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Intermediate-effect biomarkers in prevention of skin cancer

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Skin cancers, both non-melanoma and melanoma, usually progress through sequential steps towards malignant transformation, leading to mutant clones and precancerous lesions. Prevention of skin cancers relies on reduction of exposure to solar radiation and may be evaluated by measuring induction of intermediate-effect biomarkers such as sunburn cells or *p53* mutations in the epidermis, actinic (solar) keratoses, UV-induced immunosuppression or naevi.

Sunburn cells (apoptotic keratinocytes) and *p53* mutations are indicators of UV-induced DNA lesions as early steps of malignant transformation of epidermal keratinocytes. Actinic keratoses are premalignant sun-induced skin lesions, characterized as keratinized patches with aberrant cell differentiation and proliferation; they represent risk factors for basal-cell carcinoma and melanoma and are precursors of squamous-cell carcinoma. Studies in humans have investigated UV-induced immunosuppression and its modulation by topical sunscreen application, focusing on contact hypersensitivity as measured by immunization or response to haptens, or on modulation of stimulation of allogeneic lymphocytes by epidermal cells, or local release of immunomodulatory molecules such as *cis*-urocanic acid or interleukin-10.

Naevi are focal collections of melanocytes, usually found at the junction of the epidermis and dermis or at various depths in the dermis. Common acquired naevi arise after birth both spontaneously and in response to sun exposure. Most acquired naevi are clonal, while most melanocytes in non-naeval areas are not. Although it is not yet certain whether naevi represent premalignant lesions or risk factors, many melanomas arise in acquired naevi, and the number of naevi constitutes the best predictor of individual risk of melanoma. The presence of large (i.e., >5 mm) or atypical naevi (i.e., large naevi with non-uniform colour and irregular borders) is associated with elevated melanoma risk, independently of the number of smaller naevi. Children seem particularly vulnerable to sun-induced biological events involved in the genesis of melanoma, and the greatest increase in naevus numbers per unit of skin surface occurs before adolescence. Therefore, the distribution of naevi and their development in children are relevant to understanding melanoma occurrence in adults.

Introduction

Skin cancers, both non-melanoma and melanoma, usually progress through sequential steps of malignant transformation, leading from an initiated cell to a mutant clone and to a precancerous lesion. In the absence of exogenous influences, mutant clones and precancers may remain stable or tend to regress. Only on rare occasions do these precancerous lesions finally transform into an invasive cancer (Brash & Ponten, 1998) (Table 1). Epidemiological studies, and, more recently, studies of *p53* mutations in squamous-cell carcinoma (SCC) (Ziegler *et al.*, 1994) have suggested that the main etiological agent of skin cancer in humans is exposure to solar radiation, especially early in life (Autier & Doré, 1998). Clonal expansion of an initial mutated cell may be driven by sunlight exposure either by a direct effect of UV radiation on initiated target cells or through an indirect immunosuppressive effect. Immunosuppressive drugs may exert the same effect as UV radiation in facilitating the clonal expansion of precancers. Sunlight can act twice: as tumour initiator and as tumour promoter. Predisposition to sunlight-induced precancer is a multigenic trait that involves independent factors such as skin (photo)type, eye and hair colour, individual DNA repair capacity and naevus number. However, this

Table 1. Steps in the progression of skin cancers in humans

Tumour progression steps	Squamous cell carcinoma (SCC)	Malignant melanoma
Reversible (or abortive) steps	Normal keratinocyte	Normal melanocyte
	Sun-damaged epidermis (scattered keratinocytes with p53 mutations)	Sun-damaged melanocytes
	Clonal proliferation of keratinocytes with mutant p53	Naevus (clonal melanocytic proliferation)
	Actinic keratosis	Atypical (dysplastic) naevus
Irreversible steps	Carcinoma *in situ*	Melanoma *in situ*
	Squamous cell carcinoma	Early primary melanoma (radial growth phase)
		Late primary melanoma (vertical growth phase)
	Metastasis	Metastasis

latter trait results from both individual susceptibility to UV and exposure to sunlight in childhood (Autier, 1997).

