Carolina de Barros Reis Quayle

The specific roles of CPDs and 6-4PPs DNA photolesions in distinct local epithelial responses to UV light in DNA repair-deficient mice

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Supervisor: Prof. Dr. Carlos Frederico M. Menck

Co-supervisor: Prof. Dr. Gijsbertus van der Horst

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ABSTRACT

Quayle C. The specific role of CPDs and 6-4PP DNA photolesions in distinct local epithelial responses to UV light in DNA repair-deficient mice. 2013. 228 p. Ph. D thesis (Microbiology) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2013.

The ultraviolet (UV) component of sunlight is the most common environmental genotoxic factor, mainly inducing two photolesions in DNA: cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs). Photolyases are enzymes which use the energy of a photon to directly reconvert these dimers to monomers. Placental mammals do not have photolyases and count on the nucleotide excision repair (NER) pathway to remove photolesions. Deficiencies in genes involved in NER may lead to severe human syndromes, such as Xeroderma Pigmentosum (XP) and Cockayne Syndrome (CS), characterized by increased skin cancer incidence and progeria, respectively. Although the effects of UV on skin are widely known, the specific roles of each photolesion are not clear. Previous work with human fibroblasts demonstrated that, while in NER-proficient cells the removal of CPDs increases survival after UV irradiation, in NER-deficient cells both lesions play an important role in apoptosis induction. Furthermore, experiments with DNA repair-proficient mice suggested that CPDs are responsible for the majority of the deleterious effects seen after UV skin exposure. Therefore, the aim of this work is to investigate the specific roles of CPDs and 6-4PPs *in vivo*, in a system where the fast removal of photolesions by NER does not mask their function in the induction of local responses to UV irradiation. For that purpose, transgenic XPA and CSA knockout mice, expressing either CPD-photolyase or 6-4PP-photolyase, were UV exposed for 1, 25 or 36 consecutive days to a low UV dose, followed by photoreactivation. In XPA mice, ubiquitous CPD removal after acute UV exposure reduced suprabasal proliferation, partially preventing hyperplasia. Chronic UV irradiation induced significant hyperplasia, which was prevented by ubiquitous CPD removal, due to a partial reduction in basal proliferation. Furthermore, 6-4PP persistence in the genome induced significant melanin production, which was further increased by ubiquitous CPD removal. CPD removal from total epidermis prevented p53 overexpression in cluster cells, avoiding the beginning of a tumorigenic process. 6-4PP removal from basal keratinocytes partially prevented acute UV-induced hyperplasia, through basal proliferation reduction, but did not prevent chronic UV-induced hyperplasia or clustered p53 overexpression. UV-induced basal and suprabasal cell death were reduced, and skin pigmentation was prevented by 6-4PP removal from basal keratinocytes after UV exposure of XPA mice. In CSA mice, chronic UV exposure did not alter skin pigmentation but induced significant hyperplasia. CPD removal from basal keratinocytes partially prevented epidermal hyperplasia, due to a reduction in basal and suprabasal cell proliferation and an increase in basal cell death. 6-4PP removal from basal keratinocytes did not alter UV-induced hyperplasia, cell proliferation or apoptosis. These results indicate that not only the nature of the lesion, but also the total amount and location of genome damage, may play important roles in skin responses after acute and chronic UV exposure. CPDs seem to play a major role in cell proliferation, hyperplasia and tumorigenesis, whereas 6-4PPs are involved in melanogenesis and apoptosis.

Keywords: DNA photolesions. Ultraviolet radiation. DNA repair. Phtolyases. Skin. Pigmentation.

RESUMO

Quayle C. O papel específico das fotolesões de DNA CPDs e 6-4PPs em respostas epiteliais à irradiação ultravioleta em camundongos deficientes em reparo de DNA. 2013. 228 f. Tese (Doutorado em Microbiologia) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2013.

O componente ultravioleta (UV) da luz solar é o fator ambiental genotóxico mais abundante, induzindo majoritariamente duas fotolesões no DNA: dímeros de pirimidina ciclobutano (CPDs) e pirimidina (6-4) pirimidona fotoprodutos (6-4PPs). Fotoliases são enzimas que utilizam a energia de um fóton para reconverter diretamente estes dímeros em monômeros. Mamíferos placentários não possuem fotoliases, contando com a via de reparo por excisão de nucleotídeos (NER) para remover fotolesões. Deficiências em genes desta via podem causar síndromes humanas severas, como Xeroderma Pigmentosum (XP) e Síndrome de Cockayne (CS), caracterizadas por alta incidência de câncer de pele e progéria, respectivamente. Os efeitos da radiação UV na pele são amplamente conhecidos, porém os papéis específicos de cada fotolesão não estão esclarecidos. Estudo com fibroblastos humanos demonstrou que, enquanto em células proficientes em NER apenas a remoção de CPDs aumenta sobrevivência após exposição à UV, em células deficientes em NER, ambas lesões são importantes na indução de apoptose. Experimentos *in vivo* sugerem que CPDs são responsáveis pela maioria dos efeitos deletérios da exposição da pele à UV. Assim, o objetivo deste trabalho é investigar os papéis específicos de cada fotolesão, *in vivo*, em um sistema onde sua rápida remoção por NER não mascare sua função na indução de respostas locais à irradiação UV. Para tanto, camundongos transgênicos *knock out* em XPA ou CSA, expressando CPD-fotoliase ou 6-4PP-fotoliase, foram expostos por 1, 25 ou 36 dias a baixas doses de UV, seguidas por fotorreativação. Em animais XPA, a remoção de CPD após exposição aguda à UV reduziu a proliferação suprabasal, parcialmente prevenindo hiperplasia; a redução parcial em proliferação basal e suprabasal após irradiação crônica preveniu hiperplasia. A persistência de 6-4PP aumentou a concentração de melanina na epiderme, efeito exacerbado pela remoção de CPDs. Superexpressão de p53 em clusters de células foi prevenida pela remoção de CPDs, evitando processo tumorigênico. Remoção de 6-4PPs de queratinócitos basais preveniu parcialmente hiperplasia aguda através da redução da proliferação basal, porém não preveniu indução de hiperplasia crônica ou a superexpressão de p53 em clusters de células após exposição à UV. A apoptose foi reduzida e a pigmentação da pele foi prevenida pela remoção de 6-4PP em queratinócitos basais em animais XPA. Em camundongos CSA, exposição crônica à UV não alterou a pigmentação da pele, mas induziu hiperplasia. A remoção de CPDs de queratinócitos basais preveniu parcialmente a indução de hiperplasia, devido à redução de proliferação basal e suprabasal e ao aumento de apoptose basal. A remoção de 6-4PP de queratinócitos basais não preveniu a indução de hiperplasia, proliferação ou morte celular. Estes resultados indicam que, não apenas a natureza da lesão, mas também, a quantidade total e localização de dano no DNA, podem desempenhar papéis importantes nas respostas da pele à exposição aguda e crônica à radiação UV. CPDs têm papel majoritário na proliferação celular, hiperplasia e tumorigênese enquanto 6-4PPs estão envolvidos em melanogênese e apoptose.

Palavras-chave: Fotolesões de DNA. Radiação ultravioleta. Reparo de DNA. Fotoliases. Pele. Pigmentação.

1 INTRODUCTION

1. 1 Structure and instability of the DNA molecule

Gregor Mendel is considered the father of Genetics. In 1866, in his work “Versuche über Plflanzenhybriden” (Experiments in plant hybridation), the Austrian monk was the first to demonstrate, with scientific experiments, that phenotypic characteristics are inherited through hereditary entities (1). In 1869 Friedrich Miescher isolated DNA for the first time (2). Almost a hundred years later, McCarty and Avery proved that the deoxyribonucleic acid (DNA) molecule is responsible for the transmission of inherited information (3). In 1949 Erwin Chargaff showed that DNA is composed of an equal percentage of the nucleotide bases guanine (G) and cytosine (C), as well as of adenine (A) and thymine (T) (4).

It was only in 1953 that the double-helix structure of the DNA molecule was discovered by James Watson and Francis Crick (5). This finding rendered them the Nobel Prize in 1962. Marshall, Casey and Nirenberg unraveled the universal genetic code in 1967 (6) and, two years later, Beckwith´s group isolated the first gene (7). In 1973, the first nucleotide sequencing method was developed by Sanger and co-workers, a two-dimentional chromatography technique (8). This laborious methodology has been thoroughly refined in the past forty years to what is now known as “third generation sequencing techniques”, which include several different methods capable of sequencing an entire bacterial genome in only one day, reviewed in (9).

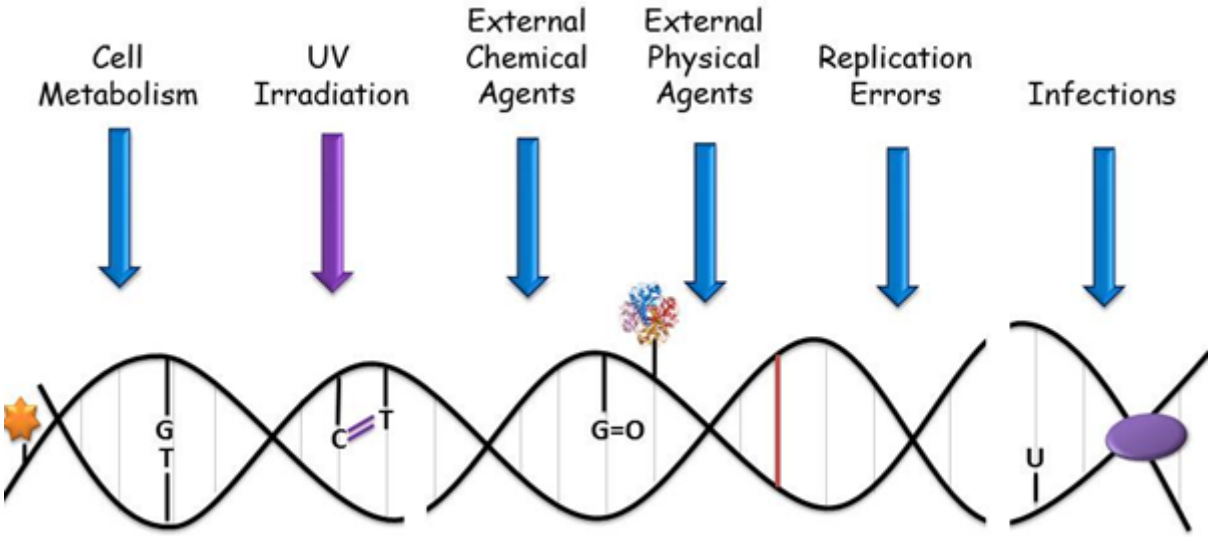
The chemical structure of DNA is well-known today. The double-helix is composed of repeated subunits, the nucleotides. Each nucleotide consists of one phosphate connected to a deoxyribose sugar, which is linked to a nucleotide base: A, C, G or T. On the same double-helix strand, each nucleotide is ligated to the next by covalent bonds in their phosphate groups. Each base is specifically paired to the opposite one in the complementary strand of the double-helix. On a canonic DNA double strand, A pairs with T and G pairs with C, via hydrogen bonds (10).

The DNA molecule contains all the genetic information of an organism and, therefore, the maintenance of its integrity is of the upmost importance to preserve cell functions and to guarantee the individual´s survival. Thus, one might expect the DNA

molecule to be very stable and resistant to injuries. However, DNA is very unstable and is constantly under attack from endogenous and exogenous chemical and physical agents which are capable of damaging its structure.

Different agents may cause several types of damages to DNA. Figure 1 presents some of the most common DNA damaging agents and DNA lesions.

Figure 1 - Common DNA damaging agents and DNA lesions



The most common endogenous and exogenous DNA damaging agents are presented in the upper part of the figure. Some of the most usual chemical and physical alterations found in DNA are represented in the DNA double-strand molecule (adapted from “I Winter course - DNA damage responses: implications in aging and cancer”).

The present work focuses on ultraviolet radiation (UV), the damages it causes in the DNA molecule and the cellular and tissue responses to those damages.

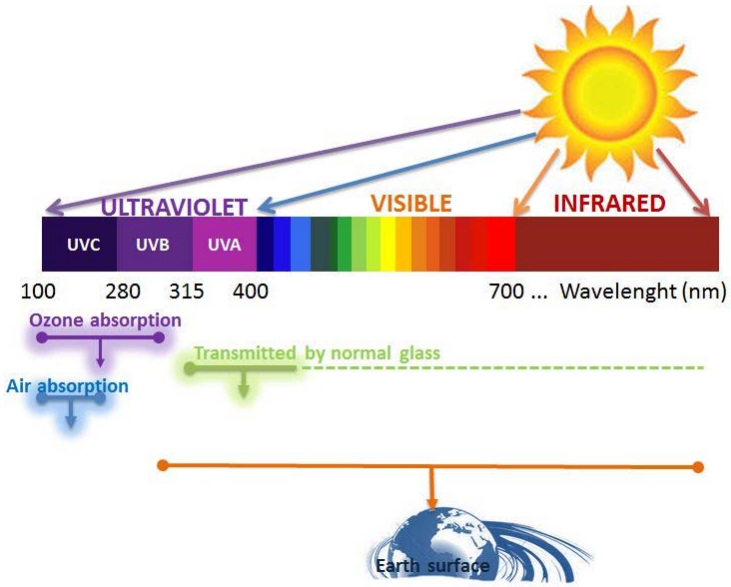
1.2 Ultraviolet radiation (UV)

1.2.1 General characteristics of UV light

Sunlight is responsible for supplying natural energy to Earth´s biological system, enabling the photosynthetic process. However, the ultraviolet component of the sun has clear detrimental effects which correlate with skin cancer incidence, reviewed in (11).

UV light is comprised of wavelengths from 100 to 400 nm and is subdivided into three bands: ultraviolet A (UVA), ultraviolet B (UVB) and ultraviolet C (UVC). The UVC band is composed of wavelengths from 100 to 280 nm and is totally blocked by the Earth´s ozone (O_3) layer and atmosphere [vaporized water, oxygen gas (O_2) and carbon dioxide and monoxide]. The UVB spectrum is comprised of wavelengths from 280 to 315 nm, 95 % of which is blocked by the Earth´s ozone layer. UVA consists of wavelengths from 315 to 400 nm and its totality reaches the planet´s surface (12). Figure 2 presents a scheme of the solar spectrum of light and the penetration of each wavelength through the Earth´s ozone layer and atmosphere.

Figure 2 - Schematic representation of the solar light spectrum and its penetration in Earth´s surface



Different ultraviolet bands have distinct penetration capacities in the Earth´s atmosphere: UVC (100-280 nm) is totally blocked by the ozone layer and atmosphere; 95 % of UVB (280-315 nm) is blocked by the ozone layer; and virtually the totality of UVA (315-400 nm) reaches the planet´s surface.

Four billion years ago the Earth´s atmosphere was anaerobic. The lack of O_2 allowed the totality of the UV spectrum to reach the planet´s surface. UV radiation was therefore a very strong selective agent for organisms, which had to adapt to this extreme condition. It is believed that this scenario was one of the biggest challenges for the terrestrial occupation by organisms. It is speculated that the rate of DNA damage in the ocean´s surface was a thousand times greater than today (13).

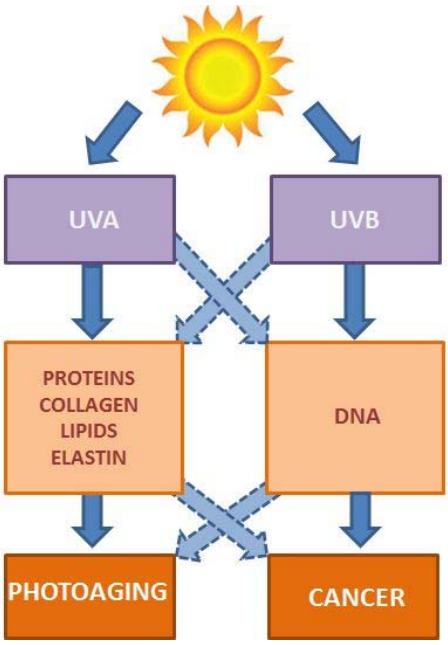
Almost a billion years later sunlight began to be used as an energy source for the photosynthetic process, which in turn began to fill the planet´s atmosphere with O_2. This modification of the Earth´s atmosphere allowed for the development of

aerobic organisms, believed to be the main reason for the explosion of the Animal Phylum, about five hundred and forty million years ago, reviewed in (14). Furthermore, UVC light reacted with the O_2 in the outer layers of the atmosphere, dissociating it´s molecules and permiting O_3 formation. This gas was trapped in layers between 10 and 50 Km from the surface and formed the ozone layer, about four hundred million years ago. As previously mentioned, the present concentration of ozone in the Earth´s atmosphere is largely responsible for blocking the totality of UVC rays and the majority of UVB light from reaching the Earth´s surface, reviewed in (15).

