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# Deciphering the role of xeroderma pigmentosum C (XPC) protein in base excision repair (BER) and oxidative stress

Nour Fayyad

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## **THÈSE**

Pour obtenir le grade de

**DOCTEUR DE L'UNIVERSITE GRENOBLE ALPES**

Spécialité : **BIS - Biotechnologie, instrumentation, signal et imagerie pour la biologie, la médecine et l'environnement**

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Préparée au sein du **DRF/IRIG/DIESE/SyMMES/CIBEST-CEA, Grenoble**

Dans l'**École doctorale Ingénierie pour la santé la Cognition et l'Environnement (EDISCE)**

## **Déchiffrage du rôle de la protéine XPC dans la réparation par excision de base (BER) et le stress oxydant**

Thèse soutenue publiquement le **30 Juin 2021**, devant le jury composé de :

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## Abstract

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**X**xeroderma pigmentosum C (XPC) protein initiates global genome-nucleotide excision repair (GG-NER) pathway to remove UV-induced DNA lesions such as pyrimidine (6-4) pyrimidone photoproducts [(6-4) PPs] and cyclobutane pyrimidine dimers (CPDs). XPC deficient (XP-C) patients show a persistence of such lesions triggering high skin cancer incidences. They also suffer from internal cancers that could be due to the accumulation of oxidative DNA damage. Such base lesions, including 8-oxoguanine (8-oxoGua), are usually repaired by the base excision repair (BER) pathway. Despite growing evidence about how XPC enhances the activity of several BER DNA glycosylases, the effect of XPC mutations on other BER factors and their activities is still elusive. Herein, we seek to answer this open question by characterizing normal and XP-C fibroblasts derived from patients, optimizing the conditions, and dividing our project into two parts.

In part one, we showed a global downregulation of BER's genes in XP-C cells post-UVB compared to normal controls. Furthermore, the major proteins linked to oxidative DNA damage repair (OGG1, MYH, and APE1) were downregulated. This led to an ineffectiveness of BER in excising UVB-induced oxidative DNA damage. In part two, we investigated whether balancing the cellular redox state by treating XP-C cells with different drugs could boost their BER's activity post-UVB. We showed that nicotinamide (NIC) and N-acetyl cysteine (NAC) pretreatments increase glutathione level, decrease ROS level, and enhance BER's gene expression and activity. Meanwhile, buthionine sulfoximine/dimethylfumuate (BSO/DMF) pretreatment depletes glutathione level, increases ROS level, and impairs BER's gene expression and activity.

Based on these results, we propose that the pretreatment with drugs that could enhance glutathione's level may protect XP-C cells from an imbalanced redox state that affects the DNA repair. This could pave the way for therapeutic strategies for XP-patients and other DNA repair-deficient patients.

Future work is required to check the efficiency of such treatments on 3D reconstructed skin and *in vivo* models. Additionally, studying the interactome linking XPC and glutathione signaling could be interesting.

**Keywords:** *Ultraviolet irradiation-B (UVB), xeroderma pigmentosum C (XPC), nucleotide excision repair (NER), bulky lesions [CPDs and (6-4) PPs], base excision repair (BER), oxidative DNA lesions (8-oxoguanine), reactive oxygen species (ROS), oxidative stress, glutathione (GSH), nicotinamide (NIC), N-acetylcysteine (NAC), buthionine sulfoximine/dimethyl fumarate (BSO/DMF)*

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## Résumé

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La protéine Xeroderma pigmentosum C (XPC) initie la réparation globale du génome par excision de nucléotides (GG-NER) pour éliminer les lésions de l'ADN induites par les rayonnements UV, telles que les photoproduits de pyrimidine (6-4) [(6-4) PPs] et les dimères de cyclobutane de pyrimidine (CPDs). Les patients déficients en XPC (XP-C) présentent une persistance de ces lésions, déclenchant ainsi une forte incidence de cancers cutanés. Ces patients souffrent également de cancers internes qui pourraient être dus à l'accumulation de lésions d'oxydation de l'ADN. Ces dernières, dont la 8-oxoguanine (8-oxoGua), sont généralement réparées par excision de bases (BER). Malgré les preuves, de plus en plus tangibles, concernant l'implication de la protéine XPC dans l'activité de plusieurs glycosylases clés de la voie BER, l'effet des mutations de *XPC* sur les autres facteurs de cette voie reste encore peu connu. Le but de ce travail de thèse est de répondre à cette question ouverte en caractérisant la modulation de la voie BER dans les cellules normales et les cellules XP-C issues de patients.

Dans un premier temps, nous avons montré un effondrement global de l'expression de plusieurs gènes importants de la voie BER dans les cellules XP-C par rapport aux cellules témoins après irradiation aux UVB. En outre, les principales protéines liées à la réparation des dommages d'oxydation de l'ADN (OGG1, MYH, et APE1) ont été dérégulées. Cela a conduit à une inefficacité du BER dans l'excision des purines oxydées induites par les UVB. Dans un deuxième temps, nous avons cherché à savoir si la modulation de l'état redox en traitant les cellules avec différents médicaments pharmacologiques pouvait restaurer l'activité de BER après irradiation aux UVB. Nous avons montré que les prétraitements par le nicotinamide (NIC) et le N-acétyl cystéine (NAC) augmentent le niveau de glutathion, diminuent la génération des espèces réactives de l'oxygène (ROS), et augmentent l'activité du BER après irradiation aux UVB. Cependant, le prétraitement à la buthionine sulfoximine/diméthylfumate (BSO/DMF) inhibe le glutathion, augmente la production des ROS, et diminue l'activité du BER.

Sur la base de ces résultats, nous pourrions proposer que le prétraitement avec des médicaments qui pourraient augmenter le niveau de glutathion puisse protéger les cellules XP-C d'un état redox déséquilibré qui affecte la réparation de l'ADN. Cela pourrait ouvrir la voie à des stratégies thérapeutiques pour les patients XP et d'autres patients souffrant des maladies génétiques de réparation de l'ADN.

Des travaux futurs sont nécessaires pour vérifier l'efficacité de ces traitements au niveau de la peau reconstruite en 3D et sur des modèles pré-cliniques *in vivo*. En outre, l'étude de l'interactome reliant XPC et la signalisation du glutathion pourrait être intéressante.

*Mots-clés : Rayonnement ultraviolet B (UVB), xeroderma pigmentosum C (XPC), réparation par excision de nucléotides (NER), lésions de l'ADN [CPD et (6-4) PP], réparation par excision de bases (BER), lésions d'oxydation de l'ADN (8-oxoguanine), espèces réactives de l'oxygène (ROS), stress oxydatif, glutathion (GSH), nicotinamide (NIC), N-acétylcystéine (NAC), buthionine sulfoximine/fumarate de diméthyle (BSO/DMF)*



*"I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale."*

*~ Marie Curie*

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## *List of Abbreviations*

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### A

Adenosine triphosphate= ATP  
Apurinic/aprimidinic (AP)  
endonuclease= APE1

### B

Base excision repair= BER  
Basal cell carcinoma= BCC

### C

Cyclobutane pyrimidine dimers= CPDs

### D

Deoxyguanosine triphosphate= dGTP  
Deoxyribnucleic acid= DNA  
Dihydrorhodamine 123= DHR123  
Dimethylfumurate= DMF  
Dimethyl sulfoxide= DMSO  
5,5-dithio-bis-(2-nitrobenzoic acid)=  
DTNB  
3-(4,5-dimethylthiazol-2-yl)-2,5-  
diphenyltetrazolium bromide = MTT  
DNA damage response= DDR  
DNA polymerase  $\beta$ = POL $\beta$   
Double-strand breaks= DSBs  
Dulbecco's Modified Eagle Medium=  
DMEM medium

### E

Ethylenediaminetetraacetic acid= EDTA

### F

Fetal Bovine Serum= FBS  
Flap endonuclease 1= FEN1  
Formamidopyrimidine glycosylase= FPG

### G

Growth Arrest and DNA Damage  
Inducible Alpha= GADD45a  
Global genome excision repair= GG-NER  
Glucradlehdye-3-phosphate  
dehydrogenase= GAPDH  
Glutathione= GSH  
Glutathione disulfide= GSSG

Glutathione peroxidase= GPx  
Glutathione-S-transferases= GST  
Guanine= G

### H

Hydrochloric acid= HCL  
hydrogen peroxide= H<sub>2</sub>O<sub>2</sub>  
hydroxyl radical= •OH

### L

L-buthionine sulfoximine= BSO  
Ligase III= LIG3

### M

Melanoma= MSC  
Metaphosphoric acid= MPA  
MutY DNA glycosylase= MYH

### N

N-acetylcysteine= NAC  
NADPH oxidase= NOX  
Nicotinamide= NIC  
Nicotinamide adenine dinucleotide=  
NAD<sup>+</sup>  
Nicotinamide adenine dinucleotide  
phosphate= NADPH  
2-(N-morpholino)ethanesulfonic acid=  
MES  
Nonmelanoma skin cancer= NMSC  
Nucleotide excision repair= NER  
Nuclear factor E2-related factor 2= Nrf2

### O

8-oxo-7,8-dihydroguanine= 8-oxoGua  
8-oxoguanine DNA glycosylase= OGG1

### P

Paraformaldehyde= PFA  
Phosphate-Buffered Saline= PBS  
Poly(ADP-Ribose) Polymerase= PARP  
Polymerase Chain Reaction= PCR  
Pyrimidine (6-4) pyrimidone  
photoproducts= (6-4) PPs



## R

Reactive oxygen species= ROS  
Ribonucleic acid= RNA

## S

Single-strand breaks= SSBs  
Sirtuin 1= SIRT1  
Singlet oxygen=  $^1\text{O}_2$   
Sodium n-Dodecyl Sulfate= SDS  
Superoxide anion=  $\text{O}_2^- \cdot$   
Squamous cell carcinoma= SCC  
Superoxide dismutase= SOD

## T

Triethanolamine= TEAM  
Tumor protein 53= P53

## U

Ultraviolet radiation B= UVB  
Ultraviolet radiation A= UVA

## X

Xeroderma pigmentosum C= XPC  
X-ray repair cross-complementing protein  
1= XRCC1

Non-Small Cell Lung Cancer Cancer Gene Expression Regulation Mutation Phenotype Skin

