UNIVERSITÀ DEL PIEMONTE ORIENTALE

UNIVERSITÀ DEGLI STUDI DEL PIEMONTE ORIENTALE

Department of Health Science

Ph.D. program in Medical Sciences and Biotechnology

Cycle XXXIV

Evaluation of vitamin B3, D3 and E as photoprotectors against UVB-induced damages on primary keratinocytes and fibroblasts

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ACCADEMIC YEARS 2018-2021

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Summary-english

Non-melanoma skin cancers (NMSCs) are one of the most frequent cancers diagnosed in the elderly Caucasian population. During the last 50 years, NMSCs incidence has notably increased due to changes in lifestyle and more consciousness about skin cancers. Among several factors that contribute to NMSCs development, ultraviolet (UV) radiations are the main responsible for cancer degeneration. Indeed, UV can damage cell DNA through direct absorption or indirectly by ROS production, which oxidize DNA bases causing mutations and mismatches. Besides, UV inhibit immune response, activate local inflammation, trigger the production of metalloproteinases, and induce cell senescence. Most of the modifications induced by UV contribute to photocarcinogenesis and photoaging of the skin. In order to prevent UV-induced damages especially on high-risk patients, the use of inexpensive chemopreventive molecules with low side effects could help to prevent NMSC development. So, this thesis aims to provide new insights on the role of vitamin B3, D3, and E, in their active forms, as photoprotectors against UVB-induced damages on human primary keratinocytes and fibroblasts. We firstly demonstrated that keratinocytes isolated from field cancerization are more sensible to UV radiations in comparison with normal keratinocytes. Nevertheless, nicotinamide (NAM) at 25 µM given 24h before irradiation efficiently protected keratinocytes from UVB damages reducing ROS production and DNA damages and blocking the inflammatory response. Then, we proved that NAM and vitamin D3 analog, calcipotriol, can protect human fibroblasts from photoaging by inhibiting ROS release, reducing DNA damages, restoring cell cycle, and decreasing cell senescence. Overall, our results suggest that NAM could be an efficient photoprotector against photocarcinogenesis and photoaging on both keratinocytes and fibroblasts. Moreover, vitamin D3 seems to be protect efficiently fibroblasts from premature aging, while vitamin E, at 1 µM concentration, showed less efficacy. Our results might suggest that nicotinamide and vitamin D3 could be used for non-invasive treatment of field cancerization and chronically photoexposed skin areas, in order to prevent NMSC relapse and to reduce demand for surgical treatment.

Summary-italiano

I tumori cutanei non melanocitari (NMSCs) sono le neoplasie cutanee più frequenti nella popolazione caucasica. Durante gli ultimi 50 anni, l'incidenza dei NMSCs è particolarmente aumentata, dovuto principalmente al cambiamento di abitudini della popolazione e a una maggiore sensibilizzazione sui tumori cutanei. Tra i diversi fattori che contribuiscono allo sviluppo dei NMSCs, l'esposizione ai raggi ultravioletti (UV) è la causa principale. Infatti, gli UV sono in grado di danneggiare il DNA delle cellule tramite assorbimento diretto o tramite la produzione di ROS, che interagiscono con i nucleotidi del DNA dando origine a mutazioni. Inoltre, gli UV inibiscono la risposta immunitaria, attivano una risposta infiammatoria locale, inducono la produzione di metalloproteasi e favoriscono la senescenza cellulare. Molte delle modificazioni indotte dai raggi UV contribuiscono alla fotocarcinogenesi e al fotoinvecchiamento della pelle. L'utilizzo di molecole con proprietà fotoprotettrici, economiche e con pochissimi effetti collaterali, potrebbe contribuire alla prevenzione dello sviluppo di tumori cutanei, specialmente nei pazienti ad alto rischio di NMSC. Perciò, lo scopo di questa tesi è quello di testare il ruolo della vitamina B3, D3 ed E, nelle loro isoforme attive, come molecole fotoprotettrici contro i danni indotti dai raggi UVB in cheratinociti e fibroblasti primari umani. Innanzitutto, abbiamo dimostrato che i cheratinociti isolati dal campo di cancerizzazione risultano essere più sensibili ai raggi UV in confronto con i cheratinociti di controllo. Tuttavia, il trattamento con nicotinamide (NAM) 25 µM 24 ore prima dell'esposizione ai raggi UV, ha protetto i cheratinociti riducendo la produzione di ROS e danno al DNA e bloccando la risposta infiammatoria. Successivamente, abbiamo dimostrato che sia NAM che calcipotriolo, analogo della vitamina D3, proteggono i fibroblasti primari umani dal fotoinvecchiamento inibendo il rilascio di ROS, riducendo i danni al DNA, ripristinando il ciclo cellulare e diminuendo la senescenza cellulare. Complessivamente, i nostri risultati suggeriscono che la vitamina B3 può proteggere dalla fotocarcinogenesi e fotoinvecchiamento cheratinociti e fibroblasti mentre, la vitamina D3 sembra proteggere i fibroblasti dal fotoinvecchiamento. Infine, è emerso che la vitamina E, alla concentrazione utilizzata, risulta essere la meno efficace. In conclusione, dall'analisi dei nostri risultati si può affermare che le vitamine B3 e D3 potrebbero essere utilizzate come trattamento non invasivo del campo di cancerizzazione e della cute cronicamente fotoesposta in modo da prevenire la ricaduta e ridurre il numero di interventi chirurgici necessari per il trattamento dei NMSC.

Abbreviations

6-4PPs- pyrimidine pyrimidone (6-4) photoproducts

AK- actinic keratosis

BCC- basal cell carcinoma

BER- base excision repair

CAL- calcipotriol

CPDs- cyclobutane pyrimidine dimers

CTCF- corrected total cell fluorescence

DCFDA- 2',7'–dichlorofluorescin diacetate

DDR- DNA damage repair

FC-HPKs- field cancerization-human primary keratinocytes

GPX-1- glutatione peroxidase-1

HDFs- human dermal fibroblasts

IF- indirect immunofluorescence

MMP- matrix metalloproteinase

MTT- 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide

NAM- nicotinamide

NER- nuclear excision repair

NHEKs- normal human epidermal keratinocytes

OGG1- 8-Oxoguanine DNA Glycosylase

PARP-1- poly-ADP-ribose-polymerase-1qRT-PCR- quantitative reverse transcription-PCR

SASP- senescence-associated secretory phenotype

SA-β-GAL- senescence associated-β-galactosidase

SCC- squamous cell carcinoma

SOD-1- superoxide dismutase-1

VDR- vitamin D receptor

α-T- α-tocopherol

INTRODUCTION

INTRODUCTION

❖ Anatomy and function of the skin

The skin is the largest organ and covers entirely the human body forming a physical barrier able to protect internal tissues from mechanical impacts, toxins, infections, radiations, and chemicals. The skin consists of three layers (Figure 1):

a) Epidermis: is the outermost part of the skin. The epidermis is mainly composed of keratinocytes distributed in five layers.

b) Dermis: located beneath the epidermal layer, consists of connective tissue, which is composed of fibroblasts, collagen, and fibres providing strength and elasticity to the skin. Moreover, are present different components such as hair follicles, mechanoreceptors, blood, and lymphatic vessels, sweat, and sebaceous glands.

c) Hypodermis: is the lowermost layer of the skin and is mainly composed by fibroblasts, adipose cells, and macrophages. It is the major site for adipose tissue storage and contains blood vessels and nerves (Sadick et al, 2014).

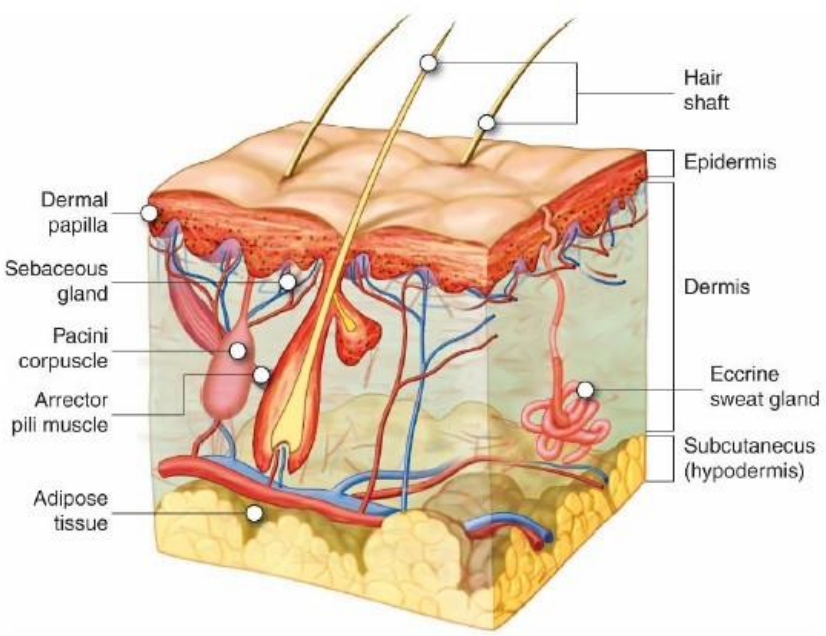


Figure 1: *Anatomy of skin. Skin is composed by three layers: epidermis, dermis and hypodermis. Epidermis is mainly composed by keratinocytes distributed in 5 layers. Dermis is mainly composed by fibroblast and connective tissue. Hypodermis is mainly composed by adipocytes, blood vessels and nerves.*

- **Epidermis**

The epidermis is the outer part of the skin and is a stratified epithelium composed of different cell types such as keratinocytes (90-95%), melanocytes (responsible for melanin production), Langerhans cells (dendritic cells), and Merkel cells (mechanoreceptors) (Yousef et al, 2021). Epidermal cells are arranged in five continuous layers, as shown in Figure 2:

a) Basal layer: is the deepest layer of the epidermis, composed by cuboidal to columnar mitotically active stem cells. This layer is joined to the basement membrane through hemidesmosomes. Basal cells are responsible for tissue renewal undergoing a specific cell differentiation. Indeed, the basal layer is composed by 10% of stem cells, 50% of amplifying cells, and 40% of postmitotic cells, which undergo terminal differentiation and migrate into the squamous layer (Sadick et al, 2014).

b) Spinosum layer**:** it is above the basal layer and is typically five to ten cell layers thick. It is composed by irregular keratinocytes joint together with desmosomes that give strength and mechanical resistance to cells, maintaining the structural integrity of the skin (Barbieri et al, 2014). Cells of the spinosum layer produce lamellar granules which contain lipids, cholesterol, fatty acids as well as enzymes, and are considered the first sign of keratinization. These granules expel their contents on the surface of the skin, imparting barrier-like properties (Sadick et al, 2014).

c) Granular layer: it is in the uppermost layer of the epidermis and usually is 3-5 cells thick. Granular layer-keratinocytes produce keratohyalin granules and lamellar granules, which contain profilaggrin (*i.e.*, the precursor of filaggrin) and glycolipids. Filaggrin cross-links with keratin filaments providing strength and structure, creating a permeability barrier to water, and facilitating cell adhesion to the stratum corneum. Since granular keratinocytes mature and migrate to the stratum corneum, they begin to dissolve their nucleus and organelles (Barbieri et al, 2014).

d) Stratum corneum: it is the most external layer, which is 15-20-cell layers thick, and serves as a barrier against external agents, infections, dehydration, chemicals, and mechanical stress. In this layer, keratinocytes are fully differentiated or keratinized, and under physiological condition, they do not have nuclei nor organelles. Here,

desmosomes begin to disappear or become non-functional, leading to desquamation, an important process necessary for cell renewal and maintaining skin homeostasis (Barbieri et al, 2014).

In addition to these layers, it is possible to observe under microscope the ***stratum lucidum***, a thin clear layer of dead cells that is present in thicker skin areas, like palms and soles.

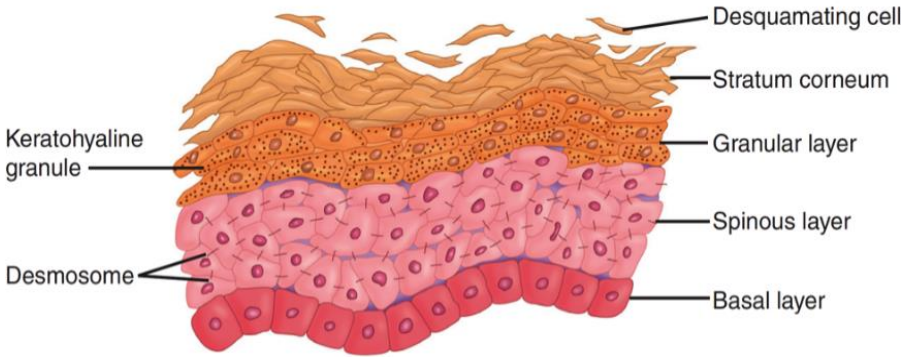


Figure 2*: human epidermis layers (Baumann, L. S., & Baumann, L. (2009). Cosmetic dermatology. McGraw-Hill Professional Publishing)*

- **Dermis**

The dermis is an elastic, compressible, and supportive connective tissue which lies between epidermis and subcutaneous tissue. It provides nutriment to the epidermis which is attached by the basement membrane. Dermis is largely composed by collagen in which there are cells, nerves, blood vessels, and other fibres. The thickness of the dermis varies, from about 5 mm on the back to about 1 mm on eyelids, depending also on the patients age and anatomic location. Based on fibres' arrangement, dermis is divided in two parts:

- Papillary dermis: It is the upper part of the dermis and creates projections into the epidermis that increase the surface of contact cushioning the body against mechanical injury.
- Reticular dermis: It is the lower part of the dermis made of collagen bundles, vascular vessels, and nerves.

Fibroblasts are the primary cell type in the dermis, and they produce collagen, elastin, and other enzymes like collagenase (MMP-1) fundamental for collagen turnover.

Nevertheless, immune cells, like mast cells, lymphocytes, and macrophages, are also present in the dermis (Kanitakis, 2002; Baumann et al, 2009).

❖ Non-melanoma skin cancer

Non-melanoma skin cancers (NMSCs) are one of the most frequent cancers diagnosed in the elderly Caucasian population. NMSCs encompass basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs), which together account for about 95% of total skin tumours. (Madan et al, 2010). NMSCs are characterized by different behaviour, growth, and metastatic capability, even though both BCC and SCC have a good prognosis if diagnosed at early stages (Didona et al, 2018).

BCCs arise from epidermal basal cells and account for 75% of NMSC cases in immunocompetent patients. It is the least aggressive NMSC with a low degree of malignancy, slow growth (months to years), and low metastatic rate (<0.5%), despite the ability of local invasion, tissue destruction, and recurrence in higher-risk subtypes (Didona et al, 2018; Kim et al, 2019). Indeed, people affected by BCC have an elevated risk of developing new BCC lesions as well as other cancers like melanoma or SCCs (Madan et al, 2010). BCCs develop primarily on sun-exposed skin areas and is rarely found on palmoplantar surfaces (Didona et al, 2018).

SCCs develop from atypical proliferative squamous cells with rapid growth (weeks to months) and show a metastatic rate of 0.1-9.9%, which is influenced by body localization, poor differentiation, large tumor and thickness (Chen et al, 2013). In addition, SCCs are characterized by a noticeable potential for recurrence, depending on tumor size, patient's immune system, degree of differentiation, and anatomic localization. SCC mortality rate is the highest among NMSCs and it accounts for about 75% of deaths due to keratinocyte carcinomas (Chen et al, 2014). As for BCC, the most important risk factor is ultraviolet radiation exposure. Indeed, SCCs usually arise from chronically sun-exposed areas, like head and neck (55%), hand and forearms (18%), and less frequently on legs (<13%) (Didona et al, 2018).

Whilst BCCs develop *de novo*, SCCs usually arise from pre-cancerous lesions as actinic keratosis (AKs) through a multistage process (Samarasinghe et al, 2012). In particular, AKs are extremely common, affecting more than 40% of the adult population, with a variable prevalence depending on the geographic area. AKs arise from chronically photo-exposed skin and 0.025-16% of AKs evolve to SCCs every year. Considering that each

patient carries several AKs, the annual risk of developing SCC has been reported between 0.15% to 80% (Didona et al, 2018). Histologically, AKs are characterized by cellular atypia especially on basal layer, affecting cell differentiation which leads to increased keratinization (Fernandez, 2017). AKs and SCCs have a similar genetic profile. Indeed, both lesions, as well as sun-damaged but normal-appearing skin, present p53 mutations induced by UV, which lead to cell cycle alterations and impaired apoptosis (Chen et al, 2013). Other modifications have been observed on AKs like mutations on CDKN2A, RAS, and tumor suppressor genes and modulation on associated telomerase activity (Padilla et al, 2010). AKs can remain stable, regress, relapse, or progress, but their behaviour is unpredictable. Progressive AKs are resistant to standard therapies because of deep migration of altered cells and are more prone to evolve into invasive SCC (Didona et al, 2018).

- **<u>Epidemiology</u>**

NMSC generally occurs in people older than 50 years. A yearly increase of 3-8% has been recorded since 1960 (Madan et al, 2010), and BCC and SCC prevalence has increased by 35% and 133% respectively during the last 20 years (Leiter et al, 2019). The rising incidence of NMSCs could be due to different factors. First, skin cancer prevention campaigns may have improved consciousness about NMSCs, improving diagnosis and awareness in physicians. Second, the use of sunbeds over the past two decades and changes in lifestyle, like outdoor activities, might enhance ultraviolet exposure in the younger population, leading to rise in NMSCs cases (Apalla et al, 2017). Moreover, the average age of the population is increasing worldwide, which could lead to an extended chronical exposure to UV rays. Finally, the thinning of the ozone layer of the atmosphere has led to an increased concentration of ultraviolet radiation on earth, contributing to the development of skin cancers (Ciążyńska et al, 2021; Cavinato et al, 2017).

However, because of the exclusion of NMSCs from traditional cancer registries, and variation in data capture among countries, incidence data are still underestimated. The Global Cancer Observatory (GLOBOCAN) estimated around 1.200.000 new cases of NMSCs in 2020 on both sex and all ages (Figure 3), representing the 6.2% of total cancer diagnosed with an incidence rate approximately 2

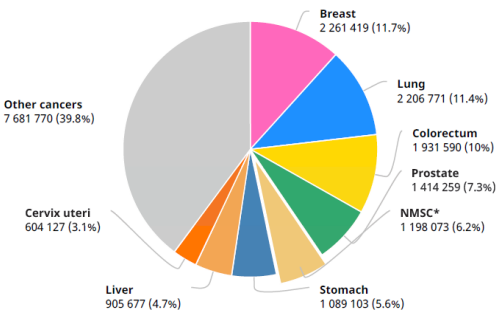
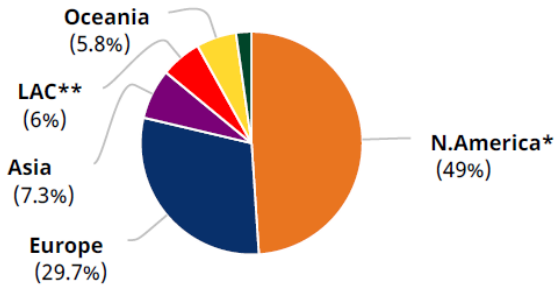


***Figure 3**: number of new cases in 2020, both sexes, all ages (GLOBOCAN 2020)*

times higher in men than women. The highest incidence was recorded in North America, which accounts for 49% of cases, followed by Europe (29.7%), Asia (7.3%) and Oceania (5.8%). In particular, in Europe the major incidence was recorded in western countries (Sung et al, 2020).