Prevention of skin cancers will largely result from reduction in exposure to solar radiation and may be evaluated by measuring induction of intermediate-effect biomarkers such as induction of sunburn cells or *p53* mutations in the epidermis, actinic (solar) keratoses, UV-induced immunosuppression or naevi (Tables 2 and 3).

Non-melanoma skin cancer

SCC of the skin results from chronic exposure to sunlight and progresses by stages (Table 1): sun-damaged epidermis with individually disordered keratinocytes; clones of keratinocytes harbouring a mutant *p53* tumour-suppressor gene within a normal epidermis; actinic (solar) keratoses, i.e., keratinized patches with aberrant cell differentiation and proliferation (these dysplastic lesions may still spontaneously regress), carcinoma *in situ*; SCC and metastasis. The set of *p53* mutations in tumours is more restricted than in precancers, suggesting the existence of additional events and selection in the progression from precancer to invasive cancer (Ziegler *et al.*, 1994; Brash & Ponten, 1998).

Basal-cell carcinoma (BCC) of the skin seems to arise without a precancer, probably from stem cells in the bulge region of the hair follicle, and contains mutations of the *Ptch* gene and, less frequently, of *p53*. Its relationship to sunlight exposure is less clear but it may, like malignant melanoma, be associated with intermittent recreational exposure rather than chronic exposure.

Sunburn cells and p53 *mutations*

Sunburn cells are identified in conventionally stained epidermal biopsies as keratinocytes with a dense, pyknotic nucleus and a homogeneously eosinophilic cytoplasm. They are keratinocytes that have sustained a lethal dose of UV radiation (apoptotic keratinocytes). It has long been observed that sunburn cells may be produced, in the absence of erythema, by doses of UV radiation below the minimum erythemal dose (Grove & Kaidbey, 1980). Hence, formation of sunburn cells in the epidermis provides a quantifiable end-point of acute damage by UV radiation.

A study conducted in healthy volunteers of skin phototypes I to III, exposed to a dose of UV radiation equivalent to the individual sun protection factor (SPF) of an SPF-15-labelled sunscreen, over areas of the middle of the back protected by

Table 2. Modulation by sunscreens of intermediate-effect biomarkers of non-melanoma skin cancer in humans

Biomarker	Comments	Sunscreen effects	Reference
Sunburn cells	Apoptotic keratinocytes identified in conventionally stained epidermal biopsies	Prevention of occurrence of sunburn cells with high-SPF sunscreen	Kaidbey (1990)
p53	Transient overexpression in response to UV radiation. Persistent nuclear accumulation in cells with *p53* mutation (diffuse pattern). Keratinocyte clones with mutated *p53* can be identified as sharply demarcated areas (compact pattern)	Decrease in overexpression of *p53* in non-sun-exposed skin following UV exposure	Ponten *et al.* (1995)
		Significant reduction in *p53*-positive keratinocytes in sun-protected skin after application of sunscreen to chronically sun-exposed skin during a summer	Berne *et al.* (1998)
Actinic keratoses	Keratinized patches with aberrant cell differentiation and proliferation induced by chronic sun exposure in a dose-dependant manner. May spontaneously regress in the absence of UV exposure	Regular application of a broad-spectrum high-SPF sunscreen prevent the development of actinic keratoses and even enhances the regression of preexisting keratoses	Thompson *et al.* (1993) Naylor *et al.* (1995)
Immunosuppression	UV irradiation (especially UVB) induces a local and systemic depression of cell-mediated immunity/-contact hypersensitivity	Prevention of UVB-induced suppression of induction to contact hypersensitivity to DNCB	
		Reduction of UV-induced suppression of response to nickel patches in nickel-allergic subjects, or to recall antigens in normal subjects	Whitmore & Morrison (1995); Serre *et al.* (1997); Hayag *et al.* (1997) Damian *et al.*, 1997; Moyal (1998)