Cancer Odds Ratio Genotype Case-Control

Studies Cisplatin Haplotypes Biomarkers, Tumor Melanoma Genetic Association

Studies Ultraviolet Rays beta-Galactosidase Asian Continental Ancestry

Group Polymorphism Chromosome 3 Endonucleases Adolescents Transcription

Factors Genetic Predisposition *Preamble* Cells Staging DNA Repair

Enzymes Alleles Sulfides Statistics as Topic Antineoplastic

Agents RTPCR DNA-Binding Proteins Polymerase Chain

Reaction Lung Cancer Colorectal Cancer DNA Damage Messenger

RNA Fibroblasts Breast Cancer DNA Repair Childhood

Cancer Protozoan Proteins Squamous Cell Carcinoma

The skin is considered as the primary external barrier protecting the body against biomolecules damage and mutations due to solar, acute and chronic, ultraviolet (UV) radiation. However, a failure in such guardianship leads to skin inflammation, hyperpigmentation, photoaging, and skin cancer (melanoma and non-melanoma) (Ryu et al. 2010; Biniek, Levi, and Dauskardt 2012). 300,000 melanoma and over 1 million non-melanoma cancer cases were reported worldwide in 2018 ("Skin Cancer" 2018). These malignancies are mostly due to accumulated UV-induced DNA damage. For example, Australia has the highest rates of skin cancer, majorly due to the exposure to high UV radiation (Olsen et al. 2015). The nature of such DNA lesions depends on the wavelength of the incident photons. Ultraviolet B (UVB, 280-320 nm), the most energetic solar radiation at the earth's surface, induces the formation of bulky lesions, cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts [(6-4) PPs] (Cadet and Douki 2018). Almost fifty percent of the UVB-induced macromolecular damage is attributable to the formation of reactive oxygen species (ROS), which lead to oxidative DNA damage (Wölflle et al. 2011). Less energetic but 20-time more intense ultraviolet A (UVA, 320-400 nm) induces the formation of CPDs alongside a wide variety of oxidatively generated lesions such as single-strand breaks and oxidized bases. Among those, 8-oxo-7,8-dihydroguanine (8-oxoguanine, 8-oxoGua) is the most common oxidative premutagenic DNA lesion (Cadet and Douki 2018). If left unrepaired, 8-oxoGua causes G:C to T:A transversion in proto-oncogenes, such as *KRAS* and *P53*, promoting internal tumorigenesis (lung, breast, ovarian, gastric, and colorectal cancers) (Vodicka et al. 2020; Yoshihara et al. 2014). This may be due to the failure of specific DNA repair pathways to recognize and repair such DNA lesions at distinct cell cycle phases, leading to genomic instability. Base excision repair pathway (BER), a highly conserved pathway from bacteria to humans, is accountable for repairing the vast majority of endogenous base damage, including alkylation, deamination, depurination, single-strand breaks (SSBs), and most importantly oxidized purines, including 8-oxoGua, through long or short-patch sub-pathways (Krokan and Bjørås 2013; Wallace 2014). BER removes approximately 40,000 endogenous base lesions per human cell per day (Wallace 2014). Despite that these small base lesions do not significantly distort the DNA helix structure, they are considered mutagenic (Krokan and Bjørås 2013). Several polymorphism variants and mutations in different BER components favorize the development of numerous internal cancers, such as colorectal, lung, colon, breast, ovarian, and bladder cancers (Wallace 2014). Interestingly, such types of cancers are also present once XPC protein is lost or mutated.

XPC is an initiator of the global genome-nucleotide excision repair pathway (GG-NER). It recognizes bulky lesions, such as CPDs and (6-4) PPs throughout the whole genome.

Evidence had shown a correlation between XPC, BER, and internal cancers. XP-C patients were diagnosed with hematological, brain glioma, gastric, thyroid, lung, and gynecological cancers (Yurchenko et al. 2020; Zebian et al. 2019). In parallel, *xpc*<sup>-/-</sup> mice models demonstrated high incidences of liver and lung malignancies (Yurchenko et al. 2020; Zebian et al. 2019). This could be explained by XPC-BER interactions. For instance, multiple studies showed that XPC interacts with DNA glycosylases [3- methyladenine DNA glycosylase (MPG), thymine DNA glycosylase (TDG), single-strand selective monofunctional uracil DNA glycosylase (SMUG1), 8-oxoguanine DNA glycosylase (OGG1)] and Apurinic/aprimidinic endonuclease (APE1) (Zebian et al. 2019; de Melo et al. 2016). However, little is mentioned about how other BER factors could adapt to XPC mutations and whether we could manipulate BER's background activity. In the first part of our project: we monitored the outcome of XPC mutations on BER and showed that it hinders BER's global gene expression and excision activity. ***Could this be via an upregulated oxidative stress level? And could a balanced redox status enhance BER's effectiveness?*** To answer such questions, we experimented with the second part of our project. We treated the normal and XP-C cells with pharmacological (antioxidant/oxidant) treatments to modulate the redox state and check whether this could impact BER's activity.

Based on our results, we were able to critically examine and monitor the cross-link between BER and XPC in a detailed manner, considering the different aspects, including several XPC mutations, different cell lines, treatments, etc.... Also, we propose that enhancing the antioxidant capacity by upregulating glutathione level could boost BER's efficiency in XP-C cells. By this, we could pave the way for therapeutic strategies that could target not only XP patients per se but also other DNA repair-deficient patients to improve and boost their background DNA repair.

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*Bibliographic Review*

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*Part of this bibliographic introduction was published as a chapter in a book entitled "Immunology and Cancer Biology" (refer to Annex). However, we wanted to be original within the manuscript, so we paraphrased and changed between the chapter and the introduction.*

## **1. Chapter One: ROS, Oxidative stress, and the skin**

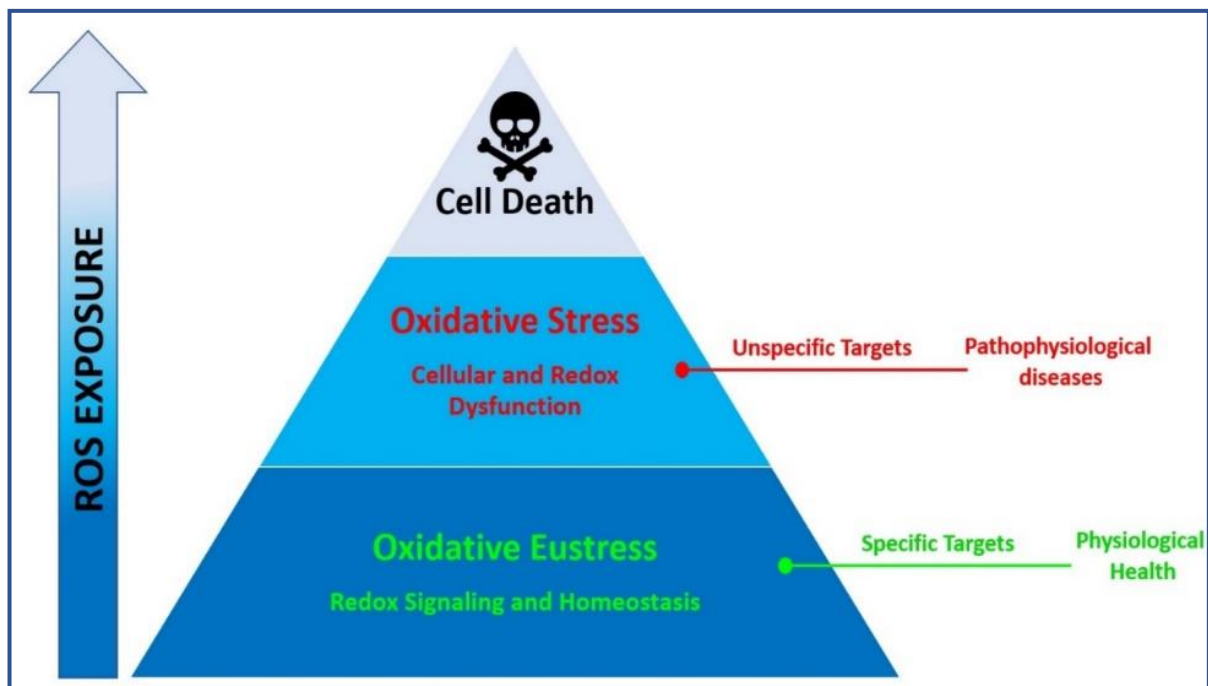
### **1.1. Definition and origin**

Reactive Oxygen Species (ROS) are generated in several cellular compartments, including the plasma membrane, peroxisome, endoplasmic reticulum, in the cytoplasm, and most prominently, on the membranes of the mitochondria. This could be in the form of radicals with a free electron (superoxide, hydroxyl, peroxy, and alkoxy radicals...) or chemically stable non-radicals (hydrogen peroxide, peroxyxynitrite, hypochlorous acid, and ozone...) as the main mechanism in photoaging and carcinogenesis (Li, Jia, and Trush 2016; Di Meo et al. 2016; Deng et al. 2018). ROS could also be generated via exogenous sources, including irradiation (UV and IR), alkylating agents, pollutants, toxins, drugs, smoking, etc....

Among ROS, superoxide anion ( $O_2^{\cdot-}$ ), hydroxyl radical ( $\cdot OH$ ), and hydrogen peroxide ( $H_2O_2$ ) are the most common in triggering oxidative stress (Birben et al. 2012). In 1985, the concept of unbalanced oxidants/antioxidants was used amongst the industry to explain such a phenomenon. They had invested billions of dollars in research to test antioxidants as free radical scavengers to diminish oxidative stress. However, such a supplementation failed to provide health benefits and rather contributed to a transition to what we now know about ROS (Go and Jones 2017). As shown in figure 1, at normal conditions, oxidative eustress is a continuous low level of ROS production as a regulatory mediator in different signaling processes, including inflammation, cell cycle regulation (entry to S-phase), adenosine triphosphate (ATP) production, the energy required for a multitude of cellular responses and functions, and as a detoxifying natural defense (Di Meo et al. 2016; D. Wu and Cederbaum 2003; Roy et al. 2017; Bae et al. 2011). It was reported that ROS are involved in normal muscle contraction where they behave as signals to modulate adaptations of muscle to exercise and as T lymphocytes' enhancers by promoting interleukin-2 (IL-2) production and regulating  $T_{regulator}/T_{effector}$  balance (Roy et al. 2017). This enhances local inflammation and immune response. Macrophages and neutrophils are other sources of ROS where they contain reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzymes that will generate  $O_2^{\cdot-}$  and  $H_2O_2$  to protect the body from infections (D. Wu and Cederbaum 2003). Upon their interaction with cellular chloride ions, hypochlorite (an active ingredient in bleach) will be produced to destroy pathogens (D. Wu and Cederbaum 2003).

Similarly, non-phagocytic cells (fibroblasts, keratinocytes, endothelial cells, pancreatic cells, and cardiac myocytes) release ROS to regulate different cellular transductions (cell cycle, growth, differentiation, NADPH oxidase) (Ahmad et al. 2017; Hirobe 2014a).

Once an imbalance occurs between ROS and antioxidants, the accumulation of ROS shifts from being advantageous to detrimental by which oxidative distress, high-level supraphysiological oxidative stress, ensues (Di Meo et al. 2016; Sies 2019). It is the major cause of human morbidity and mortality (Go and Jones 2017). Oxidative stress leads to oxidative macromolecular damages (lipids, proteins, and DNA) due to the unique electronic properties of ROS's excited oxygen electron, consequently leading to various pathological disorders (Li, Jia, and Trush 2016; Di Meo et al. 2016; Heck et al. 2003). Moderate oxidative stress causes altered cellular function (the main contributor in carcinogenesis), whereas overt oxidative stress triggers cell death (oncosis, apoptosis, and autophagy) (Li, Jia, and Trush 2016). Also, it oxidizes the triple guanine repeats at the end of the telomeres provoking their breakage and suppression (Barrera 2012).



*Figure 1. Effect of different ROS exposure concentrations on the cell. At steady state, ROS are controlled and addressed to specific targets for redox signaling and homeostasis (oxidative eustress). Higher ROS doses lead to disrupted redox signaling and molecular damage causing severe pathophysiological diseases (oxidative distress). If left unreduced, ROS will accumulate in cells leading to their destruction and death.*

Although we focused in our studies on oxidants/antioxidants balance from radical/radical scavenger balance to study oxidative stress, it is worth mentioning that in the 2000s, scientists started talking about thiol/disulfide systems as an attributive to oxidative stress by regulating

the redox status of proteins and molecules containing thiol/disulfide groups, including glutathione (GSH). This will contribute to cellular signaling and proteins' modifications at structural and functional levels (Go and Jones 2017).

Such ROS defense mechanisms act as memory protective systems that are responsible for adapting the genome to the variable environmental resources and challenges until their flexibility decreases with aging. Moreover, they are highly selective and systematic. For instance,  $H_2O_2$  is targeted by NADPH oxidase without any detectable changes in the thiol/disulfide systems (Thioredoxin "Trx" or GSH/GSSG systems....) (Go and Jones 2017).

Understanding such a mechanism via studying the human exposome could herald new therapeutic strategies that prevent and manage diseases, aging, DNA repair, and regeneration (juvenile treatments....).