In consideration of the frequent extension of chronic actinic damage to large skin areas, patients with a primary carcinoma have a 40-50% of risk to develop one or more cancerous lesions within 5 years (Madan et al, 2010). Consequently, patients frequently require numerous treatments, impacting negatively on health system costs. For instance, in Australia NMSCs are the costliest cancers due to their very high frequency (Chen et al 2013; Apalla et al, 2017).



Population	Number
*Northern America	586 575
Europe	356 180
Asia	87 040
**Latin America and the Caribbean	71 319
Oceania	69 922
Africa	27 037
Total	1 198 073

***Figure 4**: incidence of NMSCs in 2020 (GLOBOCAN 2020).*

- **<u>Risk factors</u>**

As mentioned above, the risk of NMSC development is a combination of different factors, like skin type, immunosuppression, ultraviolet radiation exposure, body sites, age.

- **Skin type**. Fitzpatrick scale is a semi-quantitative evaluation of skin colour divided in six phototypes based on basal complexion, melanin level, inflammatory response to UV and cancer risk (D'Orazio et al, 2013). People with Fitzpatrick skin type I and II (light skin, eyes, and hair colour) have a higher susceptibility to skin cancers (Didona et al, 2018). Since sun rays are strongest at the equator, and the ozone layer is thinner (Lim et al, 1999), people living in countries at lower latitudes with high doses of solar irradiance, and with these skin phenotypes are more susceptible to skin cancers (Madan et al, 2010).
- **Ultraviolet exposure**. Cumulative ultraviolet exposure is one of the most important extrinsic factors responsible for skin cancer development (Didona et al, 2018). Indeed, UV can directly damage the DNA, trigger the production of

reactive oxygen species (ROS), suppress the immune system, and activate local inflammation (Nishisgori, 2015). More than 80% of NMSCs develop on chronically sun-exposed body sites like hands, ear, nose, cheek, lip, and scalp (in bald people) (Leiter et al, 2019). BCC development is associated with intensive UV exposure during childhood and adolescence, while SCCs arise after chronic UV exposure in the earlier decades (Samarasinghe et al, 2012; Leiter et al, 2019). The increased use of sunbed, changes in clothing style and outdoor occupations and activities, has raised up the number of new cases diagnosed yearly and reduced the age of onset (Apalla et al, 2017; Leiter et al, 2019).

- **Immunosuppression**. Immunosuppression is another important factor that increases the risk of skin cancer development. Indeed, transplant recipients treated with immunosuppressors, have a 10-fold higher risk of developing BCCs and 65-250-fold of developing SCCs. This is due to reduced surveillance from immune cells in detecting and clearing cancer cells (Chen et al, 2013). UVB seems to be the most critical player on immune system impairment (Schade et al, 2005).
- **Viral infection**: Human papilloma virus (HPV) infection in the immunosuppressed patients is considered as co-factor for SCC pathogenesis. Indeed, HPV can alter cell cycle progression, DNA repair, and immune surveillance, leading to expansion of mutated clones UV-induced (Didona et al, 2018).

▪ **Treatment**

NMSCs are divided in low and high risk depending on the size and location of the lesion, immunosuppression status, histological differentiation, and subtype. Surgery is usually the first choice of treatment, and Mohs micrographic surgery (MMS) is the gold standard treatment for all high-risk lesions. Topical treatments are used for precancerous lesions, like actinic keratosis. Other techniques available for the treatment of NMSC are cryotherapy, electrodessication, photodynamic therapy, radiotherapy (Savoia et al, 2015).

❖ Photocarcinogenesis and photoaging

Chronic exposure to ultraviolet radiation leads to development of skin malignancies or photocarcinogenesis (Elmets et al, 2013) and premature skin aging or photoaging

(Gilchrest, 2013). Both these UV-induced modifications contribute to development of the field cancerization, which can evolve into malignant lesions like NMSCs.

- **Photoaging**

Ultraviolet radiations are responsible for photoaging which consists of chronic sun damages that induce histological and functional modifications into the skin (Fisher et al, 2002). This process is influenced by skin type, age, geographic location, sun exposure in relation to occupation and lifestyle, and photoprotective practices, like the use of sunscreens. Indeed, people lightly pigmentated and chronically exposed to solar light could experience the highest degree of photoaging (Han et al, 2014). Moreover, the intense use of tanning beds accelerates skin aging. Photoaged skin presents macroscopic and microscopic modifications in both dermis and epidermis and is characterized by epidermal thickening, deep wrinkles, loss of elasticity, and dryness (Fitsiou et al, 2020). In the epidermis, UV induce apoptosis of basal stem cells of the epidermis causing epidermal atrophy and delayed wound healing (Gilchrest, 2013). On the other hand, in the dermis, UV cause a reduction of the extracellular matrix, degradation of collagen fibres leading to decreased tissue elasticity and resistance (Han et al, 2014).

- **Photocarcinogenesis**

Cancer development is a multistage process in which molecular and biological changes occur on target cells. UV-induced damage, oxidative stress, inflammation, immunosuppression, and cell dysregulation all participate in cancer progression (Pihl et al, 2021). Photocarcinogenesis is mainly composed of three stages: initiation, promotion, and progression. Tumor initiation is a rapid and irreversible process in which ultraviolet radiations, in particular UVB, damage the DNA on target genes which alters cell metabolism and induces the production of reactive oxygen species (Afaq et al, 2005). Instead, tumor promotion is a lengthy process in which initiated cells accumulate mutations that lead to clonal expansion, giving rise to pre-malignant and malignant lesions, like AKs (Afaq et al, 2005). In this stage, reactive oxygen intermediates are the main responsible for biochemical changes, like DNA damage, proinflammatory cytokines release, and nitric oxide production. Moreover, UV induces immunosuppression on the skin (Elmets et al, 2013). Tumor progression is the final stage in which pre-malignant and malignant lesions

are converted into an invasive and potentially metastatic malignant tumor (Afaq et al, 2005).

The intervention on cancer development could be more appropriate and practical during tumor promotion since is a reversible event, at least in the early stages, and requires repeated and prolonged exposure to ultraviolet. Moreover, tumor promotion is a fundamental step in photocarcinogenesis, and the long time that occurs between initiation and progression offers a large window of opportunities for intervention (Afaq et al, 2005).

❖ Ultraviolet radiation

Ultraviolet radiations are part of the electromagnetic spectrum emitted by the sun and fall between visible light and gamma radiation. UV rays can be divided into UV-A, -B, and -C depending on energy and wavelength. UVC (100-280 nm) have the highest energy and shortest wavelength, UVB (280-315 nm) have mid-wave and mid-energy, and UVA (315-400 nm) have the lowest energy and the longest wavelength. Since UVC are absorbed by atmospheric ozone layer, solar UV radiations are composed by UVA (90-95%) and UVB (5-10%). As shown in Figure 5, UV rays penetrate the skin in a wavelength-dependent manner. Indeed, UVA can penetrate deeply into the dermis reaching fibroblasts while UVB are almost completely absorbed by the epidermis, with a little portion absorbed by the dermis (D'Orazio et al, 2013).

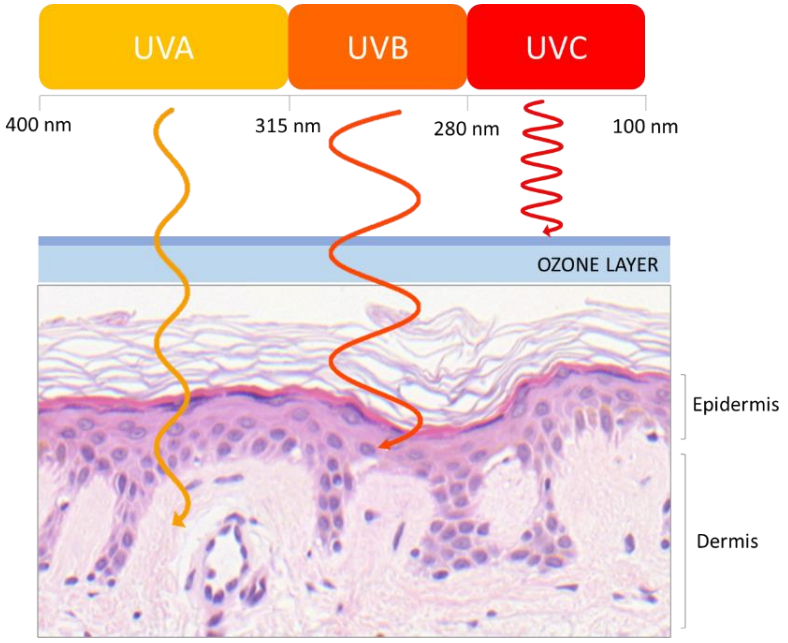


***Figure 5**: UV radiation penetrates differently into the skin depending on energy level and wavelength.*

Exposure to sunlight has both positive and negative effects on human skin. Indeed, UVB induce vitamin D synthesis, which is important for immune system and bone straightness, and UV regulate circadian rhythm, improve mood, and induce cosmetic tan. Furthermore, UV are used for the treatment of some inflammatory and immune-mediated skin diseases, like psoriasis, vitiligo and atopic dermatitis because of their immunosuppression function (Juzeniene et al, 2012, Mancuso et al, 2017). However, both UVA and UVB can do harm causing detrimental effects depending on time of exposure. Indeed, acute exposure to UV causes sunburn, phototoxicity and erythema while chronic exposure leads to photocarcinogenesis (D'Orazio et al, 2013). Due to their physical properties, UV can exert different effects on cells and molecules.

- **DNA damage**

Both UVA and UVB can directly or indirectly damage the DNA structure. Indeed, UVB photons are directly absorbed by DNA molecules causing multiple types of DNA damages, including single-strand breaks, cyclobutane pyrimidine dimers (CPDs) (Ikehata et al, 2011), and pyrimidine-pyrimidone (6-4) photoproduct (6-4PP) (Hung et al, 2020; Elmets et al, 2013). DNA alterations might occur on cancer-related genes causing cell cycle dysregulation, upregulation or downregulation of signal transduction pathways leading to cancer development (Nishisgori, 2015). All these modifications trigger damage response pathways on keratinocytes and fibroblasts like activation of p53 which blocks cell cycle and promotes DNA repair through nucleotide excision repair (NER) system (D'Orazio et al, 2013). On the other hand, UVA indirectly damage the DNA through the formation of ROS that react with nucleotide and cause mutations. Indeed, nucleotides are highly sensitive to free radical injury that can oxidize guanine at the 8^{th} position producing 8-hydroxy-2'-deoxyguanine (8-OHdG) mutagenic product, which tends to pair with adenine instead of cytosine. However, an enzyme of the base excision repair (BER) pathway called OGG1 (8-oxoguanine DNA glycosylase), cleaves mutated nucleotides and removes oxidative DNA damage (Ichihashi et al, 2003). Therefore, both UVB and UVA contribute to photocarcinogenesis (Nishisgori, 2013).

- **ROS and oxidative stress**

ROS are physiologically produced by cells in the mitochondria and antioxidants, such as glutatione peroxidase, superoxide dismutase and catalase, control their production (Rinnerthaler et al, 2015). However, the exposure to external stimuli, like UV, boost ROS production through a rapid radiolysis of water molecules (Klaunig, 2018). As consequence, an imbalance between free radicals and antioxidants occurs, leading to oxidative stress. The main ROS species produced by UV are superoxide anion, hydrogen peroxide and hydroxyl radical (Pizzino et al, 2017), and they can interact with several cellular components leading to cellular alterations.

ROS have been considered the initial step of photoaging and indirectly contributors to photocarcinogenesis (Fisher et al, 2002), affecting both epidermis and dermis, even though effects on dermis are better studied (Rinnerthaler et al, 2015). Indeed, ROS target and damage proteins and lipids within cells activating signal responses, cytokine release, transcription factors and inhibition of phosphorylases. In particular, in the dermis, ROS activate AP-1 transcription factor which inhibits collagen production and induces the expression of MMPs, like MMP-1 and MMP-3, which together degrade dermal collagen and inhibit the production of collagen (Han et al, 2014). ROS can also damage the DNA which cause the activation of p53/p21 growth-suppressive pathway which arrests the cell cycle allowing DNA repair machinery to remove mutations. However, if mutations are not removed, cells can undergo senescence. The accumulation of senescent cells in the skin contributes to the disruption of tissue integrity (Cavinato et al, 2017). All these modifications perpetrated by UV, as shown in Figure 6, facilitate angiogenesis and metastasis, and boost photooxidative stress contributing to skin carcinogenesis (Bosch et al, 2015). Since antioxidant activity in the skin is affected by age and exposure to UV (Poljšak et al, 2012), exogenous supplementation of antioxidants might counteract UV-induced ROS release, restoring normal oxidative condition within cells and tissues (Ernst et al, 2013).

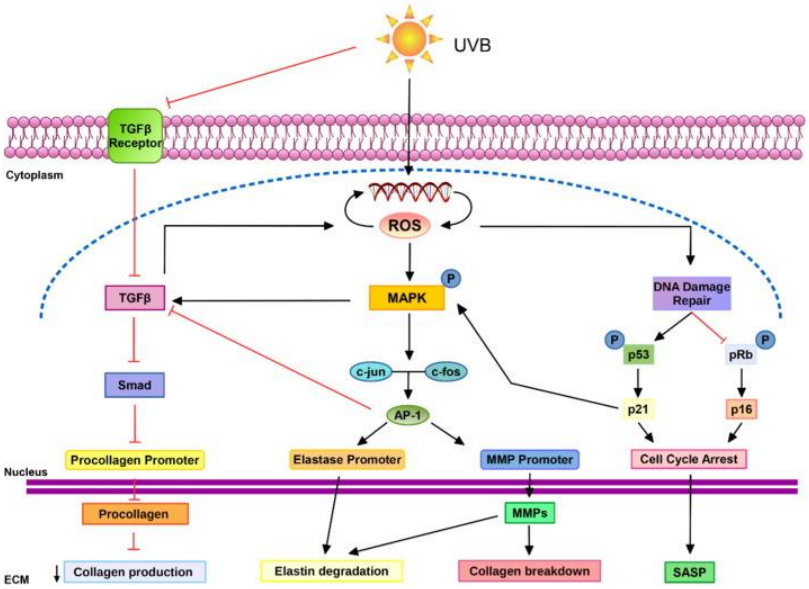


***Figure 6**: Signalling pathway activated by UVB rays (Cavinato et al, 2017). UVB act over many signalling pathways that regulate cell cycle progression, transcriptional regulation of genes involved in extracellular matrix remodelling. ROS production induced after UV exposure are the main trigger of all these modifications.*

- **Immunosuppression**

UVB can promote immunosuppression in the skin by inducing apoptosis on Langerhans cells and reducing their ability as antigen-presenting cells, enhanced by immunosuppressive cytokines released by keratinocytes. The main consequence is not only a suppressed response to external threats, like bacteria and fungi, but also a reduced immunosurveillance against mutated cells, allowing tumor to grow (Schwarz, 2005).

- **Inflammation**

UVB exposure elicits both acute inflammatory responses, as erythema and edema, and chronic photoaging as well as photocarcinogenesis (Terui et al, 2000). The first step of UV-induced inflammation is a vasodilatory phase through the production of prostaglandins and nitric oxide (NO). The second phase is the inflammatory phase in which keratinocytes release inflammatory cytokines and chemokines, like IL-1β, IL-8, TNF-α, that recruit leukocytes to the irradiation site, contributing to ROS production and oxidative stress development (Terui et al, 2000; Pihl et al, 2021). Inflammatory cytokines activate inducible Nitric Oxide Synthase (iNOS) enzyme which is responsible for NO production (Nishisgori, 2015).

- **Cell cycle arrest and senescence**

In response to severe DNA damages, cells enter in a permanent cell cycle arrest called senescence through the activation of tumor suppressor pathways. Besides cell cycle arrest, senescent cells secret several proinflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α), chemokines and matrix metalloproteinases (MMPs) known as senescence-associated secretory phenotype (SASP). In physiological condition, cell cycle arrest allows cells to repair DNA damages preventing cancer development and promotes tissue remodelling (Hernandez-Segura et al, 2018; Fitsiou et al, 2020). However, chronic exposure to damage stimuli, like UV, causes an accumulation of aberrant mutations that cannot be properly corrected leading to cellular senescence. The amassing of senescent cells within tissues affects regenerative capacities and contributes to aging and cancer development (Hernandez-Segura et al, 2018). UV-induced DNA damage, in particular double-strand DNA breaks, causes the activation of the DNA damage repair (DDR) system within cells. Persistent DDR activation triggers a series of signal transduction cascades that leads to the phosphorylation of p53 (p-p53) which activates a transcriptional network of proteins that initiate cell cycle arrest, apoptosis, and senescence (Harris et al, 2005). In particular, p-p53 activates the transcription of p21, an inhibitor of cycling dependent kinases-2 (CDK2), triggering cell cycle arrest (Hernandez-Segura et al, 2018). In addition, chronic exposure to UV triggers the activation of $p16^{(INK4a)}$/pRB pathway that contributes to senescence since p16 is an inhibitor of CDK4 and CDK6 (Herranz et al, 2018). As a consequence of cell cycle arrest, cells produce and release SASP which can have both tumor-suppressing and tumor-promoting functions (Faget et al, 2019). Indeed, SASP induces senescence to neighbour cells, contributing to control cell replication. However, pro-inflammatory cytokines released by senescent cells activate inflammation, which contributes to carcinogenesis. Moreover, MMPs can disrupt the extracellular matrix, favouring angiogenesis and metastasis (Herranz et al, 2018).

❖ Field of cancerization

The field of cancerization (FC) is defined as a tissue area exposed chronically to a carcinogenic stimulus in which cells have accumulated some but not all phenotypic alterations required for malignancies. (Curtius et al, 2017; Willenbrink et al, 2020). These phenotypical changes could be associated with increased cell growth, immune invasion, and decreased cell death; nevertheless, cells morphology may appear normal (Vanharanta et al, 2012). This concept was first described by Slaughter et al in 1953, analyzing histological atypia on the tissue surrounding oropharyngeal carcinomas (Slaughter et al, 1953). Indeed, they found that apparently normal tissue surrounding oral SCC had microscopic atypia, and that multiple secondary tumors arisen within this area. This phenomenon is due to the growth of a "mutant clone" of cells which is predisposed to evolve into neoplasm (Braakhuis et al, 2003). Nowadays, the concept of field cancerization is extended to other malignancies, such as skin, and also lung, colon, breast, bladder, and others.