The percentage of UVA and UVB light which reaches the planet´s surface not only varies according to the latitude and longitude, but also with season, altitude and weather conditions (16).

Organic molecules are capable of absorbing different UV bands depending mainly on the characteristics of their chemical bonds. For instance, aromatic protein bonds and nucleic acids are very effective in absorbing UVB, whereas UVA is mainly absorbed by other chromophores, such as the reduced form of nicotineamide-adenine-dinucleotide (NADH), riboflavins and hemoglobin (17). Figure 3 represents the most common molecules which absorb UVA and UVB in the skin.

Figure 3 - Molecules which most commonly absorb UVA and UVB in skin



Because of their physical properties, UVA and UVB´s energy are better absorbed by different organic molecules: UVA is mainly absorbed by proteins, collagen, elastin and lipids, whereas UVB is mainly absorbed directly by DNA. However, as indicated by the doted arrows, UVB and UVA can also interact with other molecules.

The absorption of radiant energy may produce a photo-chemically reactive molecule, rendering molecules to be changed or damaged (11). UV light is considered the most genotoxic physical agent present in our environment today, reviewed in (11,18).

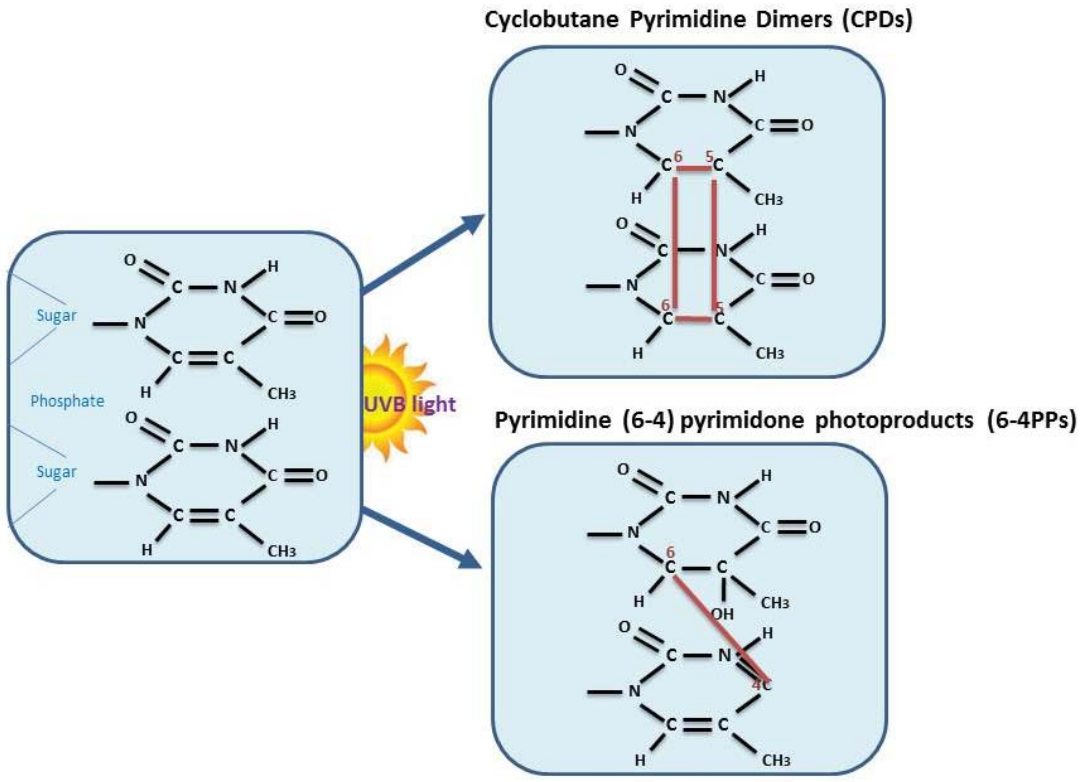
1.2.2 UV-induced photolesions in DNA

It has been known since 1877 that sunlight is capable of destroying bacteria (19). In 1885, it was proven that the germicide effect was caused by UV light from the sun (20). However, it was only in 1946 that the correlation between UV light and skin cancer was established (21).

When the photons of UV radiation are directly absorbed by the DNA molecule, two photolesions may be formed: the cyclobutane pyrimidine dimers (CPDs) and the pyrimidine (6-4) pyrimidone photoproducts (6-4PPs). The CPD lesions are characterized by two covalent bonds between carbons 5 and 6 of two adjacent pyrimidines in the same DNA strand, directly forming a cyclobutane ring. The 6-4PP

lesions are formed due to one covalent bond between carbons 4 and 6 of two adjacent pyrimidines in the same DNA strand, reviewed in (22). Figure 4 shows the chemical structure of each photolesion.

Figure 4 - Chemical structure of UV-induced photolesions

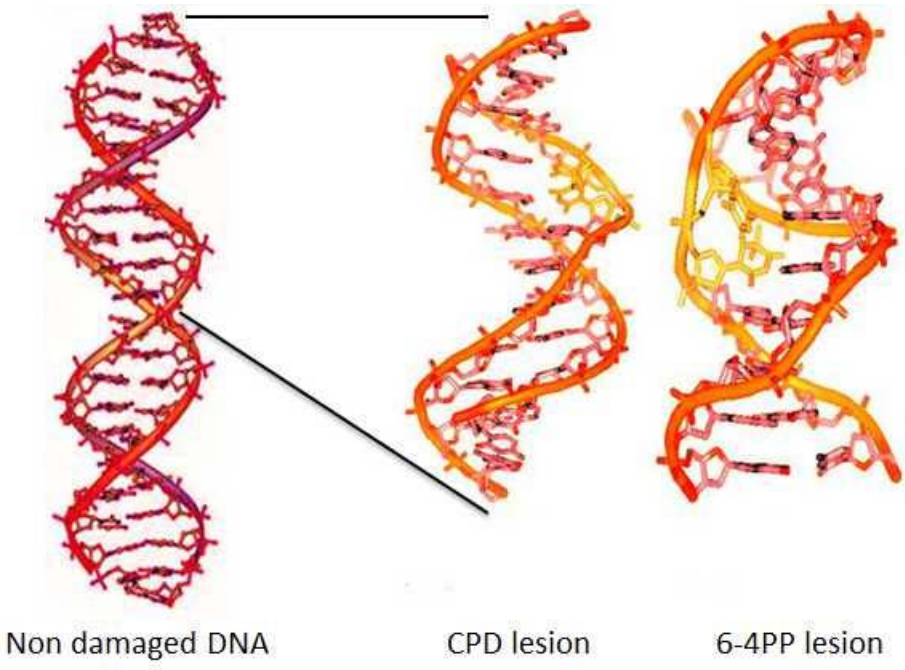


UV induces DNA photolesions through photon absorption, which leads to the formation of covalent bonds between two pyrimidines which are juxtaposed in tandem in the nucleotide DNA sequence. CPDs are characterized by the formation of two bonds between carbons 5 and 6 of the pyrimidines, whereas 6-4PPs are formed by one bond between carbons 4 and 6 of the adjacent bases.

CPD lesions can be formed in four different isoform configurations: *cis-syn*, *cis-anti*, *trans-syn* and *trans-anti*, the first being the most common in double-stranded B-form DNA (23).

CPD photolesions are three to five times more frequent than 6-4PPs, depending on the precise wavelength which reaches the DNA, nucleotide composition and chromatin structure of the DNA fragment (24). Furthermore, CPDs only cause a 30^{o} double-helix distortion (25), whereas 6-4PPs cause a significantly stronger 44^{o} distortion in the DNA double-helix (26), as illustrated in Figure 5.

Figure 5 - Representation of the double-helix distortion caused by CPDs and 6-4PPs



CPD formation causes a 30° double-helix distortion whereas 6-4PP formation causes a more significant 44° distortion. Adapted from Rastogi *et al*., 2011 (27).

In the core of a nucleosome, CPDs are formed regularly on an average of every 10.3 nucleotides, with a strong preference to form away from the histone surface (28). In linker DNA, as well as in naked DNA, CPD formation is nearly uniform, with no periodicity (29,30). Thus, it seems that DNA configuration is a deciding factor for CPD formation (31). In contrast, 6-4PPs are formed with no periodicity within the nucleosomes, but are formed six times more frequently in linker DNA (32). Other DNA-protein interactions, like those common in promoters, may also increase or decrease the DNA susceptibility to specific photolesion formation, i.e., the binding of a certain protein in a specific DNA region may be simultaneously inhibitory for 6-4PP formation and attractive for CPD formation, and vice-versa (33,34).

Furthermore, pyrimidine dimers have a different formation rate depending on the bases which form the photolesion and on the flanking bases 5´ and 3´ of the lesion. In naked UVC-irradiated plasmid, the relative yield of CPD formation is T<>T:C<>T:T<>C:C<>C (68:13:16:3) (35,36).

Even though CPDs and 6-4PPs are the most common photolesions induced in DNA after UV irradiation, they are not the only ones. 6-4PP lesions are formed through an oxetane or azetidine ring intermediate between carbons 5 and 6 of the 5´ pyrimidine and carbon 4 and the oxygen of the 3´pyrimidine. This very unstable ring

eventually breaks, transferring an amino or hydroxyl group to carbon 5 of the 5´pyrimidine, forming a stable bond between carbons 6 and 4 of the adjacent pyrimidines, reviewed in (37). Further stimulation of the 6-4PP lesion with a wavelength close to 315 nm (short UVA) may induce the formation of the 6-4PP valence isomer: Dewar photoproducts (Dewar-PP). Dewar-PP are formed by a slow electrocyclization reaction, generating the β-lactam ring characteristic of this type of DNA lesion (38).

For more information on photolesion structure, formation and distribution in DNA, please refer to the following reviews: Smerdon 1991 (39) and Pfeifer 1997 (22).

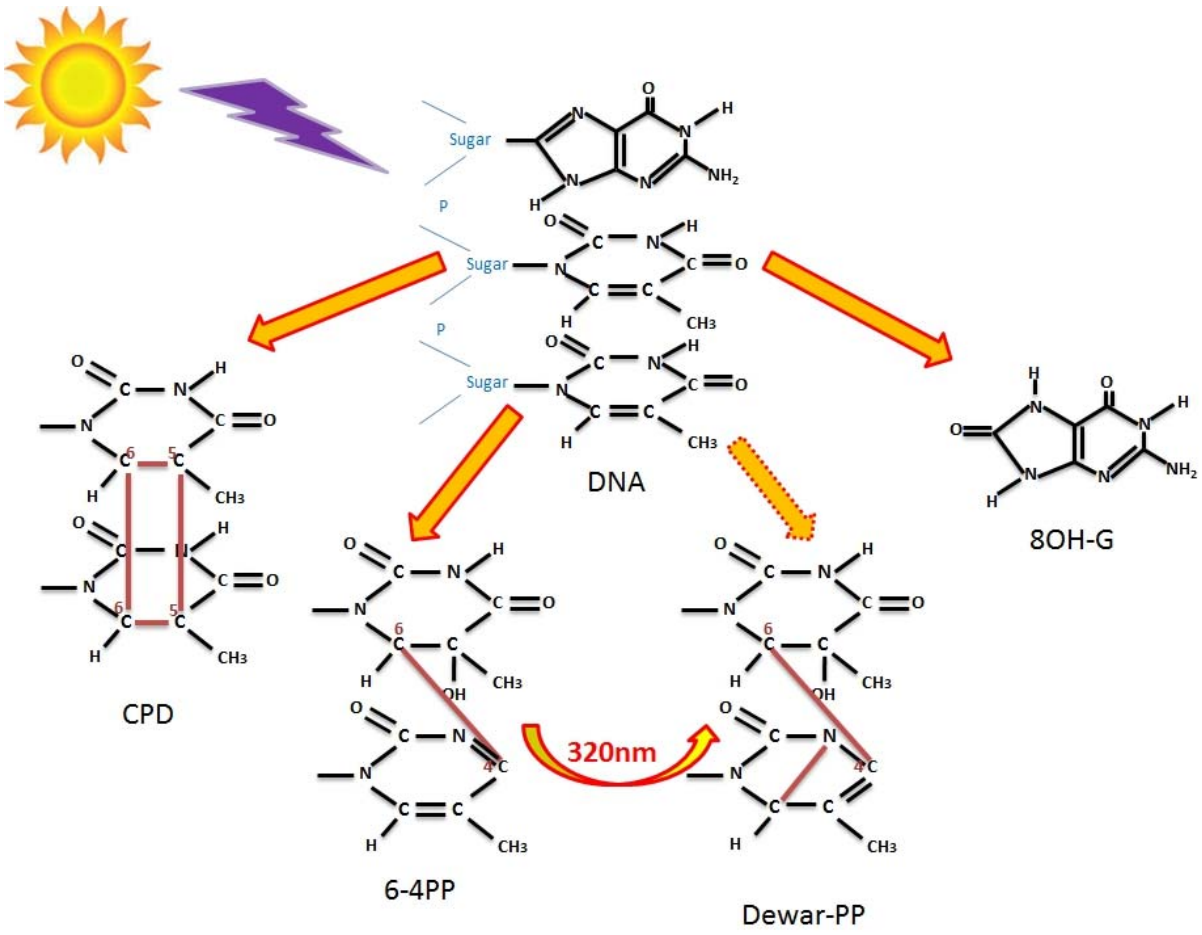
CPDs, 6-4PPs and Dewar-PP are the most common lesions induced by UVB in DNA. However, other DNA lesions can also be induced by UV light.

1.2.3 Other DNA lesions induced by UV radiation

The physical characteristics of each UV wavelength determine which organic molecules will absorb it best, reviewed in (40,41). As previously mentioned, nucleic acids and aromatic protein bonds are very effective in absorbing UVB, whereas UVA interacts mainly with chromophores (17). These specific properties directly correlate with the type of DNA damage caused by each UV wavelength.

It has been properly demonstrated in naked DNA that UVB produces a majority of CPDs, a significant amount of 6-4PP (which can be converted into the Dewar-PP isomer), and an almost undetectable quantity of oxidized lesions. UVA is also capable of directly inducing more CPDs than 6-4PPs, but at a lower frequency. On the other hand, UVA induces a significantly higher level of oxidized damage. Single strand breaks (SSBs) are virtually absent in this irradiation setup after both UVA and UVB exposures (42). Figure 6 represents the most common DNA lesions induced directly or indirectly by UVA and UVB cell irradiation.

Figure 6 - Schematic representation of the most common DNA lesions induced directly and indirectly by UVA and UVB irradiation



CPDs are the most common type of lesion induced by UVA and UVB cell irradiation, although at a lower frequency by UVA light. 6-4PPs are induced at a ~3 lower frequency than CPDs by both UVA and UVB, and may be converted to Dewar-PP by UVA irradiation. Oxidized damage, such as 8OH-G is generated at a relatively higher frequency by UVA than by UVB light. Strand breaks are generated at very low frequencies by both UVA and UVB irradiation.

Therefore, it can be concluded that UVA is capable of damaging DNA both directly, through photolesion formation, and indirectly. The indirect damages induced in DNA by UVA irradiation can be caused by two reactions, denominated type I or II. In the type I reaction, light is absorbed by endogenous chromophores, other than DNA, such as riboflavin, tryptophan or porphyrin, which interacts with the solvent or with the DNA molecule, damaging it. In the type II reaction, excited photosensitizers react with oxygen molecules, generating reactive oxygen species (ROS), which may in turn further damage DNA. Most of the damages caused in DNA through a type I or a type II reaction are oxidized lesions, such as 7,8-dihydro-8-oxoguanine (8OH-G) (43).

Alternatively, ROS can cause DNA strand breaks (44) or even interact with the nucleotide pools, producing oxidized nucleotides which can still be used in DNA transcription or replication (45,46).

Other types of DNA lesions may be formed after UV irradiation, such as 8,8-adenine dehydrodimer, pyrimidine hydrates, thymine glycol and spore photoproducts, among others. However, their formation rate is very low and/or their half-life is very short. Furthermore, some of them can only occur under very specific conditions, such as in an anhydrous environment. However, their occurrence is not part of the scope of this work and, therefore, will not be further mentioned. For a concise review please refer to chapter two of the book titled “DNA repair and Mutagenesis” (47).

Interestingly, the UV- signature mutations (C→T and CC→TT transitions) are the most common type of mutations seen in cells and tissues irradiated either with UVA or UVB light. This indicates that even though oxidized lesions may play an important part in cell responses to DNA damage, this type of lesion may not directly correlate with mutation induction after UV irradiation and its consequent genotoxicity (43,48).