#### 1.1.1. Oxidative stress targets

- **Lipids**

Phospholipids are the main components of the cellular membrane and contain polyunsaturated fatty acids that are sensitive to peroxidation. A single  $\bullet OH$  can result in multi-polyunsaturated fatty acids' peroxidation that will destroy the membranes and result in reactive products. These products may interact with the protein and DNA, leading to their damage (D. Wu and Cederbaum 2003). Lipid peroxidation products [hexanal, 4-hydroxynonenal (4-HNE), Malondialdehyde (MDA), acrolein] were identified to induce cell death (autophagy, apoptosis, ferroptosis), atherogenesis, inflammatory responses, and carcinogenesis. For instance, researchers revealed an upregulation in lipid peroxidation in colorectal, ependymal glial, thyroid cancer tissues, lung, and invasive breast carcinomas (Barrera 2012; Zabłocka-Słowińska et al. 2019). This may be due to an upregulated point mutations in tumor-suppressor genes, downregulated antioxidants' serum level, and increased inflammation level (Zabłocka-Słowińska et al. 2019).

- **Proteins**

Proteins play several roles, whether in cellular functions or signaling. They are made up of approximately 20 amino acids that are ROS sensitive. For example, histidine, methionine, and cysteine amino acids are the most susceptible to an attack by  $\bullet OH$ . Amino acids' oxidation may lead to protein physical and chemical variations (structure, cross-linkage, function); eventually, proteins will lose their functions/identity and be eliminated from cells (D. Wu and Cederbaum 2003). Nevertheless, some oxidized proteins interact with other products leading to their aggregation and not degradation (Cecarini et al. 2007). This induces cellular dysfunction,



higher oxidative stress, aging, and multiple diseases (Cecarini et al. 2007). For instance, high protein oxidation was detected in several cancer patients (gastric, colorectal, and lung) (Ma et al. 2013; Cecarini et al. 2007). This could be explained by multiple reasons, including the deficiency in DNA repair. For example, oxidation of some BER factors (OGG1, APE1, and PARP1), double-strand break repair factors (Ku), and O<sup>6</sup>-Methylguanine methyltransferase (MGMT) reduce their activity and DNA binding capacity (Alnajjar and Sweasy 2019).

- **Deoxyribonucleic acid (DNA)**

DNA is the cellular genetic material consisting of nucleotides' pairing and a sugar-phosphate backbone. It sculpts its proteins. Henceforth, any defect in the DNA can result in deleterious consequences, including miscoded, malfunctioned, or inactivated proteins. Also, Ribonucleic acid (RNA) is directly affected by the DNA's structure, stability, and expression. ROS can target the DNA, causing DNA strand breaks and nucleotide modifications. Sometimes, the cellular defense and repair mechanisms fail to repair and protect it. This leads to permanent DNA changes, potentially inimically affecting the cell (D. Wu and Cederbaum 2003).

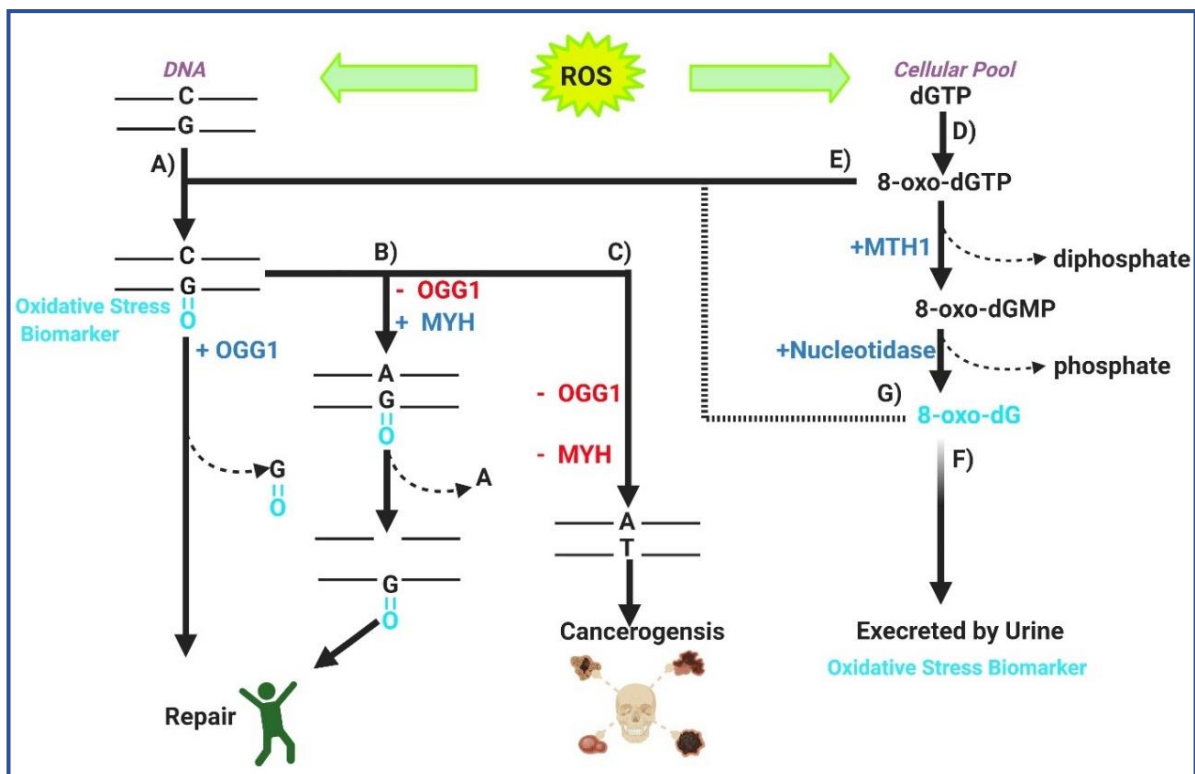
- **8-oxoGua: The main concern**

Over 100 different types of oxidative DNA modifications have already been identified in the mammalian genome. However, in the nucleus and mitochondria, 8-oxoGua is the main oxidized product due to guanine (G). Guanine has a low redox potential, making it the most vulnerable base and the most susceptible to oxidation (Nakabeppu 2014; Aguiar et al. 2013). It is estimated that approximately  $10^4$  8-oxoGua lesions per single nucleus are formed per day (Nakabeppu 2014). The strong correlation between ROS and 8-oxoGua allowed us to consider the latter as a cellular biomarker for oxidative stress and its consecutive spontaneous and induced carcinogenesis (Nakabeppu 2014; Aguiar et al. 2013). It has been shown that 8-oxoGua could form post-stress (chemicals, radiation..) via the following pathways: directly, upon the oxidation of guanine in the DNA, and indirectly (i) upon the oxidation of deoxyguanosine triphosphate (dGTPs) in the nucleotide pool into 8-oxodGTPs that will be incorporated into the DNA by DNA polymerases and/or by (ii) metabolizing 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxodG) into 8-oxodGTP that will also be incorporated to the DNA (Mundt et al. 2008). High levels of 8-oxodG were found to be excreted from cancer patients (bladder, lung, colorectal, prostate carcinomas...), leading it to be regarded as a disease and oxidative stress biomarker (Mundt et al. 2008; Sova et al. 2010). It can be detected and measured in urine or serum samples as an oxidative stress biomarker by

immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), high-pressure liquid chromatography coupled to mass spectrometric or electrochemical detection (HPLC-MS/MS; HPLC-EC) (Sova et al. 2010). Case in point, studies showed that 8-oxoGua was detected by immunohistochemistry analysis for 24 hours post-UVB exposure in epidermal cells (Kunisada et al. 2005). This may be due to the direct oxidation by UVB or the increase in the inflammatory state where neutrophils and macrophages will induce oxidative stress (Kunisada et al. 2005). Hence, it is reasonable to speculate that unrepaired 8-oxoGua is the main factor triggering carcinogenesis post-UV.

If left unrepaired, 8-oxoGua will mimic thymine (T) to be able to pair with Adenine (A), forming G:C to T:A transversion (Aguiar et al. 2013). Such transversions were detected in *RAS* oncogenes and *P53* tumor-suppressor genes in several cancers, including non-melanoma skin cancer (Kunisada et al. 2005). Therefore, the body developed a "GO-system" as a triple defense mechanism, including MTH1, MYH, and OGG1 enzymes (discussed later, in BER section) (Aguiar et al. 2013).

Briefly, as shown in figure 2, MTH1 hydrolyzes 8-oxodGTP in the nucleotide pool to its monophosphate form (8-oxodGMP), preventing its incorporation into the DNA, while OGG1 and MYH are responsible for excising 8-oxoGua opposite cytosine (C) and removing the Adenine (A) in the 8-oxoGua:A mispair, respectively (Aguiar et al. 2013).

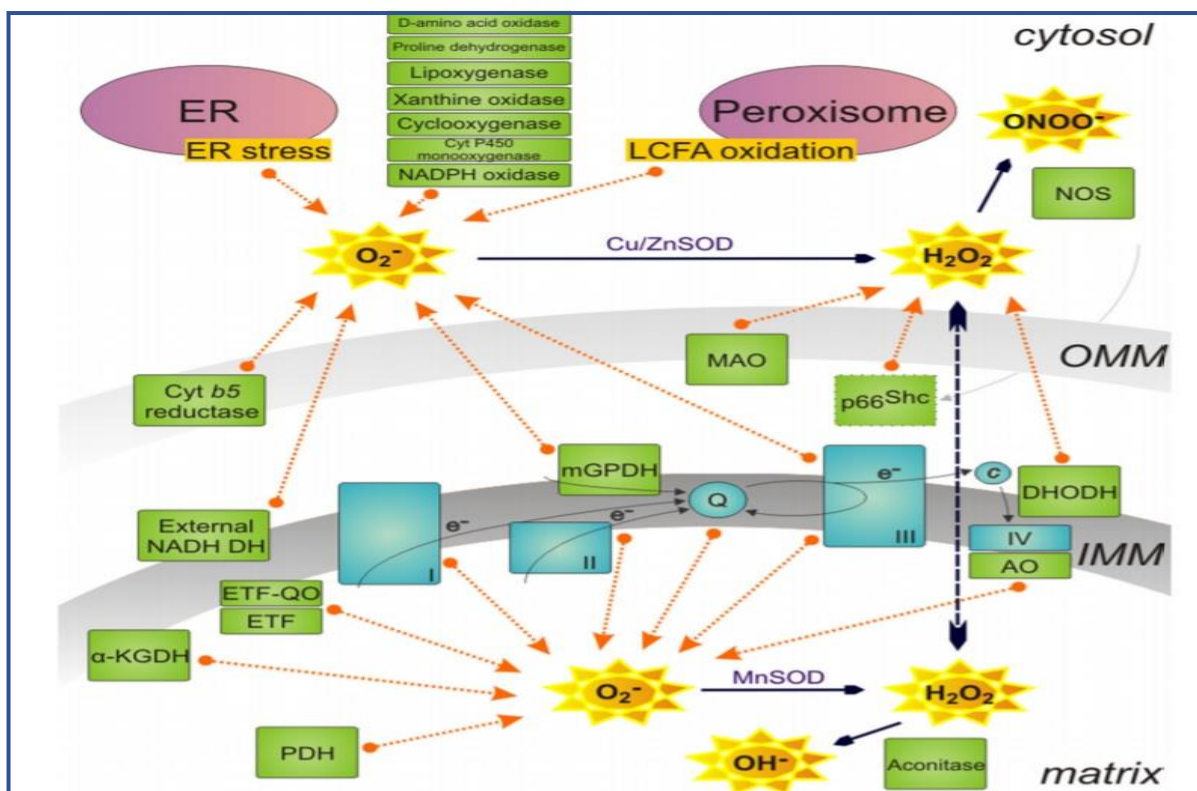


**Figure 2. ROS and its DNA damage repair.** ROS can target and oxidize guanine either in the cellular pool or at the level of DNA. A) ROS oxidizes guanine of G:C to be repaired by OGG1. If these mutations persist where Adenine (A) replaces cytosine (C), B) MYH will excise Adenine that is in front of the oxidized guanine. C) Once MYH and OGG1 are dysregulated, carcinogenesis risk elevates. This is due to the accumulation of G:C to T:A transversions mutations. D) In parallel, ROS can produce free oxidized guanine (8-oxo-dGTP). It will be targeted by the MTH1 enzyme to prevent its incorporation into the DNA. MTH1 will dephosphorylate it to have 8-oxo-dGMP. 8-oxo-dGMP will be dephosphorylated by 5' nucleotidase into 8-oxo-dG. G) Similarly to 8-oxoGua, if left not excreted, it can induce carcinogenesis. This is due to converting into 8-oxo-dGTP via multi-step enzymes (purine nucleoside phosphorylase, hypoxanthine-guanine phosphoribosyltransferase, ribonucleotide diphosphate reductase...) that could be then incorporated into the DNA if MTH1 is dysregulated. F) Upon excretion, it can be detected in the urine. Both 8-oxoGua and 8-oxodG act as oxidative stress and cancer biomarkers.