▪ Cutaneous field cancerization

The risk factors for field cancerization of the skin are similar to those for NMSC development, *i.e.,* fair skin, ultraviolet exposure, immunosuppression, age, male sex (Willenbrink et al, 2020). Nevertheless, cutaneous field cancerization is mainly caused by chronic exposure to UV radiations. Immediately after UV irradiation, a precursor cell (stem cell) acquires mutations in some target genes and divides into mutant clones (Torezan et al, 2013). Thus, the photoexposed area will be composed by different clones of mutated cells, in which the most competent mutations will dominate the field of cancerization (Curtius et al, 2017). With further exposures to UV, mutant clones accumulate more mutations, grow, and can give rise to AKs, which might evolve to SCCs (Vanharanta et al, 2012). The most altered gene found on cell of field cancerization is Tp53, which is inherited from daughter cells by the first mutated stem cell (Torezan et al, 2013). It has been demonstrated that p53-mutated clones are found on normally appearing skin but chronically exposed to UVB rays, predisposing patients to cancer (Klein et al, 2009; Jonason et al, 1996). Other genes that could be found altered on the field cancerization are NOTCH1 and NOTCH2, which are implicated in cell cycle control and other fundamental pathways for cell survival (Willenbrink et al, 2020).

In field cancerization, mutations occur not only in pre-malignant cells but also in surrounding cells, generating a cancer-promoting microenvironment. Indeed, UV radiations reach the dermis and induce a stromal reprogramming due to epigenetic mutations in some key enzymes (Curtius et al, 2017). Consequently, fibroblasts acquire a CAF-like (cancer-associated fibroblast) state resulting in reduced elastin and collagen production, overexpression of metalloproteases, and increased release of growth factors. All these modifications help mutated actinic-damaged keratinocytes to growth and to transform into neoplastic cells (Vanharanta et al, 2012; Huang et al, 2019).

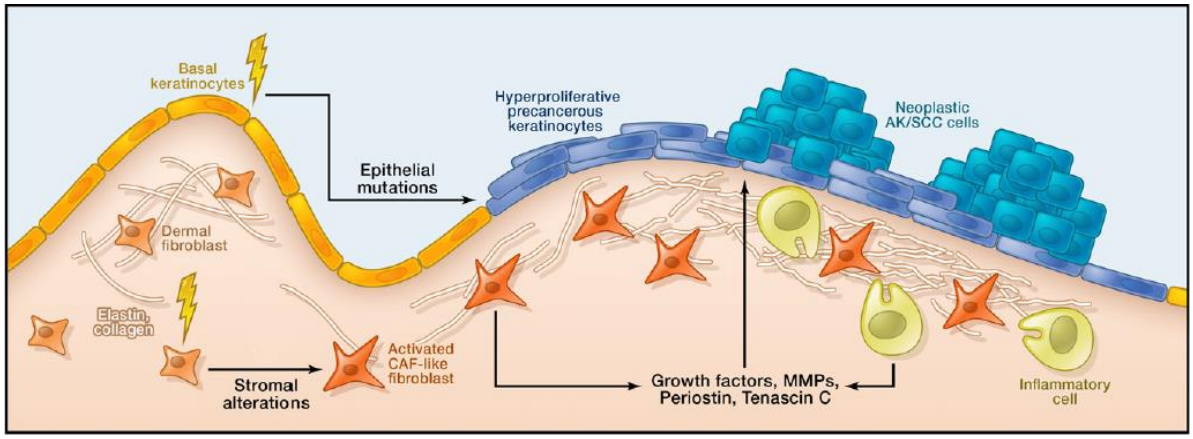


***Figure 7**: Skin field cancerization (Vanharanta et al., 2012). Exposure to UV lead to cancer-causing mutations on basal keratinocytes giving rise to AKs development. With additional mutations induced by UV, AKs can evolve to SCCs. In the dermis, chronic UV exposure induces the accumulation of mutations on fibroblasts which produce growth factors, MMPs that contribute to cancer development and field cancerization.*

- **<u>Field cancerization management</u>**

The identification and treatment of field cancerization has important therapeutic implications and clinical consequences in the treatment of precancerous lesions. Indeed, large number of mutated cells within the field cancerization increase the chance of AK development and tumor relapse (Tabor et al, 2001). AKs are one of the most diagnosed lesions in dermatology, and their incidence is expected to raise with aging of the population and the increased UV exposure (Huang et al, 2019). Moreover, AKs generally develop as multiple lesions on elderly patients, who usually carry comorbidities that can reduce treatment efficacy and feasibility. Unfortunately, there is no standard definition for cutaneous field cancerization which is still diagnosed together with AKs. However, patients with field cancerization develop malignancies that behave differently than those with multiple discrete AKs (Levine et al, 2015). Therefore, a better understanding of cutaneous

field cancerization may lead clinicians to treat patients with the correct approach (Willenbrink et al, 2020).

Patient education on the detrimental effects of UV is the ideal management of field cancerization that can minimize photodamage and stimulate the usage of sunscreen. For this purpose, WHO created in 1992 the ultraviolet index (UVI) which determines the level of solar UV radiation at Earth's surface as number from zero upwards (Chacko et al, 2021). This educational tool is an important vehicle to educate people about the risk of excessive exposure to the ultraviolet and to alert people about the need to use sun protections. Moreover, in recent years chemopreventive methods are applied to support behavioural modifications in patients with a high risk of AKs. Indeed, nicotinamide (vitamin B3) has been proposed as skin chemopreventive due to its protective effects against UV damages.

❖ Photoprotection and chemoprevention

Photoprotection against deleterious damages induced by UV could be an efficient strategy to prevent the onset of NMSCs and to treat field cancerization. Chemoprevention could be achieved by using photoprotectors which are natural or synthetic agents administered topically or systemically (Pihl et al, 2021). The most diffused topical photoprotective strategy is the use of sunscreens followed by wear protective cloths, seek shade, and avoidance of exposure to artificial sources of UV (Han et al, 2013). In particular, sunscreens can physically block or reflect UV photons by the presence of organic or inorganic filters (Bosch et al, 2015, Mancuso et al, 2017). However, sunscreens require the application of the correct amount of product and periodic replenishment, and sometimes their efficacy is limited by poor cosmetic acceptance (Bens, 2014). Therefore, especially on high-risk NMSC patients, chemopreventive molecules could be systemically administered in order to increase photoprotection. These molecules could be of natural origin or synthetic and they exert different effects including regulation of cell cycle, prevention of oxidative stress, immunomodulation, and enhancement of DNA repair. The most extensively studied agents are retinoids, obtained from vitamin A, which can efficiently chemoprevent AKs and SCCs through immunomodulation, induction of apoptosis, cell cycle modulation, and regulation of cell differentiation (Savoia et al, 2018). However, retinoids could have several side effects, limiting patient compliance (Han et al, 2014). Nowadays, several molecules have been studied through animal studies or preclinical trials in order to increase the number of chemopreventive molecules available for photoprotection.

- **Nicotinamide**

Nicotinamide (NAM), or niaciamide, is the water-soluble amide form of vitamin B3. It is assumed by daily diet since it is found in many sources like meats, liver, yeast, legumes, vegetables, cereals, coffee, and tea (Forbat et al, 2017). NAM is also a by-product of tryptophan and is metabolized by liver and excreted by kidneys (Snaidr et al, 2019). Lack of vitamin B3 leads to pellagra which is characterized by dermatitis, dementia, and diarrhoea. NAM is key cofactor for several reactions throughout the body, including its conversion into nicotinamide adenine dinucleotide (NAD) which is essential for ATP production within cells (Fania et al, 2019). NAM is also the inhibitor of the poly-ADP-ribose-polymerase (PARP)-1, an enzyme involved in DNA repair that can cause mutations whether it is overactivated and not strictly controlled (Namazi, 2007). In particular, PARP-1 enhances DNA repair through p53 activation, arresting cell cycle and allowing DNA repair enzymes to correct damaged nucleotides (Forbat et al, 2017). Whether DNA mutations are not reparable, PARP-1 induces apoptosis and cell death through NF-kB pathway, which also triggers the expression of adhesion molecules, cytokines, and inflammatory mediators (Namazi, 2007).

The use of NAM has been proposed in clinics for the treatment of several skin disorders like rosacea, acne, and also autoimmune blistering disorders due to its anti-inflammatory activity (Snaidr et al, 2019). Recently, NAM has entered in several clinical trials that evaluate its efficacy against NMSCs and its role as photoprotector (Chen et al, 2015 e 2016; Drago et al, 2016). Indeed, phase 2 (Surjana et al, 2012) and phase 3 (Chen et al, 2015) clinical trials have demonstrated that 500 mg of NAM orally administered twice a day for 4 months and one year respectively, can reduce AKs and primary NMSCs lesions in comparison with control groups, even in high-risk NMSC patients. This effect is probably due to NAM ability to hamper immunosuppression induced by UV and prevent UV-induced oxidative stress. Moreover, since NAM is the precursor of NAD, it enhances DNA repair by blocking UV-induced energy depletion and restoring ATP synthesis which is necessary for DNA repair (Snaidr et al, 2019; Forbat et al, 2017; Damian, 2017). Consistent with these hypotheses, Monfrecola et al (2013) demonstrated that HaCat cells treated with NAM and exposed to UVB showed a reduced expression of pro-inflammatory cytokines like IL-1β, IL-6, IL-8, TNF-α and an increased expression of anti-inflammatory cytokines like IL-10. Instead, Surjana et al (2013) and Thompson et al (2015) have demonstrated that NAM could prevent DNA damages induced by UV in both keratinocyte cell line (HaCaT) and *ex vivo* skin

biopsies, reducing CPDs and 8-OHdG formation (Surjana et al, 2013). Finally, Tan et al (2021) have recently demonstrated that NAM prevents UVB-induced senescence on human primary keratinocytes and restores normal cell lifespan. Taking into consideration its low cost and very few side effects at high doses, NAM could be considered a photoprotective agent able to prevent NMSC recurrence especially on high-risk NMSC and fragile patients (Snaidr et al, 2019).

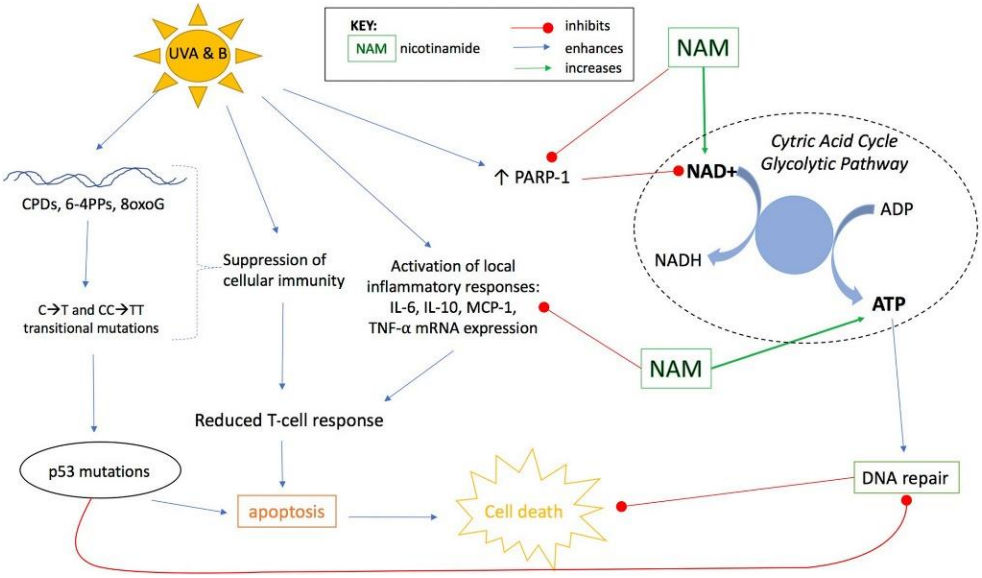


***Figure 8**: Nicotinamide and UV rays (Snaidr et al, 2019). UV rays induce activation of local inflammation, directly damage the DNA that causes an activation of PARP1. NAM can block PARP1 activity, restoring ATP levels within the cells and enhancing DNA repair. Moreover, NAM blocks pro-inflammatory cytokines expression.*

Nevertheless, all mechanisms behind NAM activities are still not completely clear. Moreover, despite the encouraging clinical results, most *in vitro* results were obtained on immortalized keratinocytes cell lines which are not as quite representative of the tissue physiology as primary cells, and studies regarding the photoprotective effect of NAM in primary keratinocytes derived from photodamaged areas are still scarce. In addition, only few papers have investigated the role of NAM as photoprotector on dermal fibroblast. Philips et al (2018) have demonstrated that NAM and its derivates were able to protect dermal fibroblast against UVA-induced senescence while Kang et al (2006) and Oblong et al (2020) have proved that NAM can delay intrinsic senescence on dermal fibroblasts. Nevertheless, nowadays there are no papers concerning the correlation between NAM and UVB-induced senescence on primary fibroblasts.

- **Vitamin E**

As the outermost organ of the body, the skin is constantly exposed to external stimuli leading to production of ROS, that can oxidase proteins, lipids, and DNA (Savoia et al, 2018). To counteract detrimental effects of ROS, the various layers of the skin are equipped with several antioxidant enzymes that prevent oxidative stress, maintaining an equilibrium between oxidants and antioxidants (Thiele et al, 2007). Vitamin E, in particular its most active form α-tocopherol, is one of the most abundant antioxidants present in the skin. Specifically, the highest α-tocopherol concentration is found on the lower stratum corneum while the lowest concentration is present in the upper layers (Thiele et al, 2007). Vitamin E is assumed by daily diet as it is found in fresh vegetables, vegetable oils, cereals, and nuts. Vitamin E exerts its antioxidant activity by scavenging free radicals and hindering oxidative stress development (Herrera et al, 2001). In our recent paper (Camillo et al, 2021), we demonstrated that fibroblasts treated with α-tocopherol in an oxidative stress condition, showed reduced ROS and NO release, normal cell cycle and lifespan, and decreased proinflammatory cytokines expression. While some antioxidants are synthetized by humans, vitamin E cutaneous levels depend on oral intake or topical administration. It has been demonstrated that UVB rays can deplete α-tocopherol concentration in the human stratum corneum by 50%, probably due to the direct absorption of UVB by α-tocopherol or by UV-induced ROS production, generating oxidative stress (Thiele et al, 2007).

The role of vitamin E as a photoprotector has been investigated in several papers, however with conflicting results. Indeed, animal models studies demonstrated that pretreatment with vitamin E before UV exposure reduces erythema and sunburn, lipid peroxidation, senescence, and oxidative stress (Thiele et al, 2007). Consistently, the same results were obtained also on cultured mouse primary keratinocytes (Maalouf et al, 2002). On the other hand, Du et al (2003) have demonstrated that human HaCaT cells treated with α-tocopherol and exposed to UVB showed normal cell viability and restored cell cycle. Jin et al (2007) have assured that human SCC cells (Colo-16) in presence of several vitamins, including vitamin E, have lower levels of ROS and apoptosis after UVB irradiation. Nevertheless, no paper has demonstrated yet a significant photoprotection against NMSC in human studies (Pihl et al, 2021). Moreover, there is a lack of knowledge about the role of vitamin E against UV-induced damage on human fibroblast.

- **Vitamin D3**

Epidermis is the primary source of vitamin D3 (cholecalciferol) which is a fat-soluble steroid hormone synthetized by a cholesterol precursor through a reaction catalysed by sunlight (Bikle, 2011). Vitamin D3 synthesis is influenced by skin type, geographical location, and personal habits. Indeed, light skin people produce more vitamin D3 in comparison with dark skin people (Kechichian et al, 2018). Vitamin D3 is also found in some animal-based food like fatty fish, milk and in some fruits and cereals (Shahriari et al, 2010). Vitamin D3 exerts different effects within cells like control of cellular proliferation, differentiation, and immunoregulation. The molecular mechanism responsible for these effects involves the binding of vitamin D to its receptor (VDR) creating a complex that goes into the nucleus, and binds to specific DNA sequences, regulating gene expression of target genes (Shahriari et al, 2010). Furthermore, Reichrath et al (2014) hypothesized that VDR interacts with p53 contributing to cell cycle arrest and allowing cells to repair DNA damages induced by UV.

Thanks to these properties, vitamin D3 is used for the treatment of several skin disorders like psoriasis, actinic keratosis, and vitiligo (Kechichian et al, 2018). Moreover, its possible role as a photoprotector has been investigated in both animal and human studies. In particular, a recent clinical trial Scott et al (2017) have demonstrated that patients treated with high doses of vitamin D3 showed reduced erythema and pro-inflammatory mediators in comparison with the placebo group and lower doses treated patients, enhancing photoprotection. However, all mechanisms activated by vitamin D3 are still not completely clear, and only few studies have been performed with human primary cells.

AIM OF THE THESIS

Nowadays, the gold standard for the treatment of NMSCs is still surgery. However, since most skin cancers arise in chronically photo-exposed sites, such as the face, and patients are predominantly elderly and affected by many comorbidities, the chance of cure is sometimes limited. A possible solution it is represented by the possibility of preventing the onset of cancer through the restoration of field-cancerization with non-invasive treatment. The search for cheap and low-side effect molecules is intriguing. Therefore, the aim of this thesis is to evaluate whether vitamins B3, E and D3 in their active forms, could be an efficient support to prevent NMSC development and relapse. In consideration of the involvement of both keratinocytes and fibroblasts in skin senescence phenomena predisposing to NMSC this elaborate is divided into two main projects:

- **Project 1**: study of the possible role of NAM as a photoprotector on human primary keratinocytes isolated from field cancerization (FC-HPKs) and normal human epidermal keratinocytes (NHEKs) evaluating oxidative stress, DNA damage, inflammation, and NO release on UVB-irradiated cells. This project has been recently published in the Journal of Investigative Dermatology (ISSN: 0022-202X; IF 8.55) titled "*Nicotinamide Attenuates UV-Induced Stress Damage in Human Primary Keratinocytes from Cancerization Fields"* (Camillo et al, 2021; doi: 10.1016/j.jid.2021.10.012).
- **Project 2**: comparison of the photoprotective role of nicotinamide, calcipotriol, a vitamin D3 analogue, and α-tocopherol on human dermal fibroblasts (HDFs) irradiated with UVB evaluating oxidative stress, cell cycle and lifespan, senescence, and DNA repair.

PROJECT 1

PROJECT 1: Nicotinamide Attenuates UV-Induced Stress Damage in Human Primary Keratinocytes from Cancerization Fields.