1.2.4 UV-induced DNA mutations

DNA wavelength absorption peaks around 260 nm (UVC), where the highest yields of CPD and 6-4PP formation are found. This relative absorption is 10^2 times smaller for UVB wavelengths and 10^{-5} for UVA (49), which directly correlates with photolesion formation rates and, consequently, to mutation induction (48).

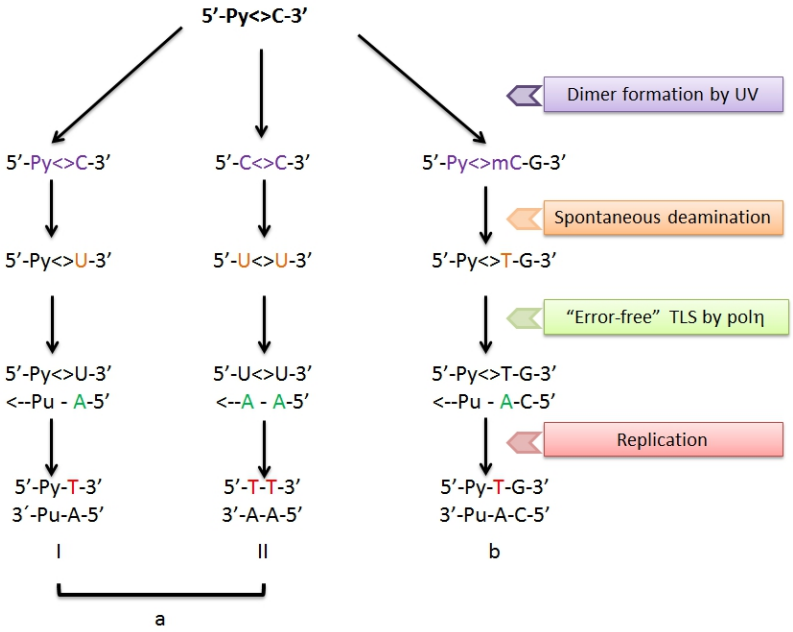
UV irradiation induces specific types of mutation at dipyrimidine sites: the most common is a base substitution of cytosine to thymine (C → T), and the relatively rare tandem base substitution of cytosines to thymines (CC → TT). These two types of mutation are known as UV-signature mutations (48,50,51).

One of the mechanisms through which UV-signature mutations occur is the cytosine deamination in the pyrimidine dimer, resulting in an uracil (U) base, followed by “error-free” translesion synthesis (TLS) of the bulky lesion by polymerase eta (pol η) during DNA replication. This TLS will insert an A opposite to the U, which, if not corrected, will result in a C → T conversion, fixating the mutation. In cases where the two pyrimidines in the dimer are C, both can undergo deamination, followed by the

TLS by Pol η, which may result in a CC → TT conversion, fixating the second type of UV-signature mutation in the DNA strand (52).

Alternatively, the deamination may occur in a methylated C (5-methylcytosine; mC), originating a T. In this case, TLS will probably add an A opposite to each T. In turn, just as in the previous model, after DNA replication this C → T conversion will be fixated as a mutation. This specific type of mutation, very common in methylated CpG islands, is known collectively as Solar UV-signature mutation (53), reviewed in (54). Figure 7 presents the aforementioned induction models for UV-signature mutations and Solar UV-signature mutations.

Figure 7 - Possible mechanisms of UV and Solar UV-signature mutations induction



Cytosine deamination of pyrimidine(s) leads to the formation of uracil(s) in the pyrimidine dimer; a) "error-free" translesion synthesis by Pol η inserts an adenine in front of each uracil. Alternatively, b) deamination of methylated cytosine leads to the presence of thymine bases in the pyrimidine dimer. Both mechanisms explain the formation and fixation of UV-signature and Solar UV-signature mutations, respectively (C → T and CC → TT). "<>" represent dimers. Adapted from Ikehata & Ono, 2011 (54).

However, TLS by Pol η alone cannot explain the UV-signature mutations, since the same mutational spectrum is seen in organisms which do not express Pol η or any homologue, such as *Escherichia coli* (*E. coli*) (55) (56). Furthermore, Xeroderma Pigmentosum Variant (XP-V) human cell lines, which fail to express a functional Pol η, present the UV-signature mutations and at a higher rate as well (57).

Therefore, at least part of the UV-signature mutations cannot be related to the Pol η activity. It is known that in *E. coli*, when polymerase 5 (Pol V) is absent, there is

a suppression of UV-induced mutagenesis (58). This enzyme is known as an “error-prone” TLS polymerase, usually inserting an A opposite to a pyrimidine dimer, which also explains the C → T and CC → TT conversions (59). It is possible that other TLS polymerases, such as polymerase Rev1 (Pol Rev1), polymerase kappa (Pol κ) and polymerase iota (Pol ι), may also play a similar role in UV-signature mutations induction, reviewed in (54).

For more detailed information on UV-photoproduct TLS, please refer to subsection 1.5 of the present work.

Furthermore, UV-signature mutations are induced mainly by CPDs (60). Even though it has been established that 6-4PP significantly contributes to the formation of UV-signature mutations (61,62), the mechanism by which these photoproducts lead to the induction of these mutations is unclear, probably involving the aforementioned “error-prone” polymerases: Pol Rev1, Pol κ and/or Pol ι (63,64).

Dewar-PP may also play an important role in UV-induced mutagenesis, since they are more stable than their isomer 6-4PP and cause a similar double helix distortion (65). However, the mechanism through which they may cause these mutations is not clear, but just like with 6-4PPs, it seems to involve “error-prone” polymerases (66).

In addition to the UV-signature mutations, the UV irradiation usually also leads to triplet mutations, especially in DNA repair-deficient backgrounds, such as alterations in the nucleotide excision repair (NER) pathway (67–69). Since CPDs are a poor substrate for NER, the lesions which are more probably involved in the induction of triplet mutations are the 6-4PPs and the Dewar-PPs. Therefore, the formation of the triplet mutations must involve “error-prone” polymerases. In the model proposed by Ikehata & Ono (54), the TLS polymerase would add a mismatched nucleotide 3´ to the dipyrimidinic site, consistent with the “error-prone” characteristic of some polymerases, such as Pol κ (63).

For more detailed information on UV-induced mutagenesis, please refer to the excellent review by Ikehata & Ono of 2011 (54).

1.3 DNA repair

Considering the relevance of genome integrity maintenance and the constant attack of endogenous and exogenous agents on DNA, it is no surprise that, during evolution, several DNA repair mechanisms have been selected in order to revert, correct, remove or adapt to the existence of such a wide variety of DNA lesions.

For instance, the Mismatch Repair (MMR) mechanism is capable of correcting wrongly paired DNA bases; Base Excision Repair (BER) deals with damaged DNA bases and SSBs; Translesion Synthesis (TLS) counts on specific DNA polymerases capable of replicating through DNA lesions; Homologous Recombination and Non-Homologous End Joining (NHEJ) deal with SSBs and, mostly, with double strand breaks (DSB) in the DNA, reviewed in (70).

Two DNA repair mechanisms are capable of dealing with the photolesions induced by UV irradiation (CPDs and 6-4PPs): photolyases and the NER pathway, which will be discussed in further detail in sections 1.3.1 and 1.3.2 of the present work, respectively.

1.3.1 Photolesion repair by photolyases

Photorepair of UV-induced DNA lesions was the first DNA repair mechanism to be discovered. In 1949, Albert Kelner was UV irradiating bacteria, searching for mutations which could lead to the production of novel antibiotics. Kelner was having trouble duplicating the mutation rates when he finally realized that the bacterial cultures which were exposed to white light after the UV exposure had lower mutation frequencies (71,72). At about the same time, Renato Dulbecco made similar discoveries working with bacteriophages and UV irradiation (73).

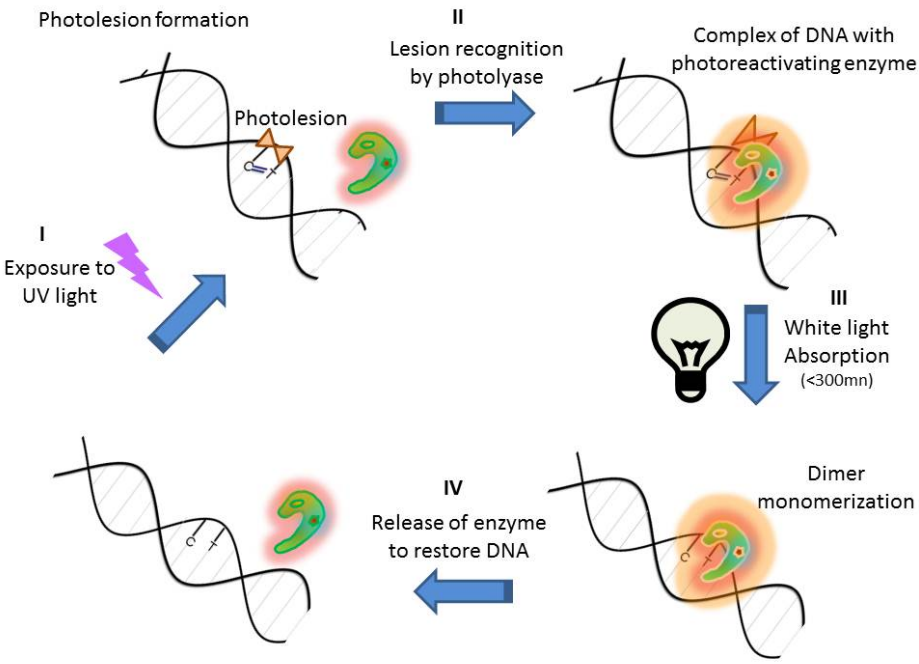
In Kelner´s second article about this subject, the process was named photoreactivation (72). Between 1958 and 1962, Claud Rupert demonstrated that the photoreactivation process actually depended on an enzyme, called photolyase (74–77). In his 1962 articles, Rupert also showed that the DNA-photolyase complex is formed in the dark and that the average turnover for lesion recovery is 1-2 minutes (76,77).

In the 80´s, the chromophores present in the photolyase enzyme were identified: all photolyases have a flavin adenine dinucleotide (FAD) as the first

chromophore. Photolyases may have an additional second chromophore, which varies from species to species: 5,10-methenyltetrahydrofolyl polyglutamate (MTHF) or 8-hydroxy-5-deazaflavin (8-HDF) (78–81). It was later proved that only FAD chromophore is essential for enzymatic activity in photolyases purified from several organisms, especially when FAD acts as a sensitizer and not the second chromophore (82–84).

Figure 8 presents a schematic representation of photolesion recognition by photolyase, lesion-enzyme complex formation in the dark and light-dependent lesion monomerization.

Figure 8 - Schematic representation of dimer photoreactivation

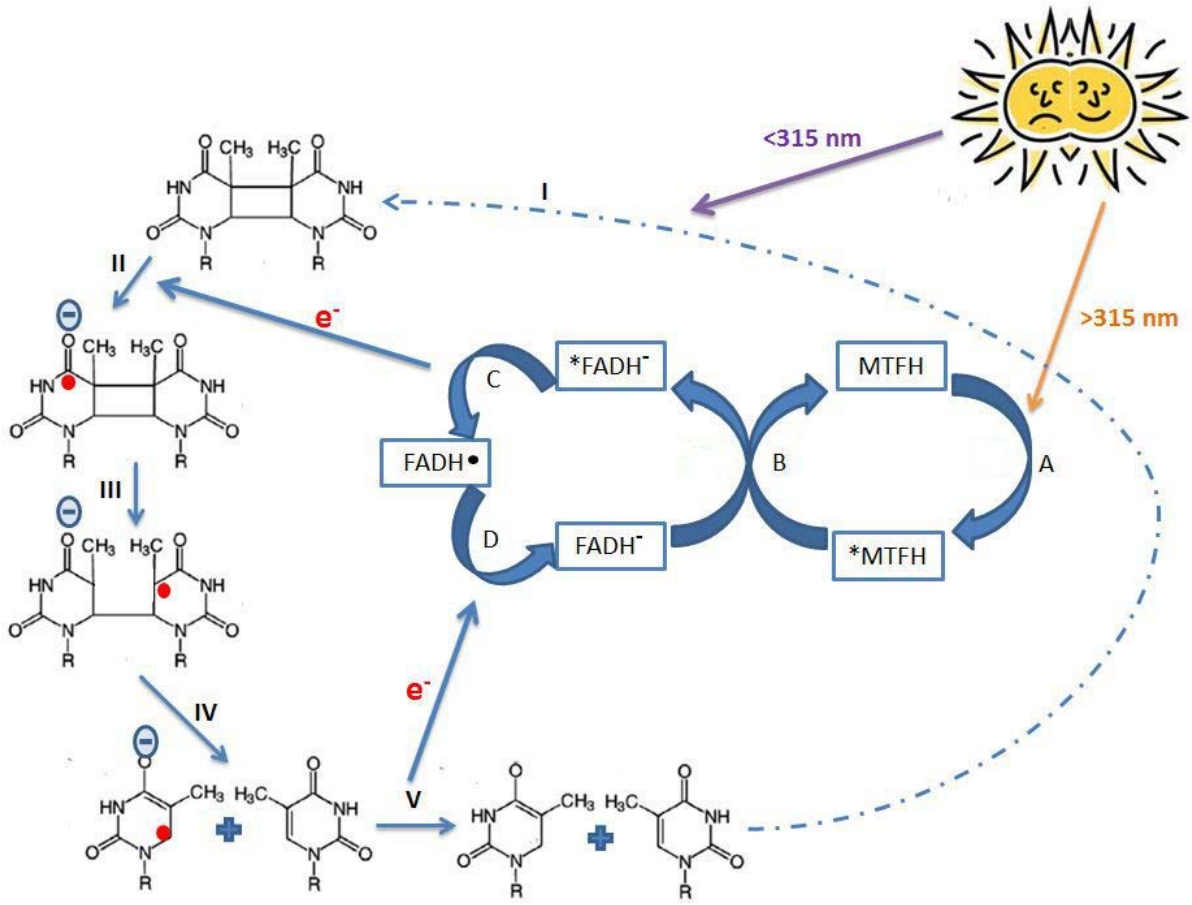


UV irradiation leads to the formation of pyrimidine dimer (I); in the dark, complex dimer-enzyme is formed (II); Photon absorption activates photolyase (III); and dimer is directly monomerized (IV); finally, the complex is undone and the enzyme is ready for another photoreactivatioin reaction.

Photolyases are capable of reconverting the pyrimidine dimers back to monomers through an electron transfer from the FAD chromophore to the lesion, breaking the extra covalent bonds which unite the dimer, allowing pyrimidine monomerization, followed by an electron transfer back to FAD. If FAD acts as a sensitizer, it will be directly excited by a photon. Alternatively, another chromophore (MTFH or 8-HDF) may first be sensitized by the photon and, in turn, excite FAD

which will transfer an electron to the dimer, as previously described. This reaction takes less than 1 ns (85) and is represented in Figure 9:

Figure 9 - Representation of chemical reactions involved in photoreactivation



A chromophore (MTFH) is sensitized by a photon (A) and transfers energy to the second chromophore (FAD) (B). A pyrimidine dimer (CPD in this case) is formed by UVB irradiation of DNA (I). FAD donates an electron (e^-) to the pyrimidine dimer (II), breaking the first (III) and then the second covalent bond which forms the cyclobutane dimer (IV); electron is transferred back to FAD, finalizing the photoreactivation process (V).

All previously mentioned studies on photoreactivation involved CPD repair by specific CPD-photolyase. It was not until 1993 that an enzyme capable of photoreactivating 6-4PPs was described in *Drosophila melanogaster* (86). CPD-photolyase and 6-4PP-photolyase are homologous, sharing an even higher amino acid sequence similarity then that between several CPD-photolyases (87).

There is strong evidence that 6-4PP-photolyase can also photoreactivate Dewar-PP, although with less affinity (88). This process begins with the Dewar-PP conversion into its 6-4PP isomer, followed by its photoreactivation, all of which occurs through 6-4PP-photolyase activity (89). CPD-photolyases (a) and 6-4PP-photolyases

(b) are very specific for their substrate: CPDs (a) and 6-4PP and Dewar-PP (b), with no cross-recognition having ever been described (87).

Both CPD-photolyases and 6-4PP-photolyases are part of the blue-light receptor family, together with the cryptochromes, which are involved in circadian rhythm control (87).

