Ultraviolet Radiation and Squamous Cell Carcinoma in Human Skin

BY

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ABSTRACT
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Ultraviolet radiation (UVR) is a major risk factor for development of skin cancer. UVR-induced DNA damage and a dysfunctional p53 protein are important steps in the development of squamous cell carcinoman in human skin (SCC). The aim of the present investigation was to analyze incidence trends of SCC in Sweden, quantify the risk of second primary cancer after SCC and further analyze the effects of UVR and p53 protein in human skin *in vivo* and *in vitro*. The effect of photoprotection by sunscreens was also evaluated.

We found that the age-standardized incidence rate of SCC in Sweden increased substantially in both men and women during the period 1961-1995, especially in men and at chronically sun-exposed skin sites. Patients with SCC are also at increased risk of developing new primary cancers, especially in the skin, squamous cell epithelium, hematopoietic tissues and respiratory organs. In experimental studies *in vivo* and *in vitro* in human skin we observed that repair of UV-induced DNA damage appears to be more efficient in chronically sun-exposed skin despite a less uniform p53 response. Non-sun-exposed skin is more homogeneous with respect to the epidermal p53 response. Keratinocytes in skin exposed frequently to the sun may be prone to react more easily to cytotoxic stress. Two different modalities of photoprotection significantly reduced the amount of DNA damage and the number of p53-positive cells. In addition, we demonstrated that a well-defined system for *in vitro* culture of explanted skin provides an excellent alternative to *in vivo* experiments.

In conclusion, this study has increased our knowledge of SCC epidemiology in Sweden and of the effects of artificial and solar UVR and sunscreens on chronically sun-exposed and non-sun-exposed sites, respectively, of human skin.

Key words: Ultraviolet radiation, squamous cell carcinoma, incidence, sunscreens, p53, DNA damage.

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ORIGINAL PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Wassberg C, Thörn M, Johansson AM, Bergström R, Berne B and Ringborg U. Increasing incidence rates of squamous cell carcinoma of the skin in Sweden. Acta Derma Venereol, 81, 268-272, 2001

II. Wassberg C, Thörn M, Yuen J, Ringborg U and Hakulinen T. Second primary cancers in patients with squamous cell carcinoma of the skin: A population-based study in Sweden. Int J Cancer, 80, 511-515, 1999.

III. Wassberg C, Bäckvall H, Diffey B, Pontén F and Berne B. UV-induced DNA damage and p53 response in chronically sun-exposed and non-sun-exposed skin: Effects of photoprotection. Submitted

IV. Bäckvall H, Wassberg C, Berne B and Pontén F. Similar UV responses are seen in a skin organ culture as in human skin *in vivo*. Exp Dermatol, accepted for publication.

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1. ABBREVIATIONS

BCC	Basal cell carcinoma of the skin
C	Cytosine
CI	Confidence interval
CPDs	Cyclobutane pyrimidine dimers
EPU	Epidermal proliferative unit
HPV	Human papillomavirus
IARC	International Agency for Research on Cancer
MED	Minimal erythema dose
MM	Malignant melanoma of the skin
NER	Nucleotide excision repair
NMSCs	Non-melanoma skin cancer
SCC	Squamous cell carcinoma of the skin
SCUP	Skin Cancer Utrecht-Philadelphia
SED	Standard erythema dose
SIR	Standardized incidence ratio
SPF	Sun protection factor
T	Thymine
TiO_2	Titanium dioxide
TT dimers	Thymine dimers
UV	Ultraviolet
UVA	Ultraviolet radiation in the A band (320-400 nm)
UVB	Ultraviolet radiation in the B band (280-320 nm)
UVC	Ultraviolet radiation in the C band (200-280 nm)
UVR	Ultraviolet radiation
XP	Xeroderma pigmentosum
ZnO	Zinc oxide

2. INTRODUCTION

2.1 Background

The incidence of skin cancer (malignant melanoma and non-melanoma skin cancer) is increasing worldwide. A major risk factor for developing skin cancer is exposure to ultraviolet radiation (UVR). UVR promotes aging of the skin and causes local and systemic immunosuppression. It also initiates and promotes carcinogenesis. The two major UV-induced types of DNA damage are the formation of cyclobutane pyrimidine dimers (CPDs), of which thymine dimers (TT dimers) are predominant, and of (6-4) photoproducts. These cellular lesions are considered to constitute the primary damage responsible for the cytotoxic, mutagenic and carcinogenic actions of solar UVR.

There are three types of invasive cancer that develop in skin: Malignant melanoma (MM), which is a tumor that originates from melanocytes; and the non-melanoma skin cancers (NMSCs), namely squamous cell carcinoma and basal cell carcinoma (BCC), both of which derive from keratinocytes. MM is an aggressive tumor that is often manifested at relatively young ages (mean 50-60 years) and sets metastases early. It is associated with high mortality if not treated at an early stage. Thus, MM differs distinctly from NMSCs, which are most common in older persons and grow less aggressively. The present thesis focuses mainly on squamous cell carcinoma in human skin (SCC).

The aim of the present investigation was to analyze incidence trends of SCC in Sweden, to quantify the risk of second primary cancer after SCC and to further investigate the effects of UVR at a cellular level by measuring the levels of TT dimers and p53 protein in human skin. The photoprotective effect of sunscreens was also evaluated by both *in vivo* and *in vitro* studies.

2.2 Normal human skin

The human skin is one of the largest organs in the body. It accounts for 16% of the body weight and has a surface area of 1.8 m^2. The most important physiological functions of

the skin are to serve as a barrier against the outer environment by protecting the body from noxious external factors, and to keep the internal systems intact.

The skin is composed of three layers: the epidermis, the dermis and the subcutis. The epidermis consists of four layers of cells, mainly keratinocytes and to a lesser extent dendritic cells (melanocytes and Langerhans' and Merkel cells). These layers also represent the stages in the differentiation/maturation of the keratinocytes. The basal cell layer (stratum basale) consists mainly of one layer of basal keratinocytes. Melanocytes make up 5-10% of the basal cell population. Melanocytes synthesize melanin, which is transferred to neighboring keratinocytes by a dendritic process. The stratum spinosum consists of daughter basal cells which have migrated upwards to form this multi-cell layer (4-10 layers) of polyhedral cells. Langerhans' cells (immunologically active cells) are mostly found in this cell layer. In the stratum granulosum, consisting of 2-3 cell layers, the cells become flattened and lose their nuclei, and in the cell cytoplasm keratohyaline granules can be seen. The uppermost layer is the stratum corneum, the horny layer, which is also the end result of the maturation of the keratinocytes. This layer consists of a sheet of overlapping polyhedral cornified cells with no nuclei, and the cytoplasm is replaced by keratin. The thickness of the epidermis is about 0.1 mm, but it may vary with the anatomical site, with the greatest thickness on the soles and palms (0.8-1.4 mm).
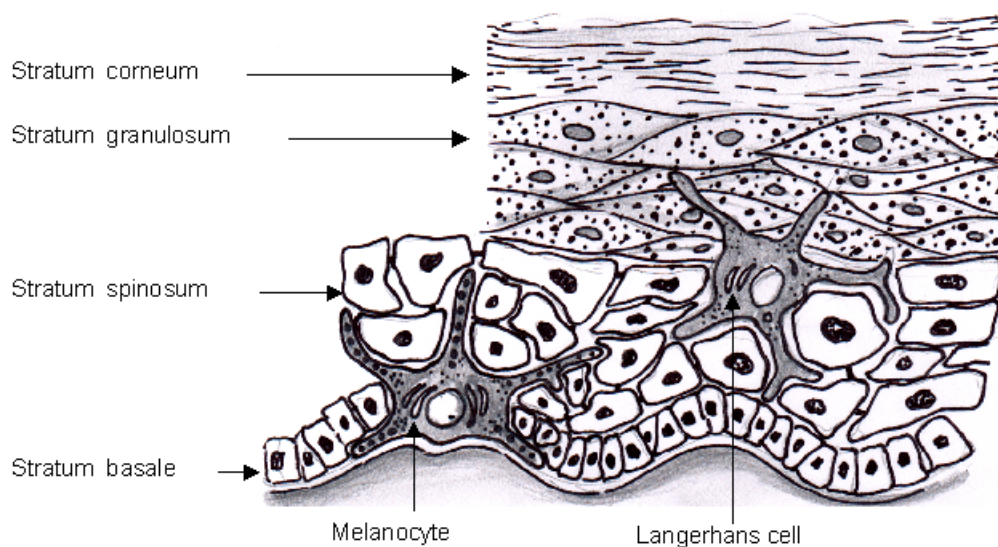

Figure 1. Anatomy of the epidermis

In the epidermis, the rate of cell proliferation in a steady state has to match the rate of cell loss from the surface in order to maintain the integrity of the tissue. Kinetic studies have shown that the differentiating time for a keratinocyte is about 14 days, by which time it has reached the stratum corneum, and that it takes a further 14 days for it to be shed away. Thus it takes 3-4 weeks for the epidermis to renew itself. This maintenance of homeostasis requires a population of stem cells that give rise to transient amplifying cells, which can undergo a limited number of cell divisions before entering the differentiation pathway and eventually becoming the terminally differentiated cells. It has been proposed that the human epidermis is built up of epidermal proliferative units (EPU), organized in a fine mosaic pattern (Asplund et al., 2001), and that each unit forms a column of cells, supported by one epidermal stem cell positioned in the basal cell layer (Parkinson, 1992; Potten, 1974).

The dermis lies immediately below the epidermis, and its main function is to serve as a strong supportive connective tissue matrix. This skin layer is mostly built up of collagen fibers running in horizontal bundles, elastin, and ground substance (glycosaminoglycans), all substances produced by the dermal fibroblasts. The thickness varies between different anatomical sites, from 0.6mm to >3 mm. Beneath the epidermis and dermis, the third and last layer of the skin is found, the subcutaneous layer, which consists of loose connective tissue and fat.

2.3 Ultraviolet radiation

The solar radiation consists of a broad spectrum of electromagnetic waves, a form of energy that can be divided into three types of wavelength bands, namely UVR, visible light, and infrared radiation. The ultraviolet part is further divided into three bands according to the nomenclature of the CIE (International Commission on Illumination): UVC (100-280 nm); UVB (280-320) and UVA (320-400 nm). Visible light occupies a band ranging from 400 nm (violet) to 700 nm (red), and infrared radiation consists of wavelengths of > 700 nm.

