reductie qua aantal actinische keratosen gelokaliseerd op de onderarmen en handruggen, terwijl wel huidirritatie werd gezien in enkele patiënten. Een kleine, maar statistisch significante toename in differentiatie parameter K10 werd gezien na behandeling met ATRA ($p = 0,049$) en een significant hogere aankleuring van het door retinoïden induceerbare K13, wanneer vergeleken werd met vehikel behandelde actinische keratosen ($p = 0,043$). Geen veranderingen werden gezien in epidermale proliferatie (Ki-67), apoptose (p53), hyperproliferatie geassocieerde keratinisatie (K16), terminale differentiatie (transglutaminase), epidermale dikte en dermale inflammatie.

In een populatie nierrecipiënten die behandeld werden met topicaal ATRA 0,02% twee maal daags voor hun actinische keratosen gelokaliseerd op de handruggen en onderarmen, werd na 6 weken behandeling geen significante verbetering gezien in dezelfde, hierboven genoemde klinische, histologische en immunohistochemische parameters; met

name geen significante inductie van K10 en/of K13. In deze populatie nierrecipiënten werd eveneens het effect van calcipotriol 0,05 mg/gram twee maal daags als monotherapie, of één maal daags 's ochtends in combinatie met topicaal ATRA 0,02% in de avond geëvalueerd. Wederom werden geen significante wijzigingen in klinische of immunohistochemische parameters gezien voor en na behandeling, zowel voor calcipotriol monotherapie als voor de combinatie therapie.

Na combinatie van de resultaten van deze studies, werden geen significante verschillen gezien in uitgangswaarden van de hierboven genoemde parameters tussen de nierrecipiënten en de niet immuungecompromitteerden. Met betrekking tot effectiviteit werd na behandeling met ATRA in actinische keratosen van immuuncompetente patiënten een toename gezien van de normale differentiatie als ook een toename van de retinoïdgeassocieerde keratinisatie. Een significant effect op epidermale proliferatie (Ki-67) en apoptose (p53), of op het aantal laesies, kon evenwel niet worden aangetoond na behandeling met gangbare concentraties ATRA. Daarom kunnen de in andere studies gerapporteerde klinische therapeutische effecten van topicaal ATRA in de behandeling van actinische keratosen mogelijkerwijs verklaard worden door veranderingen in keratinisatie in plaats van door veranderingen in epidermale proliferatie, inflammatie, en/of apoptose. Topicaal calcipotriol als monotherapie, of in combinatie met topicaal ATRA, leidde niet tot een significante verbetering van de onderzochte parameters in deze actinische keratosen. Dit in tegenstelling tot andere hyperkeratotische huidaandoeningen zoals psoriasis, waar calcipotriol effectief bleek te zijn met betrekking tot epidermale proliferatie en differentiatie, zelfs na een relatief korte behandelduur van 6 weken. Er kan evenwel niet uitgesloten worden dat langdurige behandeling met deze crèmes of applicatie met hogere concentraties, enig effect kan hebben, met name wanneer gekeken wordt in grotere patiëntenpopulaties. Aan de andere kant is het zo dat aangezien significante huidirritatie werd gezien in diverse patiënten die met ATRA behandeld werden, de vraag rijst in hoeverre hogere concentraties en langere applicatieperioden therapietrouw zullen verminderen. Daarom, bevelen wij deze applicaties niet aan voor de behandeling van actinische keratosen, maar geven de voorkeur aan andere middelen als cryotherapie en topicaal 5-fluoruracil (Efudix®).