SPF, sun protection factor

Table 3. Studies of naevus development as a function of sun protection and sunscreen use

Study type	Subjects	Exposure	End-point / Main outcome	Comments	Reference
Case–control	418 melanoma 438 controls	Never/ever use of sunscreens	Naevus count on both arms in controls increased from no use to ever use of sunscreens. Rate ratio (RR) 1.31 (95% CI, 1.19–1.43) adjusted for age, sex, hair colour, sun exposure	Increase more pronounced when subjects had ever used psoralen sunscreen. RR 2.10 (95% CI, 182–2.43)	Autier *et al.* (1995)
Cohort	357 children (170 boys, 187 girls) 7–11 yrs (median, 9 yrs)	Regular, seldom or never use of sunscreen	Whole body naevus counts at five-year interval. RR of increase in naevi ≥1 mm: 1, 0.81 (0.65–1.01), and 0.41 (0.23–0.75) for regular, seldom or never use of sunscreen.	Univariate analysis	Luther *et al.* (1996)
Retrospective cohort	631 children (321 boys, 310 girls) 6–7 yrs	Total and average sunscreen use, wearing of clothes, sun exposure.	Whole-body naevus count. Median number of naevi increased with total and average sunscreen use, and decreased with average wearing clothes. Adjusted RR of high naevus count on the trunk 1.68 (95% CI, 1.09–2.59) for the highest level of sunscreen use and 0.59 (95% CI, 0.36–0.97) for the highest level of wearing clothes	SPF of sunscreen has no effect on naevus count. Highest risk of naevi on the trunk in children using sunscreen and who never suffered from sunburns (RR, 2.21, 95% CI 1.33–3.67)	Autier *et al.* (1998)

application of an SPF-15 or SPF-30 sunscreen, showed that the SPF-30 sunscreen more efficiently prevented the formation of sunburn cells (Kaidbey, 1990).

The p53 protein plays an important role in cellular response to DNA damage. Following exposure to genotoxic agents such as ionizing or UV radiation, wild-type p53 accumulates and becomes immunohistochemically detectable. In human skin, UV irradiation induces accumulation of p53 in the epidermis; this response is rapid and transient, being detectable as early as two hours after irradiation, peaking at 24 hours and persisting for several days (Hall *et al.*, 1993). Following UV irradiation of normally unexposed skin of healthy subjects, the pattern of p53 expression in the epidermis differs according to the UV wavelength: while UVA induces p53 expression predominantly in the basal layer, UVB induces p53 expression diffusely throughout the whole epidermis (Campbell *et al.*, 1993). In contrast, mutation of *p53* leads to persistent and strong nuclear accumulation of the protein, and in human sun-exposed skin samples from the face or the dorsal surface of the hands, epidermal areas of homogeneously stained cells sharply demarcated from their surroundings and strongly reactive with antibody to p53 (compact pattern) can be detected (Ponten *et al.*, 1995), that have been shown by microdissection and DNA sequencing to reflect clonal proliferation of keratinocytes with mutated *p53* (Ren *et al.*, 1996). Mutation of *p53* plays an important role in the onset of SCC of the skin. More than 90% of human SCCs of the skin contain UV-induced *p53* mutations that are already present in actinic keratoses (Ziegler *et al.*, 1994), but are genetically unrelated to mutations present in benign clonal keratinocyte patches with *p53* mutations in sun-damaged epidermis (Ren *et al.*, 1997).

Two studies have shown that topical application of a broad-spectrum sunscreen with a sun protection factor of 15 either decreases the overexpression of wild-type p53 (a physiological response to UV-induced DNA damage) in epidermal keratinocytes following UV exposure of normal previously non-sun-exposed skin (Ponten *et al.*, 1995) or significantly reduces the number of p53-positive keratinocytes in sun-protected skin after application of sunscreen to chronically sun-exposed skin during a one-summer period (Berne *et al.*, 1998).

