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Phototherapy

photobiological aspects and therapeutical developments

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Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen, op gezag van
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door

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geboren op 4 oktober 1973 te Groningen

- aan mijn ouders
- voor Sioe Lie

Table of Contents

List of abbreviations

AP	▪	**Activator Protein**
CHS	▪	**Contact Hyper Sensitivity**
CPD	▪	**Cyclobutane Pyrimidine Dimers**
IR	▪	**Infrared**
IL	▪	**Interleukin**
JNK	▪	**c-Jun N-Terminal Kinase**
LC	▪	**Langerhans Cell**
MED	▪	**Minimal Erythema Dose**
MHC	▪	**Major Histocompatibility Complex**
MMP	▪	**Matrix metalloproteinases**
PDT	▪	**Photodynamic therapy**
PUVA	▪	**Psoralen UVA phototherapy**
RA	▪	**Rheumatoid Arthritis**
ROS	▪	**Reactive Oxygen Species**
SALT	▪	**Skin Associated Lymphoid Tissue**
SIS	▪	**Skin Immune System**
TIMP	▪	**Tissue Inhibitor of Matrix Metallo Proteinases**
TNF	▪	**Tumor Necrosis Factor**
UV	▪	**Ultraviolet radiation**
UVA	▪	**Ultraviolet radiation A**
UVB	▪	**Ultraviolet radiation B**

1

General Introduction

1.1   An Introduction to light

1.1.1 Light in a historical perspective

Light is all around us and is the most important requisite to result in the creation of life. This has been known from the early beginnings of human life. The ancient Egyptians, Sumerians and Aztecs have worshipped the sun as the bringer of life and as the God of gods. Even the Bible starts with one of the best-known religious statements after the creation of heavens and earth: "And God said: Let there be light and there was light" (Genesis 1:3), after which God creates life on earth.

As the most important life giving substance, ancient people started to attribute healing powers to the sun, like the ancient Egyptian priests and healers, who sometimes tried to increase these healing powers with a herb (Ammi majus Linnaeus) grown on the banks of the river Nile, nowadays known to contain a photoactive substance called Psoralen (1400 B.C.). Even the ancient Chinese used photoactive herbs like Bu Gu Zi (fructus Psoralea Corylifoliae). The Greek were the first to introduce the term heliotherapy as the first kind of phototherapy. Hippocrates wrote extensively about the healing powers of the sun in his Corpus Hippocraticum (460-375 B.C.). There are numerous accounts of the suns healing properties in religious and medical texts and literature (1-4).

Already during the Roman days, skin colour became the visible definer to separate the (ruling) upper class from the (working) plebs. The paler one's skin, the higher one's class. During those days, people used chalk to whiten their skin and during the mid10th century this was replaced by arsenic, both with deadly side effects due to lead or arsenic poisoning. Nowadays, in Asia men prefer women with the fairest skin, while in the Western world since the 20th century a bronzed colour is preferred (5,6).

It was not until the end of the 19th century when western medical doctors started to use sun exposure for its healing powers in special medical facilities called sanatoria for the treatment of tuberculosis of the skin (1,7). It was soon recognised that only certain parts of the light spectrum had beneficial effects on several chronic skin diseases like psoriasis and eczema and that the lack of sun exposure could lead to deficient bone development (rickets) as experienced during the industrial revolution around 1800 (1). With the invention of electricity and light bulbs and eventually specialised lamps with specific emission spectra, artificial light exposure was introduced as a medical therapy. Several diseases and natural and chemical substances were also recognised, where (combination with) light exposure had acute hazardous effects on the skin, like bulleous reactions in patients with porphyrias, or contact phototoxicity reactions with tar and plants like Hogweed and St. John's worth. It took more decennia before eventually in the 1970-80's the link between several kinds of skin cancer

and sun exposure was recognised, although Unna had already described a possible relationship in 1894 (4,8).

As ultraviolet light has major implications for our skin and body, it is of the utmost importance to gain understanding in photobiological mechanisms. First, we have to discuss some basic physical concepts.

1.1.2 The nature of light

Light represents only a small part of the electromagnetic spectrum, ranging from radioactive radiation on the one side to radio and television waves on the other side. Light is somewhere in the middle and is usually divided in infrared and visible light, UVA, UVB and UVC as shown in figure 1-1. UVB is defined as the electromagnetic spectrum ranging from 290 or to 320 nm. UVA ranges from 320 to 400 nm and visible light ranges from approx. 400 to 750 nm wavelength (9).


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        290        320          400                                  750
   UVC        UVB          UVA              Visible Light                   IR
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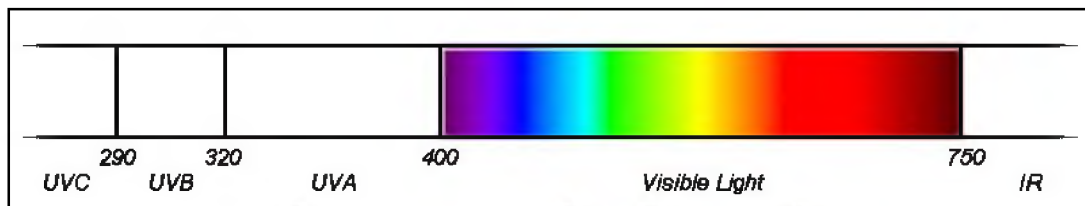
Literature is not always consistent on the term light. Sometimes it only refers to visible light (so UV and IR are referred to as radiation) and sometimes it spans the whole of the above-mentioned spectrum.

The photobiological action spectrum of light on the skin depends on the available photoactive substances, the amount of energy conveyed by the light and the penetration depth.

Skin penetration depth of light depends on wavelength and reflectance. The longer the wavelength the deeper the penetration and the larger the change in refractive index between air, skin and tissues, the more light is reflected. As a real life example of this concept one can hold a strong flashlight against the skin of the cheek or between one's thumb and forefinger. The refractive index change between air (n_D = 1.0) and stratum corneum ($n_D \approx 1.55$) is quite large, so part of the light is already reflected. The part that will penetrate the skin is red, because this is the light that can penetrate the skin this far (approximately 0.5-1 cm) (10).

One of the most important concepts of light is described with the following formula:

$$E_{photon} = h \cdot \nu \text{ (with: } h = \text{Planck constant)} = \frac{1.98 \cdot 10^{-19} \, watt \cdot seconds}{\text{wavelength in nanometers}}$$

This implies that the longer the wavelength the less energy each photon (light particle) has. So a photon of UVB has more energy than UVA or red light (11).

The ozone layer keeps UVC out of our atmosphere, so only UVA, UVB, visible light and infrared light penetrate our atmosphere. It is this spectrum that exerts the photobiological effects on the human skin. When this high energetic light penetrates the skin it encounters a magnitude of molecules on its way through the skin. Several photoactive molecules (chromophores) like bilirubin, haem and DNA can absorb these energies and shift from their ground state to states of higher energy (12-15). When photonic energy is absorbed, electrons are promoted to previous unoccupied higher energy orbits around the atomic core. Depending on the amount of photonic energy the excited state will be singlet (less energy) or triplet (more energy). Singlet excited states are more unstable than triplet excited states, but both desperately want to return to their ground state. This is only possible when electrons return to their original orbit around the atomic core, by transferring their excessive energy to the surrounding molecules as photonic energy (fluorescence), heat or by interacting with other molecules. Interaction with other molecules will usually result in arrays of biochemical chain reactions (16).

1.2 Photobiological effects of light

Photobiological effects depend not only on the wavelength, the amount of energy conveyed by the light and the depth of the penetration, but also on the type of exposure (repetitive or single).

Clinically the following acute effects can be observed:
▶ Acute excessive exposure results in sunburn, usually experienced as transitional painful erythematous skin with shedding of the upper epidermal layers (17).
▶ Repetitive exposure to lower dosages results in transitional epidermal thickening, hyperpigmentation and dryness of the exposed skin.

Chronic exposure results in the following clinical features:
▶ Wrinkling and thickening of the skin.
▶ Shift in pigmentation (freckling, lentigines, irregular hyper- and depigmentation).
▶ Photocarcinogenesis (actinic keratosis, M. Bowen, basal cell carcinoma and squamous cell carcinoma) (17).

All these effects can be interpreted as immunological effects and photodamage.

1.2.1 Immunological effects

The skin provides one of the most important defences against invading micro-organisms and malignant transformed cells. This is accomplished by an intricate system of transient and resident immunological active cells. Transient cells are predominantly circulating T-lymphocytes, polymorphonuclear granulocytes, monocytes and macrophages and are usually described with the term Skin Associated Lymphoid Tissue (SALT). Resident immune cells comprise antigen presenting Langerhans cells (LC's), immunomodulatory keratinocytes, mast cells and endothelial cells.
The amount of energy, wavelength and the type of exposure determine the immunomodulatory effects of light exposure.

UVB

Acute excessive single or repetitive exposure to UVB results in sunburn. Sunburn is immunologically characterised as immunoactivation with initially an influx of polymorphonuclear leukocytes up to 14 hours post exposure, followed by a predominantly mononuclear perivascular inflammatory infiltrate which peaks at about 14-21 hours, but also with a reduction of the density of epidermal LC's after 24 hours. Mast cells degranulate within 3-5 hours after exposure eventually leading to depletion (18,19).
Several studies have shown that chronic and repetitive exposures of UVB result in suppression of type IV hypersensitivity reactions by reducing the amount of LC's in the epidermis. Ear swelling of mice sensitised to a certain hapten was significantly less after re-exposure to the hapten after repetitive UVB exposure compared to no or only a single or repetitive UVB exposure (20,21). Several authors report that IL-1 production and MHC class II expression are also reduced, thereby increasing this immunosuppressive effect (22,23).
Prolonged exposure (> 6 weeks) to UVB decreases the number of circulating T-lymphocytes and monocytes, while increasing the number of T-lymphocytes in peripheral lymph nodes. This may be explained by the fact that the expression of adhesion molecules like ICAM-1, which play an important role in lymphocyte homing, is reduced. Cytokine production in the skin is modulated by UVB irradiation. E.g. TNF-α, a strong pro-inflammatory cytokine, is up regulated after UVB exposure, and can also impair contact hypersensitivity reactions (CHS) (24).

UVA

In contrast to UVB, exposure to UVA generally does not result in erythema and sunburn. Although several studies seem to contradict the previous statement, the effects seen in those studies are considered to be at least partly the result of incomplete shielding of the UVB and UVC output of the irradiation source (19,25-27). With no distinct erythema and sunburn, one could assume that UVA has no immunomodulatory effects at all, however UVA exposure does result in some inflammation and immunosuppression. The inflammatory response is marked by some perivascular inflammatory infiltrate, consisting of neutrophilic granulocytes influx 3 hours post irradiation, and a predominantly lymphocytic infiltration. LC's count reduces significantly too (19,22,26). This inflammatory response on UVA is most likely mediated by oxidative damage. For example glutathione, a potent endogenous radical scavenger, inhibits several immunomodulatory functions of UVA radiation in a dose dependent manner (28,29). It is assumed that products of oxidative damage can induce the production of immunomodulatory cytokines like IL-12 (30,31). Most studies on the immunomodulatory effects of UVB have concentrated on CHS reactions. So far no comparative studies between UVA and UVB have been performed on this subject.
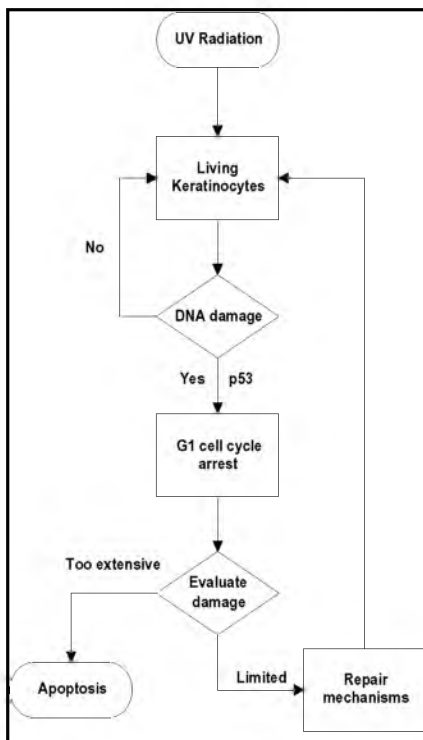
Figure 1-2: p53 repair mechanism

1.2.2   Photodamage

Photodamage can be divided in photocarcinogenesis and photoageing. Photocarcinogenesis is a complex mechanism of UV induced DNA damage, impaired repair mechanisms and failure of the skin immune system to detect malignant cells.

Photocarcinogenesis

When UV penetrates into the skin it reacts with photoreactive molecules in the skin called chromophores. One of these chromophores is DNA. Direct (UVB induced) DNA photoproducts are typically cyclobutane pyrimidine dimers (CPD), which can be thymine (T-T), cytosine (C-C) dimers or thymine-cytosine or cytosine-thymine bonds in keratinocytes, with a strong preference for thymine dimers (32,33).

Indirect photoproducts are produced by interaction with reactive oxygen species (ROS) and consist mainly of DNA strand breaks and protein cross-linking (12,13).
Normally, when DNA damage occurs, the cell cycle is halted to give time to repair the damage. One of the main factors involved is p53, a tumour suppressor gene, which can result in a G1 cell cycle arrest following activation (34). During this cell cycle arrest, damage is evaluated and repaired, which results in increased cell survival, or when the damage is regarded too extensive, the cell is terminated by apoptosis (35-38). Nuclear excision repair enzymes, like p16, play a major role in repair processes (38). Figure 1-2 summarises the photobiological effects of UV on keratinocytes. Repair can be complete or incomplete. Incomplete repair results in living keratinocytes with damaged DNA, which can result in uncontrolled cell growth (38). Cell surface antigens are absent or changed, which trigger the skin immune system to remove these cells. As described in paragraph 1.2.1 the immune system is impaired in skin following chronic exposure to UV light. This increases the risk of photocarcinogenesis (8,39-41).

Photoageing

Photoageing is the process of induction of enzyme systems resulting in increased destruction and remodelling of the intercellular matrix leading to decreased elastin and collagen content (42,43). This process gives the skin a wrinkled look. Other processes that takes place in photoageing are changes in pigmentation and atrophy of the skin (44).
Chemical reaction with oxygen containing molecules result in the formation of superoxide anion radicals (O_2-) and singlet oxygen ($^1O_2$). These reactive oxygen species are highly unstable and react with surrounding molecules, thereby activating biochemical cascades or damaging structures like collagen. Wlaschek et al. showed that $^1O_2$ induces the production of collagenase-mRNA (44). Furthermore, ROS has the capacity to strongly inhibit tissue inhibitors of metalloproteinases (TIMP's) (45).
UVB activates MAP-kinases like c-Jun amino-terminal kinase (JNK), p38 en ERK via lipidperoxidation products. Subsequent phosphorylation of c-Jun proteins and combination with c-Fos proteins produce activator protein (AP)-1 complexes. Binding with the AP-1 response element (AP-RE) induces the transcription of several MMPs (46). MMPs are extracellular matrix degrading proteins (see table 1-1) and form, combined with their inhibitors (TIMP's), an intricate extracellular matrix remodelling system.
Prolonged and excessive degradation of the extracellular matrix eventually results in fragmentation of collagen and elastin fibres and thus in wrinkled skin.

Table 1-1: Overview of currently known MMPs and substrates (adapted from McCawley et al. (47))

MMP subgroup	MMP number	Synonym	Matrix substrate
Collagenases	MMP-1	collagenase-1	collagen I, II, III, VII, X, gelatin, entactin, aggrecan, tenascin.
	MMP-8	collagenase-2	
	MMP-13	collagenase-3	
Stromelysines	MMP-3	stromelysine-1	proteoglycans, laminins, fibronectin, gelatin, collagen. III/IV/V/IX/X/XI, fibrin / fibrinogen, entactin, tenascin, vitronectin.
	MMP-10	stromelysine-2	
	MMP-11	stromelysine-3	laminin, fibronectin, aggrecan.
Gelatinases	MMP-2	gelatinase A	gelatin, elastin, fibronectin, collagen I, IV, V, VII, X, XI, laminin, aggrecan, vitronectin.
	MMP-9	gelatinase B	
Minimal domain	MMP-7	matrilysine	proteoglycans, laminins, fibronectin, gelatin, collagen. III/IV/V/IX/X/XI, fibrin / fibrinogen, entactin, tenascin, vitronectin
	MMP-26	matrilysine-2 / endometase	gelatin, collagen IV, fibronectin, fibrinogen
Membrane associated	MMP-14	MT1-MMP	gelatin, fibronectin, vitronectin, collagen, aggrecan.
	MMP-15	MT2-MMP	
	MMP-16	MT3-MMP	
	MMP-24	MT5-MMP	
	MMP-17	MT4-MMP	gelatin.
	MMP-25	MT6-MMP	gelatin, collagen IV, fibrin, fibronectin, laminin-1.
	MMP-23		gelatin.
Other MMPs	MMP-12	metalloelastase	elastin, fibronectin, fibrin/fibrinogen, laminin, proteoglycan.
	MMP-19	RASI	gelatin, tenascin, fibronectin, collagen IV, laminin, entactin, fibrin/fibrinogen, aggrecan.
	MMP-20	enamelysin	COMP.
	MMP-28	epilysin	amelogenin, aggrecan, COMP.

1.3 Immunosuppression and responses to ultraviolet radiation

1.3.1 General aspects

The immune system is of relevance in acute and chronic effects of ultraviolet radiation.
Acute effects of UV radiation compromise the inflammatory responses, characterised by pain, erythema and blistering (17,19,41). Cyclooxygenase inhibitors and corticosteroids suppress these responses (48-51). On the other hand, systemic immunosuppressive treatments may counteract the "off-switch" of UV-induced inflammation. Indeed, it is important that during methotrexate or cyclosporin A treatment patients should be warned for prolonged UV induced burns. Therefore, in

patients with immunosuppressive treatments sun exposure should be reduced.
Chronic effects of ultraviolet light combined with immunosuppression comprise a
vast increase of the number of actinic keratosis, with increased development of basal
cell carcinoma and squamous cell carcinomas (non-melanoma skin cancer) (52,53).
The combination of UV induced DNA damage and (UV) immunosuppression is
likely to result in photocarcinogenesis. This is particularly noticeable in patients with
impaired or deficient DNA repair, like patients with xeroderma pigmentosa, but also
in renal transplant patients with long-term immunosuppressive medication (52,54-
56). In all these patients highly increased numbers of skin cancer develop on UV
exposed skin, like face, hands, fore-arms, shoulders, lower-legs and feet.

1.3.2 Single target therapies in chronic inflammatory diseases

In recent years pharmaceutical companies are more and more focusing on single
target therapies for chronic inflammatory T-cell mediated diseases such as
psoriasis and rheumatoid arthritis as possibilities arise to create specific antibodies
against proteins and receptors which are relevant in the pathogenesis. Selective
immunomodulation may increase safety with regard to infections and carcinogenesis.
Several antibodies have been engineered against T-lymphocyte surface molecules
such as LFA3TIP (Alefacept, Amevive®, Biogen), which blocks the co-stimulatory
pathway between Antigen Presenting Cells and CD2 on T-lymphocytes, IDEC-114
(IDEC Pharmaceuticals), which blocks the co-stimulatory pathway CD80-CD28 on
antigen presenting cells and T-lymphocytes, and anti-CD4 antibodies (OKTcdr4a,
Immunoclone, Johnson & Johnson) (57,58). Other manufacturers focus on
intracellular T-lymphocyte signalling blockers, such as Pimecrolimus® (SDZ ASM
81, Novartis) and Tacrolimus (FK506) (59,60). Clinical and laboratory studies have
shown that these therapies have promising results in the treatment of e.g. psoriasis.
Recently anti-TNF-α and TNF-α receptor constructs have been registered for the
treatment of rheumatoid arthritis (RA), juvenile chronic arthritis and Crohn's disease
(61,62). Several placebo-controlled studies have also reported clinical efficacy
of these compounds in the treatment of psoriasis (63-65). The major concerns of
immunosuppressive treatments are infections and carcinogenesis. In this thesis the
question is addressed whether an anti-TNF-α approach influences photodamage and
photocarcinogenesis. As presented in section 1.2.1 TNF-α plays an important role in
UVB induced inflammation and apoptosis. Chapter 3 will discuss the effects of anti-
TNF-α on these mechanisms.

1.4   Phototherapy

1.4.1   General aspects

Several skin disorders can be treated with phototherapy (UVB) or photochemotherapy (PUVA). PUVA is the combination of UVA with a systemic or topical application of a psoralen (P). In particular psoriasis, affecting 2% of the world population, may be treated with phototherapy after topical treatments have failed to be effective (66). In recalcitrant atopic dermatitis, narrow band UVB (311 nm) and high dose UVA1 have become treatment options. Photo (chemo)therapy can be given as a monotherapy or combination therapy (67,68). The combination of photo (chemo)therapy with systemic retinoids is a highly effective approach for patients with cutaneous T-cell lymphoma and patients with sever psoriasis (69,70).

1.4.2   Vitiligo, a neglected area in UVB phototherapy

Vitiligo is a chronic skin disease characterised by depigmented areas on the skin due to the absence of melanocytes (3). Descriptions are found in several ancient and classic texts, e.g. the Atharva Veda, one of the most important scriptures in Indian history (dated 1500 BC), relates of king Pandu, who was covered by white spots (kilas or palita). Due to visual similarities, vitiligo patients were often mistakenly treated and outcasted as lepers.

The aetiology and pathogenesis of vitiligo are still unknown. Based on clinical findings in patients several theories have been proposed: the genetic theory as 25-30% of the patients have affected family members, the auto-immune theory as up to 77% of the patients have auto-antibodies against melanocytes or one or more other auto-immune diseases, the neurogenic theory for segmental vitiligo, which follows dermatomes, the melanocytes self-destruction theory as radical scavenger deficiencies have been detected in several patients, and last but not least the convergence theory which combine all previous mentioned theories (71). With advancing knowledge of melanogenesis and melanocyte biochemistry and physiology, several new theories have been suggested. All these new theories share the common idea that a deficiency in one of the biochemical essential compounds plays a role in the pathogenesis of vitiligo, like pseudocatalase, calciumchlorid, vitamin B12 and folic acid (72-74). These theories are based on the fact that in some patients a deficiency of pseudocatalase and/or calciumchlorid has been detected in or surrounding vitiligo lesions. Overall vitamin B12 and folic acid deficiencies in patients have not been reported. In several large groups of vitiligo patients serum concentrations were however low (72,75). Some authors speculate that this deficiency could be a common feature of vitiligo (72,75).

Vitiligo is a disease with minimal physical disabilities. Melanocytes have an important function in protecting the skin against the deleterious effects of UV irradiation, by producing melanosomes, which absorb most of the energy of UV irradiation and play an important part in determining skin colour (76). Vitiligo lesions are thus prone for sunburn, however there is still some controversy whether such also results in photocarcinogenesis and skin ageing (77-80). Several authors suggest that vitiligo patients are protected against photocarcinogenesis as epidemiological studies have shown that the incidence of skin malignancies is less in vitiligo patients and for this reason promote the almost unlimited use of phototherapy as a repigmenting therapy (78). It is however possible that because patients are more prone to sunburn they avoid sun exposure more, thus minimising the chance of photocarcinogenesis.

Psychosocial disabilities play an important role in vitiligo patients, especially in representative occupations and dark pigmented races (81,82). Treatment thus focuses on reducing the contrast between pigmented and depigmented skin. There are several options available. The easiest and most used option is to cover the lesions with clothing or camouflaging makeup. Some people tattoo their lesions. These options do not result in repigmentation. Repigmenting therapies are divided in surgical transplantation or phototherapy.

The following transplantation techniques can be used:

► Minigrafting: punch biopsies are taken from normal pigmented skin and transplanted in depigmented lesions.

► Split-skin grafting: sheets of normal pigmented epidermal skin are transplanted in depigmented lesions.

► Suction blister roof grafting: the roofs of artificially created suction blisters are transplanted in depigmented lesions.

► Cultured cell grafting: melanocytes are extracted from skin punch biopsies and cultured in a laboratory. These are harvested and sowed in depigmented lesions.

Transplantation techniques are often combined with phototherapy to stimulate the growth of the transplanted melanocytes (71,83,84).

Psoralen UVA (PUVA) phototherapy has been used to treat vitiligo since the ancient Egyptians (3). At this moment PUVA is the main treatment for vitiligo in most countries and is being regarded as the most effective repigmenting therapy, sometimes applied as topical photochemotherapy (bath or cream PUVA) but mostly used as systemic photochemotherapy (oral PUVA). The exact mechanism of repigmentation under photochemotherapy is still unknown. Several authors suggest that by decreasing the amount of LC's and immunosuppression the autoimmune phenomenon can be halted, and thereby changing the balance of de-/repigmentation and thus allowing the skin to repigment again (3,85).