## De relevantie van systemisch acitretine in de behandeling van actinische keratosen en verrucae vulgares in nierrecipiënten

Er is beschreven dat systemische retinoïden, zoals acitretine en isotretinoïne, een chemopreventief effect hebben, aangezien ze klinisch het aantal actinische keratosen kunnen reduceren en bovendien de ontwikkeling van non-melanoma huidkanker kunnen inhiberen. Met name in niertransplantatiepatiënten kan de betekenis van systemische retinoïden van belang zijn, gezien het grote aantal actinische keratosen dat normaliter aanwezig is in deze patiënten, alsmede vanwege de hoge incidentie van maligniteiten in deze populatie. Er is echter weinig informatie aanwezig over histologische en immunohistochemische effecten van systemische retinoïden in de behandeling van actinische keratosen. Zoals reeds eerder werd genoemd, zou klinische verbetering zonder verbetering qua dysplastische en proliferatieve karakteristieken deze premaligne actinische keratosen kunnen maskeren en dus zowel arts als patiënt kunnen misleiden. Daarom is het belangrijk om naast klinische parameters eveneens histologische en immunohistochemische parameters te evalueren in patiënten met actinische keratosen die behandeld werden met systemische retinoïden.

In **hoofdstuk 3** staan de resultaten vermeld van 26 nierrecipiënten die behandeld werden met oraal acitretine in een gerandomiseerde setting. Alle patiënten werden behandeld met een aanvangsdosering van 0,4 mg/kg/dag gedurende de eerste 12 weken, zoals data verkregen uit andere studies adviseerden. Na de eerste 12 weken behandeling zou de helft van de patiënten continueren met 0,2 mg/kg/dag, terwijl de andere helft van de patiënten continueerde met de aanvangsdosis van 0,4 mg/kg/dag. Echter, in tegenstelling tot andere studies, was een dosisreductie reeds in de eerste 12 weken noodzakelijk vanwege mucocutane bijwerkingen. Wij concludeerden dat met het oog op tolerantie 0,25-0,30 mg/kg/dag wellicht een betere aanvangsdosis is. Acitretine bleek veilig te kunnen worden gebruikt in deze patiënten; er deden zich geen ernstige bijwerkingen voor welke gerelateerd waren aan acitretine. Achteruitgang van de nierfunctie trad niet op tijdens acitretine gebruik. Klinische parameters lieten zowel een significante verbetering zien qua dikte van de actinische keratosen ($p < 0,01$) als een daling in het aantal laesies met de typische erythematosquameuze kenmerken ($p < 0.0001$) in beide groepen. Hoewel wij deze niet gescoord hebben, leken ook verrucae te verbeteren gedurende de studie. Een significante afname in het aantal non-melanoma huidkankers werd niet gevonden gedurende de studie periode, vergeleken met het jaar voorafgaand aan de studie. Visual analogue scores demonstreerden dat de patiënten over het algemeen een positief effect bemerkten van acitretine behandeling reeds in de eerste 2-3 maanden met betrekking tot ruwheid van de laesies ($p = 0,002$) en algemeen welbevinden ten aanzien van de huid ($p = 0,001$). Na de eerste maand van behandeling werd geen additioneel effect bemerkt na continuering met acitretine tot aan het einde van de studieperiode. Tussen de beide groepen werden geen verschillen gezien in klinische parameters en bijwerkingen, hetgeen suggereert dat een lage onderhoudsdosis van 0,2 mg/kg/dag na een 3 maanden durende initiatie met een hogere dosis adequaat is om het klinische effect te onderhouden.