1.1 Materials and methods

1.1.1 Patients

We enrolled 30 patients (22 males, 8 females) who presented to our Department (Dermatology Unit of A.O.U Maggiore della Carità di Novara, Italy) with clinical signs and personal history of intrinsic skin aging and dermatoheliosis, affected by precancerous skin lesions, dysplastic nevi, non-melanoma skin cancers (NMSCs) and/or cutaneous melanoma (CM). Informed consent was obtained from each donor.

All skin lesions presented clinical and dermoscopic criteria for surgical removal (Kirby et al, 2015; Nishisgori, 2015; Stockfleth, 2017; Willenbrink et al, 2020). During the surgical procedure, skin biopsies including epidermis and dermis were performed, at a distance not exceeding 2 cm from the resection margins. All the cutaneous lesions removed were histologically analyzed, confirming histological radicality and histotype.

1.1.2 Human primary keratinocytes isolation

FC-HPKs were isolated from the perilesional skin biopsies that were washed in ethanol 70% and physiological solution three times, cut in small pieces, and incubated at 4°C overnight in Dispase II 2 mg/ml (Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 1% Penicillin/Streptomycin (P/S). Then epidermis and dermis were separated with sterile tweezers. The epidermis was incubated in 0.25% Trypsin/0.02% EDTA solution (Euroclone, Pero, Milano, Italy) at 37°C, 5% CO_2 for 20 minutes. Through mechanical digestion, FC-HPKs were isolated, filtered using a 100 µm cell strainer, and centrifuged at 1000 rpm for 10 minutes at 4°C. Pellet was resuspended in Keratinocyte Basal Medium (KBM-2; Lonza, Basel, Swiss) and incubated for 3 days at 37°C, 5% CO_2. The medium was changed every two days with Epilife (Gibco-Thermo Fisher, Waltham, Missouri, USA) supplemented with Human Keratinocytes Growth Supplements (HKGS, Gibco), 1% P/S, and 1% Gentamycin/Amphotericin (Gibco).

Adult pooled NHEK cells (C-12006; PromoCell, Heidelberg, Germany) were cultured in KBM-2 with Supplements kit (PromoCell) as recommended by the manufacturer.

1.1.3 Cell treatment

Nicotinamide (Sigma-Aldrich) was prepared with double distilled water at a final concentration of 5, 25, and 50 µM and added in cell cultures for 18, 24, and 48 hours. Cells were then washed twice in phosphate-buffered saline (PBS) and irradiate in PBS with 400 mJ/cm^2 using a UVB (280-320 nm) lamp VL6M (Montepaone, Torino, Italy) with a peak at 312 nm; the UV spectrum was measured with a quantum photo/radiometer (HD9021, Montepaone).

1.1.4 Cell viability

Cell viability was analyzed using MTT assay as previously described (Camillo et al., 2018). NHEK and FC-HPK cells (1x10^4 cells/well) were plated on coated 96-wells microplate and treated with NAM and UVB. PBS was removed and was added a 0.2 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) solution. Then, cells were incubated for 3 hours at 37°C. Formazan crystals were solubilized with dimethyl sulfoxide (DMSO, Sigma-Aldrich) and absorbance was measured at 570 nm using Victor X Multilabel Plate Readers (PerkinElmer, Milano, Italy).

1.1.5 Intracellular ROS quantification

Intracellular ROS quantification was performed using the DCFDA-Cellular ROS assay kit (Abcam, Cambridge, UK) following the manufacturer's instructions. NHEKs and FC-HPKs (2.5x10^4 cells/well) were seeded into a coated 96-wells microplate and treated as previously described. After irradiation, cells were washed twice with PBS and incubated for 45 minutes at 37°C with 10 µM DCFDA solution. The probe was replaced with fresh PBS and the fluorescence was read with Victor X Multilabel Plate Readers (PerkinElmer) at 495/529 nm excitation/emission.

1.1.6 Single cell gel electrophoresis (Comet assay)

Comet assay was performed following Fang protocol (2015). NHEKs and FC-HPKs (2x10^5) were seeded into a coated 6-wells microplate and treated as previously described. After irradiation, cells were harvested and resuspended in 1% low melting agarose (Fisher Molecular Biology, Trevose, PA, USA) and solidified on precoated microscope slides. Then, slides were incubated in alkaline lysis buffer (pH >13) overnight at 4°C. After electrophoresis (12 V, 40 mA, 25 minutes) in the appropriate buffer, slides were stained with 10 µg/ml of Propidium Iodide for 20 minutes in the dark. Pictures of cells were taken

using fluorescence microscope Leica DS5500B (Leica, Wetzlar, Germany) and quantification of the tail DNA % was performed using the automated CometScore 2.0 software (TrikTek).

1.1.7 Quantitative Real-time RT-PCR

NHEKs and FC-HPKs ($4x10^5$ cells/well) were plated on a coated 6-wells microplate and treated as previously described. Cells were detached mechanically and resuspended in 500 µl of Trizol for total RNA isolation. RNA was quantified at the spectrophotometer (Nanodrop, Thermo Fisher) by measuring the optical density at 260 and 280 nm. Reverse transcriptase and cDNA synthesis were performed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, CA) according to the manufacturer's instructions. A two-step cycling quantitative real-time RT-PCR was performed in a volume of 10 µl per well in a Multiply Optical Strip (Sarstedt, Germany) containing SensiFast SYBR No-ROX kit (Bioline, London, UK), forward and reverse primer 400 nM, and 1 µl of cDNA template. Primers used are indicated in the Table 1 below. GAPDH was used for data normalization and relative quantification was determined by the 2ΔCT method. Data are expressed as fold change versus CTRL cells.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
GAPDH	AACGTGTCAGTGGTGGACCTG	AGTGGGTGTCGCTGTTGAAGT
iNOS	GTTCTCAAGGCACAGGTCTC	GCAGGTCACTTATGTCACTTATC
OGG1	GCGACAAGACCCCATCGAAT	CCGGAAAAAGTTTCCAGCCAG
SOD-1	GTGGGCCAAAGGATGAAGAGA	ATAGACACATCGGCCACACC
GPX-1	CCAGTTTGGGCATCAGGAGAA	CGAAGAGCATGAAGTTGGGCT
IL-1β	ACAGATGAAGTGCTCCTTCCA	GTCGGAGATTCGTAGCTGGAT
IL-8	ATGACTTCCAAGCTGGCCGT	TCCTTGGCAAAACTGCACCT
TNF-α	CATGATCCGGGACGTGGAGC	CTGATTAGAGAGAGGTCCCTG

***Table 1**. List of primers used for qRT-PCR*

1.1.8 Intracellular nitrate concentration

Intracellular nitrate quantification was performed using the Measure-IT High Sensitivity Nitrite Assay Kit (Thermo Fisher) following the manufacturer's instruction. NHEKs and FC-HPKs ($4x10^4$ cells/well) were plated into a coated 24-wells microplate. Cells were treated as previously described. After irradiation, cells were washed and incubated for 20 minutes at RT with 200 µl of double distilled water for lysis. Meanwhile, a 96-wells microplate was prepared with 100 µl of working solution where 10 µl of cell supernatant

was resuspended and incubated at RT for 10 minutes. 5 µl of quantification developer were added to each well and the plate was read at 365/450 nm excitation/emission using Victor X Multilabel Plate Readers (PerkinElmer).

1.1.9 Indirect immunofluorescence

NHEKs and FC-HPK cells (2.5 x10^5) were seeded on rounded sterile glass and treated as previously described. Cells were washed with PBS and fixed with 4% paraformaldehyde at 4°C for 10 minutes. Then, cells were incubated with blocking buffer (PBS + 0.1% Triton+ 5% BSA) for 1 h at RT. Primary antibodies mouse anti-OGG1 (1:200, Novusbio) and rabbit anti-iNOS (1:250, Thermo Fisher), diluted in PBS + 0.1% Triton + 2% BSA, were added to samples for 2h at RT. Cells were incubated with secondary antibodies anti-rabbit (Alexa Fluor-488, Thermo Fisher) and anti-mouse (Alexa Fluor-546 added with DAPI 1:1000, Sigma-Aldrich) prepared in PBS + 0.1% Triton + 2% BSA for 45 minutes at RT. Finally, cells were mounted with glycerol on a microscope slide. Pictures were taken with fluorescent microscope Leica DS5500B (Leica) and quantification was performed using ImageJ Software and indicated as corrected total cell fluorescence (CTFC) calculated with the formula: integrated density- (area of the selected cell * mean fluorescence of backgrounds).

1.1.10 Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 (California, USA). Data were expressed as mean ± SEM of *n* independent experiments. Statistical significance was assessed by one-way ANOVA multiple comparisons with Dunnett's test or Student's *t*-test for different groups of treatment comparisons. Statistical significance was defined as $p < 0.05$.

1.2 RESULTS

1.2.1 Effects of NAM and UVB on cell viability

To evaluate the possible cytotoxic effects of NAM and UVB exposure, cell viability was assessed by MTT assay on both NHEK and FC-HPK cells with and without UVB. No significant differences were found among all treatment conditions, indicating that stimulations did not affect cell viability (Figure 1a, b, c, d).

1.2.2 Effects of NAM on ROS release and antioxidant expression

As a result of UV exposure, ROS level increases within cells (Jin et al, 2007). Hence, we analyzed whether NAM could counteract ROS production comparing NHEKs and FC-HPKs before and after UVB irradiation. As shown in Figure 2a, ROS basal level in FC-HPKs was noticeably higher in comparison with NHEK cells, but NAM treatment did not affect ROS levels in both FC-HPKs and NHEKs. If subjected to UVB irradiation, NHEKs and FC-HPKs behaved in a different manner (Figure 2b). Interestingly, NHEK did not show any variation in ROS production even on only UVB-treated cells. On the other hand, FC-HPKs presented significant higher levels of ROS in comparison with untreated cells, and lower levels in 24-48h NAM pretreated cells.

To understand whether UV exposure could affect antioxidant expression, we measured gene expression of glutathione peroxidase-1 (GPX-1) and superoxide dismutase-1 (SOD-1) in both NHEKs and FC-HPKs. In accordance with ROS results, NHEK cells did not show significant modulation in GPX-1 (Figure 2c) and SOD-1 (Figure 2e) gene expression. Conversely, FC-HPKs presented a significant increase in gene expression of these antioxidants after UV exposure. NAM pretreatment scarcely reduced GPX-1 (Figure 2d) and SOD-1 (Figure 2f) mRNA expression.

1.2.3 Effects of NAM on OGG1 expression in irradiated keratinocytes

UV radiations and ROS can directly damage the DNA, through the formation of 8-OHdG bases. Thus, we investigated OGG1 expression on irradiated keratinocytes. First, we analyzed and quantified protein expression through indirect immunofluorescence in NHEKs (Figure 3a, c) and FC-HPKs (Figure 3b, d). OGG1 expression significantly raised in the presence of UVB radiation on both cells. As expected, NAM-treated cells presented a considerably lower level of OGG1, with a significant reduction in FC-HPKs treated with NAM

for 24 and 48h before UVB irradiation. No differences were found based on concentrations and treatment time.

Then, we evaluated OGG1 gene expression through qRT-PCR. As shown in Figure 3e, OGG1 mRNA levels were higher in NHEK cells after UVB irradiation, while NAM seemed to restore gene expression almost to untreated cells mRNA level. However, no significant differences were found. On the other hand, OGG1 gene expression on FC-HPKs (Figure 3f) was well-modulated by NAM, especially on 24 and 48h treated cells.

1.2.4 NAM enhances DNA repair on irradiated keratinocytes

To understand whether the modulation of OGG1 expression was correlated with the presence of DNA damage within the cells, we performed comet assay on NHEKs (Figure 4a) and FC-HPKs (Figure 4c) cells treated with NAM and exposed to UVB. Tail DNA % was used as a parameter to indicate DNA damage. Figure 4b shows that NHEKs treated with NAM before UVB irradiation presented a lower level of tail-DNA, in which NAM 5 µM given for 24h and NAM 25 µM given for 48h showed better results. Likewise, FC-HPKs (Figure 4d) showed decreased DNA damages in presence of NAM, with better results after 18 and 24h of treatment. On both cell types, NAM 5µM given for 48h was not able to protect cells against UV damages.

1.2.5 Effects of NAM on pro-inflammatory cytokines expression

UVB exposure induces the production of some pro-inflammatory cytokines, leading to inflammation (Snaidr et al., 2019). Here we analyzed IL-1β, IL-8, and TNF-α mRNA expression on both NHEK and FC-HPK cells treated with NAM and irradiated with UVB. As shown in Figure 5, proinflammatory cytokine gene expression was boosted by UVB irradiation in both types of keratinocytes, with significant differences on IL-1β (Figure 5b) and TNF-α (Figure 5f) on FC-HPKs. In general, NAM seemed to modulate more efficiently gene expression on FC-HPKs rather than NHEK cells (Figure 5a, c, d). Indeed, IL-1β gene expression on FC-HPKs (Figure 5b) was significantly lower on 24 and 48 h pretreated cells, while on NHEKs (Figure 5a) NAM action seemed similar but without significant differences. Furthermore, NAM was ineffective in the modulation of TNF-α gene expression on NHEKs (Figure 5e), while on FC-HPKs induced a consistent reduction in gene expression with the treatment given for 18h (Figure 5f). Still, NAM did not show any modulation on IL-8 gene expression on both NHEKs (Figure 5c) and FC-HPKs (Figure 5d).

1.2.6 Nicotinamide modulates iNOS expression and inhibits NO release

Because of UV-induced inflammation, iNOS is activated, leading to NO release (Nishisgori, 2015). For this reason, we first evaluated iNOS gene and protein expression on NHEKs and FC-HPKs treated with NAM and exposed to UVB radiation. Lastly, we assessed the intracellular NO production measuring indirectly the nitrate (NO_2) concentration. Protein expression on NHEKs (Figure 6a) and FC-HPKs (Figure 6c) cells was evaluated through indirect immunofluorescence. In general, iNOS expression was considerably higher in presence of UVB radiation, while NAM efficiently reduced iNOS expression on both cell types, as shown in Figures 6b and c. Comparing the CTCF quantification, it seems that iNOS expression was higher on FC-HPKs than in NHEKs. These results were confirmed also by gene expression quantification, performed with qRT-PCR (Figure 6e, f).

Consistently with iNOS expression, we found a higher level of NO on FC-HPKs (Figure 6h) than in NHEKs (Figure 6g). Moreover, NAM given for 24 and 48h before UVB irradiation significantly reduced NO production, which is correlated to a decreased iNOS expression.

Figure 1

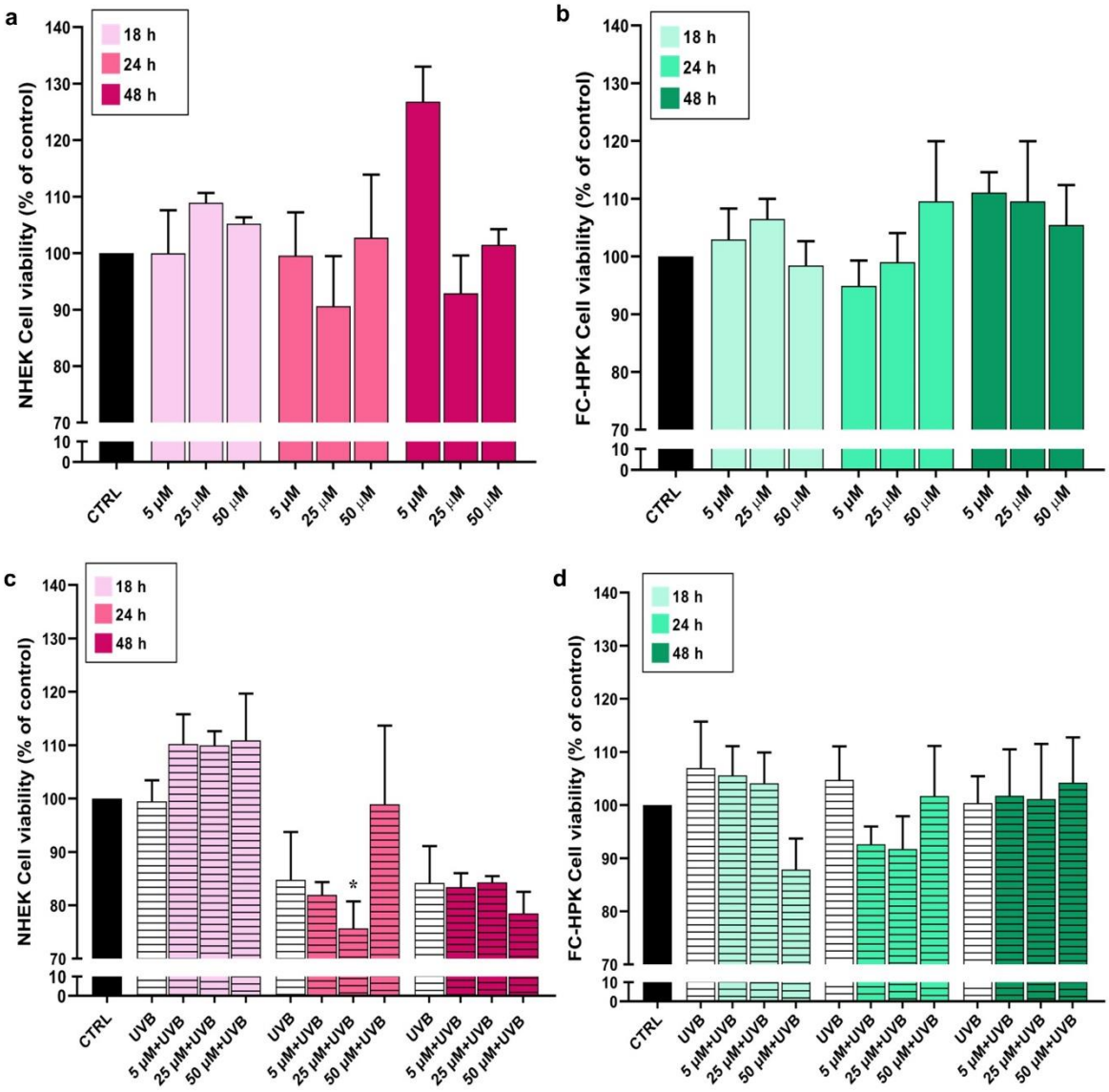


Figure 1. NHEK and FC-HPK cell viability. Cell viability was evaluated on NHEKs (**a-c**) and FC-HPKs (**b-d**) treated with NAM (5, 25 and 50 µM) for 18, 24 and 48 h without/with UVB exposure (400 mJ/cm^2). Data are expressed as means ± SEM of five independent experiments. *p <0.05, vs CTRL. CTRL, untreated cells.