Photochemotherapy is generally considered a modified phototoxic treatment with

hazardous side-effects. Recent studies have shown that photochemotherapy with treatment schedules with over 200 exposures significantly enhance the risk of skin cancer (86-88). It is however still unclear whether vitiligo patients are more or less prone to photocarcinogenesis.

Recently, narrow band UVB phototherapy has been designated as a safe and effective way to treat vitiligo patients (89). However, large controlled studies on the efficacy of narrow band UVB have not been performed yet. Furthermore, several authors suggest that the addition of vitamin B12 and folic acid may enhance photo-repigmentation (72,90). Chapter 4 will take a closer look at this new treatment in terms of efficacy and quality of life and investigates the claim of enhanced photo-repigmentation with the addition of vitamin B12 and folic acid.

1.5 Aims

The aims of this thesis are:
I.   Further elucidation of the photobiological effects, in particular regarding photodamage of UVA, broadband UVB, narrowband UVB and visible light.
II.   To find out to what extent anti-TNF-α treatment modulates the response to UVB challenge
III.   To find out the clinical efficacy of narrow UVB treatment in vitiligo and the impact of this treatment on quality of life.

Questions:
I.a   What is the difference with respect to photodamage comparing UVA and UVB?
I.b   What is the difference regarding photodamage comparing broad band and narrow band UVB?
I.c   Is visible light capable in inducing photodamage?
II.a   What is the effect of anti-TNF-α treatment on the photobiological effect after a single broad band UVB challenge
III.a Is narrow band UVB an effective treatment for vitiligo vulgaris?
III.b Does the addition of vitamin B12 and folic acid modulate the efficacy of narrow band UVB phototherapy in the treatment of vitiligo vulgaris?
III.c Does long-term narrow band UVB phototherapy for vitiligo vulgaris influence the quality of life?

1.6   Methodology

Figure 1-3: Waldmann 7001k cabin

1.6.1   UV and visible light challenge models

To address questions 1.1 up to 2.1 several UV and visible light challenge models were used.

All UV irradiations were performed with Waldmann UV phototherapy units (Waldmann 7001K, Waldmann Medizintechnik, Villingen-Schwenningen, Germany) equipped with broadband UVB (Philips UV21, Philips B.V., Eindhoven, the Netherlands), narrow band UVB (Phillips TL-01) or UVA (Philips UV6) lamps (see figure 1-3). Each lamp has its' own emission spectra and wave distribution. Figures 1-4 to 1-6 show the emission spectra of these lamps and the relative wave distribution.
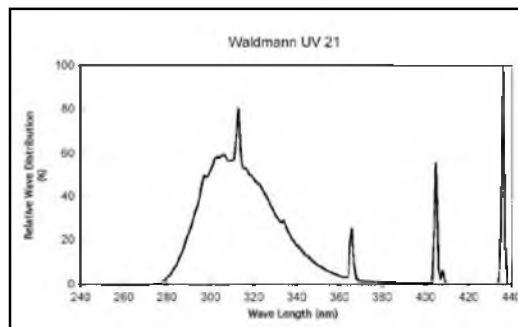
Figure 1-4: UV21 Broad band UVB distribution
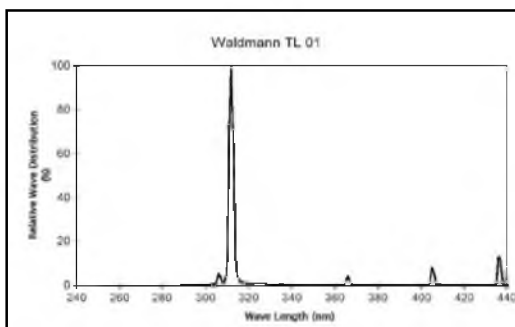
Figure 1-5: TL-01 Narrow band UVB distribution
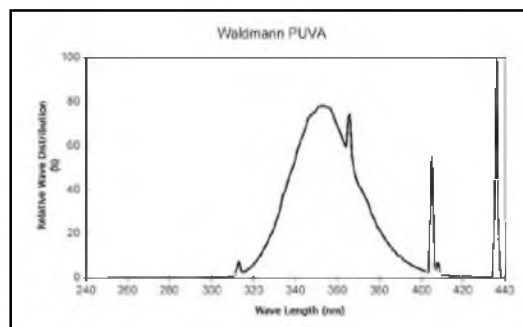
Figure 1-6: UV6 Broad Band UVA Distribution

All UV challenge models used single dose irradiation of 2-3 Minimal Erythema Doses (MED) with punch biopsies of unirradiated skin and irradiated skin at several time points (usually 4, 7, 24 or 48 hours) post exposure.

Minimal Erythema Dose was defined as just perceptible erythema 24 hour after

irradiation and was assessed by irradiating six circular areas of 2.5 cm in diameter located horizontally just above the buttocks with an incremental dosage scheme (91).

Table 1-2: Fitzpatrick's Skintypes	
Skin type	**Description**
I	Always burns easily, never tans
II	Always burns easily, tans minimally
III	Burns moderately, tans gradually and uniformly (light brown)
IV	Burns minimally, always tans well (moderately brown)
V	Rarely burns, tans profusely (dark brown)
VI	Never burns, deeply pigmented

Depending on skin types, as defined by Fitzpatrick (see table 1-2), and the used UV source an appropriate dosage scheme was selected (see table 1-3) (92,93). After MED assessment, the skin on the unexposed buttocks was irradiated once with an appropriate dose set by the biological response (i.e. dose related to the individual MED).

Table 1- 3: MED Dosage schemes according to skintype	
Skin type	**MED dosage scheme**
Broad band UVB	
I-II	0.05-0.09-0.12-0.16-0.20-0.27 J/cm^2
III-IV	0.05-0.10-0.15-0.20-0.30-0.40 J/cm^2
Narrow band UVB	
I-II	0.50-0.70-0.90-1.10-1.30-1.50 J/cm^2
III-IV	0.50-0.70-0.90-1.10-1.30-1.50 J/cm^2

Figure 1-7: Waldmann PDT 1200 Unit

High dose visible light has been given in repeated exposures with a Waldmann PDT 1200 L (Waldmann Medizintechnik, Villingen-Schwenningen, Germany, see figure 1-6) equipped with a Philips 1200 MSR metal halogen lamp (Philips B.V., Eindhoven, the Netherlands), which has an emission spectrum of 560-780 nm after filtering with the build-in dichroic cut-off filters (DT Rot and Calflex-3000, Balzers Optik, Nurnberg, Germany). Irradiations are also given on the buttocks, as these areas of the skin have had only minimal exposure to light.

After irradiation, biopsies were taken at predefined time points.

Whether UVB, UVA or visible light was given, in all cases the surrounding skin was protected with light impenetrable cloth.

1.6.2 Immunohistochemical analysis

Processing of biopsies

Punch biopsies (Stieffel, Offenbach am Main, Germany) were taken with 1% Xylocain and Adrenalin anesthesia and, depending on the immunohistochemical staining, embedded in Tissue-Tec OCT compound (Miles Scientific, Naperville, USA) and snap frozen in liquid nitrogen or embedded in paraffin after a 4-hour fixation in formaldehyde.
All biopsies were cut in 6-7 µm thick slides and stained with H & E or with immunohistochemical markers and counter stained with Mayer's haematoxylin (Sigma, St. Louis MO, USA).

Histological analysis
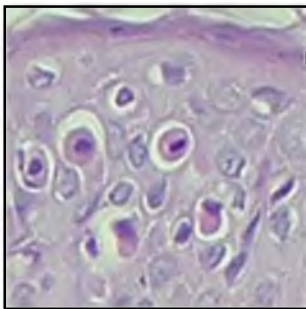
Figure 1-8: Sunburn cells

H & E stained slides were assessed for sunburn cells (apoptosis), perinuclear vacuolisation and perivascular inflammation. Sunburn cells are defined as epidermal cells with pyknotic nuclei and a strong eosinophilic cytoplasm. The sunburn cell is a specific marker for apoptotic keratinocytes. Apoptotic keratinocytes are a characteristic of UV irradiated skin. Sunburn cells were quantified and scored as the amount of positive cells in the epidermis. Inflammation was scored on a semi-quantitative scale (0: none present, 1: minimal, 2: moderate and 3: pronounced amount/staining) (94,95).

Immunohistochemical analysis

The immunohistochemical antibodies, mentioned in table 1-4, were used to asses photodamage and photo ageing. Most markers were stained using the Avidin-Biotin Complex amplification technique. Only Langerhans cell staining was performed with the peroxidase-anti-peroxidase amplification technique. Both techniques are described more in detail in the following chapters.
Epidermal nuclear stainings like p53, c-jun and p16 were scored as amount of positive cells/mm length or per 1000 cells. A similar approach was used for the cytoplasmatic

stainings T6, and Melan A. Inflammatory infiltrate was subdivided in lymphocytes, polymorphonuclear leukocytes and monocytes/macrophages. These groups were scored as the percentage of positive cells in the perivascular infiltrate on a validated semi-quantitative scale as reported before (0 = 0%, 1= 1-24%, 2 = 25-49%, 3 = 50-74%, 4 =75-100%).

Table 1-4: Overview of used antibodies and dilutions

Antibody	Target	Dilution
Monoclonal murine anti-human p53 protein (Dakopatts, Glostrup, Denmark)	(wild type and mutant) p53 protein	1:200
PhosphoPlus c-Jun (Ser63) II Antibody kit (New England Biolabs Inc., Beverly, MA, USA)	phosphorylation on point 63 of c-Jun	1:50
Monoclonal murine anti-elastin antibody (Sigma, St Louis, USA)	Elastin fibers	1:500
Ab-4 Mouse anti-human p16 (Neomarkers, Fremont, USA)	P16	1:100
Monoclonal murine anti-human MMP-1 (Oncogene, San Diego, USA)	MMP-1	1:15
DAKO T-11 (Dakopatts, Copenhagen, Denmark)	T-lymphocytes	1:100
DAKO T6 (Dakopatts, Copenhagen, Denmark)	Langerhans cells	1:100
DAKO anti-elastase (Dakopatts, Copenhagen, Denmark)	PMN's	1:100
WT14 (UMCN, Nijmegen, the Netherlands)	Monocytes/macrophages	1:100
Monoclonal murine anti-human Melan A (MART-1, Oncogene, San Diego, USA)	Melanocytes	1:50

1.6.3 Studies during anti-TNF-α induced immunosuppression

Recently, adalimumab, a fully human chimeric anti-TNF-α antibody (Knoll AG, Ludwigshafen, Germany) has been introduced for the treatment of rheumatoid arthritis (96). The antibody is administered subcutaneously and maximum clinical efficacy can be observed 2 weeks after the first administration. In this thesis the effects of the first subcutaneous administration of anti-TNF-α (0.5 mg/kg) on the UVB response are studied. Minimal erythema dose assessment was performed before and 2 weeks after the administration of the antibody. Biopsies were taken from unirradiated and 2 MED irradiated skin before and 2 weeks after administration of the antibody for immunohistochemical analysis.

1.6.4 Studies in Vitiligo

In order to answer the questions regarding phototherapy in vitiligo, a clinical trial was performed in which long term stable vitiligo patients were randomised in two groups, one with vitamin B12 and folic acid twice daily and one without. All patients were irradiated with narrow band thrice weekly.

Clinical Assessment

There is no standardised and validated scoring method for evaluating the clinical efficacy of therapies in vitiligo like the Psoriasis Area Severity Index (PASI) for psoriasis treatment evaluation. Several studies have reported their own systems for scoring repigmentation during treatment (97-99). These scoring methods have their limitations as they only evaluate the overall final result as a percentage on a x-point scale. The following methods are the most used.

► If overall repigmentation is more than 80%, these patients are scored as complete repigmentation.
► Other scores divide the scale in 3 (1 = <30%, 2 =30-60%, 3= 60-100%).

However, the following aspects of vitiligo seriously limit the validity of these approaches. The most important mode of repigmentation is the perifollicular repigmentation, which causes substantial inter-observer variation. Furthermore, there is a lot of variability between and within vitiligo patients with respect to body region. It is generally known that specific regions do pigment easily while others do not repigment at all. Therefore, overall scores do not provide clinical relevant information. Surprisingly, there is no study available, which evaluates repigmentation per region. Although quality of life scoring during treatment is a common aspect of e.g. psoriasis or atopic dermatitis treatment evaluation, this has never been reported in vitiligo patients, although it is known that vitiligo patient generally do suffer from the psychosocial implications of their disease.

To overcome the mentioned shortcomings we evaluated repigmentation per region during and at the end of the study and evaluated the quality of life after treatment.

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2

Studies on photobiology

2.1   The time course of the in vivo response to oxidative stress following UVA and UVB exposure

2.1.1 Abstract

Background Ultraviolet radiation induces a range of photobiological effects, such as an inflammatory response, photodamage and photoageing. It has long been hypothesised that UVA exerts it effects through oxidative processes, while UVB directly damages cell structures.

Objectives The aim of the present study is to elucidate the differences and possible relationships between the time course of UVA and UVB induced photodamage, inflammation and oxidative damage in vivo.

Patients/Methods Fourteen volunteers were challenged with one MED UVB or 60 J/cm^2 UVA on the lower back. At several successive time points biopsies, tapestrips and ethanol extractions were collected from irradiated and non-irradiated skin. These samples were analysed for photodamage markers (p53, c-Jun, apoptosis), inflammatory markers (T-lymfocytes, monocytes, polymorphonuclear leukocytes, Langerhans cells) and markers for the oxidative response (lipid peroxidation, catalase).

Results Both UVA and UVB induce markers for photodamage and inflammation. Although generally comparable effects were seen, UVB showed an earlier and more pronounced induction of photodamage markers and an earlier influx of an inflammatory infiltrate. Remarkably, lipid peroxidation increased by 882% one hour after UVA irradiated compared to an 111% increase in UVB irradiated skin. Catalase activity, as a maker for the oxidative defence, decreased by 83% after UVA exposure without returning to baseline levels within the studied period.

Conclusions Although maximal infiltrate accumulations were comparable, UVB showed earlier induction of markers for inflammation, suggesting that UVB and UVA challenges were equivalent in the present experimental approach with respect to inflammation. A more pronounced induction of markers for photodamage was seen following UVB as compared to UVA. In contrast, markers for oxidative damage were expressed substantially more following UVA exposure as compared to UVB exposure. It is hypothesised that the early induction of oxidative damage following UVA postpones photodamage and inflammation as compared to the dynamics of UVB.

2.1.2 Introduction

Exposure to ultraviolet radiation can have deleterious effects, like sunburn, photoageing and skin cancer. Understanding the underlying mechanisms is important

when studying photobiological profiles of different UV spectra. Photobiological processes are mediated by type I and type II photochemical reactions. Type I reactions are induced directly by UV irradiation, whereas type II reactions are indirect and mediated by reactive oxygen species and other oxidative products (1). UVB is considered to induce mainly type I reactions, while UVA is responsible for type II reactions. The present study compares UVA and UVB photoresponses and focuses on time course relationships between oxidative processes, photodamage and UV induced immunomodulation.

Acute excessive UV exposure may result in damage of cellular structures. This damage activates protective mechanisms such as the upregulation of the tumour suppressor gene p53, which allows the cell to repair the damage or if the damage is too extensive to undergo apoptosis. P53 expression is induced 3 hours after UVA and UVB irradiation, reaches its maximum at 12 hours and disappears 72 hours after exposure. P53 is expressed in all cell layers of the epidermis after UVB exposure, whereas after UVA challenge the expression is limited to the basal layers (2-4). Apoptotic keratinocytes have the histological appearance of sunburn cells. They appear 6 hours after irradiation, peak at 24 hours, and are mainly seen after shortwave UV irradiation (UVC and UVB). Sunburn cells appear only sporadically in UVA irradiated skin (3,5,6). C-Jun is a marker for the early induction of photoageing and is called an early response gene. It is induced 15 minutes post irradiation and peaks at approximately 8 hours after UVB irradiation. C-Jun expression after UVA follows a similar pattern but is less pronounced (7).

The inflammatory responses following exposure to UVA and UVB are characterised by an influx of polymorphonuclear leukocytes up to 14 hours after the irradiation. This is followed by an influx of T-lymphocytes, which concentrate perivascularly. Futhermore epidermal Langerhans cell counts reduce within one hour after irradiation, with complete depletion 24 hours after UVB exposure (8-10). A single dose of 100 J/cm^2 UVA may also reduce the amount of Langerhans cells up to 75% after 24 hours (11). A comparative study on the above-mentioned photoresponses between UVA and UVB is not yet available.

Oxidative processes play a major role in UVA induced photobiological processes, which eventually result in photodamage and photoageing. Several authors have shown that UVA and UVB induce the formation of reactive oxygen species (12,13). Recently, Sander et al. demonstrated in vivo a dose dependent protein oxidation and antioxidant depletion by UV radiation. These observations are compatible with the demonstration of oxidative products and depleted antioxidant systems in photoaged skin (14). These observations suggest that photoageing is associated with protein oxidation and antioxidant enzyme depletion. Recently, it was shown that catalase, belonging to the anti-oxidant system, is induced selectively by UVA, whilst UVB does not induce any catalase activity (15). This observation suggests that there may be substantial differences between UVA and UVB with respect to oxidative responses.

To study processes in vivo, usually biopsies for cellular and molecular studies are taken. Recently, Maes et al. described several non-invasive techniques to measure oxidation products in the upper cell layers from the skin, such as squalene and squalene hydroperoxide (16). Giacomoni et al. described a new technique to study catalase, an antioxidant defence, at the skin surface by analysing catalase activity on corneocytes removed by adhesive tape (17,18). These two non-invasive techniques permit a simplified reproducible analysis of the oxidative defence mechanisms in the skin; however so far these parameters have not been studied simultaneously with parameters for photodamage and inflammation.

In the present study we combined immunohistochemistry and the two above described non-invasive techniques to investigate the time course of photodamage, inflammation and oxidative damage of UVA and UVB irradiation. Furthermore, this study compares the photobiological responses between UVA and UVB in one experimental study.

The Minimal Erythema Dose (MED) of UVB and a dose of 60 J/cm^2 UVA were chosen as the irradiation dose. Several studies use MED for UVA, however in our experience no erythema is induced with proper UVB/C shielding when using UVA lamps. The other studies indicate that the mean "UVA MED" approaches the dose of 60 J/cm^2 used in the present study (19-21). Preliminary experiments revealed that irradiation with a dose of up to 60 J/cm^2 is possible without inducing a more than mild transient erythema, directly following exposure.

2.1.3 Material and Methods

Patients and UV irradiations

Fourteen healthy volunteers with Fitzpatrick Skintype II/III without a history of recent excessive sun exposure or tanning were included after written informed consent was obtained. The volunteers were divided at random in 2 groups of 7 subjects, an UVA and an UVB irradiated group. UVB irradiation was performed in a Waldmann 7001 cabin equipped with Phillips UV21 broadband UVB lamps. Minimal erythema dose testing was performed in each 'UVB irradiation' volunteer and was defined as the dose which can induce a just perceptible erythema 24 hours after irradiation with broad band UVB. UVA irradiation was performed with a 2100 W Dermalight 2020 sol 5 (Dr. K. Hönle Medizin technik, München, Germany) equipped with an infrared and UVB filter (Coimex Trading BV, Hattem, the Netherlands). The volunteers were irradiated at an area of 10 x 20 cm on the left side of the lower back/buttock with one MED broad band UVB or with 60 J/cm^2 UVA depending on their randomisation. The right side of the lower back/buttocks was designated as control side.

In UVB irradiated volunteers and UVA irradiated volunteers tapestrips, extractions and biopsies were collected 1, 4 and 24 hours and 1, 4, 7, 24 and 48 hours after irradiation respectively. The biopsies were embedded in Tissue Tek OCT compound

(Miles Scientific, Naperville, USA), snap frozen in liquid nitrogen and stored at -80 °C until further analysis. Sections of 6 μm were cut, air dried and fixed in acetone and again stored at -80 °C.

Monoclonal antibodies

The following monoclonal antibodies were used for immunohistochemical analysis of photodamage: Monoclonal Murine anti-human (wild type and mutant) p53 protein (1:200) (Dakopatts, Glostrup, Denmark) and PhosphoPlus c-Jun (Ser63) II Antibody kit (1:50) (New England Biolabs Inc., Beverly, MA, USA) phosphorylation on point 63 of c-Jun. HE stainings were performed to evaluate epidermal UV induced damage and inflammation. Analysis of inflammatory infiltrate was done by assessment of T-lymphocytes, Langerhans cells, polymorphonuclear leukocytes (PMN) and monocytes/macrophages respectively using the monoclonal antibodies DAKO T-11 (1:100) (Dakopatts, Copenhagen, Denmark), DAKO T6 (1:100) (Dakopatts, Copenhagen, Denmark), DAKO anti-elastase (1:100) (Dakopatts, Copenhagen, Denmark), WT14 (1:100) (UMC St Radboud, Nijmegen, the Netherlands).
The non-invasive techniques to study oxidation did not use antibodies but measure enzyme activity.

Immunohistochemical staining

The slides were air dried and immersed in a phosphate buffered saline (PBS). All slides were incubated for 15 minutes with 20% normal horse serum (Vector Laboratories, Burlingame, USA) in 1% bovine serum albumin (BSA, Organon Technika, Boxtel, the Netherlands) in PBS and then by 1 hour incubation with the primary antibody (p53, c-jun, T11, WT14 and anti-elastase). The slides were washed in PBS and incubated with rabbit anti-mouse biotinylated IgG (ABC kit-mouse, Vector Laboratories, Burlingame, USA) for 30 minutes. The sections were washed again in PBS and incubated with anti-rabbit biotinylated IgG for 30 minutes.
All slides were washed in PBS and incubated with avidin biotin complex solution (ABC kit-mouse, Vector Laboratories, Burlingame, USA) for 30 minutes and labelled with metal enhanced DAB substrate (Pierce, Rockford, USA) for visualisation.
The slides were counter-stained with Mayer's haematoxylin (Sigma, St. Louis MO, USA) and after dehydration with 100% ethanol and histosafe mounted in Permount (Fisher Scientific, New Jersey, USA).
For T-6 an indirect peroxidase technique was used. The slides were washed in phosphate-buffered saline and incubated for 1 hour at room temperature with the primary antibody. The slides were washed twice in PBS and incubated for 30 minutes with rabbit-anti-mouse-immunoglobulins (Dakopatts, Copenhagen, Denmark) conjugated with peroxidase, diluted 1:50 in PBS and containing 5% human AB

serum. After two further washes in PBS and preincubation with sodium acetate buffer (pH 4.9) the slides were stained with 3-amino-9-ethyl-carbazole for 10 minutes. The slides were counter-stained with Mayer's haematoxylin and mounted in glycerol gelatin (Sigma).

Histological examination

The histological examination was performed blindly by two observers. Photodamage was measured by counting the number of p53 positive nuclei per mm length of section. The epidermal expression of Langerhans cells, c-Jun expression, apoptosis and dermal inflammation were scored on a semi-quantitative scale (0: none present, 1: minimal, 2: moderate and 3: pronounced amount/staining). T-cells, monocytes/ macrophages and polymorphonuclear leukocytes were assessed by scoring the percentage of these inflammatory cells in the dermal infiltrate on a semi-quantitative scale (0: 0%, 1: 1-4%, 2: 5-24%, 3: 25-49%, 4: 50-74%, 5: 75-100%).

Measurement of lipid peroxides at the skin surface

For the evaluation of skin surface lipid peroxides ethanol washes were collected from irradiated and non-irradiated sites on the lower back and stored at -80°C until further analysis. Using a HPLC technique as described by Maes et al. squalene and squalene hydroperoxide levels were measured (16). Briefly: squalene and squalene hydroperoxide were separated from other lipids on a reversed phase C18 column. Squalene was detected at 220 nm, while squalene hydroperoxide was detected after post-column reaction with iron (II) and sulfosalicylic acid. The lipid peroxide value was defined as mol% squalene hydroperoxide over residual squalene.

Measurement of catalase activity on tape strippings from stratum corneum

For the evaluation of antioxidant enzyme activity in the stratum corneum twenty successive D-squame® tape strippings (CuDerm Corporation, Dallas, TX, USA) were collected from the lower back in the UVA irradiated subjects and stored at -80°C until further analysis. Enzyme activity was evaluated as described before (15,22). Briefly: the assesment of catalase activity is based on the ability to reduce hydrogen peroxide using methanol as proton donor, leading to the formation of formaldehyde, which can be detected after reaction with Purpald (Sigma-Aldrich Corporation, St.Louis, MO, USA). Specific catalase activity was obtained after normalisation to the total protein content on the same tape stripping, which was quantified as the total amount of amino acids after acid hydrolysis at high temperature.