### 1.1.2. Endogenous sources for most common ROS

- Superoxide radical ( $O_2^{\cdot-}$ )

It is formed by the addition of an extra electron to the molecular oxygen. This is mediated by xanthine oxidase, a cytosolic and peroxidase enzyme, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, found in granulocytes, monocytes, and macrophages, or by mitochondrial electron transport system, during the process of oxidative phosphorylation (OXPHOS), in which molecular oxygen ( $O_2$ ) is reduced to water in the electron transport chain (Birben et al. 2012; Snezhkina et al. 2019) (figure 3).



**Figure 3. Summary of the ROS production from different cellular sources and some of the main types** (Koňáriková and Prokisch 2015). Most of the ROS are produced in/by the mitochondria, including the respiratory chain that will release ROS to the matrix, or cytosol from the inner mitochondrial membrane (IMM) and outer mitochondrial membrane (OMM), respectively. Other proteins or organelles can also contribute to ROS

production. Respiratory chain complexes are displayed in blue, other ROS contributors in green, organelles in violet.

Mitochondrial glycerophosphate dehydrogenase (mGPDH), ketoglutarate dehydrogenases (-KGDH), electron transfer flavoprotein (ETF), ETF ubiquinone oxidoreductase (ETF Qo), pyruvate dehydrogenase (PDH), alternative oxidase (AO), dihydroorotate dehydrogenase (DHODH), external NADH dehydrogenase (NADH DH), protein p66Shc, cytochrome (cyt) b5 reductase, monoamine oxidase (MAO) and nitric oxide synthase (NOS).

Generally, around 1 to 3 percent of electrons leak from the system and produce superoxide (Birben et al. 2012). As shown in figure 4, complex I (sites IQ and IF), Q oxidoreductase, pyruvate dehydrogenase, and 2-oxoglutarate dehydrogenase produce superoxide radical ( $O_2^{\cdot-}$ ) to the mitochondrial matrix (MM) while complex III (site IIIQo) and glycerol 3-phosphate dehydrogenase generate ROS into the intermembrane mitochondrial space (IMS) (Snezhkina et al. 2019). Another mitochondrial site of  $O_2^{\cdot-}$  production is the cytochrome (CYP) catalytic cycle that gives rise to  $O_2^{\cdot-}$  and  $H_2O_2$  byproducts. Other mitochondrial proteins, such as NADH-cytochrome b5 reductase and complex II (succinate dehydrogenase) were also shown to generate  $O_2^{\cdot-}$  in the mitochondria (Snezhkina et al. 2019; Koňářiková and Prokisch 2015).

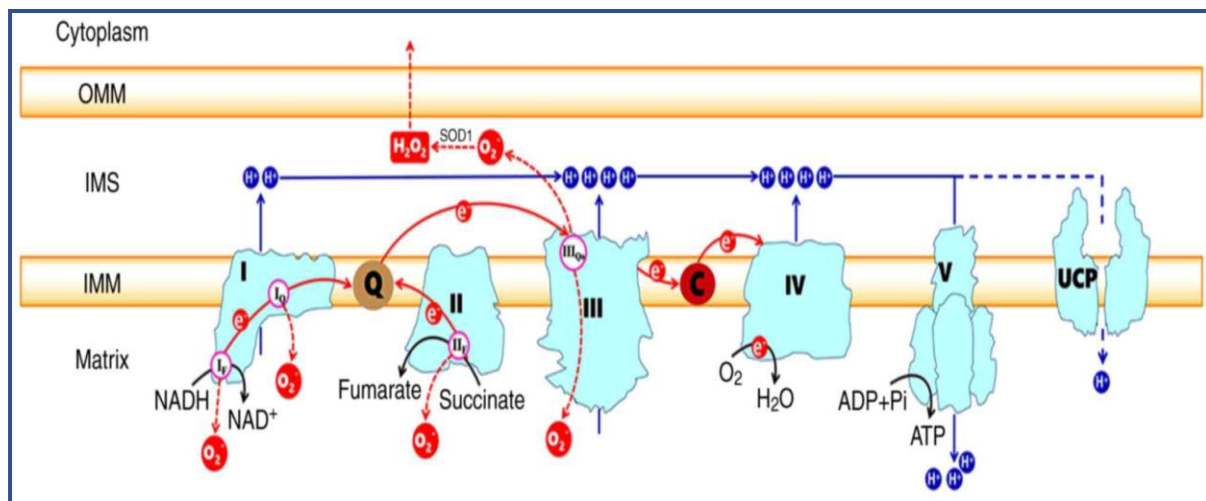
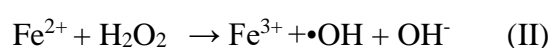
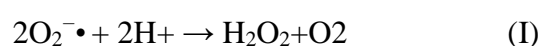


Figure 4. **Electron transport chain with electron and proton leakage** (R.-Z. Zhao et al. 2019). Electrons derived from oxidizable substrates pass through CI/III/IV or CII/III/IV in an exergonic process that drives the proton pumping into the IMS of CI, CIII, and CIV that may drive ATP synthesis at CV or be consumed by UCP.  $O_2^{\cdot-}$  are produced in IF and IQ of CI, IIF of CII, and IIIQo of CIII. It will be released by the latter into the IMS to be converted into  $H_2O_2$  by superoxide dismutase 1 (SOD1) that may diffuse into the cytoplasm. The red, black, and blue arrows represent electron pathways, substrate reactions, and proton circuits across the IMM, respectively. The complexes (I, II, III, IV, and V) are cyan-colored. Q= ubiquinone, C=cytochrome C, IMM=inner mitochondrial membrane, IMS=intermembrane space, OMM=outer mitochondrial membrane, and UCP=uncoupling protein.

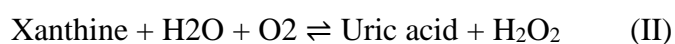
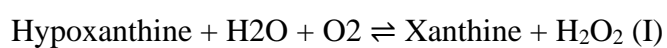
- **Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)**

More than 30 cellular enzymes have been recognized in the mitochondria, peroxisome, and endoplasmic reticulum to produce H<sub>2</sub>O<sub>2</sub> (figure 3) (Sies 2019; Snezhkina et al. 2019).

At the mitochondrial membrane, manganese superoxide dismutase (Mn-SOD) converts the O<sub>2</sub><sup>-•</sup> to H<sub>2</sub>O<sub>2</sub> [reaction I. (I)] that will be further converted to a hydroxyl radical (•OH) via Fenton reaction, by removing an electron from the participating metal ion (Snezhkina et al. 2019) [reaction I. (II)]. In parallel, CYP enzymes, members of the cytochrome (CYP) catalytic cycle, metabolize organic substrates to produce O<sub>2</sub><sup>-•</sup> and H<sub>2</sub>O<sub>2</sub> byproducts (Snezhkina et al. 2019). Similarly, monoamine oxidases (MAO) and dihydroorotate dehydrogenase produce this ROS byproduct (Snezhkina et al. 2019; Koňářiková and Prokisch 2015). Additionally, studies have identified several H<sub>2</sub>O<sub>2</sub>-producing flavin oxidases [NADPH oxidase (NOX), xanthine oxidase (XOD)] in the peroxisome. As shown in reaction II, xanthine oxidase (XOD) catalyzes the oxidation of xanthine and hypoxanthine to uric acid and O<sub>2</sub><sup>-•</sup> and H<sub>2</sub>O<sub>2</sub> byproducts (Snezhkina et al. 2019; Del Río and López-Huertas 2016).



**Reaction I.**



**Reaction II.**

- **Hydroxyl radical (•OH)**

•OH is a highly reactive and aggressive ROS that has a short lifespan. It interacts with organic and inorganic biomolecules, including DNA, proteins, lipids, sugars, and metals. This leads to their oxidative damage upon hydrogen abstraction, addition, and electron transfer. •OH are emerged from Fenton reaction [reaction I. (II)] as discussed in the H<sub>2</sub>O<sub>2</sub> part, where H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-•</sup> interact in the presence of free iron or copper ions, and from water radiolysis (water molecules decomposition) (D. Wu and Cederbaum 2003; Pisoschi and Pop 2015).

### 1.1.3. Exogenous sources

Several sources were identified to trigger ROS production, especially based on the lifestyle we are living. These include cigarette smoke, ozone exposure, hyperoxia, and most importantly irradiation (ultraviolet and ionizing) (Birben et al. 2012).

- **Cigarette smoke and alcohol consumption**

Cigarette smoking and alcohol are part of the lifestyle of most people despite their identified toxicity. First, second, and third-hand cigarette smoke (mainstream and side stream) are toxic due to ROS production, such as  $O_2^{\cdot-}$ ,  $H_2O_2$ , and other reactive radicals, mainly by the combustion process (Birben et al. 2012; J. Zhao and Hopke 2012). Additionally, the inhalation of the smoke activates immune cells, neutrophils, and macrophages, further increasing the oxidant-ROS injury (Birben et al. 2012). Similarly, alcohol consumption, under certain conditions as chronic or acute exposure, can lead to excessive free radicals generation, including  $\cdot OH$ , and/or reduction of the antioxidants' activity resulting in oxidative stress and peroxidation of cellular molecules (D. Wu and Cederbaum 2003). This is due to increased cytochrome P450 2E1 (CYP2E1) enzyme, conversion of xanthine dehydrogenase into xanthine oxidase form, and increased free iron in the cell (D. Wu and Cederbaum 2003).

- **Air pollutant: ozone exposure**

Air pollution induces inflammation-related cascade and oxidative stress in the lung, vascular, and heart tissues (Lodovici and Bigagli 2011). An increase in the ambient outdoor ozone exposure leads to the generation of ROS, such as  $O_2^{\cdot-}$ ,  $H_2O_2$ ,  $\cdot OH$ , nitric oxide (NO), peroxyxynitrite, and hypochlorous acid that will cause lipid peroxidation, reduction in pulmonary functions, and release of some inflammatory mediators (Birben et al. 2012; Voter et al. 2001).

- **Radiation**

- **Ionizing radiation (IR)**

IR originates from natural sources, such as soil, water, vegetation, and fabricated sources, such as x-rays and medical devices. It has been found that IR induces cells to accumulate in the G2/M phase leading to high cellular and mitochondrial oxidative stress (Yamamori et al. 2012). IR leads to the instantaneous formation of water radiolysis products, including ROS, upon series of reactive combinations ( $H_2O \rightarrow \cdot H$  and  $\cdot OH$ ) (Yamamori et al. 2012). In parallel, it interacts with  $O_2$  to convert radicals to  $H_2O_2$  that will further interact with free metal ions (Fenton reaction), such as Fe and Cu, to induce oxidative stress (Birben et al. 2012). Researchers showed that plasma membrane-bound NADPH oxidase enzymes generate  $O_2^{\cdot-}$  and  $H_2O_2$ . Consequently, signal cascades are activated, leading to P53-dependent cell death (Birben et al. 2012). IR is also able to form Guanine (G) radicals that contribute to oxidatively damaged genetic code and a transient decrease in the intracellular level of glutathione (GSH) antioxidant (Birben et al. 2012).