The earth's atmosphere, especially the stratospheric ozone layer, plays a major role in filtering the UVR reaching the surface of the earth. UVC is completely filtered by the ozone layer, and UVB is partially filtered, while UVA is only minimally affected by the ozone layer. The intensity of the UVR reaching ground level, i.e., terrestrial UVR, depends on the time of day, variation in the stratospheric ozone with latitude and season, geographical latitude, surface reflection, altitude, the elevation of the sun above the horizon, air pollution, and on climatic factors such as cloudiness, seasons, and day-to-day variations. Of the terrestrial UVR in the Stockholm area in Sweden, approximately 2% consists of UVB and 98% of UVA (Ulf Wester at the Swedish National Institute of Radiation protection, personal communication). Current data indicate that a worldwide decline in stratospheric ozone has occurred during the last two decades and is expected to continue well into the next century as a result of which more UVR will reach the earth (Urbach, 1997). The decline in the ozone layer in Sweden occurred mostly during the 1980s and 1990s, and this layer has now stabilized (Weine Josefsson, SMHI, Sweden, personal communication).

The biological effects of UVR are dependent on the amount of photon energy emitted and the penetration into the skin. UVC is the region with the shortest wavelengths and the highest photon energy. The terrestrial UVR that is most carcinogenic in humans is UVB, which emits most of its energy in the epidermis and induces erythema, burns and DNA damage and eventually skin cancer. UVA penetrates deeper into the dermis and induces collagen breakdown, causing aging and wrinkling of the skin. Animal studies have demonstrated that high doses of UVA over a long period of time can also cause skin cancer (Strickland, 1986; Zigman et al., 1976).

When human skin is exposed to UVR, about 5% of the UVR is reflected from the surface of the stratum corneum and about 10% is scattered in the epidermis (Anderson and Parrish, 1981). The remaining UVR is absorbed in melanin and by other molecules such as DNA, proteins, and porphyrins. These UVR-absorbing molecules are called chromophores. DNA absorbs UVR maximally at wavelengths from 245-290 nm (Tornaletti and Pfeifer, 1996). DNA is not capable of absorbing UVR at wavelengths longer than 320 nm, but the longer wavelengths can cause damage to genetic material

by reacting with other chromophores, which transfer the energy further towards the DNA, a process called photosensitization. Melanin absorbs UVR throughout the whole ultraviolet (UV) spectrum without a distinct maximum wavelength.

The UV dose absorbed by the skin can be quantified and the doses are often expressed as Standard Erythemal Dose (SED) (Diffey et al., 1997). One SED is defined as the dose that just elicits erythema in unexposed buttock skin in the most sensitive of healthy Caucasians, and has been found to be 10 mJ/cm^2. An exposure of about 1.5 SED is required to produce just perceptible erythema in individuals who burn easily and never tan (skin type I), about 2 SED in those who burn easily but tan minimally (skin type II), and 3 SED in those who will burn but tan readily (skin type III). In Denmark, one SED is equivalent to the dose received in approximately 15 minutes of sun exposure around noon on a clear and sunny day in July, and the maximum possible dose during a day is 45 SED (Vainio and Bianchini, 2001). In one day in Southern Europe, however, during holidays, for example, usually not more than 20 SED is received, as a dose of 2-3 SED is needed to induce erythema in white skin. A number of studies from different countries reviewed in the latest version of "Handbooks of cancer prevention-Sunscreens" from the International Agency for Research on Cancer (IARC) (2001) (Vainio and Bianchini, 2001) indicated that indoor workers received an annual dose of about 200 SED, while outdoor workers received two to three times this dose.

2.4 Photocarcinogenesis

2.4.1 UVR-induced DNA damage, repair and mutation

UVR is able to induce mutagenic photoproducts or lesions in DNA between adjacent pyrimidines by dimer formation on the same DNA strand. These dimers are of two main types: CPDs, most commonly TT dimers, and of (6-4) photoproducts between adjacent thymine or cytosine residues (Fig. 2). CPDs are formed between the C-4 and C-5 carbon atoms of any two adjacent pyrimidines; and (6-4) photoproducts are formed between the 5-prime 6 position often between T-C and C-C residues. CPDs are produced about three times as often as (6-4) photoproducts, but it should be noted that (6-4) photoproducts

are repaired much more quickly than CPDs in mammalian cells (Tornaletti and Pfeifer, 1996). It has been suggested that large structural distortion in the DNA helix induced by (6-4) photoproducts may ensure favorable recognition by the repair machinery (Young et al., 1996). Studies on the mutation frequency of photoproducts have shown that (6-4) photoproducts appear to be more mutagenic than CPDs (Gentil et al., 1996; Kim et al., 1995; Smith et al., 1996). If not repaired, UV-induced DNA lesions can lead to permanent mutations in the DNA sequence. These mutations are in the form of C→T and CC→TT transitions, known as UV "signature" mutations.
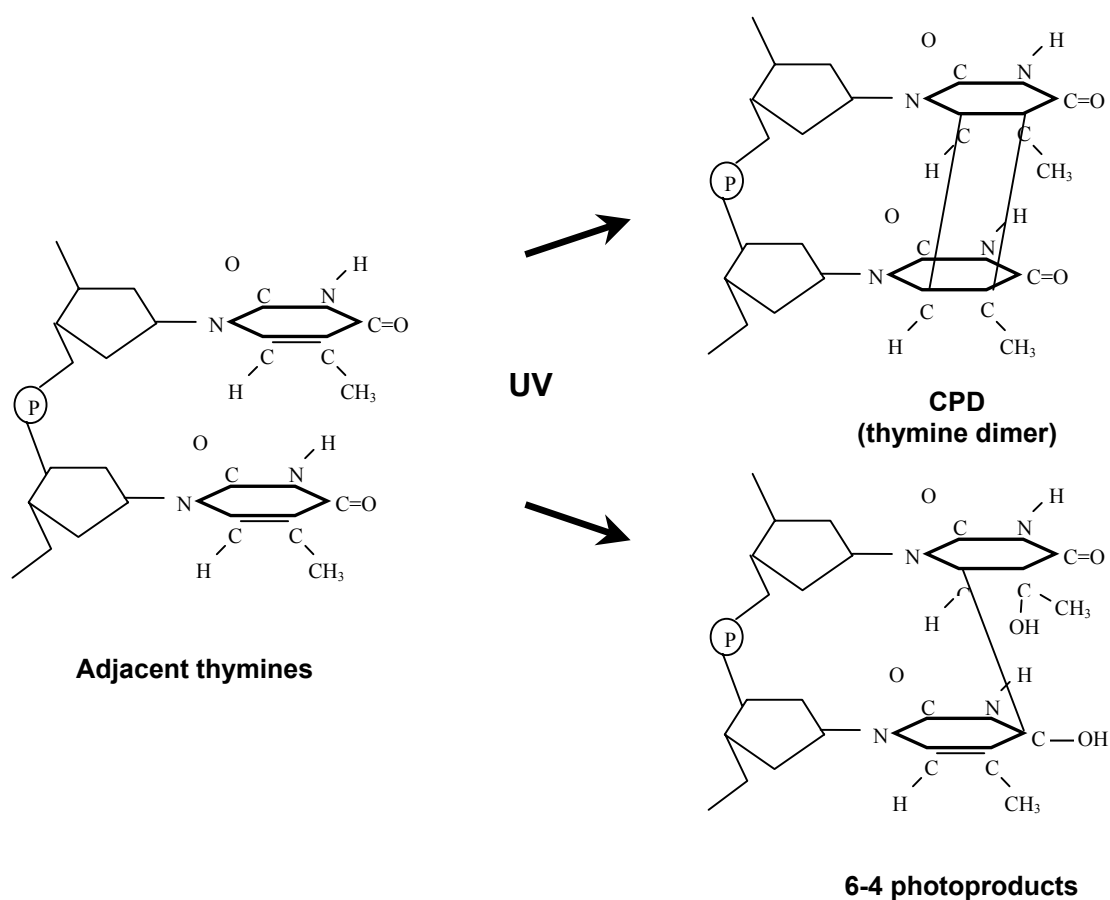

Figure 2. Structure of the two main types of UV-induced photoproducts. Reproduced with the permission from Helena Bäckvall.

The nucleotide excision repair (NER) system executes removal of CPDs and 6-4 photoproducts. About 30-50% of the CPDs in human keratinocytes are removed within 24 hours after exposure to UVR, while more than 50% of all 6-4 photoproducts are removed within 6 hours (Bykov et al., 1998; Bykov et al., 1999). However, there are large inter-individual variations in the degree of induced DNA photodamage and in the repair kinetics in human skin, and the DNA repair capacity may diminish in older populations ($\geq$50 years of age) (Xu et al., 2000). Error or deficiency in NER, as in patients with xeroderma pigmentosum (XP), results in an increased number of mutations in the genome. XP patients have a 1000 times greater risk of developing skin cancer compared to healthy subjects (Kraemer et al., 1994).

Other photoproducts in addition to CPDs and (6-4) photoproducts are produced by UVR, but these account for a much lower proportion (<1%) of all UV-induced photodamage and little is known about their mutagenicity (Tornaletti et al., 1993).

Researchers in the field of experimental UVR carcinogenesis have developed a wavelength dependency spectrum or "action" spectrum of the induction of SCC, based partly on animal experiments and partly on descriptive and analytical studies in humans (de Gruijl et al., 1993). Their work resulted in a "SCUP-m" action spectrum for mice and a "SCUP-h" for humans (SCUP=Skin Cancer Utrecht-Philadelphia). The SCUP-h action spectrum of skin cancer induction in humans (de Gruijl and Van der Leun, 1994) was calculated by correcting the SCUP-m spectrum for differences between mice and humans in epidermal UVR transmission. For UVB, the maximal carcinogenic efficiency and induction of CDPs in human skin are found at 290-300 nm, with decreasing efficiency from 300 to 350 nm.

2.4.2 p53

The human p53 tumor suppressor gene is located on the short arm of chromosome 17. This gene encodes a 53 kDa DNA-binding protein consisting of 393 amino acids. The p53 protein can be divided functionally and structurally into three domains: the N-terminal transcriptional activation domain, the DNA-binding core domain, and the

multifunctional C-terminal domain. The p53 protein forms tetramers, which are the functional forms.

There are indications that p53 plays an important role in maintaining the genomic stability in cells by regulating a number of genes that eventually lead to one of two major end-points: cell cycle regulation (cell cycle arrest) and apoptosis (programmed cell death) (Fig. 3) (Harris, 1996; Lane, 1992). In cells in which the outcome is cell cycle arrest, an increase in the p21 WAF1 protein and its downstream proteins contributes to the arrest of cells in G1. In certain cells the outcome is apoptosis, which is mediated by activation of BAX and down-regulation of Bcl2. In 1984 it was documented that UVR exposure of non-transformed cells stimulated accumulation of p53 caused by post-translational stabilization (Maltzman and Czyzyk, 1984). Previous studies in human skin have shown that p53 protein accumulates in keratinocytes after a single erythemogenic dose of UVR from different artificial UV sources, peaking at 8-24 hours after exposure and subsiding within 5 to 15 days (Hall et al., 1993; Pontén et al., 1995). This acute, reversible p53 response is interpreted as a beneficial reaction to DNA damage that facilitates repair of CPDs and (6-4) photoproducts or leads to apoptosis of severely damaged keratinocytes.

p53 is the most frequently altered gene in human cancers (>50%) (Hollstein et al., 1991). In the development of SCC, mutations in the p53 gene leading to a dysfunctional p53 protein seem to be an early and crucial event (Ziegler et al., 1996). SCC precursor lesions such as actinic keratosis often display mutation patterns similar to those in invasive SCC, with 80-90% consisting of UV "signature" mutations in the p53 gene (Ziegler et al., 1994). Sequencing data from a large number of cases of actinic keratosis and SCC in human skin showed that p53 was mutated in 60% and 90% of the cases respectively (Brash et al., 1991; Campbell et al., 1993; Jonason et al., 1996; Ren et al., 1996; Ziegler et al., 1994; Ziegler et al., 1993).