Figure 2

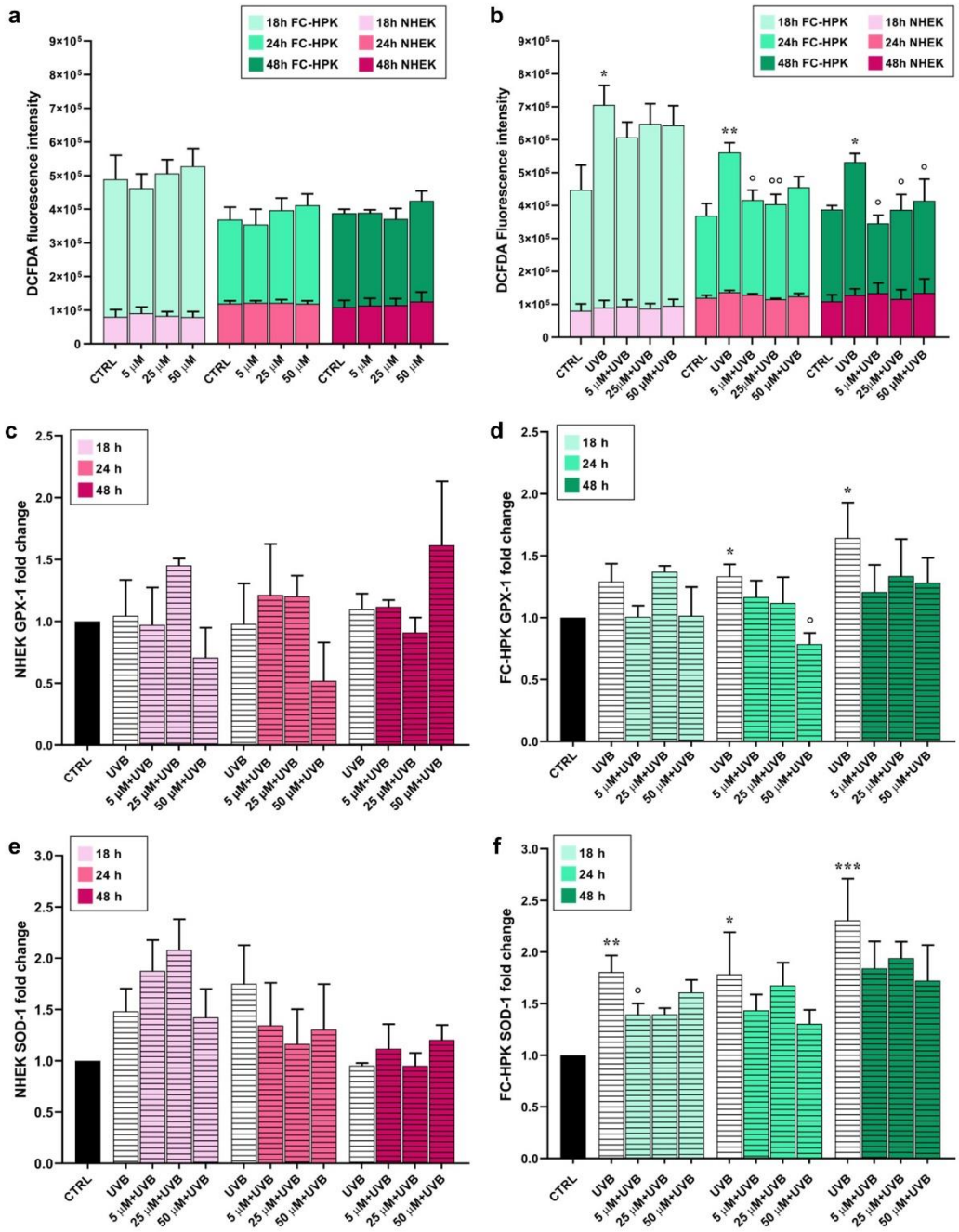


Figure 2. Effects of NAM on ROS production and antioxidant expression in UVB-irradiated NHEK and FC-HPK. NHEKs and FC-HPKs were treated with NAM (5, 25, and 50 µM) for 18, 24, and 48h before UVB irradiation (400 mJ/cm^2). (**a**) intracellular ROS production in NHEKs and FC-HPKs at basal level. (**b**) intracellular ROS level in NHEKs and FC-HPKs after UVB irradiation. GPX-1 mRNA expression on NHEKs (**c**) and FC-HPKs (**d**). SOD-1 mRNA expression on NHEKs (e) and FC-HPKs (f). Data are expressed as means ± SEM of four independent experiments. *p <0.05, **p <0.01, ***p <0.001 vs CTRL; °p <0.05, °°p <0.01 vs UVB. SOD-1, superoxide dismutase 1; GPX-1, glutathione peroxidase 1. CTRL, untreated cells.

Figure 3

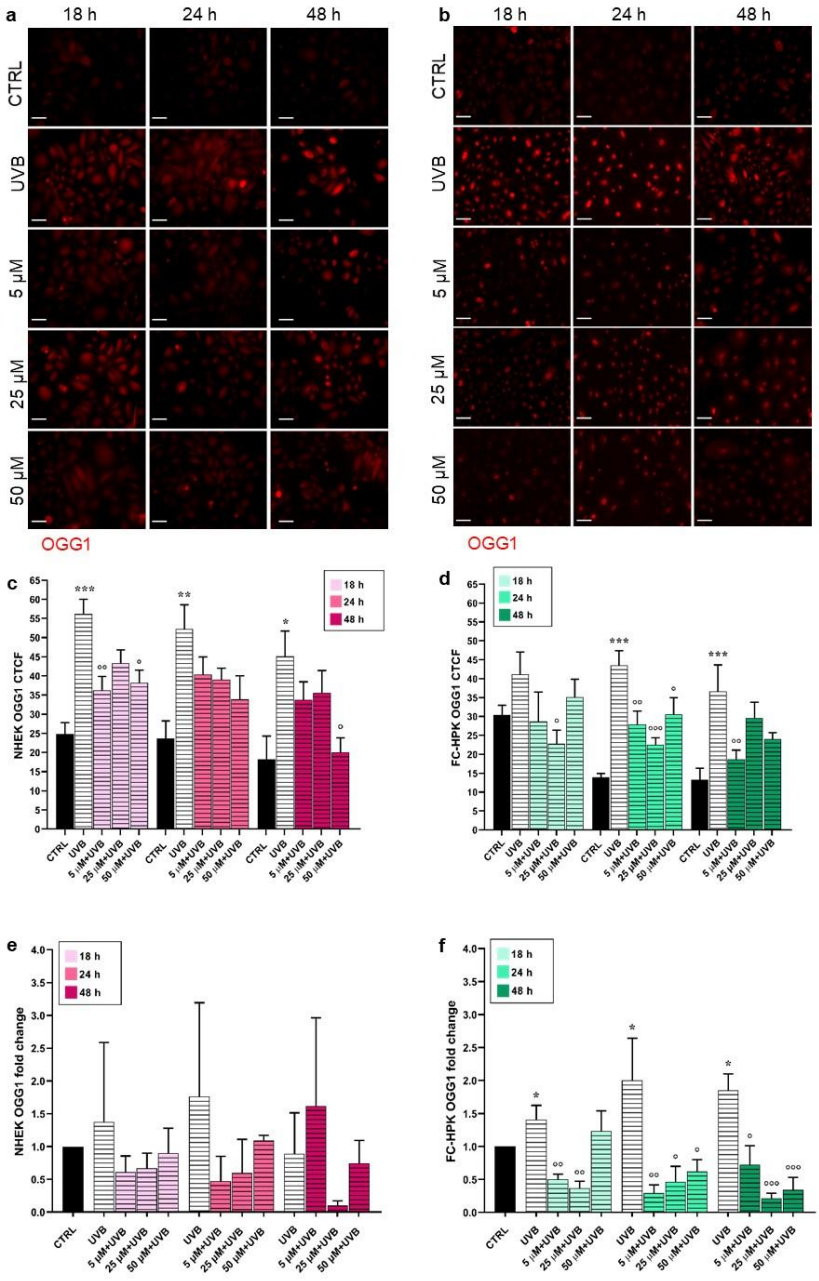


Figure 3. **Effects of NAM on OGG1 protein and gene expression on UVB-irradiated keratinocytes.** OGG1 expression was evaluated on NHEKs and FC-HPKs treated with NAM (5, 25, and 50 µM) for 18, 24, and 48 h before UVB irradiation (400 mJ/cm^2). (**a-b**) Representative IF staining of OGG1 on NHEKs and FC-HPKs respectively of three independent experiments. (**c-d**) NHEKs and FC-HPKs OGG1 IF quantification through CTCF. Data are expressed as means ± SEM of twenty different cells measured of three independent experiments (**e-f**) OGG1 gene expression through qRT-PCR on NHEKs and FC-HPKs respectively. Data represent means ± SEM of four independent experiments. *p <0.05, **p <0.01, ***p <0.001 vs CTRL; °p <0.05, °°p <0.01, °°°p <0.001 vs UVB. CTCF, corrected total cell fluorescence. CTRL, untreated cells.

Figure 4

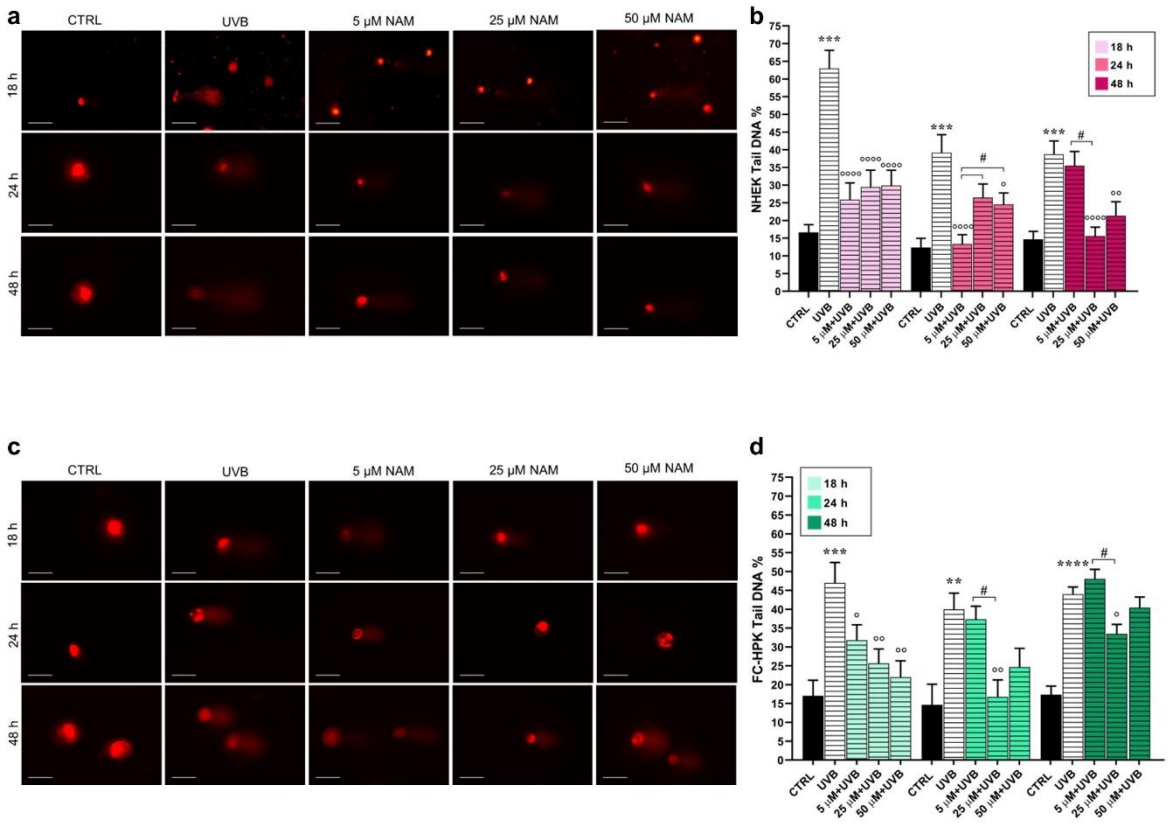


Figure 4. **NAM enhances DNA damages repair on irradiated keratinocytes**. Comet assay was performed on NHEKs (**a**) and FC-HPKs (**c**) treated with NAM (5, 25 and 50 μM) for 18, 24 and 48h before UVB irradiation (400 mJ/cm^2). Data are representative of three independent experiments. Tail DNA % quantification on NHEKs (**b**) and FC-HPKs (**d**) was used as parameter of DNA damage. Scale bar 50 μm Data are expressed as means ± SEM of three independent experiment. **p <0.01, ***p <0.001 vs CTRL; °p <0.05, °°°p <0.001, °°°°p <0.0001 vs UVB. Square brackets indicate significance between groups (#p <0.05). CTRL, untreated cells.

Figure 5

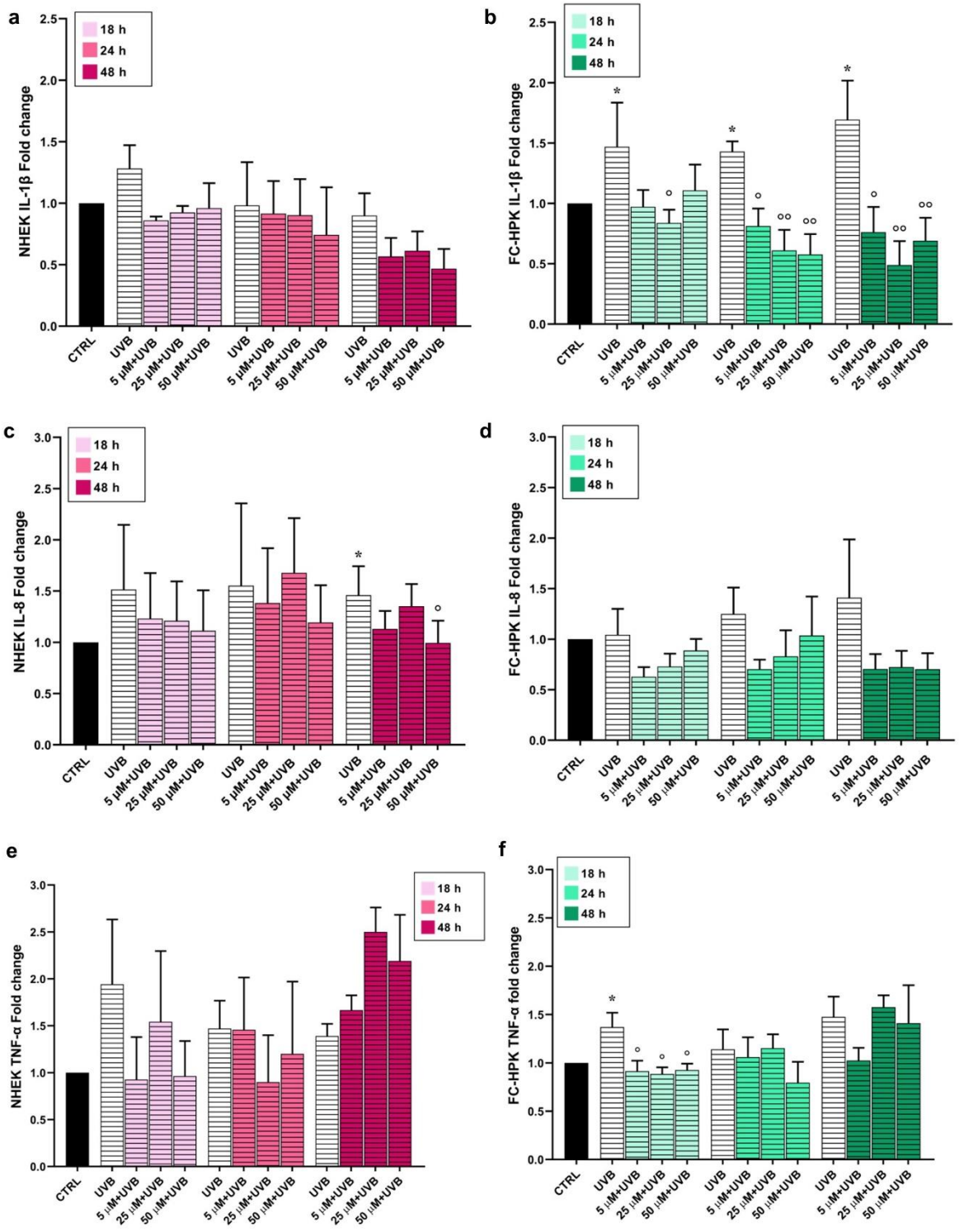


Figure 5. **Effects of NAM on pro-inflammatory cytokines expression in UVB-irradiated keratinocytes**. NHEKs and FC-HPKs treated with NAM (5, 25 and 50 µM) for 18, 24, and 48h before UVB irradiation (400 mJ/cm^2). mRNA expression of (**a**) IL-1β, (**c**) IL-8 and (**e**) TNF-α on NHEKs and (**b**, **d**, **f**) FC-HPKs. Data are expressed as means ± SEM of four independent experiments. *p <0.05 vs CTRL; °p <0.05, °°p <0.01 vs UVB. CTRL, untreated cells.

