GUSTAVO SATORU KAJITANI

In vivo effects of DNA lesions in Nucleotide Excision Repair deficient mice

Tese apresentada ao Programa de Pós-Graduação Interunidades em Biotecnologia da Universidade de São Paulo, Instituto de Ciências Biomédicas para obtenção do Título de Doutor em Biotecnologia.

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CERTIFICADO

Certificamos que o protocolo registrado sob nº **121** nas fls. **11** do livro **03** para uso de animais em experimentação, sob a responsabilidade do Prof(a) Dr(a)) **Carlos Martins Frederico Menck,** Coordenador (a) da Linha de pesquisa "*Efeito da fotorremoção específica de lesões induzidas por luz utravioleta em camundongos deficientes em reparo de DNA*" do qual participam o(s) aluno(s) **Gustavo Satoru Kajitani,** está de acordo com os Princípios Éticos de Experimentação Animal adotado pela Sociedade Brasileira de Ciência de Animais de Laboratório (SBCAL) e foi aprovado pela *COMISSÃO DE ÉTICA NO USO DE ANIMAIS* (CEUA) em **04.11.2013, com validade de 4 anos**.

São Paulo, 06 de novembro de 2013.

p/ Bueno

Prof. Dr. WOTHAN TAVARES DE LIMA
Coordenador-CEUA- ICB/USP

Profa. Dra. ANA PAULA LEPIQUE
Secretária- CEUA - ICB/USP

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“The rock is still rolling.”

Albert Camus - The Myth of Sisyphus

RESUMO

Kajitani, G.S. In vivo effects of Nucleotide Excision Repair related DNA lesions. [Tese (Doutorado em Interunidades em Biotecnologia)]- Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2018.

A molécula de DNA, responsável por carregar informações genéticas, está sob constante estresse químico e físico, proveniente de fontes endógenas e exógenas, que pode levar à formação de lesões no DNA. Para lidar com esses danos, células dispõem de mecanismos de reparo, sendo as lesões que distorcem a molécula de DNA reparadas pela via de Reparo por Excisão de Nucleotídeos (NER). Deficiências em genes da via NER podem levar à doenças humanas, como o Xeroderma Pigmentosum (XP) e a Síndrome de Cockayne (CS), caracterizadas principalmente por um grande aumento na incidência de câncer de pele e por neurodegeneração relacionada à um fenótipo de envelhecimento precoce, respectivamente. Para melhor compreendermos dessas doenças, assim como os efeitos sistêmicos das lesões de DNA relacionadas à via NER, são utilizados modelos de camundongos nocaute (KO), assim como fontes exógenas geradoras de danos no DNA, como a radiação ultravioleta (UVR). Neste trabalho, usamos dois modelos deficientes em NER para estudar os efeitos *in vivo* de lesões relacionadas à via NER, sendo o primeiro um modelo que mimetiza XP e o segundo CS. No primeiro modelo, XPA KO, estudamos o efeito das principais lesões geradas por UVR, os dímeros de pirimidina ciclobutano (CPDs) e os pirimidina (6-4) pirimidona fotoprodutos (6-4PPs) em queratinócitos. Para tanto, utilizamos fotoliases, enzimas capazes de reparar especificamente ou lesões do tipo CPD (CPD-phl) ou 6-4PP (6-4PP-phl). Observamos que em camundongos XPA KO, a remoção de CPDs foi capaz de inibir completamente a proliferação de células epidermais induzidas por UVR, enquanto a remoção de 6-4PPs reduziu, porém não impediu esse efeito. A remoção de lesões do tipo CPD ou 6-4PP foi capaz de diminuir os efeitos de morte celular e extravasamento de leucócitos na pele induzida por UVR em níveis similares, indicando que CPDs têm um maior impacto que 6-4PP sobre o efeito de hiperplasia, enquanto ambos tipos de lesão possuem efeitos similares na indução de apoptose e inflamação por UVR em camundongos XPA KO, tendo os queratinócitos um papel central na regulação desses efeitos. No segundo modelo, estudamos os efeitos de lesões relacionadas ao envelhecimento em camundongos duplo nocaute para os genes CSA/XPA (CX), previamente descrito como tendo morte prematura e neurodegeneração. Apesar de termos encontrado evidências de falhas na barreira hematoencefálica (BBB) nesses animais, não encontramos indícios de disfunção nas células endoteliais. Descobrimos, no entanto, um aumento significativo de marcadores de neuroinflamação, assim como ativação de astrócitos

e microglia, os dois principais tipos celulares relacionados à ativação de inflamação no cérebro, indicando que a neuroinflamação pode estar relacionada à neurodegeneração e defeitos da BBB encontrados neste modelo. As descobertas nesses modelos deficientes em NER podem ajudar a elucidar o papel in vivo das lesões de DNA em relação à resposta de morte e proliferação celular, assim como demonstra novos impactos da DDR sobre a indução de inflamação, com esses efeitos tendo implicações na etiologia de XP e CS, assim como fenômenos associados a danos no DNA como câncer e envelhecimento.

Palavras chave: Danos no DNA. Reparo por excisão de nucleotídeos. Inflamação. Morte Celular. Proliferação Celular.

ABSTRACT

Kajitani, G.S. In vivo effects of Nucleotide Excision Repair related DNA lesions. Ph.D thesis [(Interunits in Biotechnology)] - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2018.

The DNA molecule, responsible for carrying genetic information, is under constant chemical and physical stress, both by endogenous and exogenous sources, which may lead to the formation of DNA lesions. These damages are dealt with using several different DNA repair mechanisms, with lesions that distort the DNA molecule are repaired by the Nucleotide Excision Repair (NER) pathway. Deficiencies in the genes related to NER may lead to human syndromes, such as Xeroderma Pigmentosum (XP) and Cockayne Syndrome (CS), characterized mainly by a severely increased skin cancer incidence and premature aging like (progeroid) neurodegeneration, respectively. In order to further study these diseases, as well as the role of NER-related DNA lesions in generating cellular and systemic effects, knockout (KO) mice models are often used, as well as exogenous DNA damaging sources, such as ultraviolet radiation (UVR). In this work, we used two NER deficient models to study in vivo effects of NER-related lesions, the first KO model mimicking XP and the second one CS. In the first model, we studied the effect of the main UVR generated photolesions, cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) on keratinocytes, one of the main cell types in the epidermis. In order to study the effect of each lesions, we used photolyases, enzymes that specifically repair either CPD (CPD-phl) or 6-4PP (6-4PP-phl) lesions. We observed that in XPA KO mice, CPD removal in keratinocytes was able to completely inhibit UV induced epidermal cell proliferation and hyperplasia, while the removal of 6-4PPs in keratinocytes reduced, but not abolished these effects. The removal of either CPDs or 6-4PPs in keratinocytes was able to reduce UV induced cell death and leukocyte extravasation on similar levels, indicating that CPDs have a greater impact than 6-4PPs regarding the hyperplasia effect of UV irradiation, and that both types of DNA lesions have similar effects on promoting apoptosis and inflammation in XPA KO mice, with keratinocytes having a central role in regulating these effects. In the second model, we studied the effect of aging related lesions on CSA/XPA double knockout (CX) mice, previously established as exhibiting premature death and neurodegeneration. Although we found evidence of blood brain barrier (BBB) defects in CX mice, we did not find cell autonomous vascular dysfunction. However, we discovered a significant increase of neuroinflammation markers, as well as activation of astrocytes and microglia, the two main endogenous inflammation related cell types of the brain, which indicates that neuroinflammation could play a role in the neurodegenerative and BBB phenotype observed

in this model. The findings in these two NER deficient models help elucidate the *in vivo* role of DNA lesions regarding cell death and proliferation response, as well demonstrating novel impacts of DDR on inflammation induction, with these effects having implications on the etiology of XP and CS, as well as DNA damage associated biological phenomena such as cancer and aging.

Keywords: DNA damage. Nucleotide Excision Repair. Inflammation. Cell death. Cell proliferation.

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6-4PP-phl – “6-4PP photolyase
6-4PP – “Pyrimidine (6-4) Pyrimidone
AMPK – “Adenosine Monophosphate-activated Protein Kinase
APC – “Allophycocyanin
ATM – “Ataxia Telangiectasia Mutated
ATP – “Adenosine Triphosphate
ATR – “Ataxia Telangiectasia and Rad3 related
BBB – “Blood Brain Barrier
Bcl-2 – “B-cell lymphoma 2
BER – “Base Excision Repair
BrdU – “5-Bromo-2´-Deoxyuridine
BSA – “Bovine Serum Albumin
CD – “Cluster of Differentiation
Chk1-2 – “Checkpoint kinase 1 and 2
CPD – “Cyclobutane Pyrimidine Dimer
CPD-phl – “CPD photolyase
CSA/CSB – “Cockayne Syndrome complementation groups A and B
CS – “Cockayne Syndrome
CX – “CSA and XPA double knockout mouse model
DAB – “3,3'-Diaminobenzidine
DDB – “Damage Specific DNA Binding Protein
DDR – “DNA Damage Response
Dewar-PP – “Dewar photoproduct
DMEM – “Dulbecco's Modified Eagle's medium
DNA – “Deoxyribonucleic Acid
DNA pol – “DNA polymerase
E. coli – “Escherichia coli
EC – “Endothelial Cell
EDTA – “Ethylenediamine tetraacetic acid
EGF – “Epidermal Growth Factor
EGFR – “Epidermal Growth Factor Receptor
ERCC1 – “Excision repair cross-complementing 1
ERK – “Extracellular signal–regulated kinase
FAD – “Flavin Adenine Dinucleotide
FBS – “Fetal Bovine Serum

GAPDH – “Glyceraldehyde 3-phosphate dehydrogenase

GFAP – “Glial fibrillary acidic protein

GG-NER – “Global Genome Nucleotide Excision Repair

H&E – “Hematoxylin & Eosin

HR – “Homologous Recombination

Iba1 – “Ionized calcium binding adaptor molecule 1

ICAM-1 – “Intercellular Adhesion Molecule 1

IGF-1 – “Insulin-like Growth Factor 1

IL – “Interleukin

IVIS – “In vivo imaging system

JNK – “c-Jun N-terminal kinase

MED – “Minimal Erythemal Dose

MMP – “Matrix metalloproteinase

MMR – “Mismatch Repair

MO – “Monocyte

MPO – “Myeloperoxidase

MRI – “Magnetic Resonance Imaging

NAD+ - “Nicotinamide adenine dinucleotide

NE – “Neutrophil

NER – “Nucleotide Excision Repair

NF-κB – “Nuclear Factor kappa B

NHEJ – “Non-homologous End Joining

NLRP3 – “Nod-like receptor protein 3

NMN – “Nicotinamide Mononucleotide

LY – “Lymphocyte

OCT – “Optimal cutting temperature

P53 – “Tumor protein p53

PARP-1 – “Poly (ADP-ribose) polymerase 1

PBS – “Phosphate Buffered Saline

PCR – “Polymerase Chain Reaction

PFA – “Paraformaldehyde

PGC-1α – “Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PI3K – “Phosphoinositide 3-kinase

Pol η – “DNA polymerase eta

qRT-PCR – “Quantitative real time polymerase chain reaction

RNA pol I/II – “RNA polymerase I and II

RNA – “Ribonucleic Acid

ROS – “Reactive Oxygen Species

RPA – “Replication Protein A

RPMI – “Roswell Park Memorial Institute

RPM – “Rotations per minute

RT – “Room Temperature

SASP – “Senescence Associated Secretory Phenotype

SA-βgal – “Senescence Associated Beta galactosidase

TC-NER – “Transcription Coupled Nucleotide Excision Repair

TFIIH – “Transcription Factor II H

TLS – “Translesion Synthesis

TNF – “Tumor Necrosis Factor

TTD – “Trichothiodystrophy

UVA-UVC – “Ultraviolet A to C

UV-DDB – “UV damage DNA binding protein

UVR – “Ultraviolet Radiation

UVSSA – “UV Stimulated Scaffold Protein A

UV – “Ultraviolet

VCAM-1 – “Vascular cell adhesion molecule 1

VEGFR2 – “Vascular endothelial growth factor receptor 2

VEGF – “Vascular Endothelial Growth Factor

VLCAD – “Very long-chain specific acyl-CoA dehydrogenase, mitochondrial

WBC – “White Blood Cell

XPA-G – “Xeroderma Pigmentosum complementation groups A to G

XP-V – “Xeroderma Pigmentosum Variant

XP – “Xeroderma Pigmentosum

XRCC1 – “X-ray repair cross-complementing protein 1

Preface

This work aims to better understand the *in vivo* effects of DNA damage in Nucleotide Excision Repair (NER) deficient models, especially regarding their role on cell proliferation, cell death and inflammation. Mouse experiments were performed in the Institute of Biomedical Sciences of the University of São Paulo (São Paulo, SP, Brazil) from December 2013 to December 2016 and in Harvard T.H. Chan School of Public Health (Boston, MA, USA) from February 2017 to January 2018.

This thesis was organized into 5 chapters, with the intention of publishing 2 manuscripts for publication in scientific magazines based on Chapters 2 and 3. Chapter 1 is a general introduction on DNA damage, the NER pathway and known responses to DNA damage. Chapter 2 focus on the effects of the two main DNA lesions caused by ultraviolet radiation on NER deficient, XPA knockout mice. Chapter 3, based on a collaborative work with the Mitchell lab from Harvard School of Public Health, is centered on the characterization, especially regarding endothelial dysfunction and inflammation of a CSA/XPA double knockout mouse model that displays early aging-like features. Chapter 4 displays additional data regarding the previous and other NER deficient models. Chapter 5 presents this thesis general conclusions, followed by a list of references used for this thesis and the articles published during this doctorate as attachments.

SUMMARY

Chapter 1 - General Introduction

1.1 The DNA molecule - structure and function

The discovery of the structure of the DNA molecule was reported over 60 years ago, on 1953, in a seminal paper by Drs. James Watson & Francis Crick (1) – a breakthrough that rendered them the Nobel prize in Chemistry. Due to its importance to several fields in Biology, Chemistry, Medicine and numerous other areas, that finding is often considered to be one of the most important discoveries of the last century (2). The story of the DNA as the life molecule, however, began many years before the report of its canonical double helix structure, on the 19th century. DNA was first identified in the 1860s, in a research regarding the key components of white blood cells. A substance with different properties to proteins was found, then named as “nuclein”, as it was believed that this substance was present in the cell nucleus (3). Further studies confirmed nuclein as present in the nucleus and revealed its acidic nature (4). The structure of the sugar present in the acid, along with its placement inside the cell and its acidic properties caused this substance to be renamed as “DeoxyriboNucleic Acid” (DNA). The chemical components that make up the DNA molecule were identified, with it being composed of phosphate, sugar and four nitrogenous bases: adenine (A), cytosine (C), guanine (G) and thymine (T), with those components, arranged in the order of phosphate, sugar and base, forming a unit named as “nucleotide” (5). However, in spite of the DNA components being identified, its biological role and chemical structure was still unknown.

Meanwhile, other important biology discoveries were being unraveled. A fibrous structure within the nucleus of cells was discovered, being named “chromatin”, due to its eye catching color after being stained with a basic dye - this structure was later renamed “chromosomes”, with the “chromatin” name being repurposed as the molecular complex of DNA, RNA and proteins that make up the chromosomes in eukaryotic cells (6). Later developments provided evidence for the chromosome theory of inheritance, which postulates that the genetic material is located within the chromosomes, a theory that helped explain the mechanism of the mendelian inheritance laws, unifying cell biology with genetics (7).

The end of the 19th century and the beginning of the 20th century was a period of intense debate about which molecule was responsible for containing genetic information - For a long time, it was believed that proteins were responsible for this function, with the very word protein being coined due to the belief that it had a primary function for cell biology (8). Moreover, the DNA molecule has a much simpler composition than proteins - only four nucleotides, as opposed to more than 20 amino acids, which led many researchers to believe that it could not have a key role in genetic inheritance (9). However, a series of experiments conducted in the middle of the 20th century indicated that, instead of proteins, DNA was responsible for the function of carrying the genetic information (10). Although those results were not conclusive, further discoveries evidenced the role DNA as the genetic material (11).

Further clarification came soon after, with the paper by Watson and Crick on the structure of the DNA molecule, which proposed its structure as a double helix containing two strands of DNA, with base pairing happening on a complementary manner - Adenine pairing with Thymine and Cytosine pairing with Guanine by hydrogen bonds, with the intra strand structure being maintained by bonds between the phosphate groups and the sugar backbone, with these linkages being performed in the third (3') and fifth (5') carbon of the deoxyribose ring (Figure 1.1). This paper was soon followed by another, suggesting that for DNA replication, the two DNA strands would function as the basis for the daughter strands (12), a phenomena that came to be known as the semi-conservative DNA synthesis (13). It is worth noting that, as with all discoveries in science, this discovery would not have been possible without several others preceding it - not only the ones cited beforehand, but also exceptionally the photo 51, taken and interpreted by Rosalind Franklin (14), and the discovery of the 1:1 base pairing ratio of Adenine to Thymine and Cytosine to Guanine by Erwin Chargaff (15).

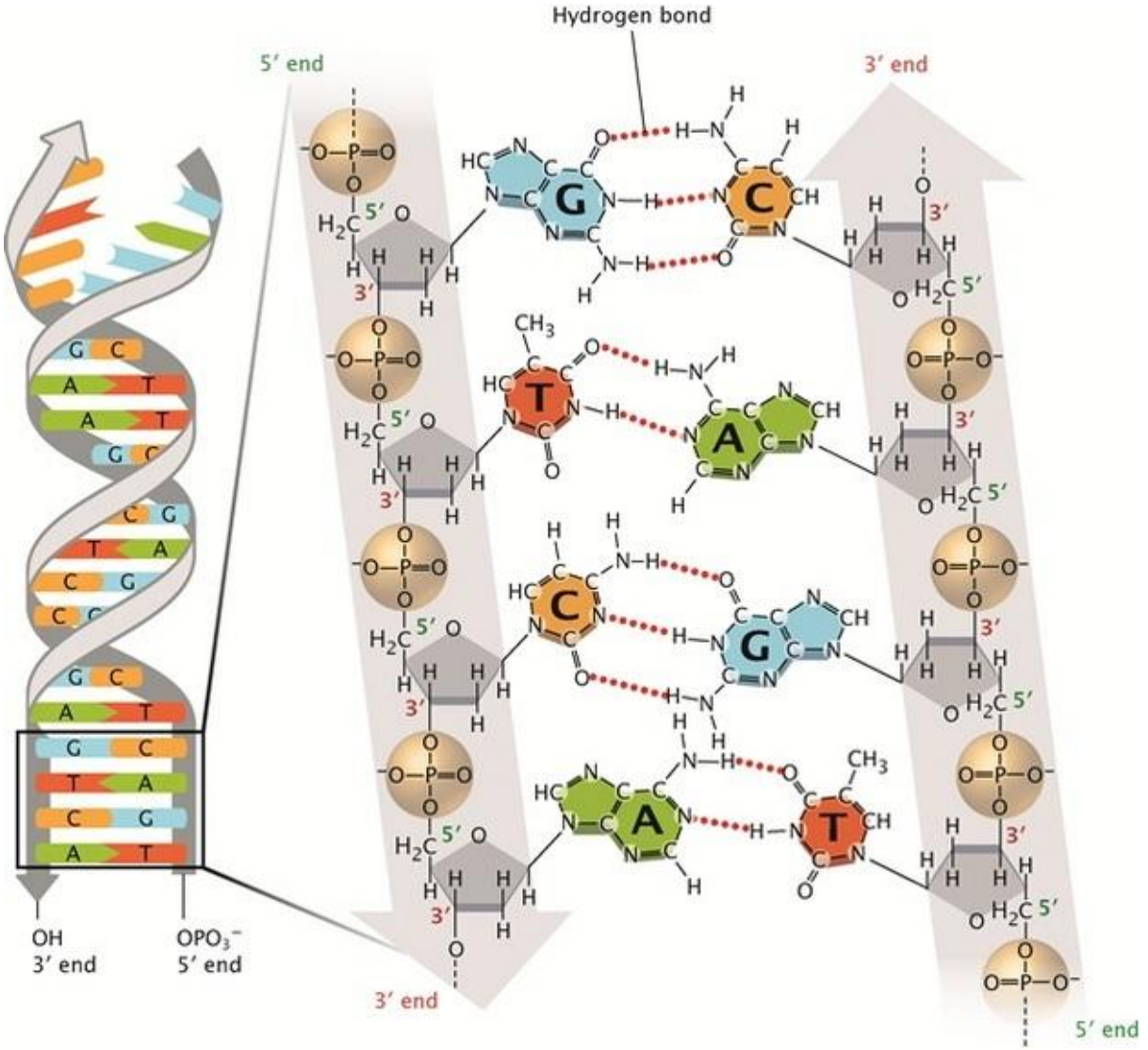

Figure 1.1. Structure of the DNA molecule. The double helix with 3' and 5' ends, alongside each DNA strand chemical structure and base pairing model. Adapted from Pray, 2008 (16).

After the confirmation of Watson and Crick suggestions on the DNA structure and replication mechanism, alongside the identification of its function as a master blueprint for coding proteins [17], DNA was solidified as the molecule that holds the genetic information. The finding of the DNA as the genetic molecule holds great importance in the life sciences, being considered a landmark that paved the way for numerous subsequent discoveries and opening up the field of molecular biology and its subfields, such as molecular biotechnology, gene editing, RNA biology being opened up afterwards.

The canonical structure of the DNA double helix, alongside the canonical A-T and C-G base-pairing, however, are prone to several spontaneous or environmental induced modifications. This was also a surprising discovery - because of the

importance of this molecule, it was assumed that it ought to be extraordinarily stable in order to maintain a high degree of fidelity required of a master blueprint (17). However, it was soon discovered that this was not the case.

1.2 DNA damage and Repair

After DNA was established as the molecule responsible for carrying the genetic information, it was generally thought that it was be incredibly chemically stable in order to maintain the integrity of the information held within it. Although that holds true in some states, such as in frozen and/or fossilized DNA (18), DNA is also an incredibly dynamic molecule and subject to constant change and stress when under life conditions - a finding that was something of a surprise at the time of its discovery (17). Francis Crick himself later recognized the importance of DNA maintenance through mechanisms such as DNA repair (19):

"we totally missed the possible role of …(DNA) repair although… I later came to realize that DNA is so precious that probably many distinct repair mechanisms would exist"

In physiological conditions, DNA is under constant stress, being subjected to several different kinds of chemical and physical stresses capable of inducing various types of modifications on the DNA structure (20). These stresses may be caused by the external environment, but may also be generated spontaneously by cell metabolism, such as reactive oxygen species (ROS) formation during cellular respiration. Moreover, the DNA replication and transcription machinery themselves can create stressful situations capable of destabilizing the DNA structure and generating mismatched base pairing, DNA breaks and other forms of chemical modifications. These modifications are broadly considered to be DNA lesions (17). Each and every primary component of DNA - bases, sugar and phosphodiester bonds are liable to being damaged. Amongst those modifications, there are lesions such as single and double DNA strand breaks, base modifications such as alkylation, methylation, and oxidation, non-canonical base pairings, base dimerization, intra or interstrand crosslinks, protein-DNA crosslinks, RNA:DNA structures and non-canonical DNA structures, such as G-quadruplex structures. All of these different

kinds of modifications have distinct effects on DNA and cell metabolism, involving distinct but often overlapping pathways in order to deal with these types of damage (21,22).

Much like the discovery of DNA itself, knowledge on these lesions, as well as the pathways responsible for dealing with them, collectively termed the DNA Damage Response (DDR), was built in a non-sequential manner. In fact, DNA damaging agents, such as, X-rays, were found to be mutagenic before the establishment of the DNA as the life molecule (23). Moreover, publication of Alexander Hollaender in 1939 had identified that the UVR induced mutagenesis in fungi was coincident of that of nucleic acids, also indicating that nucleic acids were the components of genes (24). The first DNA repair mechanism, the light dependent “reactivation” of viruses - thereafter named photoreactivation, was also found before 1953 (25). Obviously, since the DNA was not yet established as the genetic molecule, these discoveries were not linked to it at the time, but further findings helped elucidating the molecular structure and mechanism behind these lesions and their repair, such as the discovery of the photolyases enzymes in 1958 (26), responsible for the photoreactivation mechanism first discovered in 1949. Studies on many distinct models done by numerous different groups have had roles on building the DDR field, with Figure 1.2 containing some of the main discoveries in a timeline.

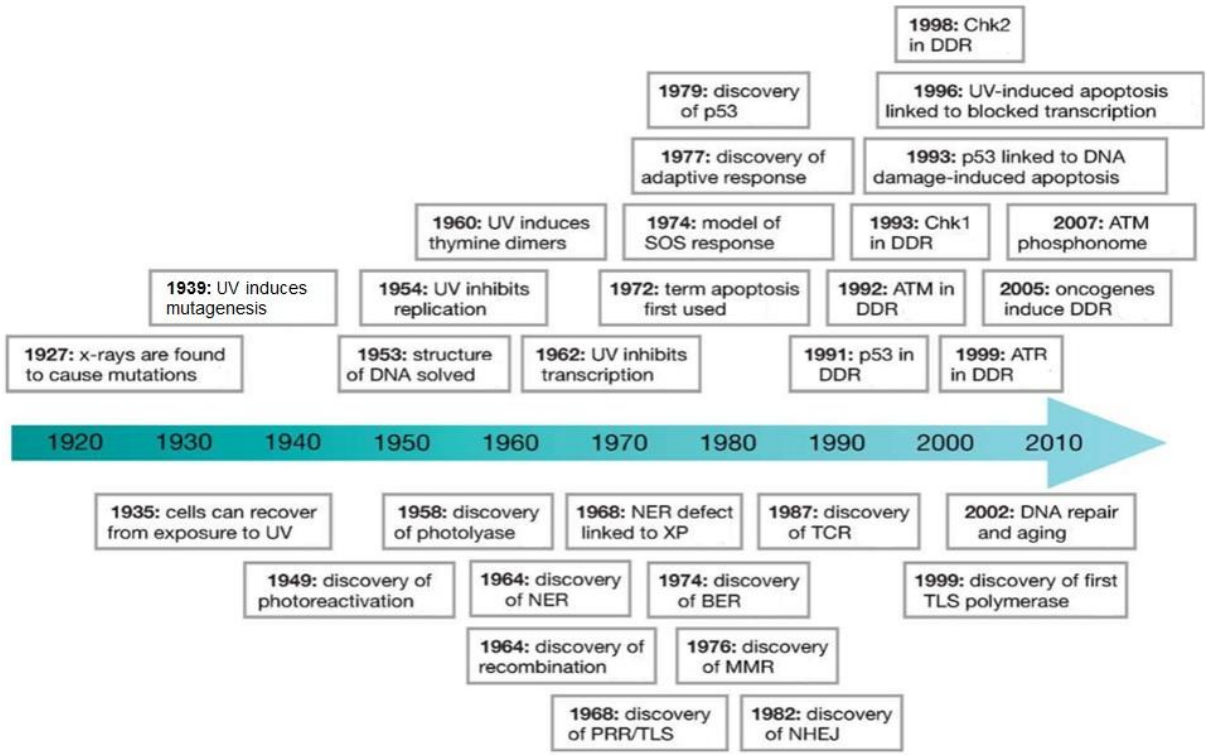

Figure 1.2. Timeline of major discoveries regarding the DNA damage response. Findings on effects of DNA damage are shown above the time arrow, and below it are the findings on DNA repair and tolerance mechanisms. Adapted from Ljungman, 2010 (27).