Statistical evaluation

The data have been analysed using the Statistica Statistical '98 package 5.1. Data are expressed as means ± SEM. For statistical analysis of the immunohistochemical part one-way ANOVAs were performed to asses significant changes in expression during the time after irradiation. If the expression changes significantly after irradiation Duncan's post hoc comparisons test was performed to establish at which timepoints significance was reached. For the non-invasive techniques a Student t-test (paired observations, two tailed distribution, two sample unequal variance). Possible correlations were analysed with the Pearson product-moment correlation. A p-value < 0.05 was considered significant.

2.1.4 Results

Both UVA and UVB irradiation were well tolerated by the volunteers. 60 J/cm^2 UVA only resulted in a transient slight heat erythema and some immediate pigment darkening.
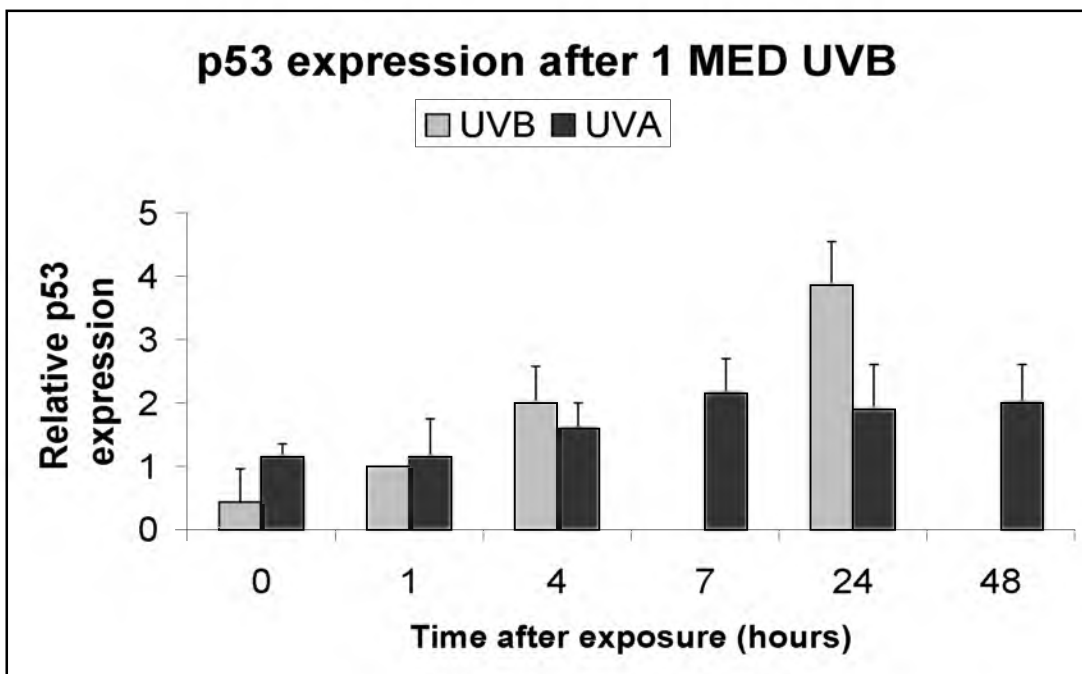
Figure 2-1-1 Time course of p53 expression after UVA and UVB exposure. UVB induces an earlier and more pronounced expression of p53.
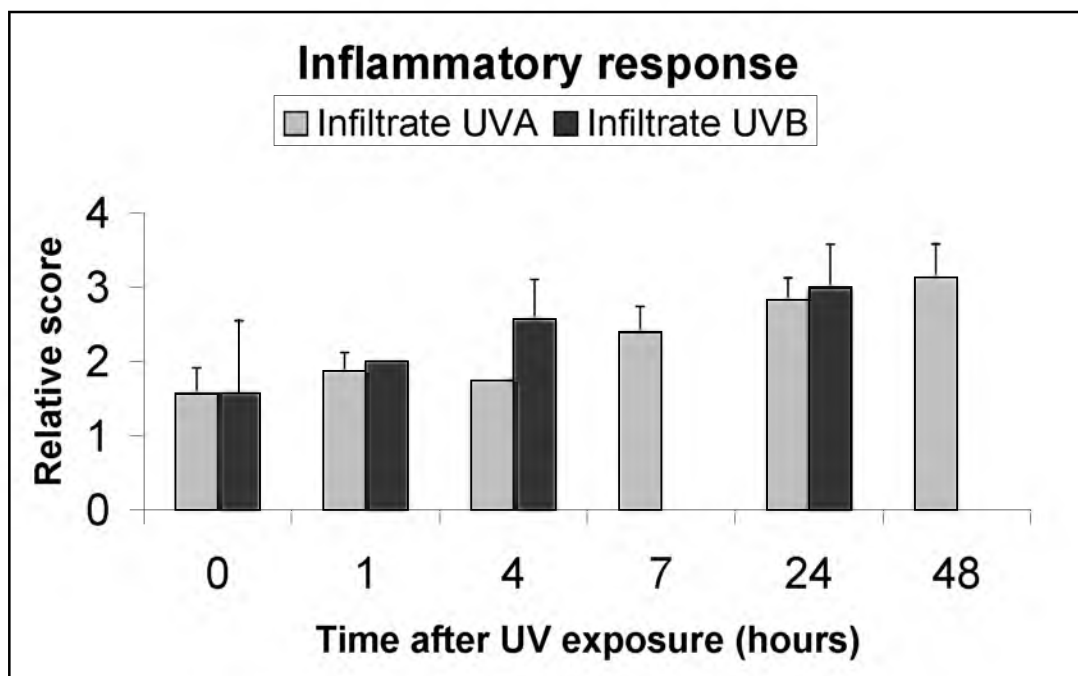
Figure 2-1-2 Time course of inflammatory response after UVA and UVB exposure. The UVB induced inflammatory response reaches significance at 4 hours post irradiation whereas UVA reaches significance 7 hours post irradiation.

Photodamage

P53 expression showed a significant increase in both UVA and UVB irradiated individuals (p<0.01). Seven hours after UVA exposure significance was reached (p<0.01). In UVB exposed skin significance was reached 4 hours after exposure (p<0.01), which peaked 20 hours later (p<0.01), without returning to baseline levels within the studied time frame (Figure 2-1-1).

Phosphorylated c-Jun also showed a significant increase in expression (p<0.01) starting 24 hours after irradiation for UVA (p<0.01) and 4 hours after irradiation with UVB (p<0.01), which also did not return to normal levels within the study period. UVA as well as UVB irradiation did not induce keratinocyte apoptosis at the used dosages, as no sunburn cells were seen at any of the time points.

Inflammation

Both UVB and UVA induced dermal perivascular inflammation (p<0.01), which was more pronounced and earlier in UVB irradiated skin. UVA induced inflammation reached significance 7 hours post irradiation and peaked at 48 hours (p<0.01),
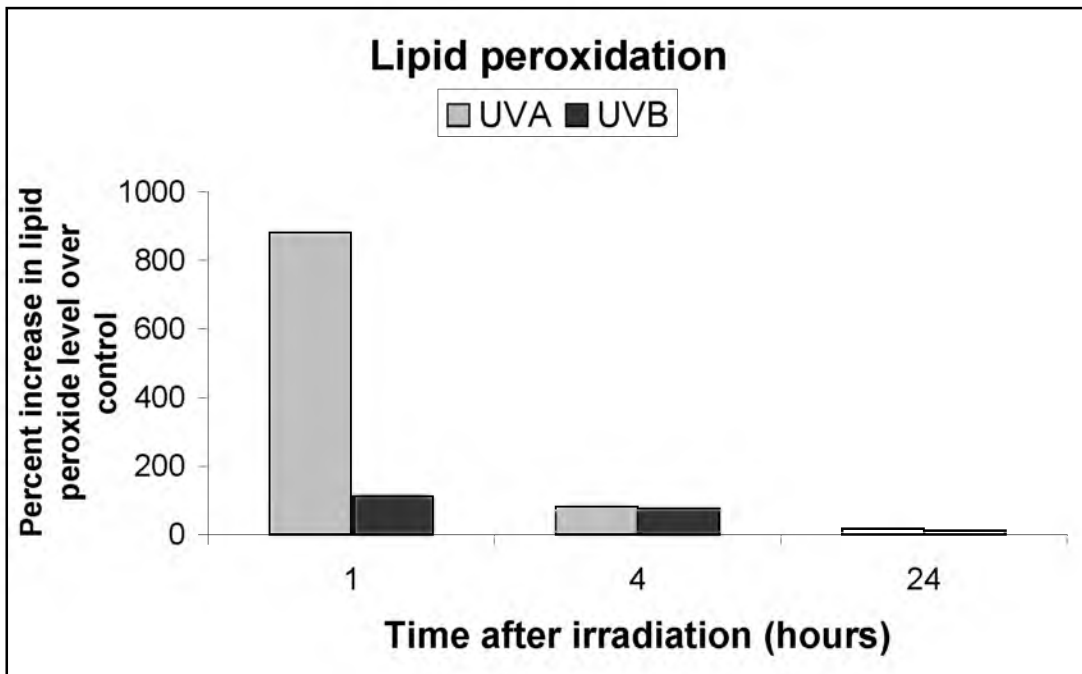
Figure 2-1-3 Time course of lipid peroxidation levels in UVA and UVB irradiated skin. An up to 882% increase in lipid peroxidation is seen 1 hour after UVA irradiation compared to only 111% in UVB irradiated skin.

whereas UVB induced inflammation reached significance 4 hours after irradiation and increased at least up to 24 hours after irradiation ($p<0.01$) (Figure 2-1-2). The increase in perivascular infiltrate was mainly attributed to the influx of T-lymphocytes in both UVB ($p<0.01$) and UVA ($p<0.01$) irradiated skin. The perivascular infiltrate did not return to baseline levels. We did not detect any change in the number of Langerhans cells, polymorphonuclear leukocytes and monocytes/macrophages count.

Oxidative processes

Lipid peroxidation was evaluated as the amount of squalene hydroperoxide (SO) on the skin surface as detected by HPLC. UVA induced a 882% increase in SO levels within 1 hour after irradiation ($p<0.01$), after which the SO levels gradually returned to baseline levels at 24 hours. During this decrease, no significant difference with the control was seen (Figure 2-1-3).

In contrast, UVB irradiation only resulted in a 111% increase in SO levels ($p<0.01$), which returned to baseline levels within 4 hours after irradiation (Figure 2-1-3).

Catalase activity, as a marker for antioxidant defence, decreased by 69% 1 hour after and 83% 3 hours after UVA irradiation. We did not observe normalisation 48 hours after UVA irradiation (Figure 2-1-4).
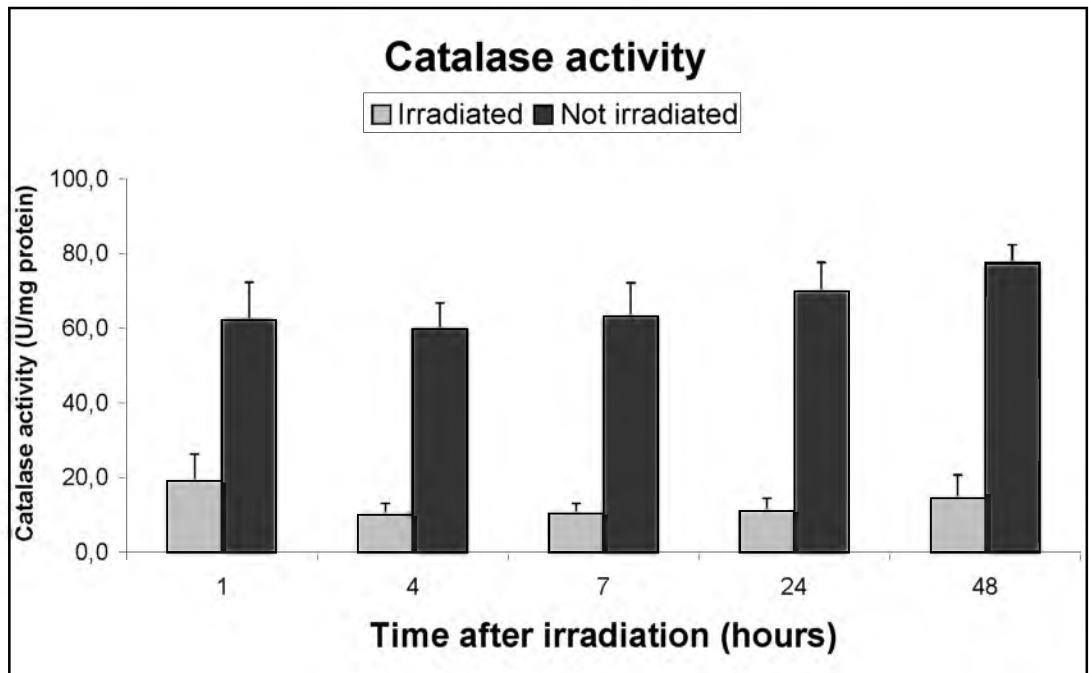
Figure 2-1-4 Time course of catalase activity in UVA irradiated and non-irradiated skin. A significant decrease in catalase activity in UVA irradiates skin is observed, which did not return to baseline levels within the studied timeframe.

Correlations

There were no statistically significant correlations between lipid peroxidation or catalase activity and the markers for photodamage and inflammation.

2.1.5 Discussion

The present study reconfirms that UVB as well as UVA induce expression of p53 and phosphorylated c-Jun (3,4,7). For UVB, the expression was more pronounced than for UVA. Furthermore, UVA induced p53 expression declined 24 hours after irradiation, while UVB induced p53 seemed to increase further. Phosphorylated c-Jun (early response gene) showed an earlier expression after UVA as well as UVB irradiation. At the used irradiation dosages of 1 MED UVB and 60 J/cm^2 UVA no sunburn cells were detected. This finding is in concordance with studies by Gilchrest et al. (20), although other investigations have described the appearance of sunburn cells at erythematogenic UVA doses (3,5,6).
Perivascular and upper dermal infiltrate with predominantly polymorphonuclear leukocytes and T-lymphocytes was also observed. No significant change in polymorphonuclear leukocytes and monocytes/macrophages ratio in the perivascular

inflammation was seen. No depletion of Langerhans cells was noted. This could probably be attributed to the low and single challenge dosage of UVA and UVB. It has been reported before that repeated low dose (1.5 MED) UVB and (0.5 MED) UVA can reduce the epidermal Langerhans cell count by at least 50% (8,19).

Lipid peroxidation as measured by the squalene peroxide levels was more pronounced in UVA irradiated skin (up to 882% increase) compared to UVB irradiated skin 1 hour after irradiation. Four hours after irradiation the mean peroxide levels of the irradiated side were still increased compared the non-irradiated control side, although the values were not significantly different. Within the following hours, the squalene peroxide levels returned to similar levels as the controls. Catalase (an important oxygen radical scavenger) activity in the upper epidermis, decreased with 69% within 1 hour after UVA irradiation and did not return to normal levels within 48 hours. This is in concurrence with our previous report, where we have shown that recovery of catalase activity occurs in 3-4 weeks after UVA exposure at an age-dependent rate (15). Interestingly, UVB exposure did not affect catalase activity (15). Recently, Sander et al. demonstrated for the first time that photoaging is indeed associated with protein oxidation (14). Furthermore, reactive oxygen species can result in DNA damage (23). It could thus be hypothesised that the UVA and UVB induced production of reactive oxygen species may influence photoageing (e.g. c-Jun) and photodamage (p53, apoptosis).

There was an intriguing delay in expression of the p53 and c-Jun markers after UVA irradiation. It could be hypothesised that this delay can be attributed to the preference of UVA for type II (oxidative pathway) photochemical reactions. It has been shown that oxidative stress can damage keratinocytes and induce expression of p53 and c-Jun (24,25). Furthermore, type II (indirect) reactions take more time to damage cells than the direct type I reactions. The present study shows that oxidative processes are more involved in UVA induced photobiological processes. These processes may account for the delay in c-Jun and p53 expression.

This study shows that no statistically significant correlation exists between any of the oxidative damage markers and photoaging, photodamage and inflammation markers, as calculated by Pearson product-moment correlations.

In the present study parameters for photodamage, inflammation and oxidative changes, following UVB and UVA challenge, have been studied in one single experimental approach. The MED for UVB can be determined easily. However, the MED for UVA is difficult to assess as relatively high doses are required, which imply that slight UVB contamination in these UVA sources confuse the MED substantially. Therefore it is virtually impossible to generate equivalent doses of UVB and UVA with respect to erythema. In the present study we approached the UVA dose range, which has been suggested to be the MED. This range has been reported to be 14-56 J/cm^2 (19-21).

Assuming that the present study has approached equivalent challenges of UVB and

UVA, several remarkable differences between UVA and UVB were observed. Firstly a highly significant and substantial difference was observed between UVA and UVB with respect to the induction of lipid peroxidation (fig. 2-1-3). Another difference was the relatively early induction of p53 and c-Jun following UVB exposure as compared to UVA exposure.

In conclusion, UVB showed an earlier and pronounced induction of markers for photodamage compared to UVA in this in vivo model. Therefore, induction of photodamage is not restricted to UVB but also is a hazard of UVA. Furthermore, inflammation was also earlier induced in UVB irradiated skin. Remarkably, lipid peroxidation proved to be a highly characteristic early phenomenon of UVA induced damage, which was far less expressed following UVB. Therefore, skinageing is a serious hazard of UVA exposure.

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2.2   The differential effect of broad band versus narrow band UVB with respect to photodamage and cutaneous inflammation

2.2.1 Abstract

Background Since Fischer reported on the superiority of 313 nm UVB compared to broad band UVB in the treatment of psoriasis, narrow band UVB has become the main phototherapeutical modality in several countries. There is some discussion about the safety and photobiological effects of narrow band UVB. In the present study narrow- and broad band UVB have been compared with respect to parameters for photodamage and inflammation.

Patients/methods Fourteen healthy volunteers were randomized in two groups. Both groups were irradiated with 3 Minimal Erythema Doses (MED) of narrow or broad band UVB respectively. Before and 4, 24 and 48 hours after irradiation 6 mm biopsies were taken for immunohistochemical analysis of p53, apoptosis and p16 (photodamage parameters) and T-cells, polymorphonuclear leukocytes and Langerhans cells (inflammatory cells).

Results Mean MED for narrow band UVB was 8.125 times higher than broad band UVB. Significant changes in expression were seen for all parameters except for p16. P53, apoptosis, T-cells and polymorphonuclear leukocytes increased, while Langerhans cell count decreased significantly. No significant differences were seen between the narrow and broad band UVB.

Conclusions In conclusion: following irradiation of 3 MED narrow band UVB and broad band UVB safety parameters for carcinogenesis and inflammation were induced to the same extent. As narrow band UVB is more effective than broad band UVB, the present study suggests superiority of narrow band UVB as a treatment with a better benefit risk ratio.

2.2.2 Introduction

Broad band UVB (290-320 nm) and Psoralen UVA photochemotherapy have been the main phototherapeutic treatments for several chronic inflammatory skin diseases for many decades. In 1976 Fischer demonstrated that narrow band (313 nm) UVB was more effective in the treatment of psoriasis than broad band UVB (1). This finding was confirmed by Parrish & Jaenicke (2). Inspired by these results a new fluorescent bulb, the TL-01 lamp, was developed by Philips which emits almost exclusively 311/312 nm UVB. Narrow band UVB is not only more effective than broad band UVB, but it also approaches the efficacy of PUVA photochemotherapy with less side effects (3,4). At present, narrow band UVB is replacing broad band UVB and even PUVA in several countries as the main modality in phototherapy for most chronic

inflammatory skin diseases.

As narrow band UVB excludes the short wavelengths of the UVB spectrum, and is closer to UVA, higher doses are needed to induce a photobiological effect (5). Therefore, in the treatment of psoriasis, where near-erythematogenic doses are recommended, individual exposures are at a higher dose, while the duration of a treatment course is shorter with less exposures (6,7). Cumulative dosages for narrow band UVB are generally higher compared to doses with broad band UVB (7,8).

A major question remains to be answered: To what extent is narrow band UVB different from broad band UVB with respect to photodamage, ageing and inflammation?

The aim of the present study was to investigate whether these two UVB modalities induce different photobiological effects to markers for photocarcinogenesis and inflammation.

Markers for photocarcinogenesis are p53, p16 and apoptosis. Several reports have indicated that narrow band UVB may be more photocarcinogenic than broad band UVB (9-11), while others contradict these (12). Single exposure to high dose broad and narrow band UVB has been shown to cause a substantial induction of p53, a tumor suppressor gene, and apoptosis (histologically seen as sunburn cells) (13,14). P16 is a nucleotide excision repair protein, which is most strongly expressed in melanocytes after shortwave UV irradiation (15,16). It is unknown if there are any differences in expression of these markers between broad and narrow band UVB.

Markers for inflammation are T6, T11 and elastase. UVR exposure results in an inflammatory reaction with at first a predominantly neutrophil perivascular infiltrate. Subsequently Langerhans cells migrate from the epidermis into the dermis (14,17,18). There are no data available on differences in expression between broad and narrow band UVB with respect to this matter.

In this present study doses of 3 MED of narrow band UVB and broad band UVB were given to healthy volunteers. At 4, 24 and 48 hours after exposure punch biopsies were taken and immunohistological assessment was performed on the following markers: p53, p16, apoptosis, T6, T11, elastase.

2.2.3 Material and Methods

Patients and UVB irradiation

Fourteen healthy volunteers with Fitzpatrick's skin type II and III were included after their written informed consent was obtained. The volunteers were randomised in a broad band UVB and a narrow band UVB group. The irradiation was performed in a Waldmann UV 7001 K phototherapy cabin equipped with broad band UVB lamps or narrow band UVB lamps. Minimal erythema doses (MED) were assessed and the volunteers were irradiated with 3 MED broad or narrow band UVB, depending on their randomisation. At 4, 24 and 48 hours after irradiation punch biopsies were

obtained from unirradiated skin and the irradiated areas on the buttocks under local anaesthesia with 1% Xylocain and Adrenalin. Biopsies were embedded in paraffin after a 4-hour fixation in formaldehyde. The study was approved by the ethical committee of the University Medical Centre Nijmegen, the Netherlands.

Minimal erythema dose assessment

MED was defined as the UVB dose required to achieve a just perceptible erythema 24 hours after irradiation. Six circular areas of 2.5 cm in diameter located horizontally just above the buttocks were irradiated with increasing doses of broadband UVB. For patients with skin type II, the following broad band UVB range was used: 0.05-0.09-0.12-0.16-0.20-0.27 J/cm^2 and for skin type III: 0.05-0.10-0.15-0.20-0.30-0.40 J/cm^2. Narrow band UVB ranges were identical for skintype II and III: 0.50-0.70-0.90-1.10-1.30-1.50 J/cm^2.

Monoclonal Antibodies

The following monoclonal antibodies were used to evaluate photodamage: Monoclonal Murine anti-human (wild type and mutant) p53 protein (1:200) (Dakopatts, Glostrup, Denmark) and Ab-4 Mouse anti-human p16 (1:100) (Neomarkers, Fremont, USA). HE stainings were performed to evaluate epidermal UV induced damage and inflammation. Analysis of inflammatory infiltrate was done by assessment of T-lymphocytes, Langerhans cells and polymorphonuclear leukocytes (PMN) respectively using the monoclonal antibodies DAKO T-11 (1:100) (Dakopatts, Copenhagen, Denmark), DAKO T6 (1:100) (Dakopatts, Copenhagen, Denmark) and DAKO anti-elastase (1:100) (Dakopatts, Copenhagen, Denmark).