- **Ultraviolet radiation (UV) : photooxidative Stress**

Terrestrial life is dependent on solar radiation, including UV, visible (light), infrared, ionizing, and microwave radiations. Approximately 5 percent of the solar radiation is UV that is subdivided into UVA (315–400 nm), UVB (280–315 nm), and UVC (100–280 nm) (Humans 2012). Based on WHO and reports, all UVC and most of UVB are absorbed by the ozone stratospheric ozone. Hence, the UV radiation reaching the earth's surface comprises about 95 percent UVA and 5 percent UVB (Humans 2012; "WHO | Ultraviolet Radiation and Health" n.d.). Nowadays, due to the digital revolution and increased interest in the detrimental and beneficial effects of UVR, the sun has not become the sole source of UV, rather with the advent of artificial sources, additional exposure sources have increased (indoor tanning lamps, phototherapy equipment...) (Humans 2012).

- a. **Solar radiation: the hero**

Moderate exposure to solar radiation is recommended due to its pleasant consequences. Tanning makes people feel better and more relaxed since sunlight improves their energy and elevates their mood and complacency (Juzeniene and Moan 2012). This mood enhancement and relaxation is also linked to the production of an opioid  $\beta$ -endorphin via stimulating proopiomelanocortin (POMC) promoter in keratinocytes (Juzeniene and Moan 2012). Gruesomely, frequent and chronic UV exposure may result in a tanning addiction (Juzeniene and Moan 2012). Vitamin D is another beneficial effect of exposure to UVB sunlight. It is produced upon cholesterol metabolism in the liver and kidney to process calcium, for normal teeth and bone development, lower diabetic and cardiovascular risk, and to regulate at least 1,000 different genes governing virtually every tissue in the body to reduce depression and enhance immunity (Powers and Murphy 2019; Mead 2008)... The American Journal of Clinical Nutrition states that calcium and vitamin D reduce expected cancer incidence rates by 50 to 77 percent in postmenopausal women (Mead 2008).

- b. **Solar radiation: the villain**

Scientists are concerned about the dark side of solar radiation since it is considered as a complete carcinogen. Repeated exposure to extensive solar radiation increases the risk of skin cancer and photoaging (Mead 2008). This is due to the production of oxidative and bulky lesions (discussed in section 1.1.2. and afterward). For example, ROS, one of the main UV-products, can lead to DNA damages, henceforth, single-and/or double-strand breaks, base modifications, mutagenesis (in proto-oncogenes and tumor suppressor genes; *P53*, etc.), and carcinogenesis. Unfortunately, organic sunscreens deceived people by not acting as protectors

rather enhance UV-induced ROS once penetrating the epidermis (Shen et al. 2014). Due to such dramatic events, several skin cancers arose becoming the most common cancer worldwide, especially in white-skin residents (figure 5) (Mead 2008). However, skin cancer may not develop instantaneously rather after a latency of time from being exposed to the carcinogen to appear markedly with age. It is estimated that UV causes almost 65 percent of melanoma and 90 percent of non-melanoma skin cancers (D'Orazio et al. 2013). In 2018, more than 0.2 million and 1 million global cases of melanoma and non-melanoma skin cancer cases were reported, respectively ("2020-Campaign-Report-GC-Version-MPA\_1.Pdf" n.d.).

### *b.1. Non-melanoma skin cancer (NMSC)*

Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are the two most common subtypes of NMSC, arising from epidermal keratinocytes, with almost 2 to 3 million global cases diagnosed every year (figure 5) (Narayanan, Saladi, and Fox 2010). Other rare NMSCs include Merkel cell carcinoma, sebaceous carcinoma, and apocrine adenocarcinoma (Ciążyńska et al. 2021). NMSC incidences represent 96 percent of all skin malignancies and are triggered mainly by UVB-irradiation due to the accumulation of the DNA bulky lesions, CPDs and (6-4) PPs (Ming et al. 2011). They are mainly found in sun-exposed areas, as head and neck regions, and are inversely proportional to skin pigmentation in the population (Narayanan, Saladi, and Fox 2010). That is why incidences differ between distinct latitude regions and are high in tropical areas (Lomas, Leonardi-Bee, and Bath-Hextall 2012). Although BCC and SCC share many similarities, they have significant etiological differences as different incidence rates (Lomas, Leonardi-Bee, and Bath-Hextall 2012). Even though BCCs account for more than 80 percent of all NMSCs, SCCs are 10-fold higher in metastasis and mortality risks upon chronic UVB exposure (Narayanan, Saladi, and Fox 2010).

Both NMSC types are less fatal than melanoma due to their tendency to remain confined to their primary site of disease, making it easier to extirpate the tumors by resection, microsurgery, or cryosurgery (D'Orazio et al. 2013).

#### ❖ Basal cell carcinoma (BCC)

BCC's development is mainly sporadic, driven by mutations initiating *P53* tumor suppressor gene molecular alterations and the activation of an intracellular hedgehog pathway that will induce basal cell proliferation. These mutations may be due to reduced clearance of UV-induced DNA lesions upon low-penetrance genetic polymorphisms of DNA repair enzymes (Feller et al. 2016). For example, nevoid basal cell carcinoma is characterized by the development of multiple BCCs. It has been recently linked to a concomitant downregulation



of BER's gene expression and activity, resulting in accumulated oxidative DNA damage that could explain BCC patients' clinical phenotype (Charazac et al. 2020).

#### ❖ Squamous cell carcinoma (SCC)

SCC's development is a multi-step process starting from a precursor skin outer layer lesions called actinic keratosis (AKs) (Feller et al. 2016). It originates from keratinocyte stem/progenitor cells of the basal cell layer of the epidermis. Its metastasis is achieved by the secretion of matrix metalloproteinases (MMP)-2/9, higher tumor thickness, alteration of the cell cycle, cell proliferation, DNA repair, etc (Feller et al. 2016; Feraudy et al. 2010). Malignant keratinocytes of SCC show UV-induced signature mutations, as found in *P53*, as proof of a direct link between UV and its development, RAS and Src kinases activation, and NFκB blockade (Feller et al. 2016; Feraudy et al. 2010). In addition, SCCs tumor cells had been shown to harbor more UVA (G to T) than UVB (C to T) signature mutations, suggesting a direct role of UVA-induced oxidized lesions in human skin carcinomas (Pfeifer and Besaratinia 2012).

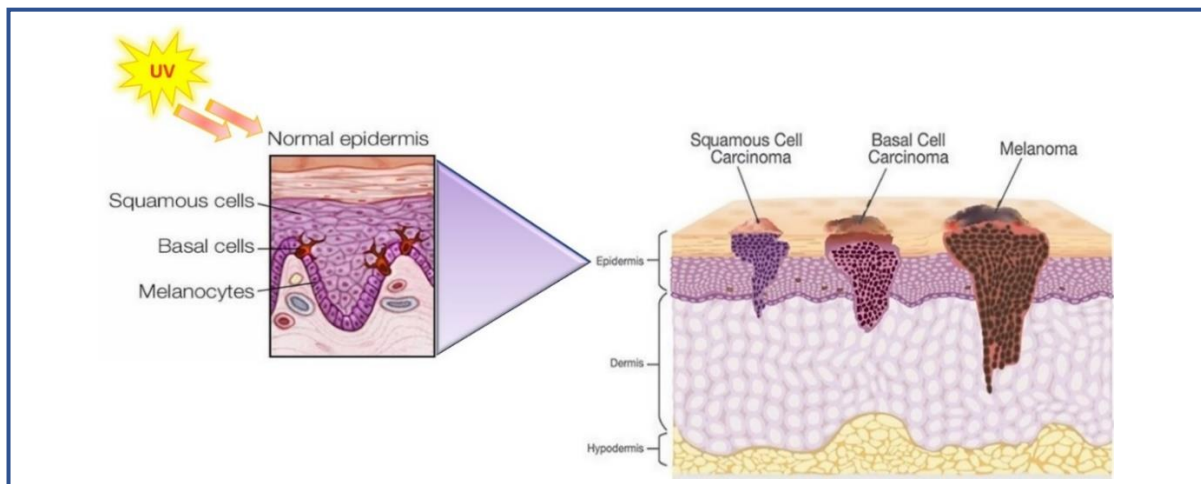
#### *b.2. Melanoma (MSC)*

Melanoma is less common than NMSC but is the most lethal and invasive skin cancer (figure 5) (Watson, Holman, and Maguire-Eisen 2016). It is estimated that more than 287,000 new cases occur worldwide each year, where it is curable if detected at early stages by nevi surgical excision (D'Orazio et al. 2013; "2020-Campaign-Report-GC-Version-MPA\_1.Pdf" n.d.). Once it is lately diagnosed, metastasis, poor diagnosis, and lethality ensue (Narayanan, Saladi, and Fox 2010). In 2020, 100,350 American patients were diagnosed with melanoma and 6,850 patients died from the disease (Siegel, Miller, and Jemal 2020). Advanced treatments (targeted therapy, immunotherapy...) showed a temporarily enhanced prognosis in metastatic patients but did not entirely prevent secondary resistance or relapse (Davis, Shalin, and Tackett 2019). Nevertheless, a better understanding of melanomagenesis's genetic and mechanistic basis paves the way to enhance treatments to achieve a specific and more lasting effect (Davis, Shalin, and Tackett 2019).

Melanomagensis results from a combination of constitutional (genetic..) and environmental factors (UV..) (Watson, Holman, and Maguire-Eisen 2016). Some of the constitutional non-modifiable risk facts: (i) a high number of nevi, skin moles, (ii) a family history of melanoma, and (iii) skin phototype (melanin degree in hair/eyes/skin). Meanwhile, the major environmental factor is chronic and intensive UV exposure, particularly during childhood (Watson, Holman, and Maguire-Eisen 2016). These events lead to several mutations in genes linked to cell growth, proliferation, and metabolism (*BRCA1*, *BRAF*, *PTEN*, *P53*...),

dysregulating signaling cascades (constitutive activation of the MAPK signaling cascade...); ultimately induce cancer metastasis and invasion (Feller et al. 2016; Watson, Holman, and Maguire-Eisen 2016; Davis, Shalin, and Tackett 2019).

The link between UV and melanoma has been controversial. Some say that UV-induced signature mutations' are not common in melanoma despite the link between UV irradiation and its development. Therefore, it is evident that UV by itself does not necessarily cause MSC; instead, an accumulation of different factors could lead to such a complex aetiopathogenesis (Feller et al. 2016). For example, several gene mutations (*BRAF* and *CDKN2A*) and progressive DNA damage are the characteristics of indirect UV-induced oxidative damage and loss of melanosomes' membranes' integrity with consequent leakage of ROS into melanoma cells' cytoplasm (Feller et al. 2016). Meanwhile, other studies confirmed by exon and genome sequencing the presence and importance of unrepaired UV-signature mutations (C > T or CC > TT transitions at dipyrimidine sites) at the non-transcribed regions and active promoters of melanoma cell lines' genome and CPDs in UV-induced melanoma. This may be due to the S-phase 6-4 PPs and CPDs repair deficiency (80 percent less repair compared to control) and ATR (Rad3-related kinase) depletion, where ATR is a DNA repair protein acting downstream NER (Budden et al. 2016; Chhabra et al. 2019).