Considering the multitude of these sorts of damage, it is not surprising that organisms possess several molecular pathways able to deal with them. Some of main DNA repair pathways and the types of DNA lesions they are associated with are represented in figure 1.3. Notably, although there are established types of DNA repair generally considered to be the canonical DNA repair pathways, new pathways and kinds of damage are still being discovered, alongside their effects (28). Moreover, besides DNA repair, cells have other ways to deal with harmful agents, such as antioxidant defenses (29), physical blockage of damaging agents (30), and other molecular stress response mechanisms to better deal with or tolerate DNA damages (31).

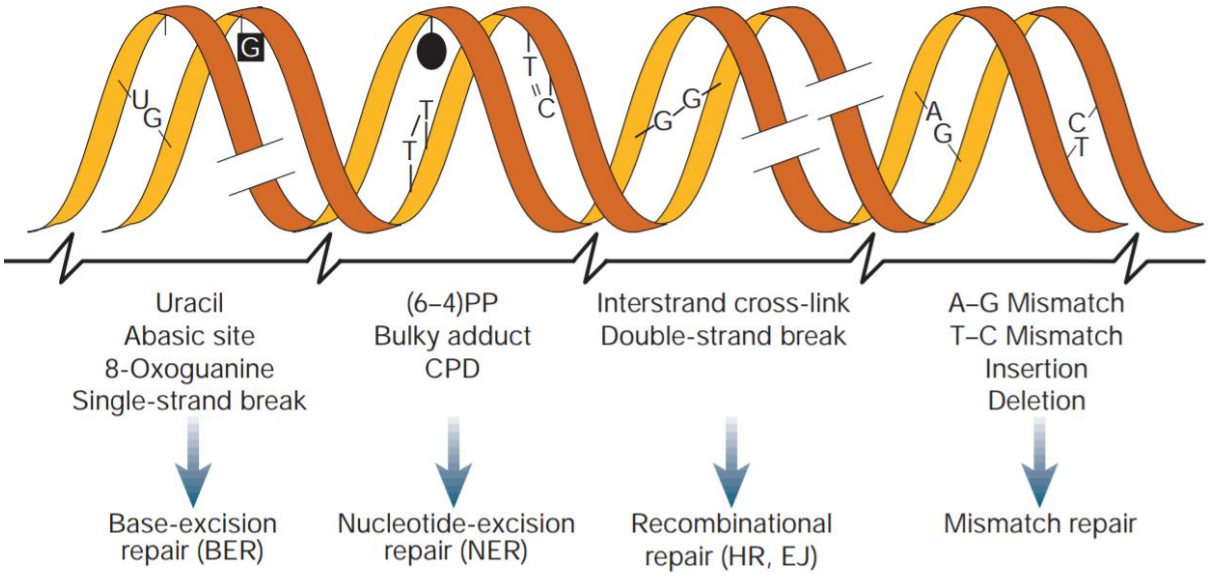

Figure 1.3. DNA lesions and repair pathways. Representation of the DNA molecule and damages that may occur to it, with the respective repair pathways responsible for dealing with these lesions displayed below them. Hoeijmakers, 2001 (32).

In this work, we will focus on a specific DNA repair pathway, responsible for repairing a wide array DNA helix distorting lesions, the Nucleotide Excision Repair (NER) pathway.

1.3 Nucleotide Excision Repair - Associated damages and diseases

The Nucleotide Excision Repair (NER) pathway was first hinted at in 1958, when an *E. coli* strain sensitive to ultraviolet (UV) irradiation was discovered (33). At the time, it was already known that UV radiation (UVR) had genotoxic and mutagenic properties (27), with this *E. coli* strain being a valuable and attractive tool for studying how organisms deal with genetic damage (34). By using methods for the measurement of DNA synthesis in this bacteria, it was found that DNA replication in the UVR sensitive strain was inhibited by minute amounts of UV irradiation when compared to a regular strain, linking UVR mediated DNA damage (also known as photolesions) to the blockage of DNA synthesis (35). It was then postulated that the recovery of DNA synthesis in the non-sensitive bacterial strand must have been due to some sort of repair of the DNA damage, with this kind of damage then rightfully presumed to be pyrimidine dimers (34,35). Furthermore, this repair mechanism was able to operate independently of light - an important observation, considering that

another, light dependent repair system (photoreactivation), had been discovered beforehand (36). It was then hypothesized that this damaged DNA was excised by a lesion removal pathway, with a new strand being synthesized to replace it. This was later confirmed, with small DNA fragments containing thymine dimers being present in UV irradiated bacterial cell cultures (37) and with the discovery of a non-semiconservative mode of DNA replication, with this unscheduled DNA synthesis following UV irradiation involving short, single-stranded DNA sections (38). This pathway that repairs lesions that distort the DNA double helix, including photolesions, and involves the enzymatic excision of chemically altered nucleotides later came to be known as the Nucleotide Excision Repair pathway.

The NER pathway was subsequently further characterized in *E. coli*, with the related proteins and their functions in the pathway being identified. This helped to better elucidate the molecular mechanisms of the NER pathway, uncovering the types of lesion it is capable of repairing and revealing the necessary steps for the lesion removal and repair, with these being the recognition of the lesion, dual excision of the DNA fragment containing the lesion, resynthesis of the excised section and ligation of the newly synthesized fragment to the pre-existing DNA strand (39).

The *E. coli* proteins involved in the NER pathway are generally conserved within prokaryotes, as well as the mechanism itself. Surprisingly, despite the eukaryote NER genes not being related to the prokaryote ones, the mechanism itself is strikingly similar, containing the same steps, although the eukaryotic NER is significantly more complex, involving more than 30 different proteins, as opposed to the 7 proteins in *E. coli* (40).

NER genes are generally conserved in Eukarya (41), with most of the studies regarding its mechanism being performed in humans and mice models, due to their biomedical significance. The eukaryotic NER (hereafter referred to simply as NER), pathway contains two subpathways for lesion recognition, the Transcription-Coupled Repair (TC-NER) and Global Genome Repair (GG-NER), with the signal for TC-NER being the stalling of a RNA polymerase (RNA pol I and II), with this subpathway being exclusive to transcriptionally active genes. Cockayne Syndrome proteins A and B

(CSA and CSB) and UV-stimulated scaffold protein A (UVSSA) proteins are involved in the removal of the arrested RNA pol II and signaling for the excision complex. The human GG-NER scans the genome for DNA lesions by utilizing the Xeroderma Pigmentosum C (XPC)-human RAD 23 homolog B (hHR23B) complex with the aid of another protein complex, the UV-DDB, composed of a dimer containing DDB1-DDB2 (DDB2 also being known as XPE), with this complex acting upon specific types of damage, such as the photolesion cyclobutane pyrimidine dimer (CPD). The excision complex is then recruited to the lesion site after the damage is recognized by either subpathway (31,42).

The excision complex involves a large number of proteins, with the XPA protein having a key, scaffolding function for the assembly of other NER factors. Another central player during this step is the transcription factor II H (TFIIH) complex, which contains several subunits required for NER, including the helicases XPB and XPD and the endonucleases XPG and the excision repair cross complementing 1 (ERCC1)-XPF complex. XPB functions in a 5'-3' direction, while XPD acts in 3'-5', thereby unwinding the DNA molecule, with replication protein A (RPA) proteins being recruited to stabilize single stranded DNA. The ERCC1-XPF endonuclease then cleaves the damaged strand 5' to the lesion site, leading to the initiation of the synthesis of a new DNA strand by polymerase δ, κ or Pol ε assisted by the proliferating cell nuclear antigen (PCNA) protein, followed by 3′ incision by XPG. Finalizing the NER, DNA ligase I or DNA ligase IIIα/XRCC1 then ligates the newly synthesized strand to the previous DNA (31,42). A simplified model of the human NER is shown below in figure 1.4.

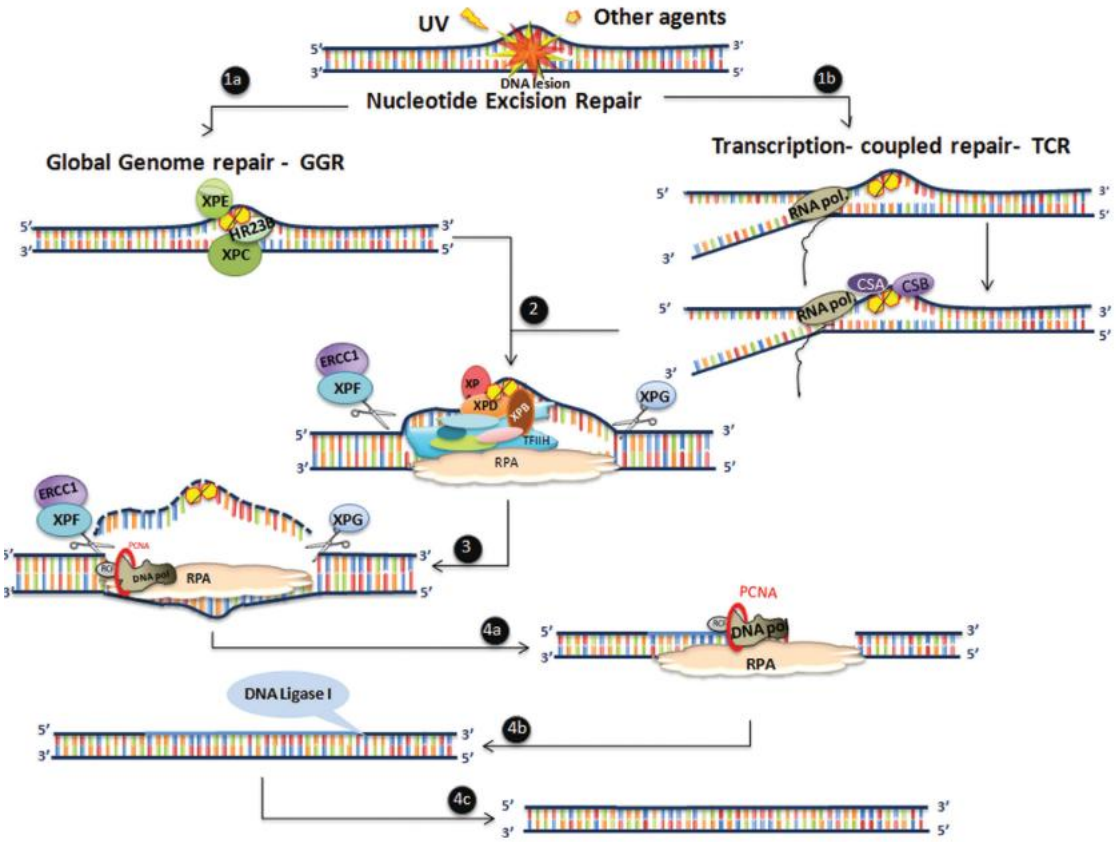

Figure 1.4. The Nucleotide Excision Repair pathway. A simplified model displays the two recognition subpathways, TC-NER and GG-NER and the steps following lesion recognition - the formation of the excision complex, removal of DNA fragment containing the lesion, resynthesis and ligation of the new DNA. Menck & Munford, 2014 (31).

Mutations in NER related genes may result in a plethora of different disorders, as shown in figure 1.5 X. These include Xerodema Pigmentosum (XP), Cockayne Syndrome (CS), Trichothiodistropy (TTD), UV-Sensitive Syndrome (UVSS), Cerebro-oculo-facio-skeletal syndrome (COFS), and combinations of these phenotypes (43). The symptoms, affected organs and age of onset of these diseases vary a lot, though they usually include cancer and/or progeroid (early aging-like) features, such as neurodegeneration. In fact, these two features are also commonly associated with diseases related to other, non-NER repair pathways, which serves to demonstrate the importance of DNA repair systems to cancer and aging. A review of these genetic diseases and their relative DNA repair pathways can be found in (44). In this work, we will focus on two of the most important NER-related disorders, Xeroderma Pigmentosum and Cockayne Syndrome.

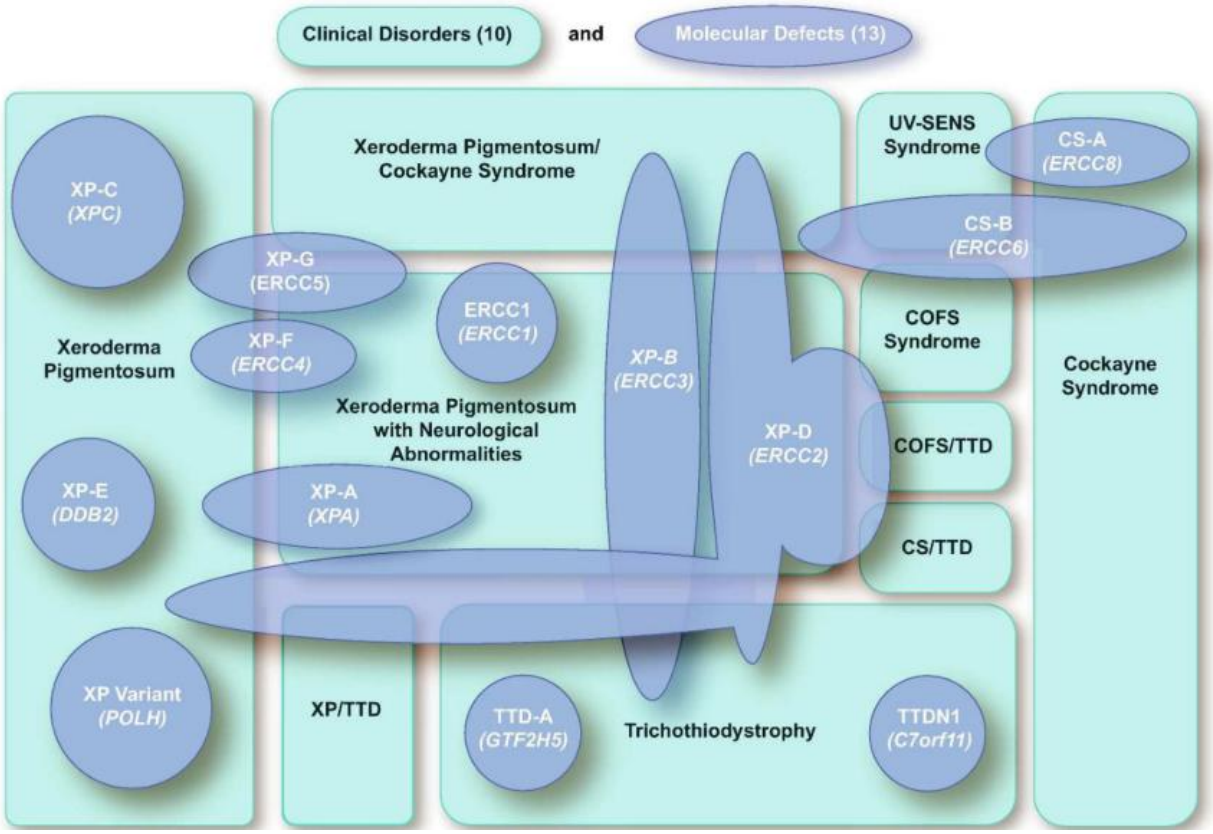

Figure 1.5. NER related disorders. Overview of syndromes caused by mutations in NER genes, and how the clinical phenotypes may overlap. DiGiovanna & Kraemer, 2012 (43).

1.3.1 Xeroderma Pigmentosum, photolesions and skin cancer

Xeroderma Pigmentosum is a rare, autosomal recessive disease with an estimated incidence of one per one million people, depending on the population (45). It manifests clinically as increased cancer risk of sun exposed areas, such as skin and mucous membranes of the eyes and mouth, having >2,000-fold increase in the incidence of skin cancers before the age of 20, photosensitivity, with pronounced burning and blistering of the skin after sun exposure, actinic damage to the skin (46). Unrelated to the sun-exposure, some XP patients also express progressive neurologic degeneration, with cerebral atrophy and primary neuronal degeneration with around 25% of XP patients having neurological symptoms, which include hearing loss, swallowing difficulties and mental retardation (43).

Our current understanding of XP starts on the 19th century in 1874, when Dr. Moriz Kaposi first described XP patients as having a dry, thin and wrinkled

appearance of their skin, alongside its checkered pigmentation, with these characteristics being responsible for the coining of the term XP, with Xeroderma meaning “dry skin”. On the 20th century, with the advancement of cell culture techniques, alongside many other discoveries related to molecular biology, including DNA repair, it was found that cells from XP patients presented hypersensitivity to UVR, and that these cells had a deficiency in the NER pathway, being unable to correctly remove photolesions from the genome (43).

The many XP NER related genes were initially found due to cell fusion studies, based on complementation of genes and then repair resynthesis measurements (unscheduled DNA synthesis), which demonstrated the presence of seven XP complementation groups (XP-A to XP-G). These genes were later cloned and identified. These cellular models paved the way for unveiling the mechanisms of the human NER pathway by way of the function of the proteins encoded by the *XPA-XPG* genes, and the order that they act for the repairing of the lesions (31). A variant complementation group was also found, named XP-V, described as having functional NER, but are defective for the *POLH* gene, which codifies for the DNA polymerase eta (pol η) protein, first described to participate in a postreplication repair process, then found to be a polymerase that takes part in a DNA damage tolerance mechanism, now known as Translesion Synthesis (TLS). Thus, the pol η does not repair the damaged strand, but rather bypass the lesion. The TLS mechanism acts using several different DNA TLS polymerases, with each having a different preferential substrate, with pol η having a higher affinity and replication fidelity for pyrimidine dimers formed by UVR (47). XP-V patients generally have similar, although milder symptoms to the XPA-XPG patients, including high skin cancer predisposition and photosensitivity. However, in contrast to many of the NER-deficient patients, they do not exhibit any form of neurological abnormalities, with UVR-induced photolesions being related to the skin symptoms of all types of XP patients (48).

UVR is electromagnetic radiation often defined having a wavelength range of 100 to 400 nm and can be subdivided into UVA (from 315 to 400 nm), UVB (280–315 nm) and UVC (100-280 nm). Broader definitions may also include smaller wavelengths down to 10 nm (classified as either Vacuum UV or Extreme UV), though

these are less commonly used for biological models, as they require vacuum for transmission and do not reach the biosphere (49). Although UVC is blocked by the ozone layer, it is often used as a UVR model for biological processes due to its ease of use and its characteristics as being “clean” source of DNA damage, as it directly causes the same DNA photolesions as UVB, a more physiologically relevant DNA damaging source (50). Sunlight is responsible for Earth’s environmental UVR, as the electromagnetic spectrum emitted by the Sun encompasses X-rays, UVR, visible light and infrared light, with UVA and UVB being the only bands of UVR capable of passing through the ozone layer and reaching the biosphere. Though UVB is partially blocked by the ozone layer, it is biologically significant due to its properties of directly generating DNA photolesions (51).

UVR is capable of damaging the DNA molecule both directly and indirectly, due to its absorption by numerous cellular components, including DNA, proteins, RNA and other organic compounds (52,53). The type of molecule affected by UVR depends on the UV wavelength, with nucleic acid, such as DNA and RNA, being very effective in absorbing UVB, and chromophores and proteins such as collagen, elastin and melanin generally having a higher absorbance for UVA (54). Though in this work we will focus on the direct DNA lesions caused by UVB, UVR is also capable of generating DNA damage by indirect intracellular mechanisms through the production of reactive oxygen species (ROS) after photosensitized chromophores react with oxygen molecules (54). ROS oxidize DNA in a plethora of ways, often reacting with DNA bases and in some cases, breaking the phosphate-sugar bond, thus generating DNA strand breaks (55). These ROS are mainly induced by UVA irradiation, with UVB playing a minor role in generating them (56).

The direct UVR induced DNA damage, the pyrimidine dimers, are formed after UVR is absorbed by thymine or cytosine, which generates covalent bonds between that pyrimidine with an adjacent pyrimidine of the same DNA strand via photochemical reaction. The main DNA photolesions formed by UVR absorption are the Cyclobutane Pyrimidine Dimers (CPDs) and the Pyrimidine (6-4) Pyrimidone photoproducts (6-4PPs). These lesions are not only chemically different (as represented in figure 1.6), with covalent bonds forming between different carbons of

the pyrimidines (57), but also differ in their formation rate, with CPDs being 3 to 5 more frequently generated than 6-4PPs (58,59).

The sites of formation also differ between these two kinds of lesions, 6-4PPs having a smaller generation at either CT or CC sites than CPDs, and 6-4PPs being formed in a generally less uniform manner than CPDs at nucleosomes, CPDs generally being formed once every 10 nucleotides at this site. 6-4PPs are also more liable to being further changed by UVA, which may induce the formation of a 6-4PP variant, the Dewar photoproducts (Dewar-PPs) (57). Furthermore, CPDs and 6-4PPs cause a different distortion on the DNA molecule, 6-4PPs being able to warp DNA in a more pronounced way in comparison the CPDs. This, in turn, affects its repair rate by NER, being able to more easily recognize the more distorted DNA, with rate of repair of 6-4PPs being significantly higher in NER proficient mammalian cells (60).

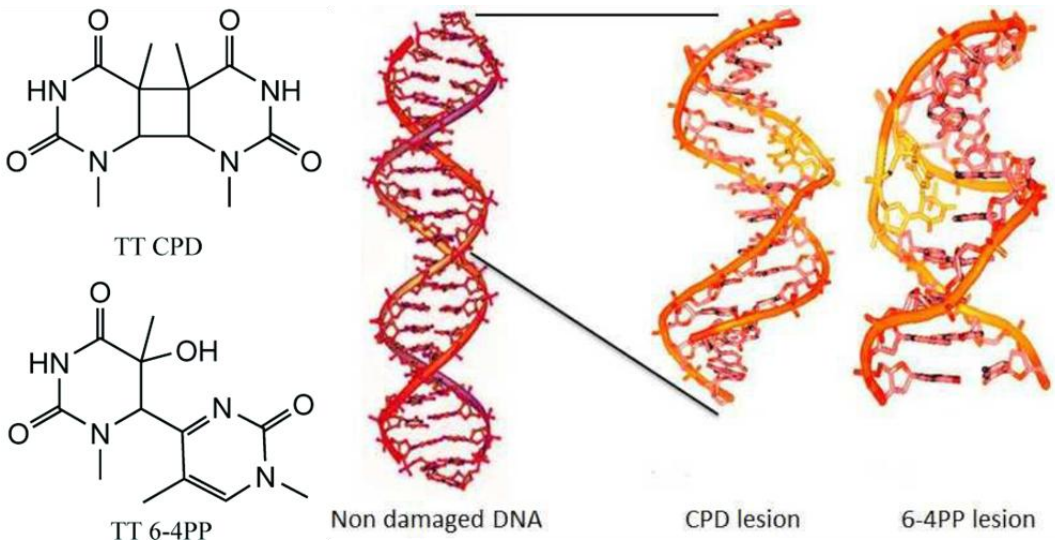

Figure 1.6. UV-induced DNA lesions. Molecular structure of CPD and 6-4PP lesions between thymines and the distortions they cause to the DNA double helix structure. Adapted from Rastogi et al., 2010 (57).

Although NER is generally thought to be found in all species, it is not the only pathway organisms rely to deal with photolesions. Photolyases, enzymes of the first discovered DNA repair mechanism (the photoreactivation), are found throughout all nodes of the evolutionary tree of life, including bacteria, archaea, plants, fungi, animal. They were lost in some groups however, such as placental mammals (which includes mice and humans) (61). The photorepair by photolyases is performed in a

light-dependent manner using a single enzyme in a process named direct repair. These enzymes bind to the DNA lesion, then through a flavin adenine dinucleotide (FAD) group, present in the enzyme, absorbs UVA/blue light (310–500 nm), enabling an electron transfer from the FAD chromophore to the DNA damage. The covalent bonds that make up the lesion are broken, followed by an electron transfer back to the FAD group (62). There are different kinds of photolyases, those mainly being CPD or 6-4PP photolyases, with CPD photolyases being more common throughout evolution. Interestingly, these photolyases are highly specific, with CPD photolyases repairing specifically CPDs and 6-4PP photolyases repairing 6-4PPs, which in turn makes them valuable tools for understanding the role of each lesion, with the removal of one photolesion enabling the observation of the effects of the remaining lesion (63).

UV induced lesions have a high biological significance due to their genotoxic and mutagenic properties, with NER deficient disorders, such as Xeroderma Pigmentosum and Cockayne syndrome further evidencing the importance of NER related DNA damages, with these lesions having a very important role regarding tumorigenesis and activation of other biological processes, such as inflammation and cell death (64,65).