Immunohistochemical Staining

Six-micrometer paraffin sections were dewaxed with histosafe (Adamas, Amerongen, the Netherlands), and dipped in a series of graded ethanol from 100% to 50% and rehydrated in demineralized water. For p53 and p16 the slides were pretreated in a 10 mM citrate buffer (high temperature microwave retrieval technique by Cattoretti) at 450 W in a microwave oven for approximately 9 minutes and left to cool for 45 minutes. The slides were then air dried and immersed in a phosphate buffered saline (PBS).
All slides were incubated for 15 minutes with 20% normal horse serum (Vector Laboratories, Burlingame, USA) in 1% bovine serum albumin (BSA, Organon Technika, Boxtel, the Netherlands) in PBS and then by 1 hour incubation with the primary antibody (p53, p16, T11 and anti-elastase). The slides were washed in PBS and incubated with rabbit anti-mouse biotinylated IgG (ABC kit-mouse, Vector
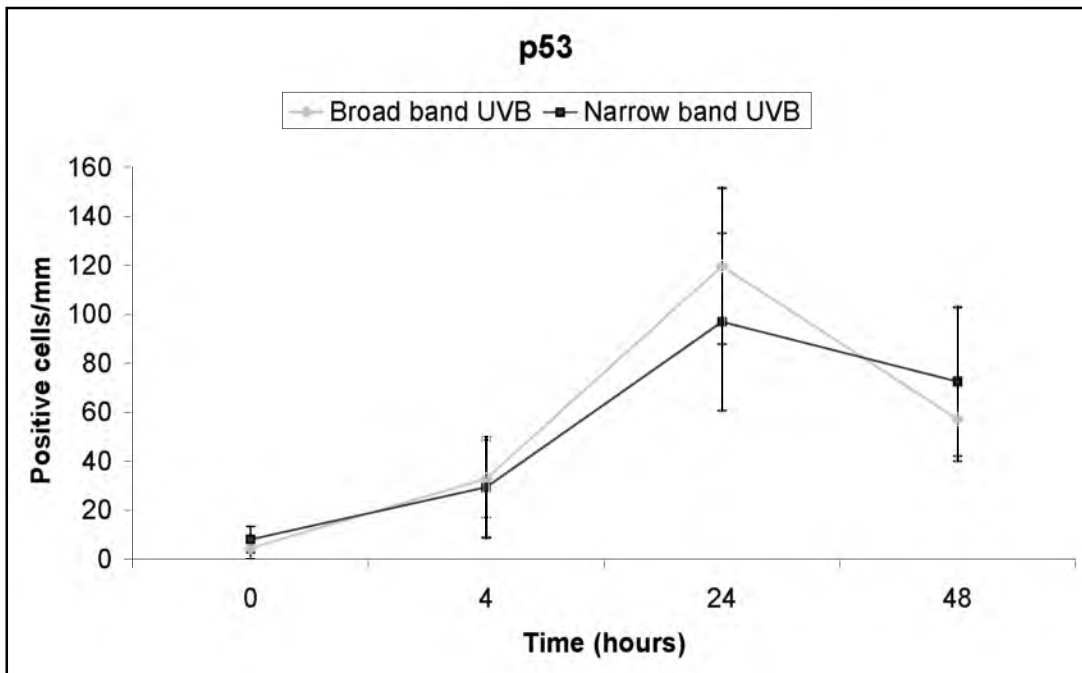
Figure 2-2-1 Mean p53 expression (+/- SD) for broad and narrow band UVB increases significantly up to 24 hours post irradiation. No significant difference between both groups is seen.

Laboratories, Burlingame, USA) for 30 minutes. The sections were washed again in PBS and incubated with anti-rabbit biotinylated IgG for 30 minutes.

All slides were washed in PBS and incubated with avidin biotin complex solution (ABC kit-mouse, Vector Laboratories, Burlingame, USA) for 30 minutes and labelled with metal enhanced DAB substrate (Pierce, Rockford, USA) for visualisation.

The slides were counter-stained with Mayer's haematoxylin (Sigma, St. Louis MO, USA) and after dehydration with 100% ethanol and histosafe mounted in Permount (Fisher Scientific, New Jersey, USA).

For T-6 an indirect peroxidase technique was used. After dewaxing, the slides were washed in phosphate-buffered saline and incubated for 1 hour at room temperature with the primary antibody. The slides were washed twice in PBS and incubated for 30 minutes with rabbit-anti-mouse-immunoglobulins (Dakopatts, Copenhagen, Denmark) conjugated with peroxidase, diluted 1:50 in PBS and containing 5% human AB serum. After two further washes in PBS and preincubation with sodium acetate buffer (pH 4.9) the slides were stained with 3-amino-9-ethyl-carbazole for 10 minutes. The slides were counter-stained with Mayer's haematoxylin and mounted in glycerol gelatin (Sigma).

Figure 2-2-2 Mean sunburn cell count (+/- SD) for broad and narrow band UVB increases significantly up to 48 hours post irradiation. No significant difference between both groups is seen.
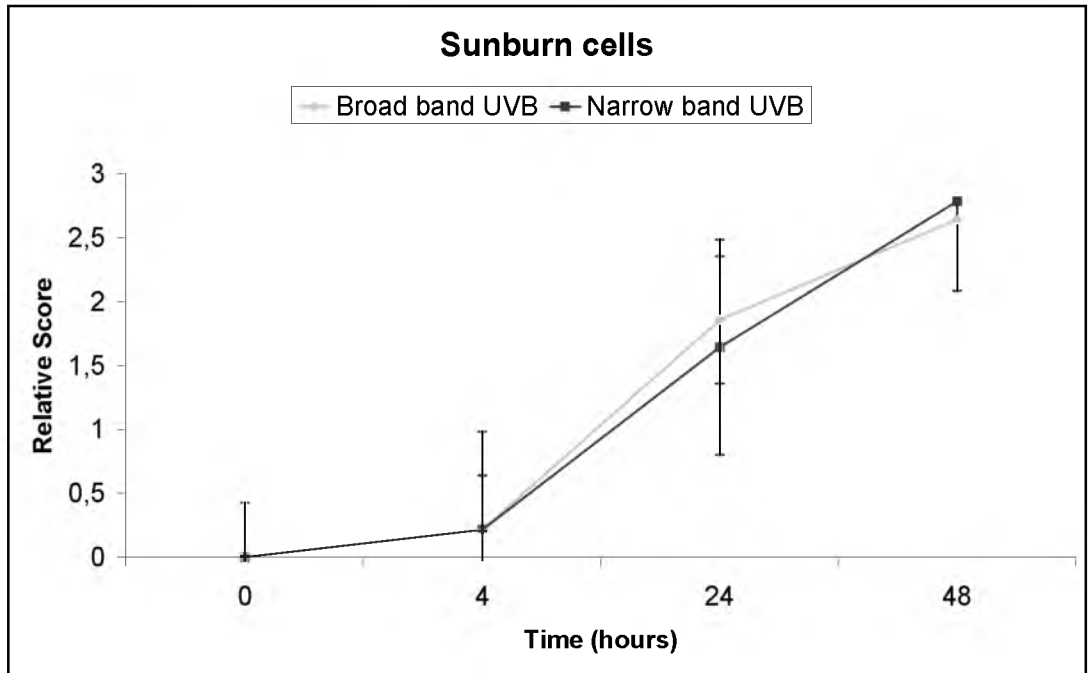
Histological examination

The histological examination was performed blindly by two observers. Photodamage was measured by counting the number of p53 positive nuclei per mm length of section. The epidermal expression of p16, Langerhans cells and apoptosis as well as dermal inflammation were scored on a semi-quantitative scale (0: none present, 1: minimal, 2: moderate and 3: pronounced amount/staining). T-cells and polymorphonuclear leukocytes were assessed by scoring the percentage of these inflammatory cells in the dermal infiltrate on a semi-quantitative scale.

Statistical evaluation
The data have been analysed using the Statistica Statistical package. Data are expressed as means ± SEM. For statistical analysis two way ANOVAs were performed, if applicable followed by Duncan's post hoc comparisons test. A p-value < 0.05 was considered significant.

2.2.4 Results

UV exposure was well tolerated by the volunteers, although a pronounced erythema
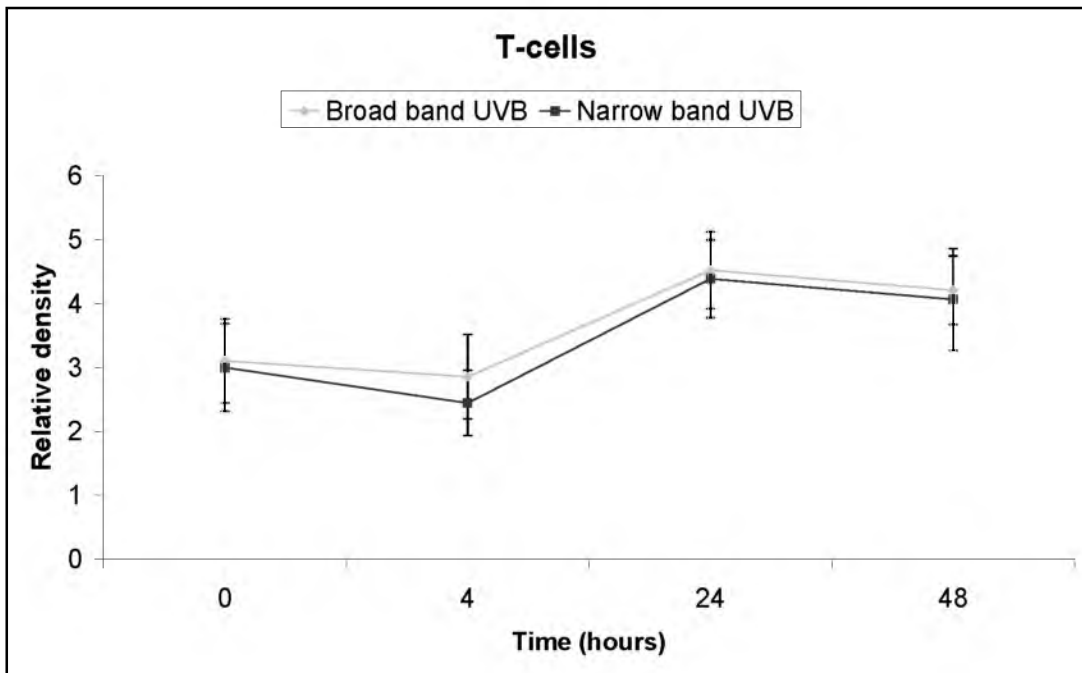
Figure 2-2-3 Mean T-cell score (+/- SD) for broad and narrow band UVB increases significantly 24 hours post irradiation. No significant difference between both groups is seen.

was visible on the irradiated areas. No visible change in pigmentation was observed. Mean MED for broad band UVB was significantly lower than for narrow band UVB (p=0.0000). The difference between the mean doses of broad band and narrow band UVB to induce a just perceptible erythema was 1.14 J/cm². Narrow band UVB MED doses were 8.125 times higher than broad band UVB MED doses.

Broad band and narrow band UVB at equivalent MED doses were equally potent in inducing p53 (p=0.8815), apoptosis (p=0.9131) and p16 expression (p=0.9835). The number of sunburn cells continued to increase significantly up to 48 hours after irradiation (p=0.0000). In contrast, p53 expression reached a maximum at 24 hours post irradiation, followed by a decline (p=0.0000) (see figure 2-2-1 and 2-2-2). P16 expression did not show any significant change following UVB exposure (p=0.5212).

Both broad and narrow band UVB showed a moderate but significant increase in perivascular dermal infiltrate up to 48 hours after irradiation (p=0.0000), predominantly consisting of T-cells (p=0.0000) and polymorphonuclear leukocytes (p=0.0000). In contrast, a significant decrease in epidermal Langerhans cell count was observed from 4 hours up to 48 hours post broad and narrow band UVB exposure.

Figure 2-2-4 Mean PMN score (+/- SD) for broad and narrow band UVB increases significantly 24 hours post irradiation. No significant difference between both groups is seen.
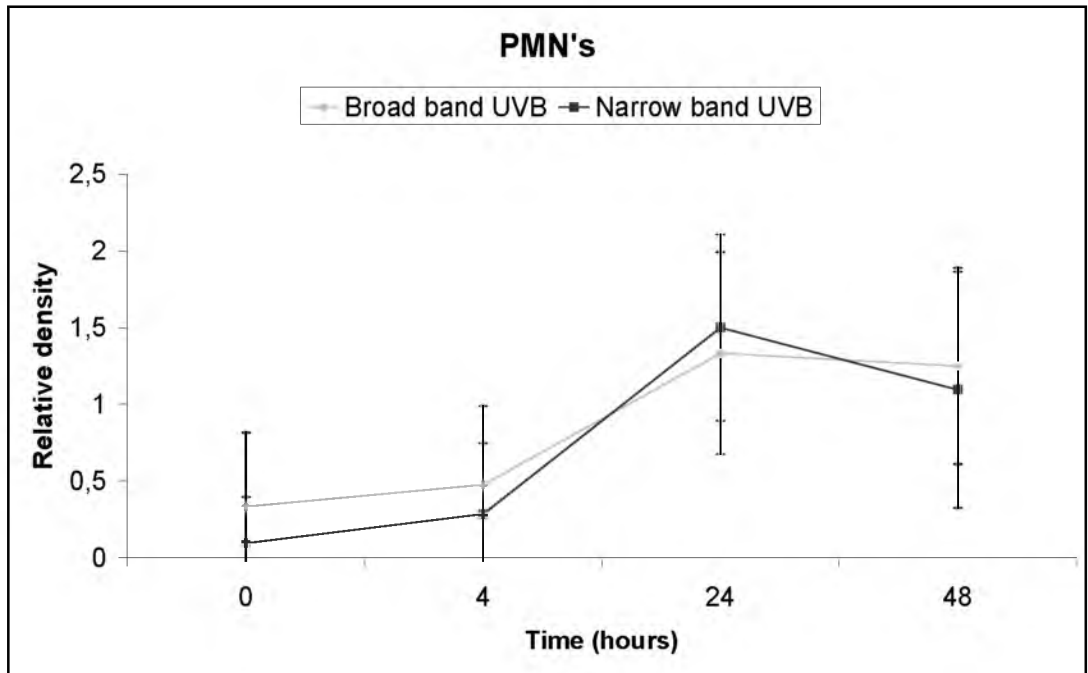
No significant differences in accumulation of T-cells and PMNs or decrease of Langerhans cells between broad and narrow band UVB were observed ($p>0.2947$) (see figure 2-2-3 to 2-2-5).

2.2.5 Discussion

The present study shows that mean MED doses were 8.125 times higher for narrow band UVB compared with broad band UVB. Several other studies have also shown that MED doses were significantly higher for narrow band UVB ranging from 2.8-4.52 times higher in healthy volunteers and up to 10.8 times higher in hairless mice (5,11,19).

In treating several chronic inflammatory diseases such as psoriasis narrow band UVB is regarded as more effective compared to broad band UVB and as equally effective with less side effects as compared to PUVA (3,7).

Previous studies in mice however have shown that narrow band UVB may be more carcinogenic than broad band UVB (9-11). The median tumor induction time of repeated near erythematous doses of narrow band UVB in albino Skh-1 hairless mouse (hr/hr) was 14 weeks compared to 21 weeks with broad band UVB irradiation. The present study did not show any differences in p53, p16 and apoptosis in vivo

Figure 2-2-5 Mean Langerhans cell count (+/- SD) for broad and narrow band UVB decreases significantly from 4 hours and onwards post irradiation. No significant difference between both groups is seen.
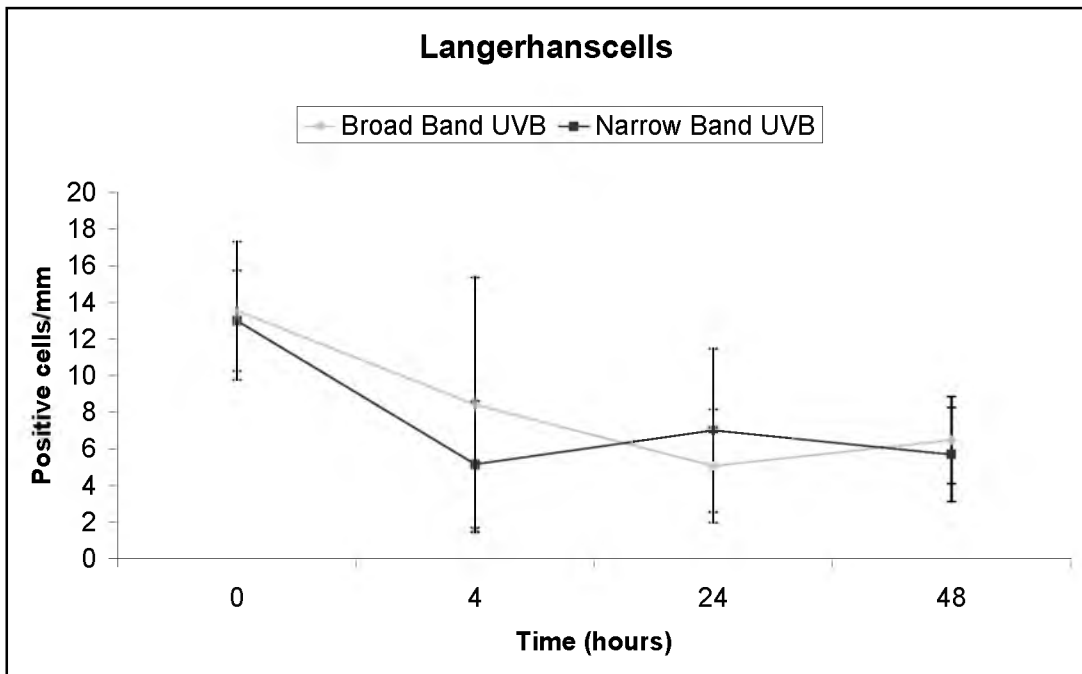
in healthy human volunteers and therefore seems to confirm previous experimental mouse studies that did not show enhanced narrow band UVB photocarcinogenesis (12,20).

With respect to inflammation the present study confirms that a single high dose challenge of both broad and narrow band UVB induces a moderate perivascular influx of inflammatory cells, predominantly T-lymphocytes and polymorphonuclear leukocytes, with a pronounced reduction of epidermal Langerhans cells (21).

The present study demonstrates that after irradiation with 3 MED narrow band and broad band UVB similar effects on inflammation and photodamage are induced. The higher efficacy of narrow band UVB in the treatment of several skin diseases compared with broad band UVB and PUVA results in a reduced number of irradiations needed to induce a remission (6). The present study indicates that irradiation with narrow band and broad band UVB at 3 times the MED has a similar safety profile. Therefore, it can be concluded that narrow band UVB has an improved benefit ratio as compared to broad band UVB.

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2.3   High dose long wave visible light induces perinuclear vacuolization in vivo but does not result in early photoageing and apoptosis

2.3.1   Abstract

With the advancing widespread use of photodynamic therapy, questions arise about the necessity to protect the adjacent healthy skin from high dose long-wave light. The aim of the present study is to investigate the effects of high dose visible light on the skin of healthy volunteers with focus on apoptosis, DNA damage, inflammation, melanogenesis and induction of matrix metalloproteinases (MMP). Fourteen healthy volunteers were included and irradiated daily on their buttocks with 1300 kJ/m^2 long wave visible light (560-780 nm) on 5 consecutive days with a cumulative dose of 6500 kJ/m^2. In each volunteer six biopsies were taken before and 24 hours after irradiation on day 1, 2, 3 and 5 and on day 8 and 12. Frozen and paraffin sections were investigated by measuring parameters for photodamage (apoptosis, p53, phosphorylated c-Jun), and skin ageing (phosphorylated c-Jun, MMP-1, elastin content) and melanogenesis (melan-A). Although no sunburn cells were seen, a significant increase in perinuclear vacuolization was noted ($p<0.0003$) from day 5 till 7 days after the last irradiation. There was no expression of phosphorylated c-Jun, whereas the expression of p53, Melan A, MMP-1 and elastin content did not change. High dose visible light induces a significant increase in perinuclear vacuolization, but does not result in apoptosis, photodamage or early induction of skin ageing.

2.3.2   Introduction

Studies in photodermatology have been focused mainly on the ultraviolet light (UV) part of the electromagnetic radiation spectrum. It is known that UV light contains sufficient energy to result in readily discernible biological effects in the skin. Visible light has up till recently been regarded to be without any significant photobiological effect on the skin. With the recent developments in photodynamic therapy, visible light has become increasingly interesting for researchers, especially in combination with appropriate photosensitizers, such as 5-amino-levulinic acid. The spectrum of the visible light used in dermatological PDT ranges from 560 till 780 nm. With the emerging widespread use of photodynamic therapy the safety question for surrounding normal healthy skin and personnel arises. As visible light has always been regarded as harmless for healthy skin, almost no research has been done whether this supposition is correct.

Most visible light research has focused on the near UV spectrum ranging from 400-450 nm. As most effects in photobiology are not induced by one specific wavelength

but by a whole range of wavelengths it is attractive to hypothesize that near UV irradiation might have similar but perhaps less potent effects than UVA irradiation. Several studies have shown that near UV irradiation results in UVA-like effects, mainly consisting of type II reactions such as DNA strand breaks due to singlet oxygen species generation mediated by endogenous photosensitizers like billirubin (1,2). But what about the effect of electromagnetic radiation in the red-light spectrum as used in PDT? Again it is attractive to speculate that similar effects can be seen in the red-light spectrum. 5-Amino levulinic acid, as used in PDT, is an endogenous substance and a precursor of the endogenous photosensitizer protoporphyrin IX. Protoporphyrin IX produces singlet oxygen species after irradiation with light in the Soret band range. Therefore it is possible that red light may result in singlet oxygen mediated damage. Studies on the photobiological effects of visible light are sparse. Edström et al. have shown that high dose visible light (400-800 nm) can result in an increased p53 expression in vivo. The doses used are rather low and given over 4 weeks. The number of volunteers was small with only 3-4 biopsies per time point (3-5).

UVA irradiations may induce photoageing. Indeed with prolonged UV exposure skin wrinkling occurs due to loss of collagen content and elastin fragmentation (6,7). Early markers for this process are phosphorylated c-Jun and MMP expression (8). Photoageing in a later phase is marked by elastosis and elastin fiber fragmentation. High dose UVA-1 has remarkable effects on morphea, which may be regarded, at least to some extent as a strongly increased photoageing process. It is attractive to speculate that high dose visible light may have similar effects, with respect to skin ageing.

In the present study the effects of high dose visible light on skin of healthy volunteers are investigated with respect to melanogenesis (Melan A), skin ageing (phosphorylated c-Jun, MMP-1, elastin content) and photodamage (apoptosis, p53, phosphorylated c-Jun). No Minimal Erythema Dose testing was performed as visible light, which includes the used wavelength range, is not known to induce erythema, furthermore a recent study showed that the physical dose determines the amount of photodamage and not the biological dose as defined by MED's (9).

2.3.3 Material & methods

This study was approved by the regional Ethical Committee. Fourteen young healthy volunteers (mean age 23.3 range 19-32) with skin type I-III were included in this study after giving written consent. To avoid age-related changes in the photobiological response of the skin only non-smoking volunteers under the age of 35 years, without a history of skin disease or prolonged sun or UV exposure, and without clinical visible photodamaged skin were included.

Irradiation was given on 5 consecutive days with a Waldmann 1200 L PDT lamp

(Herbert Waldmann GmbH & Co., Villingen-Schwenningen, Germany), which uses a 1200 watt Philips metal halogen lamp (MSR 1200 HR, Philips B.V., Eindhoven, the Netherlands), which has an emission spectrum of 560-780 nm after filtering with the build-in dichroic cut-off filters (DT Rot and Calflex-3000, Balzers Optik, Nurnberg, Germany). The surrounding skin was protected with light impenetrable cloth. Each day 1300 kJ/m^2 was given on the sun protected area of the buttocks to a cumulative dose of 6500 kJ/m^2. Before each irradiation the lamp was allowed to warm up for 10 minutes. To minimize the number of biopsies the volunteers were randomized in a paraffin and cryo embedding group for microscopical analysis. On days 1, 2, 3 and 5, 3 mm punch biopsies were taken immediately before the irradiation, in addition 3 mm punch biopsies were taken on days 8 and 12, under local anesthesia with 1% Xylocain and Adrenalin. Depending on randomization, biopsies were snap frozen in Tissue-Tec OCT compound (Miles Scientific, Naperville, USA) or embedded in paraffin after a 4-hour fixation in formaldehyde.

Monoclonal antibodies

The following monoclonal antibodies were used on paraffin sections: monoclonal murine anti-human (wild type and mutant) p53 protein (1:200) (Dakopatts, Glostrup, Denmark), PhosphoPlus c-Jun (Ser63) II (1:50) Antibody kit (New England Biolabs Inc., Beverly, MA, USA), which detects phosphorylation on point 63 of c-Jun, monoclonal murine anti-elastin antibody (1:500) (Sigma, St Louis, USA) and monoclonal murine anti-human Melan A (1:50)(MART-1, Oncogene, San Diego, USA). Hematoxylin and eosin (H&E) staining was performed on paraffin embedded sections to evaluate epidermal UV induced damage and inflammation. Staining for monoclonal murine anti-human MMP-1 (1:15) (Oncogene, San Diego, USA) was performed on cryo sections. As controls biopsies 24 hours after irradiation were taken from two MED UVB irradiated healthy skin and prolonged (approx. 5000 kJ/m^2) UVA-1 irradiated skin was used as controls. Keratinocyte apoptosis was evaluated by identifying sunburncells in the epidermis.