Actinic keratoses

Actinic, or solar, keratoses are premalignant, sun-induced skin lesions, characterized as keratinized patches with aberrant cell differentiation and proliferation. They represent risk factors for BCC and melanoma and are precursors to SCC, although the rate of transformation is low (Green & O'Rourke, 1985; Marks *et al.*, 1988a, b). Strong clinical and experimental evidence supports their being precursors of SCC of the skin. Approximately 60% of SCCs are thought to arise in actinic keratoses (Marks *et al.*, 1988a). Similarly to SCC, they have a strong link to sun exposure and share many features with their malignant counterpart, including a dose–response relationship with cumulative sun exposure (Vitasa *et al.*, 1990) and an increased incidence in immunocompromised individuals such as transplant patients (Blohme & Larko, 1984).

Two randomized, placebo-controlled studies have investigated the prevention of new actinic keratoses and the reduction of pre-existing keratoses by sunscreens in high-risk populations with actinic keratoses or even non-melanoma skin cancer. The Australian study (Thompson *et al.*, 1993), conducted over a period of six months (one summer) in 431 subjects, as well as the North American study (Naylor *et al.*, 1995), conducted on a smaller number of subjects (50) over a two-year period, both showed that regular application of a high sun protection factor broad-spectrum sunscreen significantly reduced the total number of actinic keratoses. The Australian study further showed that this reduction in total number of actinic keratoses stemmed from a reduction in the development of new solar keratoses and an increased remission rate of keratoses present at baseline.

Immunosuppression

Experimental studies have shown that UV irradiation, and more especially UVB, induces local and systemic suppression of cell-mediated immunity that plays an important role in the control of growth of UV-induced malignant tumours (Kripke, 1994). Although there is no direct evidence that UVB-induced immunosuppression plays a role in the growth of sunlight-associated skin malignancies in humans, there is abundant circumstantial evidence; for instance, immunosuppressed

patients such as transplant recipients have greatly elevated rates of non-melanoma skin cancer incidence (Granstein, 1995).

Recent studies have investigated UV-induced immunosuppression and its modulation by topical sunscreen application in humans, focusing on contact hypersensitivity as measured by immunization or response to haptens, on modulation of stimulation of allogeneic lymphocytes by epidermal cells, or on release of immunomodulatory molecules such as *cis*-urocanic acid or interleukin-10.

Dinitrochlorobenzene (DNCB) is a potent contact sensitizer to which spontaneous sensitization is rarely encountered in human populations and has been widely used for evaluation of immune capacities of patients. Typically, sensitization to DNCB is induced by applying to the skin in a Finn chamber a small patch of filter paper containing 30–50 μg DNCB in acetone solution; the patch is removed after 48 hours. The sensitization induced is tested two weeks after the first contact with DNCB by application of challenge patches containing a range of concentrations of DNCB (usually from 3.125 to 12.5 μg). The challenge patches are removed after 48 hours and the contact hypersensitivity reactions are assessed 24 hours later.

Several studies have shown that in healthy volunteers an erythemal UV exposure significantly impairs the afferent arm of the contact hypersensitivity reaction, and that the application of a high sun protection factor sunscreen can at least partially prevent the UV-induced suppression of contact hypersensitivity, without itself interfering with the contact sensitization, probably by preventing the decrease in epidermal Langerhans cells at the irradiated site that usually follows exposure to UVB (Whitmore & Morison, 1995; Hayag *et al.*, 1997; Serre *et al.*, 1997). However, these tests result in the permanent sensitization of the subjects to a potent allergen, and hence may cause an allergic risk, even if the DNCB molecule is rarely encountered in everyday life.