1.3.2 Cockayne Syndrome, endogenous lesions and aging

Cockayne Syndrome is another rare, recessive, autosomal NER related genetic disease, characterized by photosensitivity, developmental abnormalities, such as cachetic dwarfism and arrested sexual development, and most notably premature aging-like (progeroid) features, including kyphosis, thin hair, fat loss, osteoporosis and neurological symptoms, which may have contributions of both abnormal development and progressive degeneration (66). CS neurodegeneration differs from XP neurodegeneration, CS patients displaying dysmyelination, vascular abnormalities, cerebellar ataxia, microcephaly, brain calcification and mainly purkinje cell death, as opposed to XP, which displays cerebral atrophy and primary neuronal degeneration. CS neurodegeneration features often result in progressive hearing and visual loss, ataxia, psychomotor delay and mental retardation (67,68). Moreover, CS patients, unlike XP patients, do not have an increase in mutation rate nor in cancer

incidence (69). CS is a highly heterogeneous disease, being divided into 3 subtypes, depending on the severity and age of onset of the symptoms. The most common genes associated with CS are the TC-NER *csa* and *csb* genes, though variant forms of the syndrome, such as XP/CS may also be caused due to mutations in *xpb*, *xpd* and *xpg* genes (70).

Although CS was first described in 1936 by Edward Cockayne (71), its first connection to DNA repair deficiency only surfaced in 1977, with initial studies revealing that although CS cells had the unscheduled DNA synthesis associated with NER, they still were hypersensitive to UVR. It was later revealed that these cells had a slow recovery of RNA synthesis after UV irradiation, which suggested an issue with transcription of the damaged strand, leading to the hypothesis and later comprehension that these patients had an impaired TC-NER (72). These findings explain the photosensitivity in CS patients, with it being caused by a failure to correctly repair UV induced photolesions in a similar way to XP. However, whether the deficiencies in TC-NER directly contribute and are alone able to explain the progeroid features of the disease is still an ongoing debate (73–75).

It was initially assumed that TC-NER dysfunction was the main cause of CS, as it was hypothesized that endogenous lesions, unrepaired by TC-NER and accumulated throughout CS patients lifespan were responsible for dysregulation of transcription and eventual cell death. Though it was a reasonable proposition at the time of its inception, emerging new data are now challenging this hypothesis (74). One of the main arguments against it is the recent discovery of the molecular mechanism of UV-Sensitive Syndrome (UVsS), a genetic syndrome whose patients also lack TC-NER due to mutations in either *uvssa*, *csa* or *csb* genes, but are only photosensitive for UV-induced photolesions and so far do not display any signs of progeroid or neurodegenerative features (76,77). And although the mutations in CSA or CSB that cause UVsS are different from those of CS patients, the outcome regarding their TC-NER deficiency of UV induced lesions is the same (74).

Although there are types of endogenous DNA lesions that are substrates for NER but not other repair pathways, such as cyclopurine deoxynucleosides (78), some DNA-protein crosslinks (79) and DNA lesions resulting from lipid peroxidation

such as the malondialdehydedeoxyguanosine adduct M1G (80), the main endogenous lesions hypothesized to cause CS have yet to be identified, which further brings speculation as to what is the molecular cause of CS. Other possible explanations for the molecular causes of CS are mitochondrial and metabolic alterations, as well as transcriptional abnormalities (81). CS proteins are involved in the regulation of these processes, with CS proteins being detected in mitochondria, having a role in the autophagy of damaged mitochondria (82) and CS cells having an altered fatty acid oxidation rate (83). Moreover, RNA metabolism is heavily altered in CS cells, with both RNA pol II and recently RNA pol I being implicated in this transcriptional alterations. Finally, a more recent theory implicates the persistence of protein complexes, such as NER machinery (75) or the transcription factors at damaged sites (84) as a main feature of NER-related neurodegeneration.

In spite of these more recent speculations as to the molecular cause of CS, the canonical explanation still refers to endogenous DNA damage having a central role in this disease and its early aging-like features, as well as aging in general, a theory also supported by several other evidences, including, but not limited to, different mouse models. In a similar way to the human progeroid diseases, these models have a deficiency in one or more NER related genes, such as the $Ercc1^{-/\Delta}$, $Csb^{m/m}/Xpa^{-/-}$, $Csb^{m/m}/Xpc^{-/-}$, $Xpd^{TTD}/Xpa^{-/-}$, $Xpg^{-/-}$ and $Csa^{-/-}/Xpa^{-/-}$ mice (85). All of these mice models display similar characteristics that generally mimic those of progeroid diseases, with decreased lifespan, smaller body size, lipodystrophy and neurodegeneration. Though shortened lifespan alone can be a misleading point regarding premature aging, as it is sensitive to artefacts regarding genetics and environmental sources of variation, the aforementioned and other age-related pathologies and additional aging parameters points to DNA damage having a causal role in progeroid features and being related to aging in general (86), with the mechanisms that links DNA damage to aging and cancer being discussed in the following topic.

1.4 Cellular and Molecular effects of NER-related DNA damage

DNA damage can have several different effects depending on the type of lesion and repair pathway involved (87). As previously mentioned, NER-related DNA damage are capable of distorting the structure of the DNA double helix, with the main effect being the stalling of RNA and DNA polymerases, thereby having an effect on transcription and replication (88). The stalling of these processes and persistence of lesions have major implications for cells, being able to activate a series of cellular reactions to genomic stress, with these effects collectively being called the DNA damage response (DDR) (89).

Cellular DDR often involves the signaling of the damage through different kinases, such as Ataxia Telangiectasia Mutated (ATM) and Ataxia Telangiectasia and Rad3 Related (ATR), which are in turn able to activate several other responses, including cell cycle arrest, DNA repair, transcriptional changes and programmed cell death. ATM and ATR have been shown to phosphorylate over 400 different substrates in order to deal with genomic stress (90). Among these, we highlight the tumor suppressor protein p53, a highly versatile protein with several different post-translational modification sites capable of regulating a plethora of cellular processes, including the DDR-related aforementioned ones, such as apoptosis and cell cycle arrest (91). The importance of p53 is further demonstrated by its significance to carcinogenesis regulation, with about 50% of tumors having a p53 mutation (92). DNA damage, DDR and its players therefore have a profound effect on cell homeostasis, with these molecular and cellular aspects having a large influence on the two previously discussed topics of cancer and aging. Amongst these aspects, we will focus on mutagenesis and carcinogenesis, cell death, senescence and inflammation.

1.4.1 Mutagenesis, carcinogenesis and cell proliferation

Mutagenesis has been shown to be the main cause of tumorigenesis, especially when occurring in tumor suppressor genes (such as p53) (93) or proto-oncogenes (such as some ras genes) (94). Mutations may occur spontaneously, due to replicational errors inserted by a replicative DNA polymerase (such as DNA pol ε).

These spontaneous mutation are generally uncommon, however, since the error rate of these polymerases are exceptionally low, estimated to be in the magnitude of 1×10^{-6} to 1×10^{-8} (95). Bulky DNA lesions and their causative sources, such as UVR, are able to severely increase the mutational rate due to their effect of blocking the DNA replication machinery. In order to deal with this arrest, other, more error-prone polymerases, capable of bypassing the damage through translesion synthesis (TLS) are recruited to the lesion site (31). TLS polymerases have a generally higher mutation rate, depending on the substrate having a rate of $1x10^{-2}$, over 4 orders of magnitude higher than replicative polymerases with proofreading activity (96).

The TLS process allows the cell to continue replicating its DNA, thereby impeding more severe effects, such as a DNA double strand break caused by replication stress (97,98). The mutational load and type of mutation varies with the type of damage and TLS polymerase recruited to deal with the lesion, with UVR generally producing a C>T transition mutation due to the action of error-prone TLS polymerases (99). Moreover, in order to properly occur a mutation, two rounds of DNA synthesis are necessary: one to first insert an erroneous base pairing and the other to fixate the mutation by inserting the base complementing to the lately acquired one (17). Mutations may or may not have effects on protein conformation, depending on where and how they happen, with the most prominent effects being either gain or loss of function (100).

As previously stated, mutations in tumor suppressor or proto-oncogenes are responsible for the process of tumorigenesis. These genes generally have a role regarding cell proliferation or processes that regulate it, such as cell death or growth arrest, though others have been recently implicated as well, such as genes that regulate cell metabolism and inflammation, with several hallmarks of cancer having been established (101). Still, as cancer is majorly a cell proliferation disease, the most traditional proto-oncogenes are generally growth factor receptors (such as EGFR) and related genes, as the proteins encoded by those genes have a major role in activating complex signaling cascades that result in activation of the cell cycle, such as MAP kinases and/or ERK.

Importantly, cell proliferation can also be activated as a part of DDR depending on the cell type, and may have important physiological roles such as a protective or restorative function, depending on the context. The signaling cascades that contribute to cell proliferation are often in a tightly regulated balance in order to impede tumorigenesis process, with organisms having a plethora of failsafe mechanisms to deal with this possibility. One of these mechanisms is the programmed cell death, with the balance of cell proliferation and cell death, amongst other factors often being implicated in the balance between cancer and aging related disorders (64) (figure 1.7).

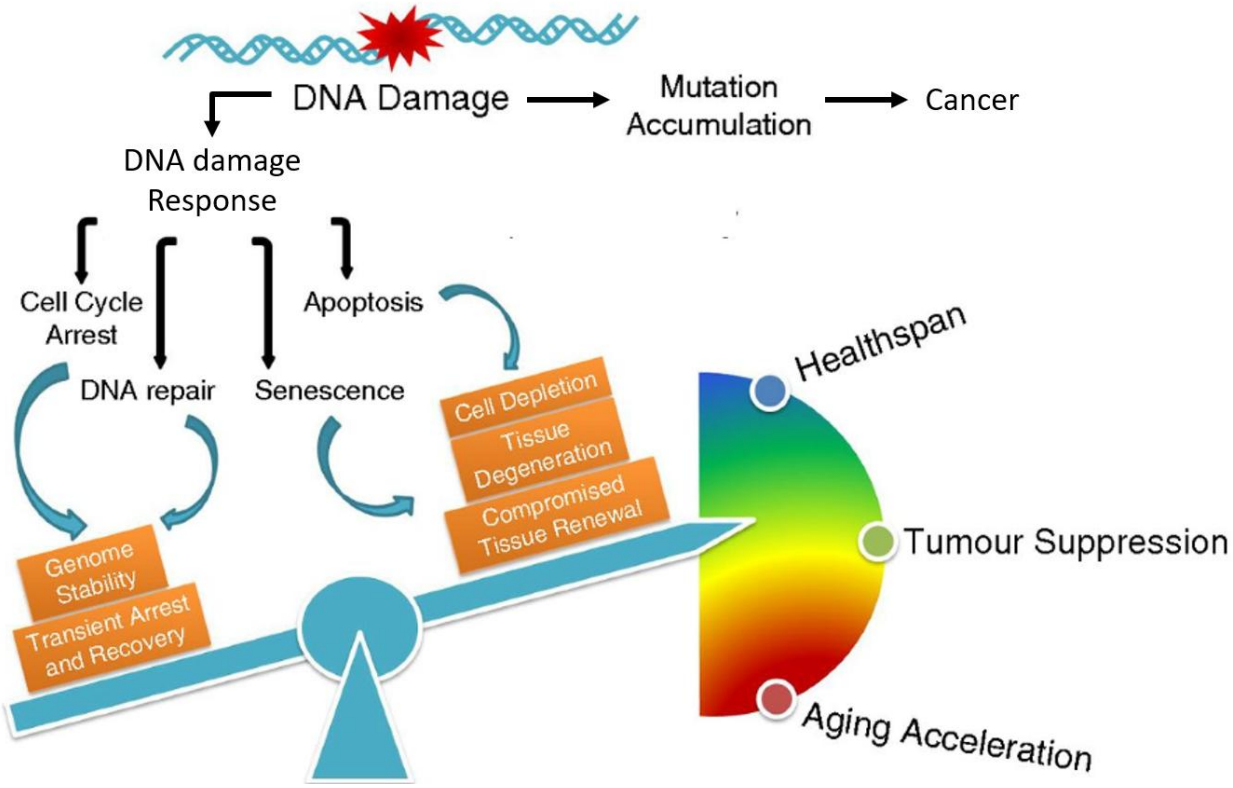

Figure 1.7. DNA damage, cancer and aging. Possible consequences of genomic damage, with repair failure affecting tumorigenesis by the accumulation of mutations. Another possible negative consequence is the triggering of senescence or apoptosis, which contributes to aging. Adapted from Ou & Schumacher, 2018 (102).

1.4.2 DNA damage induced cell death

Cell death has been implicated in several different biological processes, including, but not limited to, development, anti-tumorigenesis, inflammation initiation and resolution, several different disorders and aging. The type of cell death is important to further understand these phenomena, with cell death mechanisms being intrinsic or extrinsic, programmed or accidental. Regarding DNA damage associated cell death, the main type is apoptosis, a programmed and regulated cell death mechanism in which morphological changes, such as rounding-up of the cell, reduction of cellular volume, chromatin condensation, nuclear fragmentation and plasma membrane blebbing (103). Due to modifications in the plasma membrane structure, such as the exposure of the membrane lipid phosphatidylserine, macrophages are able to recognize and phagocyte *in vivo* apoptotic cells, thereby maintaining tissue homeostasis, as the cell contents do not spill into the tissue and activate other molecular signals, such as pro-inflammatory pathways (104).

Apoptosis is orchestrated through the activation of several specific caspases, a family of protease enzymes that, during apoptotic cell death, initiate and execute this program by producing a cascade reaction, cleaving hundreds of substrates in the process, destabilizing cell organelles and activating DNases, thus producing DNA fragmentation and cell death (105). DNA damage activates mainly the intrinsic apoptosis pathway, mediated by overactivation of p53, permeabilization of the mitochondrial membrane and activation of caspases, such as initiator caspases 8 and 9 and effector caspase 3. Importantly, DNA damage induced apoptosis signaling is often counter balanced by pro-survival factors, such as EGF signaling, NF-κB and Bcl-2, with cell fate being determined by the amount, site and type of lesion, as well as the cell capacity to repair it (106). For instance, in NER proficient cells, CPD lesions are a more significant apoptosis inducer than 6-4PPs, while in NER deficient cells, both lesions are able to induce apoptotic cell death in a similar proportion, despite CPDs having a higher formation rate than 6-4PPs. Furthermore, the cell type and epigenetic background also influence the impact of DNA damage on cell death, with keratinocytes having a higher survival rate than fibroblasts when exposed to UVB irradiation (107,108) and some neurons being more resistant than others to oxidatively generated damage depending on the brain region (109).

Cell death has key roles in maintaining organism homeostasis in numerous different ways, with DNA damage induced apoptosis being a key regulator of tumorigenesis (101). However, programmed cell death pathways have also been implicated in normal aging and progeroid syndromes, by promoting immune system decline, sarcopenia and neurodegenerative diseases (110). DNA lesions and their responses are an important regulator of these processes (111), with the level of signaling and of DNA damage possibly leading the cell to its demise. Another possibility that also impacts aging and disease and can be a resultant of damaged DNA is cell senescence (112).

1.4.3 Senescence and inflammation

Cell senescence, unlike cell death or mutagenesis, is a concept still under heavy debate as to its exact classification and hallmarks (113). While initially introduced to describe age-related changes in aging eukaryotic cell cultures, especially the exhaustion of their mitotic potential due to an intrinsic mechanism (114), cell senescence has grown into a field of its own, with the accumulation of senescent cells being a hallmark of both cancer (101) and aging (112), and with the concept of what is a senescence state being further expanded upon, including characteristics other than permanent cell cycle arrest, such as resistance to apoptosis induction, deregulation of cell metabolism and an abnormal secretory phenotype, with senescent cell generally secreting molecules such as IL-1 α, IL-1B, IL-6 and IL-8 through the activation of NF-κB, a pro-survival, anti-apoptotic, pro-inflammatory mechanism. The senescence associated secretory phenotype (SASP) is, however, a very heterogeneous characteristic of senescent cells, depending on the cell type and context, often secreting anti-inflammatory, immunosuppressive factors as well, such as IL-10 and IL-13 through other types of signaling, such as the Jak2/Stat3 pathway (113).

The mechanisms of the induction of cell senescence have also been expanded upon, with DNA damage caused by endogenous and exogenous sources having been shown to induce this state, with persistent DDR being considered a hallmark among senescent cells and having the involvement of cell cycle arrest

proteins such as p21 and, notably, $p16^{InkA}$ (113). Additionally, DNA repair deficient progeroid models have been shown to accumulate early senescent cells depending on the tissue, with these cells having a significant impact on organismal health (115,116). Recently, the induction of apoptosis specifically in senescent cells has been shown to rescue several of the progeroid phenotypes in a mouse model (117). The main hypothesis regarding this health improvement involves the decrease of pro-inflammatory SASP and subsequent decrease of basal, non-pathogenic induced (sterile) inflammation, as inflammation as a process has been implicated in several different aging-associated disorders, including cancer, cardiovascular and neurodegenerative diseases, as well as a decrease in lifespan (118).

Inflammation, much like cell senescence, is a concept that has recently been expanded upon (119). It is generally defined as a response of the innate immune system to stimulation by invading pathogens or endogenous damage signals involving several different cell types, such as immune, vascular, and tissue specific immuno-competent cells, such as Langerhans cells and keratinocytes in skin tissue (120,121), and astrocytes and microglia in the brain (122). Though it is classically and most often associated with defense against pathogens and/or tissue damage and repair, new data and insights have revealed a more multifaceted concept with interactions to other systems, such as metabolism and nucleic acid damage. Both lesions in DNA and RNA have been shown to elicit some pro-inflammatory cell and molecular responses through different mechanisms, such as activation of IL-1α (123), of the protein complex known as the inflammasome (124), the transcription factor NF-κB (125) and toll-like receptors (126), with these non-pathogen related (sterile) inflammation mechanisms being novel concepts, with its underlying structures and effects currently being established.

Importantly, although cell senescence and inflammation may have a negative impact on organismal health especially when under chronic situations, these two processes are also very important for tissue homeostasis, with senescence being an important anti-tumorigenesis mechanism, as it arrests cell cycle, impeding proliferation and mutagenesis of damaged cells (127), and inflammation having major roles regulating defense against pathogens and tumorigenesis (128), as well as driving tissue remodeling and restoration (129). Thus, much like cell death and

proliferation, the complexity of these biological processes must be considered when studying and developing strategies to improve organismal health.

1.5 Final considerations

DNA damage and its responses have important effects on numerous biological processes. NER deficient models, such as the ones used in this work (namely Xpa and Csa knockout mice) are important tools in order to study the role of different NER-related lesions on DNA damage induced effects such as cell proliferation, cell death, senescence and inflammation.

This work expands upon these concepts in two fronts: In Chapter 2, we investigate the role of UV induced CPD and 6-4PP lesions on keratinocyte responses of NER deficient, $Xpa^{-/-}$ knockout mice, a model for Xeroderma Pigmentosum. Meanwhile, in Chapter 3, we examine the impact of aging related effects on neurovascular dysfunction and inflammation in $Xpa^{-/-}/Csa^{-/-}$ double knockout mice, a model for Cockayne Syndrome. Chapter 4 integrates and discusses the results of the previous chapters and their biological implications.

1.6 Objectives

- Evaluate the specific effect of CPD and 6-4PP lesions generated by UVB irradiation regarding the induction of hyperplasia, cell proliferation, cell death and inflammation using NER deficient, Xpa knockout mice expressing photolyases in keratinocytes.

- Investigate the relationship between accumulation of NER-related DNA lesions and neuroinflammation and neurovascular dysfunction using a progeroid, Csa/Xpa double knockout mouse model.

Chapter 2: The effects of the photoremoval of specific lesions induced by ultraviolet irradiation in DNA repair deficient mice

2.1 Summary

Ultraviolet (UV) irradiation is considered one of the most genotoxic agents present in the environment. It is capable of damaging DNA molecules, inducing mainly cyclobutane pyrimidine dimers (CPD) and pyrimidine 6-4 pyrimidone photoproducts (6-4PP). These lesions interfere in essential cellular processes, such as transcription and replication, thereby promoting several effects in the skin, such as inflammation, dysplasia and cell death. Photolyases are enzymes that repair these lesions in a light dependent mechanism known as “photoreactivation”, in which the enzyme breaks the covalent bond that binds the dimer. Photolyases act in a direct and specific manner for each lesion. However, these enzymes are absent in placental mammals, in which the UV-induced lesions are repaired by the Nucleotide Excision Repair (NER) pathway.

In this study, we show that in NER deficient mice, keratinocyte specific *in vivo* CPD removal was able to completely inhibit UV induced epidermal cell proliferation and hyperplasia, while the removal of 6-4PPs in keratinocytes reduced, but not abolished these UV effects. Moreover, the removal of either CPDs or 6-4PPs in keratinocytes was able to reduce UV induced apoptosis and inflammation on similar levels. These findings indicate that CPDs have a greater impact than 6-4PPs regarding the hyperplasia effect of UV irradiation, while both types of DNA lesions have similar effects on promoting apoptosis and inflammation in NER deficient mice, with keratinocytes having a central role in regulating these effects.

2.2 Introduction

The DNA molecule is responsible for containing and transmitting the genetic information of all living beings. In spite of its importance for maintaining life, the DNA molecule is under constant physical and chemical stress (20). If not repaired, these lesions are capable of inducing several different biological effects, many of which can be harmful for the organism, such as cell death and mutagenesis, involved in the processes of aging and tumorigenesis. Ultraviolet (UV) irradiation is considered to be the main exogenous physical factor involved in carcinogenesis, capable of directly damaging DNA (130).

UV irradiation is a type of electromagnetic radiation present in sunlight with a wavelength spectrum ranging from 100 to 400 nm, and is classically subdivided into three main bands: UVA (315 to 400 nm), UVB (280 to 315 nm) and UVC (100 to 280 nm) (50). The Sun-emitted UV irradiation is partially blocked by the ozone layer, UVC being completely blocked, as well as the majority of UVB irradiation, with the percentages of UVA and UVB light that reaches the surface varying with the location conditions (51). Although a large portion of Sun-emitted UVB irradiation is blocked by the ozone layer, it is still biologically relevant, being capable of directly damaging DNA, generating photolesions (131). The main DNA photolesions formed by UV irradiation are the cyclobutane pyrimidine dimers (CPDs) and the pyrimidine (6-4) pyrimidone photoproducts (6-4PPs). These lesions are distortions in the DNA molecule caused by the formation of covalent bonds between adjacent pyrimidines of the same DNA strand, and may interfere in essential cell processes, such as transcription and replication (57,88).

In order to repair these types of damages, several species possess photolyases, enzymes capable of directly repairing CPDs or 6-4PPs through a process known as photoreactivation, in which the enzyme binds specifically to the photolesion and, in a light-dependent reaction, reverts them back to the original monomers (62). Moreover, photolyases act in a specific manner, with CPD-photolyases repairing only CPDs and 6-4PP-photolyases repairing only 6-4PPs. Due to their specificities, photolyases can be used as tools to study the distinct effect of each photolesion (63).

Although photolyase genes are generally found in all domains of life, they are absent in some groups, most notably placental mammals. In these organisms, CPD and 6-4PP lesions are removed by the nucleotide excision repair (NER) pathway, responsible for removing a broad variety of lesions that distort the DNA double helix structure (61). The NER pathway involves multiple intermediate steps to effectively repair the damaged strand - first by recognizing and excising the damaged strand, then synthesizing a new strand afterwards by using the non-damaged strand as its template (42). Several proteins are involved in the recognition, signaling and repair of the lesion, with several diseases being associated with deficiencies in NER-related genes, such as Xeroderma Pigmentosum (XP), a recessive, autosomal syndrome caused by mutations in several genes involved in the NER pathway (mainly XPA-XPG genes) (43), characterized primarily by high skin neoplasia risk and cutaneous sensitivity to sunlight, with XP patients often having severe sunburn and blistering of the skin after minimal sunlight exposure (132,133).

UV irradiation has been shown to induce a process of sterile inflammation, in which skin cells, such as keratinocytes, activate pro-inflammatory molecules, such as the inflammasome complex, NF-κB and cytokines such as IL-1α, IL-1β, and TNFα (123,134,135). These molecules, alongside other UV effects contribute to the expression of proteins responsible for tissue remodeling, mainly matrix metalloproteinases (MMPs) (136,137) and proteins related to leukocyte adhesion such as ICAM-1 (138). These types of molecules are integral to the inflammatory process, allowing cells such as neutrophils and macrophages to enter the skin tissue and initiate inflammation. In NER proficient mice, CPD lesions have been shown to be a major factor for this process, with the expression of CPD-photolyase being capable of reducing UV-induced inflammation in these mice (139).

Photolesions are also related to other UV-induced effects such as skin cell proliferation and cell death. CPD, but not 6-4PP removal, in NER proficient mice has been shown to inhibit the skin hyperplasia and cell death effects of UV irradiation (140). Similarly, the *in vitro* expression of CPD, but not 6-4PP photolyase, has been shown to reduce UV-induced cell death in NER proficient cells. In contrast, different *in vitro* effects of photolesions in NER deficient cells have been observed, with both

CPDs and 6-4PPs contributing to the apoptotic effect of UV irradiation [6]. However, the *in vivo* effects of each photolesion in NER deficient models has yet to be determined.

In the current study, we show that in XPA knockout, NER deficient mice, the removal of either CPDs or 6-4PPs by keratinocyte specific transgenic expression of photolyases *in vivo* is able to reduce acute UVB induced apoptosis. The removal of either photolesion also have similar reducing effects on the induction of UVB related inflammation, with both photolyases able to reduce active neutrophils in the skin and ICAM-1, but not MMP expression. Only CPD removal abolished chronic UV induced skin cell proliferation and hyperplasia, with 6-4PP removal having a smaller impact on these UV induced effects. These results indicate that both types of DNA lesions have similar effects on the induction of apoptosis and inflammation, and that CPDs have a greater effect regarding hyperplasia in NER deficient organisms.