Immunohistochemical staining

All slides were processed side-by-side. Six μm paraffin sections were dewaxed with histosafe (Adamas, Amerongen, the Netherlands), and dipped in a series of graded ethanol from 100% to 50% and rehydrated in demineralized water. The slides were pre-treated in a 10 mM citrate buffer (high temperature microwave oven retrieval technique by Cattoretti (10)) at 450 W in a microwave oven for approximately 9 minutes and left to cool for 45 minutes. The slides were then air dried and immersed in a phosphate buffered saline (PBS). For the elastin staining this step was substituted by 5 minute trypsinization with trypsine-CaCl$_2$ solution.

For p53, elastin and Melan A this initial step was followed by incubation for 15 minutes with 20% normal horse serum (Vector Laboratories, Burlingame, USA) in 1% bovine serum albumin (BSA, Organon Technika, Boxtel, the Netherlands) in PBS and then by 1 hour incubation with the primary antibody. The slides were washed in PBS and incubated with horse anti-mouse biotinylated IgG (ABC kit-mouse, Vector Laboratories, Burlingame, USA) for 30 minutes. For phosphorylated c-Jun the initial step was followed by incubation for 15 minutes in 20% normal goat serum (Vector Laboratories, Burlingame, USA) and by 1 hour incubation with the anti-c-Jun or anti-ser73-phosphorylated c-Jun antibody (1:50). The sections were washed again in PBS and incubated with anti-rabbit biotinylated IgG for 30 minutes.
All slides were washed in PBS and incubated with avidin biotin complex solution (ABC kit-mouse, Vector Laboratories, Burlingame, USA) for 30 minutes and labeled with metal enhanced DAB substrate (Pierce, Rockford, USA) for visualization.
All slides were counter-stained with Mayer's haematoxylin (Sigma, St. Louis MO, USA) and after dehydration with 100% ethanol and histosafe mounted in Permount (Fisher Scientific, New Jersey, USA).
MMP-1 staining was performed on 6 μm cryostat sections fixed in 100% acetone following the above-mentioned procedure, starting with the first incubation. After counter-staining with Mayer's haematoxylin (Sigma, St. Louis MO, USA) the sections were mounted in glycerol-gelatin.

Histological examination and scoring of immunostaining results

The histological examination was performed blindly by two independent observers (MT & TS). Photodamage was measured by counting the number of p53 and phosphorylated c-Jun positive nuclei as well as the number of sunburn cells and cells with perinuclear vacuolization both per 1000 epidermal cells. Sunburn cells were defined as epidermal cells with pyknotic nuclei and a strong eosinophilic cytoplasm and perinuclear vacuolization as epidermal cells with pyknotic nuclei with large cytoplasmatic vacuoles around the nuclei and without the eosinophilic cytoplasm. Perinuclear vacuolization was only quantified in paraffin sections. Melanin containing cells were scored by counting the amount of Melan A positive cells per 1000 epidermal cells. Inflammation, elastin content and MMP-1 were described qualitatively and semi-quantitatively.

Statistical evaluation

The data have been analyzed using the Statistica 5.5 Statistical package (http://www.statsoft.com/). Data are expressed as means ± SEM. For statistical analysis One-way Analysis of Variance (ANOVA)-analysis (repeated measures) and a Duncan-test were performed. A p-value < 0.05 was considered significant.
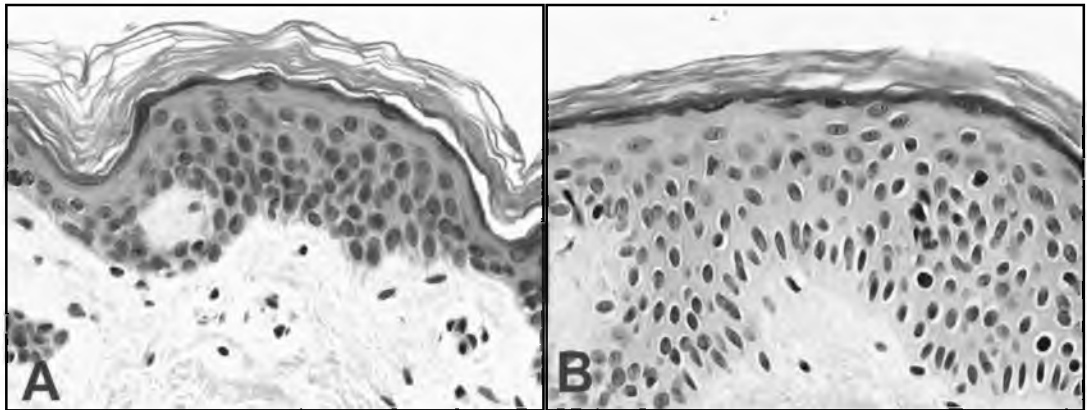
Figure 2-3-1 Perinuclear vacuolization before and 12 days after exposure of high dose long wave visible light.

2.3.4 Results

Treatment was well tolerated by all volunteers, and all described it as very comfortable. Only in one volunteer slight heat-induced erythema was noted once immediately post-irradiation.

None of the volunteers experienced other local effects. No visible change in pigmentation was seen. In the UVA-1 irradiated control a pronounced hyperpigmentation was seen with moderate wrinkling of the skin.

Although H&E staining did not show any sunburn cells, a significant increase was observed of epidermal cells with small, but not pyknotic, nuclei and perinuclear vacuolization (figure 2-3-1). This increase was significant in a One-way Analysis of Variance (p=0.0003). As can be seen in figure 2-3-2 this increase was significant in the Duncan test from day 5 onwards compared to the previous time points. On day 12 we still noted an increase of these cells.

No inflammatory infiltrate was visible.

No significant change in p53 (p=0.1864) expression was seen (figure 2-3-3). Phosphorylated c-jun expression was not seen in any of the slides, although our positive (2 MED UVB irradiated) control did stain positive. Elastin fibers showed clear presence of candelabra-like structures and no signs of elastosis or fragmentation of fibers during and after the irradiation period.

Dermal MMP-1 expression was visible in dendritic cells in all the slides, but no significant changes were seen as compared to the unirradiated control skin of each subject.

No clinical change in pigmentation was seen. This was confirmed by the Melan A staining (p=0.1923).

Perinuclear vacuolization
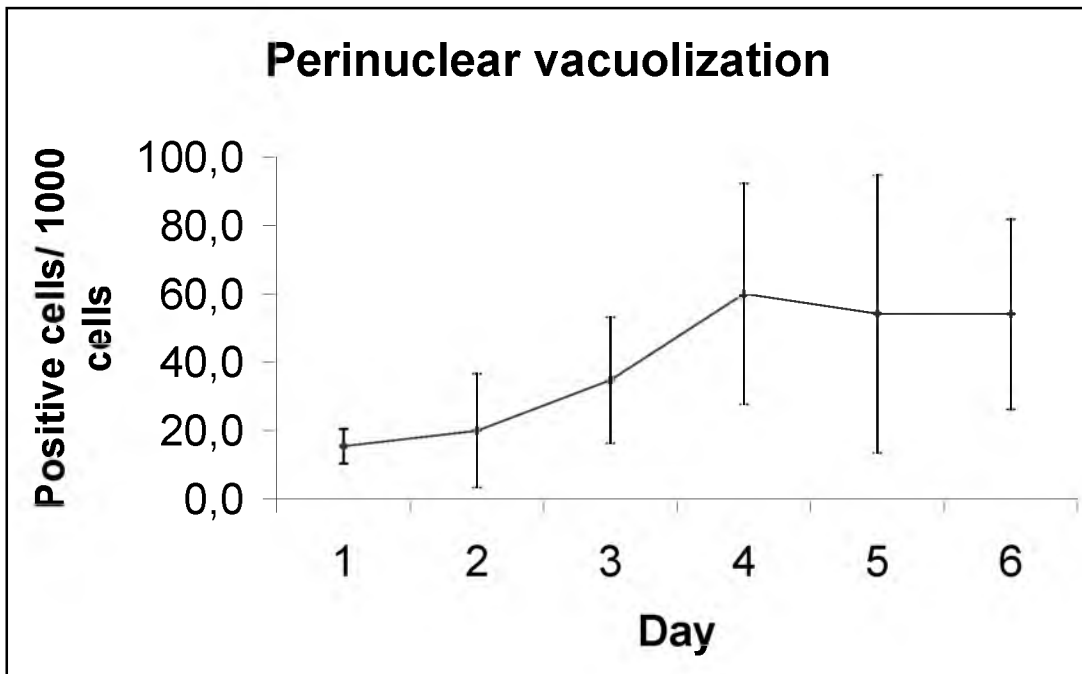
Figure 2-3-2 Mean perinuclear vacuolization (±SD) increases after exposure to high dose long wave visible light while not returning to normal up to 7 days after the last exposure

2.3.5 Discussion

To the best of our knowledge the photobiological effects of visible light on human skin have not been studied in depth before. However, several authors have speculated that short wave visible light (400-500 nm) can have similar but less pronounced photobiological effects on the skin as UVA light. These mainly consist of singlet oxygen mediated processes (indirect = type II), and not of direct effects like UVB does (direct = type I). This can be explained by the different energy levels of photons at different wavelengths as described by the following formula (11).

$$E_{photon} = h.\nu \text{ (with: } h = \text{Planck constant)} = \frac{1.98 \cdot 10^{-19} \, watt \cdot seconds}{\text{wavelength in nanometers}}$$

This formula implies that UVB with shorter wavelengths has much more energy than UVA and even more than visible light. UVB has enough energy to excite DNA molecules directly, while UVA can only exert such effect via endogenous photoactive substances (porphyrins), which result in singlet oxygen formation, which in turn reacts with DNA and other molecules (1,2). Physiologically there is only a limited amount of these photoactive substances available in the skin, which implies that after
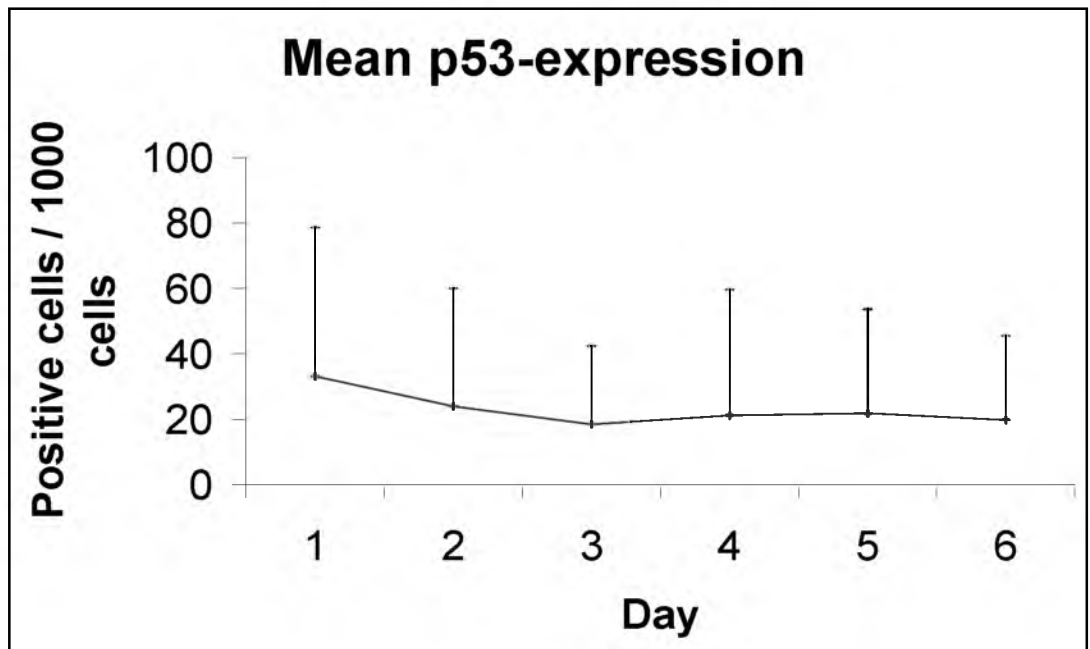
Figure 2-3-3 Mean p53 expression (+SD) does not change significantly during repeated high dose visible light irradiation (p=0.1864)

some time these substances get depleted, resulting in an only limited damage (12,13). Studies have shown that near UVA visible light can result in DNA single strand breaks (14), p53 induction (3) and immediate pigment darkening (15,16).

As PDT uses the endogenous porphyrin pathway with an excitation spectrum of 630-635 nm, it may be hypothesized that long wave visible light can excite the small amount of endogenous protoporphyrin IX and thus result in singlet oxygen mediated effects. Edstrom et al. have studied high dose visible light (3 irradiations per week, with a cumulative dose of 15.120 kJ/m^2 over 4 week period). They used a filtered Xenon lamp with an emission spectrum of 400-800 nm wavelength (4,5). In fact their study reconfirms that near UVA light can induce minimal but significant p53, Ki67 and p21waf expression (3). A limitation of their study was that each observation consisted of only 3 randomly selected volunteers from their pool of 12 subjects.

In contrast to the study of Edstrom et al. we have specifically chosen to exclude the near UVA spectrum and to look specifically at the wavelengths used in dermatologic photodynamic therapy. Our study shows that long wave visible light does not induce significant changes in apoptosis, p53 and phosphorylated c-Jun expression. However, a significant increase in perinuclear vacuolization was seen. Perinuclear vacuolization is well known to dermatopathologists as an artefact in cryo embedded sections. We however discovered this significant phenomenon in paraffin embedded sections. In the present blinded histological assessment this phenomenon proved to

be a real hallmark for the exposure to high dose of long wave visible light. Several authors have reported similar findings in UV irradiated skin, but did not discuss its significance (17,18). It can be postulated that this phenomenon in UV irradiated skin is seen as a transitional stage in the process from viable keratinocyte to apoptosis, which eventually result in clearly visible sunburn cells and additional p53 and phosphorylated c-Jun expression. It is still unclear what the significance of this phenomenon is as it was not accompanied by apoptotic cells, additional p53 and phosphorylated c-Jun expression. We however cannot entirely rule out the possibility of a heat-induced effect. Our volunteers did describe the exposure as comfortable, and in one individual slight heat erythema was visible once after an exposure. Our control for elastin and MMP-1 stainings was skin taken from a patient who received prolonged exposure in an UVA-1 phototherapy cabin, about 30 minutes after the last treatment. These paraffin sections did not show perinuclear vacuolization. In our experience, temperature rises considerably during this treatment even to an uncomfortable level. So it is unlikely that perinuclear vacuolization is heat-induced. Nowadays UVA-1 is used for the treatment of morphea, due to the accelerated degradation of intercellular matrix. This is marked by elastin fiber fragmentation and elastosis, collagen degradation and MMP-1 and c-Jun induction (19,20). C-Jun induction leads to Activator Protein-1 production, which in turn activates several matrix metalloproteinases (8). It was attractive to speculate whether high dose long wave visible light, with the advantage of a greater penetration depth due to its longer wavelength, could have similar effects as UVA-1. To prevent possible bias by age and smoking related induction of these markers, we exclusively included long-term non-smoking volunteers under the age of 40 years in the present study (19,21,22). Minimal MMP-1 expression was seen at all time points in dendritic cells in the dermis, however we did not observe significant changes in expression. There was no expression of phosphorylated c-Jun. Both results indicate that activation of degrading enzymes was not present. This is also confirmed by the elastin fiber staining which did not show changes during our study. We did not check for Tissue Inhibitors of Matrix Metalloproteinases (TIMPs) as a change in TIMP concentrations would probably not affect the detected minimal MMP-1 levels significantly. In conclusion high dose visible light phototherapy is no viable option in the treatment of sclerodermiform diseases.

Some studies have shown that visible light can induce immediate pigment darkening and melanogenesis (15,16). These studies however always included the UVA and near UVA spectrum, which can be expected to result in some immediate pigment darkening and melanogenesis. Our study shows that melanogenesis is not induced by long wave visible light.

2.3.6 Conclusion

The present study shows that high dose long wave visible light at doses as used in PDT does not cause epidermal and dermal DNA damage, inflammation, melanogenesis and early photoageing parameters. The only sign of a photobiological effect was a significant increase in epidermal perinuclear vacuolization. This perinuclear vacuolization, however, did not result in apoptosis.

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3

Modulation of the UVB response

3.1 Adalimumab, a fully human anti-TNF-α monoclonal antibody (MoAb), treatment does not influence experimental UV response in the skin of rheumatoid arthritis patients.

3.1.1 Abstract

Background TNF-α is known to play an important role in UV induced immunomodulation and photodamage. It plays a role in UVB mediated induction of apoptosis and is a strong inducer of the JNK pathway, which eventually leads to the loss of dermal collagen and elastin content. Recently chimeric anti-TNF-α has been introduced as a therapy for rheumatoid arthritis.

Objectives The aim of the present study is to investigate the effect of anti-TNF-α treatment on UV induced DNA damage, apoptosis, and induction of matrix metallo proteinases.

Patients/Methods Twelve patients with rheumatoid arthritis were included and irradiated with 2 MED broad band UVB before and after administration of 0.5 mg/kg anti-TNF-α monoclonal antibody. Twenty-four hours after irradiation biopsies were taken. Frozen and paraffin sections were stained for p53, c-Jun, phosphorylated c-Jun, sunburn cells and MMP-1.

Results No significant changes were observed in the expression of p53 and sunburn cells and MMP-1 content after treatment with anti-TNF-α, whereas a slight but significant decrease in c-Jun and phosphorylated c-Jun expression was noted ($p=0.0250$ and $p=0.0431$ respectively).

Conclusions Our results show no influence of anti-TNF-α at therapeutic doses on the UV response in patients with rheumatoid arthritis.

3.1.2 Introduction

Irradiation with UVB (290-320 nm) is known to have acute and chronic effects on the skin. Acute effects of UVB encompass inflammation, DNA damage and DNA repair mechanism as well as apoptosis ("sunburn cells"). Chronic effects of UVB are photo ageing and carcinogenesis.

In vitro and in vivo studies have shown that a spectrum of cytokines is induced by UVB irradiation (1-6). TNF-α is a proinflammatory cytokine which has been shown to play a pivotal role in UVB induced inflammation and apoptosis. TNF-α is released by keratinocytes following UVB irradiation of the skin. Moreover, in vitro studies have shown that blocking TNF-α results in reduced expression of DNA damage markers and the number of apoptotic keratinocytes significantly (2,3,7-9). Although UV carcinogenesis was not found to be enhanced in knockout mice deficient for TNF-α receptors, inhibition of apoptosis in the skin after blocking TNF-α might

have consequences for the clinical use of such treatment (10). Therefore, it is of importance to investigate whether anti-TNF-α is capable of inhibiting apoptosis in humans in vivo.

In animal studies and in keratinocyte cultures both TNF-α (11) and UVR (12) induction have shown to induce c-Jun and phosphorylated c-Jun (13,14). The transcription factor AP-1, which is the complex of c-Jun and c-Fos is also induced by UVR (15). TNF-α plays an important role in the prolonged activation of AP-1, due to its effect on the c-Jun kinases (14). Although the role of c-Jun and phosphorylated c-Jun in relation to apoptosis remains unclear, it is generally accepted that c-Jun and c-Fos play an important role in photoaging.

AP-1 induces the expression of several matrix metalloproteinases (MMPs) such as gelatinases, collagenases and stromelysin (16-19).

Chronic exposure to these MMPs result in wrinkles and skin ageing due to loss of collagen and increase in the elastin fragmentation of the skin (20-25). Up to date, the effect of anti-TNF-α treatment on the expression and activation of c-Jun, c-Fos and matrix metalloproteinases in human skin has not been studied.

Chimeric anti-TNF-α monoclonal antibodies (e.g. antibodies containing both human and mouse amino acid sequences) and TNF receptor constructs have now been registered for rheumatoid arthritis (RA), juvenile chronic arthritis and Crohn's disease. Interestingly, these therapies have also impressive effects on the skin lesions of patients with psoriatic arthritis. This opens the possibility of the future use of TNF neutralisation in inflammatory skin diseases.

Our centre participates in clinical trials with a new fully human, and potentially less immunogenic, anti-TNF-α antibody (adalimumab, D2E7, Knoll) in patients with RA. The efficacy and safety of this compound have been extensively demonstrated (26) and this agent is now undergoing phase III studies. (27-32)

In this paper, the responsiveness of the skin to UVB challenge was assessed before and two weeks after the first administration of adalimumab, in patients with active RA. This time schedule seems warranted since a single administration of this compound results in a clear clinical improvement and in a reduction of the acute phase reaction within days.

The aim of the present study was to investigate to what extent UVB irradiation effects are influenced by treatment of anti-TNF-α in vivo. We mainly focused on apoptosis, c-Jun/phosphorylated c-Jun and MMP-1 induction. The potential impact of these effects on carcinogenesis and skin aging are discussed.

3.1.3 Methods

Patients and UVB irradiation

Twelve consenting patients with longstanding refractory RA according to the revised

ARA criteria (1987) and active disease were recruited for this study (33,34). Patients underwent a 4-week washout period for disease modifying antirheumatic drugs (DMARD) and were on stable doses of NSAIDs and/or prednisone during the study. Patients had to have skin type II and III. The UVB irradiation was performed in a Waldmann UV 7001 K phototherapy cabin. Minimal Erythema doses (MED) were assessed before as well as two weeks after a single subcutaneous administration of the anti-TNF-α monoclonal antibody (0.5 mg/kg, Knoll AG, Ludwigshafen, Germany). Subsequently, these patients received a single dose irradiation of 2 MED broad band UVB (290-320 nm) on the buttocks. Three mm punch biopsies were obtained from unirradiated skin and the UVB exposed area at 24 hours after irradiation under local anaesthesia with 1% Xylocain and Adrenalin. UVB irradiation and biopsy procedures were repeated two weeks after the administration of the anti-TNF-α antibody (table 3-1). Biopsies were snap frozen in Tissue Tek OCT compound (Miles Scientific, Naperville, USA) or embedded in paraffin after a 4-hour fixation in formaldehyde. The study was approved by the ethical committee of the University Medical Centre Nijmegen, the Netherlands.

Table 3-1: Study schedule regarding UVB irradiation, biopsies and anti-TNF-α administration

Time	Action
T = 0	First MED UVB assessment
T = 24	Biopsies from 2 MED irradiated skin and unirradiated skin
T = 48	Subcutaneous administration of 0.5 mg/kg anti-TNF-α
T = 2 weeks after anti-TNF-α administration	Second MED UVB assessment
T = 2 weeks + 24 hours	Biopsies from 2 MED irradiated skin

Minimal erythema dose assessment

MED was defined as the UVB dose required to achieve a just perceptible erythema 24 hours after irradiation (35). Six circular areas of 2.5 cm in diameter located horizontally just above the buttocks were irradiated with increasing doses of broadband UVB. For patients with skin type II, the following UVB range were used: 0.05-0.09-0.12-0.16-0.20-0.27 J/cm^2 and for skin type III: 0.05-0.10-0.15-0.20-0.30-0.40 J/cm^2.

Monoclonal Antibodies

The following monoclonal antibodies were used: Monoclonal Murine anti-Human (wild type and mutant) p53 protein (1:200) (Dakopatts, Glostrup, Denmark), C-jun (1:50) and PhosphoPlus c-jun (Ser63) II (1:50) Antibody kit (New England Biolabs Inc., Beverly, MA, USA), which detects phosphorylation on point 63 of c-Jun and

monoclonal anti-human MMP-1 (1:15) (Oncogene). HE stainings were performed to evaluate epidermal UV induced damage.

Immunohistochemical staining

Six μm paraffin sections were dewaxed with histosafe (Adamas, Amerongen, the Netherlands), and dipped in a series of graded ethanol from 100% to 50% and rehydrated in demineralized water. The slides were pre-treated in a 10 mM citrate buffer (high temperature microwave retrieval technique by Cattoretti (36)) at 450 W in a microwave oven for approximately 9 minutes and left to cool for 45 minutes. The slides were then air dried and immersed in a phosphate buffered saline (PBS).
For p53 this initial step was followed by incubation for 15 minutes with 20% normal horse serum (Vector Laboratories, Burlingame, USA) in 1% bovine serum albumin (BSA, Organon Technika, Boxtel, the Netherlands) in PBS and then by 1 hour incubation with the p53 antibody (1:200). The slides were washed in PBS and incubated with rabbit anti-mouse biotinylated IgG (ABC kit-mouse, Vector Laboratories, Burlingame, USA) for 30 minutes. For c-Jun and phosphorylated c-Jun the initial step was followed by incubation for 15 minutes in 20% normal goat serum (Vector Laboratories, Burlingame, USA) and by 1 hour incubation with the anti-c-Jun or anti-ser73-phosphorylated c-Jun antibody (1:50). The sections were washed again in PBS and incubated with anti-rabbit biotinylated IgG for 30 minutes.
All the slides were washed in PBS and incubated with avidin biotin complex solution (ABC kit-mouse, Vector Laboratories, Burlingame, USA) for 30 minutes and labelled with metal enhanced DAB substrate (Pierce, Rockford, USA) for visualisation.
All slides were counter-stained with Mayer's haematoxylin (Sigma, St. Louis MO, USA) and after dehydration with 100% ethanol and histosafe mounted in Permount (Fisher Scientific, New Jersey, USA).
MMP-1 staining was done on 7 μm cryostat sections fixed in 100% acetone following the above mentioned procedure, starting with the first incubation. After counter-staining the sections were mounted in glycerol-gelatin.