De corresponderende immunohistochemische studie in actinische keratosen, zoals beschreven in paragraaf 3.2, en welke werd uitgevoerd in 33 nierrecipiënten, liet zien dat klinische verbetering van deze laesies gedurende acitretine behandeling voortkwam uit een reductie in de dikte van het stratum corneum, waarschijnlijk ten gevolge van veranderingen in het keratinisatieproces. Significante verbetering in K10 ($p = 0,02$) en inductie van K13 ($p < 0,01$) en K19 ($p = 0,05$) wijzen erop dat een directe interferentie met keratinisatie het onderliggende mechanisme is. Echter, epidermale proliferatie (Ki-67), apoptose (p53), hyperproliferatie geassocieerde keratinisatie (K16), terminale differentiatie (transglutaminase), en dermale inflammatie, bleven onveranderd. Data verkregen uit de cessatiestudie van systemisch acitretine in de behandeling van actinische keratosen (paragraaf 3.3), welke werd verricht in 9 nierrecipiënten, toonde geen significante toename in het aantal actinische keratosen of plaveiselcelcarcinomen 3 maanden nadat acitretine gestopt was. Dit in tegenstelling tot het aantal wratten dat wel significant was toegenomen na deze periode ($p = 0,02$). Induratie van de actinische keratosen nam significant toe gedurende de eerste 6 weken na stoppen met acitretine ($p = 0,004$). Een VAS score, welke het algemeen welbevinden representeerde, was significant gereduceerd 12 weken na cessatie. Met betrekking tot de immunohistochemische parameters werd een significante reductie gezien in K13 expressie 6 weken na stoppen met acitretine, terwijl de expressie van MIB-1, p53 en $p16^{INK4A}$ onveranderd bleven. Al met al leidde stoppen met acitretinebehandeling in nierrecipiënten tot klinische verslechtering binnen 3 maanden, zonder een significante toename in huidkanker en premaligne afwijkingen, en vanuit immunohistochemisch perspectief bovendien tot een significante daling in K13 expressie, zonder verandering in expressie van celcyclus geassocieerde markers.

Concluderend kan gesteld worden dat systemische behandeling met acitretine het aspect van actinische keratosen en wratten kan verbeteren door veranderingen in keratinisatie, welke bestaan uit een stimulatie van de normale differentiatie als ook een inductie van retinoïd-geassocieerde differentiatie. Acitretine heeft geen invloed op epidermale proliferatie, apoptose en dermale inflammatie in onze studies. Klinisch werd tijdens behandeling met acitretine een reductie gezien van het aantal karakteristieke actinische keratosen, welke gekenmerkt worden door erytheem, desquamatie en veelal een dikke laag hoorn, alhoewel vaak erythemateuze maculae persisteerden. Deze maculae vertoonden na histologisch en immunohistochemisch onderzoek nog immer de kenmerken van actinische keratosen, zoals dysplasie en toegenomen epidermale proliferatie. Actinische keratosen worden door acitretine behandeling dus enigszins gemaskeerd. Theoretisch zou acitretine eveneens plaveiselcelcarcinomata en andere non-melanoma huidkankers kunnen maskeren. In de praktijk blijven deze aandoeningen echter gemakkelijk klinisch herkenbaar, aangezien zij doorgaans snel progressief zijn, ondanks retinoïd behandeling. Toch zou in de langzaam groeiende plaveiselcelcarcinomen, met name die welke klinisch sterk lijken op actinische keratosen of die welke hieruit ontstaan zijn, inderdaad een maskerend effect uit kunnen gaan van acitretine. Deze data verklaren ook de plotselinge toename in aantal van (karakteristieke) actinische keratosen en plaveiselcelcarcinomen welke gerapporteerd zijn na het stoppen met acitretine in sommige studies. Behandeling gedurende lange tijd lijkt derhalve aangewezen met de laagst mogelijke, effectieve dosis.

## De betekenis van retinoïd-geassocieerde keratine expressie in (pre)maligne huidaandoeningen

K13 en K19 komen normaliter niet tot expressie in volwassen humane epidermis, maar komen wel voor in embryonale epidermis en kunnen tevens geïnduceerd worden door retinoïden. **Hoofdstuk 3** toont dat niet-retinoïd behandelde actinische keratosen eveneens K13 en/of K19 tot expressie kunnen brengen. In de studie met acitretine voor actinische keratosen in nierrecipiënten werden K13 en K19 expressie gezien in 30 %, respectievelijk 15 % van de biopten. In deze studie werden enkel patiënten geïncludeerd met een hoge prevalentie van actinische keratosen en meestal eveneens multipele plaveiselcelcarcinomen in hun voorgeschiedenis. In de studie waar actinische keratosen van nierrecipiënten werden behandeld met topicaal ATRA en/of calcipotriol, werd geen K13 expressie gezien in niet-retinoïd behandelde actinische keratosen. In deze studie namen patiënten deel met een relatief geringer aantal actinische keratosen en plaveiselcelcarcinomen. K13 expressie, in tegenstelling tot K19 expressie, werd eveneens gezien in niet-retinoïd behandelde verrucae vulgares en bovendien met een hogere incidentie in nierrecipiënten (82 %) dan in niet-immuungecompromitteerden (14 %), zoals beschreven in paragraaf 3.6. Data verkregen van de cessatie studie met acitretine in actinische keratosen toonde een significante reductie in K13 expressie 6 weken nadat acitretine gestopt was ($p = 0,02$). Echter in 1 patiënt werd na 6 weken stoppen met acitretine nog steeds een focaal expressie patroon van K13 gezien; in een andere patiënt werd dit nog in enige mate gezien na 12 weken gestopt te zijn.