Blue-light receptor family genes are widely distributed throughout all kingdoms of life, including viruses, prokaryotes and eukaryotes (both plants and animals). However, placental mammals do not have photolyase encoding genes. One possible explanation for the absence of these genes is that placental mammals developed nocturnal habits during evolution, which may have released the evolutionary pressure for their presence (90).

For more detailed information on photolyase structure, function and distribution, please refer to the following works: the book titled “DNA Repair and Mutagenesis” (70) and Eker´s 2009 review (90).

There is no DNA repair process more efficient and effective than one involving only one enzyme, composed of one single polypeptide, and requiring only one step which does not include DNA cleavages or base substitution.

However, placental mammals cannot count on direct photoreversal of UV-induced lesions, and depend exclusively on the NER pathway to remove photolesions from their genomic DNA.

1.3.2 Photolesion repair by the Nucleotide Excision Repair (NER) pathway

The first evidences of the existence of a DNA repair pathway not involving photoreactivation came in 1958, when Hill discovered an *E. coli* strain particularly sensitive to UV irradiation (91), and in 1962, when Howard-Flanders and co-workers defined a genic *locus*, also in *E.coli*, which controlled photoproduct removal (92). In 1964, simultaneously and independently, Setlow & Carrier and Boyce & Howard-Flanders demonstrated that, in this lesion removal pathway, a small DNA fragment containing the lesion was excised (93,94). Only six months later, Pettijohn and Hanawalt showed that after the excision of the damaged DNA fragment there was a form of non-semiconservative DNA synthesis (95).

In the same year, Rasmussen & Painter demonstrated that DNA repair also occurs in mammalian cells (96). In the following decade several mechanisms of DNA

repair, including BER and MMR, were discovered, reviewed in (97). In 1985, Bohr and colleagues showed that lesions present in the transcribed strand of active genes are removed faster by a NER subpathway, the transcription coupled repair (TC-NER), than lesions elsewhere in the genome, which are removed by the global genome repair (GG-NER) (98).

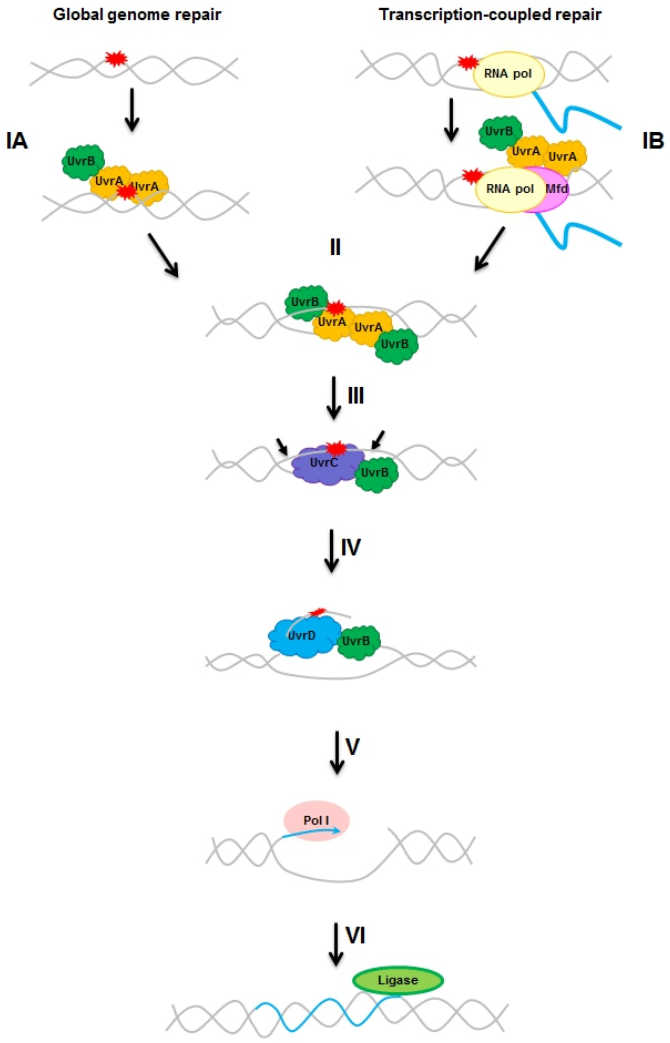
NER is a highly versatile pathway, capable of removing a wide variety of bulky lesions. Its substrates not only include UV-induced photoproducts (CPD, 6-4PP and Dewar-PP), but also oxidized damages and intrastrand crosslinks. Even though these lesions can be caused by several different endogenous and exogenous agents and have little to no chemical similarity, the recognition factors are able to identify the distortion they cause on the double-helix, despite of its origin (99).

The NER pathway is widely found in both prokaryotes and eukaryotes. In prokaryotes, only seven proteins are involved (four UV resistant proteins: UvrA, UvrB, UvrC and UvrD ; mutation frequency decline protein, Mfd; polymerase 1, Pol I; and a ligase, Lig) in the lesion removal, whereas in mammals this pathway requires the concerted action of over thirty proteins, reviewed in (99,100).

The first step in this lesion-removal system is (1) lesion recognition, followed by (2) region stabilization, (3) double-helix opening, (4) damaged fragment excision, (4) re-synthesis of the excised fragment, and (5) ligation to pre-existing contiguous DNA.

E. coli is the model organism in the study of prokaryotic NER. As schematized in Figure 10, the DNA lesion is either recognized by a stalled RNA polymerase (RNA Pol) with the aid of Mfd and $UvrA_2B$ in the TC-NER, or directly by the later protein trimer in the GG-NER. After lesion recognition, subpathways converge to the same pathway, a second UvrB is recruited and $UvrA_2$ leads to a DNA conformational change. The complex is released and only one UvrB stays at the lesion site. UvrC is recruited and forms the complex UvrBC. The fragment which contains the damage is excised about 5 nucleotides upstream and then on the eighth nucleotide downstream of the lesion by UvrC (101); UvrD aids in the removal of the excised fragment; Pol I synthesizes the DNA which is then ligated by a DNA ligase. Energy (adenosine triphosphate, ATP) is required for the NER pathway to be successful, reviewed in (99,100).

Figure 10 - Simplified model of nucleotide excision repair in *E. coli*



In the GG-NER subpathway, the lesion is recognized by the $UvrA_2B$ complex (IA). In the TC-NER subpathway, RNA Pol stalled by the lesion recognizes the damage with the aid of Mfd and the $UvrA_2B$ complex (IB). After the recognition step, subpathways converge into one pathway. $UvrA_2$ recruits a second UvrB and causes a DNA conformational change (II). The protein complex is then released, only one UvrB remains and UvrC is recruited to make incisions 5´ and a 3´ to the DNA lesion (III). UvrC is released and UvrD is recruited to aid in the removal of the damaged DNA fragment (IV). The gap is filled by Pol I (V) and the newly synthetized DNA is sealed by a DNA ligase (VI).

The proteins involved in the NER pathway are conserved inside prokaryotic and eukaryotic groups, but not between them, reviewed in (99). The eukaryotic system is significantly more complex.

In the eukaryotic GG-NER, the lesion is recognized by the complex xeroderma pigmentosum (XP) complementation group C (XPC) – human RAD 23 homolog B (hHR23B), potentially altering the double-helix structure at the damage site, allowing for the other factors to recognize and stabilize the damaged region (102). However, there is evidence that if the lesion severely distorts the DNA double-helix, XPC may not be required for its recognition (103). The UV-damaged DNA binding (UV-DDB)

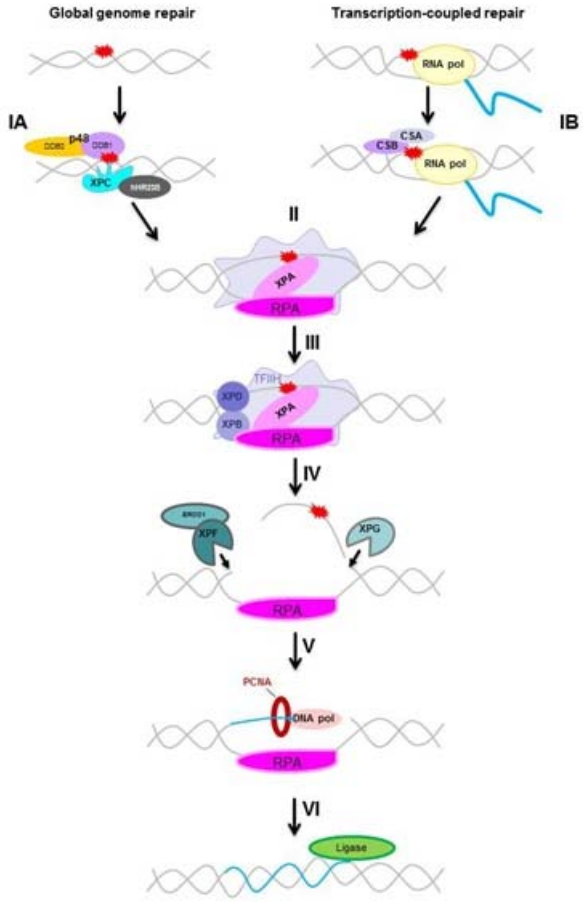
protein, also known as protein 48 (p48) or XPE, is composed of a dimer (DDB1-DDB2), and seems to aid XPC in the recognition of some specific types of damage, such as CPD (104). XPA also has affinity with damaged DNA and helps in lesion recognition and other NER factors recruitment (105).

Once XPA binds to the damage site, it recruits the transcription factor II H (TFIIH) complex (106). TFIIH is formed of nine subunits and has a basal role in transcription. Some of the TFIIH subunits required for NER are: XPB, XPD, the complex excision repair cross-complementing rodent factor 1 (ERCC1)-XPF (ERCC1-XPF) and XPG (107,108). After TFIIH is recruited, in the presence of XPG, XPC is released (106). XPB has a 5´-3´helicase activity, while XPD´s activity is 3´-5´ (109). While the helicases are opening the double-helix of the region containing the damage, the replication protein A (RPA), which has high affinity for single-stranded DNA (ssDNA), is recruited (110,111), forming a complex with XPA. The nucleases then take part, excising a 24-32 nucleotide fragment. ERCC1-XPF makes the incision 16-25 bonds 5´ of the damage (112) and then XPG cuts two to nine phosphodiester bonds 3´ of the lesion (113–115). This pre-incision complex (PIC) is then released and the gap is filled by a proliferating cell nuclear antigen (PCNA) dependent polymerase (probably polymerase delta, Pol δ, or polymerase epsilon, Pol ε) (116), with RPAs stabilizing the ssDNA (106). The gap is finally sealed by a DNA ligase, possibly Ligase I (117).

The previously described GG-NER subpathway is responsible for recognizing and removing lesions which can be situated anywhere in the genome. The other subpathway, TC-NER, differs only in the lesion recognition step and is responsible for lesion removal from the transcribed strands of active genes. In TC-NER, when RNA polymerase II (RNA Pol II) is stalled at a lesion site during transcription, it recruits the other NER factors, with the aid of Cockayne Syndrome proteins A and B (CSA and CSB). CS proteins seem to support the RNA Pol II complex allowing for its temporary removal from the lesion site (118,119). Both GG-NER and TC-NER require energy (ATP) in several of their steps.

Figure 11 presents a simplified model of the mammalian NER pathway. For more detailed information about the prokaryotic and eukaryotic NER pathways, please refer to the reviews by Wood (120) and Batty (99).

Figure 11 - Simplified model of the mammalian nucleotide excision repair (NER) pathway



In the GG-NER subpathway, the lesion is recognized by the XPC-hHR23B complex, with the aid of the p48 protein (IA). In the TC-NER subpathway, RNA Pol II stalled by the lesion recognizes the lesion site (IB). After the recognition step, both subpathways converge into one pathway. XPA and RPA are recruited and stabilize the lesion site (II). TFIIH complex (XPB, XPD, ERCC1-XPF, XPG) is recruited and DNA double-helix is unwinded by helicases XPB and XPD (III). ERCC1-XPF excises 5´ of the lesion and then XPG excises 3´ (IV). Proteins are released and the gap is filled by a PCNA dependent DNA polymerase (V) and the newly synthetized DNA is sealed by a DNA ligase (VI). Adapted from Quayle *et al*., 2011 (121).

While CPD-photolyase and 6-4PP-photolyase are able to specifically remove CPDs and 6-4PPs at the same rate (122), the NER pathway removes 6-4PPs much faster than CPDs. The totality of 6-4PPs is removed within 3 hours, whereas only 60 % of CPDs is removed in 24 hours, even for low UVC doses (10-15 J/m^2) (123,124). This difference is probably related to the different DNA distortion caused by each photolesion (27) and to the fact that XPC and XPA recognize lesions in DNA by the distortion they cause on the double-helix.

Therefore, when considering GG-NER, the more distortive the DNA lesion is, the quicker it will be recognized and removed. In this model, both types of lesion

present in the transcribed strand of active genes must be removed by TC-NER with the same efficiency, reviewed in (22,106).

Furthermore, photolesion removal by TC-NER is significantly quicker than by GG-NER: in eight hours, 80 % of CPD lesions are removed from the transcribed strands of active genes, while less than 30 % is removed from the rest of the genome (125). This difference is probably related to the more efficient surveillance exercised by TC-NER, which counts on RNA Pol II stalling as a recruiting signal for the NER proteins, and also to lesion accessibility (22).

As previously mentioned, p48 seems to aid XPC in CPD recognition (104). However, rodents, such as mice, lack this protein and are virtually unable to remove CPD lesions through GG-NER (126). Therefore, these lesions must accumulate throughout their genome, except in active genes.

When photolesions are not repaired by either photolyases nor by the NER pathway, there is a mechanism which helps cells to deal with their presence, avoiding the elicitation of other cellular responses, such as senescence and apoptosis (viewed in more detail in section 1.5 of the present work): the translesion DNA synthesis.

1.4 Translesion synthesis (TLS) of UV-induced photoproducts

Replicative DNA polymerases are not able to accommodate most damaged DNA in their active site (including CPDs, 6-4PPs and Dewar-PPs), promoting a physical blockage to strand elongation, reviewed in (127). Therefore, the presence of photolesions during cell replication may lead to replication fork collapse which, in turn, may culminate in cell death (reviewed in section 1.5 of the present work).

There is, however, a mechanism which helps cells to cope with the presence of DNA damage during replication: DNA translesion synthesis (TLS). TLS was first observed in 1968 by Rupp and Howard-Flanders, when they identified gaps after DNA synthesis in *E. coli* strains defective for DNA repair (128). The idea of TLS was already circulating in the 1970´s, but it was only in 1996 that the first TLS polymerase was identified (129), reviewed in (127).

Nowadays, several TLS polymerases are known. Some of them are “error-free” for specific types of DNA lesions, while others are considered to be “error-

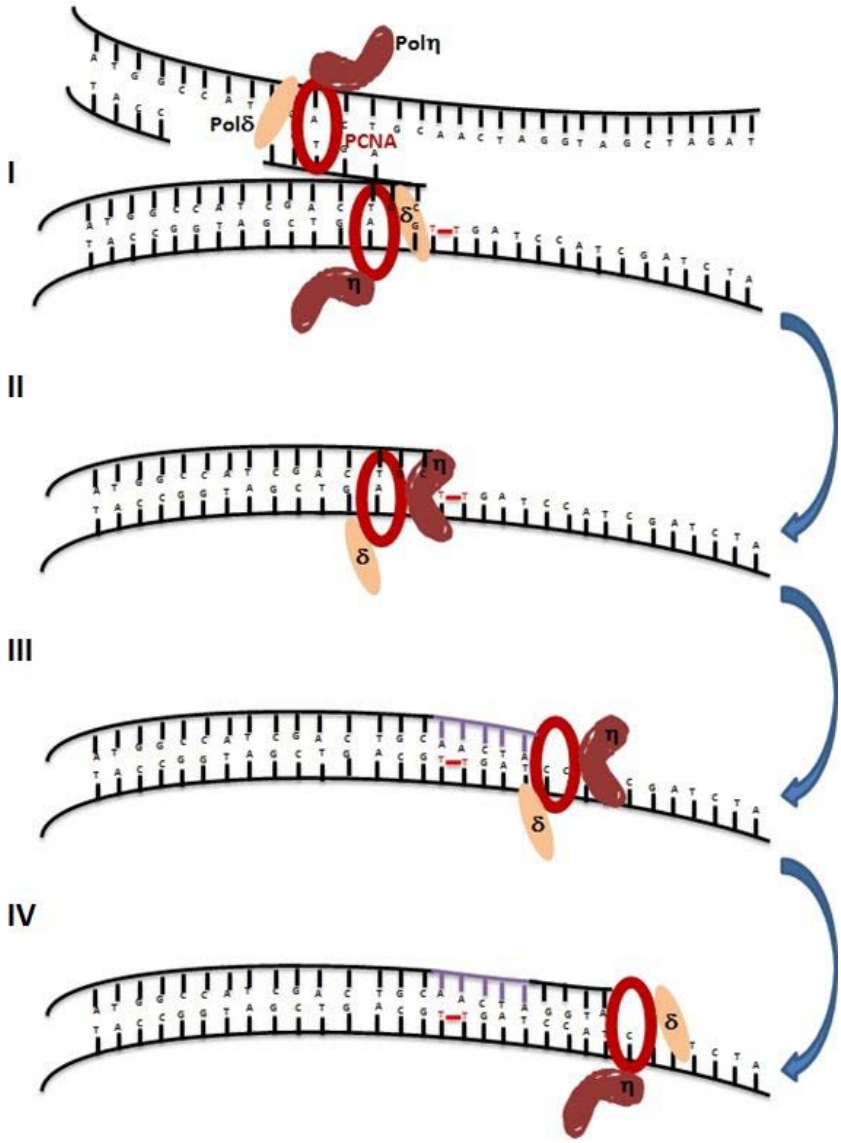
prone". TLS polymerases are found in all domains of life, which suggests they play a very important role in genome maintenance, reviewed in (130).