There are two different immunohistochemical p53 patterns in the epidermis: 1) a physiological "reactive pattern" in normal epidermis that is seen immediately after UVR, and 2) a compact "patchlike" pattern representing a clone of keratinocytes most
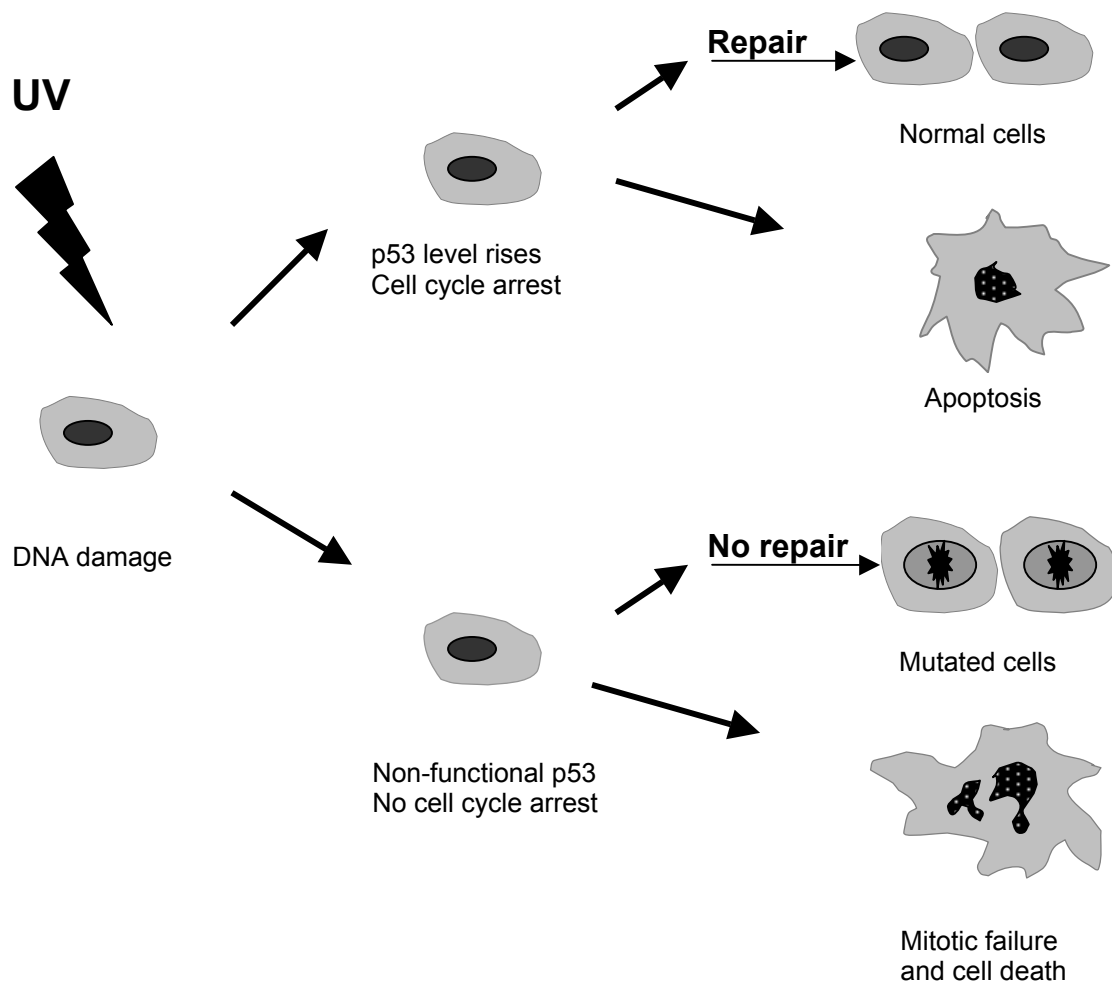
Figure 3. Schematic illustration of the function of p53 as the "guardian of the genome". In a normal cell with a normal p53 response, either the cell can enter G1 (cell cycle arrest),with consequent DNA repair, or, if the DNA damage is too great, the cell can undergo p53-dependent apoptosis. If the cell exhibits a non-functional p53, the DNA damage will not be repaired and a mutation may take place in the cell, leading to a manifest mutation in daughter cells. A non-functional p53 then divides the daughter cells, leading to cell mutation and genomic instability with mitotic failure and cell death.

often located in chronically sun-exposed skin (Ren et al., 1996). The reactive pattern is characterized by dispersed p53-positive cells in all cell layers in the epidermis, with an intermediate staining intensity, while the "patchlike" pattern representing a p53 clone is a more compact pattern of strong nuclear accumulation of immunoreactive p53 with

sharp borders against surrounding keratinocytes. p53 clones are very common in chronically sun-exposed human skin and both the size and number of these clones appear to increase with age. The biological significance of epidermal p53 clones is not clear, but approximately 70% of the p53 clones have an underlying mutation in the p53 gene (Ren et al., 1996), although no genetic link has yet been found between p53 clones and adjacent non-melanoma skin cancers (Ren et al., 1997).

2.5 Squamous cell carcinoma of human skin

2.5.1 Incidence

An increasing incidence of NMSCs has been observed worldwide (Parkin et al., 1997), especially in populations of Caucasian origin (Gallagher et al., 1990). It has been estimated that at least 2.75 million cases of NMSCs were diagnosed in the world in 1985 (Armstrong and Kricker, 1995), and in the United States about 1.3 million cases of NMSC are expected to occur in the year 2001 (Alam et al., 2001). Among the NMSCs cases in white populations, approximately 80% are BCC and 20% are SCC (Miller and Weinstock, 1994).

Few population-based national cancer registries include NMSCs among the conditions to be reported. There are several reasons for this; often no hospitalization is needed, the prognosis is favorable, and all excised skin cancer specimens are not sent for histopathological confirmation. Consequently, population-based studies of patients with NMSCs are sparse. Furthermore, the completeness of the data varies between cancer registries, and underreporting of NMSCs may be substantial (Coleman and Demaret, 1988) and also a serious obstacle to epidemiological studies. Among the Scandinavian countries, both SCC and BCC have been registered separately in Denmark (since 1978), Norway (during 1976-82) and Finland, while in Sweden and Iceland all malignant skin tumors except BCC have been reported. In Sweden SCC constitutes about 6% of all diagnosed cancers, and among both men and women it has been the malignant tumor with the most rapid increase in incidence in the last 10-year period, with an average annual increase of 3.1% in men and 3.9% in women. Data from the Swedish Cancer

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Registry have shown that between 1978 and 1997 the age-standardized incidence rates of SCC (Swedish standard population; the 1970 census) in Sweden increased from 15.4 to 30.2 per 100,000 in men and from 6.1 to 14.1 in women. In Scandinavia, there is also a south to north gradient in the incidence rates of both SCC and BCC (Magnus, 1991).

The lowest age-standardized incidence rates of NMSCs are found in populations of Asian and African origin (Parkin et al., 1997). In 1988-92, among people in Shanghai the age-standardized incidence rate of NMSCs in men was 1.7 and in women 1.1 per 100,000, and among black people in the USA these figures were 0.7 and 1.0, respectively. The highest incidence rates of NMSCs are found in Australia. SCC alone has a yearly age-standardized incidence of 250 per 100,000 Australia-wide (Marks et al., 1993), but regional differences exist. Incidence trends for SCC among people in Western Europe (Coebergh et al., 1991; Franceschi et al., 1996; Kaldor et al., 1993; Magnus, 1991; Roberts, 1990) and people of European origin (Buettner and Raasch, 1998; English et al., 1997; Marks et al., 1993) show similar patterns; i.e., a continuously rising incidence of SCC in general, especially since 1985, higher rates among elderly persons, particularly among men, and the greatest risks for head-neck sites. The much higher age-standardized incidence rates in general for SCC in men can be explained by differences in sun tanning habits, hairstyles, clothing behavior, or indoor and outdoor occupations between the sexes (Beral and Robinson, 1981). Baldness in men entails a higher dose of UV on the external ears, scalp and neck. Pearl and Scott (1986) reviewed the literature and calculated the relative tumor densities in different incidence studies of NMSCs worldwide. Tumor densities were calculated for individual anatomical sites, and such results are important in analyses of the correlation between UVR exposure and skin cancer. They found an extreme excess of both BCC and SCC on sun-exposed areas (head-neck, backs of the hands and forearms).

In a Danish study from 1991 the mortality from SCC was calculated 4.3% and that for BCC 0.1% (Osterlind et al., 1991).

2.5.2 Risk factors

The two major risk factors for developing SCC are the amount of sun exposure and the degree of pigmentation (skin type). Those at highest risk are fair-skinned individuals with skin type I-II according to Fitzpatrick´s classification (Fitzpatrick, 1988), exposed to an excessive amount of sun. High cumulative chronic sun exposure is probably the most important risk factor for SCC (Osterlind et al., 1991). This solar exposure may be occupational, but in recent times it has become more and more recreational. Occupational exposure to ultraviolet radiation has been considered to imply an increased risk for SCC among outdoor workers compared to those working indoors (Beral and Robinson, 1981). In addition to individuals with fair skin, older persons are run an increased risk of developing SCC (Alam et al., 2001; Vitasa et al., 1990). However, age as a risk factor is difficult to separate from exposure to sunlight, as the lifetime dose of sunlight is clearly related to age. A history of actinic keratosis also increases the risk of SCC (Marks et al., 1990). Salasche et al. (2000) reviewed the literature and concluded that among persons with multiple actinic keratosis, the cumulative lifetime risk of having at least one invasive SCC is substantial, possibly 6-10%, whereas Marks et al. (1988), in a prospective study of malignant transformation of actinic keratosis to SCC, found that the risk appeared to be less than 1 per 1000 per year. According to Frisch and Melbye (1995), patients who have had one SCC run an increased risk of developing a new SCC.