De meeste patiënten met K13 en/of K19 positieve actinische keratosen of verrucae vulgares in de uitgangsbiopten hadden nimmer topicale of systemische retinoïden gebruikt in het verleden. Een enkeling gebruikte ten tijde van de screening topicale of systemische retinoïden en onderging derhalve een washout periode (4 weken voor topicale retinoïden; 3 maanden voor systemische retinoïden) om te kunnen participeren aan onderzoek. Daarom zou er een andere verklaring moeten zijn voor de expressie van deze keratinen in de uitgangsbiopten. Een interessante bevinding is dat deze keratinen, wanneer aanwezig in niet-retinoïd behandelde uitgangsbiopten van actinische keratosen en verrucae vulgares, typische expressie in losliggende, individuele cellen tonen, terwijl na retinoïdbehandeling een uitgebreider, meer focaal of bandvormig expressie patroon wordt gezien.

Andere (dierexperimentele) studies wijzen er op dat expressie van laagmoleculaire keratinen, zoals K13 en K19, gezien kunnen worden in huidmaligniteiten als uiting van een meer gedifferentieerd stadium. Daarom is expressie van K13 en/of K19 in een niet-retinoïd behandelde premaligne laesie mogelijkerwijs een teken van gevorderde dedifferentiatie en herbergt deze laesie dus een groter risico om maligne te ontaarden. Mede hierom keken wij eveneens naar parameters welke positief geassocieerd zijn met actinische keratosen die een hoger risico hebben op maligne transformatie. Inderdaad vonden we statistisch significante associaties tussen K13 en/of K19 expressie enerzijds en enkele van deze parameters, waaronder Ki-67, p53, KIN, en eveneens het aantal recent geëvolueerde plaveiselcelcarcinomen. De significant hogere expressie van K13 in verrucae

vulgares van nierrecipiënten ten opzichte van deze expressie in verrucae vulgares in de normale populatie, past eveneens bij deze hypothese, aangezien bepaalde types humaan papillomavirus verondersteld wordt geassocieerd te zijn met maligne transformatie, met name in nierrecipiënten.

Al met al kan er geconcludeerd worden dat K13 en K19 mogelijk twee nieuwe parameters zijn die een rol kunnen spelen bij de identificatie van actinische keratosen die een hoog risico hebben op maligne transformatie. Het is waarschijnlijk dat K13 en K19 expressie epifenomenen zijn welke gerelateerd zijn aan 2 onafhankelijke onderliggende mechanismen: maligne transformatie en retinoïd-geassocieerde keratinisatie. Het patroon van K13 en/of K19 expressie kan een aanwijzing zijn om te differentiëren tussen deze beide mechanismen.

Toekomstige studies zouden zich kunnen richten op het gedrag van actinische keratosen en plaveiselcelcarcinomen tijdens retinoïd behandeling. In welke mate maskeren retinoïden inderdaad deze laesies? Het is bovendien relevant om ook de effectiviteit van RXR-selectieve retinoïden zoals bexaroteen, te evalueren in de behandeling van actinische keratosen. Ondanks het feit dat topicaal calcipotriol niet effectief bleek in de behandeling van actinische keratosen, zou ook de combinatie van een vitamine D3 derivaat en een RXR-selectief retinoïd interessant zijn om te onderzoeken vanwege de heterodimeer formatie van beider nucleaire receptoren.