Figure 6

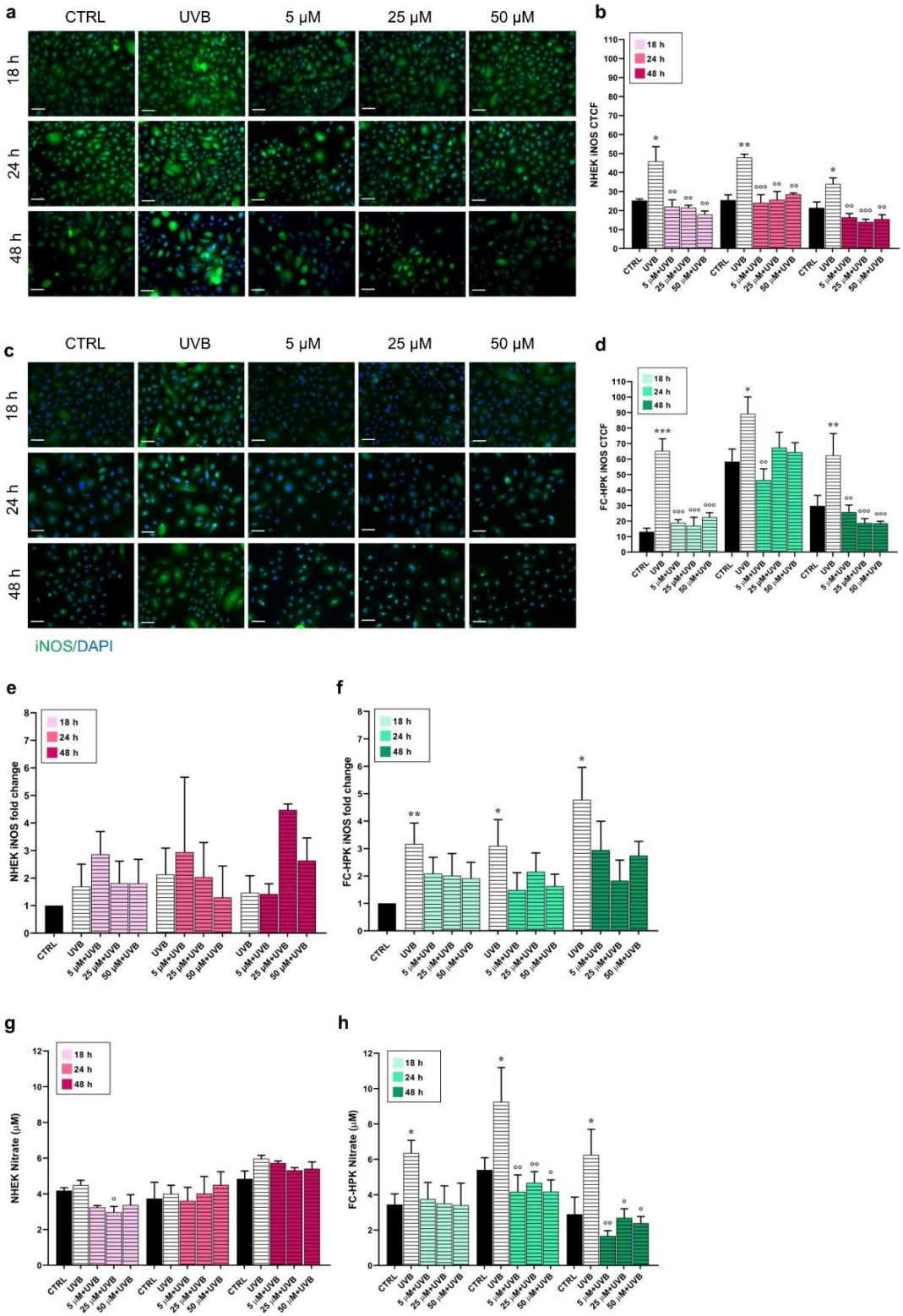


Figure 6. Effects of NAM on iNOS expression and on NO release in UVB-irradiated keratinocytes. NHEKs and FC-HPKs were treated with NAM (5, 25, and 50 µM) for 18, 24, and 48 h before UVB irradiation (400 mJ/cm^2). (**a-c**) Representative IF of iNOS and DAPI stained NHEKs and FC-HPKs of three independent experiments. (**b-d**) Immunofluorescence CTCF quantification on

NHEKs and FC-HPKs. Data are expressed as means ± SEM of twenty different cells measured from three independent experiments. Scale bar 50 µm (**e-f**) qRT-PCR of iNOS performed on NHEKs and FC-HPKs. Data are expressed as means ± SEM of four independent experiments. (**g-h**) Intracellular nitrate concentration of NHEKs and FC-HPKs. Data are expressed as means ± SEM of seven independent experiments. *p <0.05, **p <0.01, ***p <0.001 vs CTRL; °p <0.05, °°p <0.01, °°°p <0.001 vs UVB. CTCF, corrected total cell fluorescence iNOS, induce nitric oxide synthase. CTRL, untreated cells.

PROJECT 2

PROJECT 2: The role of vitamin B3, D3 and E against photoaging on UVB-irradiated human dermal fibroblasts.

2.1 Materials and Methods

2.1.1 Primary human dermal fibroblast isolation and treatment

Primary human dermal fibroblasts (HDFs) were isolated from a healthy donor, after obtaining informed consent. Healthy skin biopsy was washed in ethanol 70% and physiological solution three times, cut in small pieces and incubated at 4°C overnight in Dispase II 2 mg/ml (Sigma-Aldrich) supplemented with 1% Penicillin/Streptomycin (P/S). Then epidermis and dermis were separated with sterile tweezers. The dermis was cut in 2-3 mm^2 pieces, put on 6 well plate, covered with square sterile coverslip and incubated with 1ml of DMEM 20% FBS. Fresh culture media DMEM 10% FBS was replaced every two days, and after 3 weeks fibroblasts were detached with 0.25% Trypsin/0.02% EDTA solution (Euroclone) and subcultured until passage 17.

2.1.2 HDFs treatment

HDFs were treated with 25 µM nicotinamide (NAM) (Merck KGaA, Darmstadt, Germany), 100 nM calcipotriol (CAL) (Merck) and 1 µM α-tocopherol (α-T) (Merck) for 24h. Then, cells were washed and irradiated in PBS with 40 mJ/cm^2 UVB (280-320 nm) lamp VL6M (Montepaone, Torino, Italy) with a peak at 312 nm; the UV spectrum was measured with a quantum photo/radiometer (HD9021, Montepaone). PBS was removed and vitamins-supplemented cell media was added for 24 h.

2.1.3 MTT assay

HDFs ($7x10^4$ cells/well) were seeded into a 96-wells multiplate and treated as described above. After treatment, 0.2 mg/ml of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Merck) were added and formazan crystals were dissolved with dimethyl sulfoxide (Merck). Absorbance was read at 570 nm using Victor X Multilabel Plate Readers (PerkinElmer).

2.1.4 Cell cycle analysis

Cell cycle was evaluated through flow cytometry. HDFs ($4x10^5$ cells/well) were seeded into a 6-wells multiplate and treated as described above. Then, cell medium was collected, and cells were detached with 0.25% Trypsin/0.02% EDTA solution (Euroclone),

centrifuged, and fixed with cold 70% ethanol for 1h at -20°C. Subsequently, cells were washed twice with PBS and resuspended in 200 µl PBS with 25 µg/mL RNAse A (Immunological sciences, Rome, Italy) and 100 µg/ml propidium iodide (Immunological sciences) and incubated for 15 min at 37°C protected from light. Then, samples were transferred into tubes for flow cytometry and analyzed with Attune NxT (Life Technologies, Monza, Italy).

2.1.5 Single cell gel electrophoresis

DNA fragmentation was evaluated through Comet assay as described by Fang et al. (2015). HDFs ($2x10^5$ cells/well) were plated into a 6-wells multiplate and treated as previously described. Cells were detached using trypsin/EDTA solution, resuspended in 1% low melting agarose (Fisher Molecular Biology) and solidified on precoated microscope slides. Then, samples were incubated in lysis buffer (2,5 M NaCl, 100 mM EDTA, 10 mM Trizma Base, pH >13) overnight at 4°C. After electrophoresis (12 V, 40 mA, 25 minutes) in the appropriate buffer, slides were stained with 10 µg/ml of propidium iodide for 20 minutes in the dark. Pictures of cells were taken using a fluorescence microscope (Leica DS5500B) and quantification of the tail DNA % was performed using the automated CometScore 2.0 software (TrikTek).

2.1.6 Intracellular ROS quantification

Intracellular ROS quantification was performed using the DCFDA-Cellular ROS assay kit (Abcam, Cambridge, UK) following the manufacturer's instructions. HDFs ($2.5x10^4$ cells/well) were seeded into a 96-wells microplate and treated as previously described. Then, cells were washed twice with PBS and incubated for 45 minutes at 37°C, 5% CO_2 with 10 µM DCFDA solution. The probe was replaced with fresh PBS and the fluorescence was read with Victor X Multilabel Plate Readers (PerkinElmer) at 495/529 nm excitation/emission.

2.1.7 Quantitative real-time PCR

HDFs ($4x10^5$ cells/well) were plated in a 6-wells microplate and treated as described above. Cells were detached mechanically and resuspended in 500 µl of Trizol for the total RNA isolation. The amount and purity of RNA were quantified at the spectrophotometer (Nanodrop, Thermo-Fisher) by measuring the optical density at 260 and 280 nm. Reverse transcriptase and cDNA synthesis were performed using a high-capacity cDNA reverse

transcription kit (Applied Biosystems, Foster City, California, USA) according to the manufacturer's instructions. A two-step cycling quantitative real-time PCR was performed in a volume of 10 µl per well in a Multiply Optical Strip (Sarstedt, Nümbrecht, Germany) containing SensiFast SYBR No-ROX kit (Bioline, London, UK), forward and reverse primer 400 nM, and 1 µl of cDNA template. Primers used are indicated in the table above. GAPDH was used for data normalization and the relative quantification was determined by the 2ΔCT method. Data are expressed as fold change versus CTRL cells.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
GAPDH	AACGTGTCAGTGGTGGACCTG	AGTGGGTGTCGCTGTTGAAGT
p53	ACCTATGGAACTACTTCCTGAAA	GAGCTTCATCTGGACCTGGG
OGG1	GCGACAAGACCCCATCGAAT	CCGGAAAAAGTTTCCAGCCAG
SOD-1	GTGGGCCAAAGGATGAAGAGA	ATAGACACATCGGCCACACC
p16	CATAGATGCCGCGGAAGGT	AAGTTTCCCGAGGTTTCTCAGA
p21	TGGAGACTCTCAGGGTCGAAA	GGCGTTTGGAGTGGTAGAAATC
MMP-1	TGTCAGGGGAGATCATCG	TTCATCAAAATGAGCATCCC

***Table 2**. List of primers used for qRT-PCR*

2.1.8 Senescence-associated β-galactosidase staining

HDFs ($5x10^4$ cells/well) were seeded into a 6-wells multiplate and treated as previously described. Senescence-associated β-galactosidase (SA-βGal) staining was performed using the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, Danvers, MA) following manufacturer's instructions. Pictures were taken using optical phase contrast microscope Leica DMi1 (Leica).

2.1.9 Western blotting

HDFs ($2x10^5$ cells/well) were seeded into a 6-wells multiplate and treated as previously described. Cells were homogenized in cold RIPA lysis buffer (Merck) supplemented with protease inhibitor cocktail (Life Technologies) and phosphatase inhibitors. Proteins were quantified by BCA assay (Life Technologies) and denaturated in 2x Loading buffer (Merck) at 95°C for 5 min. 40 µg of proteins were loaded on SDS-PAGE gel at 10% acrylamide. Proteins were transferred to PVDF membrane and incubated with primary antibodies rabbit-anti-OGG1 (1:1000 Thermo Fisher), rabbit-anti-phospho-p53 (1:500, Merck KGaA) and rabbit-anti-p53 (1:1000, Merck KGaA) o.n. at 4°C. Specific secondary antibodies HRP-conjugated were added for 1 hr at RT. β-actin was used as

homogenate protein loading control. Membranes were developed using enhanced chemiluminescence method (ECL, Biorad) and acquired with ChemiDoc Imaging System (Biorad). The relative band intensity was quantified using ImageJ Software.

2.1.10 Intracellular nitric oxide

Intracellular nitrate quantification was performed using Measure-IT High Sensitivity Nitrite Assay Kit (Thermo Fisher) following the manufacturer's instruction. HDFs ($4x10^4$ cells/well) were plated into 24-wells microplate and were treated as previously described. Cells were washed and lysed with 200 µl of double distilled water. Meanwhile, a 96-wells microplate was prepared with 100 µl of working solution, and 10 µl of cell supernatant was added and incubated at RT for 10 minutes. 5 µl of quantification developer were added to each well and the plate was read at 365/450 nm excitation/emission using Victor X Multilabel Plate Readers (PerkinElmer).

2.1.11 Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 (California, USA). Data were expressed as mean ± SEM of *n* independent experiments. Statistical significance was assessed by one-way ANOVA multiple comparisons with Dunnett's test or Student's t-test for different groups of treatment comparisons. Statistical significance was defined as $p < 0.05$.

2.2 Results

2.2.1 Incidence of cell proliferation and cell viability of irradiated HDFs.

To evaluate whether UVB and vitamins might affect cell proliferation, we analysed Ki67 expression through indirect immunofluorescence (Figure 1a). As expected, UVB significantly lowered the percentage of cells expressing Ki67, suggesting a reduction of cell proliferation (Figure 1b). However, pre-treatment with NAM, CAL and α-T reverted this trend, improving cell proliferation. We also investigated the role of vitamins on cell viability after UV irradiation (Figure 1c). Interestingly, none of the vitamins have improved cell viability on irradiated HDFs. These results might suggest that vitamins are able to restore cell proliferation, but they do not influence cell viability on irradiated HDFs.

2.2.2 Vitamins reduce UV-induced oxidative stress

Since UV-induced oxidative stress is considered the primary mechanism of photoaging (Fisher et al, 2002), we quantified ROS production and oxidative stress markers. As shown in Figure 2a, NAM and CAL efficiently reduced ROS production while, surprisingly, α-T did not show any significant variation. We also tested antioxidant SOD-1 gene expression (Figure 2b), showing a relevant modulation only on NAM-treated cells. Because of AP-1 activation induced by ROS, MMP-1 is released by fibroblasts after UV exposure (Han et al, 2014). In Figure 2c we demonstrated that UV enhances MMP-1 gene expression, and all vitamins reverted this trend. Finally, we quantified nitrate concentration as a direct marker of NO production induced by UVB rays. As shown in Figure 2d, only NAM and CAL were able to reduce NO release by irradiated HDFs suggesting that treatment of cells with these vitamins before UV irradiation, might prevent oxidative stress and ROS release.

2.2.3 Effects of vitamins on DNA damage repair

Both UV rays and ROS can damage the DNA through the formation of 8-OHdG base. Since HDFs treated with vitamins showed reduced ROS levels and oxidative stress markers expression, we investigated whether also DNA damage was affected by vitamins pre-treatment through comet assay (Figure 3a) and quantification of OGG1 expression. Consistently, cells pre-treated with vitamins, in particular NAM and CAL, showed reduced levels of DNA damage (Figure 3b), correlated to a decreased gene expression of OGG1 with a significant modulation with NAM pre-treatment (Figure 3c). Protein expression is significantly increased after UVB exposure and vitamins pre-treatment seemed to revert

this trend (Figure 3d-e). However, only cells treated with CAL showed significant differences. These results may suggest that vitamins, in particular NAM and CAL, are able to prevent oxidative damages to DNA.

2.2.4 Effects of UVB and vitamins on p53/p21 pathway

In order to repair UV-induced and oxidative DNA damages, cells can activate the p53/p21 growth-suppressive pathway which leads to cell cycle arrest and senescence. In particular, p53 is activated via phosphorylation (p-p53) which upregulates p21, enforcing senescence in response to DNA damage (Cavinato et al, 2017). Thus, we evaluated if p53/p21 pathway is involved in mechanisms of HDFs vitamin-mediated photoprotection. Firstly, we evaluated p53 mRNA levels through qRT-PCR (Figure 4a). HDFs exposed only to UVB showed a significant increase in p53 gene expression; however, all vitamins were able to reduce p53 levels. Then, we analyzed protein expression of both p53 and p-p53 through western blotting (Figure 4b). Interestingly, HDFs treated with vitamins, in particular, NAM showed an accumulation of p53 (Figure 4c) but low levels of p-p53, even without a statistically significant difference. On the other hand, cells treated only with UVB showed a significant increased activation of p53. These results may suggest that since on cells pre-treated with vitamins DNA damages were lower, p53/p21 pathway is less active and, consequently, p53 accumulates within cells. This hypothesis could be confirmed by the measurement of p21 gene (Figure 4e) and protein (Figure 4f-g) expression. Indeed, on UVB irradiated HDFs, p21 expression was significantly higher in comparison with control cells while pre-treatment with vitamins lowered p21 gene and protein expression.

2.2.5 Effects of UVB and vitamins on cell cycle

After observing the activation of p53/p21 pathway by UVB and its inactivation by vitamins, we evaluated how UVB and vitamins can affect the cell cycle through flow cytometry (Figure 5a). As shown on the board of Figure 5b and on Figure 5c, UVB did not affect G1 and S cell cycle phases. However, UVB significantly reduced the number of cells on G2 phase in comparison with control cells (Figure 5d), a possible consequence of the upregulation of p21. On the other hand, vitamins, in particular NAM and α-T, showed an increased number of cells in G2 phase, which correlates with the lower level of p21 expression showed before. With flow cytometry, we also evaluated cells on Sub-G1 phase

(Figure 5e). As shown, the percentage of cells within this stage is significantly higher with UVB irradiation; however, all vitamins reversed this trend.

2.2.6 Incidence of senescence on irradiated HDFs

Because of the activation of p53/p21 pathway and cell cycle arrest, cells can undergo senescence. Therefore, we analysed the expression of SA-β-Gal (Figure 6a). As shown in Figure 6b, after UVB irradiation the number of cells expressing SA-β-Gal was significantly higher in comparison with control cells. Nevertheless, cells pre-treated with vitamins were less positive to SA-β-Gal stain, suggesting that vitamins could prevent senescence and restore the cell cycle. Finally, we quantified p16 gene expression as a marker of senescence and cell cycle arrest through qRT-PCR (Figure 6c). As expected, UVB irradiation significantly increased p16 gene expression; however, only NAM pre-treatment showed a significant reduction.

Figure 1

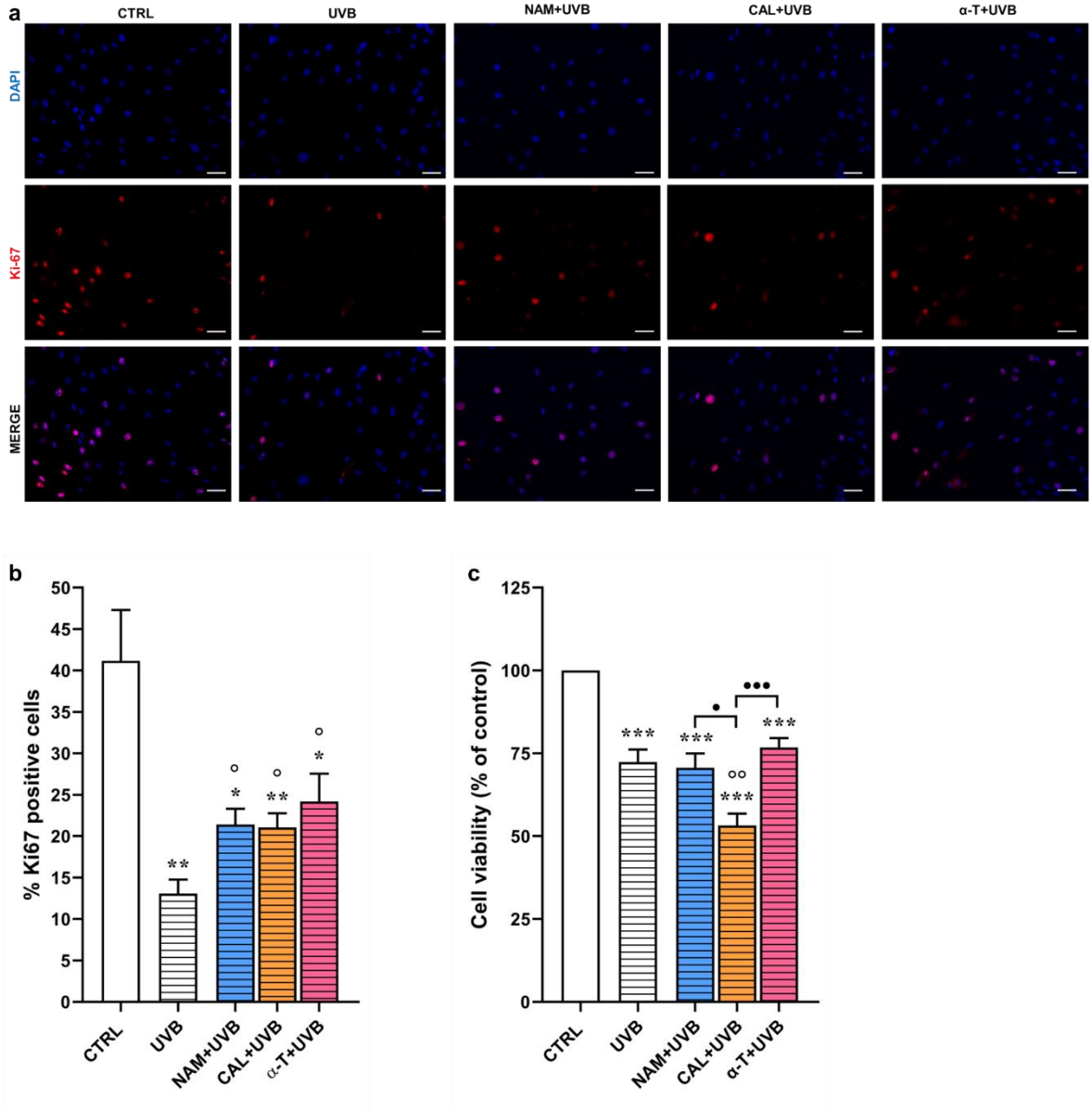


Figure 1. Effects cell proliferation and viability. HDFs were stimulated with NAM 25 µM, CAL 100 nM and α-T 1 µM for 24h, irradiated with UVB 40 mJ/cm^2 and incubated for 24 h. (**a**) Representative IF of Ki-67 and (**b**) percentage of Ki-67 positive cells of three independent experiments indicated as means ± SEM. (**c**) MTT assay for evaluation of cell viability. Data are expressed as means ± SEM of seven independent experiments. *p <0.05, **p<0.01, ***p<0.001 vs CTRL; °p<0.05, °°p<0.01 vs UVB; •p<0.05, •••p<0.001 significance between groups. CTRL, untreated cells; NAM, nicotinamide; CAL, calcipotriol; α-T, α-tocopherol.