Other risk factors are smoking, exposure to ionizing radiation, exposure to arsenic and coal-tar, scar formation (chronic ulcers and burn scars), and viruses such as human papillomavirus (HPV), especially in immunosuppressed patients. Transcriptionally active viral DNA may become incorporated into the keratinocyte genome, or the HPV may act through alteration of the p53 pathway. Recipients of organ transplants, for example kidneys, are at increased risk of developing SCC, probably because of the use of immunosuppressive medication or as a result of conditions leading to an immunocomprised status (Jensen et al., 1999). SCC in immunosuppressed patients seems to have a more aggressive course.

2.5.3 Clinical features and biology

Actinic keratosis, also called solar keratosis, is a precursor to SCC. The general clinical features of actinic keratosis are inflamed, hyperkeratotic and scaly lesions which may vary in size from 2 to 6 mm in diameter. Usually several lesions are present, and these may coalesce to form large lesions. SCC in the early stage, i.e., SCC in situ, may resemble actinic keratosis. A clinical indication of malignant transformation is thickening and tenderness on palpation, due to perineural involvement. Clinically, SCC is manifested as a proliferative exophytic tumor growing moderately rapidly over a period of months (Fig. 4). These tumors vary in size from millimeters to centimeters depending on the duration of growth. Occasionally, they can be infiltrating and firm without an exophytic component. The tumor induces an inflammatory reaction, to become crusted and erythematous. Ulceration may occur, and a patient may describe the lesion as an itchy or painful non-healing wound that bleeds when traumatized (Alam et al., 2001). SCC is often treated by primary surgery, and in skilled hands surgical excision is associated with long-term cure rates of higher than 90% (Rowe et al., 1992). An excision margin of 4 to 6 mm has been recommended, depending on the tumor size and anatomical site. Other treatments of primary SCC include curettage, diathermy and cryosurgery.

Invasive SCC has the potential to metastasize and the risk of metastasis increases with larger lesions (>2 cm in diameter), a tumor depth of >4 mm, a poorly differentiated histological appearance, and with certain anatomical skin sites. The risk of metastasis from SCC of the lip and ear varies from 10-15%, compared to 2% in tumors from other light-exposed areas (Rowe et al., 1992). Metastases from SCC appear initially most commonly in the regional lymph nodes, followed by the lungs, liver, brain, skin and bone. The long-term prognosis for survival with metastatic disease is poor, with ten-year survival rates of less than 20% among patients with regional lymph node involvement and less than 10% among those with distant metastasis (Alam et al., 2001).

SCC can also develop in pre-existing dysplastic lesions such as Bowen´s disease, arsenical keratosis, hydrocarbon (tar) keratosis, radiation keratosis, erythroplasia of Queyrat, and epidermodysplasia verruciformis. Bowen´s disease occurs as one or more

well-demarcated erythematous scaling plaques on the lower leg or trunk; the lesions are slow growing, with a histology of carcinoma in situ. The risk of malignant transformation seems to be very low. In rare cases SCC arises de novo, i.e., in skin that appears completely normal, without a precursor lesion either clinically or histologically.
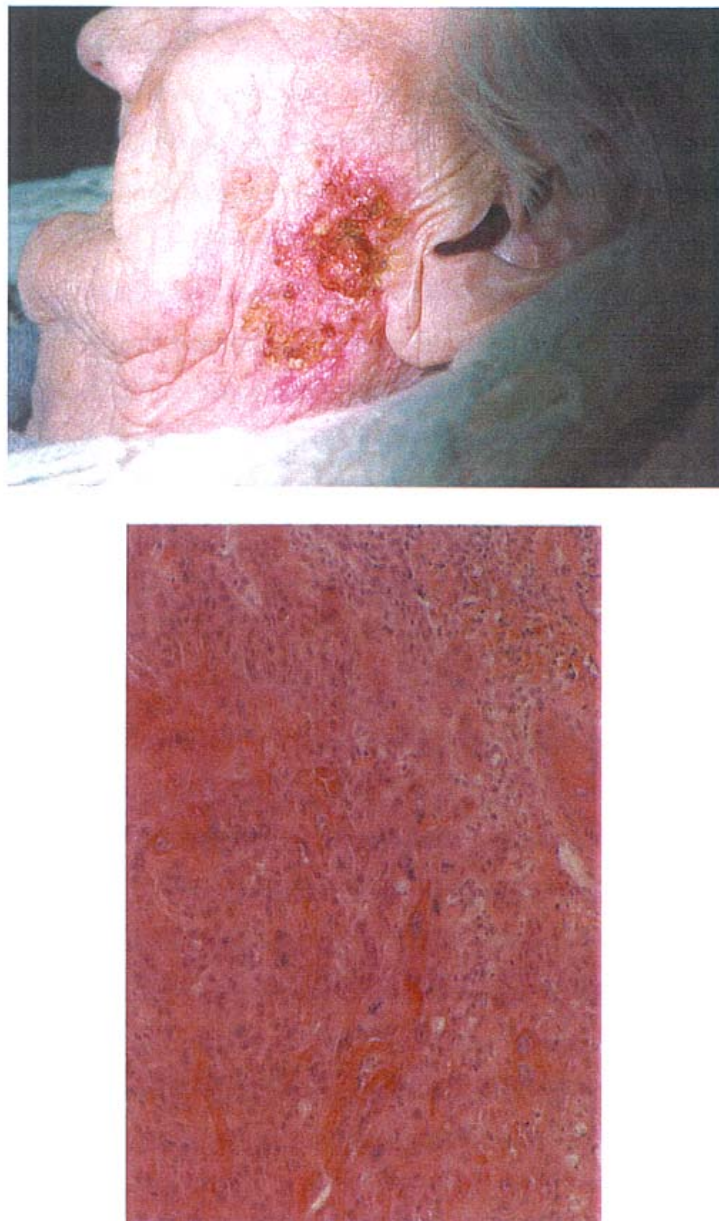

Figure 4. Two photos of squamous cell carcinoma in human skin, a clinical view (top) and an immunohistochemical view (bottom)

It has been proposed that cancer occurs through a multistep series of genetic events (hits), sometimes divided into initiation, promotion and progression as stages in cancer growth (Vogelstein and Kinzler, 1993). Carcinogenesis is initiated when a clone of cells gains a selective growth advantage through genetic events, leading to a tumor. Tumor progression implies further genetic alterations resulting in selective growth advantage in some cells within a tumor, with a consequent change to a more malignant tumor. Activation of oncogenes and inactivation of tumor suppressor genes are often involved. In the development of SCC it is proposed that UVR acts both as an initiator and a promoter. Mutations in proto-oncogenes such as ras have been described (Spencer et al., 1995), although mutations in the tumor suppressor gene p53 seem to be of great importance in this carcinogenesis. Wikonkal and Brash (1999) have suggested the tumor model for SCC illustrated in Figure5.

2.6 Photoprotection

The skin pigmentation and thickening of the epidermis are the most important factors for providing natural endogenous photoprotection (Lock-Andersen et al., 1997; Lock-Andersen and Wulf, 1997). In addition, increased photoprotection can be achieved by wearing protective clothing, hats and sunglasses and by topical application of sunscreens.

Sunscreens consist of one or several UVR-absorbing filters in a vehicle. They are designed to reduce erythema induced by UVR (sunburn) in humans, and this reduction is expressed as their sun protection factor (SPF). SPF is defined as the ratio of the smallest amount of ultraviolet energy required to produce minimal erythema on sunscreen-protected skin to the amount of energy required to produce the same erythema on unprotected skin (Vainio and Bianchini, 2001).

Sunscreen filters can be divided into two main types, organic chemical or inorganic chemical filters (Vainio and Bianchini, 2001). The organic filters only absorb UVR, while the inorganic ones both absorb and scatter UVR. The organic chemical absorbers

are generally aromatic compounds conjugated with a carbonyl group. The main groups of filter derivates currently used are: cinnamates, camphors, para-aminobenzoates
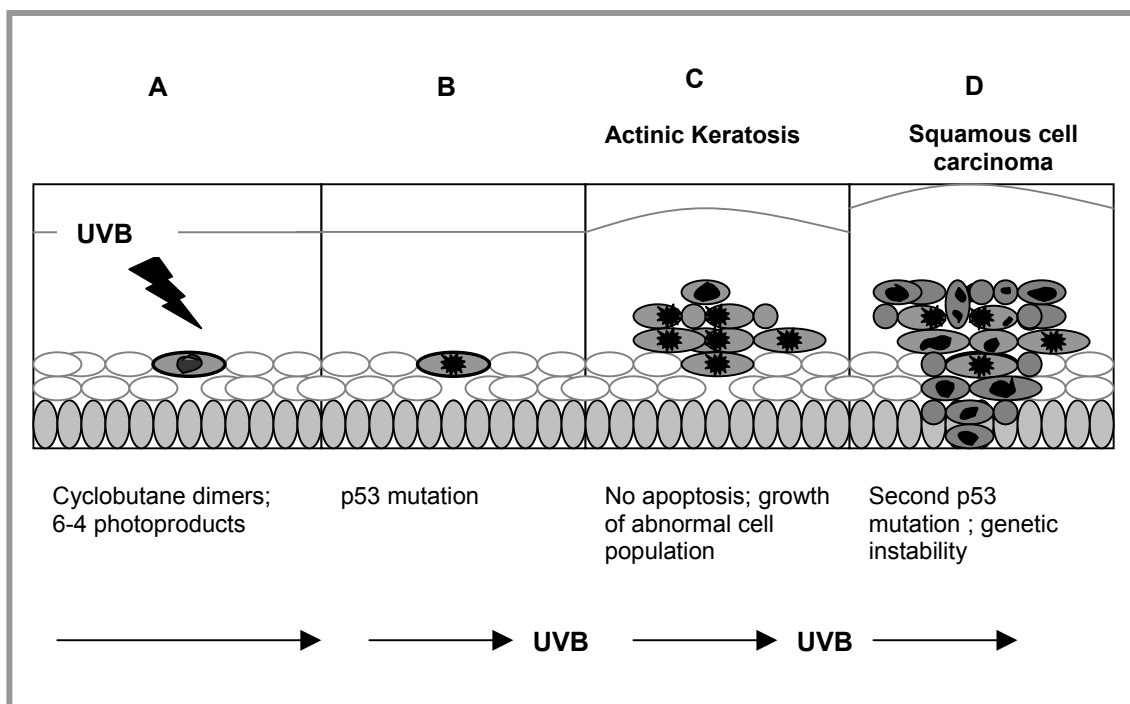

Figure 5. An unrepaired photoproduct leads to a mutation in an epidermal cell (A). If the mutation occurs in one allele of the p53 gene (B), the cell can fail to undergo apoptosis and thereby gain proliferative advantage over normal cells, with clonal expansion and development of actinic keratosis (C). Continued exposure to UVR might then inactivate the remaining functional allele of p53, which could accelerate the formation of SCC by promoting genetic instability (D).