Histological examination

The histological examination was performed blindly by a single observer. Apoptosis was quantified as the number of sunburn cells in the epidermis and MMP-1 as the number of positive cells in the dermis both on a semi-quantitative scale (0: none present, 1: minimal, 2: moderate and 3: pronounced amount). Photodamage was measured by counting the number of p53, c-Jun and phosphorylated c-Jun positive nuclei per mm length of section.
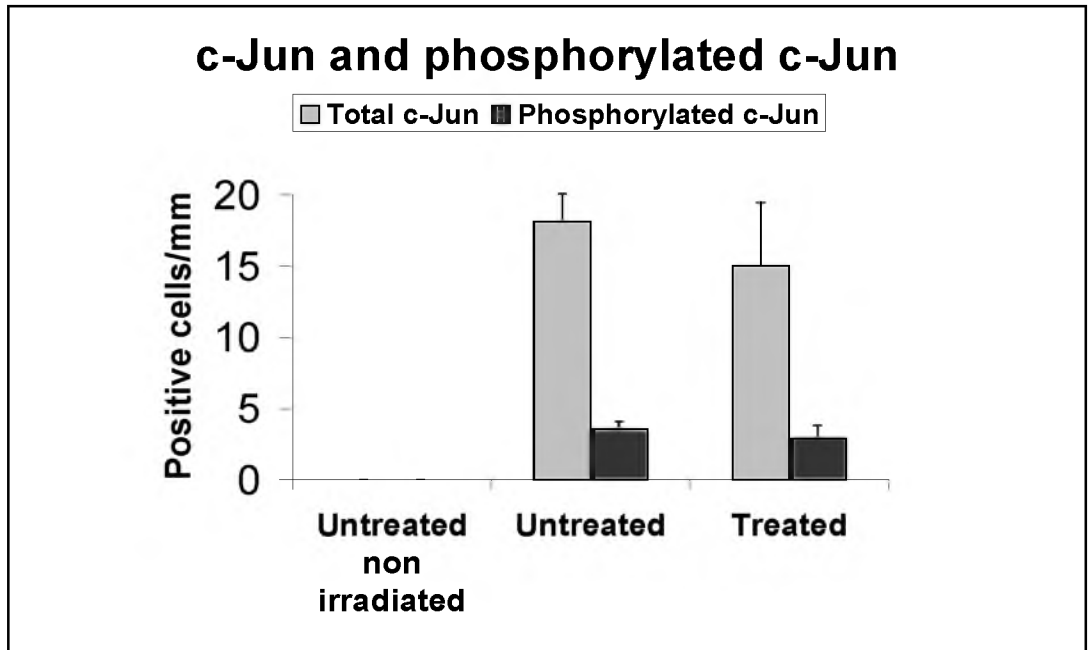
Figure 3-1 C-Jun and phosphorylated c-Jun expression in untreated and unchallanged skin and before and after 2 weeks treatment with anti-TNF-α in UVB challanged skin (mean ± SEM)

Statistical evaluation

The data have been analysed using the SPSS Statistical package. Data are expressed as means ± SEM. For statistical analysis Wilcoxon matched pairs tests were performed. A p-value < 0.05 was considered significant.
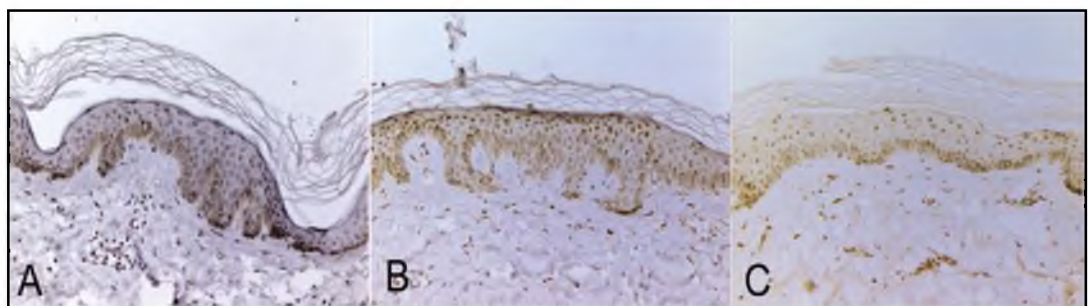
Figure 3-2 Immunohistochemistry showing c-Jun expression in untreated and unchallenged skin (A) and before (B) and after (C) 2 weeks treatment with anti-TNF-α in UVB challanged skin
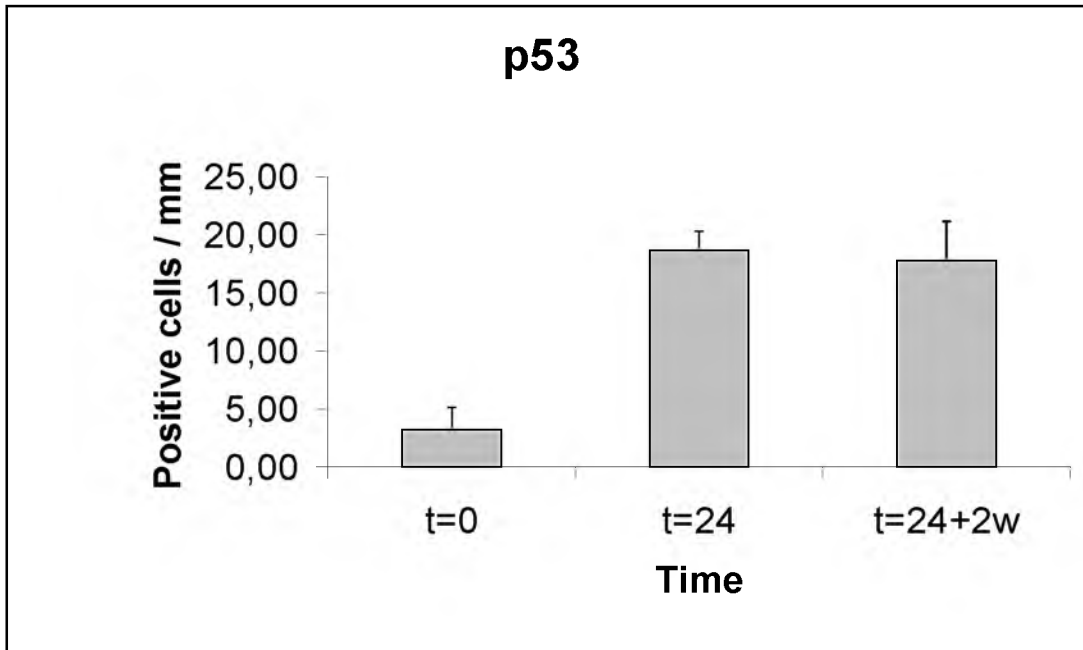
Figure 3-3 p53 expression in untreated and unchallanged skin and before and after 2 weeks treatment with anti-TNF-α in UVB challanged skin (mean ± SEM)

3.1.4 Results

Minimal Erythema Dose

MEDs ranged from 0.10 J/cm^2 to 0.30 J/cm^2. The MEDs before and 2 weeks after the subcutaneous administration of the anti-TNF-α antibody did not differ significantly (p=0.5), the medians being 0.15 J/cm^2 and 0.16 J/cm^2 respectively.

HE staining for sunburn cells

A significant rise in sunburn cells (SBC's) count was seen after irradiation (p=0.03), but no significant difference was seen after treatment with the anti-TNF-α antibody (p=0.50).

Markers for DNA damage

All markers (p53, c-jun and phosphorylated c-jun) showed a significant increase in expression after UVB irradiation (p=0.0076 for all three markers). C-Jun and phosphorylated c-Jun showed a significant but slight decrease in expression after treatment with anti-TNF-α (p=0.0250 and p=0.0431 respectively, figures 3-1

Sunburn cell and MMP-1
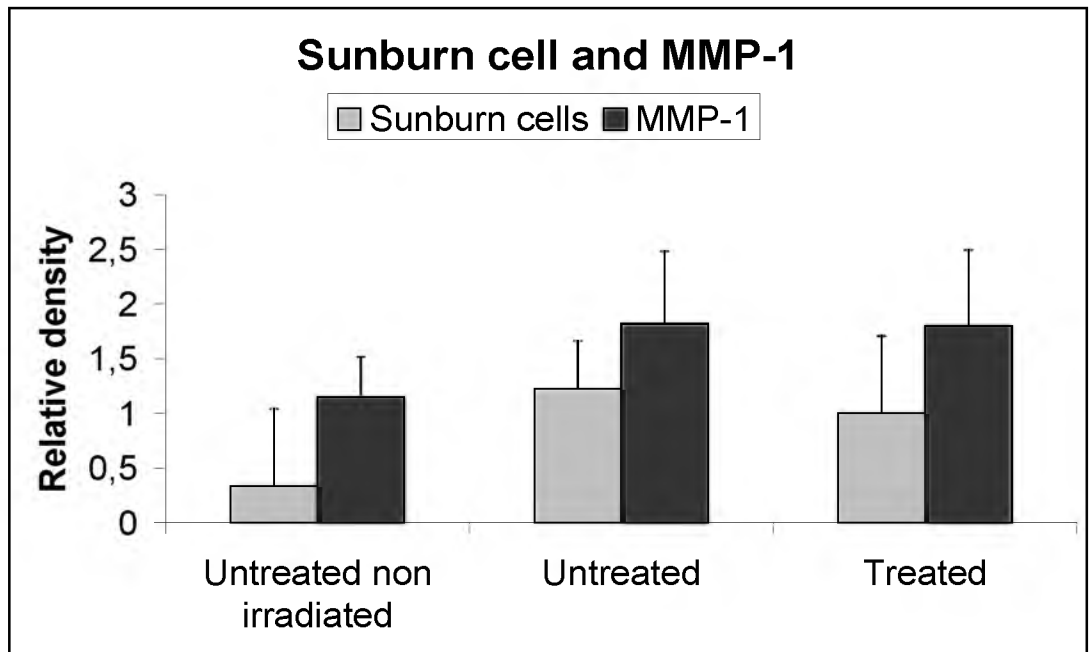
☐ Sunburn cells ■ MMP-1

Figure 3-4 Sunburn cells and MMP-1 expression in untreated and unchallanged skin and before and after 2 weeks treatment with anti-TNF-α in UVB challanged skin (mean ± SEM)

and 3-2). Initially, after UV exposure, expression of c-Jun and phosphorylated c-Jun is observed in the upper layers of the epidermis (stratum granulosum). After administration of the anti-TNF-α antibody there seems to be a slight shift towards the stratum spinosum.

P53 expression did not change significantly after treatment with the anti-TNF-α antibody.

Matrix Metallo Proteinase -1

Before irradiation there was a minimal staining of MMP-1 positive cells in the upper dermis, which increased significantly ($p=0.003$) after irradiation with broad band UVB. There was no significant change in MMP-1 expression after the administration of the anti-TNF-α ($p=0.735$)

3.1.5 Discussion

The dosage of anti-TNF-α, as used in the present study, was set by an optimal therapeutic effect in rheumatoid arthritis. Indeed 7 out of 12 patients fulfilled clinical response criteria after two weeks. In contrast, the effect of anti-TNF-α administration on UV induced skin inflammation was negligible; showing no change of minimal

erythema dose scores before and after treatment with anti-TNF-α (0.15 ± 0.04 J/cm² and 0.16 ± 0.07 J/cm²). A recent study showed that NSAIDs can reduce the UV induced erythema and in vitro even result in reduced production of TNF-α and several cytokines. In our study however, erythema was induced, although the patients were on stable doses of NSAIDs and/or prednisone. Minimal erythema doses were comparable with earlier published doses for comparable skintypes. It is feasible that NSAID doses applied in vivo did not reach enough skin penetration compared to the direct application of NSAIDs on HACAT cells.

The present in vivo study provides additional evidence of the safety of the use of anti-TNF-α in patients with severe inflammatory diseases like rheumatoid arthritis. Anti-TNF-α treatment did not influence the UVB induced skin response significantly, as markers for photo damage and early markers for photo aging are not or only slightly changed after UVB irradiation and anti-TNF-α treatment. Only the induction of c-Jun and phosphorylated c-Jun is slightly but significantly decreased after treatment with the antibody. Especially the decrease in phosphorylated (active) c-Jun was almost negligible due to the use of a semiquantitative scoring method. It was however attractive to speculate if the in-vivo inhibition of these parameters might be reflected in a decreased induction of matrix metalloproteinases (MMPs) in the skin, which in the long term might result in less photo aging in sun exposed subjects or decrease in apoptosis. MMPs also play an important role in the degeneration of cartilage in rheumatoid arthritis resulting in increased expression of MMP-1 and MMP-3 concentrations in serum. This study clearly shows that MMP-1 expression in the skin after UVB irradiation is not affected by the anti-TNF-α treatment, this in spite of the fact that MMP-1 and MMP-3 concentrations in serum decreased significantly 2 weeks after the first administration of anti-TNF-α (37).

It has been shown that anti-TNF-α reduces the number of apoptotic cells after UVR exposure in keratinocyte cultures and shaved Balb/c-mice (9). However, in these studies anti-TNF-α had been added immediately after the UV challenge. Based on the present study, it is too early to exclude entirely an effect of anti-TNF-α on apoptosis in humans in vivo, although a sustained inhibition of apoptosis has been ruled out.

It is attractive to speculate why anti-TNF-α treatment does not influence these partly TNF-α mediated processes. As most of our patients did improve remarkably from their rheumatoid arthritis it is likely that the antibody did work. One may hypothesize that skin penetration of the antibody is impaired. This however seems unlikely because of the remarkable effects of anti-TNF-α treatment in psoriasis and arthritic psoriasis patients. The anti-TNF-α antibody seems to accumulate in areas with extremely enhanced TNF-α expression. In our patients this area were the joints instead of the skin.

In conclusion, the present study demonstrates that, except for a slight but significant decrease in c-Jun and phosphorylated c-Jun, anti-TNF-α treatment does not significantly influence our UVB induced photo response model in the skin of

rheumatoid arthritis patients. Further studies are however indicated to study the long-term effects of anti-TNF-α on skin ageing and carcinogenesis and in inflammatory skin conditions.

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4

Narrow band UVB phototherapy for vitiligo

4.1 The treatment op vitiligo vulgaris with narrow band uvb (311 nm) for one year and the effect of addition of folic acid and vitamin B12

4.1.1 Abstract

Narrow band UVB is succeeding PUVA as the main treatment of vitiligo vulgaris in several European countries. Deficiency states for vitamin B12 and folic acid in some vitiligo patients have prompted researchers to look into the efficacy of these vitamins in the treatment of vitiligo. In the present controlled study we investigated the value of narrow band UVB phototherapy in the treatment of vitiligo and the possible additive effect of vitamin B12 and folic acid.

Twenty-seven patients with long-term stable vitiligo were included and randomised in an 'UVB only' (UVB) or 'UVB combined with vitamin B12 and folic acid' (UVB+) group. Patients were irradiated thrice weekly for one year, whilst repigmentation was carefully monitored. In 92% (25/27) of the patients up to 100% repigmentation was seen. Repigmentation was notable in lesions on the face, neck and throat, lower arm, chest, back and lower legs, whilst repigmentation on hands and wrists and feet and ankles proved to be minimal. Maximum repigmentation rates did not differ significantly between the UVB group and the UVB+ group.

Our study reconfirms that narrow band UVB phototherapy is an effective treatment for vitiligo and shows that co-treatment with vitamin B12 and folic acid does not improve the outcome of treatment of vitiligo with narrow band UVB phototherapy.

4.1.2 Introduction

Vitiligo has been known for centuries and is a disease characterised by white patches, due to a loss of melanocytes (1,3). Although several treatments were suggested during the ages, it was not until the 1900's that the first effective treatment was found, photochemotherapy (4,6). Psoralen and UVA irradiation (PUVA) treatment has been the only successful treatment for decades and in some countries PUVA still is the main treatment for generalised vitiligo. Recently, new advances in phototherapy have provided new treatment possibilities. Especially narrow band UVB has been a major breakthrough and is succeeding PUVA as the main treatment in several countries (7,8). So far, however, controlled studies on the efficacy of narrow band UVB are not available. It should be taken into consideration that UVB therapy only is effective after prolonged treatment and that long-term placebo-controlled studies for approaches where ample evidence is available are difficult to justify. In order to further substantiate the evidence for the efficacy of long-term narrow band UVB therapy, a study in patients with longstanding stable vitiligo is of importance.

Research into the aetiology and pathomechanisms of vitiligo has provided some insight in the biochemical pathways involved. This resulted in the development of combined treatments of phototherapy with several biochemical compounds as pseudocatalase, calciumchlorid and phenylalanine (9,14).

It has been suggested that vitamin B12 and folic acid may play a role in the melanin synthesis pathway, as the pteridine part of folic acid and N-N-methylene tetrahydrofolate as well as the vitamin B12 dependent formation of methionine could interfere with processes leading to depigmentation and pigmentary dilution (15,17). Several reports suggest that a deficiency of vitamin B12 and folic acid can be a common characteristic of vitiligo, while others contradict them (18,20). Therefore it is attractive to hypothesise that vitamin B12 and folic acid may contribute to the efficacy of phototherapy in vitiligo.

The aim of the present study is to confirm the therapeutic value of narrow band UVB in the treatment of patients with stable vitiligo and secondly to evaluate the additional effect of vitamin B12 and folic acid on the treatment of vitiligo vulgaris with narrow band UVB phototherapy using a non-blinded approach comparing UVB and vitamin B12 and folic acid with narrow band UVB.

4.1.3 Material & methods

Patients

Twenty-seven patients, male and female, age 18 and above, with stable vitiligo vulgaris (at least 1 year no changes) and Fitzpatrick's skintype II-IV from our outpatient clinic were included in the study, after written informed consent had been obtained. Patients using any medical treatment for their vitiligo at the moment were excluded. Other exclusion criteria were patients with a history of skin cancer or dysplastic naevus syndrome, photosensitivity or use of photosensitising medicines, psychiatric or epileptic disorders, known renal failure and known allergies to substances in the trial medication. Table 4-1-1 shows their demographic data.

Treatments

The patients were randomised in two narrow band UVB treatment groups. The first group only received narrow band UVB phototherapy, the second group received twice daily vitamin B12 (cobalamin) 1000 g sustained release tablets (Orthica B.V., Weesp, the Netherlands) and folic acid 5 mg tablets (Dumex, the Netherlands). These doses were chosen in concordance with a previous study by Juhlin & Olsson (21). Both groups were irradiated with narrow band (311 nm) UVB as generated by Philips TL-01 lamps in a Waldmann 7001 cabin (Waldmann Medical Technology, the Netherlands). Irradiation was given 3 times weekly for 12 months and started with

0.10 J/cm^2 exposure, which increased with 0-30% every next visit on an individual basis.

Table 4-1-1: Patient characteristics

Characteristics	Control group	Vitamin group
No. of patients	13	14
Skin type II	5	3
Skin type III	7	10
Skin type IV	1	1
Age mean ± SD (Range)	41.6±8.7 (20-51)	46.8±10.2 (29-68)
Sex, M/F	6/7	5 / 9
Duration of disease mean ± SD (Range)	10.8±9.9 (1-30)	18.3±13.1 (1-47)
Percentage of body surface affected mean ± SD (Range)	8.4±5.6 (2-18)	10±7.5 (3-26)

All the vitiligenous areas were carefully monitored monthly during the study period, including before and after photographs. The percentage of repigmentation was visually estimated in all areas. As primary efficacy criterion we selected the areas showing the most active repigmentation. This was designated as "maximum repigmentation".

Treatment was continued for one year after start of phototherapy or discontinued earlier if full repigmentation was reached before that.

Treatment outcome was visually scored as the percentage of repigmentation of depigmented lesions.

Statistical analysis was performed with a one way ANOVA test on SPSS 7.5 for Windows.

4.1.4 Results

The mean cumulative dose of narrow band UVB given to each patient was 126.68 ± 88.64 J/cm^2 (11.6-401.76 J/cm^2). Twenty-five of the 27 patients showed prominent repigmentation on most lesions of the body. In each group one patient showed less than 5% repigmentation after 4 months of phototherapy and these patients were advised to discontinue phototherapy treatment.

As shown in Table 4-1-2 more than 90% repigmentation (median values) is reached on the face, neck and throat, lower arm, chest, back and lower legs, while less than 25% repigmentation (median values) is seen on the hands and wrists and feet and ankles. Fig. 4-1-1 shows the maximum repigmentation in vitiligenous lesions for both groups.
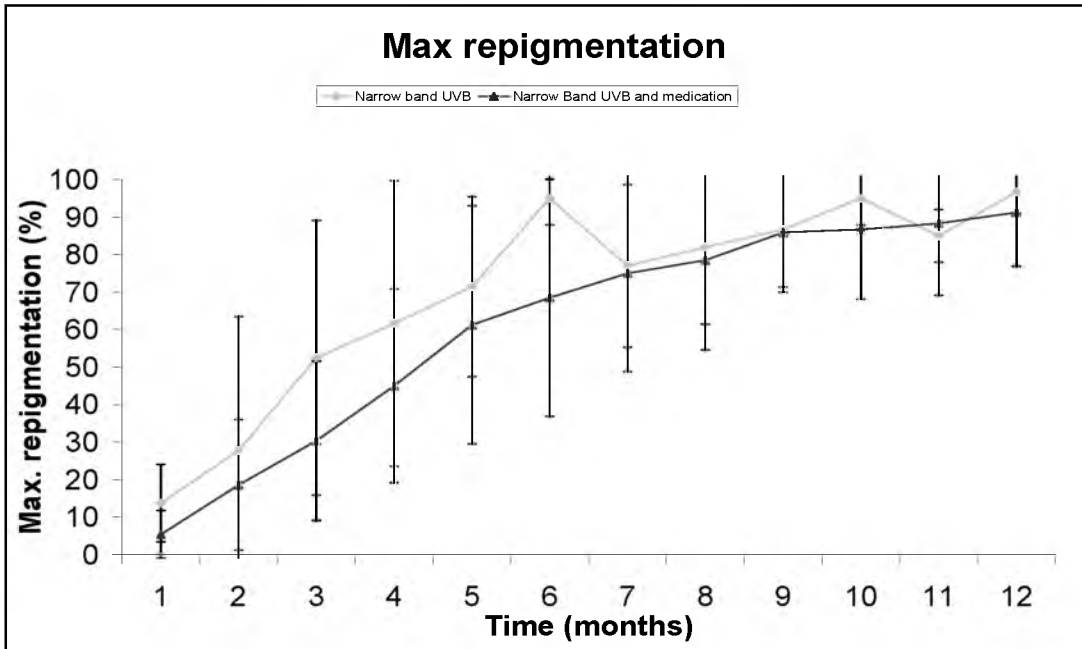
Figure 4-1-1 Course of maximum repigmentation during one year narrow band UVB phototherapy with (◊) or without (▲) medication.

Table 4-1-2: Percentage of repigmentation in different body areas of all the patients

Area	Number of patients	Repigmentation (%) Mean ± SD	Repigmentation Median (Range)
Face	19	71.8 ± 35.5	90 (5-100)
Neck/Throat	7	68.6 ± 40.7	90 (5-100)
Upper Arm	4	65.0 ± 31.0	65 (30-100)
Elbow	12	50.4 ± 29.8	45 (10-100)
Lower Arm	7	80.0 ± 24.5	90 (30-100)
Hand/Wrist	18	35.6 ± 31.5	22.5 (10-100)
Axilla	17	37.4 ± 31.9	40 (0-100)
Chest	9	71.1 ± 35.9	90 (20-100)
Abdomen	3	80.0 ± 10.0	80 (70-90)
Back	10	84.0 ± 27.5	92.5 (10-100)
Groin	14	70.4 ± 25.5	77.5 (20-100)
Upper Leg	6	55.0 ± 40.9	70 (0-100)
Knee	5	62.0 ± 41.5	80 (0-100)
Lower Leg	12	85.0 ± 16.1	90 (60-100)
Foot/Ankle	12	12.1 ± 10.8	10 (0-40)

No significant difference in repigmentation was reached at any of the monthly measurement points between the two groups ($p \geq 0.175$). No additional effect of vitamin B12 and folic acid on repigmentation was seen either on less responsive areas like the hands and feet ($p \geq 0.23$).