**Figure 5. A modified scheme showing the different skin cancers (SCC, BCC, and melanoma) arising post-solar irradiation** ("Types of Skin Cancer: Can You Spot Them?" .2018 ; ThingLink n.d.). Keratinocytes form in the deep basal layer as basal cells. Then, they gradually migrate upwards upon differentiation becoming squamous cells. Upon sun exposure, different skin cancers occur. SCC resembles the disease of cells on the skin's surface, BCC starts at the basal cells, and melanoma arises in the pigment cells-melanocytes.

### c. UV components

It acts as the main interest in research due to activating various signaling cascades and to severe consequences. Upon UV-induced DNA damage, NF $\kappa$ B/PI3-K/MAPK/P53/ATR signaling cascades will be activated to stimulate cell cycle arrest and DNA repair. In case of repair failure or extreme damage, mutations accumulate, leading to skin disorders (inflammation, hyperpigmentation, aging, melanoma, non-melanoma skin cancer), internal cancers, and other pathologies (Ryu et al. 2010; Budden et al. 2016).

#### *c.1. UVA*

UVA comprises over 95 percent of UV radiation incidents. Even though it is weakly absorbed by the DNA, it can lead to lethal damages in the latter and other skin biomolecules, especially after the surge in human exposure to UVA due to outdoor leisure. These damages occur upon an interaction with endogenous photosensitizers, i.e., endogenous chromophores (flavins, porphyrins, NADPH oxidase, and melanin...), henceforth, triggering ROS generation that will interact with guanine at the DNA level leading to 8-oxoGua (Birben et al. 2012; Douki et al. 2003; Brem et al. 2017). H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>-•</sup>, NO, <sup>•</sup>OH, and singlet oxygen (<sup>1</sup>O<sub>2</sub>) have been detected in UVA-initiated responses where <sup>•</sup>OH has a minor contribution to the formation of oxidative DNA lesions on the contrary to <sup>1</sup>O<sub>2</sub> (Douki et al. 2003; Valencia and Kochevar 2008). UVA may promote the formation of <sup>•</sup>OH via the photosensitized production of <sup>1</sup>O<sub>2</sub>, inducing a wide range of further DNA damage (Douki et al. 2003). <sup>1</sup>O<sub>2</sub>, the first excited state of oxygen, and O<sub>2</sub><sup>-•</sup> are formed by the electron transfer from the excited state of the photosensitizer exclusively during irradiation due to their short lifetimes. However, this duration is enough to produce additional ROS that will extend after UVA (Mundt et al. 2008). NOX1 mainly produces ROS in the mitochondria and plasma membrane in most cellular sources, including keratinocytes. This was proven by Valencia et al., who used siRNA against NOX1 to show that UVA-induced ROS decreases. Once O<sub>2</sub><sup>-•</sup> is formed by NOX1 and released to the extracellular space, it undergoes dismutation into H<sub>2</sub>O<sub>2</sub> that can diffuse into the same cell, or nearby cells, to elicit responses and further ROS from endogenous sources. Valencia et al. proposed that NOX1 is activated by one of two possible mechanisms: (i) <sup>1</sup>O<sub>2</sub> increases [Ca<sup>2+</sup>] or (ii) ceramide that will activate Rac, a member of the Rho family of small GTPases, consequently activating NOX1 (Valencia and Kochevar 2008). Surprisingly, this ROS and its oxidative DNA damage are not the major consequences of UVA. The use of alkaline modified gel electrophoresis (with repair enzymes) and HPLC-MS/MS showed that single-strand breaks, oxidized DNA damage (essentially 8-oxoGua), and CPDs (mainly TT CPDs) are formed in a 1:1:3:10 ratio, as

presented in figures 6 and 7 (Douki et al. 2003). These CPDs are likely formed by photosensitization; triplet energy transfer from an excited chromophore mainly to thymine (due to its lowest triplet state energy). Photosensitization processes may be involved in photocarcinogenesis, particularly in the induction of melanoma (Mouret et al. 2006). However, UVA induces neither 6-4 (PP)s nor their Dewar isomers (Douki et al. 2003).

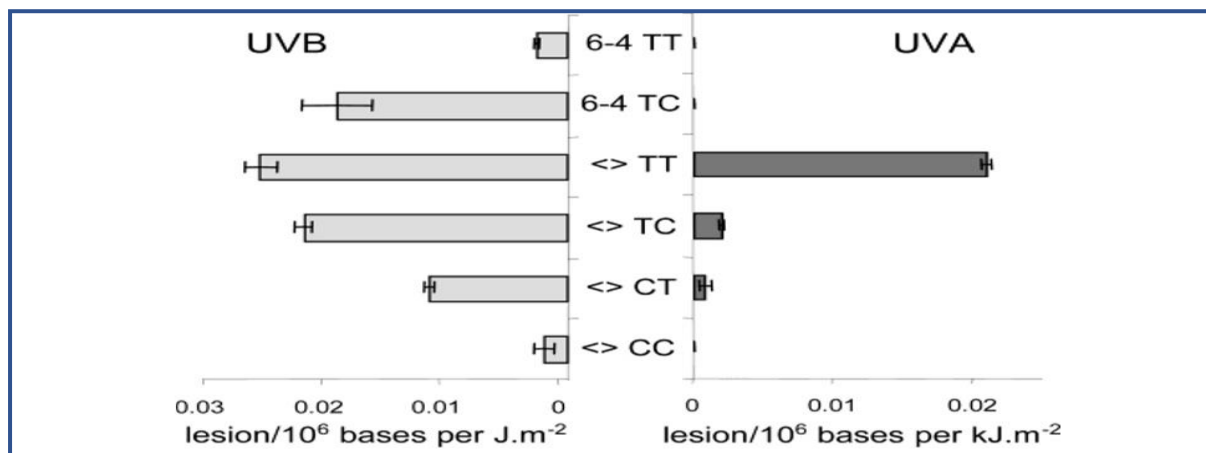


Figure 6. *Distribution of CPDs (<math>\langle \rangle</math>) and (6-4)PPs (6-4) at the four possible bipyrimidine sites within the DNA upon UVA vs UVB (Douki et al. 2003). Results represent the yield of formation ( $\pm$ SD) obtained by linear regression of lesion level with respect to the applied dose.*

### c.2. UVB

UVB generates ROS and bulky lesions [CPDs and (6-4) PPs] (figure 7). Although it represents almost 5 percent of the solar UV-radiation, it is expected to induce the most significant damage due to its absorption by the DNA in the skin's epidermis, which in turn will induce DNA damage response (DDR) network (Douki et al. 2003; Hosseini et al. 2018). DDR will trigger different cellular fates based on the type and extent of damage and DNA repair capacity. If left unrepaired, apoptosis, disorders, and skin cancer may form, primarily NMSC (BCCs and SCCs) (Ryu et al. 2010; Hosseini et al. 2018). A study had shown, by flow cytometric analysis of the fluorescent intensity following DCFH<sub>2</sub>-DA staining, that UVB-irradiation upregulates intracellular ROS in dermal fibroblasts and keratinocytes compared to sham control (Ryu et al. 2010; Deng et al. 2018). Such an irradiation has been shown to generate excessive ROS quantities that swiftly overpower the antioxidant defense mechanisms (H. R. Rezvani, Ged, et al. 2008). The production occurs straightway and 2-3 hours post-irradiation (H. R. Rezvani, Ged, et al. 2008). These ROS include  $\cdot$ OH,  $O_2^{\cdot-}$ ,  $^1O_2$ , and  $H_2O_2$  (Deng et al. 2018). Upon UVB, catalase could also be converted into reactive intermediates by shifting between its catalytic and peroxidative activity that antioxidants will detoxify (Heck et al. 2003). Once antioxidants are limited, ROS accumulate to induce oxidative stress (Heck et al. 2003). In parallel, UVB

has been shown to stimulate several signaling pathways that contribute to ROS production. Ryu et al. suggested that ROS generation is linked to the BLT2-NOX1 pathway in a P53-independent manner (Ryu et al. 2010, 2). This may lead to apoptotic cell death via a ROS–ASK1–JNK/p38 kinase signaling cascade (Ryu et al. 2010). Nonetheless, it is not the only pathway; the EGF-linked signaling cascade had been shown to participate in UVB-generated ROS in keratinocytes (Ryu et al. 2010).

Furthermore, direct absorption of UVB by DNA results in dimerization between adjacent pyrimidine bases producing CPDs and (6-4) PPs (Douki et al. 2003). CPDs are four-membered ring lesions formed by the coupling of two covalent bonds between adjacent pyrimidines, while (6-4) PPs consist of a covalent bond between carbon at the 6<sup>th</sup> position of one ring and that at the 4<sup>th</sup> position of the ring on the adjacent 3' pyrimidine (Amaro-Ortiz, Yan, and D'Orazio 2014). It is estimated that 10<sup>5</sup> UV-induced DNA damages (single-strand DNA breaks, bases, bulky lesions) are produced in every skin cell per day, leading to UV-signature mutations (D'Orazio et al. 2013; Hoeijmakers 2009). Exposure of keratinocytes to the sun for one day can also induce inflammation and oxidative damage (Hoeijmakers 2009). The resulting bulky photoproducts distort the DNA helix, which will halt transcription and DNA replication if left unrepaired (Budden and Bowden 2013). Upon chronic exposure to UVB, (6-4) PPs may be converted into their Dewar isomers (Douki et al. 2003). In UVB-irradiated cells, as shown in figure 6, TT and TC lesions were the most reactive sites observed in both photoproducts, where the overall ratio of CPDs to (6-4) PPs was 3:1. They are known to be mutagenic events contributing to skin tumors due to their high proportion in mutated *P53* (TC to TT or CC to TT transitions). CPDs at CC and (6-4) PPs at TT are infrequent UVB-induced photolesions (Douki et al. 2003; Mouret et al. 2006).

### *c.3. UVA-UVB interactions*

UVA and UVB have been suggested to have a synergetic effect. Notably, UVA showed an enhancement of UVB-induced immune responses' suppression and cytotoxicity. This may be due to that UVA damages DNA repair proteins compromising the DNA repair (non-homologous DNA end joining, OGG1 and MYH glycosylases of BER, NER...) by which the DNA becomes more vulnerable to the toxic effect of UVB (Karran and Brem 2016). Some studies showed that UVA and UVB-produced CPDs, if left unrepaired, strongly contribute to skin cancer mutations, most profoundly, mutation hotspots in the *P53*-tumor suppressor gene leading to skin cancer (Pfeifer and Besaratinia 2012). On the other side, exposure to both UV

irradiations can upregulate cytokines and increase T-regulatory cells' activity to prevent autoimmune diseases (Mead 2008).

#### c.4. UVC

Even though the atmospheric ozone layers absorb UVC, researchers used artificial lights in their studies due to UVC's action as an anti-microbial approach. Some studies showed that UVC produces ROS, such as  $H_2O_2$  and  $^1O_2$ , eventually inducing 8-oxoGua (Gomes et al. 2005). In addition, UVC has been categorized as the most lethal range of wavelengths strongly absorbed by microorganisms resulting in nucleic acids' damage; therefore, it is considered germicidal (Dai et al. 2012). This is due to pyrimidine molecules' dimerization that will block replication and prevent microorganisms from growth. This became the glimmer of hope towards inactivating antibiotic-resistant microorganisms. Scientists have started testing it *in vivo* and clinical trials locally on infected areas without damaging the surrounding cells (Dai et al. 2012). Interestingly, 222 nm short-wavelength UVC has shown to be not absorbed by mammalian cells and is efficient in eliminating viruses and bacteria (Narita et al. 2020).