(PABA), salicylates, benzophenones, dibenzoyl methanes, and anthranilates. The first four compounds are principally UVB absorbers, while the last three are principally absorbers of UVA. Occasionally, benzophenones are also called broad-band absorbers.

The inorganic filters most frequently used are zinc oxide (ZnO) and titanium dioxide (TiO_2). These are opaque materials and they are generally used in combination with organic absorbers to achieve a higher SPF. The main advantages of organic chemical absorbers are that they are soluble in vehicles that are water-resistant. The disadvantages are a risk of irritant or allergic/photoallergic reactions and an unpredictable photostability (Tarras-Wahlberg et al., 1999). Inorganic filters offer broad-band protection and entail a very low risk for allergic or photoallergic reactions, but they are cosmetically less acceptable.

The European standard recommended sunscreen application dose is 2 mg/cm^2, however it has been shown that people apply only 20-50% of recommended dose (Stenberg and Larko, 1985; Wulf et al., 1997). Consequently, the protection from the product can be as low as 20-50% of that expected from the SPF given on the label.

Sunscreens are able to prevent photocarcinogenesis in mice (Ananthaswamy et al., 1999). In humans it has been reported that use of sunscreen is associated with a reduction in the incidence of actinic keratosis (Naylor and Farmer, 1997; Thompson et al., 1993). One large 4.5 year randomized controlled study on the use of a broad spectrum SPF 16 sunscreen showed a significant reduction in the total number of SCC (Green et al., 1999).

3. PRESENT INVESTIGATION

3.1 Aims of the investigation

The specific aims of the present investigation were as follows:

➜ to monitor the incidence rates of squamous cell carcinoma in Sweden from 1961 to 1995 according to sex, age, anatomical site and unit surface area; also, to analyze the rates on the basis of age-period-cohort modeling (Paper I);

➜ to quantify the short-term and long-term risks of developing a second primary cancer among patients with an earlier diagnosis of squamous cell carcinoma, with special focus on hypotheses regarding common risk factors or relation to tissue of similar origin (Paper II);

➜ to investigate the cellular levels of TT dimers, p53 protein and Ki-67 antigen (proliferation marker) in human skin at differently sun-exposed anatomical sites after artificial UV irradiation and after natural sun-exposure during a summer in Sweden (Paper III);

➜ to optimize and utilize a skin organ culture model to investigate repair kinetics and responses to UVR in human skin at different sun-exposed anatomical sites and to determine whether UVR responses seen *in vivo* can be reproduced *in vitro* (Paper IV);

➜ to evaluate the protective efficacy of photoprotection with respect to DNA damage, p53 protein, and proliferation (Papers III-IV).

3.2 Materials and methods

3.2.1 Cancer registry and coding practices

The Swedish Cancer Registry at the National Board of Health and Welfare was founded in 1958 as a population-based registry and has since published cancer incidence reports on a regular basis. The completeness of the Register has been estimated to be almost

complete (Mattsson and Wallgren, 1984), an achievement due to compulsory reporting both by the clinician and by the pathologist, who must report separately any diagnosis of cancer made on pathological and cytological specimens according to the World Health Organization (WHO) International Classification of Diseases, Seventh edition (ICD-7) (WHO, 1957). However, the stage of the disease at diagnosis or treatment is not recorded.

3.2.2 Statistical methods in studies I and II

Study I (Paper I): A descriptive and analytical study

A total of 39,805 cancer cases (24,890 men and 14,915 women) were registered by the Swedish Cancer Registry with the site denoted "NMSCs" or "skin (melanoma excluded)" from 1 January 1961 through 31 December 1995. The patients mainly had SCC. Age-standardized incidence rates, age-specific incidence rates and incidence rates per 100,000 unit surface area were calculated for the following skin sites: trunk, upper extremities, lower extremities, and head and neck sites (eyelid, external ear, face, scalp-neck). To further explain the incidence rates, multivariate analysis was performed with age-period-cohort modeling.

Age-standardized incidence rate: The incidence rates can be adjusted for age distribution, e.g, to the Swedish standard population (the 1970 census) or to the world standard population. In order to facilitate international comparisons, we used the world population as reference. The direct method of standardization was used, which meant that the age-specific cohort rates obtained were applied to the theoretical world standard age distribution (Fleiss, 1981).

Age-specific incidence rates: Rates were estimated as the average rate per year during each 5-year period of time, starting with 1961-1965 and ending with 1991-1995, using the age groups 0-39, 40-59, 60-79, $\geq$ 80 years.

Age-standardized incidence rates per unit surface area: The rates per 100,000 unit surface area for a specified site were calculated by dividing the age- and site-specified

incidence rates of each gender and calendar year by the proportion of the anatomical site area compared to the whole body area.

Age-period-cohort modeling: This method was used to further explain the observed time trends (Clayton and Schifflers, 1987; Clayton and Schifflers, 1987). Variation in cancer rates over time could be due to secular (period) or generational (cohort) effects. In some cases neither period nor cohort alone could describe the data and both effects were considered in the model. The models were considered were; age, age+drift, age+cohort, age+period and age+cohort+period. By determining the difference in deviance, hierarchial models can be compared using the chi-square distribution. Goodness-of-fit tests for the models were performed and the model fit was evaluated in terms of the deviance. When the deviance is close to the degree of freedom, the model may be considered adequate.

If there is a sudden change in all age groups simultaneously, then the age-period model will describe the data well. Such a change might occur as a result of a change in population exposure to a late-stage carcinogen, affecting all age groups equally, or following changes in the completeness of registration, in coding practices, or in diagnostic activities. The introduction of a screening program that is equally applied and equally effective over all age groups will have the same effect. The age-cohort model will provide a better description of incidence trends when the carcinogenic exposure affect certain generations or cohorts.

Study II (Paper II): A retrospective cohort study

All patients with a first SCC diagnosed between 1 January 1958 and 31 December 1992 were selected from the Swedish Cancer Registry. A total of 25,947 cases were found, 16,252 men and 9,695 women. We calculated person-years at risk from the date of diagnosis of SCC to the closing date of the follow-up, which was the date of death or the date of diagnosis of a second primary cancer or the end of the follow-up on 31 December 1992. The standardized incidence ratio (SIR; see below) was calculated for men and women separately by age groups and years of follow-up. Analyses were performed for risk of second primary cancer at all sites and at special individual sites on

the basis of hypotheses regarding common risk factors or relation to tissue of similar origin (skin, aerodigestive, digestive, and hematopoietic and lymphoproliferative tissues, and tissue of squamous cell epithelium and ectodermal origin).

The standardized incidence ratio is defined as the ratio between the observed number of cases with a new second primary cancer and the expected number of cases. To estimate the expected number of cases of second primary cancers, we multiplied the number of person-years at risk by the national age-specific incidence rates for the cancer site in question in 5-year age groups for each calendar-year of diagnosis.

3.2.3 Studies III and IV

Study III (Paper III): Six healthy volunteers (V1-V6; two men, four women; age 47-75, mean 57 years) entered the study. All of them were of Caucasian origin, two with skin type II and four with skin type III (Fitzpatrick, 1988). All six volunteers took part in two experiments including artificial UV irradiation and sun exposure during six summer weeks in Sweden.

Study IV (Paper IV): Skin explants were sampled from 23 healthy volunteers and patients. They were of both sexes, with ages ranging from 20 to 90 years. All subjects were Caucasians with skin type II-III (Fitzpatrick, 1988). Chronically sun-exposed skin from eight patients was used for optimization of the organ culture model. Non-sun-exposed skin from ten female patients (four < 30 years and six > 60 years) and chronically sun-exposed skin from five patients (> 60 years) was used for the study of DNA repair kinetics and the epidermal response to UVR.

Artificial UV irradiation: The source of irradiation was a SUPUVASUN 3000 (Mutzas, Germany) device equipped with a SUN filter emitting a broad band of UVB, UVA and near-infrared irradiation. At a treatment distance of 50 cm, the effect was 0.03 mW/cm^2 of UVB and 65 mW/cm^2 of UVA. In study III, artificial UV irradiation was performed in May 1998 and all volunteers received the same UV dose, 40 mJ/cm^2 of UVB and 85

J/cm^2 of UVA at each irradiated area. In study IV, the skin explants received a UV dose of 30 mJ/cm^2 of UVB and 63.75 J/cm^2 of UVA.

In study III (Paper III), UV irradiation was performed on chronically sun-exposed skin (dorsal forearm) and non-sun-exposed skin (buttock) with and without photoprotection. A topical sunscreen lotion with an SPF of 15 (Coppertone 15^{TM}, Schering-Plough, Stockholm, Sweden) and containing both UVB and UVA absorbers {benzophenone 3 (Eusolex 4360), butyl methoxydibenzoylmethane (Parsol 1789) and octyl methoxycinnamate (Parsol MCX)} was applied on 4 cm^2 areas of the forearm and buttock 15 min before UV irradiation. The layer thickness used, 2 mg/cm^2, is equivalent to that used by the manufacturers for SPF determination (COLIPA) [1994 #126]. Another, circular area 9 mm in diameter was subjected to total photoprotection by coverage with blue denim fabric (SPF 1700) attached to the skin by a 25 mm diameter circular occlusive dressing (Actiderm^{TM}, Convatec, Bristol-Meyers Squibb, Princeton, NJ, USA). Twenty-four hours after irradiation, 3 mm punch biopsy specimens were obtained under local anesthesia from photoprotected (sunscreen and denim fabric) irradiated, non-protected irradiated, and non-irradiated (control) skin on the forearm and buttock.

Sun exposure in Sweden: During six summer weeks in Sweden (July - August) the volunteers used photoprotection identical to that in the experiment above, i.e., topical sunscreen with SPF 15 and blue denim fabric, on the dorsal forearm. They were instructed to apply the sunscreen *ad libitum* every morning on a defined area of 4 cm^2. The denim fabric was changed two to four times a week. Twenty-four hours after the last sun exposure, 3 mm punch biopsy specimens were obtained; one each from skin protected with sunscreen and blue denim fabric, and one from unprotected skin. (Paper III)

Quantification of UV dose: The total UV dose received during the sun-exposure periods was measured by letting the volunteers wear a polysulphone badge on their wrist. The badges were changed every one to three days depending on the weather. The UV dose for every badge was measured at the Regional Medical Physics Department, Newcastle,

UK, by a conventional spectrophotometer at 330 nm, and the total dose for each individual was calculated and expressed in SED. (Paper III)

Organ culture model in study IV

Chronically sun-exposed skin (facial) and non-sun-exposed skin (breast) were collected. The skin was cut with a razor blade, creating skin explants composed of epidermis supported by a thin rim of dermis. Skin explants were placed on cell culture inserts with the dermis side down on a membrane in contact with medium (Dulbecco's minimum essential medium supplemented with 5% fetal bovine serum (FBS) and 1% penicillin.). Organ culture was performed in a humidified incubator containing 5 % CO_2 and 95 % air. The skin explants were cultured for 24 hours prior to UV irradiation.