The general principle by which TLS polymerases are capable of replicating through DNA damage is that after the replicative polymerase (Pol ε or Pol δ) is stalled at the DNA damage site, PCNA suffers a series of modifications (ubiquitilation, sumoylation and/or phosphorylation), reducing its affinity with that polymerase and increasing the affinity for a specific TLS polymerase, which is then recruited to the site of the lesion. In combination with PCNA, this TLS polymerase will replicate the damaged site and, possibly, extend the patch for a few nucleotides before the replicative polymerase resumes the DNA duplication process. Alternatively, depending on the type of lesion and on the TLS polymerase involved, a second TLS polymerase, or even the replicative polymerase, may make the patch extension, reviewed in (127).

The present work focuses on Pol η, which is capable of transposing *cys-syn* T<>T CPD lesions with accuracy. Pol η is part of the Y polymerase family and was first identified in yeast in 1999 (131) and, later in the same year, in humans (132,133).

The active site of Pol η is particularly large and can accommodate both bases of the pyrimidine dimer. It also stabilizes the T<>T, the most common type of CPD, so that two As can be paired with two Ts. To ensure that replication is accurate after Pol η is released and a replicative polymerase resumes the DNA synthesis, it also adds three nucleotides after the lesion while still stabilizing the dimer, guaranteeing that there is no DNA distortion after the translesion patch (134,135). Figure 12 presents a simplified model for the translesion of T<>T by Pol η, a process considered "error-free" (~2 % errors), reviewed in (135).

Figure 12 - Simplified translesion mechanism of Pol η of T<>T lesion



The replicative polymerase is stalled at the presence of T<>T dimers (I). It is substituted by the TLS "error-free" Pol η (II), which is capable of adding two As pairing the two Ts and three more bases before the replication fork switches back to the replicative polymerase.

When the photolesion in question is 6-4PP or Dewar-PP, or when Pol η is not present, it seems that other TLS polymerases take place, in a more "error-prone" fashion. There is strong evidence suggesting that the polymerases involved in this process are Pol κ or Pol ι, in combination with polymerase zeta (Pol ζ) (63,64,136,137). It has recently been shown that Pol ζ is not only involved in "error-prone" TLS but also in "error-free" (< 2 % errors) 6-4PP TLS, independently of Pol κ or Pol ι (138).

For more detailed information about the structure and action mechanism of TLS polymerases, including Pol η, please refer to the following reviews: Livneh, 2010 (139) and Sale, 2012 (127).

1.5 Cellular responses to UV-induced photoproducts

The main regulators of cellular responses to DNA damage are ataxia telangiectasia mutated (ATM), ATM and RAD3 related (ATR) and DNA protein kinase (DNA-PK). It is known that ATM and DNA-PK generally respond to DSB, whereas ATR is mainly activated by the presence of ssDNA, reviewed in (27).

ATR is the principal regulator of the cell responses against UV-induced DNA damage, mostly through RPA signaling in ssDNA regions at stalled replication forks. This DNA structure also recruits the ATR-interacting protein (ATRIP) and the 9-1-1 complex [RAS-related associated with diabetes (RAD) 9 (RAD 9), RAD 1 and hydroxyurea sensitive homolog to *S. pombae* 1 (HUS 1)] which in turn recruits topoisomerase binding protein 1 (TOPBP1). This protein then activates ATR through ATRIP, reviewed in (140).

Activated ATR is capable of phosphorylating a series of effector signals which may lead to specific cell cycle checkpoint activation, recruitment of DNA repair systems, senescence induction, different types of cell death (such as apoptosis or necrosis), or autophagy. It is possible that several of these responses are elicited at the same time or in sequence. For instance, the presence of DNA damage may induce checkpoint activation followed by the recruitment of a DNA repair pathway; if the damage is not satisfactorily removed, the cell may undergo apoptosis, reviewed in (27).

One of the main targets of ATR is checkpoint 1 (CHK1). Phosphorylated CHK1 phosphorylates cell division cycle protein 25 (CDC25), whose consequent degradation will lead to G1/S and/or intra-S phase checkpoint activation, preventing cells to enter mitosis, reviewed in (27).

Another ATR target is the transcription factor protein 53 (p53), a central regulator of DNA damage response. P53 controls a series of UV-responses, from checkpoint activation, to DNA repair proteins recruitment, and even apoptosis and cell proliferation. This broad number of roles that p53 plays in cell responses to UV irradiation is due to a high diversity of targets. For instance, p53 interacts with the

CDK inhibitor protein 21 (p21), which arrests cells in the G1/S checkpoint, reviewed in (27).

Checkpoint activation is particularly important since it allows cells time to recruit DNA repair proteins to the DNA damage site, preventing the cell to attempt to replicate with damaged DNA. Replication of damaged DNA may lead to mutation, or cell death induction, reviewed in (141).

The UV-induced mutation pathways have been previously explained in section 1.2.4 of the present work. It has also been previously explained how dipyrimidine lesions can cause transcription fork arrest (142) (section 1.4), which in mammal cells is usually repaired by TC-NER (section 1.3.2). By the same principle, it is also possible for photolesions to stall replication fork (subject of the previous section).

Considering that photolesions are capable of stalling transcription forks, they may be able to alter the transcription pattern of a given cell, with severe consequences, depending on the genes involved (143,144).

However, if the photolesion stalls a replication fork, and it is not repaired, it may lead to fork collapse, which can cause DSBs. This type of DNA break can not only lead to genomic instability, chromosome rearrangements and mutations, but it is also a very strong apoptotic signal (145).

UV irradiation can induce cell death through several mechanisms, including necrosis when the injury is too severe for the cell to deal with. Most commonly, when lesions are not efficiently removed, apoptosis is induced in a p53/p21/B-cell lymphoma 2 (BCL2) associated protein X (BAX) dependent manner, reviewed in (146).

1.6 NER deficiency related disorders

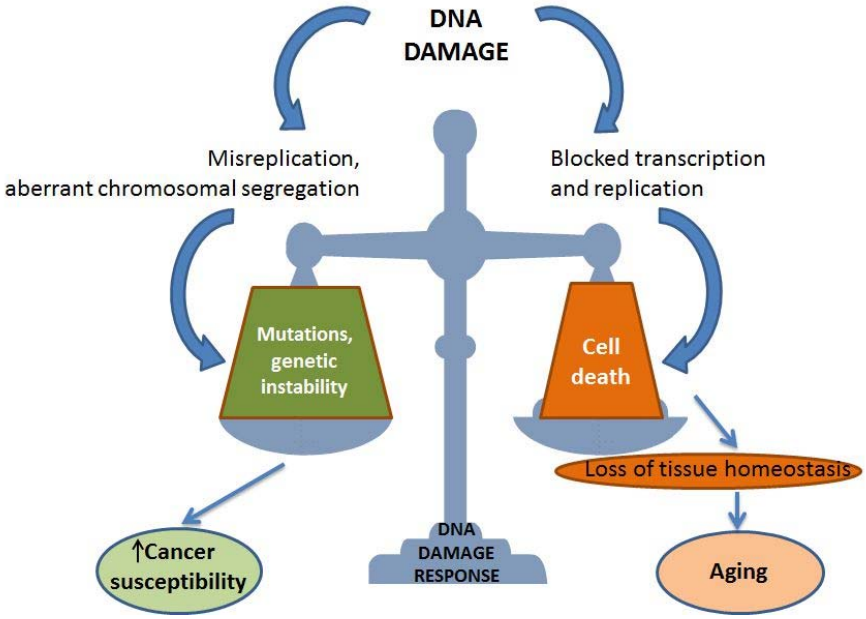
If the fact that DNA Repair is spread throughout all kingdoms of life is not enough proof of the importance of these mechanisms, the existence of several disorders related to different deficiencies in DNA Repair should provide enough evidence. These include Xeroderma Pigmentosum (XP), Cockayne Syndrome (CS), Trichothiodystrophy (TTD), Werner Syndrome (WRN), Bloom Syndrome (BLM), Rothmund-Thompson Syndrome (RT), Fanconi Anemia (FA), Ataxia Telangiectasia (AT) and Hutchinson-Gilford Syndrome (HGPS).

All of the previously mentioned conditions may, at least in some cases, manifest themselves with progeria. The term “progeria” comes from the Greek “pro” (before) and “géras” (old age) and means “segmented premature aging”. The term “segmented” is added to emphasize that not all tissues from the organisms necessarily age at the same speed, meaning that, in some diseases, only a few organs may be affected.

Even though since the late 1950´s the theories from Failla and Szillaard suggested that normal aging was probably related to genomic mutations, the intimate connection between DNA repair deficiencies and aging was not well understood for a long time, reviewed in (147–149). It was only in the late 1990´s that Hoeijmakers´ group was able to establish this link, analyzing data from several mouse models with DNA repair deficiencies and progeria.

Comparing the deficiencies and phenotypes of NER deficient animals Hoeijmakers´ group observed that TC-NER deficient animals tended to have increased cell death and progeria, as did CS and TTD patients. On the other hand, GG-NER deficient mice tended to accumulate genetic mutations and have enhanced cancer susceptibility, as observed in most XP patients. With these observations, they were able to conclude that the link between DNA repair and aging was the DNA damage response (DDR): cell death culminated with loss of tissue homeostasis and consequent aging, whereas low cell death rate and consequence genetic mutation accumulation led to an enhanced cancer susceptibility, reviewed in (141,150,151). Figure 13 represents the delicate balance between cancer and aging.

Figure 13 - The delicate balance between aging and cancer



DDR regulates the cell´s response against DNA damage: if the lesion is too severe, it may lead to cell death and consequent loss of tissue homeostasis (right side of the scale); on the other hand, if lesions are not removed and the cell does not die, it may accumulate damages that can induce mutations, which will increase cancer susceptibility (left side of the scale).

The present work will focus on two NER deficiency related diseases, investigating the correlation between their cancer susceptibility and UV light: XP and CS.

1.6.1 Xeroderma Pigmentosum (XP)

Xeroderma pigmentosum (XP) is a rare autosomic recessive disorder which affects ~1:200.000 people. It is characterized by photosensitivity, actinic skin, increased cancer risk (>1000) in sun-exposed areas (skin, mucous membranes and eyes) and, in some patients, neurologic degeneration. Children are normal at birth and the external symptoms increase with sun exposure: freckles start to appear and eventually the skin starts to show poikiloderma (areas of hyperpigmentation, hypopigmentation, atrophy and/or telangiectasias). The first skin cancer usually appears between 9 and 10 years of age, almost 60 years before the average occurrence in the normal population (152), reviewed in (153,154).

XP was first described by Moriz Kaposi in 1874, who worked with four patients (155). In 1883, Albert Neisser reported two XP siblings with progressive neurological

degeneration (156). Today it is known that about 25 % of XP patients present this specific symptom (152).

In 1964, Gartler showed that XP cells presented hypersensitivity to UV (157), which was correlated with DNA repair deficiencies four years later by Cleaver (158). In 1969, it was demonstrated that photoproducts were not removed by XP cells and that they lacked an efficient NER pathway (159–162).

By 1999, all genes involved in XP development had been identified and isolated, reviewed in (154). Cell fusion experiments showed that there are seven XP complementation groups (A-G) (163–167) and a variant form (XP-V), which is NER-proficient but defective for the polymerase H (*POL H*) gene (Pol η) (168).

For more detailed information about XP clinical features, etiology and molecular aspects, please refer to the reviews of Lehmann (153) and DiGiovanna (154), or to Ahmad & Hanaoka and Balajee´s books (169,170).

Several mouse models with different XP deficiencies have been created (171). In the present work, two XP mouse models were used: XPA and XPC. Detailed information about these two lineages are given in the next subsections of the present work.

1.6.1.1 XPA mouse model

XPA patients present a very severe phenotype, since deficiency in this protein completely abolishes NER activity. These subjects present high cancer incidence in sun exposed areas and, in some cases, neurodegeneration.

XPA mice were generated substituting exons 3 and 4 of this gene with a resistance cassette to neomycin in embryonic stem (ES) cells. These animals are knockout (KO) for XPA. 50 % of XPA embryos die after the 13th day post coitum. However, the embryos which come to term develop normally, with a normal lifespan (172).

When UVB irradiated (310 J/m^2), these animals presented a very similar response to that of XPA patients exposed to sunlight: erythema and hyperplasia, solar keratosis and skin tumors (squamous cell carcinomas, SCC), in a higher intensity and incidence than the heterozygous littermates. Eye abnormalities were also observed (172).

XPA mice are also sensitive to other DNA damaging agents, such as 7,12-dimethylbenz[*α*]anthracene (DMBA) and benzo[*α*]pyrene (B[*α*]P). Furthermore, with old age these animals present a higher incidence of internal cancer when compared to wild type (WT) animals, reviewed in (170).

1.6.1.2 XPC mouse model

XPC patients may have a mild phenotype, since the absence of this protein only disrupts a subpathway of NER, the GG-NER. In this case, lesions present on the transcribed strand of active genes are still efficiently removed. These patients present an intermediary increase in cancer incidence in sun exposed areas and no neurodegeneration (173).

XPC mice were generated through inactivation of exon 10 via insertion of a resistance cassette to neomycin by homologour recombination in ES cells. KO animals were born at a mendelian rate and present a normal lifespan (173).

Furthermore, animals do not present a lower minimal erythema dose (MED) compared to WT mice, unlike XPA mice (174). However, just like XPC patients, XPC mice present an increased cancer predisposition on UV exposed areas (175). This indicates that damage in the transcribed strand of active genes may be the trigger for erythema and edema but not for cancer predisposition.

1.6.2 Cockayne Syndrome (CS)

Cockayne Syndrome (CS) is a rare autosomal recessive disorder, with an incidence of ~1:200.000 births. CS patients are characterized by mild photosensitivity, with no increase in cancer incidence, and progressive ataxia, neurodegeneration and progeria, which results in a reduced lifespan: an average of 12 years, reviewed in (176,177).

CS was first described in 1936 by the pediatrician Edward Cockayne (178). The patient´s apparent sensitivity to UV light was proven with UV irradiation of cultured cells in 1977 (179). The comprehension that CS patients have a defective TC-NER came from the observation that their cells present a normal level of unscheduled DNA synthesis (UDS) (180), but a very slow recovery of RNA synthesis (RRS) (181) after UV irradiation .

Deficiencies in several genes can lead to a CS phenotype: *CSA*, *CSB*, *XPB*, *XPD* and *XPG*. Mutations in *CSA* or *CSB* hinder TC-NER. Mutations in *XPB*, *XPD* or *XPG* can affect the whole NER pathway. Therefore, it is not surprising that, depending on the specific protein motif which is affected, patients with mutations in any of these three genes can present different phenotypes: XP, CS, TTD or a combination (XP/CS, XP/TTD or CS/TTD). Considering that these proteins also seem to have other roles in the cell might help to explain this heterogeneity. For instance, CSB also seems to be involved in DNA repair of oxidized damage and chromatin remodeling; XPB and XPD are helicases, part of the TFIIH transcription complex; and XPG is a nuclease, also part of TFIIH.

Most UV Signature mutations found in skin tumors in mice and humans are C → T conversions in non-transcribed strands. Interestingly, in CSB patients and CSB mice, these transitions are mainly found in the transcribed strands of active genes. Furthermore, unlike CSB patients, CSB mice present an increase in cancer susceptibility, which may be due to the lack of CPD removal by GG-NER (182). Contradictory data suggests that, specifically in keratinocytes, DDB-2 is expressed in sufficient levels to ensure CPD removal and prevent carcinogenesis (183). However, enhanced DDB-2 expression increases mice resistance to UV-induced carcinogenesis (184).