Nickel is a frequent contact allergen in the general population. Up to 15% of women and 5% of men develop allergic contact dermatitis when exposed to nickel. UV radiation suppresses the allergic response of these individuals to patch testing with nickel, and clinical improvement of nickel allergy occurs after whole-body irradiation. This model has been developed into a technique for evaluating the immune protection afforded by sunscreens, and since neither UV irradiation nor sunscreens significantly affect erythema induced by a skin irritant, sodium lauryl sulfate, nickel patch testing appears to be a valid means of assessing UV-induced immunosuppression in humans and its modulation by sunscreens (Damian *et al.*, 1997). The fact that even with suberythemal UV irradiation, suppression of allergic response to nickel was abolished only by broad-spectrum sunscreens suggests that UVA plays an important role in UV-induced immunosuppression.

Alternatively, UV-induced immunosuppression can be assessed in normal subjects by using the delayed hypersensitivity response to common recall antigens (Moyal, 1998).

Another minimally invasive technique to explore UV-induced immunosuppression and its modulation by sunscreens is offered by the mixed epidermal cell–lymphocyte reaction (MECLR), in which epidermal cells obtained by the suction blister method after localized UV irradiation are used to stimulate allogeneic lymphocytes *in vitro*. However, conflicting reports have shown that the ability of sunscreens to interfere with UV-induced modulation of cell-mediated immune responses critically depends on the UV irradiation protocol and assay end-points that may involve different mechanisms of UV-induced immunomodulation (Van Praag *et al.*, 1991; Hurks *et al.*, 1997).

Numerous mediators are released by keratinocytes upon UV irradiation. *cis*-Urocanic acid, formed by photoisomerization in the epidermis of *trans*-urocanic acid, accumulates in the stratum corneum and is considered an important mediator of local immunosuppression resulting from exposure to UV radiation. Formation of *cis*-urocanic acid can be measured in stratum corneum strippings and has been used to evaluate photoprotection by sunscreens (Krien & Moyal, 1994; de Fine Olivarius *et al.*, 1999). More recently, interleukin-10 (IL-10) mRNA expression has been studied by reverse transcription polymerase chain reaction in epidermal cells obtained from suction blisters (Hochberg & Enk, 1999).

Melanoma

Cutaneous melanoma represents the end of a continuum starting from sun-damaged melanocytes in

the epidermis and progressing through a common acquired naevus which may show cellular atypia, an atypical (dysplastic) naevus with architectural atypia, melanoma *in situ*, early (radial growth phase) primary melanoma with no competence for metastasis, late (vertical growth phase) primary melanoma, and finally to metastatic melanoma (Table 1).

Although the pathogenesis of melanoma is far from fully understood (Gilchrest *et al.*, 1999), the main exogenous etiological factor of melanoma appears to be intermittent (recreational) sun exposure, especially in childhood (Autier & Doré, 1998). It has been proposed that melanomas arising before the age of 50 years occur electively in body areas intermittently exposed to sunlight, while melanomas arising at a later age occur in body areas more likely to be chronically sun-exposed (Elwood & Gallagher, 1998).

Naevi

Naevi are focal collections of non-dendritic melanocytes, usually found at the junction of the epidermis and dermis (junctional naevi) or at various depths in the dermis (compound or dermal naevi). Common acquired naevi arise after birth, both spontaneously and in response to various factors, particularly exposure to the sun (Harrison *et al.*, 1994).

Many, if not most, cutaneous melanomas arise in acquired naevi, and it has recently been shown that most acquired naevi are clonal, while most melanocytes in non-naeval areas are not (Robinson *et al.*, 1998). Although it is not clear whether acquired naevi represent actual premalignant lesions or risk factors, the number of common naevi in any given individual, which is determined by genetic factors and sun exposure, constitutes the best predictor of individual risk of melanoma (Boyle *et al.*, 1995; Berwick & Halpern, 1997). The presence of large naevi (e.g., those with one dimension >5 mm) or of atypical naevi (e.g., large naevi with non-uniform colour and irregular borders) is also associated with higher melanoma risk, independently of the number of smaller naevi (Bataille *et al.*, 1996; Tucker *et al.*, 1997). Large naevi are not uncommon in children, but atypical naevi are rare in North American or European children before onset of puberty (Greene *et al.*, 1985).