During UV irradiation the culture plates were kept on ice to avoid overheating. An SPF 15 sunscreen lotion, the same as was used in study III, was applied at the recommended dose 5 min before UV irradiation. After UV irradiation the skin explants were further incubated at 37°C and harvested after 4, 24 and 48 hours. Control skin explants were incubated under the same conditions as the UV-irradiated skin.

Immunohistochemistry in studies III and IV

Biopsy specimens were fixed in 4% buffered formalin for one to three days, paraffin-embedded and cut into 4 μm thick sections. Sections were deparaffinized in xylene and rehydrated in a series of graded alcohol. The sections were permeabilized by microwave treatment at 750 W for 2x5 min in 0.01 M citrate buffer (pH 6). After 30 min treatment with 0.3% hydrogen peroxide solution to exhaust endogenous peroxidase activity, slides were incubated with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) to block non-specific binding. TT dimers, p53 protein, and expression of Ki-67 were detected by use of different primary antibodies (30 min incubation in a humidified chamber at room temperature). To detect TT dimmers, a primary monoclonal mouse antibody, KTM-53 (Kamiya Biomedical Company, USA, code MC-0621, dilution 1:5000 in PBS containing 1% BSA), was used. For detection of p53 protein the sections were incubated with D-07 (Dako, code M7001, dilution 1:200 in PBS containing 1% BSA), a monoclonal mouse antibody recognizing both wild-type and mutated forms of

p53. To detect proliferating cells, a primary monoclonal mouse antibody MIB-1 (Immunotech, code 0505, dilution 1:50 in PBS containing 1% BSA), recognizing the Ki-67 antigen, was used. After incubation of either of the three above-mentioned primary antibodies, a biotinylated rabbit anti-mouse antibody (Dako, code E0354, dilution 1:200 in PBS containing 1% BSA) was used as a secondary antibody. The immune reaction was visualized by avidin/biotin complex (Dako code K0355, dilution 1:200 in PBS containing 1% BSA) with 0.004% hydrogen peroxide as a substrate and DAB (diaminobenzidine) as a chromogen. Negative controls were performed by omitting the primary antibody. Mayer´s hematoxylin was used for counterstaining in studies III and IV. In study IV, the immunostaining of MIB-1 to detect Ki-67 protein was performed in an automated immunostainer (Ventana Medical System Inc., Tucon, AZ, USA). These slides were counterstained in Harris hematoxylin.

Scoring of TT dimers, p53 and Ki67

Counting of TT dimer-, p53- and Ki-67-immunoreactive keratinocytes in the epidermis was carried out in each tissue section under a light microscope at 400x magnification. Three random microscopic fields were counted, covering about 700 keratinocytes per specimen. No distinction in staining intensity was made. All the counting was performed blindly by one person: CW in study III and HB in study IV. All cells per microscopic field were counted, both negative and positive immunoreactivity for p53, TT dimers and Ki 67, and the results are presented as percentages of counted positive immunoreactive keratinocytes out of the total number of counted keratinocytes. In study IV, Ki-67-immunoreactive keratinocytes were counted using an image analysis system.

3.3 Results

3.3.1 Study I

The age-standardized incidence rates of SCC increased substantially in both men and women during the study period 1961-1995, from 4.4 to 23.1 per 100,000 (+425%) in men and from 4.1 to 10.1 per 100,000 (+146%) in women (Fig. 6). The mean annual percentage increase during the entire study period was 3.1% in men and 2.6% in

women. We further separated the total study period into two time periods, 1961-1980 and 1981-1995, and the highest average annual percentage increase was seen during the latter period, 4.3% in men and 4.0% in women. The highest incidence was seen for the head-neck site, with an increase during the whole study period from 3.1 to 14.6 per 100,000 (+370%) in men and from 2.3 to 5.0 per 100,000 (+119%) in women. The incidence rates for specified head-neck sites showed that the face and external ear were the subsites with the most elevated rates in men, while in women the face showed the greatest increase and no sharp increase in incidence rate was observed for the scalp-neck or external ear. SCC was most common among elderly persons and the age-specific incidence rates displayed the highest increase over time in the oldest age groups, i.e., 60–79 and 80 years and older. When the incidence rates were calculated per 100,000 unit surface area, i.e., taking site area into account, the highest rates were seen for the external ear and eyelid in men and the eyelid and face in women. Multivariate analyses showed that the age+period+cohort effect model in men best explained the incidence rate, while in women the age+period effect model was adequate.
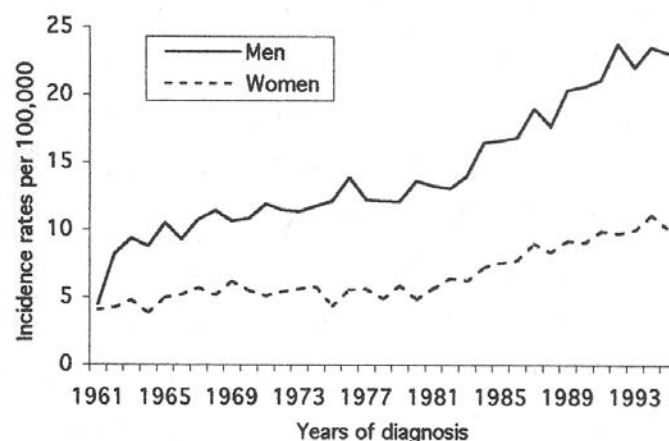
Figure 6. Age-standardized incidence rates of squamous cell carcinoma (all sites) in men and women in Sweden 1961-1995 (World population as reference).

3.3.2 Study II

Among a total of 137,312 person-years at risk, a subsequent second primary cancer was found in 5,706 patients (22%), 4,043 men and 1,663 women. The expected number of cases was estimated to be 2,651 and SIR was 2.15 [95% confidence interval (CI)=2.1-2.2]. Thus, patients with a diagnosis of SCC in Sweden run twice as high a risk of developing new primary cancer compared to the general population. The risk was most marked within the first year after diagnosis (SIR=2.7; 95% CI=2.6-2.8), but was also significantly elevated during a period of 15 years or longer after diagnosis. The highest risks were found for men below the age of 60 years at diagnosis (SIR=2.5; 95% CI=2.2-2.8) and especially during the first year of follow-up (SIR=9.2; 95% CI=6.9-12.2). When a second primary SCC was excluded, SIR was reduced to 1.30 (95% CI=1.25-1.34).

An increased relative risk for a developing new primary tumor at several specified sites was found:

1. Skin: The highest risk was found for a second primary SCC of the skin (SIR=15.6; 95% CI=15.0-16.3) and the risk of malignant melanoma was markedly elevated (SIR=3.0; 95% CI=2.5-3.7).

2. Aerodigestive tissues: An increased risk was found for second primary cancers in the mouth (SIR=2.0; 95% CI=1.2-3.2), nasopharynx (SIR=3.0; 95% CI=1.2-6.2), hypopharynx (SIR=2.7; 95% CI=1.5-4.7), and trachea-bronchus-lung-pleura (SIR=1.7, 95% CI=1.5-1.9), for both men and women. The risk of developing cancer of the trachea-bronchus-lung-pleura was significantly elevated up to 9 years after the diagnosis of SCC.

3. Hematopoietic and lymphoproliferative tissues: The risk of developing a second cancer in some hematopoietic and lymphoproliferative tissues was almost doubled: non-Hodgkin´s lymphoma (SIR=1.9; 95% CI=1.6-2.3), Hodgkin´s disease (SIR=2.1; 95% CI= 1.2-3.2), lymphatic leukemia (SIR=1.8; 95% CI= 1.4-2.3), and myeloid leukemia (SIR=2.0; 95% CI= 1.4-2.7), and the risk for these cancers was significantly elevated up to 4 years after diagnosis of SCC. No excess risk was noted for multiple myeloma.

4. <u>Squamous cell epithelium</u>: A significantly increased risk of a second primary cancer was found for most sites of squamous cell epithelium. The highest risk was observed for lip cancer (SIR=5.2; 95% CI=4.2-6.3), especially in women (SIR=10.5; 95% CI=6.3-16.4). Other sites with a significantly increased risk were the esophagus (SIR=1.5; 95% CI=1.1-2.0), cervix uteri (SIR=2.2; 95% CI=1.4-3.2), vulva-vagina (SIR=2.3; 95% CI=1.4-3.5), and respiratory organs (SIR=1.7, 95% CI=1.5-1.9).

5. <u>Digestive tissues</u>: Slightly increased rates (SIR=1.0-1.5) were noted for second tumors in digestive tissues (stomach, small intestine, biliary passages and liver, colon, rectum-anus), while no excess risk of pancreatic carcinoma was found.

6. <u>Tissues of ectodermal origin</u>: A high SIR (SIR=5.5; 95% CI=3.7-8.0) was observed for second primary cancers in salivary glands. No increased risks were observed for second primary cancers of the breast or tumors of the endocrine glands.

3.3.3 Study III

Artificial UVR induced a large number of TT dimer-positive keratinocytes in unprotected skin, but significantly more TT dimers were found in non-sun-exposed skin (mean=32%) compared to chronically sun-exposed skin (mean=17%) (Fig. 7). Both sunscreen and blue denim fabric gave almost total photoprotection, with <1% TT dimer-positive keratinocytes. Very few TT dimer-positive keratinocytes were found in unprotected skin after six summer weeks in Sweden. This was probably due to the fact that the final days of both sessions were cloudy, allowing time for the TT dimers to be repaired.

In all subjects, a single dose of artificial UVR increased the number of p53-positive keratinocytes in unprotected skin, both in non-sun-exposed (mean=30%) and chronically sun-exposed (mean=33%) skin compared to the controls (mean=3%). Figure 8 shows the individual p53 levels 24 hours after artificial irradiation in non-sun-exposed skin (Fig. 8 A), chronically sun-exposed skin (Fig. 8 B) and in chronically sun-exposed skin after six summer weeks in Sweden (Fig. 8 C) set in relation to the degree of

photoprotection. There were large interindividual variations in p53, especially in chronically sun-exposed skin. The two kinds of photoprotection reduced the p53-positive cells significantly compared to those in unprotected skin. However, we observed more efficient inhibition of the epidermal p53 response in non-sun-exposed skin compared to chronically sun-exposed skin. The total UV dose received from 6 weeks of natural sun was significantly correlated to the amount of p53-positive keratinocytes. In addition, a total of two p53 clones were found in both photoprotected and unprotected dorsal forearm skin in the two oldest volunteers after the summer session.