For more detailed information on CS, please refer to the reviews of Friedberg (176) and Hanawalt (177).

Several mice models with the same mutations as CS patients have been created. However, in the present work, only CSA animals have been used and will be described in detail.

1.6.2.1 CSA mouse model

CSA patients present mild photosensitivity, with no increase in cancer predisposition, but with several progeroid characteristics, including progressive neurodegeneration (176,177).

CSA mice were generated with the insertion of a hygromycine resistance gene in exon 2 of the *Csa* gene in ES cells. Animals were born at a mendelian rate (185).

Like CSA patients, CSA mice also show mild photosensitivity but, unlike the patients, present increased predisposition to skin cancer after UV irradiation and

almost no progeroid characteristics, with the exception of age-related retinopathy. CSA mice have a normal lifespan (185).

This observed difference in the phenotypes of mice and men may have a correlation with the fact that rodents lack the p48 protein and therefore have no GG-NER of CPDs.

1.7 UV radiation and the skin

The skin is the largest organ of the human body and it functions as an interface between internal and external environments. Because of this characteristic, the skin is under constant attack from genotoxic exogenous agents, such as UV light. Therefore, the last part of this introduction will focus on skin responses to UV light.

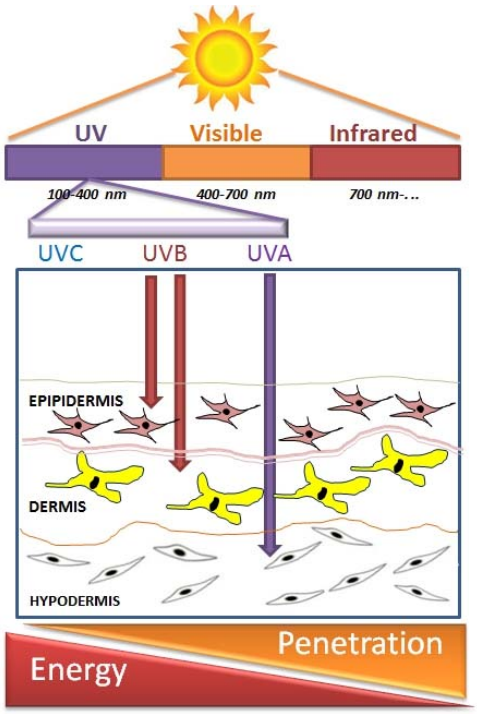
1.7.1 UV penetration in the skin

Electromagnetic radiation, such as UV light, is capable of transporting energy (E!) without carrying particles. It obeys the same physical laws as visible light, suffering diffraction, reflection, polarization and interference. The electromagnetic waves loose E! while propagating in any vehicle. When withstanding the interface between two environments (such as the skin/air surface), part of the light´s E! will be reflected and part transmitted to the second environment (in this case, the skin), where it may be absorbed. How far the light will go in a given vehicle depends not only on its E!, but mainly on the characteristics of the environment, reviewed in (186).

For instance, the UVB wavelength (280-315 nm) is strongly absorbed by nucleic acids and the protein´s aromatic amino acids, both present in high levels in the epidermis. Therefore, UVB light is totally absorbed by this external skin layer, virtually never reaching the dermis. On the other hand, UVA wavelengths are not efficiently absorbed by the structures present in the epidermis and go straight through. In the dermis, UVA photons are scattered several times by collagen bundles, before being absorbed by other chromophores, such as NADH, riboflavins and hemoglobin, reviewed in (17).

Figure 14 represents the UV light spectrum and the penetration capacity of each UV wavelength in the skin. To be noted: the penetration spectrum of human and mouse skin is similar.

Figure 14 - UV light spectrum and the penetration of each wavelength in the skin



UVC light is totally blocked by the ozone layer and the atmosphere. The great majority of UVB is absorbed by the epidermis whereas almost all of UVA is absorbed in the dermis. The greater the energy of the wavelength, the lower its penetration capacity in the skin. These properties apply to human and mouse skin.

To better understand the interaction between UV light and the skin, it is very important to comprehend the skin structure.

1.7.2 Skin structure

The epithelial tissue serves as interface between the environment and the inside of our bodies. Therefore, its integrity is fundamental not only to conserve the integrity of higher organisms, but also to preserve essential body functions, such as temperature regulation and substance absorption and excretion.

The epithelial tissue is constituted on its outside layer by the epidermis, originated from the ectoderm, and more internally by the dermis and the hypodermis, both originated from the mesoderm.

The hypodermis is mainly composed of adipocytes, but also by fibroblasts, blood vessels and nerves. It is basically responsible for thermoregulation, nutrient hoarding and protection against mechanical trauma.

The dermis is comprised of fibroblasts, which secrete collagen and elastin, and by blood and lymphatic vessels, muscle bundles and nerves. The dermis is the conective tissue responsible for the structural and functional support of the skin, also ensuring epidermal nutrition and oxygenation.

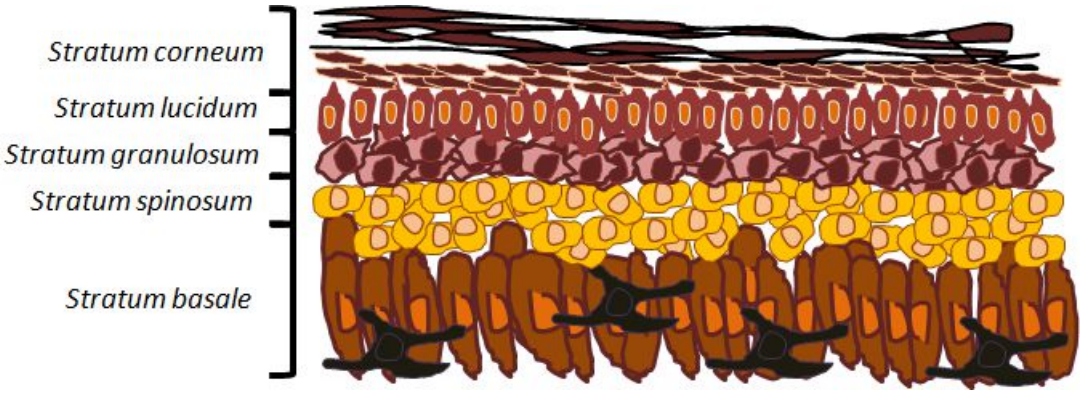
The epidermis is formed by keratinocytes, melanocytes, and Langerhans´ and Merkel´s cells. Keratinocytes are the cells responsible for synthetizing keratins, the proteins of the intermediate filament, which are characterized as being resistant and impermeable. Only the keratinocytes of the epidermal basal layer, which are the cells in contact with the basement membrane (just above the dermis), are capable of proliferating. Once these cells divide, they start migrating to the outer layer of the epidermis. During this period, cells change the type and amount of keratin they produce and accumulate, which leads to the loss of cell function and cell flattening, culminating in cell death on the stratum corneum.

Proliferating keratinocytes in contact with the basement membrane form the *stratum basale* or germinative. These keratinocytes present an elongated morphology and low keratin levels. In the *stratum spinosum*, cells are rich in desmosomes, the keratin content starts to increase and they become more oval-like. The keratinocytes in the *stratum granulosum* present lozenge morphology and are rich in filaggrin granules. In the *stratum lucidum*, cells no longer present a nucleus and accumulate more keratin. Finally, in the *stratum corneum*, cells are no longer metabolically active, are very compact and extremely rich in keratin.

Melanocytes are mainly found in the deeper epidermal layers. These cells are responsible for producing melanin (eumelanin and pheomelanin). Each individual presents a different level of each type of melanin, which is responsible for characterizing the different skin tones. Melanin is synthesized and exported to keratinocytes in melanosomes. Melanin production can be stimulated by UV light, which will be discussed in detail in the next subsections.

Figure 15 represents the epidermal layers formed as keratinocytes migrate to the epithelial surface, loosing nuclear and metabolic activities.

Figure 15 - Epidermal layers formed through keratinocyte migration and differentiation



Only the keratinocytes in contact with the basement membrane (*stratum basale*) are capable of proliferating. These cells then start a journey of migration and differentiation towards the outer layer of the epithelium, loosing nuclear and metabolic activities and accumulating keratin. Cells in the *stratum spinosum* and *granulosum* still present a nucleus, which is lost in the *stratum lucidum*. In the *stratum corneum*, cells are no longer metabolically active. Melatinocytes are represented as dark cells in the *stratum basale*.

For more detailed information about skin anatomy and physiology please refer to Bolognia´s book, chapter 1 in particular (187).

Mouse skin structure is fundamentally the same as human skin. However, each *stratum* is constituted of fewer layers and the intermediate ones are very hard to observe. In total, the mouse epithelium presents an average of 2-3 layers (188). Human epidermis is almost ten times thicker: 50 µm for human epidermis (187) compaired witht 6 µm for mouse epidermis (189). This characteristic makes mouse skin more sensitive to UV light. Still, despite differences in the intensity of the effects observed in the skin of these two animals, the acute and chronic responses to UV light are essentially the same.

The epidermis, in direct contact with the environment, is highly exposed to a series of harmful physical and chemical agents and its most important function is to protect complex organisms from injuries caused by exogenous factors, such as UV light.

Factors such as skin phototype, body region and age influence skin sensitivity to UV and determine the intensity of acute and chronic responses. For instance, fair-skinned people, as well as children and the elderly present a higher sensitivity to UV. The same is true for areas with a more delicate and thinner skin, such as the face and neck, reviewed in (190).

1.7.3 Acute effects of UV light in the skin

Acute skin responses to UV light usually peak around 24 hours after UV irradiation and can be observed up to 72 hours after exposure. They include, but are not restricted to, apoptosis, inflammation and hyperplasia (epidermal thickening). These responses are UV dose dependent and elicited by UVA and, with greater intensity, UVB (191).

These responses seem to be p53-related: p53 levels peak approximately 12 hours after UV exposure. This transcription factor is activated by the presence of DNA damage and leads to cell cycle checkpoint activation, followed by an arrest in cell cycle progression in the G1 phase. Alternatively, when injuries are too severe to be repaired, p53 may elicit a programed cell death response via p21/BAX/BCL2 response.

All of the aforementioned acute responses are also observed in p53 KO animals, but with a 12-24 hour delay (192). Therefore, evidence suggests that p53 is not essential but presents a stimulatory effect in acute skin responses to UV light (192).

Immediate responses to UV light also include vitamin D synthesis, inflammation, erythema (skin redness) and edema (skin swelling).

1.7.3.1 <u>Sunburn cells</u>

Sunburn cells are apoptotic cells characterized by pyknotic nucleus and shrunken cytoplasm. The UV-induced cell death is usually observed from 6 to 72 hours, peaking 36 hours after exposure. The dying cells start to migrate to the outer epidermal layers, eventually co-localizing with the hyperkeratosis areas. There is a UV-dose effect in the induction of sunburn cells and high levels of cell death can be observed as macroscopic peeling (192).

Interestingly, after UV irradiation, despite the high number of dying cells, what is observed is epidermal thickening and not shrinkage. This phenomenon is observed because of a secondary response which starts approximately 12 hours after UV irradiation, when p53 levels decrease: cell proliferation (193).

1.7.3.2 Hyperplasia

Hyperplasia is an increase of epidermal thickness due to the rise of the total number of keratinocytes, leading to an augmentation in the number of epidermal layers. In hyperplastic skin tissue, it is common to observe an enlargement of cell size.

The hyperplastic response seems to be independent of genetic background and is pigment-independent. This reaction is considered the most effective skin photoprotection defense mechanism in individuals who tan poorly (190). The epidermal thickening physically blocks UV light, diminishing the total amount of rays which can reach deeper epithelial layers, protecting the proliferative cells from its damaging effects.

The observed hyperplasia is the result of the combination of two cell responses: cell death of the previously existing keratinocytes, and cell proliferation in the basal epidermal layer (192). The hyperplastic response starts 12 hours after exposure, when p53 levels start to decrease. It peaks in 48 hours, and is observed up to 72 hours after UV irradiation (192). This response seems to be dependent on the epidermal growth factor (EGF), since epithelium lacking its receptor (EGFR) does not present hyperplasia after UV exposure (194,195). Typically, the reversion of the hyperplastic response starts 96 hours after the proliferative stimulus has been removed (193).

1.7.3.3 Erythema and edema

Erythema (skin redness) and edema (skin swelling) walk hand-in-hand and are caused initially by infrared and UVA rays, which reach the dermis and cause vasodilation and consequent increase of blood content in the skin. This response starts only a few minutes after UV exposure and fades within 4 hours, unless there has been enough damage to start an inflammatory response which will sustain vasodilation. Edema and erythema induction is UV-dose dependent and may even lead to blistering, when the damage is too severe, reviewed in (190).

1.7.3.4 Inflammation

UV-induced inflammation starts a few hours after UV exposure and peaks 6-24 hours after exposure (196) and includes, as a consequence of lipid peroxidation and DNA damage, increased production of prostaglandins (PG), tumor necrosis factor alpha (TNF-α), nuclear factor kappa beta (NF-κβ), histamine, kinins and inflammatory cytokines (interleukins – IL - 1α, 1β and 6), reviewed in (197,198).

Together, these molecules regulate the expression of adhesion molecules (intracellular adhesion molecule 1, ICAM-I, and endothelial-leucocyte adhesion molecule, ELAM-1) in vascular endothelium and keratinocytes, recruiting mononuclear cells and neutrophils. As a consequence, vasodilation, lymph infiltration and inflammation can be observed. The inflammatory process increases the formation of ROS and there is evidence that this may aid in the launching of the tumorigenic process through the increase of DNA damage levels, reviewed in (197,198).

1.7.3.5 Vitamin D production

Vitamin D is crucial for the maintenance of endogenous calcium levels and skeleton mineralization. The vitamin D precursor (pre-vitamin D or colecalciferol) is formed in the epithelium with the aid of UV-irradiation. The active form of this vitamin, calcitriol, is then produced in the kidneys and liver.

It is well known that UV light exposure can lead to several types of skin cancer. However, recent works present evidence that vitamin D production may aid not only in skeletal health, but also in skin cancer prevention and regression. Therefore, even though low UV exposition is sufficient for a satisfactory calcitriol production (10-15 min exposure, 2-3 times/week), extreme care has to be taken when advising people to “stay out of the sun”, since this seems to be another delicate balance, reviewed in (199).

1.7.3.6 Pigmentation

There are three types of UV-induced pigmentation: the immediate pigment darkening (IPD), the persistent pigment darkening (PPD) and the late tanning

response. IPD starts 1-2 minutes after UV irradiation, lasting no more than a few minutes. PPD is frequently confused with tanning, but consists of a phenomenon similar to IPD, starting a few minutes after UV exposure and lasting up to a few days. Both IPD and PPD are also known as Meirowsky phenomena and are caused by photoxidation of melanin and its precursors. IPD pigment is more grayish, whereas PPD pigment is brownish; none of them seem to have photo-protective properties, reviewed in (198,200). The IPD and the PPD capacities are independent of the tanning ability of the individual, even though higher melanin content in the skin increases the IPD and PPD responses.

Late skin pigmentation (or tanning) starts 2-3 hours after UV irradiation and peaks in 3 weeks. This late response correlates with melanin production by melanocytes and its distribution to surrounding keratinocytes and will be discussed in more detail in the next sections of the present work.

1.7.4 Chronic effects of UV light in the skin

Chronic effects comprise delayed local and systemic responses to UV light which can be observed from 2 days up to several months or years after UV exposure. These chronic responses include photoaging, immunosuppression, persistent hyperplasia, delayed pigmentation and tumorigenesis. Hyperplasia and pigmentation are considered photoprotective/photoadaptive responses and are mostly related to the overexpression of genes related to cell cycle control, DNA damage response or apoptosis, such as *p53*, *GADD45* and *BAX* (201,202).

1.7.4.1 Photoaging

Aging is the progressive decline of function and reserve capacity of all organs in the body, due to the loss of tissue homeostasis. Skin photoaging is the superposition of intrinsic aging and environmental factors, such as chronic UV exposure. Photoaged skin is characterized by several macro and microscopic changes, including variation in epidermal thickness and pigmentation, dermal elastosis, alterations in the collagen composition and levels, inflammatory infiltrates, vessel ectasia, and wrinkling, reviewed in (203).

Several types of local damages and responses contribute to the development of skin photoaging, such as genomic and mitochondrial DNA damage, protein oxidation, telomere shortening, UV-signaling pathways and loss of tissue homeostasis, reviewed in (203).