# Modulatie van UV-geïnduceerde inflammatie door retinoïden

## Modulatie van UVB-geïnduceerd erytheem, in het bijzonder de minimale erytheem dosis (MED), door topicaal all-trans retinoic acid

Ondanks het feit dat de functie van RAR-selectieve retinoïden in combinatie met foto(chemo)therapie veelvuldig onderwerp van studie is geweest in veel dermatologische aandoeningen en ondanks het feit dat deze combinatietherapie heden ten dage veel gebruikt wordt, is er weinig informatie beschikbaar over de UV-modulerende eigenschappen van retinoïden Het is bekend dat retinoïden inflammatie induceren en kunnen leiden tot een fenomeen genoemd *"retinoïd dermatitis"*, met kenmerken als toegenomen desquamatie en erytheem van de huid. Verder kan retinoïd behandeling leiden tot een afname in epidermale dikte, en kan dus op deze wijze de effectiviteit van foto(chemo)-therapie met name in hyperkeratotische huidaandoeningen versterken via toegenomen penetratie van UV-licht door de huid.

In dit proefschrift vonden we een significante toename in erytheem na een week behandeling met twee maal daags topicaal all-trans retinoic acid 0,05% op normale huid ten opzichte van haar vehikel. Bovendien werd een typisch patroon van perifolliculair erytheem gezien, welke geïnterpreteerd kan worden als retinoïd geïnduceerde dermatitis. Na UVB belichting werden er geen significante verschillen gevonden in MED waarden tussen met ATRA voorbehandelde huid, met vehikel voorbehandelde huid en niet voorbehandelde huid, zowel 24 uur als 48 uur na belichting. Met betrekking tot de intensiteit van het erytheem na behandeling met elk der bovengenoemde voorbehandelingen werd eveneens geen significant verschil gezien. Concluderend zijn er, los van een toename in (perifolliculair) erytheem ten gevolge van retinoïd behandeling, geen aanwijzingen dat topicaal ATRA de mate van UVB-geïnduceerd erytheem na een eenmalige belichting kan beïnvloeden.

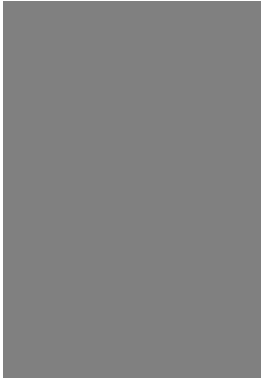
## Modulatie van UVB-geïnduceerd erytheem, in het bijzonder de minimale erytheem dosis (MED), door oraal bexaroteen

Het is waarschijnlijk dat RXR-selectieve retinoïden een innovatie vormen in retinoïd behandeling van een breed scala aan huidaandoeningen. Aangezien RAR-selectieve retinoïden frequent gecombineerd worden met foto(chemo)therapie ter versterking van het effect van PUVA of UVB behandeling, is het interessant te speculeren in welke mate RXR-selectieve retinoiden eveneens foto(chemo)therapie versterken. Daarom is het belangrijk te weten of RXR-selectieve retinoiden UV-geïnduceerd erytheem moduleren.

In dit proefschrift is het effect beschreven van het RXR-selectieve retinoïd bexaroteen op UVB-geïnduceerde inflammatie door bepaling van de MED en de graad van erytheem. Eenmalige expositie aan een reeks van 9 in intensiteit toenemende belichtingen met breedspectrum UVB op de onderrug werd goed verdragen en geen fotosensitiviteitsreacties werden gezien.