No increase in proliferation was observed after artificial UVR or after six summer weeks in Sweden. However, increased levels of Ki-67 positive cells were found after one week of exposure to sunlight in Spain.
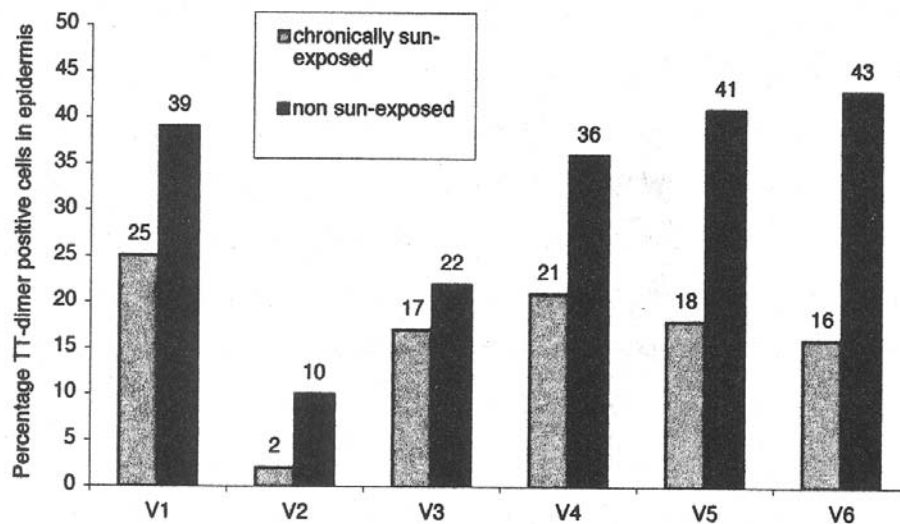
Figure 7. TT dimers in non-protected chronically sun-exposed and non-sun-exposed skin after artificial UV irradiation in volunteers V1-V6.
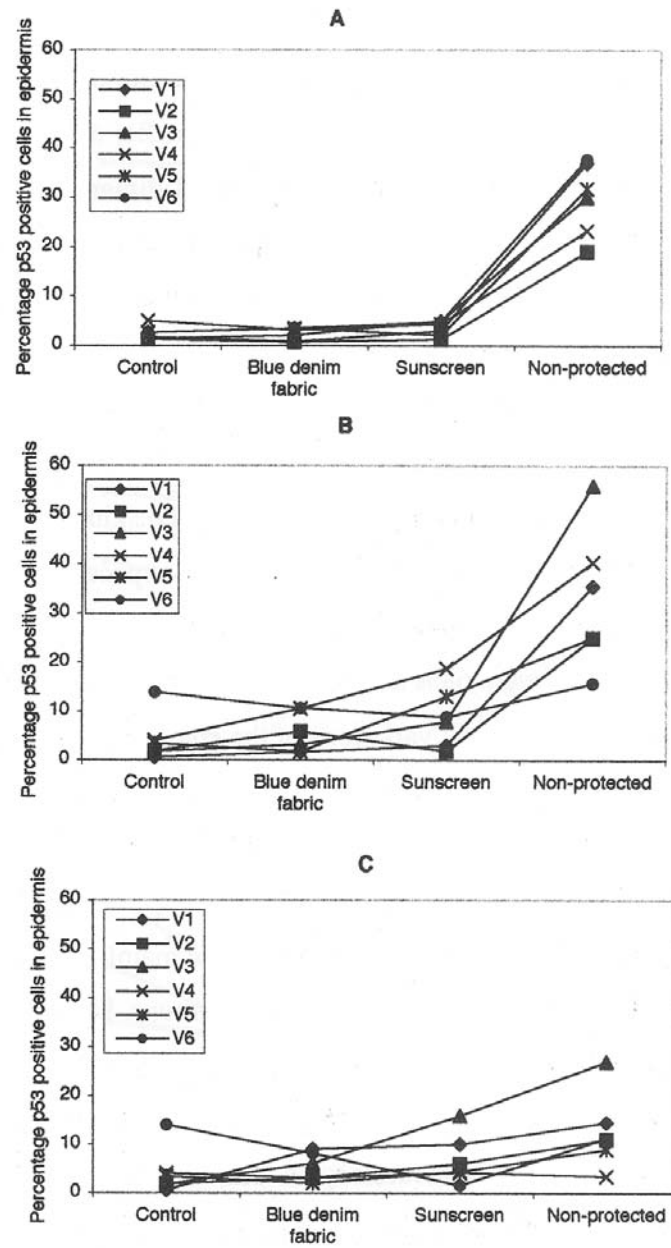
Figure 8. This figure (A-C) shows the percentage of p53-positive cells in non-sun-exposed (A) and chronically sun-exposed (B) skin with different degrees of photoprotection after artificial UV irradiation. p53-positive cells in chronically sun-exposed skin with different degrees of photoprotection after 6 weeks of sun exposure in Sweden are also shown (C).

3.3.4 Study IV

Organ-cultured skin showed a normal histological structure up to five days, but after 96 hours in culture, several scattered pyknotic cells were found in the epidermis. Increased levels of p53 and Ki-67 were observed after 6 hours in culture but both parameters returned to normal within 24 hours in culture. Four hours after artificial UV irradiation, the keratinocytes showed strong immunoreactivity for TT dimers. The graphs in Figure 9 A-C show the induction and removal of TT dimers at different time points (4, 24, 48 hours post-irradiation) in three groups. Gradual repair during the incubation period resulted in only a few residual TT dimers after 48 hours. We observed differences in the formation of TT dimers between individuals as well as differences in repair efficiency in skin of different anatomical sites. The repair appeared to be more efficient in chronically sun-exposed skin compared to non-sun-exposed skin. The mean reduction of TT dimers from 4 hours to 48 hours in chronically sun-exposed skin was 82% in subjects > 60 years of age, while in non-sun-exposed skin of subjects < 30 years old and > 60 years old the reduction were 68% and 59% respectively. Similarly to the variations seen with TT dimers, interindividual variations of the epidermal p53 response were also observed after artificial UV irradiation, as summarized for the three groups in Figure 8 D-F. Despite this variation, a similar pattern was found. An increase in p53 protein after 4-24 hours was observed and the p53-positive cells were equally distributed in all cell layers. The positive cells still present after 48 hours were mainly found in the basal layer. There was no clear difference between the three groups. However, chronically sun-exposed skin appeared to have a more variable p53 response compared to non-sun-exposed skin. Topical sunscreen nearly abolished the TT-dimer formation and clearly reduced the amount of p53-positive keratinocytes. The numbers of Ki-67-positive cells did not differ between the three skin groups and no significant increase or decrease in positive cells was observed after UV irradiation.
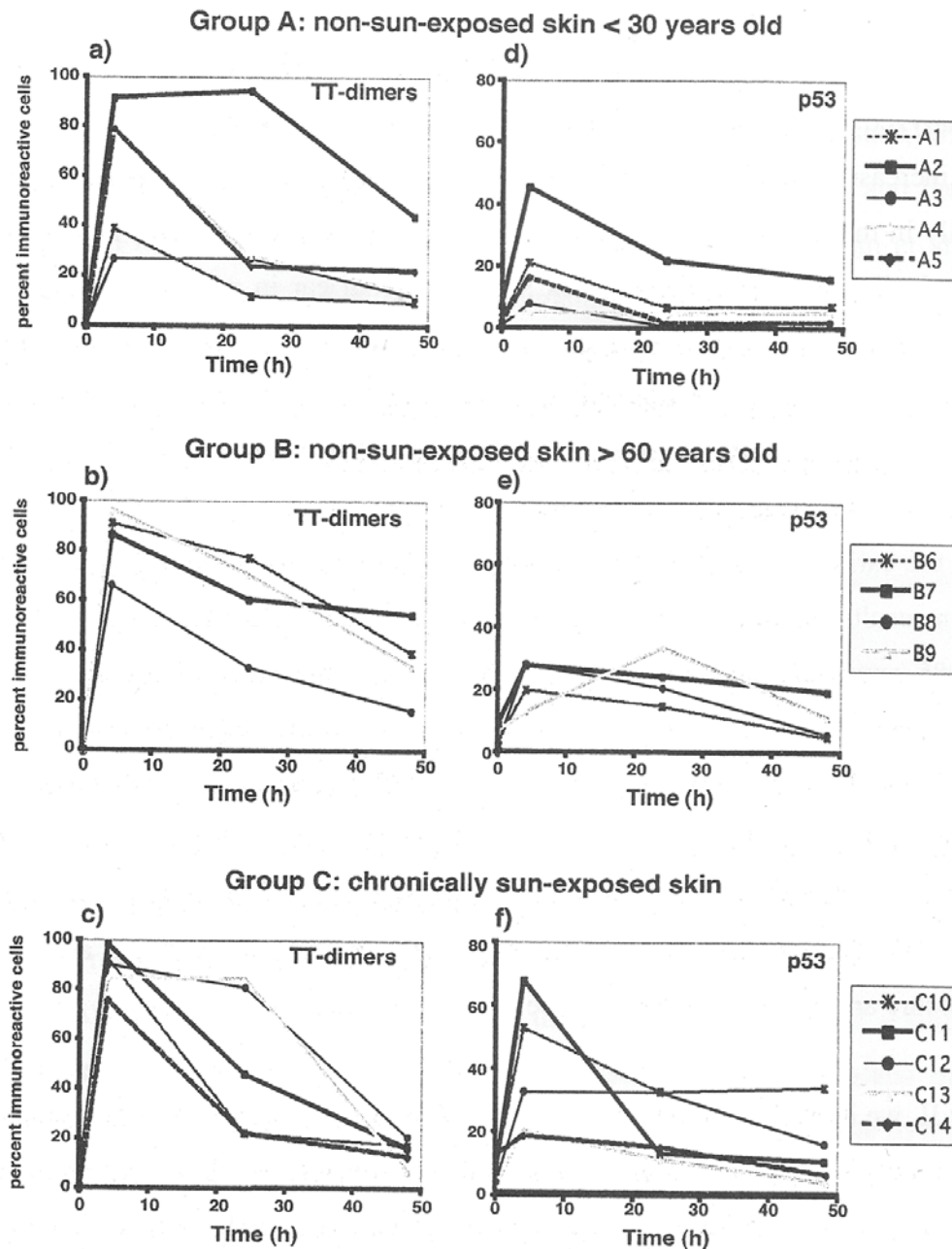
Figure 9. Graphs showing the formation and repair of TT dimers and induced p53 after UV irradiation of organ-cultured skin explants from 14 patients, divided into three skin groups (A-C). The left panel of the figure (a-c) shows the percentage of TT dimer-positive cells in a) non-sun-exposed skin from subjects < 30 years of age, b) non-sun-exposed skin from subjects > 60 years of age, and c) chronically sun-exposed skin. The right panel of the figure (d-f) shows the induction of p53 in d) non-sun-exposed skin in subjects < 30 years of age, e) non-sun-exposed skin in subjects > 60 years of age and f) chronically sun-exposed skin.