Children seem particularly vulnerable to sun-induced biological events possibly involved in the genesis of melanoma (Holman & Armstrong, 1984; Autier & Doré, 1998). Also, the greatest increase in naevus numbers per unit of skin surface takes place before adolescence (English & Armstrong, 1994). Therefore, the distribution of naevi and their development in children is relevant to understanding melanoma occurrence in adults.

A recent study by our group (Autier *et al.*, 2000) assessed the body-distribution of naevi, with particular reference to differences in sun exposure by body-site, in 649 European children aged 6–7 years from Brussels (Belgium), Bochum (Germany), Lyon (France) and Rome (Italy). Counts of naevi of size 2–4.9 mm and ≥5 mm were performed using a standard method. The numbers of naevi 2–4.9 mm and of naevi ≥5 mm were strongly correlated, especially on the trunk. For naevi 2–4.9 mm, the highest relative densities were found on the face, back, shoulders and the external surface of the arms. Lowest relative densities were found on the hands, legs, feet and abdomen (Figure 1). The relative density of naevi ≥5 mm was higher on the trunk than on any other body site (Figure 2). Similar body distributions were observed in both sexes and at each centre. The body-site distribution of naevi 2–4.9 mm seemed to parallel the usual sun exposure patterns of young European children. It is suggested that the development of naevi ≥5 mm might be a marker of the vulnerability of melanocytes to the harmful effects of solar radiation. Such vulnerability would be maximal on the trunk, and would decrease distally, with melanocytes of the hands and feet having the lowest vulnerability. The number of naevi acquired on a specific area of skin would result from the combined effects of local vulnerability to solar radiation and local sun exposure history. The origin of acquired body-site differences in the susceptibility of melanocytes to UV radiation is unknown, although it seems to parallel the body-site density of sensory innervation.

Several studies have investigated the development of naevi as a function of sun protection or sunscreen use (Table 3). In a case–control study conducted in Europe in 418 melanoma cases and 438 controls (Autier *et al.*, 1995), it was noted that the use of sunscreen was associated with a higher density of pigmented lesions of the skin in controls. The naevus count on both arms in

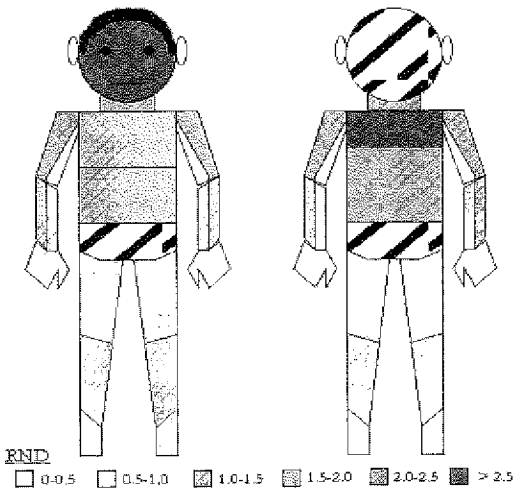

Figure 1. Relative densities of naevi of size ≥ 2 mm in 6–7-year old European children.

RND = relative naevus density.

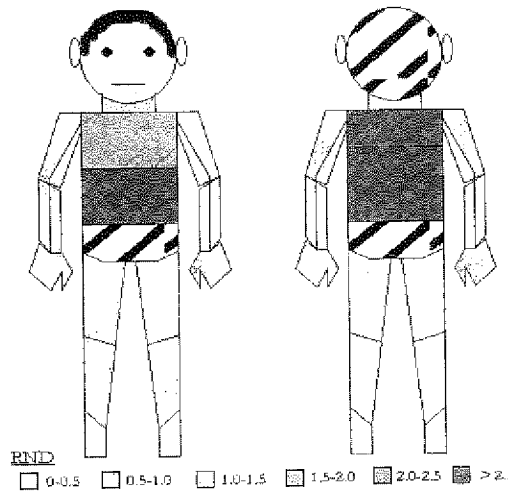

Figure 2. Relative densities of naevi of size > 5 mm in 6–7-year-old European children.