Prevention of skin photoaging can be achieved by limiting sun exposure and regular use of sunscreens that protect against both UVA and UVB. Other products which antagonize the UV-signaling pathway, such as tretinoin and antioxidants, or that aid in the reduction of metalloproteinase levels and in collagen reconstitution, may help in the treatment of this condition. A better comprehension of the photoaging process, as well as the development of preventive and palliative methods, aids not only in improving skin appearance of middle-aged people and beyond, but also helps to reduce the burden of skin cancer, reviewed in (203).

1.7.4.2 Immunosuppression

In 1974, Kripke published the first evidence that UV light has an impact in the immunologic system (204), which helped to explain a series of previous data and successful light treatments against psoriasis and tuberculosis (205). A clear sign UV-induced immunosuppression is the enhanced susceptibility to several types of infection after UVB exposure (206–208).

Immunological responses usually arise from a series of signals which compose an intricate network. In photo-immunosuppression, one of the main signals seems to be DNA damage in cells present in the epithelium, including those from the immune system, such as Langerhans cells (LCs). In fact, removal of CPDs, but not 6-4PPs, is capable of preventing UV-induced immunosuppression. Interestingly, lesion removal from only keratinocytes did not produce the same results (209).

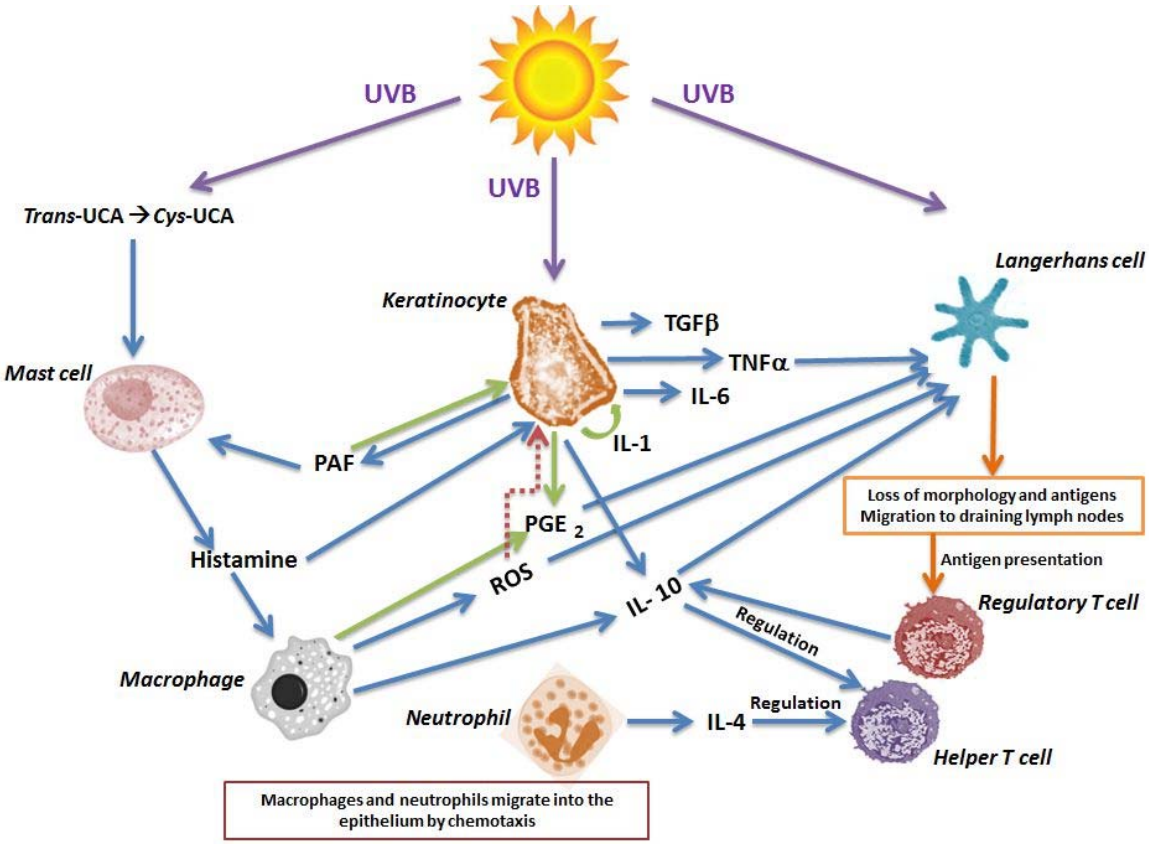
An increase in local immunosuppression is obtained by TC-NER blockage (CSB animals) (210), but an increase in systemic immunosuppression is only achieved with the simultaneous inactivation of TC-NER and GG-NER (XPA animals) (211).

The exact pathway through which UVB induces immunosuppression is still not clear and may vary depending on the wavelength in question, exposure duration, previous exposures, skin area and local pigmentation.

The presence of photoproducts and oxidized damages in DNA cause keratinocytes to secrete TNF-α, interleukin (IL) 10 (IL-10), IL-6, IL-1, transforming growth factor beta (TGF-β) and the platelet activating factor (PAF). TNF-α and IL-10 prevent LCs to differentiate into dendritic cells and stimulate their migration to draining lymph nodes.

UV converts *trans*-uronic acid (UCA) to *cys*-UCA, which induces mast cells (MCs) to release histamine. Histamine, IL-1 and PAF promote PGE_2 secretion by keratinocytes. Macrophages (Mas) and neutrophils migrate into the epithelium by chemotaxis 72 hours after injury. Histamine induces MAs to secrete IL-10 and produce ROS. ROS increase the production of several of the aforementioned signaling molecules, probably through DNA damage, reviewed in (212). Interferon gama (INF-γ), IL-4, IL-10 and IL-12 regulate the levels of helper T lymphocytes (Ths) (213). Reduced levels of Ths and increased levels of regulatory T lymphocyte (Treg) are major markers of UV-induced immunosuppression, reviewed in (214,215). Figure 16 presents a simplified version of the known pathways which regulate UV-induced immunosuppression.

Figure 16 - Pathways which regulate UV-induced immunosuppression



DNA damage causes keratinocytes to secrete TNF-α, IL-10, IL-6, IL-1, TGF-β and PAF. TNF-α and IL-10 prevent Langerhans cells to differentiate and stimulate their migration to draining lymph nodes. UV converts *trans*-UCA to *cys*-UCA, which induces mast cells to release histamine. Histamine, IL-1 and PAF promote PGE_2 secretion by keratinocytes. Macrophages and neutrophils migrate into the epithelium by chemotaxis. Histamine induces macrophages to secrete IL-10 and produce ROS. ROS increase production of signaling molecules, which increase levels of regulatory T cells. IL-4 and IL-10 also regulate the levels of helper T lymphocytes.

UVB-induced immunosuppression can be prevented with low UVA exposure (216), probably through the induction of INF-ɣ and IL-12 (217). IL-12 seems to increase GG-NER, thus reducing the levels of remaining photodamage in DNA (218).

It is important to highlight that UV-induced immunosuppression has an important role in tumor development since the immune system surveillance may detect and combat early tumors. For instance, the absence of IL-10 prevent Treg-induced immunosuppression, reducing tumor induction in mice (219).

1.7.4.3 Persistent hyperplasia and dysplasia

Hyperplasia is not only an acute response against UV-induced skin damage. As one of the most important epidermal responses against this type of injury, hyperplasia persists for as long as the damaging agent is present (220). If tissue homeostasis is preserved, the hyperplastic response starts to be reversed 96 hours after the stimulus has been removed (193).

However, when tissue homeostasis is lost, hyperplasia becomes persistent. Histologically, there is no difference between acute and chronic hyperplasia, but in persistent hyperplasia signs of loss of tissue homeostasis are usually seen, such as dysplasia. Dysplasia is characterized by the loss of tissue morphology, usually presenting alteration of cell differentiation patterns in the epidermis.

It is important to emphasize that an acute stimulus, if strong enough, can also lead to persistent hyperplasia and dysplasia.

1.7.4.4 Delayed skin pigmentation (tanning)

Two types of melanin pigmentation may be observed in the skin: a) constitutive, which is the genetically determined skin color observed in different individuals; and b) induced, which is a reversible increase in tanning as a response to specific stimulatory agents, such as UV radiation, reviewed in (190).

According to Fitzpatrick (221), human skin can be classified in six different phototypes (I – VI). Phototype I is the most sensitive, with individuals presenting white skin and a great tendency to sunburn and no capacity to tan. As the scale progresses sun sensitivity decreases: the tendency to sunburn disappears and the ability to tan increases. Phototypes V and VI are the least sensitive, with individuals presenting brown and black skin, respectively, reviewed in (198,222).

Tanning capacity is genetically determined and depends on the ability of melanocytes to produce melanin, the total amount of melanin formed within each melanosome, and melanosome distribution. Fair-skinned individuals present clustered small melanosomes (0.6 – 0.7 μm) which are only partially melanized; while dark-skinned individuals present bigger melanosomes (1 μm) which are heavily melanized and more evenly dispersed. Therefore, fair-skinned individuals (skin photoypes I and II) are less efficient in producing visible pigment than dark-skinned

individuals (phototypes V and VI), who have an epithelium more efficient in absorbing light, reviewed in (198).

Tanning becomes visible 3-5 days after UV exposure. There is an increase in melanin production, related to the elevation of melanocyte activity and number, augmentation of melanocyte dendrites elongation and branching, and to an increase in melanosome quantity and size. After melanin is produced, melanosomes are exported to keratinocytes where they are diffusely distributed, but group above the nucleus forming a cap, reviewed in (198).

Curiously, UVA-induced melanin tends to stay in melanosomes located in the basal epidermal layer, whereas UVB-induced melanin is distributed to the upper keratinocyte layers, reviewed in (198). There is further evidence that the tanning mechanisms differ between UVA and UVB stimulation. In cell culture, UVB-induced melanogenesis requires the presence not only of melanocytes, but also of keratinocytes, suggesting that signaling molecules, such as endothelin 1 (ET-1) and IL-1α, may be required to elicit this process. In UVA-induced melanin production, keratinocytes are not required but oxygen is, which suggests that ROS formation may be required in this process, reviewed in (223).

Furthermore, it has been shown that UVA-induced pigmentation requires the presence of melanin pigment and depends on the production of oxidized damage in melanocytes. ROS can damage DNA (base oxidation and SSB), lipids, proteins and other molecules, initiating a signaling pathway which will culminate in melanogenesis. On the other hand, UVB-induced melanogenesis is pigment-independent and relies on direct DNA damage (6-4PPs and CPDs), reviewed in (224). Despite their different mechanisms of melanogenesis induction, UVA and UVB exposures have the same general outcome: increase of melanin production. Interestingly, UVA and UVB have an additive property for both erythema and pigment induction, reviewed in (223)

The aforementioned changes in melanocytes and melanosomes are regulated by a series of direct and indirect effects of UV on melanocytes. UV damages keratinocytes´ DNA, which leads to the expression of many citokynes, such as ET-1, granulocyte-macrophage colony-stimulating factor (GMCSF), basic fibroblast growth factor (bFGF) and stem cell factor (SCF), which are recognized by melanocytes and regulate melanogenesis. Furthermore, DNA damage in keratinocytes also leads to an increase in p53 levels, provoking higher POMC expression and, consequently, of its derivatives: alpha melanocyte stimulating hormone (αMSH), adrenocorticotropic

hormone (ACTH) and beta endorphin. αMSH is recognized by its receptor in melanocytes, MC1R, which stimulates adenylate cyclase (AC). Cyclic adenosine monophosphate (cAMP) is increased by AC, consequently raising protein kinase A (PKA) levels. PKA phosphorylates the cAMP response element-binding protein (CREB), raising microphthalmia transcription factor (MITF) expression. In turn, MITF induces the expression of a series of proteins related to the melanogenic pathway, such as tyrosinase (TYR), L-3,4-dihidroxyphenylalanine (DOPA) chrome tautomerase (DCT) and dihydroxyindole-2-carboxylic acid (DHICA) oxidase (TYRP1). Alternatively, a series of other pathways may lead to an increase in melanogenesis, in a MITF dependent or independent manner, reviewed in (222).

Evidence suggests that not only the presence of DNA damage but, more importantly, its repair is responsible for iniciating the aforementioned melanogenic pathway. Nucleotide fragments produced during NER are capable of inducing melanogenesis. For instance, the addition of small damaged DNA fragments *in vitro* and *in vivo* was proved capable of stimulating melanin production (225,226).

Several investigations confirm the central role of p53 in melanogenesis, mostly through the increase of POMC expression (227,228). Curiously, POMC null mice can still produce normal melanin levels, indicating that this is not the only pathway involved in melanogenesis induction (229). Furthermore, after UV exposure, keratinocytes secrete ET-1, colony stimulating factor 2 (CSF2), v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog 1 (KIT1), PGE_2 and NO. These factors are recognized by melanocytes and/or their precursors (melanoblasts), inducing cell proliferation and differentiation; dendricity increase; melanogenesis; and melanosome formation, transport and density, reviewed in (198,223,230–232).

Melanogenesis inhibition is achieved through the secretion of a series of factors by keratinocytes, such as IL-1α, TNFα, interferons and bFGF. A fine tuning between all these molecules is responsible for melanogenesis control, reviewed in (223).

Interestingly, αMSH may also play an important role in preventing UV-induced apoptosis, aiding in the removal of photolesions from keratinocytes and melanocytes, in a NER-dependent manner (233).

After melanogenesis is stimulated, three pathways control the production of eumelanins and pheomelanin, reviewed in (234).

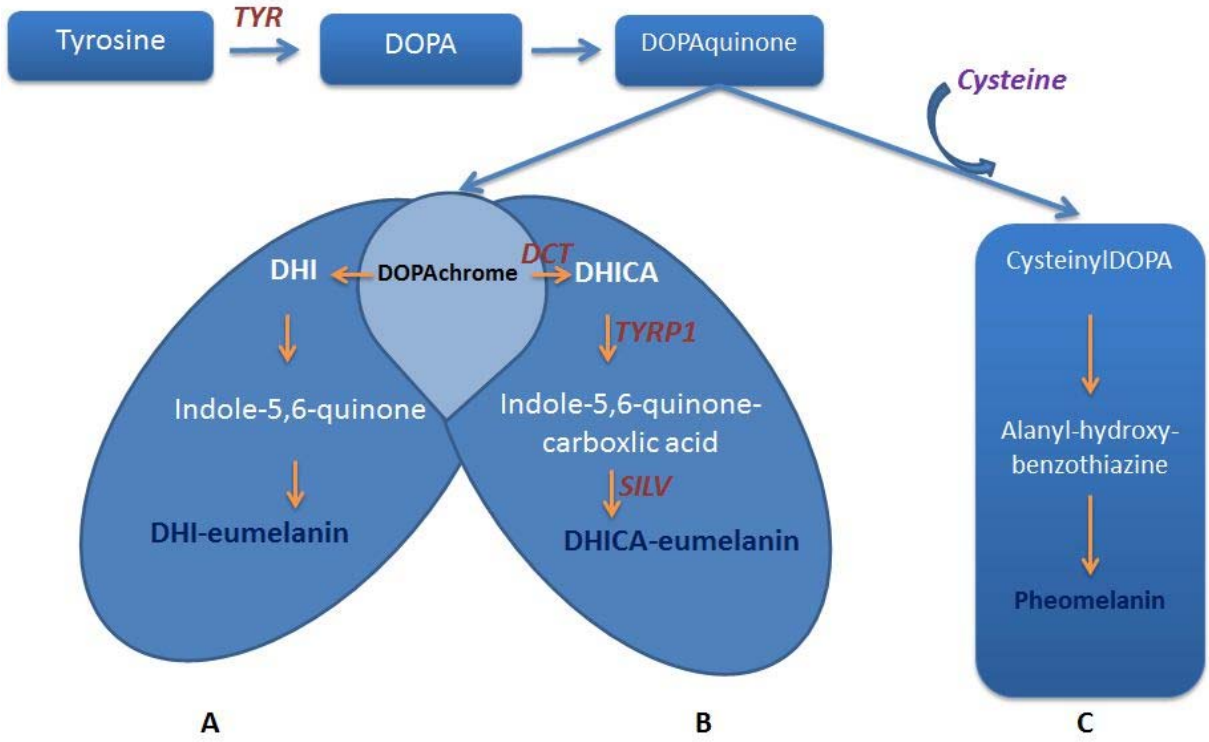
Dihydroxyindole-eumelanin (DHI-eumelanin) is produced by the Raper-Mason pathway. TYR converts tyrosine to its dihydroxylated form, DOPA. The subsequent reactions are spontaneous: DOPA oxidazes to DOPAquinone, which then cyclizes to DOPAchrome. DOPAchrome decarboxylates to DHI, which oxidazes to indole-5,6-quinone. Polymerization of these quinone and indole-quinone intermediates originates the dark black pigment known as DHI-eumelanin.