3.4 General Discussion

Two epidemiological population-based studies are presented in papers I and II. In study I we analyzed the incidence trends of SCC in Sweden over a period of 35 years. A rapidly increasing incidence of SCC was found in chronically sun-exposed skin sites, especially in men but also in women. The highest rates per unit surface area were seen for chronically sun-exposed head-neck sites - external ear in men and the eyelid and face in women. The noticeable differences in age-standardized incidence rates between men and women can be explained by differences in sun-tanning habits, hairstyles, clothing behavior or indoor and outdoor occupations between the sexes. Baldness in men entails a higher dose of UV on the external ears and scalp and neck. Our findings support the earlier observation that almost all SCC occurs on chronically sun-exposed skin. Our results, i.e., continuously rising incidence rates for SCC in general, especially after 1985 onward, higher rates among the elderly, a higher incidence men, and the most elevated risks at head-neck sites, are consistent with previous reports on Western European populations (Beral and Robinson, 1981; Coebergh et al., 1991; Franceschi et al., 1996; Kaldor et al., 1993; Levi et al., 1995; Magnus, 1991; Roberts, 1990) or populations of European origin (Buettner and Raasch, 1998; English et al., 1997; Marks et al., 1993). The most likely explanations for this pattern are increased accumulated sun exposure and an increasing incidence among the elderly.

In study II, we investigated the relative risk of developing a second primary cancer after a diagnosis of SCC. We tested different hypotheses concerning relative risks at different anatomical sites on the basis of reported results regarding risk factors. Our analysis showed that patients with a first SCC run twice as high a risk of developing a new cancer compared to the general population in Sweden. The risk was most marked within the first year of diagnosis, but was also significantly elevated during a period of 15 years or longer after the diagnosis. The highest risk was found among patients in whom SCC was diagnosed before the age of 60 years. Increased risks were found for several specified sites. The risk of a new skin cancer was very high, supporting the notion that patients with SCC should avoid excessive sun exposure because of the high risk of developing second primary malignancies of the skin. Furthermore, regular skin examinations, especially during the first five years after a diagnosis of SCC, should

promote early detection of new skin tumors. The high risk for lip cancer in women (SIR=10.5; 95% CI=6.3-16.4) may be due to a combination effect of smoking and UV exposure. We found an increased risk of cancers of aerodigestive tissues, which are usually related to smoking. A significant association between smoking and the risk of SCC has been reported previously (Frisch and Melbye, 1995; Grodstein et al., 1995) (De Hertog et al., 2001). Elevated risks for immunorelated cancers in SCC patients and vice versa have been observed in Finland, Denmark, and Switzerland (Frisch and Melbye, 1995; Levi et al., 1997; Teppo et al., 1985). For non-Hodgkin's lymphoma the association may be due to exposure to UVR (Adami et al., 1995). UVR has been found to exert both local and systemic immunosuppressive effects and to promote the occurrence of malignant tumors (Kripke, 1990). It is possible that immunosuppression, either resulting from UVR or from other causes such as immunosuppressive treatments, is the common etiological factor. We also found an increased risk for second primary cancer of the vulva-vagina and the cervix. The association between SCC and cancer of the cervix has been observed previously, and the roles of HPV (Franceschi et al., 1996) and tobacco smoking have also been discussed (De Hertog et al., 2001; Levi et al., 1997). Finally, a strong association between SCC and tumors of the salivary glands was found. This relationship has been reported earlier (Frisch and Melbye, 1995; Teppo et al., 1985). An ectodermal origin and radiation therapy are two suggested relationships, but the roles of UVR and HPV can also be discussed. The salivary gland may also harbor HPV (Lambropoulos et al., 1997), which may possibly play a role in malignant transformation of squamous cells at different locations, as mentioned above. Thus, the incidence rates of SCC have increased in Sweden, markedly since 1985 and in the oldest age groups. SCC may be regarded as a risk marker for later development of other malignant diseases. Risk factors common to different tumor types, predominantly UV exposure but probably also smoking, HPV or an intrinsic tumor susceptibility might explain the increased risk of developing other cancer among SCC patients.

In studies III and IV, we performed experimental studies *in vivo* and *in vitro* in human skin. A major and novel finding in both of these studies was that significantly more TT dimer-positive cells were induced in unprotected non-sun-exposed skin compared to chronically sun-exposed skin *in vivo* and that repair of TT dimers-positive cells

appeared to be more efficient in chronically sun-exposed than in non-sun-exposed skin *in vitro*. The smaller number of TT dimer-positive cells found in chronically sun-exposed skin could reflect a lower degree of UV-induced damage due to differences, for example in melanin pigmentation and skin thickness compared to non-sun-exposed skin. There may also be differences in repair of UV-induced DNA damage as well as in p53 induction, depending on previous sun exposure. That is, skin areas subjected to chronic sun exposure may exhibit an earlier or stronger p53 response resulting in more efficient repair of DNA damage. Perhaps keratinocytes in chronically sun-exposed skin are "primed" to react more easily to cytotoxic cell stress. The mechanisms of such possible "priming" are unknown.

In study III, both modalities of photoprotection significantly reduced the amount of TT dimer- and p53-positive cells. Another novel finding was that the p53 response was reduced to a larger extent in non-sun-exposed skin than in chronically sun-exposed skin, indicating that the photoprotection was more efficient in inhibiting the p53 response in non-sun-exposed skin than in chronically sun-exposed skin. Such differences may have important clinical implications. Possibly analysis of the p53 response in sunscreen-treated chronically sun-exposed skin would provide a better measure of sunscreen efficiency than the response in non-sun-exposed skin. Our experimental studies support earlier results of large interindividual variations in the levels of p53 and TT dimer (Berne et al., 1998; Ling et al., 2001; Pontén et al., 1995). Despite the large interindividual variation in p53, we found a significant correlation between the total UV dose delivered during six summer weeks in Sweden and the p53 levels in unprotected dorsal forearm skin. We found two p53 clones, both in chronically sun-exposed skin, from the two oldest volunteers. It is known that p53 clones are very common in chronically sun-exposed human skin, and both the size and number of p53 clones appear to increase with age. The biological significance of epidermal p53 clones is not clear, but it has been shown that 70% of analyzed clones exhibit a p53 gene mutation (Ren et al., 1996). A leading hypothesis suggests that keratinocytes with a mutated p53 gene are more resistant to UV-induced apoptosis and thus will clonally expand in skin subjected to repetitive UV exposure. It is tempting to interpret p53 clones as forerunners of neoplasia. Perhaps a p53 clone represents a first step toward SCC or BCC or both,

despite the fact that no genetic link has yet been found between p53 clones and adjacent non-melanoma skin cancers. The long-term effect of sunscreens on the incidence of p53 clones may prove to be an important indicator of the efficiency of sunscreens in preventing skin cancer.

The SPF 15 sunscreen (broad-spectrum; UVA+UVB absorber) used in the experiments in studies III and IV significantly decreased TT dimer formation and p53-positive cells in human skin. There are contradictory reports, however, as to whether a broad-spectrum sunscreen protects better against CPD formation than a UVB sunscreen (Fourtanier et al., 2000; Ley and Fourtanier, 1997; Young et al., 2000). Previous observations have shown that sunscreens used in recommended doses reduce UV-induced DNA damage (Freeman et al., 1988; van Praag et al., 1993) and inhibit p53 mutations and SCC formation in mice skin (Ananthaswamy et al., 1997). In addition, animal experiments have yielded evidence that sunscreens may provide some protection against UV-induced immunosuppression (Wolf et al., 1993; Kripke et al., 1992).

In study IV, we demonstrated that a skin organ culture model provides a relevant tool for analysis of epidermal responses to UVR and that the results are similar to those obtained *in vivo*. Induction of DNA damage and of p53 protein by different UV sources has previously been investigated both *in vivo* and *in vitro* (Bykov et al., 1998; Bykov et al., 1998; Bykov et al., 1999; Davenport et al., 1999; Ling et al., 2001; Muramatsu et al., 1992; Pontén et al., 1995; Ponten et al., 2001; Young et al., 1996; Young et al., 1998), but simultaneous analysis of p53 protein induction, TT dimer formation and DNA repair using human skin explants has not previously been reported. One interesting finding in study IV was that basal cells appeared to be repaired more efficiently than superficially located cells. One reason for this might be that there is more extensive DNA damage in superficial keratinocytes. Another possibility is that there is a difference in repair kinetics coupled to differentiation, a notion consistent with the finding in an earlier study that DNA repair (NER) was p53-dependent in basal undifferentiated cells (Li et al., 1997). Further, we suggest that skin organ culture can be used for analyzing differences in epidermal responses to UV radiation not only between

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chronically sun-exposed and non-sun-exposed human skin, but also between different stages/forms of NMSC in order to investigate the multistep pathway of skin carcinogenesis.

In summary, these studies have increased our knowledge of SCC epidemiology in Sweden and of the effects of artificial and solar UVR and sunscreens on chronically sun-exposed and non-sun-exposed sites, respectively, of human skin.

3.5 Conclusions

1. There has been an increasing incidence of squamous cell carcinoma in Sweden in the last three decades, with an especially rapid rise during the last fifteen years, particularly in chronically sun-exposed skin sites in men. Accumulated sun exposure and an increasing incidence among the elderly most likely explain the observed trends. In the light of these findings, it is important to inform the population about skin cancer and the benefits of sun-protective behavior. (Paper I)

2. Patients with SCC are at increased risk of developing new primary cancers, especially in the skin, squamous cell epithelium, and aerodigestive, hematopoietic and lymphoproliferative tissues. Risk factors common to SCC and a new cancer, such as UV exposure, smoking and immunosuppression might partly explain our findings. A coexistent intrinsic susceptibility among SCC patients to develop cancer is also possible. SCC may also be regarded as a risk marker for later development of other malignant diseases. (Paper II)

3. Repair of UV-induced DNA damage appears to be more efficient in chronically sun-exposed skin, despite a less uniform p53 response to UVR, whereas non-sun-exposed skin is more homogeneous with respect to the epidermal p53 response. Skin keratinocytes that have been frequently exposed to the sun may be prone to react more readily to cytotoxic stress. (Paper III)

4. A well-defined system for *in vitro* culture of skin explants provides an excellent alternative to *in vivo* experiments. We suggest that the skin organ culture model is well suited for mapping DNA damage and repair mechanisms in non-sun-exposed skin and chronically sun-exposed skin, and could be used for analysis of actinic keratosis, SCC and BCC. Furthermore, the model can be useful for investigating the protective effects of sunscreens on a cellular level and under standardized experimental conditions. (Paper IV)

5. Both modalities of photoprotection used in these studies significantly reduce the number of TT dimer- and p53-positive cells. It is concluded from studies III and IV that both inter- and intra-individual variations occur in the epidermal responses to UV-induced DNA damage, both in chronically sun-exposed skin and in non-sun-exposed skin. (Papers III and IV)

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