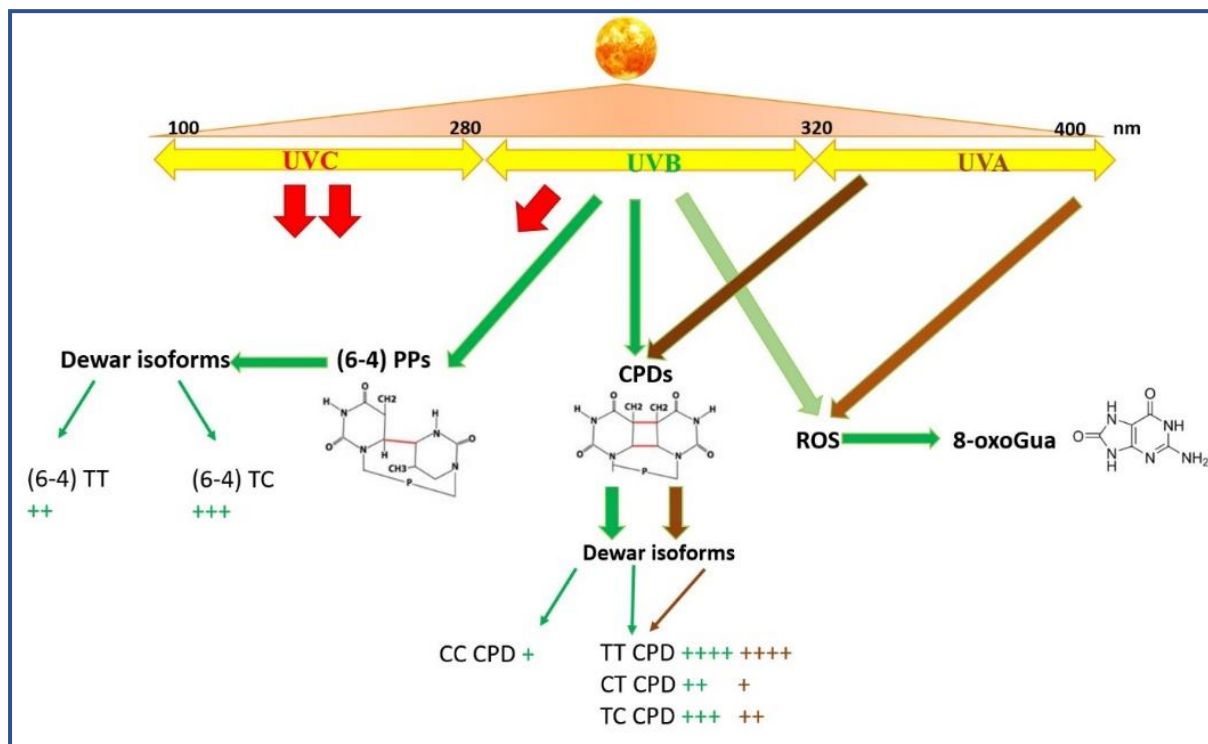
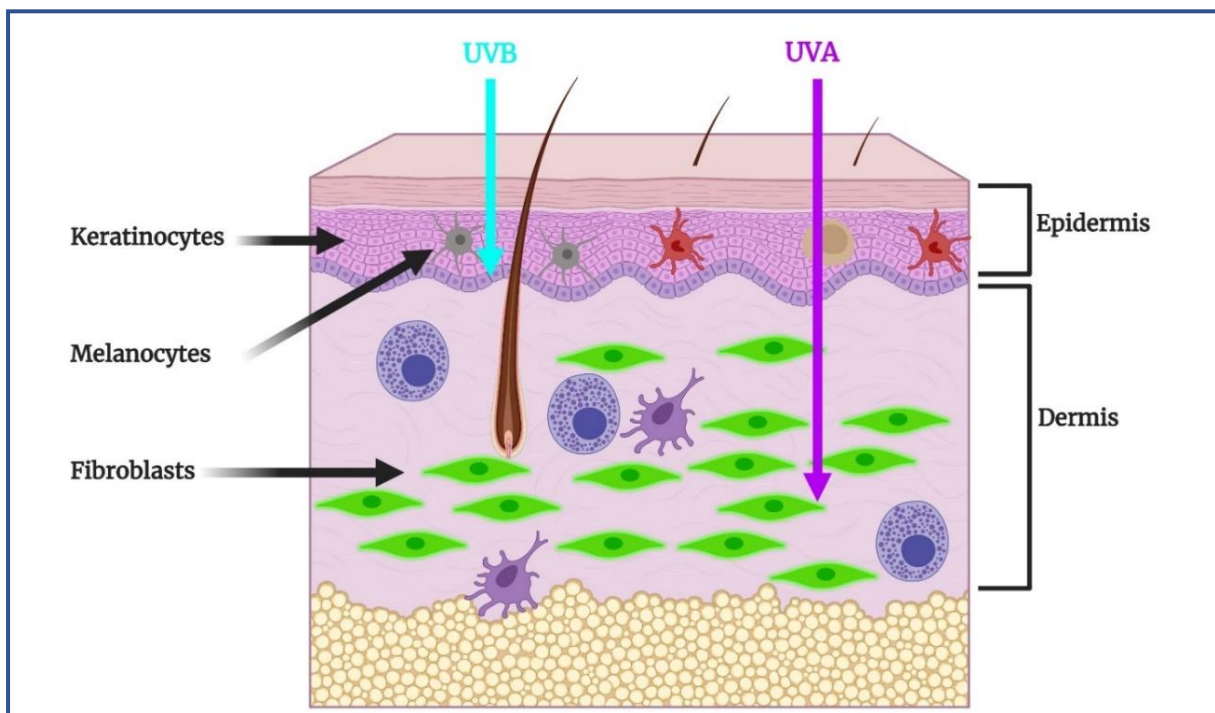


Figure 7. A scheme presenting the effect of UV on DNA. UVC is blocked from penetrating the earth due to the ozone layer, which makes it the least impactful. UVB is partially blocked and can form (6-4) PPs (TT and TC), CPDs (CC, TT, CT, and TC), and oxidative DNA lesions (8-oxoGuanine). UVA produces CPDs (TT, CT, and TC) and oxidative DNA lesions (8-oxoguanine). The red arrow represents blockage by the ozone layer; the green arrow represents UVB; the brown arrow represents UVA. The degree of color represents the difference in lesions, while + represents how much the isoforms are confluent in UVB vs UVA.

#### d. Skin: UV-barrier

The skin comprises two primary layers, the epidermis and dermis, consisting of epithelial, mesenchymal, glandular, and neurovascular components. Usually, it is surmised that the upper epidermal layers' biological and physical characteristics protect the basal layer against UV penetration and the subsequent DNA damage, while the dermal layer participates in many skin physiologic responses (Mouret et al. 2006; D'Orazio et al. 2013). Interestingly, UVA-induced DNA lesions, including CPDs, are not efficiently prevented solely by skin, where UV poses a double risk to the skin by both increasing the biomechanical driving force for damage and decreasing skin's natural ability to resist (Biniek, Levi, and Dauskardt 2012; Mouret et al. 2006). This leads to acute (erythema....) and chronic (photoaging....) conditions and various skin cancers upon photochemical reactions (Biniek, Levi, and Dauskardt 2012). Reconstruction of human skin models by adding a differentiated epidermal layer (keratinocytes) over a dermal layer (fibroblasts) was the first silver lining behind investigating the biological effects of UVA and UVB on epidermal keratinocytes and dermal fibroblasts, at physiological state (Bernerd, Marionnet, and Duval 2012). As shown in figure 8, UVB only penetrates the epidermis, whereas UVA can penetrate the dermis despite that UVB has higher energy than the latter (Shen et al. 2014). Thus, excessive UVB could lead to skin burns, DNA damage and promotes skin cancers (Mead 2008).



*Figure 8. Skin layer components and UV. UVB penetrates the epidermis while UVA penetrates further into the dermis. The epidermis and dermis contain many cells, most importantly, keratinocytes + melanocytes and fibroblasts, respectively.*

### *d.1. Skin layer components*

#### ❖ Fibroblasts

Although fibroblasts are heterogeneous and found in most tissues, we will focus on those present dominantly in the dermal skin layer due to their vital role in dermal-epidermal interactions, homeostasis, skin diseases, and carcinomas. Once activated, they synthesize and secrete extracellular matrix (collagen, proteoglycans, growth factors, fibronectin, and proteases) for skin's structural integrity through acting as an autocrine and paracrine loop (Tracy, Minasian, and Caterson 2016; Ghetti et al. 2018). Primarily, fibroblast-secreted factors [epidermal growth factor (EGF), keratinocyte growth factor (KGF), and insulin-like growth factor (IGF)] promote epidermal keratinocytes' proliferation, differentiation, survival, repair, and migration for epidermal homeostasis and photocarcinogenesis resistance post-acute-UV exposure. This was well elaborated by Fernandez et al. They showed that UVB-induced CPDs were higher and more persistent in primo-keratinocytes culture than keratinocytes and fibroblast co-culture before and after irradiation. Concurrently, keratinocyte apoptosis was reduced in the presence of fibroblasts (lower cleaved caspase-3 and elevated phosphorylated Bad) that coincide with the fact that growth factors synthesized by fibroblasts suppress UV-induced apoptosis in surrounding cells. Also, fibroblasts' secretions stabilize P53, which is known to orchestrate DNA repair, cell cycle, and apoptosis. Hence, dermal fibroblasts reduce keratinocytes' UVB-induced cell death and enhance their DNA repair (Fernandez et al. 2014). For instance, UVB or oxidative stress-induced fibroblasts' senescence decreases their production of IGF-1. This leads to less activated IGF-1R on keratinocytes and failure of protection against UVB. Thus, UVB will induce keratinocytes' apoptosis triggering aging, while survived keratinocytes will keep dividing and accumulating mutations triggering nonmelanoma skin cancer (Lewis et al. 2010). Interestingly, supplying type-2 diabetic patients with high concentrations of insulin, which share a similar molecular structure of IGF-1, was shown to activate IGF-1R and 2.5-fold decreased age-dependent skin cancer incidents (Lewis et al. 2010).

#### ❖ Keratinocytes

90-95 percent of the epidermal layer is composed of keratinocytes. They are characterized by the expression of cytokeratin, keratin, filaggrin, desmosomes, and tight junctions to form an effective physicochemical barrier against stress exposure [bacteria, viruses, chemicals, and radiation (IR, UV)] (D'Orazio et al. 2013; Hirobe 2014). They also act as UV barriers due to their accumulation of melanin pigments produced by melanocytes (D'Orazio et al. 2013).



Keratinocytes are implicated in regulating melanocytes' function by producing several factors, including SP-1 transcription factor and  $\alpha$ -Melanocyte-stimulating hormone ( $\alpha$ -MSH) that are responsible for stimulating melanogenesis (Hirobe 2014).

Keratinocytes' DNA harbors up to  $10^5$  UV-photoproducts [CPDs, and (6-4) PPs] per day (Kasraian et al. 2019). Such photoproducts give rise to the UV-signature mutations, C > T and/or CC > TT. In parallel, UV-induced ROS will cause further DNA damage, promoting mutagenesis. In response to UV-DNA damage, photoproducts, and oxidative lesions, P53- $\alpha$ MSH-induced DNA repair mechanisms will be alerted and activated (Feller et al. 2016). Several hours post-UV exposure, epidermal keratinocytes replicate, leading to the thickening of the epidermis to protect the skin from future UV penetration. However, upon excessive UV-dose, thereby unreparable DNA damage, keratinocytes activate their P53 dependent-apoptotic pathway (D'Orazio et al. 2013).