2.3 Materials and Methods

2.3.1 Mice lines

XPA knockout mice expressing CPD or 6-4PP photolyase were obtained by generational crossing between the XPA mice described in (141) with transgenic photolyase mice, both CPD and 6-4PP photolyases genes being expressed under the control of the keratinocyte specific K-14 promoter (142). All strains used in this project were kindly donated by Drs. Hoeijmakers and van der Horst (Erasmus University, Rotterdam, The Netherlands) and had a Black6J or Black6J/SKH-1 (hairless mice, mutated in the *hairless* gene) background, established models for UV irradiation (141,143). XPA KO mice were maintained by homozygous crosses, while photolyase expressing genes and the *hairless* gene were maintained by heterozygous crosses. All animals used for experiments were 8 to 10 week old, with no difference observed between males and females. Housing, breeding, genotyping and experimentation were performed in accordance with the regulations established by the ethical committee of the Institute of Biomedical Sciences of the University of Sao Paulo.

Genotyping of the mice was performed by extracting DNA from mice tail followed by polymerase chain reaction (PCR) of the target genes. For the tail DNA extraction, a 0.2 cm tail snip was obtained while weaning the mice (approximately 3 week old), followed by tissue digestion in 500 µL of lysis buffer [0.1 M Tris pH 8 (Sigma-Aldrich, St. Louis, MI, USA)]; 0.005 M EDTA (Sigma-Aldrich); 1 mL SDS 10%; 0.2 M NaCl (Sigma-Aldrich); 42.5 mL of H_2O, 0.4 mg/mL RNAse (Invitrogen); 0,4 mg/ml Proteinase K (Invitrogen)] at 55ºC overnight. The digested tail solution is then incubated in 500 µL Phenol:Chloroform:Isoamyl acid (Life Technologies, Carlsbad, CA, USA,) (25:24:1), followed by 10 min centrifugation at 14,000 RPM at 4ºC. The supernatant was then collected and mixed with isopropanol (Merck, Whitehouse Station, NJ, USA). This solution was again centrifuged for 10 min at 14,000 RPM at 4ºC, followed by a single 75% ethanol wash. The supernatant was then discarded, and after drying the DNA pellet overnight, it was resuspended in 100 µL H_2O;

PCR of the *xpa*, *hairless*, CPD-photolyase and 6-4PP photolyase genes are described in supplementary table S2.1 and S2.2, and primer sequences used for this reaction in supplementary table S2.3.

Following PCR, 3 µl of Bromophenol blue solution [0.25% bromophenol blue, 40% sacarose (Merck), 59.75% H_2O] was added to the amplified DNA products, which was electrophoresed on a 2% agarose (Sigma-Aldrich) gel in 0.5x TBE buffer (40 mM Tris-Cl, 45 mM boric acid, 1 mM EDTA) at 80 V. Expected band sizes for genotyping of the *xpa* gene are 300 bp for wild type and 250 bp for knockout, while for the *hr* gene are 400 bp for the wild type and 250 bp for the mutant. CPD and 6-4PP transgenic photolyases expected band sizes are approximately 300 bp.

2.3.2 Mice irradiation and photoreactivation

Mice were irradiated with a Philips TL12-40W UVB lamp, using a UV dosimeter (VLX-3.W, Vilber) to measure the amount of UVR. No UVC (254 nm) irradiation were detected, with UVA (365 nm) irradiation being below <0.05 J/m^2/s. Immediately after UV irradiation, mice were photoreactivated by 3 h, using four white lamps Polylux XL F36W/840. Minimal erythemal dose (MED) was determined as 20

J/m^2 of UVB by analyzing the macroscopic induction of erythema, wounding, skin peeling, skin thickening and pigmentation.

2.3.3 Chronic irradiation of hairless XPA KO mice

XPA KO, hairless mice expressing either CPD or 6-4PP photolyase were irradiated for 30 consecutive days with a 1 MED UVB (20 J/m^2) dose followed by 3 h photoreactivation, with animals being observed daily. After 48 h of the last day of irradiation, mice were euthanized and 1 cm^2 mice dorsal skin was collected, with a 5 mg BrdU peritoneal injection being performed 2 h prior to euthanasia and harvesting for cell proliferation analysis.

2.3.4 Tissue fixation for histology analysis

Skin samples were fixed in 4% formaldehyde (Merck) at 4^oC overnight. The samples were then dehydrated by sequential immersion for 1 h in each of the following solutions at room temperature: PBS 1X, 50% Ethanol (Merck), 70% ethanol, 80% ethanol, 90% ethanol, 2x 100% ethanol and 2x xylene (Sigma-Aldrich). After dehydration, samples were twice incubated in paraffin 60^oC for 1 h each. Samples were then mounted in paraffin blocks and kept at RT until 5 µm cuts from skin tissue were obtained using a microtome and placed on Starfrost (Knittel-Glaser) slides with 10% ethanol at 50^oC until total fluid evaporation. For fixation on the slide, skin sections were maintained at 37^oC overnight and stored at RT until staining.

2.3.5 Quantification of epidermal thickness

Tissue slides were deparaffinized and hydrated through sequential immersion in Xylene (100% twice), Ethanol (100% twice, 95%, 70% and 50%) and dH_2O under room temperature. Slides were then stained with hematoxylin and eosin. Stain excess was washed under indirect water flow, and tissue was subsequently dehydrated through immersion in Ethanol and Xylene. Slides were then mounted using Entellan and Menzel-Glass coverslips.

In order to quantify epidermal thickness, an Axiovert 200 (Zeiss) optical microscope was used, with a 100x objective. Epidermal thickness was defined as the distance between the end of outer layer of epidermis and the basal lamina, wherein the epidermis meets the dermis. Invagination sites, such as sweat glands and hair follicles were disconsidered in this analysis. Three measurements were performed per field, using three fields in each slice, and three slices per animal, with a total of twenty-seven measurements per animal. The Axiovision Rel. 4.8 (Zeiss) software was used for quantification.

2.3.6 Tissue cell proliferation

In order to detect epidermal cell proliferation, we performed immunohistochemistry for BrdU detection. Tissue slides were deparaffinized and hydrated as previously described, then incubated for 30 min in 50% METOH 1% H_2O_2 (30%, Merck) at room temperature (RT) for endogenous peroxidase inactivation, followed by two PBS washes. Samples were then incubated in pepsin (18 U/ml) diluted in 100 mM HCl at 37^{o}C for 30 min, followed by two PBS washes and incubation at 56^{o}C for 20 min in 1 M HCl. pH was neutralized with 100 mM sodium borate in PBS (pH 8.5), followed by three PBS washes. Slides were then incubated in blocking solution (5% FBS in 1% PBS/BSA) for 10 min, at RT, followed by incubation with anti-BrdU (M0744, DAKO), diluted 1:100 in blocking solution overnight at 4^{o}C. Slides were then washed in PBS and incubated for 1 h with HRP anti-mouse (Sigma-Aldrich, A9044), diluted 1:100 in blocking solution. Substrate reaction was done with 3,3'-Diaminobenzidine (DAB, Spring) until nuclei were stained. Counter staining was performed with hematoxylin (Merck). Slides were mounted with Entellan and coverslips. Images were obtained with Axiovert 200 Optic Microscope (Zeiss) under 100x objective using Axiovision Rel. 4.8 (Zeiss) software. We performed three blind measurements per skin tissue of $BrdU^{+}$ basal and suprabasal cells, analyzing 3 slices per animal. Quantification of BrdU positive cells was performed by calculating the ratio between stained basal layer cells and total basal layer cells, while quantification of suprabasal BrdU positive cells was done by the ratio between these cells and the total number of basal layer cells.

2.3.7 Acute irradiation of XPA KO mice for *in vivo* assessment of inflammation and cell death

XPA KO, photolyase expressing, mice were anesthetized and shaved 24 h before irradiation. Mice were then irradiated with a single 200 J/m^2 (10 MED) dose. In order to assess inflammation induction by UVB light, mice were injected with anti-ICAM-1/DiD fluorophore (excitation 640 nm, emission 680 nm) nanoparticles and. Following 6 and 24 h of irradiation, mice were inoculated with the XenoLight RediJect Chemiluminescent Inflammation Probe (PerkinElmer) probe, in order to detect active, myeloperoxidase expressing neutrophils. Probe fluorescence and chemiluminescence were detected using the In vivo imaging system (IVIS) Spectrum (PerkinElmer), located in the Core Facility Center for Research Support of the University of Sao Paulo (CEFAP-USP). We also used the MMPSense 645 FAST probe (PerkinElmer) in order to detect several MMPs (2, 3, 7, 9, 12, and 13).

Following 48 h of irradiation, mice were assessed for *in vivo* cell death by using Annexin-V/DiD fluorophore nanoparticles, injected intravenously X h previous to detection by IVIS Spectrum. When imaging, mice were kept under anesthesia using isoflurane. Image analysis was performed with Living Image 4.0 (PerkinElmer) software. Radiances were normalized using non-irradiated control mice.

2.3.8 Statistical analysis

Data were expressed as mean +- standard deviation, and analyzed with one-way ANOVA followed by Bonferroni's multiple comparison test. p value < 0.05 was considered significant, with "*" indicating $P \leq 0.05$, "**" $P \leq 0.01$ and "***" $P \leq 0.001$.

2.4 Results

2.4.1 Photorepair of 6-4PPs reduces, while CPD removal completely inhibits UVB induced hyperplasia and cell proliferation in chronically irradiated XPA KO mice

XPA knockout, NER deficient, mice show hypersensitivity to UVR, with low doses producing excessive skin abrasion. Therefore, a Minimal Erythemal Dose (MED) was established before initiating any irradiation experiments. The MED for our conditions was found to be 20 J/m^2, and was used as a biological parameter henceforth. In order to assess the role of CPDs and 6-4PPs on the induction of hyperplasia, mice received over the course of 30 days a single dose of 20 J/m^2 UVB irradiation followed by a 3 h photoreactivation, in order to activate the photolyase (CPD or 6-4PP) in keratinocytes. Morphological changes in epidermal thickness were quantitatively analyzed from H&E stained sections (Figure 2.1A). As expected, XPA mice not expressing any photolyase under chronic UVR developed hyperplasia, used as a positive control. Mice expressing CPD-photolyase in keratinocytes, on the other hand, did not show increased epidermal thickness, evidencing the causative role of CPD lesions for this UVR effect. Surprisingly, the removal of 6-4PPs in keratinocytes also affected hyperplasia induction, with the epidermal thickness being reduced in 6-4PP-photolyase expressing mice when compared to the positive control, which implies that 6-4PPs can also contribute for UVR induced hyperplasia.

Furthermore, UVR induced cell proliferation was analyzed in these chronically irradiated mice by quantifying BrdU positive cells in both basal and suprabasal epidermal cells (Figure 2.1B), corroborating the hyperplasia analysis. Keratinocyte CPD photoremoval completely prevented the UVR cell proliferation effect, both in basal and suprabasal epidermal layers, while the removal of 6-4PPs attenuates this effect in the basal layer and fully inhibits it in the suprabasal epidermal layer.

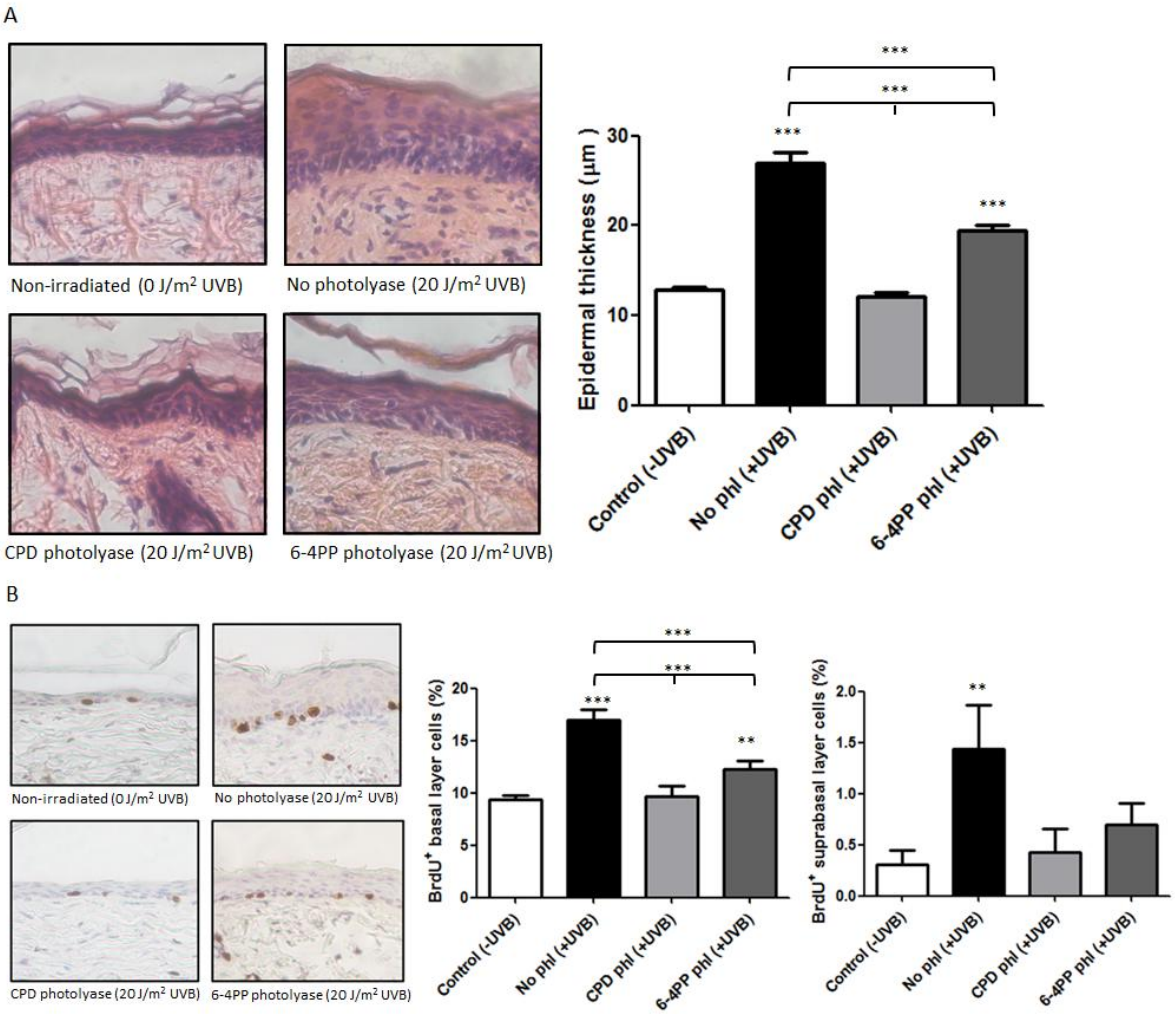

Figure 2.1: CPD removal in keratinocytes abrogates UVR induced hyperplasia and cell proliferation, while 6-4PP removal decreases these effects in XPA KO mice. (A) Epidermal thickness of XPA KO mice daily irradiated or not with UVB (20 J/m^2) followed by photoreactivation of CPD or 6-4PP Photolyase (phl) for 30 days. Quantitative analysis of epidermal thickness was performed by perpendicular measurements of the tissue extension in skin sections stained with H/E (n = 4), with representative images (40x) shown. **(B)** Quantification of cell proliferation in the basal and suprabasal layers of chronically UV irradiated XPA KO mice, with representative images (40x). Tissues were stained for BrdU$^+$ cells by immunohistochemistry counterstained with hematoxylin (n = 4).

2.4.2 Photorepair of either CPDs and 6-4PPs reduces UV induced apoptosis in XPA KO mice

Apoptotic cell death was analyzed *in vivo* through nanoparticles linked to Annexin-V and DiD-fluorophore, with this probe being validated using dexamethasone, a potent inducer of apoptosis of thymus cells (144) (Supplementary figure S2.1). Annexin-V is a molecule that binds to phosphatidylserine, phospholipid that is exposed when cells trigger apoptosis (145). A higher UVB dose (200 J/m^2)

was used to better visualize the acute, UV induced effects using *in vivo* probes. Our results show that 48 h after UVB irradiation, the expression of CPD-photolyase in keratinocytes of XPA KO mice was able to significantly reduce the apoptotic signal when compared to XPA mice not expressing photolyases, suggesting the participation of CPDs in UVB-induced *in vivo* cell death. Interestingly, a similar result was obtained with 6-4PP expressing mice, suggesting that both CPDs and 6-4PPs participate in apoptosis triggering events following UV irradiation in these NER deficient mice (Figure 2.2).

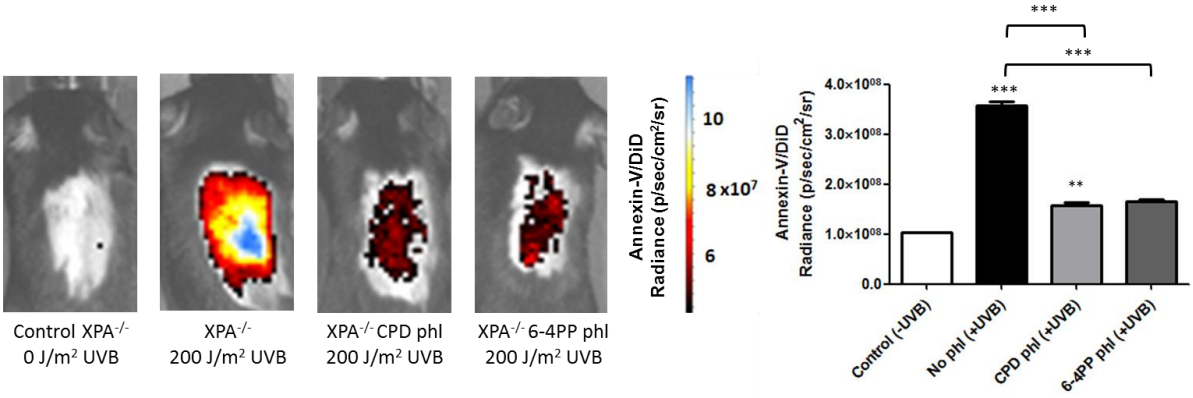

Figure 2.2: Effect of keratinocyte specific photorepair of CPDs and 6-4PPs on UVR induced apoptosis in XPA KO mice. Apoptosis was analysed by *in vivo* imaging using Annexin-V/DiD fluorophore containing nanoparticles, 24 h after UVB (200 J/m^2) irradiation. Radiance of DiD containing nanoparticles were quantified in the central region of XPA KO mice exposed dorsal skin, n=3.

2.4.3 CPD and 6-4PPs photoremoval have similar effects on acute UVB induced inflammation in XPA KO mice

UV-induced leukocyte infiltration was measured *in vivo* 6 and 24 h after UVB (200 J/m^2) irradiation and photoreactivation. ICAM-1, a cell surface protein responsible for neutrophil adhesion, was measured using a nanoparticle containing anti-ICAM-1 antibody and DiD fluorophore, validated in Supplementary figure S2.2 using lipopolysaccharide (LPS). We also used a commercial chemiluminescent probe capable of detecting Myeloperoxidase (MPO), an enzyme most expressed by active neutrophils and a key mediator of inflammation-dependent oxidative stress. Interestingly, both CPD or 6-4PP removal in keratinocytes had a similar effect 6 h after UVB irradiation in reducing the expression of cell membrane protein ICAM-1

(Figure 2.3A), responsible for leukocyte transmigration into tissue. Similarly, CPD and 6-4PP photoremoval also lessened the infiltration of active neutrophils in the skin, as measured by *in vivo* myeloperoxidase (MPO) expression 6 and 24 h after UVB irradiation (Figure 2.3B and C), which indicates that these two photolesions participate in the inflammatory event of leukocyte tissue extravasation following UVR. We also measured *in vivo* the presence of Matrix Metalloproteinases (MMPs), enzymes that modulate innate immunity, using a commercial probe capable of detecting several kinds of MMPs. Interestingly, unlike our previous results, the removal of neither photolesion was able to reduce the UV induced MMP tissue presence 24 h after irradiation (Figure 2.3D), what could be explained by a saturation effect.

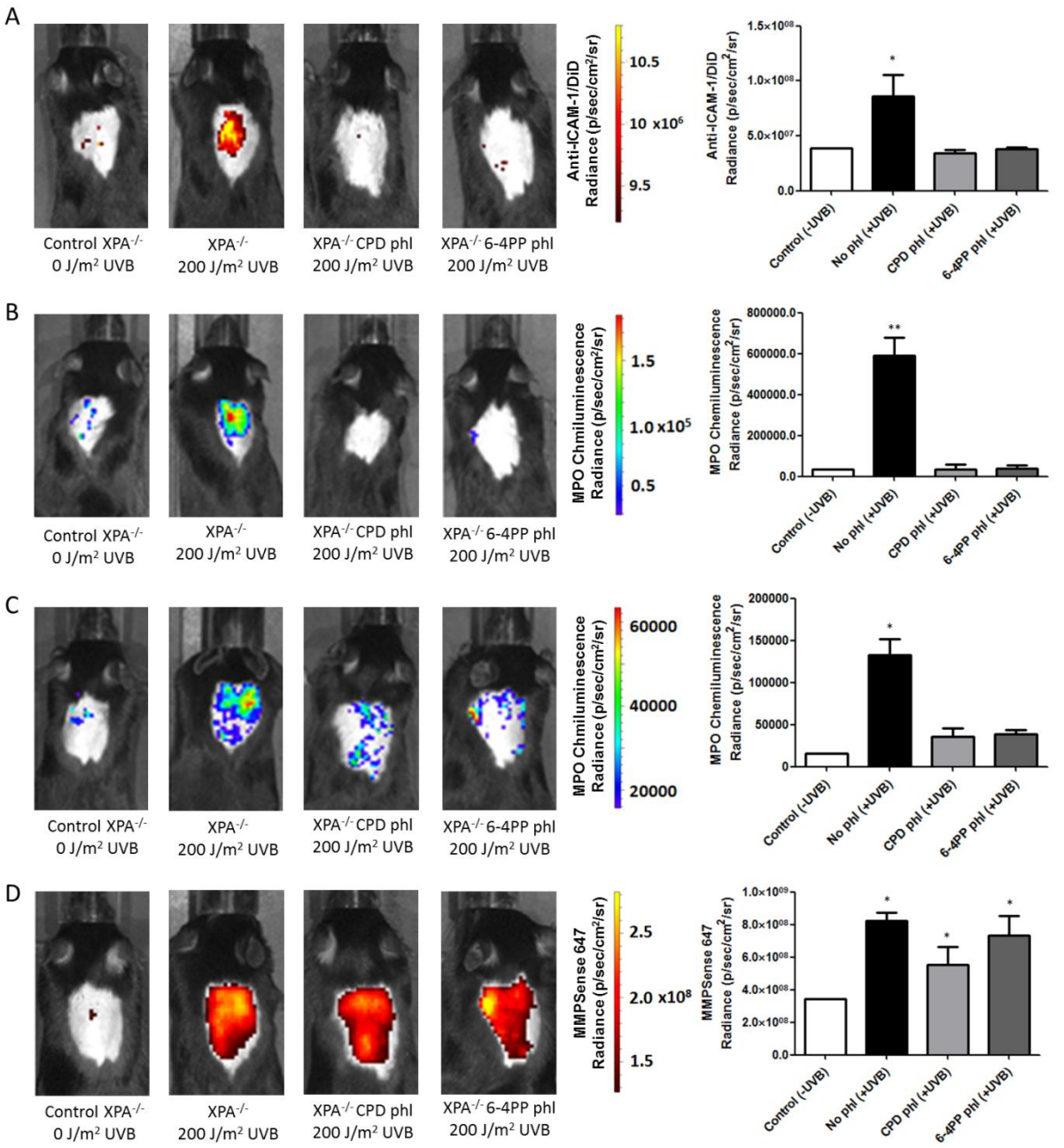

Figure 2.3: In vivo effects of keratinocyte specific photorepair of CPDs or 6-4PPs in XPA KO mice after a single, high UVB irradiation dose. (A) *In vivo* imaging of ICAM-1, an inflammation marker, coupled to a DiD fluorophore containing nanoparticle. Imaging was performed in photolyase (CPD or 6-4PP phl) expressing XPA KO mice 6 h after irradiation with 200 J/m2 UVB, n=4. **(B and C)** MPO, an active neutrophil marker, was measured *in vivo* 6 (B) and 24 h (C) after UVB irradiation of XPA mice by using a chemiluminescent probe, n=2. **(D)** MMPsense, a fluorescent probe that detects MMP2, 3, 7, 9, 12, and 13, was used to measure *in vivo* the presence of MMPs in mice skin 24 h after UVB irradiation, n=2. Radiance quantifications of fluorescent and chemiluminescent probes were performed in the central region of the mice exposed dorsal skin with equivalent sized regions of interest.

2.5 Discussion

In this work, we used NER deficient, XPA KO mice in order to investigate the *in vivo* effects of the photoremoval of CPDs or 6-4PPs in keratinocytes after ultraviolet (UV) irradiation. We observed that in these conditions, the removal of CPDs is capable of completely inhibiting the UV hyperplasic effect, while the removal of 6-4PP is able to reduce it. These results corroborate similar data previously obtained by our group (146), in which XPA KO mice expressing CPD-photolyase in all cell types under a β-actin promoter also inhibited the UV induced hyperplasia effect in NER deficient mice. As such, the analysis in the present work show not only the importance of both photolesions for these effects, but also the central role of keratinocytes regarding UV radiation (UVR) on skin, in line with previous findings (142).

UV irradiation induces the activation of the EGFR (endothelial growth factor receptor) protein, capable of activating important survival and cell proliferation pathways, such as the PI3K/Akt, JNK and ERK pathways. UV induced EGFR activation has also been shown to downregulate p53 and p21, thereby inhibiting apoptosis and cell cycle blockage (147). UV irradiation also enables the translocation of EGFR to the cell nucleus, promoting the transcription of the Cyclin D1 gene, which in turn stimulates cell proliferation, thereby leading to hyperplasia (148). In line with our hyperplasia results, the removal of CPD and 6-4PP photolesions in keratinocytes was able to reduce UVR induced cell proliferation, with CPD removal completely inhibiting it. Alhough we have shown that 6-4PPs influence this UV induced effect, and CPD-photolyase mice still contain the remaining 6-4PPs, it is possible that these remaining photolesions do not reach a threshold capable of initiating signaling for cell proliferation. We suggest that DNA damage associated pathways (including p53, PI3K/Akt, JNK and ERK) possibly interact with and regulate EGFR induced cell proliferation and hyperplasia, events related to UVR induced carcinogenesis (149).