RND = relative naevus density.

control subjects significantly increased from those reporting no sunscreen use to those who ever used sunscreen.

A study of naevus development was conducted in Germany among schoolchildren (Luther *et al.*, 1996). Naevus counts were performed in 866 children between the ages of 1 and 6 years (median age, 4 years), and a second time after a five-year period in 424 children of whom the parents had agreed to participate. Univariate analysis showed a significant relationship between the regular use of sunscreen and a high increase in numbers of melanocytic naevi. This association was interpreted by the authors as resulting from a tendency of children who use sunscreen regularly to have more cumulative sun exposure.

A retrospective cohort study examined the number of naevi in 631 6–7-year-old European children in elementary schools in Brussels, Bochum, Lyon and Rome, according to their sun exposure history, physical protection, sunscreen use and sunburn history from birth to the moment of skin examination (Autier *et al.*, 1998). In all study locations, the median numbers of naevi tended to increase with total or average sunscreen use during holidays, whereas the reverse was true for average wearing of clothes in the sun. Median naevus counts increased with both increasing sun exposure and average sunscreen use. After adjustment for sun exposure and host characteristics, the relative risk for a high naevus count on the trunk was 1.68 (95% confidence interval, 1.09–2.59) for the highest level of sunscreen use and 0.59 (95% confidence interval, 0.36–0.97) for the highest level of wearing clothes while in the sun. The average sun protection factor of the sunscreen used had no demonstrable effect on naevus counts. The highest risk of naevi on the trunk associated with sunscreen use was seen in children who never suffered from sunburn (relative risk, 2.21; 95% confidence interval, 1.33–3.67).

Conclusion

Few intermediate end-points related to the biology of tumour progression can be reliably used as biomarkers to assess chemoprevention of skin cancers.

Assessment of sunburn cell formation or of *p53* mutation induction in the epidermis provides information on the early steps of non-melanoma skin cancer. However, due to their invasive measurement, these biomarkers would be difficult to use in large-scale prevention trials. Actinic keratoses, precursors to SCC, share a number of

features with their malignant counterpart, including a dose-dependent link to cumulative sun exposure, and can be easily measured clinically. They have therefore been measured in large-scale trials in humans that have shown that a reduction of actinic keratoses can be obtained by use of sunscreens. The use of such an intermediate-effect biomarker in chemoprevention studies may be regarded as validated by a recent Australian trial showing a reduction in risk of SCC, but not of BCC, in a prospective controlled trial of sunscreen use in the prevention of non-melanoma skin cancer (Green *et al.*, 1999).

Naevi and melanomas share a number of common features and it is thought that long-term prospective studies on melanoma occurrence could be replaced by prospective studies of naevus development. In this respect, the maintenance of a low number of naevi should be considered in the evaluation of the efficacy of protection against long-term harmful effects of sunlight exposure. However, the prevalence of common acquired naevi exceeds by far that of melanoma, and future studies should focus on more precise identification of subjects at risk, for example by considering markers of DNA damage susceptibility or of DNA repair proficiency such as the inherited susceptibility to induction of chromatid breaks by an UV-mimetic mutagen, 4-nitroquinoline-1-oxide (Wei *et al.*, 1996; Wu *et al.*, 1996). In public health, it is increasingly evident that a reduction in the incidence of melanoma will be preceded by a reduction in naevus numbers. The evolution of naevus number at different ages could serve as an indicator of the likely trend of melanoma incidence in the coming years.

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