Fig. 4-2-2 shows an example of an average response on a patient treated with UVB phototherapy alone. In all patients transient erythema was observed occasionally, usually experienced as localised prickling of the skin within 24 hours after irradiation. However these responses were mild and never induced aggravation of the lesions (Koebner phenomenon).

4.1.5 Discussion

So far no standardised scoring method for evaluating the effect of repigmentation therapies is available. A PASI like score, as used for psoriasis, would be a great step forward to evaluate and compare the effect of repigmentation between different therapies. Some studies use a rather inaccurate graded scoring method (less than 30%, 30-70% and more than 70% repigmentation, or total, partial and no repigmentation) (7,22,25). Our study shows that only slight repigmentation or no repigmentation at all was seen on the dorsa of hand and feet and the inner wrist, while other areas like the face, chest, extensor surfaces of the lower arm and leg, back and neck/throat often repigment rather easily and completely (Fig. 4-1-2 and Table 4-1-2). Previously resistance of hands and feet and the inner wrist has been suggested for PUVA phototherapy, although this study did not provide a systematic quantitative analysis (25). On the other hand some studies claim to achieve full repigmentation, again not specifying the responses per body region (10).

In view of the substantial intra individual variability, we have avoided the pitfall of a general score and instead chosen for the approach of scoring repigmentation
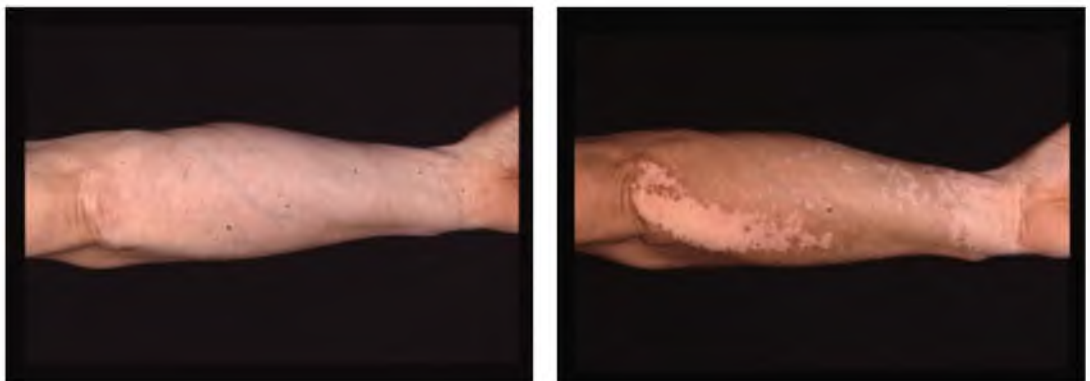
Figure 4-1-2 Before and after photographs of a vitiligenous area on the lower arm and wrist of a patient. Note the minimal repigmentation on the elbow and wrist. Patient was treated with narrow band UVB alone. Photographs were taken with an interval of 12 months.

visually per lesion and to select retrospectively the maximum repigmentated area in each patient as the primary efficacy criterion. As additional efficacy criterion we used the repigmentation in all body areas. A modified Psoriasis Area Severity Index (PASI) score, with special attention to less responding areas, possibly assisted with computerised measurement of the total reduction in vitiligenous areas could have provided more insight in the efficacy of vitiligo treatments.

Although no large studies and no controlled studies have been done, narrow band UVB has been proven in some studies to be effective in inducing repigmentation in patients with vitiligo (8,23,26). Our study shows that narrow band UVB treatment results in an up to 100% repigmentation of depigmented lesions in 92% (25/27) of patients with long-term stable vitiligo vulgaris. During this treatment period no relapse was seen and besides some prickling sensations usually on depigmented areas of the skin and occasional phototherapy induced erythema no major side effects were seen.

There have been reports that folic acid and vitamin B12 can play an indirect role in repigmentation. Both substances have been indicated in being able to supplement deficiency states of tyrosine and methionine, which have been regarded as possible mechanisms for depigmentation in vitiligo and pigmentary dilution as seen in homocysteinuria (21). Others report possible deficiencies of folic acid and/or vitamin B12 in vitiligo patients (18,20), however recently a large controlled study by Kim et al. did not show any difference in serum levels compared with a normal control group (19). This is in concordance with previous findings by Juhlin & Olsson (21). We therefore decided not to measure serum levels of these compounds in our patients.

The study by Juhlin & Olsson showed an improvement of vitiligo with solar UV exposure when combined with vitamin B12 and folic acid (21). This study however did not include a control group and was conducted without standardised UV exposure.

The present study reconfirms the efficacy of narrow band UVB phototherapy in vitiligo but did not show any advantage of the addition of vitamin B12 and folic acid.

A possible explanation for the discrepancy between the studies could be that we have used an optimal dose of narrow band UVB for a long period of time, which by its high efficacy hides any effect of vitamin B12 and folic acid and which are thus not needed.

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4.2 Quality of life in vitiligo patients after treatment with long-term narrow band UVB phototherapy

4.2.1 Abstract

Long term treatments for chronic diseases like vitiligo need to be evaluated for their clinical efficacy. Assessment of the quality of life, however, may provide the most relevant information on the actual benefit for these patients. In this study we evaluated quality of life after long-term narrow band UVB for the treatment of vitiligo.

All patients, with long-term stable vitiligo vulgaris, who were treated at our clinic during the last four years received a specifically for this study designed Quality of Life questionnaire, which included questions about general wellbeing, camouflage and psychosocial aspects. 71.4% of the patients responded. Most patients indicated an improvement on a psychological level, but an increase in camouflaging.

The present study shows that, after long-term narrow band UVB phototherapy, skin appearance does not play a major role in the life of vitiligo patients, while well being only improved in a minority of patients.

4.2.2 Introduction

Vitiligo is a disease characterised by loss of melanocytes, resulting in depigmentation of skin and hair. Vitiligo can be a socially disabling disease, which affects quality of life.

Aetiology and pathogenesis are still largely unknown. Treatments can be classified in four main groups: Topical corticosteroids, phototherapy, melanocyte transplantation and camouflage. Good clinical results have been achieved with narrow band UVB phototherapy (1,2). In general, treatment has to be given 2-3 times weekly for about 2 years to achieve a substantial repigmentation of affected areas. However, some areas, such as the hands and feet are recalcitrant to these treatments and full repigmentation of these areas is rare (in press). Treatment results can be expressed in terms of degree of repigmentation. However, assessment of quality of life will provide the most relevant information on the actual benefit of a treatment in patients with chronic diseases such as vitiligo.

The most important feature of quality of life measurements is that it is only an indication of the quality of life at the time-point the measurement is taken. Several general validated instruments have been introduced like the General Health Questionnaire (GHQ) (3), Medical Outcomes Study 36-item Short-Form Survey (SF-36) (4) and other structured diagnostic interviews. Due to shortcomings in these instruments for the dermatological setting, several disease specific questionnaires were developed such as the Psoriasis Disability Index (5). Comparison between

different skin diseases, however, is still not possible.

In 1994 Finlay and Khan proposed the Dermatology Life Quality Index (DLQI) (6) and in 1996 Chren et al. proposed the Skindex (7). The DLQI consists of 10 questions which focus on 7 aspects of quality of life (symptoms, feelings, daily activities, leisure activities, work or school, personal relationships and treatment), while the Skindex compromises 61 questions focusing on 8 aspects of quality of life (cognitive effects, social effects, physical limitations, physical discomfort, depression, fear, embarrassment and anger) (8,9). Recently the 61 questions version was shortened to a more practical 16 questions version (10,11).

QOL problems in vitiligo patients can be categorised in 6 major categories: choosing clothes, use of sunblocks, use of camouflage, avoidance of activities, reactions of others and emotional reactions (12). The DQLI addresses most of these aspects and is the easiest and most practical approach for questionnaires, although the Skindex is probably more accurate and detailed.

The best way to evaluate the QOL after a treatment is to take a baseline questionnaire (before treatment) and another after having finished the treatment. In the present study the patients were asked retrospective to compare their current QOL with the past. Therefore the outcome of the present study can only provide preliminary conclusions.

With the retrospective nature of our study in mind we developed a new and unvalidated quality of life questionnaire to evaluate the effects of long-term narrow band phototherapy UVB. We modified the DQLI to fit the retrospective nature of our study and added a few extra questions about camouflage and general wellbeing.

The aim of the present study was to investigate the effect of long-term narrow band UVB phototherapy on the quality of life in adult vitiligo patients.

4.2.3 Material & methods

All vitiligo patients who had received long-term narrow band UVB phototherapy during the last 4 years were approached to participate in an anonymous retrospective comparative quality of life questionnaire. During that 4 year period none of the patients had received psychological counselling and no attempts, besides clinically treating the vitiligo, were made to influence the quality of life. All patients received a letter explaining the study, a freepost return envelope and a questionnaire. The questionnaire (appendix I) consisted of 14 multiple-choice questions regarding general wellbeing and health (1-3), social and psychological aspects (4-12) and camouflage (13-14). The questions were scored on a Likert scale to evaluate the answers. The rule the higher the better applies here. A positive number (1 and 2) denotes an improved quality of life after long-term narrow band UVB phototherapy as compared with the situation before treatment, using retrospective analysis. The numbers -1 and -2 denote a deterioration of the situation. The number zero denotes no

difference or that the question was not relevant/applicable for that patient.

The maximum score for the questionnaire is 28 points and the minimum -28. The maximum sum of points (cumulative for all respondents) per question is 2 times the number of respondents.

All data were collected and analysed using version 10.0 of the SPSS Statistical Analysis package.

4.2.4. Results

Forty-two patients with vitiligo, who were treated with long-term narrow band UVB phototherapy in our clinic during the past 4 years were approached. Thirty patients (71.4%) returned the questionnaire. The answers are displayed as means and cumulative sum scores (maximum score = 60) per question in table 4-2-1 and as cumulative sum scores (maximum score = 28) per patient in table 4-2-2.

Twenty-one out of these patients had a mean score above zero, which denotes an improvement. In 23.3% (7/30) a mean score above 1 was recorded, which is a significant improvement after phototherapy. The question about how patients evaluate their health had the best overall score with a mean of 0.86, while the last question about camouflage showed the lowest score with a mean of -0.3, which indicates that most people had to camouflage more after phototherapy treatment than before. Actually all, except the last question showed an overall score above zero (as shown in table 4-2-1).

Table 4-2-1: Mean and cumulative sum scores per question

Qtn	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10	Q11	Q12	Q13	Q14
Mean	0.87	0.27	0.40	0.07	0.33	0.17	0.07	0.07	0.03	0.10	0.30	0.40	0.03	-0.30
Sum	26	8	12	2	10	5	2	2	1	3	9	12	1	-9

The higher the better. Maximum mean score = 2 and minimum mean score = -2. Maximum sum score = 60 and minimum sum score = -60.

26.7% of the patients reported a cumulative sum score below zero (score -1 to -12), indicating a loss of quality of life to some degree, and 70 % reported an improvement (score 1 to 20), while 1 patient indicated that his quality of life was not affected by the phototherapy (shown in table 4-2-2).

General health was generally good (7 excellent, 18 good) with only a slightly improvement after phototherapy (3 much better, 3 slightly better, 22 comparable). The skin influences life less after phototherapy in 30% of the patients (7 much less, 2 slightly less, 16 comparable).

Case	p1	p2	p3	p4	p5	p6	p7	p8	p9	p10
Sum	6	1	10	8	2	1	1	1	-12	5
Case	p11	p12	p13	p14	p15	p16	p17	p18	p19	p20
Sum	-3	1	1	2	-2	11	-1	10	2	16
Case	p21	p22	p23	p24	p25	p26	p27	p28	p29	p30
Sum	-7	5	-3	-1	-6	1	20	8	7	0

Table 4-2-2: Cumulative sum score per patient.

The higher the better. Maximum sum score = 28 and minimum sum score = -28

Phototherapy had only a minor effect on the social level. Only 23.3 % noticed less influence on leisure activities after treatment, 13.3% had less problems with sports, 6.7% with work or study, 6.7% with relationships, one person even had a better sex life after treatment. 16.7% had less problems with starting relationships or getting new friends. With respect to psychological aspects, only 16.7 % had less physical and emotional problems after phototherapy. 26.7% did feel less stared at by people, and 36.7% had less negative feelings or felt insecure.

Interestingly 26.7% never had problem with sports, 43.3% never had problems with work or study, 43.3% never had problems with relationships or with starting new ones, 53.3% never had sexual problems due to their vitiligo (indicated question was not applicable). However, more people had to camouflage their lesions as 20.7% had to choose appropriate clothing compared with 17.2% who improved and even 30% needed more time to camouflage the lesions compared with only 10% who needed less time to camouflage lesions.

4.2.5 Discussion

Quality of life research in vitiligo has always focused on the impact of the disease on social, psychological and physical wellbeing. As far as we know this is the first study to focus on the impact of treatment on the quality of life in adult patients with vitiligo. Njoo et al. have described the favourable effects of narrow band UVB on the quality of life in children with vitiligo before (13).

Quality of life and subjective assessment of the seriousness or extent of a disease is highly variable between patients. In our experience, especially in visible diseases like vitiligo, there seems to be a discrepancy between clinical diseases severity and a patients own experience. Due to this phenomenon, it is possible that minimal lesions could account for a serious impact on the quality of life. Furthermore, a slight improvement could result in a huge improvement of the quality of life. Therefore, we did not take the extent or severity of the vitiligo and the clinical efficacy of the treatment in account.

The present study showed a response rate of 71.4% and provides a representative

impression of the study population. In fact the response rate of the present study is very high as compared with the 39% response of the mass questionnaire by Kent and Al-Abadie in 1996 in members from the UK Vitiligo Society (14,15). However, our study included only a small number of patients. Our response rate is similar to the 65-70% response rate in a questionnaire under patients who attended two vitiligo clinics (16).

The present study shows that QOL improves following long-term narrow band phototherapy as 70% of the patients had a mean score above zero, 23.3% had an even more pronounced improvement with mean scores above one.

Former QOL studies in vitiligo patients have always focused on the general QOL scores, without discussing separate items. Kent and Al'abadie however did describe 6 items that influence vitiligo patients lives within 3 weeks preceding their questionnaire. They focused on choice of clothing, use of sunblocks, use of camouflaging make-up, avoidance of activities, reactions of others and emotional reactions. Our study shows that after narrow band UVB phototherapy, psychosocial items improved, while in other patients camouflaging and choice of clothes were negatively influenced. One of the major problems with phototherapy in vitiligo is that depigmented skin is not affected selectively, causing marked pigmentation of the uninvolved skin as well. Thus during phototherapy normal skin may tan easily in contrast to lesional skin which can result in increased contrast in skin colour and probably in a more increased use of camouflage cosmetics till full repigmentation is reached. Treatment resulted in smaller, but more visible lesions as commented by some patients. One other factor is that repigmentation is usually very limited on visible parts of the skin like dorsum of hands and feet. This could also explain the mean overall score of -0.3 for the question about the time needed for camouflaging.

A large group of our patients never had problems with their sex life (43.4%) or with work or studying (53.3%). It is remotely possible that some people who answered 'no change' did mean not applicable. In this case the percentages could change to 96.6% and 93.3% respectively. Similar responses were also seen in the study by Kent and Al'abadie, as none of their respondents noted these problems.

Patients generally rated their health as good to excellent, but phototherapy only accounted for a small improvement (20% of patients) in general wellbeing. An interesting finding was that after phototherapy, skin appearance influenced more (30% of patients) peoples' lives less negatively. Most people found that their health had improved after phototherapy. This seems to be in concordance with the fact that the cumulative sum score/question was 12 for the question about the extent of insecurity and negative feelings caused by the skin, which indicates a reduction in these feelings.

Due to the retrospective nature of this study it is not possible to rule out biasing phenomena like cognitive dissonance, as people tend to recall what they were like before very inaccurately. Longitudinal studies are warranted to study the effects of

long term narrow band UVB on the quality of life.

4.2.6 Conclusion

This study using a non-validated QOL questionnaire (based on the DQLI) showed that skin appearance does not play a major role in the lives of vitiligo patients after narrow band UVB phototherapy (also indicated by reduced feelings of insecurity and negative feelings) and that only in a minority of patients general wellbeing improved. Furthermore phototherapy increased the necessity to camouflage. It should be noted that this study was performed shortly after completion of the phototherapy treatment. Long-term studies are warranted to correct for the increased contrast bias and to evaluate long-term effects on quality of life.

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APPENDIX I
Quality of life questionnaire

Instruction: This questionnaire concerns your point of view on your health. With these questions we can evaluate a possible improvement in health status and social abilities.

Please answer every question by marking the answer in the appropriate mentioned way. If you are not sure how to answer the question, please give the best possible answer.

1.   How would you generally rate your health?
(complete only one answer)
►   Excellent   ………………………………………………………..2
►   Good   ………………………………………………………...1
►   Moderate   ………………………………………………………-1
►   Poor   ………………………………………………………-2

2.   How would you generally rate your health now compared with before the phototherapy?
(complete only one answer)
►   Much better than before phototherapy   ………………………...2
►   Slightly better than before phototherapy   ………………………...1
►   Comparable with before phototherapy   ……..………………...0
►   Slightly worse than before phototherapy   ………………………-1
►   Much worse than before phototherapy   ………………………-2

3.   To what extent does your skin influence your life nowadays compared with before phototherapy?
(complete only one answer)
►   Much less than before phototherapy   ………………………...2
►   Slightly less than before phototherapy   ………………………...1
►   Comparable with before phototherapy   ………………………..0
►   Slightly more than before phototherapy   ………………………-1
►   Much more than before phototherapy   …………….…………-2
►   Not relevant   ………………………………………………..0

4.   To what extent did your physical health and emotional problems inhibit your social activities with friends, family and others compared with before phototherapy?
(complete only one answer)
►   Much less than before phototherapy   ………………………...2
►   Slightly less than before phototherapy   ………………………...1
►   Comparable with before phototherapy   ………………………..0
►   Slightly more than before phototherapy   ………………………-1
►   Much more than before phototherapy   ………………………-2
►   Not relevant   ….……………………..………………...0

5.   To what extent does you skin influence your leisure activities negatively compared with before phototherapy?
(complete only one answer)
►   Much less than before phototherapy   ………………………...2

►   Slightly less than before phototherapy   ………………………1
►   Comparable with before phototherapy   ………………………0
►   Slightly more than before phototherapy   ………………………-1
►   Much more than before phototherapy   ………………………-2
►   Not relevant   ….……………….…………………....0

6.   To what extent does your skin interfere with sports compared with before phototherapy?
(complete only one answer)
►   Much less than before phototherapy   ………………………..2
►   Slightly less than before phototherapy   ………………….…..1
►   Comparable with before phototherapy   ………………….…..0
►   Slightly more than before phototherapy   ………………….…..-1
►   Much more than before phototherapy   ………………….…..-2
►   Not relevant   ….……………….…………………....0

7.   To what extent does your skin nowadays restrain you from working or studying compared with before phototherapy?
(complete only one answer)
►   Much less than before phototherapy   ………………………..2
►   Slightly less than before phototherapy   ………………….…..1
►   Comparable with before phototherapy   ………………….…..0
►   Slightly more than before phototherapy   ………………….…..-1
►   Much more than before phototherapy   ………………….…..-2
►   Not relevant   ….……………….…..……………....0

8.   To what extent does your skin nowadays play a role when problems arise with friends or family compared with before phototherapy?
(complete only one answer)
►   Much less than before phototherapy   ………………………..2
►   Slightly less than before phototherapy   ………………….…..1
►   Comparable with before phototherapy   ………………….…..0
►   Slightly more than before phototherapy   ………………….…..-1
►   Much more than before phototherapy   ………………….…..-2
►   Not relevant   ….……………….…………………....0

9.   To what extent does your skin nowadays play a role in sexual problems compared with before phototherapy??
(complete only one answer)
►   Much less than before phototherapy   ………………………..2
►   Slightly less than before phototherapy   ………………….…..1
►   Comparable with before phototherapy   ………………….…..0
►   Slightly more than before phototherapy   ………………….…..-1
►   Much more than before phototherapy   ………………….…..-2
►   Not relevant   ….……………….…………………....0

10.   To what extent does your skin nowadays play a role in finding new friends and new relationships compared with before phototherapy??
(complete only one answer)
►   Much less than before phototherapy   ………………………..2

►	Slightly less than before phototherapy	………………….....1
►	Comparable with before phototherapy	………….…………..0
►	Slightly more than before phototherapy	………………….…-1
►	Much more than before phototherapy	………………….…-2
►	Not relevant	………………….….......……………....0

11.   To what extent do you nowadays feel stared at by people in your neighbourhood compared with before phototherapy??
(complete only one answer)

►	Much less than before phototherapy	………………….…...2
►	Slightly less than before phototherapy	………………….…...1
►	Comparable with before phototherapy	………………….…...0
►	Slightly more than before phototherapy	………………….…...-1
►	Much more than before phototherapy	………………….…...-2
►	Not relevant	………………….…......……………....0

12.   To what extent does your skin nowadays lead to insecurity or negative feelings compared with before phototherapy?
(complete only one answer)

►	Much less than before phototherapy	………………….…...2
►	Slightly less than before phototherapy	………………….…...1
►	Comparable with before phototherapy	………………….…...0
►	Slightly more than before phototherapy	………………….…...-1
►	Much more than before phototherapy	………………….…...-2
►	Not relevant	………………….…......……………....0

13.   To what extend does you skin nowadays influence your choice of clothes compared with before phototherapy?
(complete only one answer)

►	Much less than before phototherapy	………………….…...2
►	Slightly less than before phototherapy	………………….…...1
►	Comparable with before phototherapy	………………….…...0
►	Slightly more than before phototherapy	………………….…...-1
►	Much more than before phototherapy	………………….…...-2
►	Not relevant	………………….…......……………....0

14.   How much time do you need camouflaging vitiligo lesions compared with before phototherapy?
(complete only one answer)

►	Much less than before phototherapy	………………….…...2
►	Slightly less than before phototherapy	………………….…...1
►	Comparable with before phototherapy	………………….…...0
►	Slightly more than before phototherapy	………………….…...-1
►	Much more than before phototherapy	………………….…...-2
►	Not relevant	......………………….…......……………....0

5

Summary, discussion and future prospects

5.1   Introduction

The aims of this thesis have been described in section 1.5 and comprise:

I.   Further elucidation of the photobiological effects, in particular regarding photodamage of UVA, broadband UVB, narrowband UVB and visible light.

II.   To find out to what extent anti-TNF-α treatment modulates the response to UVB challenge

III. To find out the clinical efficacy of narrow band UVB treatment in vitiligo and the impact of this treatment on quality of life.

In this chapter, the contributions will be summarised and the questions as formulated in the introduction will be answered.

5.2   Further elucidation of the photobiological effects, in particular regarding photodamage of UVA, broadband UVB, narrowband UVB and visible light.

5.2.1   What are the differences between the photobiological responses of UVA and UVB, especially with respect to oxidative processes?

As shown in chapter 2, UVA and UVB affect different photochemical pathways to induce photobiological responses. Generally, UVB exerts its effects by type I reactions, thereby directly damaging cell structures and molecules. UVA, on the other hand, exerts its effects by indirect type II reactions, mediated by reactive oxygen species (1). Although this hypothesis is generally accepted, no in vivo data were available to support this hypothesis. Recently however, Sander et al. showed that UVA induced oxidative products are associated with long-term effects of UVA exposure, such as photoageing (2).

In chapter 2 the photobiological and oxidative effects after a single exposure to UVA and UVB have been investigated. UVA and UVB both induce inflammation in the upper dermis, as shown by the increase in inflammatory infiltrate. This increase is most pronounced in UVB irradiated skin and is mainly attributed to an increase in T-cells. Although the used irradiation dose was quite low and did not result in the induction of sunburn cells, p53 and c-Jun expression were observed. P53 expression reached an earlier and higher expression in UVB compared to UVA irradiated skin. A similar expression pattern was seen for c-Jun; a statistically significant increase was reached 4 hour after UVB exposure and 24 hours after UVA irradiation.

With the performed non-invasive techniques it was possible to demonstrate a pronounced induction of oxidised lipids in UVA irradiated skin one hour after irradiation, as measured by squalene peroxide levels. This induction was significantly

enhanced in UVA irradiated skin compared to UVB irradiated skin. Furthermore, catalase activity, an endogenous antioxidant system, was impaired by 80% 4 hours after irradiation with UVA. A previous study showed that UVB does not influence catalase activity (3).