Figure 2

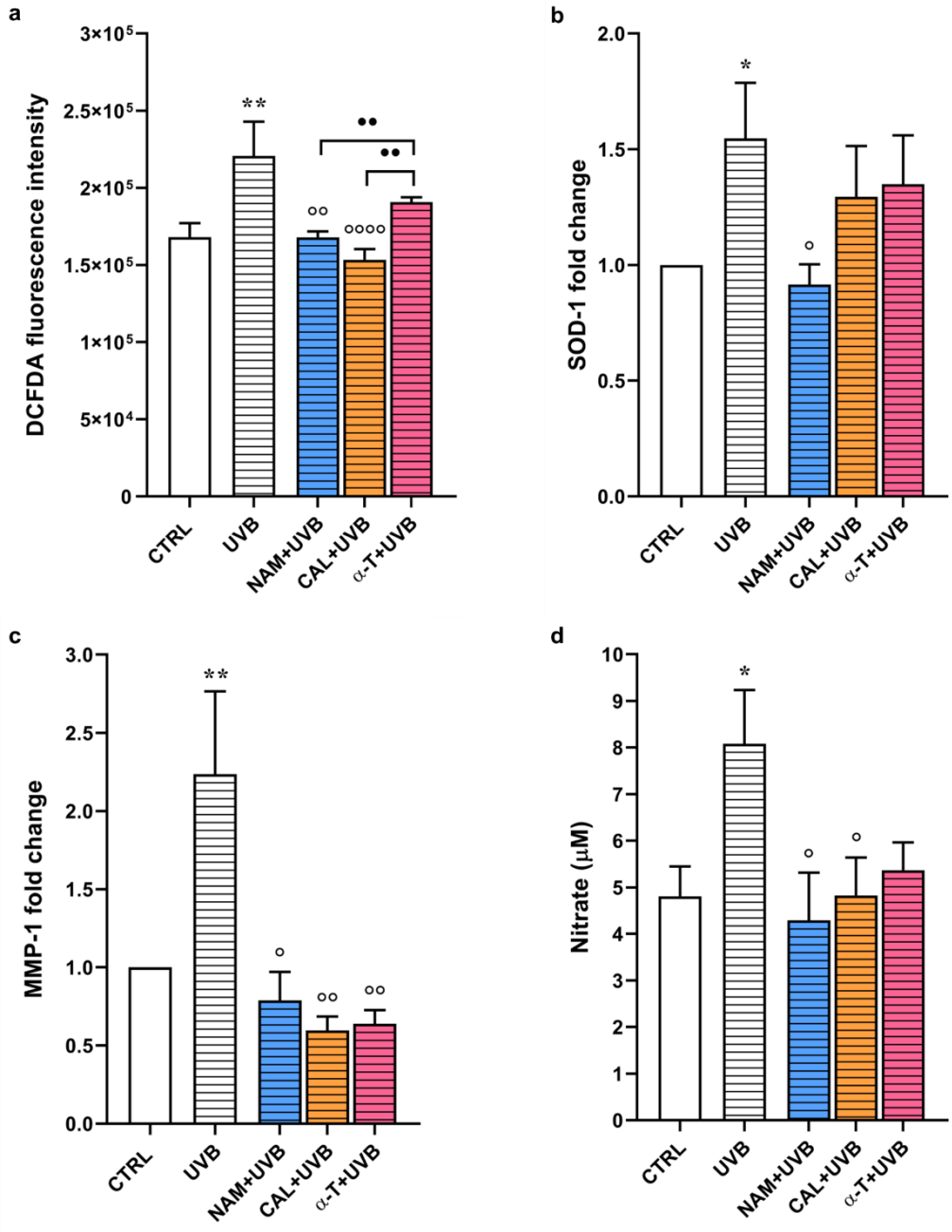


Figure 2. Effects on ROS production, antioxidants expression and intracellular nitrate concentration. HDFs were stimulated with NAM 25 µM, CAL 100 nM and α-T 1 µM for 24h, irradiated with UVB 40 mJ/cm^2 and incubated for 24 h. (**a**) Intracellular ROS quantification using DCFDA fluorescent probe. Data are expressed as means ± SEM of four independent experiments. (**b**) Gene expression of SOD-1 and (**c**) MMP-1. Data are expressed as means ± SEM of seven independent experiments. (**d**) Intracellular nitrate concentration. Data are expressed as means ± SEM of five independent results. *p <0.05, **p<0.01, vs CTRL; °p<0.05, °°p<0.01, °°°°p<0.0001 vs UVB; ••p<0.01 significance between groups. CTRL, untreated cells; NAM, nicotinamide; CAL, calcipotriol; α-T, α-tocopherol.

Figure 3

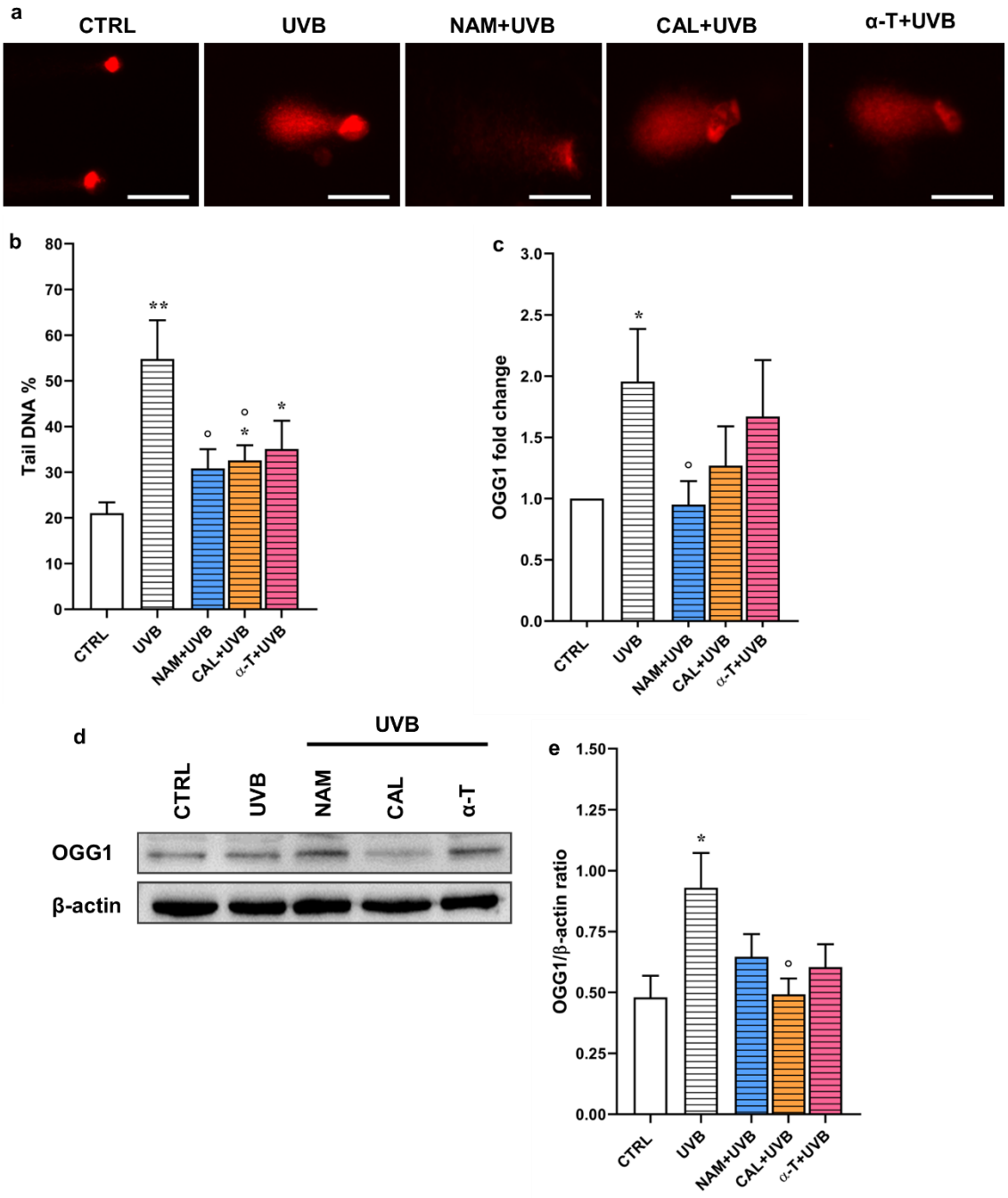


Figure 3. Effects on DNA damage level and OGG1 expression. HDFs were stimulated with NAM 25 µM, CAL 100 nM and α-T 1 µM for 24h, irradiated with UVB 40 mJ/cm^2 and incubated for 24 h. (**a**) Representative comet assay and (**b**) tail DNA % quantification of six independent experiments expressed as means ± SEM. (**c**) OGG1 gene expression. Data are expressed as means ± SEM of seven independent experiments. (**d**) Representative western blotting and (**e**) densiometric analysis of OGG1 protein expression vs β-actin of five independent experiments represented as means ± SEM. *p<0.05, **p<0.01 vs CTRL; °p<0.05 vs UVB. CTRL, untreated cells; NAM, nicotinamide; CAL, calcipotriol; α-T, α-tocopherol.

Figure 4

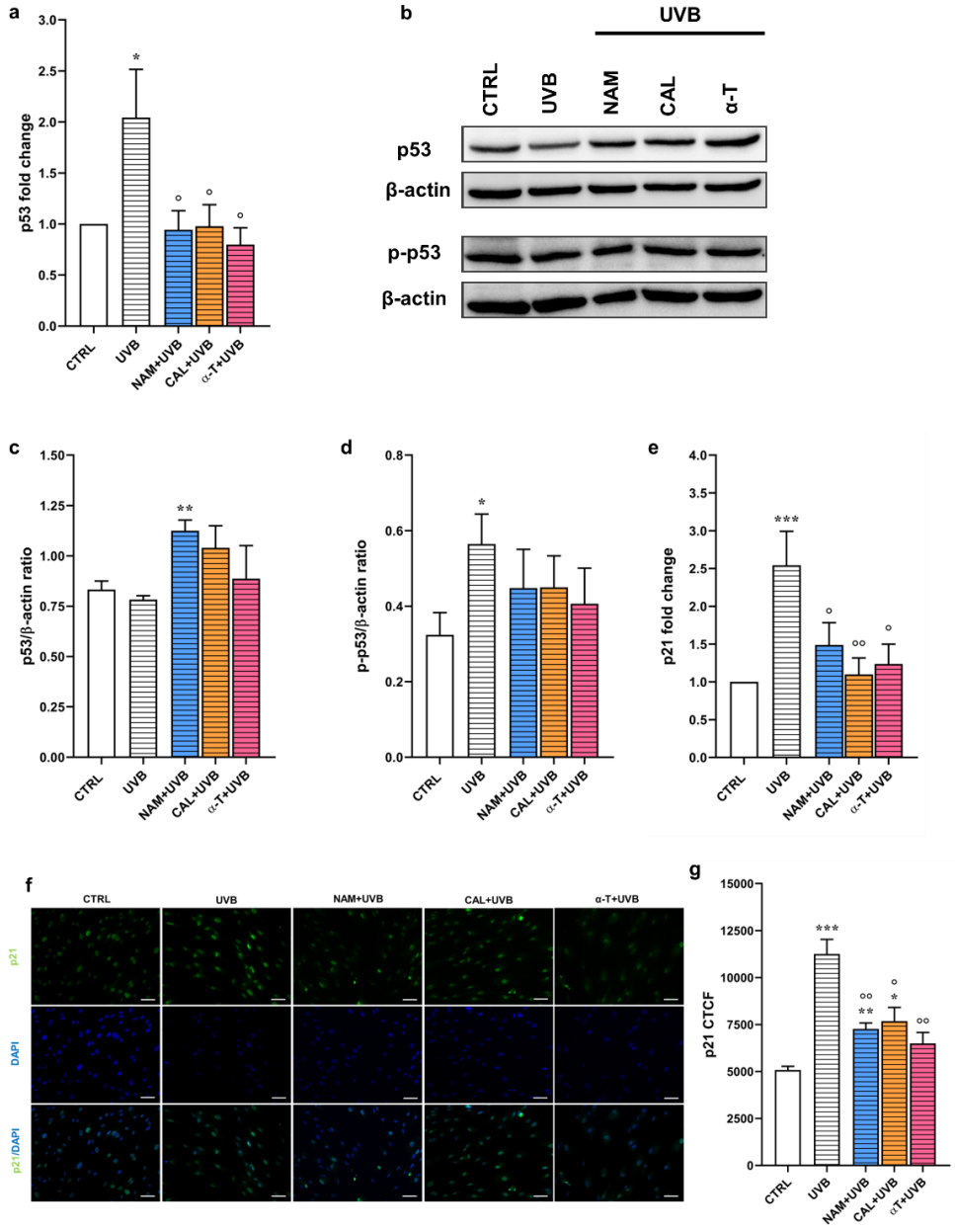
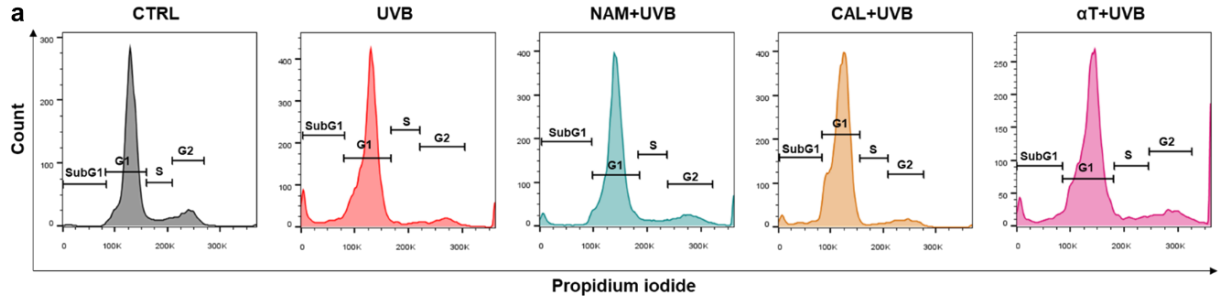


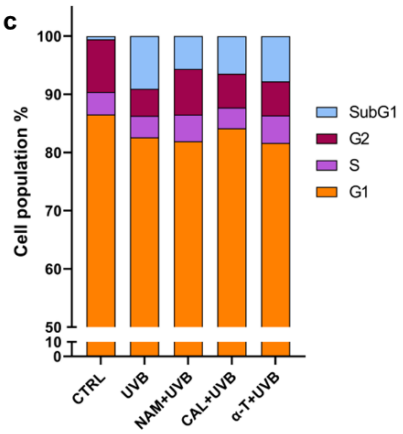
Figure 4. Effects on p53/p21 pathway. (**a**) p53 gene expression of seven independent experiments indicated as means ± SEM. (**b**) Representatives western blotting of p53 and p-p53 protein expression. Densiometric analysis of p53 (**c**) and p-p53 (**d**) is represented as means ± SEM of five independent experiments. (**e**) p21 gene expression of seven independent experiments expressed as means ± SEM. (**f**) Representative IF of p21 stain and (**g**) CTCF quantification of p21 IF expressed as means ± SEM of four independent experiments. For CTCF quantification, at least 20 cells were measured for each experiment. *p<0.05, **p<0.01, ***p<0.001 vs CTRL; °p<0.05, °°p<0.01 vs UVB. CTRL, untreated cells; NAM, nicotinamide; CAL, calcipotriol; α-T, α-tocopherol.

Figure 5



b

Sample	SubG1 (%)	G1 (%)	S (%)	G2 (%)
CTRL	0.53±0.14	86.54±1.96	3.88±0.58	9.04±0.66
UVB	9.04±0.90	82.61±1.82	3.70±0.69	4,65±0.89
NAM+UVB	5.65±0.78	81.94±1.92	4.57±0.48	7.85±0.94
CAL+UVB	6.46±1.30	84.16±3.03	3.58±0.46	5.80±0.99
α-T+UVB	7.77±1.11	81.64±2.93	4.73±0.78	5.85±1.03



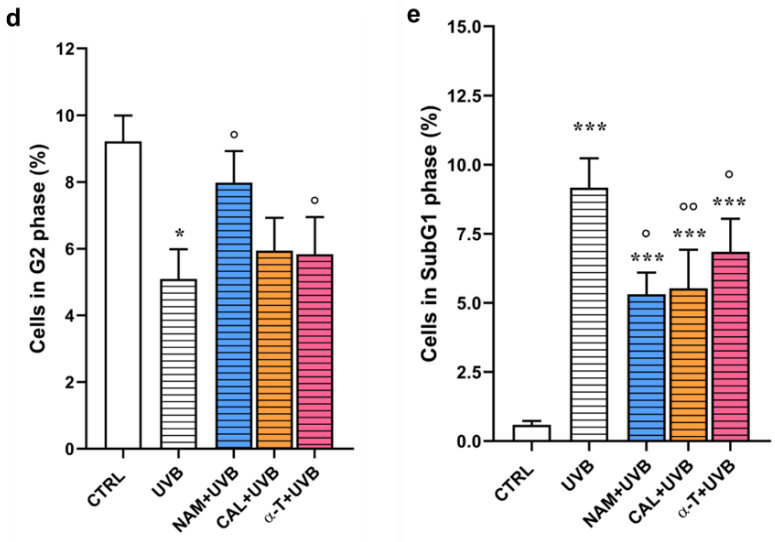


Figure 5. Effects on cell cycle phases. HDFs were stimulated with NAM 25 µM, CAL 100 nM and α-T 1 µM for 24 h, irradiated with UVB 40 mJ/cm^2 and incubated for 24 h. (**a**) Representative FACS analysis of cell cycle using propidium iodide of seven independent experiments. (**b**) Percentage of cells in each cell cycle phase indicated as means ± SEM and (**c**) graphical representation. (**d**) Percentage of cells on G2 phase and (**e**) on Sub-G1 phase. Data are expressed as means ± SEM of seven independent experiments. *p<0.05, ***p<0.001 vs CTRL; °p<0.05, °°p<0.01 vs UVB. CTRL, untreated cells; NAM, nicotinamide; CAL, calcipotriol; α-T, α-tocopherol.