Apoptosis is the main type of cell death induced by UV irradiation (150), with UVR induced DNA damage activating the p53 related cell death pathway (151,152). The results indicate that in NER deficient models, both CPD and 6-4PP lesions contribute to the induction of this UVR effect, with the removal of these lesions lead

to a reduction in the UVR-induced apoptosis in the mice skin. These data, along with previous studies by (140), with NER proficient mice expressing either CPD or 6-4PP photolyase, corroborate previous *in vitro* studies (88), in which the removal of CPD lesions in NER proficient cells resulted in a reduction of apoptosis and 6-4PP removal did not, while in XP-A, NER deficient cells, the removal of either lesions resulted in a reduction of apoptosis. In a similar way to the UV pro-inflammatory effects, these results have implications regarding the role of these photolesions in cell death promotion, possibly depending not only on the number of lesions but also on the type of lesion caused by UV irradiation and on the cells where these lesions are present.

Previous studies show the importance of UVR on inflammation induction, with UV irradiation being able to induce several different kinds of pro-inflammatory effects, such as activation of the inflammasome and of inflammation-related cytokines and transcription factors such as NF-κB and p38 (153,154). Moreover, the removal of CPDs in NER proficient animals was able to reduce the pro-inflammatory effects of UV (139), linking photolesions to inflammation. Our study further characterizes this link by showing that both CPDs and 6-4PPs have a role in these effects on NER deficient mice, with the removal of either one in keratinocytes causing a reduction in UVB induced neutrophil infiltration and activation in the skin, in line with previous results showing that keratinocytes having a major role in regulating UVR related inflammation (134,155) and suggesting that the inflammatory responses might be dependent not only on the quantity of lesions but also on the type of lesions. Although CPD lesions are generated in a higher rate (3 to 5 fold) than 6-4PPs (58), the removal of either lesion resulted in a similar inflammation preventing effect.

In spite of the anti-inflammatory effect of photorepair, the removal of these photolesions did not show significant effects regarding the level of Matrix Metalloproteinases (MMPs) in the skin following UV irradiation, though the methodology used in this study did not differentiate between different kinds of MMPs. The different types of MMPs have generally distinct effects, participating in both inflammation initiation and resolution (156). For instance, MMP2, MMP3 and MMP9 have a role in activating the pro-inflammatory cytokines TNF-a and IL-1B, while MMP3 may also participate in the degradation of mature IL-1B depending on the context (157). One should be cautious, however, on the conclusion that DNA lesion

photoremoval is not related to induction of MMPs in these mice. The strong induction observed may be due to over saturating levels of detection, and thus, it is possible that a small reduction on the photoreactivate animals could be simply not be detected. Therefore, further studies regarding specific MMPs are required in order to better elucidate the role of CPDs and 6-4PPs on the induction of these molecules.

In summary, by using XPA KO, keratinocyte specific photolyase expressing mice, we were able to demonstrate that both CPD and 6-4PP lesions participate in UV related effects such as hyperplasia, cell proliferation, inflammation and apoptosis using *in vivo* NER deficient models, with keratinocytes having a major role regarding these effects. These results corroborate previous studies concerning photolesion effects on apoptosis and hyperplasia and have novel implications regarding DNA damage as a pro-inflammatory stimuli. Moreover, these discoveries have important implications for XP patients, incapable of repairing UV induced photolesions. These patients not only have a much higher skin carcinogenesis predisposition, but also have different mutation spectra in skin tumors (158). This could be related the pro-inflammatory effects of both photolesions, with inflammation being a critical factor in tumour progression and being able to damage DNA by releasing oxidizing agents (159). Furthermore, unlike NER proficient models, in which CPD lesions are the main photolesion responsible for triggering the studied effects, NER deficient models have both CPDs and 6-4PPs participating in these effects, with 6-4PPs possibly having a different role in XP tumorigenesis (88), with the tumors from these individuals having different causative lesions compared to the rest of the population. Additional investigations on the molecular mechanisms of the activation of the aforementioned UV effects in NER deficient models could shed a light on XP carcinogenesis and how the photolesions interact with the multitude of the molecular pathways involved in these UVR responses.

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Conflict of interest statement. None declared.

2.7 Supplementary Material:

Target gene	Primers (pM)	DNTPs (pM)	Buffer 10x (µL)	Taq Polymerase (U)	H2O (µL)
Photolyases & *hairless*	0.25	0.25	2.25	0.5	q.s. 20
xpa	0.8	0.2	3.75	0.625	q.s. 25

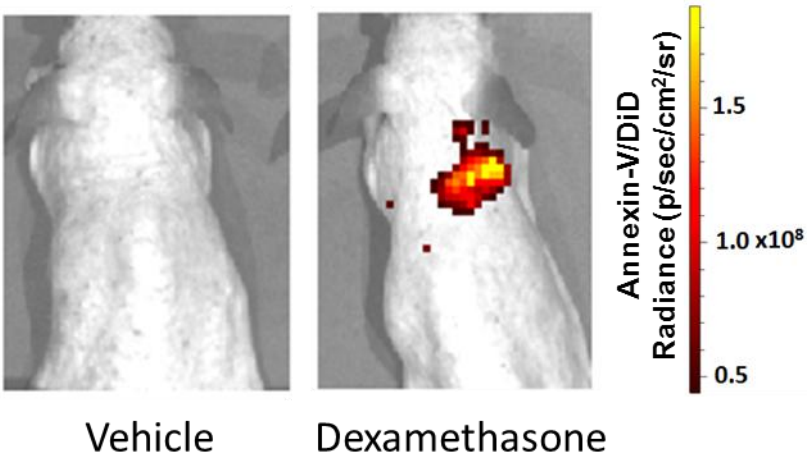
Supplementary Table S2.1: Genotyping PCR reagents concentration for CPD and 6-4PP photolyases, hairless and XPA genes.

Target gene	Steps												
	1		2		3		4		5	6		7	
	T (°C)	t (s)	T (°C)	t (s)	T (°C)	t (s)	T (°C)	t (s)	Repetitions of steps (2-4)	T (°C)	t (s)	T (°C)	t (s)
Photolyases & *hairless*	95	60	95	30	58	30	72	30	30	72	600	4	∞
xpa	95	120	95	30	62	30	72	60	35	72	300	4	∞

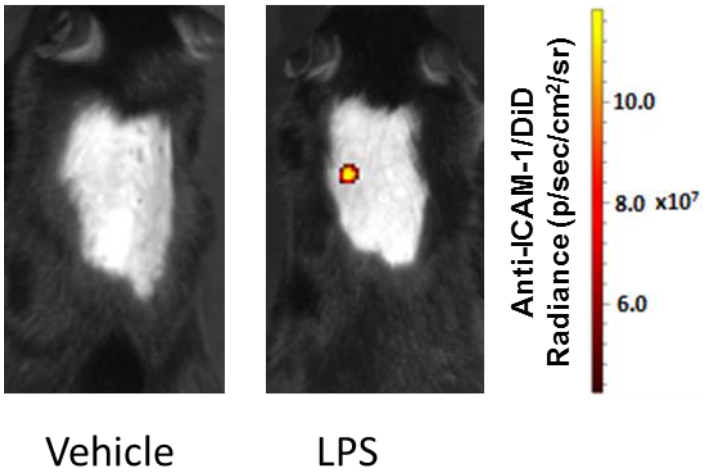
Supplementary Table S2.2: Temperature cycles used for genotyping PCRs.

Target gene	Primer	5'->3' Sequence
CPD photolyase	CPD P1	TGAGACTCATCTCCCAGGAC
	CPD P2	CACCAATGCCATGTGTTTGC
6-4PP photolyase	6-4PP P1	GCACGATTCAGCAAGCAAGG
	6-4PP P2	CGGTACCTCTACCTATTTGAGTT
hairless	HR P1	GCGTTACTGCAGCTAGCTTG
	HR P2	TGTAGCCTGTGGTCGCATAG
	HR P3	CTCCTGTTTGCTTGGTCATC
xpa	XPA-PGK2 154	GGCCACTTGTGTAGCGCCAA
	XPA26 155	GTGTCAGGCATAAGATCTATGACAA
	XP47 156	AGGCAAGCACCTGCAGCTGT

Supplementary Table S2.3: Primer sequences for genotyping PCRs.



Supplementary Figure S2.1: Apoptosis detecting nanoparticles validation. Hairless mice received 100 µL of 10 µM mg dexamethasone, a potent inducer of apoptosis in the thymus, injected intraperitonially and imaged after 18 h, with Annexin-V/DiD nanoparticles injected intravenously 3 h prior to imaging.



Supplementary Figure S2.2: Validation of ICAM-1 binding nanoparticle. Black6J mice were injected subcutaneously with 100 µL of 10 mg/ml LPS in shaved dorsal skin and had in vivo imaging performed 6 h following irradiation, with anti-ICAM1/DiD nanoparticles injected 3 h prior to imaging.

Chapter 3 - Neurovascular dysfunction and neuroinflammation in a Cockayne syndrome mouse model

3.1 Summary

Cockayne Syndrome (CS) is a rare, autosomal genetic disorder characterized by premature aging-like features, such as cachectic dwarfism, retinal atrophy and progressive neurodegeneration. The underlying genetic defect in CS lies in genes associated with the transcription-coupled arm of the nucleotide excision DNA repair (NER) pathway, although how defective DNA repair leads to the particular symptoms of CS is not yet clear.

In this work, we used a mouse model of severe CS with total loss of NER, termed the CX model, which recapitulates several CS-related phenotypes, resulting in premature death of these mice at approximately 20 weeks of age. Although CX mice exhibit a severe progeroid phenotype, we found no evidence of a cell autonomous vascular dysfunction in these mice. In spite of this observation, we detected a blood barrier dysfunction and a significant increase in inflammatory markers in the brains of these animals, which indicates that neuroinflammation could play a role in the neurodegenerative phenotype observed in this model as well as in the human disease. These findings have implications for the etiology of this disease and could contribute to the study of novel therapeutic targets for the treatment of Cockayne Syndrome patients.

3.2 Introduction

The DNA molecule is under constant physical and chemical stress, capable of generating DNA damage (20). These lesions, if not correctly repaired, may induce several biological processes, many of which can be detrimental to organism health. Notably, DNA damage and its repair have been implicated in the process of aging - DNA damage accumulates over a lifetime, and is able to trigger processes such as senescence, cell signaling alterations and cell death, all of which are associated with aging (64,112). Furthermore, dysfunctional DNA repair genes may lead to genetic diseases characterized by segmental progeroid syndromes, having some premature aging phenotypes, such as altered endocrinal axis and tissue degeneration (160). These diseases are often associated with accelerated aging of the brain, being characterized by a progressive and profound neurodegeneration (161). There are numerous genetic syndromes associated with DNA repair deficiencies, such as Trichotiodystrophy, Xeroderma pigmentosum, and Cockayne syndrome (162).

Cockayne syndrome (CS) is a rare, autosomal, recessive disorder caused by mutations in one of two genes involved in the transcription-coupled nucleotide excision DNA repair (TC-NER) pathway, CSA or CSB, characterized by a wide range of symptoms, including cachectic dwarfism, lipodystrophy, photosensitivity, and multiorgan degeneration (70). Regarding neurodegeneration in CS, various neurological abnormalities are observed, such as loss of Purkinje cells in the cerebellum, reduced numbers of oligodendrocytes, demyelination of central and peripheral nervous tissue, brain calcification, and microcephaly (161). It is worth noting that although CS has been extensively studied, the mechanisms underlying the progeroid/neurodegenerative phenotype are not yet fully understood, and there are currently no therapies for CS patients (71).

In order to study the relationship between DNA repair deficiency and progeroid syndromes, various knockout mouse models have been developed and characterized. These include the Ercc1$^{-/\Delta}$ (163), XPD/XPA (164), CSB/XPA (165) and CSA/XPA (termed “CX”) (166). These models exhibit several phenotypes that mimic those of human CS, such as reduced weight and size, indicating postnatal developmental defects, progressive loss of adiposity, kyphosis, abnormal gait,

hindlimb paralysis and premature death (166). Other notable neurological symptoms in these mice models often include reduced cerebellar size and decreased white matter, loss of Purkinje cells and patchy areas of myelin loss (167,168). Interestingly, although similar, these models exhibit some differences among themselves. For instance, Ercc1$^{-/\Delta}$ fibroblasts exhibit early senescence, while XPD/XPA does not (163,164).

Besides neuron-specific DNA repair defects (169), other factors have been implicated in the neuropathology of CS - Vascular dysfunction and neuroinflammation have both been proposed to be involved in the progressive neurodegeneration of CS, as cell abnormalities related to these processes have been found in CS patients and in progeroid mice models (115,170,171). Endothelial cells (ECs) form the inner lining of blood vessels, having essential roles in every organ, including the brain, wherein they are responsible for processes such as the formation and maintenance of the blood brain barrier, energy metabolism and inflammation (172,173). Besides endothelial cells, other brain cell types are involved in the inflammation of this organ, including neuron and glial cells, such as astrocytes and microglia. There are many evidences that neuroinflammation can play a role in neurodegenerative diseases concerning myelin abnormalities, neuron morphology, synapse elimination and cell death (174–177). Regarding DNA repair related effects, the oxidative stress generated by a neuroinflammatory response is expected to generate lipid peroxidation products stemming from myelin oxidation – these products can damage neuronal DNA, thus causing neuronal loss (161).

In this work, we found that the CX mouce model does not present cell autonomous EC dysfunction. Nevertheless, we observed an increase in brain vascular permeability, EC senescence, vascular activation/inflammation gene expression, upregulation of active NF-κB, and an increase in active astrocytes and microglia in CX mice brains. These results indicate that neuroinflammation is likely a primary factor contributing to the neuropathology of these animals, providing a novel insight to the etiology of this complex disease.

3.3 Materials and Methods

3.3.1 Mice lines

Knockout mice of the *csa* and *xpa* genes and double KO (CX) strains have been described previously, with genotyping, care and housing of the mice strains being performed as described before (166). Briefly, in order to perform genotyping of the CX colony, DNA was extracted by boiling mice ear punch tissue at 100°C with 50 mM NaOH, followed by neutralization with 1 M Tris-HCl. PCR was performed as per supplementary tables S3.1-3. Expected band sizes for wild type and knockout alleles for *xpa* genotyping are 300 bp and 200 bp, while for *csa* they are 230 bp and 140 bp, respectively. All strains used in this project (WT, CSA KO and CX) had a Black6J background. Animals were maintained by heterozygous crosses. Housing, breeding, and experimentation were performed in accordance with the regulations established by the Harvard Medical Area Institutional Animal Care and Use Committee (IACUC).

3.3.2 Pharmacological Interventions

Axitinib (VEGFR2 specific inhibitor) were supplemented at a daily dose of 30 mg/kg/d in food, a dose previously described as having phenotypical effects on mice (178).

3.3.3 Brain vascular permeability

In order to assess the permeability of the blood brain barrier, mice were injected intravenously with 200 µl of PBS-2% Evans blue (Sigma-Aldrich, St. Louis, MI, USA), sacrificed 1 h later, and perfused intracardially with PBS 1X. Brains were then harvested and sectioned to investigate the inclusion of Evans blue into the brain tissue.

3.3.4 IgG Western Blot

IgG infiltration, another marker of blood brain barrier permeability, was measured via western blot in order to assess the presence of this protein in mice

brains. Brain protein was isolated by grinding tissue in NP-40 buffer containing protease inhibitors and dithiothreritol (DTT). After isolation, protein concentration was quantified and normalized using Pierce BCA Protein Assay Kit (Thermo-Fisher, Waltham, MS, USA), boiled in Sodium dodecyl sulfate (SDS) buffer and separated through polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes and blotted for mouse IgG (P 0447, Dako, Santa Clara, CA, USA) and GAPDH (sc-137179, Santa Cruz Biotechnology, Dallas, TX, USA) .

3.3.5 Gene expression analysis by qPCR

RNA was isolated from brain tissue with RNA bee (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol, with tissue being grinded in RNA bee on ice, followed by ethanol washes. RNA was precipitated with isopropanol, and quantified with a Nanodrop 2000 spectrophotometer (Thermo Fisher). cDNA was synthesized using 1 μg of total RNA using the Verso cDNA kit (Thermo Fisher). qRT-PCR was performed with SYBR green master mix (BIORAD, Hercules, CA, USA). Fold changes were calculated by the ΔΔCt method using β-actin or GAPDH as standard, and normalized to the experimental control. Primer sequences are as follows:

β-actin F:5'-AGCTTCTTTGCAGCTCCTTCGTTG-3'; R:5'-TTCTGACCCATTCCCACCATCACA-3'
GAPDH F: 5'-AACTTTGGCATTGTGGAAGG -3'; R: 5'-ACACATTGGGGGTAGGAACA-3'
ICAM-1 F:5′-GCCTCCGGACTTTCGATCTT-3′; R: 5′-GTCAGGGGTGTCGAGCTTTG-3′
P-Selectin F: 5′-CCCTGGCAACAGCCTTCAG-3′; R: 5′-GGGTCCTCAAAATCGTCATCC-3′.
Tnfα F: 5'-AGGGTCTGGGCCATAGAACT-3'; R:5'-CCACCACGCTCTTCTGTCTAC-3'
VCAM F: 5′-AGTTGGGGATTCGGTTGTTCT-3′; R: 5′-CCCCTCATTCCTTACCACCC-3′.

3.3.6 Primary endothelial cell culture

Endothelial cell lines were obtained from lungs of WT, CSA KO and CX mice strains in order to assess endothelial cell proliferation capacity, migration and ICAM-1 activation *in vitro*. For the establishment of the cell lines, animals were anesthetized with isoflurane, followed by euthanasia by cervical dislocation, followed by the removal of the lungs. The lungs were then mechanically dissociated, followed by

chemical digestion (200 U/ml Collagenase type II, 200 U/mL Collagenase type IV, 1 U/mL Dispase in DMEM) in order to create a single cell suspension. In order to purify for endothelial cells, we used EasySep Mouse APC Positive Selection Kit (Stem Cell Technologies) after incubation with anti-CD31-APC antibody, as per manufacturer instructions. Cells were seeded at a density of 1 x 10^5 cells/mL in Vasculife complete medium (Lifeline Cell Technology, Frederick, MD, USA). Cultures were incubated in humidified atmosphere with 5% CO_2 and 5% O_2. Cells were lifted after reaching approximately 80% confluency by incubation with 0.25% trypsin and counted using a hemocytometer. At least 3 distinct cell lines were used per group per experiment.

3.3.7 Wound healing assay

In order to measure migration capacity of endothelial cells, a single scratch wound was created using a sterile p200 pipette tip on a confluent field of cells, 24 h after seeding (100,000 cells per well in 24-well plate) in a serum-free condition. Repopulation across the scratch wound was recorded by a phase-contrast microscopy for up to 48 h using a digital camera. Wound closure was determined at each time point from digital images using ImageJ software.

3.3.8 Aortic ring assay

In order to assess the angiogenesis capacity of the different mice strains, we performed the aortic ring assay, performed according to the published protocol (Baker et al., 2011). Briefly, we obtained ~0.5 mm wide rings from mice thoracic aortas, which were then embedded in 50 µL of growth factor reduced Matrigel (Corning) in a 96-well plate. Vessel sprouting was stimulated by complete Vasculife media. The media was replaced every two days, and images were taken using a phase-contrast microscope coupled to a digital camera. The length of sprouts originating from aortic rings was quantified by ImageJ software. Aortic rings were collected from at least 3 mice per group and the assay was performed using 3 technical replicates.

3.3.9 Senescence Associated β-galactosidase staining

One of the most established assays to measure the presence of senescent cells in a given tissue is the Senescence Associated Beta galactosidase (SA-βgal) staining. The assay was performed as per manufacturer instructions (Cell Signaling Technologies, Danvers MS, USA). Briefly, aortas and perigonadal fat were removed and washed twice with PBS 1X, followed by fixation with fixation solution for 10 min, washed with PBS 1X, and incubated with β-galactosidase staining solution.

3.3.10 VEGF ELISA

Serum VEGF levels were measured in order to verify the activity of the VEGFR2 inhibitor, Axitinib. Blood was collected before euthanasia by heart puncture and centrifuged at 1200 RPM for 10 min. Serum was collected and stored in -80oC conditions until the assay was performed. We used Mouse VEGF Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA), as per manufacturer instructions. Briefly, each sample was diluted 5 fold, with 50 µL of the diluted sample being added to the ELISA microplate along with 50 µL of the ELISA diluent. After a 2 h incubation, each well was washed 5 times, followed by a 2 h incubation with 100 µL of mouse VEGF conjugate. Wells were then washed again 5 times, followed by 30 min incubation with 100 µL substrate solution, and 100 µL stop solution. Absorbance was measured with a plate reader set to 450 nm and correction to 570 nm.

3.3.11 Immunohistochemistry

Mice brains were harvested and fixed overnight with 4% PFA at 4oC. The tissue was then washed twice with PBS 1X, followed by 30% sucrose in PBS 1X incubation at 4oC until brains sunk. Brains were then embedded in OCT and cut in a cryotome. Mice gastrocnemius muscle was obtained and embedded in OCT, then cut in a cryotome. For immunostaining, we used 18 um coronal brain sections and 5 um muscle sections, placed in Superfrost VWR slides (VWR International). GFAP, Iba1, CD68 and CD31 were immunostained by first fixing the slides with 4% PFA for 10 min, followed by PBS 1X washes. The tissues were then permeabilized with 0.02% Triton-X for 12 min, followed by PBS 1X washes and 1 h blocking by incubating

tissues with 10% FBS, 1% BSA in PBS 1X ot 10% goat serum solution. Slides were then incubated with GFAP, Iba1, CD68 or CD31 primary antibody solution overnight at 4oC. For the C3 staining, tissues were fixed with acetone for 10 min, and antigen retrieval was performed using 50% formic acid for 5 min. After incubation with the primary antibody, slides were washed with PBS 1X, followed by incubation with secondary antibody solution. Antibodies used in this project can be found in supplementary table 1. Images were taken on Axio Observer fluorescence microscope (Carl Zeiss, Oberkochen, Germany), and GFAP or Iba1 positive cells were quantified using ImageJ software.

3.3.12 Flow cytometry analysis

Flow cytometry experiments were performed on a BD LSRFortessa and analysed using FlowJo ver.10. For ICAM-1 staining in cell culture endothelial cells, cells were detached from plates using Accumax (Innovative Cell Technologies, San Diego, CA, USA) at approximately 70% confluency, followed by wash and 2 h incubation with PE/Cy7 ICAM-1 antibody (Biolegend, San Diego, CA, USA). Cells were then washed and flow cytometry was performed.

For brain tissue flow cytometry, mouse brains were dissociated in RPMI (Mybiosource San Diego, CA, USA) by gentle trituration using 10 mL pipettes, followed by a 30 min incubation in digestion buffer (200 U/mL Collagenase II, 200 U/mL Collagenase IV, 1 U/mL Dispase). This process was performed twice, in order to create a single cell suspension, which was stained for endothelial cells using APC-CD31 Antibody (Miltenyi Biotec), followed by incubation in 4oC overnight in fixation buffer (eBiosciences). Cells were then washed and incubated in permeabilization buffer (eBiosciences) for 1 hour. Brain cells were then stained for p16 and p-p65. Antibodies used in this project can be found in supplementary table 1.

3.3.13 Statistical analysis

All data are presented as mean ± SEM. One-way or two-way ANOVA followed by Tukey’s post hoc test were used for multiple comparison analysis. Kaplan-Meier method was used for survival analysis. p values of less than or equal to 0.05, 0.01,

and 0.001 (indicated by asterisks (*) when comparing within the same group and by the pound sign (#) when comparing between groups) were considered statistically significant. p values of higher than 0.05 were considered nonsignificant.

3.4 Results

3.4.1 Blood brain barrier dysfunction and endothelial cell activation in CX mice

A feature commonly associated with of neurovascular dysfunction is increased permeability of the blood brain barrier (BBB), which itself can also be detrimental to the central nervous system. In order to assess the integrity of the BBB in CX mice, we injected the mice with the Evans Blue dye, an azo dye with a high affinity to serum albumin. Under normal circumstances, albumin cannot cross the BBB due to its high molecular weight. When the BBB has been compromised, the albumin-Evans blue complex is the able to enter the brain tissue and stain it. We observed a more pronounced stain in CX mice brains, increasing in an age dependent manner (Figure 3.1A). This observation indicates that there is a neurovascular dysfunction in CX mice brain, which could potentially contribute to its neurodegenerative phenotype. Moreover, we also observed a robust increase in the levels of the IgG protein in CX mice brains, which also indicate an increase in the permeability of the BBB (Figure 3.1B).

There are several proteins commonly associated with vascular activation and inflammation, with many of them overlapping. We measured the level of gene expression of several of these factors (ICAM-1, TNFα, p-Selectin, VCAM-1) by qRT-PCR, and we found that the levels of ICAM-1 and TNFα gene expression were higher in CX animals in both tested age groups (10-13 and 17-19 week old animals, Figure 3.1C), which indicates that vascular dysfunction and/or neuroinflammation might play a role in the phenotype of these mice.