In an alternative pathway, when the enzyme DCT is present, it converts DOPAchrome to a carboxylated intermediate, DHICA, which is then oxidized by TYRP1 into indole-5,6-quinone-carboxylic acid. When this last form is further oxidized, it can polymerase, with the aid of DHICA polymerase (SILV), and form a dark brownish pigment: DHICA-eumelanin.

A third pathway is responsible for forming pheomelanin, a light brown pigment. DOPAquinone is converted to cysteinylDOPA with the addiction of the amino acid cysteine. CysteinylDOPA is converted to alanyl-hydroxy-benzothiazine, which polymerases, forming pheomelanin.

These pathways are represented in Figure 17.

Figure 17 - Melanogenesis pathways for the production of eumelanin and pheomelanin



Tyr is the limiting enzyme which converts tyrosine in DOPA, which is spontaneously converted to DOPAquinone and then to DOPAchrome. Through the Raper-Mason pathway, DOPAchrome is spontaneously converted to DHI and then to indole-5,6-quinone. The polymerization of these quinone and indole-quinone intermediate factors forms the darker form of melanin: DHI-eumelanin. On a secondary pathway, DCT converts DOPAchrome to DHICA that is then converted by TYRP1 to indole-5,6-quinone-carboxylic acid. Polymerization of these intermediate factors with the aid of Silv leads to the formation of the brownish form of eumelanin: DHICA-eumelanin. Alternatively, cysteine may be spontaneously added to DOPAquinone forming cysteinylDOPA, which is spontaneously converted to alanyl-hydroxy-benzothiazine, which polymerases into pheomelanin.

The exact mechanism for the activation of different melanogenesis pathways is not well understood. TYR levels determine total melanin production. DCT levels seem to define what type of eumelanin is formed; and environmental factors, such as cysteine availability, lead to pheomelanin production. It is believed that the two additional pathways to the Raper-Mason pathway have evolved to aid in the elimination of the intermediates generated in melanin production which are very toxic to cells. The same reason could explain why this pigment is produced inside specific vesicles, the melanosomes, which have detoxing enzymes and prevent ROS from damaging other cell structures, reviewed in (234).

Furthermore, it is also known that UV irradiation leads to increased levels of Tyr and reduced levels of DCT, TYRP1 and SILV, culminating in an increased DHI-eumelanin production, the darkest of all melanins. In fact, DHI-eumelanin is the most

efficient in protecting skin against UV light side damaging effects, followed by DIHCA-eumelanin and pheomelanin, respectively, reviewed in (234).

Contrary to popular belief, tanning has only a moderate effect in protecting the skin from the injuries caused by UV irradiation: a deep tan only increases protection by a factor of two or three. It is even argued that melanin might not have been evolutionarily selected to protect organisms from the damaging effects of UV exposition, but for camouflage and temperature maintenance, reviewed in (190). Nevertheless, fair-skinned individuals are over 10 times more sensitive to the effects of UV light than those that are dark-skinned, which puts that hypothesis in question, reviewed in (198).

However, in addition to blocking the passage of UV light, and thus protecting epithelial tissue from direct UV damage, melanin can also act as a chromophore which absorbs UV light and produces ROS, increasing cellular and indirect tissue damage. Until recently it was believed that the presence of pheomelanin was deleterious for the organism because of its low UV-blockage capacity and increased ROS production (224). However, recent evidence suggests that the higher photosensitive of skin phototype I individuals is not related to their higher pheomelanin/eumelanin ratios, but to non-pigmentary roles of MC1R, which is mutated in red hair subjects. Both eumelanin and melanin have been shown to efficiently protect cells from UV rays (235).

Melanocytes are resistant to UV-induced photodamage because of their high melanin content and are particularly resistant to UV-induced, p53 mediated, apoptosis, probably due to enhanced BCL2 (BAX suppressor) levels and upregulation of the growth arrest and DNA damage-inducible protein alpha (GADD45a), an apoptosis inhibitor, reviewed in (236). However, melanocytes have been described as having lower DNA repair capacities for oxidized damage and photolesions, which may be due to the presence of melanin (237).

1.7.4.5 Skin tumorigenesis

Skin cancer is the most common form of cancer in Brazil and in the world, constituting 25 % of all registered malignant tumors, in men and women (INCA). The incidence has been escalating due to an expansion of recreational exposure, such as sun tanning and outdoor sports; sunbed tanning (238); absent or incorrect use of

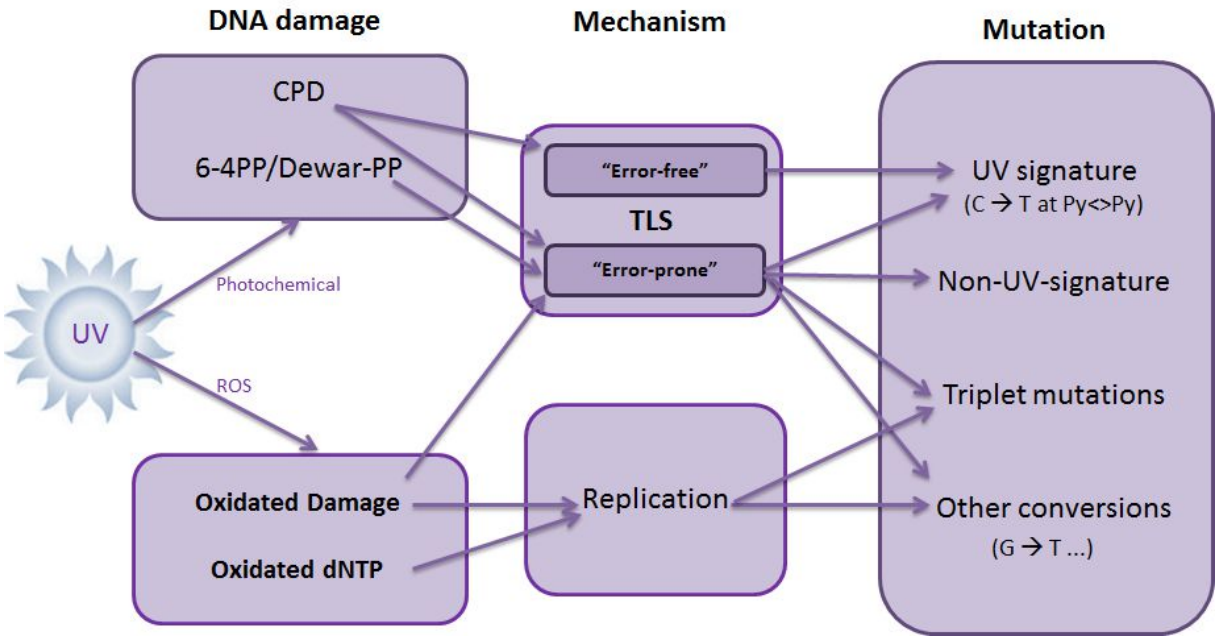
sunscreens (or use of ineffective sunscreens) (239); and to a progressive reduction of the ozone layer in certain areas, reviewed in (240).

Exposure to UV light is one of the biggest risk factors for the development of skin cancer. As explained in detail in subsection 1.2, UV light causes two types of photolesions on DNA, CPDs and 6-4PPs, in addition to indirect oxidized damage. If these lesions are not successfully removed, they can lead to mutations, which can culminate in cancer induction.

There is a set of mutations found in virtually all UV-induced skin tumors, called UV-signature mutations: C → T and CC → TT conversions. As shown in Figure 7, they are formed through deamination of pyrimidines in a photodimer followed by "error-free" TLS. Triplet mutations are also commonly found in these tumors.

Figure 18 summarizes the mechanisms through which UV-signature mutations and other UV-induced mutations may be formed in DNA.

Figure 18 - Induction mechanisms of UV- signature mutations and other UV-induced mutations



UV irradiation can damage the genomic DNA, generating CPDs, 6-4PPs and oxidized damages. TLS of CPD by "error-free" Pol η may lead to UV-signature mutations (C → T and CC → TT conversions). TLS of CPDs, 6-4PPs and oxidized damages by "error-prone" polymerases may lead to the formation of UV-signature mutations or other mutations, such as G → T and T → G conversions. Replication (without TLS) of oxidized damages or the use of damaged nucleotides during replication can lead to the formation of non UV-signature mutations. Adapted from Ikehata & Ono, 2011 (54).

Carcinogenesis is a multistep process that demands a sum of mutations in proto-oncogenes and tumor suppressor genes and/or in genes which regulate their expression. Two of the most commonly mutated genes found in UV-induced skin cancer are p53 and rat sarcoma (Ras), reviewed in (241).

During the carcinogenic process, cells have to suffer enough mutations to be able to resist cell death signals, sustain proliferative signals, deregulate cell energetics, evade growth suppression, enable replicative immortality, induce angiogenesis, avoid destruction from the immunologic system, and activate invasion and metastasis mechanisms, reviewed in (242).

A series of factors influences photocarcinogenesis: UV dose and specific wavelength, repetitive exposure, skin area exposed, age of the individual, skin type, use of sunscreens and diet, reviewed in (241).

Three types of skin cancers correlate with UV exposure: basal cell carcinoma (BCC), SCC, which are collectively referred to as non-melanoma skin cancer (NMSC), and cutaneous malignant melanoma (CMM), reviewed in (241). On average, BCC comprises 75 % of all skin cancers, followed by SCC with 21 % and CMM with 4 %.

Fair-skinned individuals (phototypes I and II), people with light-colored eyes and hair, with a tendency to sunburn and not to tan, immunosuppressed subjects, and those with a family history are considered high-risk groups for skin cancer (243).

BCC is the most frequent cancer in Caucasians, although rarely found in Orientals and Afro-Americans. It is a solid malignant skin cancer caused by the transformation of basal keratinocytes and presents a slow progression and low metastatic rates. BCC afflicts more men than women and its onset usually occurs around 69 years of age. It is more common on the head and neck and in inhabitants of the tropics. In the United States of America (USA), 750.000 new cases of BCC are diagnosed every year, reviewed in (240).

BCC is associated with intermittent UVB exposures, followed by intense sunburns, especially in childhood. Specific risk factors include mutations that lead to alterations in skin pigmentation and in vitamin D production. Mutations in p53 and in protein patched homolog (PTCH) are commonly found in BCC, reviewed in (240).

SCC is a solid malignant tumor, formed by transformed keratinocytes (not from the basal epidermal layer), with low metastatic rates and mortality. It is the second most common skin tumor in Caucasians, with a higher incidence in males. Although

UVB light is considered the most important cause of SCC, it is also commonly caused by chemical (arsenic and benzantracen) and biological agents (human papillomavirus, HPV). In the USA, approximately 200.000 new SCC cases are diagnosed every year, (244). The average age of SCC incidence is 68 years for women and 72 for men (245).

Risk factors include cumulative UVB exposition, inhabiting lower latitudes, immunosuppression, fair-skinned, people with light-colored eyes and hair, tendency to sunburn, and family history. The presence of pre-malignant lesions, the actinic keratosis, is an indicator of future development of SCC. Mutations in p53 are the most common in SCC, together with several chromosomal alterations (244), reviewed in (240).

CMM is a solid, malignant tumor, composed of transformed melanocytes, usually occurring in the epidermal-dermal junction. It comprises only 4 % of the totality of skin tumors, but it is their most lethal form, due to the high metastatic rate. The total incidence is 10-15/100.000 habitants in Europe, 45/100.000 habitants in Australia and it is estimated to inflict around 2.5-7.4/100.000 habitants in Brazil. CMM is very rare in children, presenting its higher frequency in the elderly, with an average incidence at 50 years of age. CMM incidence is a little higher in females (246).

The presence of nevi is a risk factor, especially atypical and familial nevi. Mutations in rat sarcoma (*RAS*), cluster of differentiation 14 (*CD14*), protein 16 (*p16*), apoptotic protease activating factor 1 (*APAF1*) and v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) are commonly found in CMM (246).

CMM occurs in sun-exposed areas, and it is believed that intermittent UV exposure, with sunburns in early childhood, severely increases the risk of later development. There is evidence suggesting that UVA plays an important role in melanoma induction, probably due to direct absorption by melanin (247), reviewed in (248). However, CMM has not been successfully developed in mice or other placental mammals solely with UVA exposures. Therefore, UVB exposition is still considered the factor that is mainly responsible for CMM development, reviewed in (240).

Table 1 presents the estimated incidence of NMSC and CMM in Brazil in 2010 and 2012, as estimated by the National Institute of Cancer (*Instituto Nacional de Câncer, INCA).*

Table 1 - Estimation of skin cancer occurrence in Brazil between 2010 and 2012

Gender	*Type of skin cancer/ year*	*2010*	*2012*
Men	CMM	2.960	3.170
	NMSC	53.410	62.680
Women	CMM	2.970	3.060
	NMSC	60.440	71.490
Total	CMM	5.930	6.230
	NMSC	113.850	134.170

Skin cancer incidence predictions have increased from 2010 to 2012 in Brazil. Cutaneous malignant melanoma (CMM) affects more men than women, while the reverse is true for non-melanoma skin cancer (NMSC). Data obtained from INCA.

1.8 Final Considerations

The scientific community has long accepted the intimate relationship between UV exposure, photolesion formation in DNA, mutation induction (Signature UV-induced mutations) and the development of skin tumors. However, the specific role played by each photolesion, CPDs and 6-4PPs, in cell and tissue responses to UV irradiation, such as erythema, edema, pigmentation, hyperplasia and tumorigenesis, is still not clear.

Previous works have explored this question, but employing mice proficient in DNA repair mechanisms, which may have masked the role of 6-4PPs in skin responses to UV irradiation due to fast removal of these lesions by NER. In these studies, only the removal of CPDs was capable of preventing UV-induced local and systemic responses (209,249,250).

Furthermore, an *in vitro* research revealed that, in XPA cells, the removal of CPDs as well as the removal of 6-4PPs reduced apoptosis (251). Four years later, another study using adenoviral vectors for the delivery of photolyase transgene in several DNA repair-deficient fibroblasts demonstrated that the removal of both photolesions was able to reduce UV-induced apoptosis in XPA, XPD and XPG cells. Interestingly, in WT, XPV and CSA cell lines only the removal of CPDs had an impact in cell death after UV irradiation (122). These results suggest that depending on the DNA repair status of the organism, each photolesion may play different roles.

Therefore, it becomes of interest to study the specific role of each UV-induced photolesion in skin responses to UV exposure in a system where the fast removal of

6-4PP lesions by NER does not mask its role in hyperplasia, pigmentation, cell proliferation, cell death and tumorigenesis induction. This knowledge will not only increase the base knowledge of these processes, but may also aid in the development of prevention and treatment strategies for the unwanted UV side-effects, especially for subjects with photosensitivity, such as Xeroderma Pigmentosum patients.

6 CONCLUSIONS

The present work shows an intimate connection between cell proliferation and apoptosis in the development of acute and chronic UV-induced hyperplasia and dysplasia. Chronic hyperplasia prevention depends on reduced basal cell proliferation and high basal apoptosis.

Ubiquitous CPD removal partially prevented acute hyperplasia and dysplasia despite an increase in basal proliferation, through reduced suprabasal proliferation and sustained apoptosis. After chronic UV exposure, XPA mice presented increased melanogenesis, which was further enhanced by CPD removal. Hyperplasia and p53 patch induction were prevented by CPD removal from the whole epidermis in XPA animals, through reduced cell proliferation and sustained cell death.

6-4PP removal from basal keratinocytes partially prevented acute hyperplasia and dysplasia through the reduction of basal and suprabasal cell proliferation. However, this photolesion removal did not prevent chronic hyperplasia and p53 patch induction. Melanin production was refrained by 6-4PP removal from basal keratinocytes in XPA mice.

In CSA animals, CPD removal from basal keratinocytes partially prevented chronic hyperplasia and dysplasia, through reduced basal proliferation and increased basal apoptosis. 6-4PP removal from basal keratinocytes did not alter UV-induced cell and tissue responses. UV exposure alone or in combination with photolesion removal did not induce melanogenesis in CSA mice.

These results demonstrate that, in a DNA repair-deficient background, CPDs play a major role in skin responses to acute and chronic UV irradiation, especially in cell proliferation and p53 patch induction. However, CPDs are clearly not the sole player in skin responses to UV exposure. 6-4PPs seem to play a minor role in skin responses to low UV doses, being mainly involved in apoptosis and melanogenesis.

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