Na 3 maanden behandeling met hetzij 0,5-, hetzij 3 mg bexaroteen per kg per dag werd een tendens waargenomen tot toename in de mate van erytheem en daling van de MED waarde in de 4 patiënten die 3 mg bexaroteen per kg per dag kregen; deze veranderingen waren echter niet statistisch significant. Een dosisrespons relatie tussen MED en de dosis van bexaroteen werd niet gevonden en evenmin werden er abnormale reacties gezien op UVB belichting. Er kan evenwel niet uitgesloten worden dat met name hogere concentraties bexaroteen in grotere populaties toch een effect zouden kunnen onthullen op de MED waarde en de gradatie van erytheem. Aan de andere kan suggereren de uitkomsten van deze studie dat patiënten die behandeld worden met bexaroteen 0,5 – 3 mg/kg/dag geen extra beschermende maatregelen hoeven te nemen met betrekking tot de korte termijn effecten van zonlichtexpositie en lijkt combinatietherapie van RXR-selectieve retinoiden met UV-therapie mogelijk.

Aanvullende studies gericht op de effectiviteit van bexaroteen in combinatie met foto(chemo)therapie in cutaan T-cel lymfoom en psoriasis zijn zinvol, met name daar waar het gaat om hogere concentraties bexaroteen. Naast de combinatie van orale behandeling met bexaroteen en UV-belichting zou ook topicale toediening van bexaroteen een aantrekkelijk alternatief kunnen vormen. Toekomstige onderzoeken zouden zich kunnen richten op RXR subtype selectieve retinoiden in combinatie met foto(chemo)-therapie.



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# Abstracts


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# Curriculum vitae

*Jürgen Smit werd op 9 november 1970 geboren te 's Gravenhage. Na het grootste gedeelte van zijn jeugd te hebben doorbracht in het Gelderse Zelhem, doorliep hij het Gymnasium op het St. Ludgercollege te Doetinchem en behaalde aldaar zijn VWO diploma in 1989. Aansluitend ving hij aan met de studie geneeskunde aan de Katholieke Universiteit Nijmegen. Hij behaalde zijn doctoraalexamen in februari 1997, waarop na een wachttijd van enkele maanden kon worden aangevangen met de co-schappen. In Pretoria, Zuid-Afrika, verrichtte hij zijn wetenschappelijke stage op het gebied van de gastro-enterologie en praktiseerde hij in het kader van een niet facultaire stage op de afdelingen dermatologie van het Pretoria Academic Hospital en het Kalafong Hospital. In februari 1998 behaalde hij zijn artsexamen. Na aanvankelijk voorkeur te hebben gehad voor de chirurgie en later de interne geneeskunde, werd het ijs in Zuid-Afrika definitief gebroken voor de dermatologie. Sinds maart 1998 is hij dan ook werkzaam als arts-assistent op de afdeling dermatologie van het Universitair Medisch Centrum St. Radboud te Nijmegen, alwaar hij onder leiding van Prof.dr.dr. P.C.M. van de Kerkhof en Dr. E.M.G.J. de Jong zich toelegde op de onderzoeken welke hebben geleid tot dit proefschrift. Sinds maart 2002 is hij in opleiding tot dermatoloog.*

*Naast bovengenoemde werkzaamheden was hij van juli 1999 tot en met juni 2002 hoofdredacteur van het medische tijdschrift "Arts Assistent" van de Landelijke Vereniging van Assistent Geneeskundigen (LVAG), in welke hoedanigheid hij eveneens participeerde in het LVAG-hoofdbestuur. Ook was hij actief op het gebied van de PDA-georiënteerde medische informatietechnologie. In juli 2000 nam hij het initiatief tot de oprichting van "MediCE", de Nederlandse gebruikersvereniging voor Windows CE / Pocket PC bestuurde zakcomputers in de medische sector, alwaar hij tot december 2000 de functie van voorzitter op zich nam. Na een fusie van MediCE met de Psiongebruikersvereniging uit het UMC St. Radboud te Nijmegen bleef hij tot februari 2002 aan als voorzitter van de nieuwe, platform onafhankelijke vereniging "UM2C", de Usergroup for Medical Mobile Computing. Sinds april 2002 is hij woonachtig in het Duitse Kleve, alwaar hij samenwoont met Jeanette Radstaak en hun herdershonden Aida en Noa.*