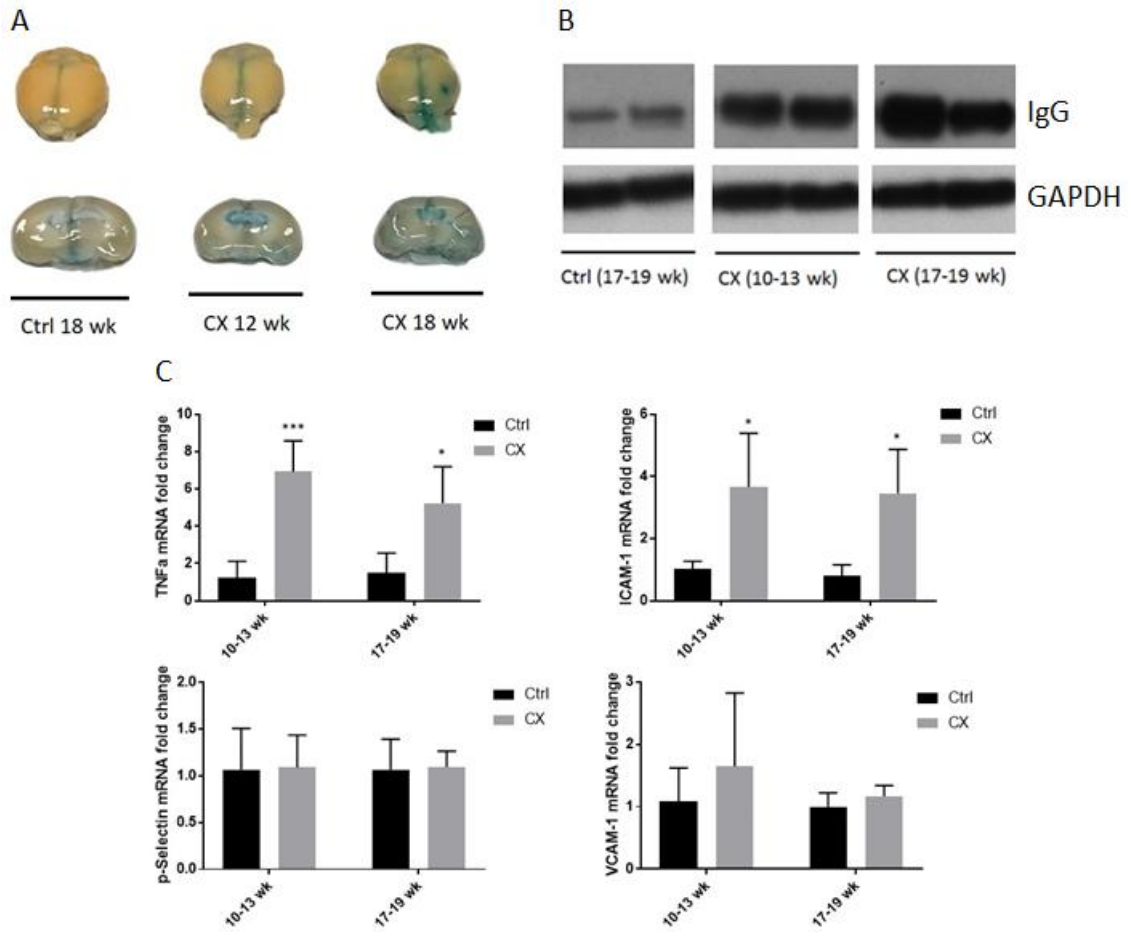


Figure 3.1. CX mice display brain vascular permeability and expression of vascular cell activation genes. (A) Evans blue stain shows higher permeability in CX brains in an age-dependent manner. (B) Western blot of IgG, another marker of permeability of the blood brain barrier in mice brain. GAPDH was used as a loading control. (C) qRT-PCR of vascular cell activation markers, with ICAM-1 and TNFα in CX mice being significantly higher than control CSA mice, in both 10-13 and 17-19 week old age groups, though there was no difference between age groups. Two way Anova, n≥4.

3.4.2 CX mice do not display cell autonomous vascular dysfunction

In order to investigate whether endothelial cell dysfunction plays a role in the CS phenotype, we obtained primary ECs lines from WT, CSA and CX mice to perform *in vitro* assays to study genotype effects in processes such as cell proliferation, migration capacity and endothelial cell activation in a cell autonomous manner. We also performed the aortic ring assay, an *ex vivo* assay to measure angiogenesis, and investigated EC senescence by the SA-βgal staining in mice aorta. We did not detect any differences between any of the genotypes regarding proliferation, migration, ICAM-1 activation, angiogenesis or senescence in endothelial cells (Figures 3.2A-E), which indicate that, in contrast to other similar progeroid

models, the CX mice do not display cell autonomous vascular dysfunction. We also performed the aortic ring assay in axitinib treated animals and found no difference regarding sprout length (Figure 3.2D), which indicates that the extracellular levels of endothelial cell growth factors provided in the media was enough to activate endothelial cell sprouting regardless of previous treatment with VEGFR2 inhibitors. As a positive control to the SA-βgal early senescence phenotype, we used CX mice fat, as it had been previously reported by the Mitchell lab that it there is a significant increase of the senescence phenotype in this tissue in CX animals (Figure 3.2F).

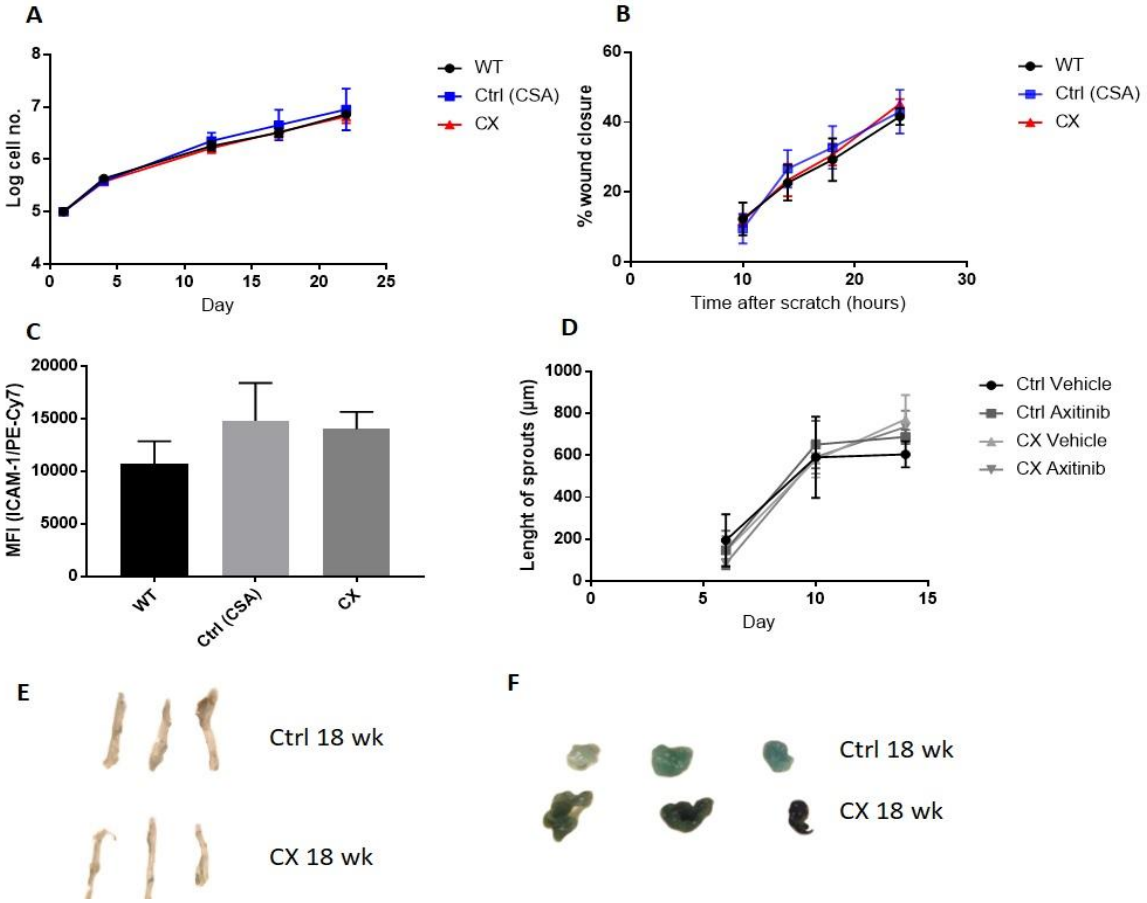


Figure 3.2. CX mice do not display cell autonomous vascular dysfunction phenotype regarding endothelial cell proliferation, migration, ICAM-1 activation, angiogenesis nor senescence. (A) Proliferation rates of CX ECs do not differ from neither the CSA nor WT ECs. (B) Migration capacity, as measured by the wound healing assay. (C) ICAM-1 expression in endothelial cells, measured by fluorescence intensity in FACS analysis. (D) Angiogenesis capacity, measured using the aortic ring assay in 12 week old animals. (E) Senescence Associated βgalactosidase staining of mice aortas. (F) Senescence Associated βgalactosidase staining of mice perigonadal fat used as a positive control for the SA-βgal assay, n≥3 for all experiments.

3.4.3 Senescence and pro-inflammatory markers in CX mice brains

Although we did not find any evidence of early vessel senescence in a cell autonomous manner, we also investigated this phenotype in CX mice brain, as it is one of the most notably affected organs of CS patients. Senescence is a process heavily affected by the extracellular milieu, often occuring in a tissue specific manner. Senescent cells are also more prone to having a pro-inflammatory phenotype, with its secreting phenotype being heavily altered. In order to investigate whether brain endothelial cells have these phenotypes, we used an *ex vivo* strategy, in which we processed the brain into a single cell suspension by physical and chemical dissociation, then immunostained endothelial cells (using anti-CD31, an endothelial cell marker), p16 (a senescence marker), and active NF-κB (a pro-inflammatory marker, by staining p-p65). We found a significant upregulation of p16 in CD31 positive cells (Figure 3.3A), and also a p-p65 upregulation in CD31 negative CX mice cells (Figure 3.3B), which indicate that there is an early senescence phenotype in CX brain ECs, and a neuroinflammatory phenotype that is not found in endothelial cells under cell autonomous conditions.

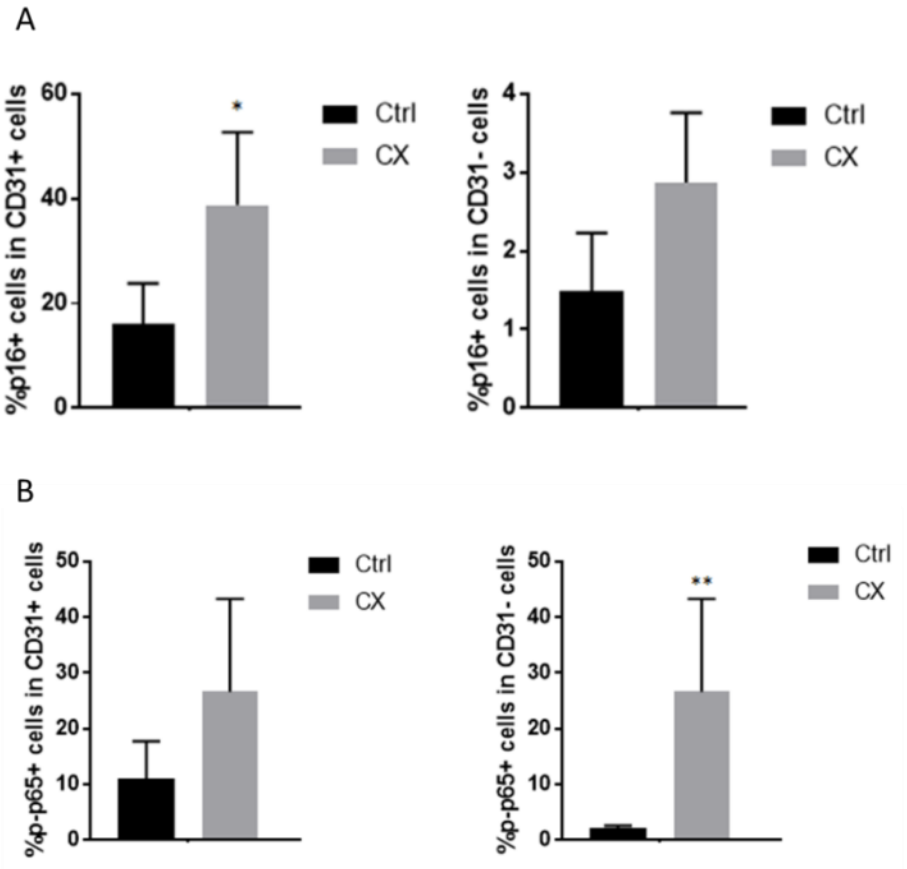


Figure 3.3. CX mice brain display p16 upregulation in endothelial cells and p-p65 in non-endothelial cells. (A) FACS analysis of endothelial and non-endothelial p16 positive cells in CX mice brains. (B) Active p65 FACS analysis of endothelial and non-endothelial cells in CX mice brains, n≥4.

3.4.4 Glial cell activation in CX mice brain

Several reports have linked glial cell activation and neurodegeneration. We investigated whether the two glial cell types involved in neuroinflammation, astrocytes and microglia, displayed abnormal activation status and increased numbers in CX mice when compared to the control (CSA) mice. We observed a markedly increase in the number of activated astrocyte by the GFAP (Figure 3.4A) and C3 staining (Figure 3.4B), with the reactive astrocyte number being maintained over the different ages. We also found that the number of microglia in this progeroid model is increased in an age dependent manner (Figure 3.4C), with these cells being in a more active state when compared to their control counterpart, as observed by Iba1 and CD68 staining (Figure 3.4D). Moreover, the inhibition of angiogenesis did not increase these phenotypes (Figure 3.4E), which indicates that the glial cell activation occurs independently of a brain vascular dysfunction.

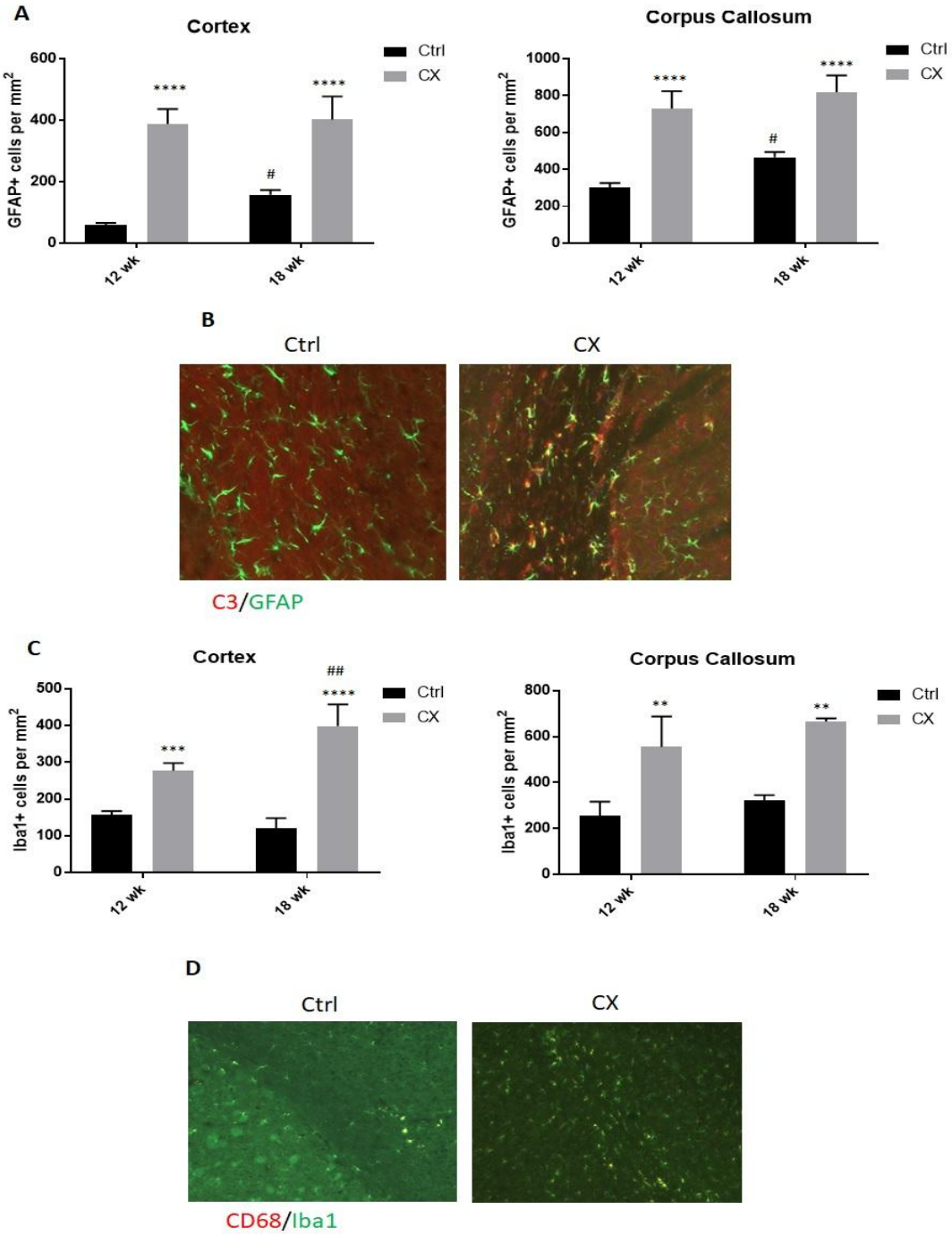


Figure 3.4. CX mice display astrocyte and microglia activation in the brain. (A) Reactive astrocytes number increase in CX brain cortex and corpus callosum, as measured by GFAP staining. (B) C3, a marker of active astrocytes co-stains with GFAP in CX mice. (C) Microglia number in CX brain cortex and corpus callosum, shown by Iba1 positive cells. (D) CD68, a marker or active microglia co-stains with Iba1 in CX mice brain, n≥4 for all experiments.

3.4.5 Axitinib treatment decreases vessel number in developing CX animals, but does not decrease gliosis in adult CX mice

Previous findings from our lab had shown that axitinib, a VEGFR2 inhibitor, caused premature death in young (4-6 week old) animals, which indicated a possible CS related vascular dysfunction (supplementary Figure 1). We investigated whether axitinib treatment caused a decrease in number of vessels in CX mice when compared to control mice. In order to assess that, we treated 6 week old mice with axitinib for 3 weeks, and as a control for the treatment, we measured VEGF levels in the serum of those animals. As shown in Figure 3.5A, serum VEGF levels increased with Axitinib treatment**,** which indicates that Axitinib was having a significant effect on these animals. The number of blood vessels in these animals in these animals was also significantly reduced, as indicated by the CD31 positive cells measured in the gastrocnemius muscle (Figure 3.5B). Moreover, the 6 week old CX mice treated with axitinib displayed significant poorer body conditions (data not shown).

In order to test the effect of axitinib regarding brain vascular dysfunction in these animals, we used older, post developmental stage animals (9 week old), and treated them with axitinib for 3 weeks. We again used VEGF serum levels as a treatment control (Figure 3.5A), and tested whether axitinib would increase the level of neuroinflammatory markers. However, we did not find an increase in astro nor microgliosis markers (Figure 3.5C), which indicates that vascular dysfunction is not a primary source of the neuroinflammation observed in the CX animals, and that the axitinib related premature death previously found was a development related effect in CX animals.

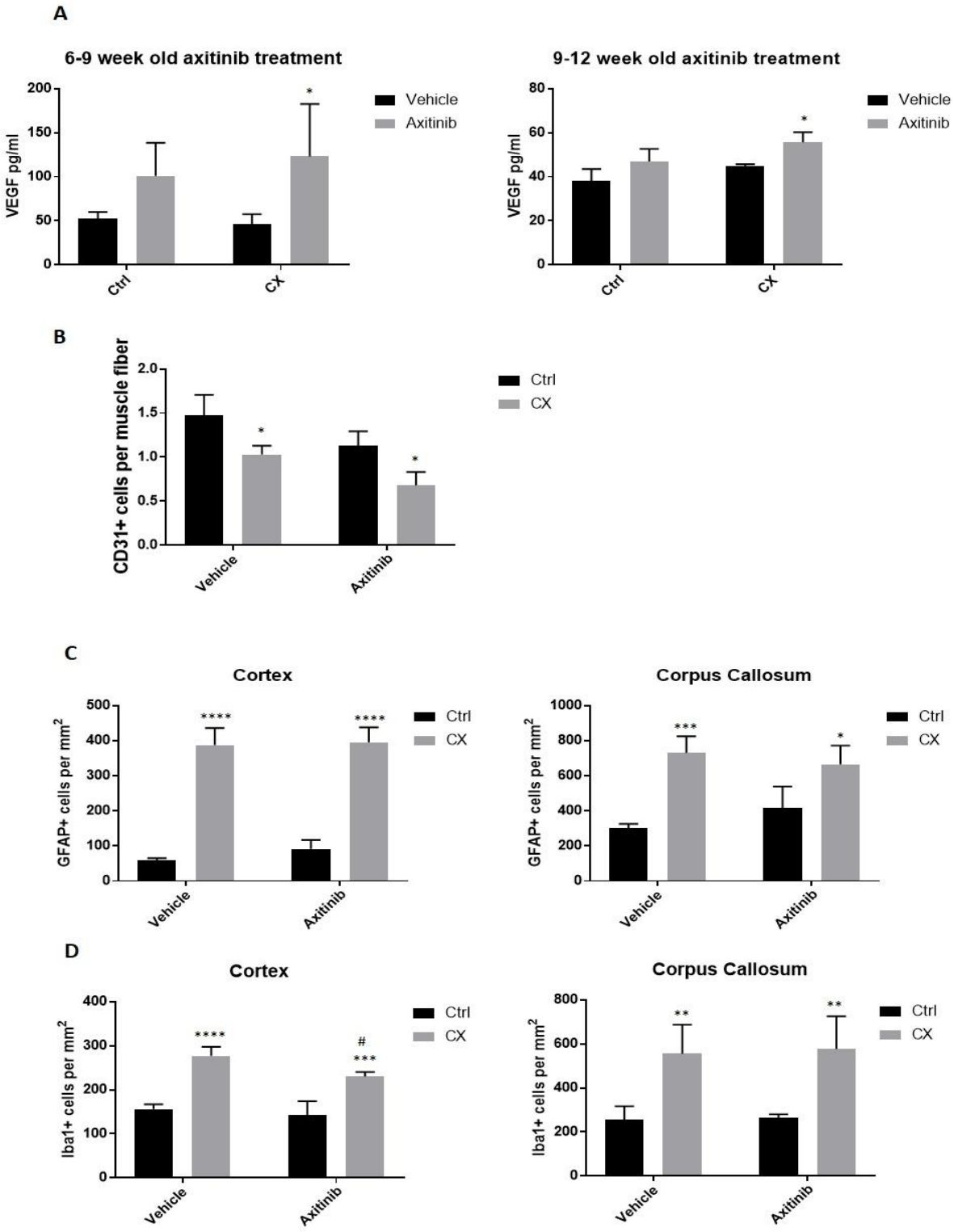


Figure 3.5. Effects of axitinib treatment in developing and adult CX mice. (A) Serum VEGF levels increase in CX animals treated with axitinib during the developmental and adult stage mice. (B) Developing CX mice display a decrease in endothelial cell number in gastrocnemius muscle after 3 week axitinib treatment. (C) Axitinib treatment in adult CX mice does not increase astrogliosis. (D) Axitinib treatment in adult CX mice does not increase microgliosis, n≥4 for all experiments.

3.5 Discussion

Cockayne syndrome is a multifactorial disease that affects several different organs, with neurodevelopmental and neurodegenerative defects being some of the most prominent phenotypes (179). Although CS is caused by mutations in DNA repair related genes, it is still yet unknown the role of DNA damage in the etiology of the progeroid phenotype presented by the CS patients (161). The proteins encoded by the CS-related genes have several functions besides transcription coupled repair, such as chromatin remodeling, ribosomal biogenesis and gene regulation (180,181). In this work, we used a double knockout (CSA/XPA, termed CX) genetic model that mimics various progeroid CS phenotypes (166) in order to investigate the role of vascular dysfunction and neuroinflammation in this progeroid model.

Reports using other similar mouse models have suggested that a vascular dysfunction may play a role in the degenerative phenotype observed in progeroid models (115). As such, we used primary endothelial cell lines to investigate whether this phenotype was also observed in our model. However, we could not find any functional difference between CX endothelial cells when compared to their controls, which indicates that vascular cells do not play a role in a cell autonomous manner regarding the CS phenotype. Nevertheless, other external factors, such as the presence of senescent perivascular fat cells, or other pro-inflammatory cells residing in a close proximity to the ECs could still generate a local vascular dysfunction phenotype (182), in both brain tissue and in other organs.

Despite there being no cell autonomous defects in CX endothelial cells, we found a higher brain vascular permeability, which indicates a blood brain barrier dysfunction in our progeroid model, alongside an increase in ICAM-1 and TNFα, both being markers of vascular activation and inflammation (183), and also an increase in p16 positive cells, a marker of senescence (184). These results suggest that these animals have a brain specific endothelial cell phenotype. We hypothesize that pro-inflammatory factors, as indicated by the higher levels of TNFα and active NF-κB, influence the brain microenvironment and other cell types, such as glial cells, in a neuroinflammatory, deleterious manner, and thus could be involved in the brain

vascular dysfunction observed in a similar way to other neuropathologic models (185,186).

Furthermore, we found an increase in reactive astrocytes and microglia, the two glial cells with pro-inflammatory capabilities, a phenotype also found in other progeroid models and during pathologic and normal aging (165,181,187). The relationship between these cell types, neuroinflammation and neurodegeneration is a very complex one, with both glial cells being versatile, multifunctional cells, involved in a variety of processes, such as energy metabolism, debri clearance, synaptic maintenance, cell signaling, anti and pro inflammatory stimuli and regulation (188). Therefore, it is important not to overstate the effects of merely an increase in the number of reactive glia, as there are numerous roles that these cells could be playing. For instance, astrocytes are the main cells that provide energy to brain cells, especially neurons (189) - as such, a possible explanation to this astrogliosis could be related to the necessity of these cells to meet the energy requirements of the other brain cells, as it has been observed that CX mice have a higher fatty acid oxidation rate and generally more active metabolism (83).