The delay in expression of the p53 and c-Jun markers after UVA irradiation could be explained by the fact that p53 and c-Jun are induced by activation of the oxidative pathway (4,5). The results mentioned in chapter 2 support this hypothesis.

5.2.2 What are the differences between broadband and narrowband UVB induced photodamage?

Narrow band UVB is gradually replacing broadband UVB phototherapy and also PUVA photochemotherapy in Europe. There is, however, discussion on the safety aspect, as several experimental studies have shown increased photocarcinogenesis in mice (6-8). As a higher dose of narrow band UVB is generally used over a shorter treatment period compared to broad band UVB phototherapy an enhanced and modified photobiological effect can be expected (9,10). This thesis shows that, an 8.1 higher narrow band UVB dose is needed to induce erythema in the skin of healthy volunteers. Acute effects of broad band UVB are inflammation, characterised by a perivascular infiltrate consisting of T-lymphocytes, polymorphonuclear leukocytes, and a significant decrease of the number of epidermal Langerhans cell (11,12), as well as a pronounced induction of p53, p16 and sunburn cells (13-15). This thesis confirms previous findings and shows that similar effects are seen in 3 MED narrow band UVB irradiated skin. No differences were observed between the photobiological responses to 3 MED broad band and 3 MED narrow band UVB exposure. Thus, an equivalent erythematogenic dose induces a similar photobiological effect, although physical doses differ a factor 8.1. This observation seems to contradict the findings of the study by Sheehan et al. who showed that the photobiological effect depends on the physical dose and not on the biological dose as measured with MED (16). This discrepancy could be explained by the difference in wavelength spectrum, as the study described in chapter 2 focuses only on 311 nm wavelength, while the study by Sheehan et al focused on the whole UVA and B spectrum (295-400 nm) (17).

5.2.3 Is visible light capable of inducing photodamage?

Visible light has always been regarded as safe and harmless at natural exposure conditions. In the last decade high dose visible light applications, such as laser and photodynamic therapy (PDT), have been introduced in dermatology. Studies on the effects of high dose visible light are sparse. As discussed in chapter 2, PDT depends on the availability of specific chromophores in the treatment area. In practise, to treat

target lesions with photodynamic therapy, it is necessary to increase the amount of locally available chromophores by applying 5-aminoleavulinic acid or derivatives (18). Chromophores such as DNA and bilirubin are naturally available in the skin (19,20). It is feasible that irradiation of these endogenous chromophores by high dose visible light, as used in PDT, could induce oxidation of surrounding molecules and lead to 'adverse' photobiological effects, such as photocarcinogenesis and photoageing. With the emerging wide spread use of this presumably safe therapy, questions concerning its safety arise. The sparse investigations on photobiological effects of visible light usually include the near-UVA spectrum of light (400-500 nm). Several studies have shown that near-UVA irradiation results in UVA-like photobiological effects, such as DNA strand breaks due to singlet oxygen species. To a lesser extent p53 induction and immediate pigment darkening are observed (21-24). Edström et al. showed that high dose visible light (400-800 nm) may induce limited p53, Ki67 and p21waf expression (25,26). Their spectrum however did include the near-UVA range (27). Therefore, a significant near-UVA effect cannot be excluded. In contrast, the study described in chapter 2, on the effects of high dose long wave visible light (560-780 nm) on skin of healthy volunteers, showed no induction of p53, c-Jun, Melan A, MMP-1 and sunburn cell formation. However, perinuclear vacuolisation was seen, which appeared 24 hours after the first irradiation, reached significance after the 4th irradiation, and did not return to normal one week after the last irradiation. So far, The significance of perinuclear vacuolisation is not clear. In UVB exposed skin a similar phenomenon can be observed. It could be hypothesised that this is a transitional stage toward apoptosis (28,29). In the study presented in chapter 2, no sunburn cells were seen for up to 11 days after the first irradiation and, as no p53 and c-jun expression was seen it is highly unlikely that an apoptosis pathway was induced.

5.3 To what extent does anti-TNF-α treatment modulate the response to UVB challenge?

5.3.1 What is the effect of anti-TNF-α treatment on the photobiological response after a single broad band UVB challenge?

TNF-α is a pro-inflammatory cytokine and has been shown to play a role in UV induced inflammation. It has been demonstrated that TNF-α expression is induced after UV irradiation (30,31). Anti-TNF-α has been introduced in the treatment of several chronic inflammatory diseases, such as rheumatoid arthritis, juvenile chronic arthritis, Crohn's disease and recently as experimental treatment in psoriasis vulgaris (32-35). It is remotely possible that single target therapies could potentially influence photobiological processes, such as photocarcinogenesis. In vitro studies have shown that suppression of the TNF-α photoresponse could reduce the expression of DNA

damage markers and inhibit apoptosis (36-39). It is unknown if similar effects in vivo occur after treatment with anti-TNF-α and whether reduced apoptosis could implicate enhanced photocarcinogenesis.

The study described in chapter 3 investigates the effects of anti-TNF-α on the broad band UVB induced photoresponse, with focus on photodamage. Rheumatoid arthritis patients were irradiated with 2 MED broad band UVB, before and after treatment with anti-TNF-α.

In the epidermis a significant increase in p53, c-jun expression and apoptosis was seen after irradiation in the epidermis. A significant increase in dermal matrix metalloproteinase-1 (MMP-1) expression was observed in the dermis. After treatment with the anti-TNF-α antibody only significant changes were seen in the expression of c-Jun and phosphorylated c-Jun expression, which slightly but significantly decreased during treatment with anti-TNF-α compared to before anti-TNF-α treatment. The production of matrix metalloproteinases is closely associated to the expression of c-Jun (40). In the study described in chapter 3 however, no change in MMP-1 expression before and after anti-TNF-α treatment was seen.

As mentioned earlier, in vitro anti-TNF-α can reduce photodamage and apoptosis. Our study shows that in vivo this effect is not expressed. We cannot exclude with certainty that a reduction might have been observed if a different time schedule was chosen, as the pharmacokinetics of the antibody in the skin is still largely unknown.

5.4   What is the clinical efficacy of narrow band UVB treatment in vitiligo and the impact of this treatment on quality of life?

5.4.1 Is narrow band UVB an effective treatment for vitiligo vulgaris?

Narrow band UVB has been introduced a few years ago as a possibly new and safe alternative for PUVA in the treatment of vitiligo (41-43). With the knowledge that photo (chemo)therapy for vitiligo is usually an intensive and protracted treatment and that especially long-term photochemotherapy may increase the risk of skin cancer significantly, a safe new and effective treatment is needed (44,45). Studies on the efficacy of narrow band UVB are sparse. These studies often use inaccurate graded scoring methods such as less than 25%, 26-50%, 51-75% and 76-100%) (46,47). These scoring methods evaluate repigmentation over the whole body and these methods do not reconcile the fact that the repigmention rate differs per body area and that some areas show complete repigmentation and some do not repigment at all. The study in chapter 4 describes a new repigmentation scoring system, which describes repigmentation percentage per body area. In this open study twenty-seven patients with long-term stable vitiligo (at least one year no new lesions or growth of vitiligo lesions) were irradiated thrice weekly with narrow band UVB phototherapy.

Irradiation dosages were increased on an individual basis with 0-30%. The percentage of repigmentation was estimated per body area. Areas with maximum repigmentation rate were designated as 'maximum repigmentation'.

This study clearly showed that narrow band UVB phototherapy is an effective treatment for vitiligo and that at least in one or more body areas in 92% of the patients up to 100% repigmentation could be reached. Furthermore it showed that 'hairless' lesions, such as dorsa of hands (knuckles and fingers), the inner wrist and feet, almost do not repigment (median repigmentation 22.5%), while other areas such as extensor surfaces of the lower arm and leg, back and throat often repigment rather easily (up to 100%). With these results in mind a revision of the generally used vitiligo repigmentation scoring methods seems warranted.

5.4.2 Does the addition of vitamin B12 and folic acid modulate the efficacy of narrow band UVB phototherapy in the treatment of vitiligo vulgaris?

Several authors have suggested that deficiencies, at the cellular level, of biochemical compounds, such as pseudocatalase, calciumchlorid and phenylalanine, which play a part in the melanogenesis pathway, could play a role in the pathogenesis of vitiligo (48-50). Some studies have shown that addition of these compounds could improve the repigmentation efficacy of phototherapy (48,51,52). In several groups of vitiligo patients, vitamin B12 and folic acid deficiencies have been demonstrated, although this is not a common characteristic to all vitiligo patients (53,54).

Although vitamin B12 and folic acid are not directly part of the melaninsynthesis pathway, indirect influence on the melaninsynthesis is likely (55). The combination of vitamin B12 and folic acid with UV exposure has been tested before in the sunny regions of Sweden (56). In this study a clear repigmentation was seen in vitiligo patients. This study however did not include a control group and did use a non-standardised irradiation protocol, i.e. expose the skin as much as possible to the Swedish sun. In the study mentioned in chapter 4, twenty-seven patients were randomised for treatment with narrow band UVB in combination with vitamin B12 and folic acid or treatment with narrow band only. Both groups were irradiated thrice weekly with narrow band UVB in our clinic. Although clear repigmentation was observed, no difference was seen between both groups.

5.4.3 Does long-term narrow band UVB phototherapy influence the quality of life in patients with vitiligo vulgaris?

Although vitiligo is not life threatening or physical debilitating, it is a very visible and socially handicapping disease as the lesions have as predilection sites the hands and

face, which can negatively influence quality of life (57-60). Studies on the efficacy of treatments for vitiligo vulgaris have always been focused on the effectiveness of therapies on repigmentation, whereas quality of life studies are sparse.

Assessment of quality of life may provide relevant information on the actual benefit of a treatment in patients with chronic diseases such as vitiligo. Quality of life research in vitiligo has mostly been focused on the impact of vitiligo on social, psychological and physical wellbeing and less on the impact of therapies on these factors (57,58,61,62). Quality of life problems in vitiligo patients can be categorised in the following categories: choosing clothes, use of sunblocks, use of camouflage, avoidance of activities, reactions of others and emotional reactions (57,58). There is no specific vitiligo oriented quality of life index. Njoo et al. studied the quality of life in children with vitiligo with the Child Dermatology Life Quality Index, a revised version of the 'adult' Dermatology Life Quality Index (DLQI) developed by Finlay and Khan (63). For the study in patients with vitiligo described in chapter 4 a slightly revised version of this DLQI was devised with more focus on visibility and avoidance. Furthermore, the questions were modified in view of the retrospective character of the study. A high percentage of the invited participants responded (71.4%), which is exceptionally high compared with the response rate of 39% in another quality of life questionnaire in vitiligo patients (57,58). This can be explained by the fact that all patients had recently finished long term phototherapy for vitiligo, with high compliance, while in the study by Kent and Al-Abadie all members of the UK vitiligo society, with or without current or recent treatment were invited (57,58). The response rate in our study was similar to that of other vitiligo studies where all patients were currently under active treatment (64).

Although our study used a non-validated retrospective Quality of Life Questionnaire, several interesting results were found. After long-term phototherapy for vitiligo, skin appearance does not play a major role in the lives of vitiligo patients anymore (as indicated by reduced feelings of insecurity and negative feelings) although only in a minority of patients general well-being improved. Furthermore, phototherapy increased the necessity to camouflage of the lesions, due to the pigment darkening of the surrounding uninvolved skin. The limited improvement of well-being and the increased necessity for camouflage seriously restricts our enthousiasm on the relevance of phototherapy in vitiligo. Long-term prospective studies are warranted to evaluate long-term effects on quality of life.

5.5   Future suggestions

Based on the findings and discussions in this thesis several suggestions for further research can be made.

The delay in UVA photoresponse compared to UVB, as described in chapter 2.1 might be caused by oxidative damage. Further studies should test this hypothesis.

As discussed before, the therapeutic efficacy of narrow band UVB is reached earlier as compared to broad band UVB, after treatment with doses with equivalent erythematogenic effects. However, the physical doses of narrow band UVB as compared to broad band UVB are substantially higher before a similar therapeutic effect is reached. The study in chapter 2.2 showed that narrow band and broad band UVB in equivalent erythematogenic doses after a single challenge have similar photodamage profiles. This supports the hypothesis that narrow band UVB is the safer treatment. However, repeated exposure and long term studies are necessary to further substantiate this hypothesis.

As described in chapter 2.3 high dose visible light seems safe, but it is unknown why perinuclear vacuolisation occurs. Long-term studies could provide more information on the significance of this phenomenon.

Based on the discussion in chapter 3 further studies on the effect of anti-TNF-α on photocarcinogenesis should be performed. In the present study we have restricted our observation to the effects in the skin 2 weeks after anti-TNF-α treatment. It is remotely possible that anti-TNF-α might have more acute or even delayed effects on the responses to UV. Further studies should clarify this issue.

As discussed in chapter 4.1 further development of a new more reliable scoring method for repigmentation efficacy, based on regional differences in repigmentation, is necessary.

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6

Nederlandse samenvatting en conclusies

6.1   Nederlandse samenvatting en conclusies

In dit proefschrift werden de effecten van diverse lichtspectra op de normale huid onderzocht alsmede de effectiviteit van smalspectrum UVB als behandeling voor vitiligo.

Hiervoor werden in de inleiding (hoofdstuk 1) een aantal vragen geformuleerd, welke in hoofdstuk 2, 3 en 4 werden beantwoord aan de hand van originele artikelen.

In hoofdstuk 2 werden de fotobiologische responsen van diverse lichtspectra onderzocht. Er is met name gekeken naar huidveroudering en beschadiging onder invloed van licht, maar ook naar inflammatie en pigmentatie. In paragraaf 2.1 worden breedspectrum UVA en UVB met elkaar vergeleken met nadruk op verschillen in oxidatieve processen en mogelijke relaties met huidbeschadiging en inflammatie. Er wordt al geruime tijd verondersteld dat UVA met name via oxidatieve processen effecten induceert. UVB veroorzaakt een direct effect. De gebruikelijke en verwachte expressiepatronen van inflammatie- en huidbeschadigingsparameters werden gezien. Opvallend was een vertraging van ongeveer 3-20 uur in de expressie van p53 en c-Jun na UVA belichting ten opzichte van UVB belichting. UVA induceerde significant meer oxidatieve schade (gemeten aan de hand van catalase verbruik en lipid oxidatie) dan UVB, welke al een uur na de UV blootstelling gemeten kon worden. Een uur na UVA blootstelling werd al een 882% stijging in squaleen hydroperoxide waargenomen tegenover een slechts 111% stijging in UVB blootgestelde huid. Een anti-oxidant systeem als catalase liet daarentegen een uur na UVA blootstelling al een 70% daling in activiteit zien, welke zich niet binnen 48 uur herstelde. Dit fenomeen werd niet bij UVB waargenomen. Gezien deze bevindingen is het waarschijnlijk dat de vertraging in p53 en c-Jun expressie aan deze oxidatieve processen kan worden toegeschreven.

In paragraaf 2.2 werden twee spectra van UVB met elkaar vergeleken, namelijk smalspectrum (311 nm) en breedspectrum (290-320). Ook hier werden huidbeschadiging, veroudering en inflammatie na UV blootstelling bestudeerd. Alle vrijwilligers werden aan 3x de minimale erytheemdosis (MED) blootgesteld. De fysische dosis voor één MED was gemiddeld een factor 8 hoger bij de smalspectrum UVB groep. Desondanks bleek dat ondanks dit grote verschil vergelijkbare expressie van markers voor huidbeschadiging, veroudering en inflammatie werden gezien. Er waren geen statistisch significante verschillen tussen beide groepen. Met de wetenschap dat smalspectrum UVB klinisch veel effectiever is gebleken in de behandeling van eczeem en psoriasis en dat over het algemeen minder belichtingen nodig zijn, kan worden geconcludeerd dat smalspectrum UVB lichttherapie hierdoor mogelijk ook veiliger is dan breedspectrum UVB.

In de afgelopen jaren worden hoge doses zichtbaar licht in toenemende mate toegepast in de dermatologie, onder andere in het kader van de fotodynamische therapie. In

paragraaf 2.3 werd gekeken naar de mogelijke effecten van hoge doses zichtbaar licht op de normale huid. Zichtbaar licht werd altijd als volledig onschadelijk betracht. Echter diverse studies hebben aan getoond dat er wel degelijk expressie van diverse huidbeschadigings- and verouderingsmarkers geïnduceerd konden worden. Met de studie beschreven in paragraaf 2.3 lieten wij zien dat deze bevindingen enigszins genuanceerd moeten worden. Met het zichtbaar licht spectrum (560-700 nm), zoals gebruikt in deze studie, werd alleen het onbekende fenomeen perinucleaire vacuolisatie gezien. Er werden geen andere markers tot expressie gebracht. De eerder beschreven veranderingen onder zichtbaar licht lijken te worden bewerkstelligd door het deel van het zichtbaar licht spectrum dat nog vrij energierijk is en dicht bij het UVA spectrum ligt (400-560 nm). In het gebruikte spectrum van deze vorm van fotodynamische therapie leek er geen relevante schade aan de omringende huid te worden toegebracht gedurende enkele of meerdere PDT-sessies. De relevantie van de perinucleaire vacuolisatie is onduidelijk.

In hoofdstuk 3 werd aandacht besteed aan de mogelijke beïnvloeding van de lichtrespons door anti-TNF-α, een nieuw geneesmiddel voor chronisch inflammatoire aandoeningen als reumatoïde artritis en M.Crohn. Inmiddels wordt deze therapie ook in trialverband binnen de dermatologie toegepast. Aangezien TNF-α een belangrijke pro-inflammatoire factor is in de UV-respons, leek het zinvol om de invloed van dit geneesmiddel op de UV-respons te onderzoeken. In het kader van een onderzoek bij patiënten met chronisch reumatoïde artritis werden patiënten, voor en na toediening van anti-TNF-α, aan UVB blootgesteld. De klinische effectiviteit van een subcutane injectie met anti-TNF-α, op het synovium van reumatoïde artritis patiënten, is na 2 weken optimaal. Daarom was er voor gekozen om de respons op UVB bestraling 2 weken na de toediening van anti-TNF-α te onderzoeken. In dit tijdschema werden behoudens een minimale maar significante toename in c-Jun expressie geen andere verschillen waargenomen. Het is niet uit te sluiten, dat een gewijzigd tijdschema, met het biopt kort na de subcutane toediening van het anti-TNF-α antilichaam, wel effecten op beschadigingsmarkers kan laten zien.

In hoofdstuk 4 werd gekeken naar de klinische en psychosociale effecten van smalspectrum UVB lichttherapie voor de behandeling van vitiligo. Tot voor kort was PUVA fotochemotherapie de behandeling bij uitstek voor vitiligo. Met de komst van smalspectrum UVB lijkt hier verandering in te komen. Recent onderzoek heeft aangetoond dat smalspectrum UVB een effectieve therapie voor vitiligo lijkt te zijn. Het grote nadeel van deze onderzoeken was het feit dat er onnauwkeurige en algemene scoringsmethodes gebruikt werden. Effectiviteit werd namelijk gemeten in vier repigmentatie gradaties (minder dan 25%, 26-50%, 51-75% en 76-100%), welke gelden voor het gehele lichaam. In de studie in paragraaf 4.1 werd rekening gehouden met regionale verschillen in repigmentatie. Dit onderzoek liet zien dat

3 keer per week smalspectrum UVB lichttherapie in 92% van de patiënten tot een volledige repigmentatie van diverse laesies kon leiden en dat diverse 'haarloze' laesies als handen, binnenzijde van de polsen en voeten slechts minimaal (<20%) repigmenteerden.

Diverse auteurs hebben aangetoond dat het toevoegen van stoffen, die een mogelijke rol bij de melanogenese spelen, in combinatie met lichttherapie tot een versnelde en verbeterde repigmentatie zou kunnen leiden. De studie in paragraaf 4.1 onderzocht de effecten van vitamine B12 en foliumzuur op smalspectrum UVB geïnduceerde repigmentatie. Enkele studies hadden aangetoond dat sommige patiënten een deficiëntie voor vitamine B12 en foliumzuur hebben en dat zonlichtblootstelling in combinatie met suppletie effectief zou zijn. Uit onze studie bleek er echter geen significant verschil met de controle groep te zijn in repigmentatie effectiviteit.

Ondanks dat vitiligo geen levensbedreigende of lichamelijk invaliderende ziekte is hebben veel patiënten, door de zichtbaarheid van de aandoening, regelmatig psychosociale problemen. De effectiviteit van lichttherapie wordt voornamelijk gemeten aan de hand van de repigmentatie en slechts zelden aan verbetering van de kwaliteit van leven. In de studie beschreven in paragraaf 4.2 werden alle vitiligopatiënten die in de periode 1998-2001 langdurig met smalspectrum UVB lichttherapie werden behandeld, gevraagd deel te nemen aan een kwaliteit van leven vragenlijst. Hierbij bleek dat de meeste patiënten sinds de behandeling minder aandacht aan hun huid en huidaandoening schonken, maar ook dat er in sommige gevallen meer gecamoufleerd moest worden. Dit valt te verklaren door een toegenomen contrast tussen gepigmenteerde en gedepigmenteerde huid na lichttherapie en het feit dat de handen voorkeurslokalisaties zijn en minder goed repigmenteren (zie paragraaf 4.1).

In hoofdstuk 5 werden alle bevindingen samengevat en bediscussieerd aan de hand van de bestaande literatuur.

Dankwoord

Dit proefschrift had niet tot stand kunnen komen zonder de samenwerking en hulp van velen. Helaas is het onmogelijk om iedereen die een bijdrage heeft geleverd bij naam te noemen. Op deze waarschijnlijk meest gelezen pagina's van het proefschrift zal ik een poging doen om de belangrijksten te vermelden.

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Rianne Gerritsen, als mijn co-promotor schreef jij grote delen van de projecten die uiteindelijk tot dit proefschrift hebben geleid. Ook jij stond open voor allerlei ideeën die tot diverse publicaties hebben geleid. Voor alle problemen, al dan niet proefschriftgerelateerd, kon ik altijd bij je terecht. Je verbaasde me aangenaam door actief deel te nemen aan een cursus behandeling van psoriasis met Chinese kruiden in Londen, iets wat ik altijd zal blijven onthouden.

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Jaappieter Wolffensperger, je was mijn eerste wetenschappelijk stage student. Voor beiden wel even wennen, maar je hebt prachtig werk geleverd (cum laude) en ik hoop dat het leven als legerarts je goed is bevallen.

Tim Smits, aanvankelijk begonnen als wetenschappelijk stagiaire (cum laude) en nu zelf promovendus op onze afdeling dermatologie. Je enthousiasme en wetenschappelijke inzicht kwamen nog net op tijd voor mij, en brachten mijn promotie in de noodzakelijke stroomversnelling. Iets wat uiteindelijk resulteerde in een langdurige samenwerking, hechte vriendschap en meerdere publicaties en presentaties.

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Arno en Wijnand, jullie zijn beiden langere tijd kamergenoten geweest. Met jullie is onze oorspronkelijke meidenkamer (Astrid, Mieke en Mai Le) toch echt een gezellige

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Curriculum vitae

Milan Tjioe werd geboren op 4 oktober 1973 in Groningen, en groeide gedurende de eerste 7 jaar op in het Groningse dorp Haren. Vervolgens verhuisde hij naar Nijmegen, alwaar hij zijn lagere en middelbare school opleiding genoot. Na het VWO-diploma behaald te hebben op het R.K. Lyceum Dominicus College studeerde hij gedurende de zomer Chinees op het Foreign Languages Institute te Beijing, China. Naast zijn studie geneeskunde aan de Katholieke Universiteit Nijmegen was hij actief als voorzitter van de Werkgroep Integratie Geneeswijzen en behaalde hij zijn Masters degree in traditionele Chinese geneeskunde bij de Nederlandse Artsen Acupunctuur Vereniging. Na zijn artsexamen in november 1997 ging hij onder leiding van Prof.dr.dr. P.C.M. van de Kerkhof en Dr. M.J.P. Gerritsen werken op de afdeling Dermatologie in het Universitair Medisch Centrum St Radboud Nijmegen, alwaar de onderzoeken werden verricht die tot dit proefschrift hebben geleid.

Naast zijn belangstelling voor de dermatologie heeft hij interesses in de medische informatie- en communicatietechnologie. In het kader hiervan is hij één van de drijfveren van medisch-zakcomputer gebruikend Nederland en één van de oprichters van de Usergroup for Medical Mobile Computing (UM2C). Sinds 1999 schrijft hij artikelen over zakcomputers voor de Amerikaanse website Geek.com.

Sinds 1 september 2002 is hij dermatoloog in opleiding.

Milan is gelukkig getrouwd met Sioe Lie Go.