Figure 6

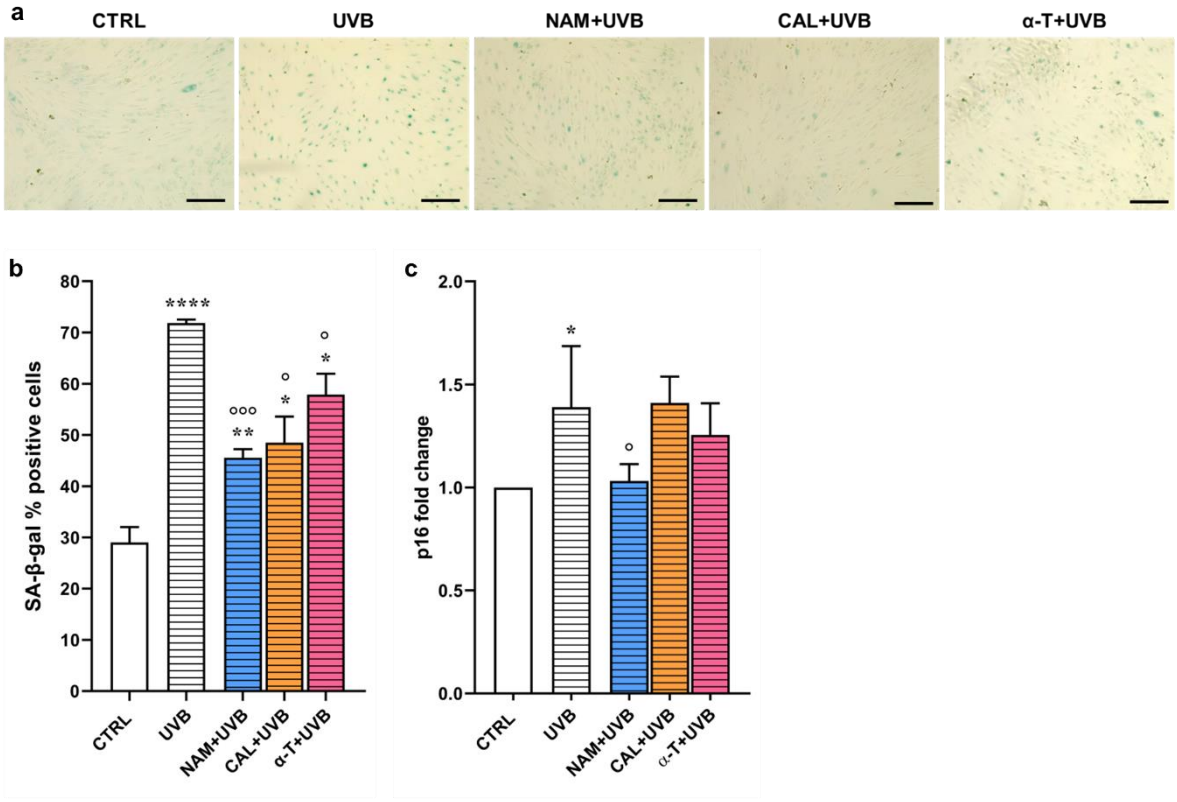


Figure 6. Effects on senescence and p16 expression. HDFs were stimulated with NAM 25 µM, CAL 100 nM and α-T 1 µM for 24 h and irradiated with UVB 40 mJ/cm^2 and incubated for 24 h. (**a**) Representative SA-β-GAL stain and (**b**) and percentage of SA-β-GAL positive cells. Data are expressed as means ± SEM of four independent experiments. (**c**) p16 gene expression. Data are expressed as means ± SEM of seven independent experiments. *p<0.05, **p<0.01, ****p<0.0001 vs CTRL; °p<0.05, °°°p<0.001 vs UVB. CTRL, untreated cells; NAM, nicotinamide; CAL, calcipotriol; α-T, α-tocopherol.

CONCLUSIONS

CONCLUSIONS AND FUTURE PERSPECTIVES

During the last 50 years, changes in lifestyle, like outdoor activities and the use of solar sunbeds, have resulted in increased sun exposure and a consequent enhancement of NMSC incidence, especially in the Caucasian population (Bilaç et al, 2014). In 2020, NMSCs were the fifth most diagnosed cancer worldwide with more than 1.000.000 cases and with an incidence rate 2 times higher in male patients. NMSCs mainly encompass BCCs, SCCs, and AKs. These are malignant lesions characterized by different clinical behaviours, differentiation, cell growth and metastatic rate, while AKs are premalignant lesions extremely common in the adult population that may evolve to SCCs. Since chronic actinic damage usually occurs on large areas of the body, NMSC patients often carry more than one lesion (Costa et al, 2015). Among several intrinsic and extrinsic factors that contribute to NMSC development, ultraviolet exposure is the main risk factor for cancerous degeneration (Khavkin et al, 2011). Indeed, NMSCs usually arise from chronically photo-exposed skin areas in which both epidermis and dermis carry several macroscopical and microscopical signs of premature aging as wrinkles, elastosis, irregular pigmentation, delayed wound healing, impaired proliferation, differentiation, and apoptosis of basal keratinocytes of the epidermis, degradation of collagen fibres, and reduction of extracellular matrix in the dermis (Bilaç et al, 2014). UVA and UVB can exert several effects within cells affecting directly or indirectly different molecules. Indeed, UVB photons are absorbed by DNA, producing CDPs and 6-4PPs complexes that may occur on cancer-related genes, as p53, leading to cell cycle dysregulation and activation of several pathways (Hung et al, 2020). Furthermore, both UVA and UVB trigger ROS production which can interact with different molecules, including DNA in which they promote the 8-OHdG base formation, leading to mutations and impairment on DNA replication (Ichihashi et al, 2003). ROS also play a crucial role in collagen metabolism by directly disrupting collagen fibres and activating MMPs, leading to connective tissue damages and favouring cancer cell infiltration and metastasis (Bilaç et al, 2014). In presence of DNA damages, cells activate different pathways to arrest the cell cycle and to repair DNA mutations, like p53/p21 and DDR system. In physiological conditions, p53 is activated by phosphorylation and enters the nucleus inducing the expression of several genes, including p21 which blocks the cell cycle through the inhibition of the cyclin kinase pathway (Karimian et al, 2016). However, p53 is mutated not only in the 50-100% NMSCs but also in AKs and sun-damaged but normal

appearing cells, facilitating the development of cancer in these areas (Chen et al, 2013). Moreover, in presence of severe DNA damages, cell cycle arrest becomes permanent, a condition defined as senescence (Schuch et al, 2017). Senescent cells produce and release SASP molecules and proinflammatory cytokines that contributes to photoaging and to the formation of a cancer-promoting environment, also called field cancerization (Faget et al, 2019). The field cancerization is a chronically photoexposed area that surrounds malignant and premalignant lesions in which cells have accumulated some but not all mutations required for cancer development (Jetter et al, 2018). Nevertheless, if these mutated cells are chronically exposed to UV rays, they might acquire more mutations giving rise to AKs formation and tumor progression (Willenbrink et al, 2020). The field of cancerization involves both epidermis and dermis. Indeed, UV-irradiated fibroblasts produce growth factors that help mutated actinic-damaged keratinocytes to transform into neoplastic cells. Recently, several preclinical studies suggested that the treatment of the field cancerization prevents the onset of NMSC, and the importance of field therapy is underlined by the most important guidelines (Kirby et al, 2015; Werner et al, 2015; de Berker et al, 2017; Fleming et al, 2017; Moscarella et al, 2020). Similarly, skin cancer chemoprevention strategies, including those based on nutrient intake, play a crucial role in controlling skin carcinogenesis (Camillo et al., 2018; Savoia et al., 2018). Retinoids are the most studied and used photoprotective molecules, however their severe systemic side effects limit the patient compliance and the possibility of clinical use. Therefore, several research groups worldwide are focusing on the study of different molecules that combine effectiveness in cancer prevention with patient compliance (Pihl et al, 2021).

NAM is the amide form of vitamin B3, it is assumed by daily diet and is the precursor of NAD, a key coenzyme essential for DNA repair and cellular metabolism (Chen et al, 2014). NAM increases energy availability, enhances DNA damages repair, and inhibits inflammatory response blocking PARP1 in irradiated HaCat keratinocytes (Surjana et al., 2013; Park et al., 2010) exposed to both UVA and UVB and in irradiated human primary keratinocytes (Pelle et al., 2003). Nowadays, NAM is proposed for the treatment of several skin disorders, and recent clinical trials have demonstrated NAM efficacy also in reducing AKs and primary NMSCs lesions even on high-risk NMSC patients. However, most *in vitro* studies have been performed on keratinocyte cell lines and there is still a lack of

information about the role of NAM as photoprotector on both primary keratinocytes and fibroblasts.

Alpha-tocopherol is the most active form of vitamin E and is one of the most abundant antioxidants presents in the skin. It is assumed by daily diet, and it is well known for its ability to protect against oxidation damage. Indeed, α-T can capture free radicals and prevents oxidative stress damages (Niki et al, 2012). As reviewed by Thiele et al, (2017), there are several papers that have evaluated the use of vitamin E as a cosmeceutical molecule with photoprotective properties. Besides, several animal and *in vitro* studies have demonstrated that α-tocopherol reduces UV-induced damages, even in the dermis (Chung et al, 2002). However, there is still a lack of knowledge about the role of α-T against photoaging, especially on human primary cells. Moreover, the role of vitamin E supplementation on different animal models has given controversial results, especially on lifespan (Ernst et al, 2013).

Vitamin D3 is synthetized by keratinocytes through a reaction catalysed by sunlight, and it is also found in some animal-based foods (Bikle, 2011). Vitamin D3 can control cell proliferation and differentiation, and immune response by creating a complex with the VDR that acts as a transcription factor (Wang et al, 2012). Present-day, vitamin D3 is used for the treatment of some skin disorders and its role as photoprotector has been investigated on both animal and human models (Pihl et al, 2021). Indeed, recent clinical trial has showed that vitamin D3 can reduce UV-induce effects on the skin (Scott et al, 2017). However, there is still a lack of knowledge about mechanisms activated by vitamin D3 and studies performed on primary cells are not available.

This thesis aims to provide new insights on vitamins effects on UV-induced damages on human primary skin cells. In particular, the first part (Project 1) evaluated the role of NAM against oxidative stress UV-induced on FC-HPKs and NHEKs, and the second part (Project 2) evaluated the role of vitamin B3, D3 and E against photoaging on HDFs.

In Project 1, we firstly measured intracellular ROS production and antioxidants gene expression on both NHEKs and FC-HPKs. Interestingly, NAM given 24 and 48h before irradiation efficiently decreased ROS levels only on irradiated FC-HPKs, while NHEKs seemed to be less sensitive to UV light. Indeed, unirradiated FC-HPKs showed 4-fold higher ROS levels in comparison with NHEK cells. Consistently, GPX-1 and SOD-1 gene expression was modulated by NAM only on irradiated FC-HPKs. Altogether, these results may confirm

that long exposure to UV radiation, as happens in elderly patients and in those with actinic damage, could trigger ROS release helping to promote the formation of cancerization field (Liao et al, 2019). Then, we analyzed the expression of OGG1, an enzyme responsible for 8-OHdG excision which is one of the markers of the oxidative DNA damage caused by the combined effect of ROS and direct exposure to UV rays (Pelle et al, 2003). Our data confirm that the OGG1 protein expression was well-modulated by NAM treatment on both types of keratinocytes, with significant results after 24 and 48h treatment. No differences were found among NAM concentrations. Instead, cells treated with 25 µM NAM, especially FC-HPKs, showed a greater gene expression reduction. In agreement with these results, we found that NAM-treated keratinocytes presented lower DNA damages with the best results at 25 µM concentration. Our findings are in line with those obtained by Surjana et al. (2013), who demonstrated that NAM at a concentration of 50 µM decreased the 8-OHdG expression in UV-irradiated HaCat cells and enhanced DNA repair after UV exposure. Since UVB light induces local inflammation and increases cytokine production (Pillai et al, 2005; Snaidr et al, 2019), we thirdly investigated pro-inflammatory cytokines gene expression. In general, NAM treatment seemed to reduce gene expression on both keratinocyte types. However, we found significant modulation of IL-1β and TNF-α gene expression only on FC-HPKs treated with NAM given for 24 - 48h and 18h respectively. Our data are partially in line with those of Monfrecola et al. (2013), who reported a decreased TNF-α gene expression in UVB-irradiated HaCat cells pre-treated with 5 mM of NAM after 6h from irradiation, without IL-8 modulation. However, in contrast with our findings, they showed that NAM was ineffective in IL-1β gene expression modulation. This discrepancy might be due to the different UVB dosages, time of treatment and to the different experimental models. Lastly, we investigated iNOS expression and NO release as marker of UV-induced damages (de Jager et al, 2017). NAM efficiently decreased protein expression on both keratinocytes but showed a slight modulation of gene expression only on FC-HPKs. Consistent with the reduction of iNOS expression, also NO release was affected by NAM treatment, especially on FC-HPKs treated with NAM for 24 and 48h before irradiation. No relevant differences were found among NAM concentrations. This data is remarkable, since exposure to UV induces an increase of NO release and iNOS expression, as previously demonstrated by Chang et al. (2003), on neonatal foreskin keratinocytes irradiated with UVB. Overall, our results suggest that keratinocytes isolated from the field cancerization

might be more sensitive to UV light than normal keratinocytes. However, NAM exposure before UV irradiation seems to protect both keratinocyte types from multiple UV-induced damages, including oxidative stress, DNA impairments and inflammation.

In Project 2, we firstly evaluated cell proliferation and viability on HDFs. As expected by literature data (Kim et al, 2016), both cell proliferation and viability were affected by UVB exposure. Interestingly, all vitamins improved cell proliferation on irradiated cells, but they did not show any effects on cell viability. Secondly, since oxidative stress is considered the initial step of photoaging (Bilaç et al, 2014), we measured ROS production and antioxidants expression. We showed that UVB increased ROS release, but this trend was reverted by NAM and CAL treatment and that antioxidant gene expression of SOD-1 and GPX-1 was significantly decreased by vitamins, especially NAM. Also nitrate concentration, considered as a marker of oxidative stress, was reduced by NAM and CAL. We also analysed MMP-1 gene expression inasmuch is induced by ROS through AP-1 transcription (Cavinato et al, 2017). We found that even MMP-1 expression was reduced by vitamins after UVB irradiation. These results suggest that vitamins, in particular NAM and CAL, are able to counteract ROS release induced by UVB and to reduce the oxidative stress condition. Because both ROS and UVB can damage the DNA, we then evaluated oxidative DNA damages and OGG1 gene expression, as marker of BER activation. We found that cells treated with NAM and CAL before UV irradiation presented less DNA damages and OGG1 expression in comparison with UVB irradiated cells, in which cell DNA appeared acutely damaged. These results correlate with the reduced ROS production elicited by vitamins, demonstrating that they protect DNA from oxidative damages by inhibiting ROS production induced by UVB. After that, we checked the activation of p53/p21 pathway in response to DNA damage (Cavinato et al, 2017). Consistently with DNA damage results, we found that p53 gene expression was enhanced by UVB and most of the p53 protein produced was found in the phosphorylated form. Moreover, we detected an increased p21 gene and protein expression, suggesting that the UV-induced DNA damages triggered the activation of p53 that induced the transcription of p21 in order to block the cell cycle and allow DNA repair (Harris et al, 2005). In accordance with previous results, HDFs treated with vitamins showed reduced p53 gene expression and activation, and consequently, lower levels of p21 expression. Therefore, the next step was the analysis of the cell cycle and phases. We discovered that with high levels of p21 expression, as found on UVB-irradiated cells,

correlated a block of the cell cycle in G2 phase. These data are in line with those reported by Greussing et al (2013) in which they have demonstrated that p53/p21 pathway was activated by UVB irradiation on HDFs. However, NAM and α-T pre-treatment restored the cell cycle. Moreover, we found that UVB increased cells in the SubG1 phase, in which are grouped death cells, and vitamin pre-treatment reduced the percentage of cells in this phase. These results might suggest that since vitamins seem to protect cells from oxidative DNA damages, the activation of the p53/p21 pathway is lower, and consequently, cell cycle and cell death are less affected. Finally, we investigated whether the arrest of the cell cycle evolved to senescence. In fact, irradiated cells showed increased production of SA-βGAL in comparison with vitamin-treated cells and higher levels of p16 gene expression, which was reduced by NAM pre-treatment. Taken all together, these results suggest that vitamins, in particular NAM and vitamin D3 attenuate photoaging on human dermal fibroblasts by reducing ROS production and consequently DNA damages, restoring cell cycle and avoiding senescence.

In conclusion, our findings could support recent encouraging results about vitamins as useful molecules for chemoprevention of NMSC and photoaging. Particularly, NAM seems to be effective on both keratinocytes and fibroblasts, showing photoprotective properties against UVB induced oxidative stress and photoaging. Similarly, vitamin D3 showed good results on photoprotection on fibroblasts, while α-tocopherol seems to be less effective at studied concentration. However, more extensive studies are needed to better elucidate the mechanism of action of these molecules, their possible interactions with antioxidants also in a clinical setting. Indeed, the analysis of the combination of different vitamins against photoaging and photocarcinogenesis on both keratinocytes and fibroblasts would enhance our knowledge about these molecules and their action. Moreover, it would be interesting analyse whether keratinocytes and fibroblasts, isolated from patients treated with NAM topically and systemically, present different behaviours and molecular profile. Finally, the use of 3D cultures will improve our understanding on the interaction between keratinocytes and fibroblasts on the field cancerization.

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