Nevertheless, considering the active, pro-inflammatory characteristics of glial cells, these phenotypes are notably correlated to numerous neurodegenerative diseases, with astrocytes and microglia being able to induce neuronal and oligodendrocyte cell death, synaptic and cell signaling dysfunction when active (174,177). Our results show that CX animals not only have a more pronounced gliosis, but also that these glial cells are in their active state, as observed by their expression of C3 in astrocytes and CD68 in microglia, markers of a toxic activation state found in other neurodegenerative models (177). Moreover, it has been observed in other models that pro-inflammatory stimuli by glial cells are related to a higher BBB permeability and dysfunction (190). We thus hypothesize that these cells possess roles in the neuroinflammatory, neurodegeneration and brain vascular dysfunction observed in these animals. Therefore, identifying the molecular mechanisms behind this CS-related glial cell activation and neuroinflammation could enable further insight to the etiology of this multifactorial disease, and thus provide novel therapeutic targets for the treatment of this syndrome.

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Conflict of interest statement. None declared.

3.7 Supplementary material

Target gene	Primers (pM)	DNTPs (pM)	Buffer 10x (µL)	Taq Polymerase (U)	H2O (uL)
xpa	0.8	0.2	3.75	0.625	q.s. 25
csa	0.66	0.2	3.25	2.5	q.s. 25

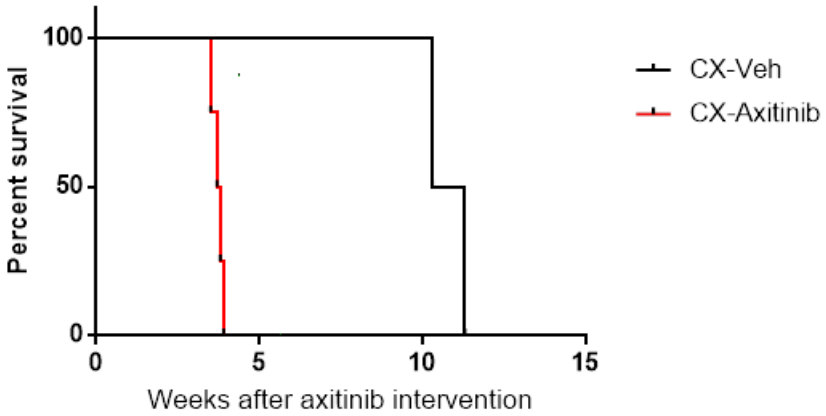
Supplementary Table S3.1: Genotyping PCR reagents concentration for *xpa* and *csa* genes.

Target gene	Steps												
	1		2		3		4		5	6		7	
	T (°C)	t (s)	T (°C)	t (s)	T (°C)	t (s)	T (°C)	t (s)	Repetitions of steps (2-4)	T (°C)	t (s)	T (°C)	t (s)
xpa	95	120	95	30	62	30	72	60	35	72	300	4	∞
csa	95	300	95	60	62	60	72	60	40	72	600	4	∞

Supplementary Table S3.2: Temperature cycles for genotyping PCRs.

Target gene	Primer	5'->3' Sequence
xpa	XPA-PGK2 154	GGCCACTTGTGTAGCGCCAA
	XPA26 155	GTGTCAGGCATAAGATCTATGACAA
	XP47 156	AGGCAAGCACCTGCAGCTGT
csa	CSA6	TCCTGGGGCTGGAGTTAAAC
	CSA7	AAAGGCAAGATTTTTCTGCA
	CSA-PGK3	TAGGGGAGGAGTAGAAGGTG

Supplementary Table S3.3: Primer sequences for genotyping PCRs.



Supplementary Figure S3.1. Survival of CX mice after axitinib treatment. Kaplan-Meier survival curves of vehicle and axitinib (30 mg/kg/d) treated CX mice, with the treatment starting with ≤ 6 week old animals.

Chapter 4 - Other effects of DNA damage in NER deficient models

4.1 Introduction

The two previous chapters address the *in vivo* effects of DNA damage regarding UV-induced photolesions on a Xeroderma Pigmentosum model and neurodegeneration on a Cockayne Syndrome model. Although the previously reported effects are the main theme of this thesis, we also studied other DNA damage and repair effects in the previously used and in other models, including a different cell proliferation and hyperplasia response to UV irradiation in TC-NER deficient CSA KO mice and further characterization of the inflammatory response to DNA damage in GG-NER deficient and CX models. We will also briefly discuss, in this chapter, about metabolic effects of DNA damage, as well as display the effects of a possible metabolism related therapeutic intervention on the longevity of the CX model.

Metabolic alterations due to endogenous DNA lesions have been implicated in some of the symptoms of progeroid syndromes such as the smaller stature, generally considered to be an adaptive response that shifts the energy expenditure from body growth to DDR (191). Some aspects of this adaptive response have been shown to be similar to the beneficial adaptive response caused by calorie restriction, including lower blood glucose and IGF-1 levels (164) This IGF-1 dampening, however, may also have a role in the etiology of the progeroid diseases, as lower IGF-1 levels have been found in human CS neuronal cells and can have a negative impact on neurodevelopmental aspects such as synaptogenesis and survival of Purkinje cells (68).

This metabolic shift is caused, in part, due to DNA repair as well as DNA Damage response being energetically costly processes, as they involve expenditure of NAD^+ and ATP for post-translational modification signaling as well as the repair process itself (192–194). DDR affects metabolism processes of oxidative phosphorylation and fatty acid oxidation through molecular pathways such as AMPK, VLCAD and PGC-1α and PARP-1 (83), the latter of which is an important enzyme for DDR signaling that uses NAD^+ as its coenzyme to PARylate substrates (192). PARP-

1 mediated NAD^+ depletion has been implicated in process including cell death and neurodegeneration, with NAD^+ supplementation being recently suggested as a possible intervention for the increase of healthspan and lifespan (195). Thus, we investigated whether a therapeutic intervention using NAD^+ supplementation through nicotinamide mononucleotide (NMN), a NAD^+ precursor, would result in a heath improvement in the CX model.

4.2 Materials and Methods

4.2.1 Mice lines

CSA and XPA KO knockout mice expressing CPD or 6-4PP photolyase (phl) and double knockout CSA/XPA (CX) mice lines were generated by generational crossing, with PCR genotyping and primers performed as described as in Chapters 2 (Section 2.2.1) and 3 (Section 3.2.1). Housing, breeding, genotyping and experimentation were performed in accordance with the regulations established by the ethical committee of the Institute of Biomedical Sciences of the University of Sao Paulo or the Harvard Medical Area Institutional Animal Care and Use Committee (IACUC).

4.2.2 CSA KO mouse skin irradiation - Epidermal thickness and cell proliferation quantification

CSA KO mice expressing either CPD or 6-4PP photolyase (phl) in keratinocytes, under the control of the K-14 promoter, received a single dose of 30 J/m^2 UVB irradiation. All four experimental CSA KO groups - Non irradiated, irradiated no photolyase, CPD phl irradiated, 6-4PP phl irradiated - contained both males and females, with $n \geq 5$ for each group. Following 46 h of UVB irradiation, mice were injected with 5 mg of BrdU, in order to quantify cell proliferation. Two hours after BrdU injection (48 h after irradiation), mice were sacrificed and had approximately 1 cm^2 of the exposed dorsal skin collected for histological and immunohistochemical analysis. Epidermal thickness and cell proliferation were quantified as described in Chapter 2 (Section 2.2.4-6). All animals used for experiments were 8 to 10 week old, with no difference observed between males and females.

4.2.3 Flow cytometry interleukin measurement of cell culture supernatant

GG-NER deficient, XP4PA (XP-C fibroblast) and its corrected counterpart XP-C^{cor} cell lines were maintained and plated in 10% FBS, 1% AB DMEM. Cells were trypsinized and 50.000 cells were plated in 35 mm2 dishes (Corning).

Plated cells were irradiated in PBS 1X with 100, 200 and 500 J/m^2 UVB doses. After irradiation, cells were kept in cell culture media. Supernatant was collected 2, 12 and 24 h after irradiation, and immediately frozen in dry ice and kept at -80^{o}C until interleukin measurement. In order to do so, we performed a Cytometric Bead Array (CBA) using the *Human Inflammatory Cytokines Kit* (BD Biosciences) as described by the manufacturer. Briefly, six kinds of beads (differentiated by their fluorescence intensity detected by the 533/30 nm filter) bound to antibodies to one cytokine (IL-1β, IL-6, IL-8, IL-10, IL-12p70, TNFα) were mixed with cell culture supernatant containing secreted cytokines, followed by incubation with a secondary antibody coupled to the phycoerythrin (PE) fluorophore. The fluorescence of the mixtures containing beads bound to cytokines was measured using the flow cytometer "BD Accuri C6" (BD Biosciences), and acquired by the equipment's software with the "*BD Accuri CBA Kit Template*" configuration. Analysis was performed using FCAP 3.0 software (BD Biosciences).

4.2.4 IL-1β and TNFα ELISA

Serum IL-1β and TNFα levels of CX and CSA control were measured by collecting blood serum and performing ELISA as in Chapter 3 (Section 3.2.9), using IL1β and TNFα Quantikine ELISA Kit (R&D Systems). Absorbance was measured with a plate reader set to 450 nm and correction to 570 nm.

4.2.5 Mouse complete blood count

Complete blood count of CX and CSA control mice was performed by collecting mouse blood from the tail into EDTA containing tubes. Samples were kept

in ice until measurement using the white blood cell hematology system Hemavet 950LV (Drew Scientific).

4.2.6 NAD^+ supplementation and lifespan measurement in CX mice

NMN was supplemented to CX and CSA control mice, starting 6 weeks after birth, at a daily dose of 400 mg/kg/d in drinking water, previously described as having phenotypical effects on mice (196). NMN in water was changed once every three days and animals were observed, weighted and had their lean and fat mass calculated by magnetic resonance imaging (MRI) every week. Animals were observed once every other day 17 weeks after birth, and were sacrificed once hindlimb paralysis was observed.

4.2.7 Statistical analysis

All data are presented as mean ± SEM. One-way ANOVA followed by Tukey's post hoc test were used for multiple comparison analysis, and t-tests were used for comparison between two groups. Kaplan-Meier method was used for survival analysis. p value < 0.05 was considered significant, with "*" indicating P ≤ 0,05, "**" P ≤ 0.01 and "***" P ≤ 0.001.

4.3 Results

4.3.1 CPD, but not 6-4PP removal, is able to attenuate UVB-induced hyperplasia and cell proliferation in TC-NER deficient mice

TC-NER deficient, CSA KO mice expressing CPD or 6-4PP photolyase in keratinocytes received a single, low UVB dose (30 J/m^2), followed by photoreactivation, in order to identify the effects of CPD or 6-4PP photoremoval. The effects regarding hyperplasia and cell proliferation were investigated 48 h after UV irradiation. We observed that, in contrast to complete NER deficiency of XPA KO mice (Chapter 2), CPD removal only attenuated both hyperplasia and cell proliferation in the basal epidermis layer in CSA KO mice, while 6-4PP had no significant effect in neither (Figures 4.1A and 4.1B). The removal of either

photolesion, however, managed to lower the amount of proliferating suprabasal layer, indicating a lower tissue dysfunction (Figure 4.1C).

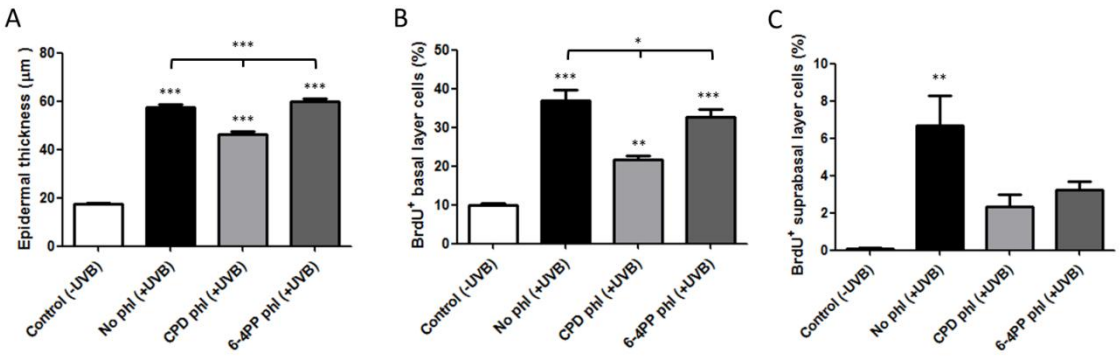


Figure 4.1. Removal of CPDs, but not 6-4PPs, is able to reduce hyperplasia in CSA KO mice. (A) Quantification of epidermal thickness of CSA mice 48 h after 30 J/m^2 UVB irradiation. (B) and (C) Percentage of UVR induced cell proliferation of the basal and suprabasal epidermal layers, respectively.

4.3.2 UVB irradiation induces cytokine secretion in GG-NER fibrolasts

Secretion of six inflammation related cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12p70, TNFα) were measured in the supernatant of two different human transformed fibroblast cell lines - XP4PA (XPC deficient) and XPC^{cor} (XP4PA corrected for XPC). Under the experimental conditions, we found secretion of only the pro-inflammatory cytokines IL-6 and IL-8 out of the six measured cytokines in these cells (data not shown). We observed an increase of these cytokines in the supernatant of IL-8 in XP4PA cells even without any UVR treatment, due to a time dependent accumulation of these molecules. In spite of this, we found that UVB irradiation (200 J/m^2) induced an increase in IL-8 in XP4PA cells, while we did not detect an increase in the secretion of this cytokine under any conditions in XPC^{cor} cells. IL-6 levels were undetectable in all experimental conditions in XPC^{cor} cells, while it showed an increase in XP4PA when irradiated with 500 J/m^2 (Figure 4.2). indicating that UVB irradiation is able to stimulate pro-inflammatory signaling regarding the release of these two cytokines, especially in the absence of DNA damage removal by GG-NER.

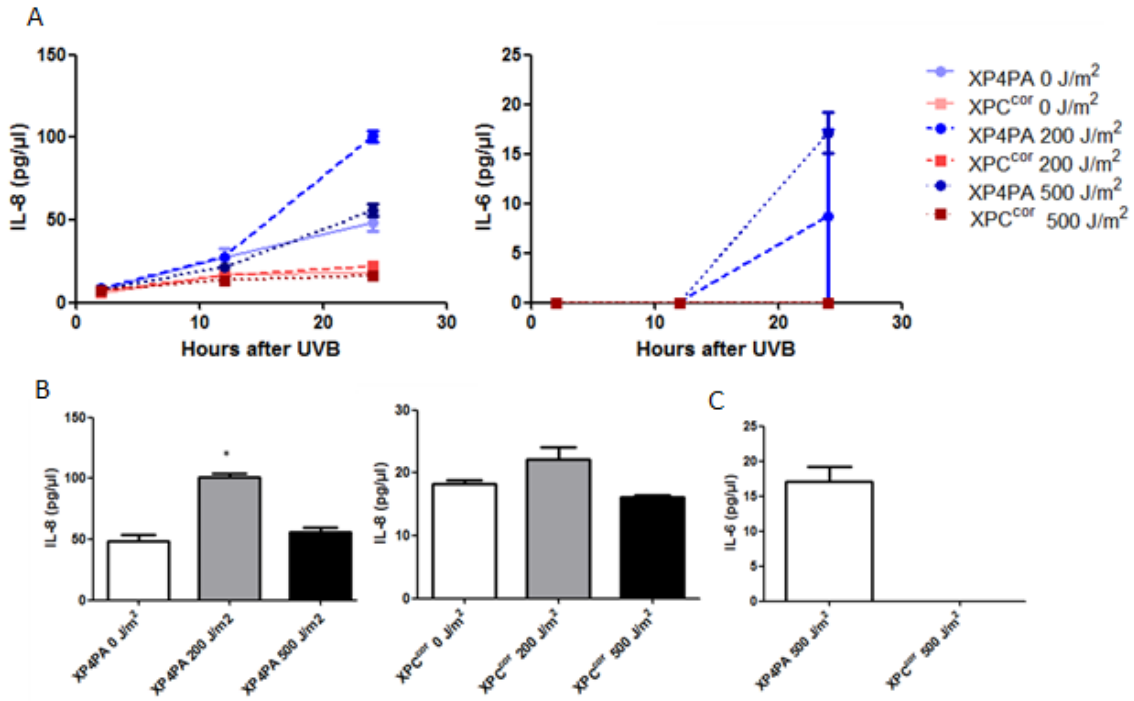


Figure 4.2. UVB irradiation induces secretion of IL-8 and IL-6 in XPC deficient cells. (A) Kinetics of IL-8 and IL-6 release in XP4PA and XPC^{cor} cell lines after 0, 200 or 500 J/m^2 UVB irradiation. (B) Secretion of IL-8, 24 h after 0, 200 or 500 J/m^2 UVB irradiation in XP4PA and XPC^{cor} cells. (C) IL-6 secretion 24 h after 500 J/m^2 in XP4PA and XPC^{cor} cells.

4.3.3 CX mice have increased circulating immune cells, but no increase in the pro-inflammatory cytokines IL-1β and TNFα

Among the inflammatory features investigated in the double mutant CSA/XPA (CX) mice, we studied possible general systemic effects on immune activation. IL-1β and TNFα are among the main pro-inflammatory cytokines secreted under pathogen and non-pathogen (sterile) induced inflammation, initiating several processes such as ICAM-1 overexpression, leukocyte extravasation, immune cell activation. Despite finding adipose tissue early senescence and neuroinflammation in CX mice (Chapter 3), we did not find any increase in these two cytokines. In contrast to this observation, we found that these mice have an increased level of circulating immune cells (leukocytosis) in general, with an increase in neutrophils, monocytes and lymphocytes (Figure 4.3). The results indicate that in spite of no cytokine signaling by IL-1β and TNFα, CX mice are under a basal, general inflammation or have priming of inflammatory cells, which in turn hints at a potentially degenerative, inflammation-related mechanism due to a stress response.

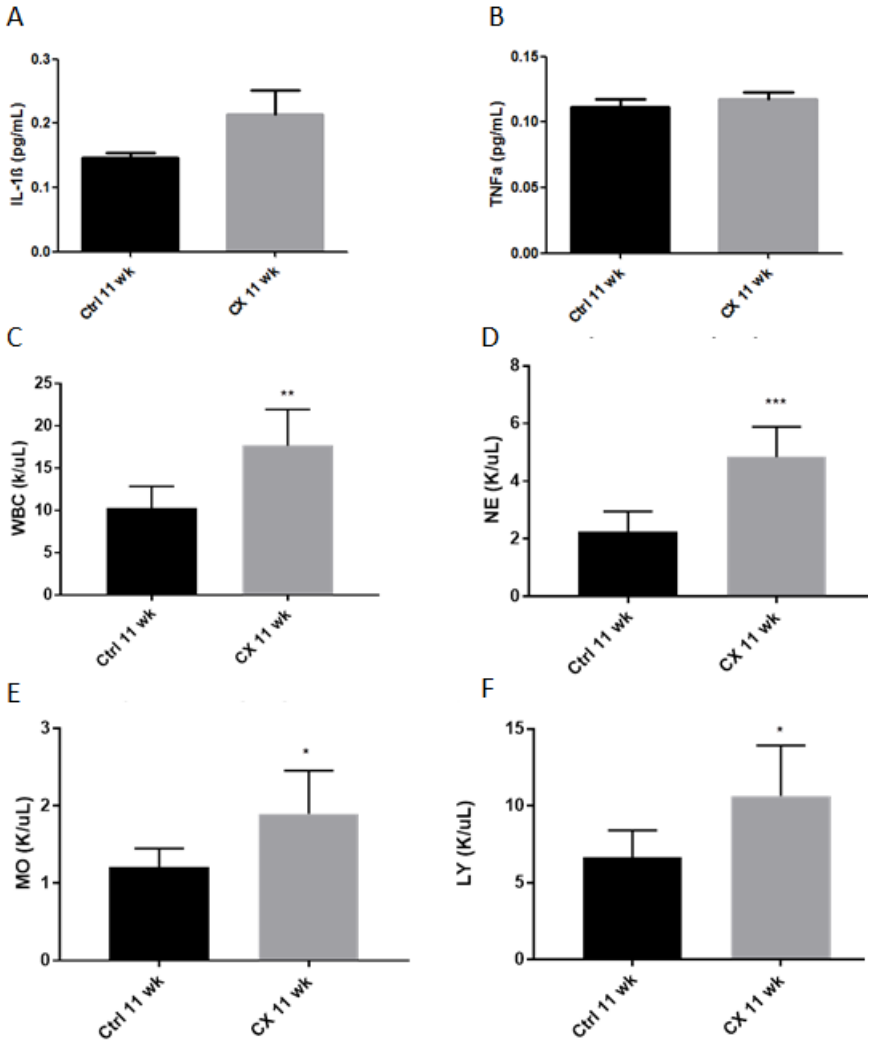


Figure 4.3. Cytokine and immune cell levels in CX mice circulating blood. (A and B) IL-1β and TNFα cytokines in CX and Control (CSA KO) plasma. (C-F) Quantification of circulating blood levels of white blood cells (WBC), neutrophils (NE), monocytes (MO) and lymphocytes (LY), respectively.

4.3.4 NAD^+ supplementation does not increase CX mice longevity

Preliminary results indicated that NAD^+ supplementation using the precursors NMN and NR had a small but positive effect on CX mice lifespan. However, in spite of previous findings, we found no increase in longevity nor in other healthspan parameters such as body weight and composition (Figure 4.4), corroborating previous results obtained in the Mitchell lab that indicated that NMN did not enhance metabolic endpoints such as glucose metabolism or ketogenesis (167).

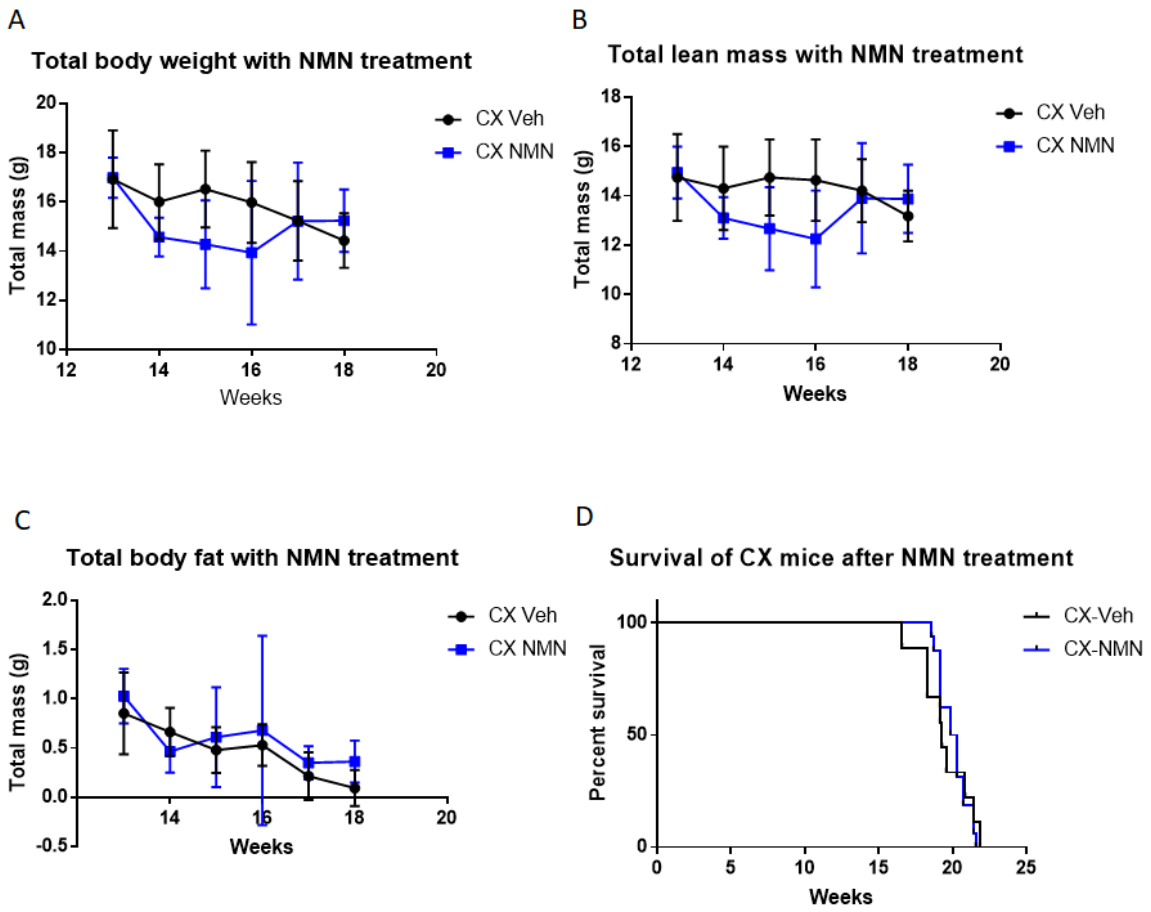


Figure 4.4. NAD+ replenishment does not alter healthspan nor lifespan of CX mice. (A-C) Measurement of healthspan parameters total body weight, lean and fat mass of treated and untreated CX mice. (D) Kaplan-Meyer survival curve of CX mice after NMN treatment.

4.4 Discussion

4.4.1 Differential effects of UV induced lesions in the epidermis CSA KO mice

TC-NER deficient, CSA KO mice displays no overt signs of neurodegeneration or neurodevelopmental defects, unlike Cockayne Syndrome patients. They do however display photosensitivity and a higher skin cancer incidence when exposed to UVR (197). As shown in this chapter, removal of CPDs, but not 6-4PPs in keratinocytes is able to reduce the hyperplasic effect of UVR after acute UV exposure, which corroborates previous data from our lab, in which we observed a similar pattern after exposing the same mice models to chronic UVR insult (146).

As discussed in Chapter 2, UVR is able to trigger skin cell proliferation and hyperplasia through an EGFR and DNA lesion dependent mechanism, with CPDs having a more prominent role in this effect, possibly due to a quantitative effect in XPA KO mice. Similarly, in CSA KO mice, CPDs were found to be more important than 6-4PPs to this UVR effect, with CPD removal reducing hyperplasia and 6-4PP removal having no effects, which contrasts with XPA KO mice, in which CPD removal able to abrogate hyperplasia, while 6-4PP was able to reduce it. Interestingly, the hyperplasic effect was notably greater in CSA KO than XPA KO mice, which may help explain the difference between the effects of photoremoval in these two models, as the baseline UV induced hyperplasia is already higher in CSA KO mice, possibly due to a different DDR signaling mechanism, initiated by transcription arrest. As mentioned in Chapter 2, this UVR induced hyperplasia is related to the activation of EGFR pathways (147), with the underlying DDR-EGFR crosstalk, as well as the role of the photolesions for this inhibition or activation of EGFR still yet to be elucidated.

4.3.2 Other aspects of DNA damage induced inflammation

In this chapter, we also characterized other aspects of both UV induced cellular and molecular aspects of inflammation, as well as CS related whole body inflammation. Although keratinocytes and Langerhans cells are generally considered to be the some of the main immune cell types in the skin (198), fibroblasts have also been shown to have secretion of inflammation-related molecules, such as interleukins and matrix metalloproteinases (199,200). Though we did not investigate further into the photolesions themselves, our results with XP-C cells further demonstrate the cell autonomous effect UV induced DNA damage has on skin inflammation, with fibroblasts also having a possible role on the effects observed in the UV irradiated mice used in Chapter 2.

Other than UVR induced cytokine release in skin cells, we also observed the induction of pro-inflammatory cytokines after treating cells with the DNA damaging chemotherapeutic drug doxorubicin (201) and cell exposure to pollution microparticles in lung cells. These microparticles have been shown to induce DNA damage (202) with the pro-inflammatory effect also being more prominent in NER deficient models *in vivo* (Alves et al., unpublished data).

Interestingly, in the CX model, we did not observe basal levels of two of the most prominent pro-inflammatory cytokines, IL-1β and TNFα. This is further confounded by our observation reported in Chapter 3, with CX mice having early adipose tissue senescence. Senescence associated secretory phenotype (SASP) usually have more pro-inflammatory cytokine profiles, including the release of IL-1β and TNFα (203). Despite of this observation, we found a higher level of circulating immune cells in the blood of CX mice. Thus, it is possible that other cytokines, such as IL-6 and IL-8 are involved in this inflammation-related phenotype, or that this leukocytosis phenotype is mediated through a non-systemic mechanism, such as a tissue-specific immune cell maturation dysfunction, with one possibility being bone marrow dysfunction, a feature observed in another NER-related progeroid model (204).

Although sparse, these data along with results reported in Chapters 2 and 3 point toward a somewhat general crosstalk between DNA damage and inflammation. However, similarly to other inflammation inducing processes, such as senescence, this relationship is still being investigated, with some effects being cell type specific, distinct for different kinds of DNA damage or depending on the extracellular context (65). Further studies will help better characterize the molecular pathways involved in this process, such as the mechanisms we propose in Chapter 5.

4.3.3 Metabolism alterations and interventions in DNA damage models

Alterations in energy metabolism are well established consequences of DNA damage, with several proteins, such as AMPK and PARP-1 participating in both DDR and metabolism regulation (192,205). PARP-1 has been linked to several DNA repair process, including NER by stabilization of the GG-NER recognition complex (206) and regulation of XPA function (207). Moreover, NAD^+ depletion by hyperactivation of PARP-1 in human XPA cells and in CX mice has been implicated in deficient mitophagy. In these models, NAD^+ supplementation reverted some of the mitochondrial phenotypes, indicating that it could be a possible treatment for CS patients (195). As our results demonstrated, however, NAD^+ supplementation using the precursor NMN was not able to improve any of our metabolic endpoints, nor the lifespan of CX mice. Furthermore, previous results from the Mitchell lab, using a triple

knockout mouse model for PARP-1/CSA/XPA (PARP-1/CX) did not significantly extend CX mouse lifespan (unpublished data). This indicates that while PARP-1 mediated NAD^+ depletion might play a role in the etiology of the disease, especially regarding the mitochondrial phenotype, it is not sufficient to rescue the overall neurodegenerative/progeroid phenotype in this model.

Interestingly, other metabolic interventions have had success in extending lifespan of progeroid models. NAD^+ replenishment has been shown to improve lifespan and healthspan in $ATM^{-/-}$ DDR deficient mouse models via mitophagy and DNA repair (208). Furthermore, calorie restriction, which causes a plethora of beneficial adaptive stress responses, including activating NAD^+ related enzymes (209), activating AMPK (210) and oxidative phosphorylation (211) has been shown to almost double the lifespan of the $Ercc1^{-/\Delta}$ and XPG KO models (212). Finally, methionine restriction, which resembles calorie restriction in some molecular aspects (AMPK, PGC-1α activation, increase in oxidative phosphorylation) also increases CX mice lifespan by over 40% (83). Thus, although NAD^+ replenishment was unable to increase lifespan of CX mice, other metabolic interventions continue to be a possible intervention for improving the health of progeroid patients, as well as counteract negative effects of aging itself.

Chapter 5 - General Discussion and Conclusions

The effects of DNA damage are numerous and depend on a number of factors, such as number, site and type of lesions, as well as cell type and *in vivo* context. In this work, we used two NER-deficient models, the Xpa and the Csa/Xpa knockout mice, in order to further understand the *in vivo* effects of NER-related lesions. Though these models differ phenotypically in several different ways, with the Xpa KO mice resembling Xeroderma Pigmentosum patients (141) and the Csa/Xpa (CX) knockout mice mimicking Cockayne Syndrome (166), they have a defect in the same molecular pathway, as well as similarities regarding higher sensitivity to DNA damaging agents (167,213). In this chapter, we will explore some of the general overlaps of these models regarding cellular and systemic effects of DNA damage - namely, cell death and induction of inflammation.

Apoptotic cell death is a known DNA damage response (DDR) mechanism that participates in the maintenance of tissue homeostasis by inhibiting tumorigenesis, clearing out dysfunctional cells and regulating inflammation (105,214). A high level of apoptosis, however, may directly or indirectly be detrimental to organism health, contributing to the aging and dysfunction of various organs, including the skin (215) and the brain (212), the two main organs studied in this thesis. Although the balance between this cell death and pro-survival pathways, as well as the amount of damage necessary to tip the balance in favor of cell death varies with cell type and context, the pathway used for this effect is generally the same for many cell types (216).

The main source for skin cell DNA damage under physiological conditions is ultraviolet radiation (UVR), an electromagnetic radiation considered to be the most ubiquitous exogenous source of DNA damage of our environment (130). In chapter 2, we describe the *in vivo* role of the two main DNA lesions generated by UVR, cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) on Xpa knockout mice expressing either CPD or 6-4PP photolyases in keratinocytes, and have observed that similarly to previous *in vitro* results (88), removal of either CPD or 6-4PP on a NER deficient background resulted in a reduction of apoptosis, indicating that both lesions contribute to the induction of

this effect. On the opposite side of apoptosis, we have UV-induced cell proliferation in epidermis, with both photolesions contributing to this process, but with CPDs having a more prominent role in this process on NER deficient mice. On NER proficient models, CPDs are markedly more important than 6-4PPs for both processes, possibly due to rapid repair of 6-4PPs (88,140).

Apoptosis has been extensively studied on NER deficient progeroid models, especially regarding its effect on neurodegenerative diseases, with $Ercc1^{-/\Delta}$, $Xpg^{-/-}$ and $Csb^{m/m}/Xpa^{-/-}$ mice having been described as displaying increased apoptosis in certain regions of the brain (168,213,217), with an alleviation of DNA damage in these models being correlated to a decrease in neuronal cell death and an improvement in neurological function (212). CX mice, the model used in chapter 3, has also been observed to have age dependent neuronal cell death (167). Furthermore, $Csb^{m/m}/Xpa^{-/-}$neuron-specific knockout mice also display a severe age dependent increase in neuronal cell death. Interestingly, although these mice display some similar phenotypes to its whole body counterpart, the neurodegenerative phenotype can only be observed at a much later life stage than every other whole body NER progeroid mouse model (169). Thus, while cell-autonomous neuronal apoptosis induced by endogenous DNA damage is important to the neurodegenerative aspects of progeroid models, it is not the only factor involved in this process. Another component to progeroid mice neurodegeneration might involve neuroglia regulated neuroinflammation, which has been implicated in numerous neurodegenerative disorders by mechanisms such as synapse elimination (174) and induction of oligodendrocyte and neuronal cell death (177).

In the previous chapters of this thesis, we speculate upon and bring evidences to the hypothesis of an interplay between DNA damage and inflammation, with DNA damage being related to the induction of proteins related to inflammation such as ICAM-1 and NF-κB, and participating in processes of neutrophil, astrocyte and microglial activation, thereby having a major role in UV-induced inflammation and neuroinflammation. Although the exact pro-inflammatory pathways have yet to be fully elucidated, we propose that DNA damage may be directly or indirectly driving these effects, with DNA damage induced inflammation being a general phenomenon

in many immune-related cell types, including keratinocytes (124), adipocytes (218), fibroblasts (219), and microglia (220).

As shown and discussed in chapter 2, UV-induced inflammation is linked to both CPD and 6-4PP photolesions, as the removal of either lesion was able to reduce inflammation markers in NER deficient XPA mice skin, and CPD removal was able to decrease UV induced inflammation in NER proficient mice. Several molecular pathways have been previously implicated for the signaling of this effect (65), such as the activation of the pro-inflammatory transcription factor NF-κB through a DDR mediated mechanism (221). Another possible mechanism for DNA damage induced sterile inflammation was described in (123), with the cytokine IL-1α appearing to have a role as a DNA damage sensor, being recruited at CPD containing sites, and with IL-1α$^{-/-}$ mice having a reduced neutrophil infiltration response in the skin after UV irradiation. The NLRP3 inflammasome protein complex, related to IL-1α and NF-κB, has also been shown to be activated by UV-induced DNA damage (124). These pro-inflammatory pathways have been proposed to be a type of UV-induced DNA damage response, with NER having a major role in regulating the pathways involved in this process (222). Moreover, UV induced inflammation has also been shown to have a role in tumorigenesis alongside UV photolesions themselves (223), with the inhibition of inflammation resulting in a decrease in cancer incidence after chronic UV irradiation in both NER proficient and deficient mice (224,225).

Regarding the progeroid related neuroinflammation, although activation of every previously mentioned pathway (NF-κB, IL-1α and NLRP3) has been linked to neurodegeneration (226–228), there have been so far fewer direct evidences of NER-related DNA damage activating a neuroinflammation signaling pathway. This is difficulted due to a lack of usable tools to specifically repair aging related DNA damage, in contrast to UV induced photolesions, which can be repaired by photolyases (63). Additionally, neuroinflammation is an emerging concept and novel area of study, still requiring and developing more precise markers to better elucidate this multifactorial process (229). Regardless, considering the amount of evidence, including this thesis chapter 3, showing that deficiency in NER generates a neuroinflammation phenotype *in vivo* (168,213), alongside other evidences showing this effect *in vitro* (171,220), it is reasonable to hypothesize that this is a general

effect caused by DNA damage in certain nervous system cells. Whether the signaling pathways of its activation and execution are similar to the UV induced ones remains to be determined.

Besides the aforementioned inflammation pathways inducible directly by DNA damage, there are other indirect ways DNA lesions can stimulate pro-inflammatory pathways, especially by causing cell senescence. One of the hallmarks of cell senescence is activation of the senescence associated secretory phenotype (SASP), partially induced by the overexpression of the pro-survival protein NF-κB, which in turn also activates the expression of generally pro-inflammatory cytokines, such as IL1, IL-6 and IL-8 (113). Surprisingly, we did not find senescence of endothelial cells in the CX model, in contrast to previous findings using the Ercc1$^{-/\Delta}$ model (115), which shows that although similar, these segmental progeroid models can have fundamentally different phenotypes in some cell types. We also found pronounced adipose tissue senescence in our model, which may contribute to general organismal inflammation and/or priming of immune cells. The adipose tissue SASP, however, may also differ between progeroid models, as there was no evidence of adipose tissue specific inflammation in CX mice (83), again unlike the Ercc1$^{-/\Delta}$ model (230).

The general findings of this thesis regarding the *in vivo* effects of NER-related DNA damages are summarized in figure 5.1. Briefly, UVR induced photolesions (CPD and 6-4PP) in keratinocytes of XPA KO, NER deficient mice are related to induction of cell death, inflammation and cell proliferation (CPDs having a more prominent role than 6-4PPs in cell proliferation). Regarding NER progeroid mice, although we found no evidence of a cell autonomous endothelial vascular dysfunction in the CX model, we report that these mice have an increase of neuroinflammation markers and a blood brain barrier defect. These observations on the consequences of NER-related DNA damage have implications on the etiology of the NER associated diseases Xeroderma Pigmentosum and Cockayne Syndrome as well as the biological processes related to these diseases – respectively, cancer and aging-related neurodegeneration.

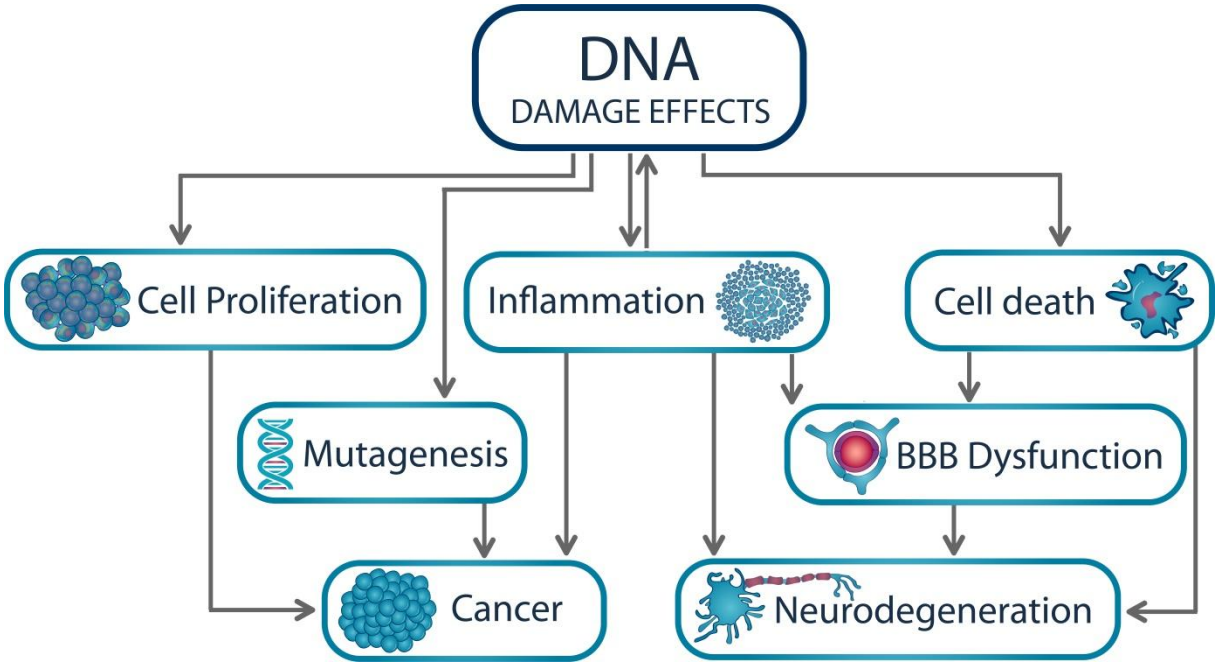


Figure 5.1. Conclusions summary. Working model of the studied DNA damage effects and how they relate to processes that affect organism health, namely Cancer and Neurodegeneration.

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Attachments: Published articles

1. Rocha CRR, **Kajitani GS**, Quinet A, Fortunato RS, Menck CFM. NRF2 and glutathione are key resistance mediators to temozolomide in glioma and melanoma cells. Oncotarget. 2016;

2. De Oliveira Alves N, Vessoni AT, Quinet A, Fortunato RS, **Kajitani GS**, Peixoto MS, Hacon SS, Artaxo P, Saldiva P, Menck CFM, Medeiros SRB. Biomass burning in the Amazon region causes DNA damage and cell death in human lung cells. Sci Rep. 2017;7(1).

3. Fortunato RS, Gomes LR, Munford V, Pessoa CF, Quinet A, Hecht F, **Kajitani GS**, Milito CB, Carvalho DP, Menck CFM. DUOX1 Silencing in Mammary Cell Alters the Response to Genotoxic Stress. Oxid Med Cell Longev [Internet]. 2018;2018:1–9. Available from: https://www.hindawi.com/journals/omcl/2018/3570526/

4. Treviño-Villarreal JH, Reynolds JS, Bartelt A, Langston PK, MacArthur MR, Arduini A, Tosti V, Veronese N, Bertozzi B, Brace LE, Mejia P, Trocha K, **Kajitani GS**, Longchamp A, Harputlugil E, Gathungu R, Bird SS, Bullock AD, Figenshau RS, Andriole GL, Thompson A, Heeren J, Ozaki K, Kristal BS, Fontana L, Mitchell JR Dietary protein restriction reduces circulating VLDL triglyceride levels via CREBH-APOA5–dependent and–independent mechanisms. *JCI Insight*. 2018 ;(21).

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Research Paper

NRF2 and glutathione are key resistance mediators to temozolomide in glioma and melanoma cells

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ABSTRACT

Cancer is a leading cause of death worldwide, and while great advances have been made particularly in chemotherapy, many types of cancer still present a dismal prognosis. In the case of glioma, temozolomide (TMZ) is the main option for treatment, but it has limited success due to drug resistance. While this resistance is usually associated to DNA repair mechanisms, in this work we demonstrate that oxidative stress plays an important role. We showed that upon TMZ treatment there is an induction of the nuclear factor erythroid 2-related factor 2 (NRF2), which is the main antioxidant transcription factor regulator in human cells. This is accompanied by an enhancement of glutathione (GSH) concentration in the tumor cells. The effectiveness of this pathway was proven by silencing NFR2, which greatly enhanced cell death upon TMZ treatment both *in vitro* and *in vivo*. Also, higher DNA damage and induced cell death was observed by combining BSO - a GSH inhibitor - with TMZ. Similar effects were also observed using *in vitro* and *in vivo* models of melanoma, thus possibly indicating that GSH has a decisive role in TMZ resistance in a wider range of tumors. Thus, a combined regimen of BSO and TMZ configures an interesting therapeutic alternative for fighting both glioma and melanoma.

INTRODUCTION

Malignant gliomas are the most common type of primary brain tumors in adults, with an incidence rate of approximately 5 cases per 100,000 inhabitants [1]. It is also one of most aggressive types of cancer. Patients diagnosed with glioma have a dismal prognosis, with a median survival rate of 15 months and a 5-year survival rate of ~2% [2]. Current therapy includes surgery for tumor resection, followed by radiotherapy and/or concomitant adjuvant chemotherapy. The main chemotherapy protocol for this type of tumor is based on temozolomide (TMZ) [3].

Metastatic melanoma shares several of glioma's features, in particular, high aggressiveness and poor prognosis. The average survival rate for melanoma patients with brain metastasis is about 4 months and a complete cure is observed in less than 1% of the patients [4]. Besides surgery and radiotherapy, melanoma patients are usually submitted to chemotherapy treatment with dacarbazine (DTIC), fotemustine or cisplatin [5], and, as it is the case with glioma, TMZ.

Nevertheless, as revealed by glioma and melanoma patients' average survival rates, current chemotherapeutic protocols have limited success. This occurs mainly due to drug resistance. Several mechanisms command resistance and many of those are tissue and/or drug specific. Thus, it is crucial to fully understand chemotherapy resistance mechanisms in order to develop new approaches to overcome it, improving the efficacy of therapy protocols.

Temozolomide (TMZ) is an alkylating agent that causes methylation on DNA bases in several positions, ultimately leading to cell death. Many DNA repair mechanisms are involved in resolution of DNA damage induced by TMZ, such as base excision repair (BER), mismatch repair (MMR) and direct repair by O⁶-methylguanine-DNA methyltransferase (MGMT). In fact, until now, the main known TMZ resistance mechanisms are related to the DNA repair capacity of the cells [6]. However, its is important to notice that due to poor drug response

SCIENTIFIC REPORTS

OPEN

Biomass burning in the Amazon region causes DNA damage and cell death in human lung cells

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Most of the studies on air pollution focus on emissions from fossil fuel burning in urban centers. However, approximately half of the world's population is exposed to air pollution caused by biomass burning emissions. In the Brazilian Amazon population, over 10 million people are directly exposed to high levels of pollutants resulting from deforestation and agricultural fires. This work is the first study to present an integrated view of the effects of inhalable particles present in emissions of biomass burning. Exposing human lung cells to particulate matter smaller than 10 μm (PM_{10}), significantly increased the level of reactive oxygen species (ROS), inflammatory cytokines, autophagy, and DNA damage. Continued PM_{10} exposure activated apoptosis and necrosis. Interestingly, retene, a polycyclic aromatic hydrocarbon present in PM_{10}, is a potential compound for the effects of PM_{10}, causing DNA damage and cell death. The PM_{10} concentrations observed during Amazon biomass burning were sufficient to induce severe adverse effects in human lung cells. Our study provides new data that will help elucidate the mechanism of PM_{10}-mediated lung cancer development. In addition, the results of this study support the establishment of new guidelines for human health protection in regions strongly impacted by biomass burning.

Most of the overwhelming amount of research on exposure to air pollution is focused on urban centers and on the role of fossil fuels as the most important source of atmospheric pollutants. However, approximately 3 billion people in the world are exposed to air pollution from biomass burning, originating from using wood or coal as cooking fuel in simple stoves, home heating with open fires, deforestation, and agricultural practices[1].

Biomass burning emits significant quantities of known pollutants hazardous to health, including several carcinogenic compounds[2]. World Health Organization (WHO) reported that in 2012, approximately 7 million people - one in eight total global deaths - as a result of exposure to air pollution[3]. Fire is a global phenomenon, and is an integral part of the earth's ecosystem[4,5].

In particular, the Brazilian Amazon region contains world's largest tropical forest and is considered, during the rainy season, one of the continental regions least affected by human activities[6,7]. However, during the dry season, high concentrations of aerosol particles from biomass burning (mainly agricultural practices and deforestation) have been documented in this region[7,8]. The combination of forest fires and human occupation has turned biomass burning into a serious public health threat. The majority of forest fires occur in the deforestation arc, a belt in the southern and western regions of the forest, directly impacting over 10 million people in the area[9]. Many

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Research Article

DUOX1 Silencing in Mammary Cell Alters the Response to Genotoxic Stress

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DUOX1 is an H_2O_2-generating enzyme related to a wide range of biological features, such as hormone synthesis, host defense, cellular proliferation, and fertilization. DUOX1 is frequently downregulated in lung and liver cancers, suggesting a tumor suppressor role for this enzyme. Here, we show that DUOX1 expression is decreased in breast cancer cell lines and also in breast cancers when compared to the nontumor counterpart. In order to address the role of DUOX1 in breast cells, we stably knocked down the expression of DUOX1 in nontumor mammary cells (MCF12A) with shRNA. This led to higher cell proliferation rates and decreased migration and adhesion properties, which are typical features for transformed cells. After genotoxic stress induced by doxorubicin, DUOX1-silenced cells showed reduced IL-6 and IL-8 secretion and increased apoptosis levels. Furthermore, the cell proliferation rate was higher in DUOX1-silenced cells after doxorubicin medication in comparison to control cells. In conclusion, we demonstrate here that DUOX1 is silenced in breast cancer, which seems to be involved in breast carcinogenesis.

1. Introduction

Cancer is the leading cause of death in economically developed countries and the second in developing countries, only behind deaths related to cardiovascular disease. In women, breast cancer is the second main cause of cancer death, exceeded only by lung cancer [1]. Breast cancer has an extensive list of risk factors associated with its development, such as age, sex, genetic predisposition, breast density, personal and familiar history of breast cancer, obesity, and early menarche [2]. Several authors suggest that a common point between various risk factors is an imbalance of redox homeostasis, which is related to the establishment and development of several tumors [3].

Reactive oxygen species (ROS), such as superoxide, hydroxyl radical, and hydrogen peroxide (H_2O_2), comprise a large group of oxygen-derived small molecules that include radical and nonradical species. ROS avidly interact with a large spectrum of cellular constituents, including small inorganic molecules, proteins, lipids, and nucleic acids, altering their structures and functions [4]. Many authors classify these molecules as harmful to biological organisms; however,

JCI insight

RESEARCH ARTICLE

Dietary protein restriction reduces circulating VLDL triglyceride levels via CREBH-APOA5–dependent and –independent mechanisms

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Hypertriglyceridemia is an independent risk factor for cardiovascular disease. Dietary interventions based on protein restriction (PR) reduce circulating triglycerides (TGs), but underlying mechanisms and clinical relevance remain unclear. Here, we show that 1 week of a protein-free diet without enforced calorie restriction significantly lowered circulating TGs in both lean and diet-induced obese mice. Mechanistically, the TG-lowering effect of PR was due, in part, to changes in very low–density lipoprotein (VLDL) metabolism both in liver and peripheral tissues. In the periphery, PR stimulated VLDL-TG consumption by increasing VLDL-bound APOA5 expression and promoting VLDL-TG hydrolysis and clearance from circulation. The PR-mediated increase in *Apoa5* expression was controlled by the transcription factor CREBH, which coordinately regulated hepatic expression of fatty acid oxidation–related genes, including *Fgf21* and *Ppara*. The CREBH-APOA5 axis activation upon PR was intact in mice lacking the GCN2-dependent amino acid–sensing arm of the integrated stress response. However, constitutive hepatic activation of the amino acid–responsive kinase mTORC1 compromised CREBH activation, leading to blunted APOA5 expression and PR-recalcitrant hypertriglyceridemia. PR also contributed to hypotriglyceridemia by reducing the rate of VLDL-TG secretion, independently of activation of the CREBH-APOA5 axis. Finally, a randomized controlled clinical trial revealed that 4–6 weeks of reduced protein intake (7%–9% of calories) decreased VLDL particle number, increased VLDL-bound APOA5 expression, and lowered plasma TGs, consistent with mechanistic conservation of PR-mediated hypotriglyceridemia in humans with translational potential as a nutraceutical intervention for dyslipidemia.

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