#### ❖ Melanocytes

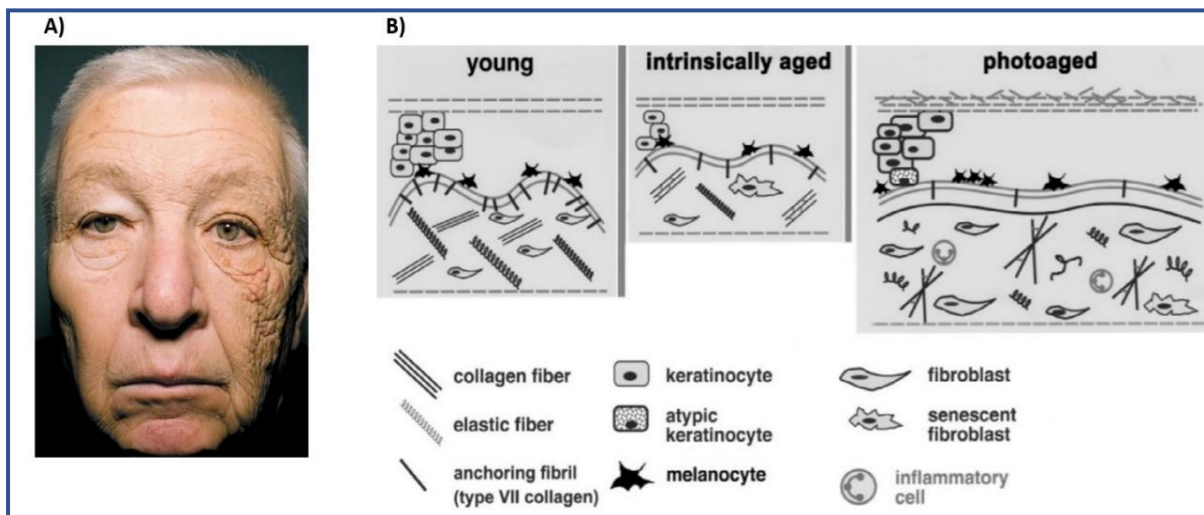
Melanocytes are melanin-producing cells found at the basal stratum-epidermal and dermal layers of the skin. As mentioned before, melanin absorbs UV waves to protect the keratinocytes from DNA damage and oxidative injury. It acts as the sole-source of skin pigmentation (D'Orazio et al. 2013; Hirobe 2014). Two different melanin types are produced: eumelanin and pheomelanin. Pheomelanin is similarly produced among individuals. It generates free radicals (ROS), promoting oxidative DNA damage, photoproducts, and melanomagenesis. The more eumelanin produced, the darker the skin, and the more it is protected from DNA damage and skin cancer risk (D'Orazio et al. 2013). Alongside UV exposure, melanocytes proliferate to produce more melanin deposit in keratinocytes as an adaptive tanning protective response. Melanogenesis is induced by melanocortin 1 receptor (MC1R) / $\alpha$ MSH/cAMP signaling pathway. This pathway will activate DNA repair mechanisms and anti-oxidants production (SOD, catalase....), thereby reducing ROS and diminishing oxidative stress and DNA damage (Feller et al. 2016; Amaro-Ortiz, Yan, and D'Orazio 2014). Melanocytes with MC1R variants have lower DNA repair capacity (NER and BER....), decreased apoptosis, more DNA oxidative damage, and photoproducts. This could increase cancer risk (D'Orazio et al. 2013; Feller et al. 2016).

#### *d.2. Photoaging*

Skin aging is the degeneration and molecular modifications of the epidermal, dermal, and subcutaneous skin layers (Shin et al. 2019). It can be a natural or induced process. Intrinsic aging is a slow normal process that affects the skin and other organs in a similar manner, in

both photo-protected and photo-exposed areas. However, as shown in figure 9, photoaging, also called dermatoheliosis, is an induced process affecting various skin layers, including dermal connective tissue and collagen, contributing to premature aging. It can be prevented or alleviated. Both aging processes may overlap in some biomolecular mechanisms (Wlaschek et al. 2001; Junyin Chen et al. 2019). An *in vitro* study showed that high concentrations of UVB-induced ROS in dermal fibroblasts contribute to such a damaging process by increasing telomere shortening, cellular senescence, and chronic-inflammatory systematic NFκB activation. Such a signaling pathway is known to induce age-related diseases. Increased levels of the  $\cdot\text{OH}$ ,  $\text{O}_2^{\cdot-}$ ,  $^1\text{O}_2$ , and  $\text{H}_2\text{O}_2$  can damage these fibroblasts leading to inhibition and degradation of the extracellular matrix and collagen. Deng et al. showed that UVB causes a significant decrease in type-1 collagen at mRNA and protein levels (Deng et al. 2018; Z. Liu et al. 2018). In parallel,  $^1\text{O}_2$  and  $\text{H}_2\text{O}_2$  are the major ROS involved in UVA-dependent induction of matrix metalloproteinases (MMP-1, -2, and -3), while  $\cdot\text{OH}$  induces UVB-dependent MMP-1 and MMP-3. By activating specific signaling cascades (MAPK...), these MMPs will contribute to elastin accumulation and collagen matrix loss and reduce metabolism, the prominent photoaging hallmarks (Wlaschek et al. 2001; Junyin Chen et al. 2019). Chen et al. reported that treatment of fibroblasts exposed to UVA with concentrated growth factors fibrin gel (5 percent), previously used for wound healing and repair, can act as ROS scavengers by upregulating antioxidants' activities (superoxide dismutase; SOD...) and restore normal cellular proliferation and migration of dermal fibroblasts. This could inhibit and/or treat photodamaged skin cells (Junyin Chen et al. 2019). Antioxidants (vitamin C),  $\alpha$ -hydroxy acids, and retinoids were shown to regulate the production of extracellular matrix by fibroblasts and collagen and improve the skin layers' histology (dermal thickness, elasticity..) (Shin et al. 2019).

Of note, *XPC* mutations (discussed later) could contribute to photoaging by producing NOX1 dependent-ROS and activating progerin and  $\beta$ -galactosidase activity (Hosseini et al. 2015).



**Figure 9. A modified scheme representing intrinsic aging vs photoaging.** A) A 69-year-old man was diagnosed with unilateral photoaging. He drove a business truck for 28 years, such that UV infiltrated through the window glass to penetrate the epidermis and upper dermis layers. This led to gradual, asymptomatic thickening and wrinkling of the left side of his face (Gordon and Brieva 2012). B) schematic representation characterizing young, intrinsically aged, and photoaged skin. Young skin contained a balanced cellular composition and distribution (keratinocytes, melanocytes, fibroblasts...), while intrinsically aged skin had an overall reduction in the cell count and a decrease in epidermal and dermal thickness. Conversely, photoaged skin presents an increase in the thickness of epidermal and dermal compartments, acanthosis, mild hyperkeratosis, elastic fibers, inflammatory cells, and inhomogeneous distribution of melanocytes (Wlaschek et al. 2001).

#### 1.1.4. ROS and signaling pathways

ROS play a multi-role in the body: a protector, initiator of different signaling cascades, and a striker. Nuclear ROS (nROS), cytosolic ROS (cROS), and mitochondrial ROS (mROS) act as signaling molecules regulating various signaling pathways, including growth, differentiation, progression, and cellular death (J. Zhang et al. 2016). ROS regulate over 500 putative protein targets containing cysteine residues. For instance, ROS stimulate PDGF and EGF growth factors for normal tyrosine kinase signaling (Finkel 2011). Oxidants, mainly  $H_2O_2$ , are produced nearby or diffuse into the intended target, thereby achieve some measure of overall signaling specificity (Finkel 2011). Classically, ROS were considered part of the body's immune defense mechanism, where neutrophils release them for destructing exogenous pathogens (J. Zhang et al. 2016). As a case in point, scientists reported the role of mitochondrial oxidants in the formation of the NLRP3 (NOD-like receptor pyrin domain-containing 3) inflammasome, ultimately leading to caspase-induced cell death (Finkel 2011). Moreover, ROS induce several signaling pathways. Mitogen-activated protein kinases (MAPKs) induce appropriate physiological response towards stress, including cellular proliferation, differentiation, inflammation, and apoptosis (W. Zhang and Liu 2002). In response to oxidative stress, ROS activates several MAPK family members. Apoptosis signal-regulating kinase 1 (ASK1) is activated by tumor necrosis factor (TNF). This will induce its dissociation from

thioredoxin (TRX) to activate downstream effectors, including the c-Jun N-terminal kinase (JNK) and the p38 MAPK pathway required for cell death. PKG, cAMP dependent-PKA, and JNK are activated by intermolecular disulfide bond formation, intramolecular disulfide bond, and phosphatase inhibition, respectively (Finkel 2011; P. D. Ray, Huang, and Tsuji 2012). Other activated cellular pathways engaged in cellular apoptosis and proliferation involve NF $\kappa$ B, phosphoinositide-3-kinase- (PI3K-) Akt, and ataxia-telangiectasia mutated (ATM) pathways (J. Zhang et al. 2016; Finkel 2011). In the presence of ROS, Akt can stimulate the Nrf2/KEAP1/ARE pathway. This pathway is well-known as a major model of cellular defense against oxidative stress where Nuclear factor E2-related factor 2 (Nrf2) heterodimerize with transcription factors of the Maf family to bind upstream the cis-regulatory antioxidant response element (ARE) sequence in the promoter region of cytoprotective genes to stimulate various antioxidant enzymes (SOD, GST...) and to reduce oxidative DNA damage and mediate redox homeostasis. In the absence of stress, Kelch-like ECH-associated protein 1 (KEAP1) negatively regulate Nrf2 in the cytoplasm by tagging it for proteasomal degradation (David et al. 2017; Ming et al. 2011). A recent study showed that KEAP1 inhibition and Nrf2 pathway activation could be done via Nardochinoid C. Such a product could be a safer substitute to the few available Nrf2 activators with some side effects (Luo et al. 2018). However, other experiments (human cell culture, animal models, clinical trials, etc...) are needed in the future to confirm its therapeutic efficacy and efficiency (Luo et al. 2018).

Also, ATM protein kinase is involved in the homeostatic feedback loop regulated by and regulating ROS. It can modulate NADPH synthesis through pentose phosphate shunt and regulate mitochondrial biogenesis. In parallel, H<sub>2</sub>O<sub>2</sub> can oxidize its C-terminal cysteine residue for activation, thereby preventing ataxia, immunodeficiency, premature aging, and cancer predisposition due to activating a response to DNA double-strand breaks (Finkel 2011).

P53's cysteine residues act as targets for ROS, but it can also regulate ROS in turn. In response to ROS, P53 is known to upregulate transcription of *NADPH* and several antioxidant genes (*SODs*, *Gpx1*...) (Sies 2019). Once the delicate balance between expressed ROS and antioxidants is disturbed, ROS-induced tumor-promoting events will begin (Liou and Storz 2010). This is done by upregulating the expression of cell cycle cyclins (cyclin B2, cyclin D3, cyclin E1, and cyclin E2..) to expedite G1 to S phase transition and promote tumor cell metastasis and invasiveness (Liou and Storz 2010). Surprisingly, at high ROS levels, P53 will step out to reduce the antioxidants' and their transcription factors' expression tipping the cellular fate towards death (Srinivas et al. 2019).

## 1.2. Defense mechanisms against oxidative stress

Several protective mechanisms were studied in aerobic organisms to detoxify or prevent ROS. It could be by natural metabolic processes, including series of antioxidant proteins [SOD, glutathione peroxidase (GPx), catalase, Nrf2] or synthetic/natural products (J. Zhang et al. 2016). Such products could act as ROS scavengers or antioxidants such as alpha-tocopherol, selenium, ferulic acid, flavonoids (strawberry, pomegranate....), lipid-soluble carotenoids (lycopene, beta-carotene), and vitamins (A and C) (Amaro-Ortiz, Yan, and D’Orazio 2014). It is worth mentioning that the antioxidants state differs amongst cell types. For example, although keratinocytes show less sensitivity to UV-DNA damage than lung or skin fibroblasts, they have triple the level of GSH compared to the fibroblasts (Morley et al. 2003).

### 1.2.1. Enzymatic defense mechanism

- **Superoxide dismutases (SODs)**

In mammals, SODs are categorized based on their location and the metal ions they require for their function into three variants: (i) Cu/Zn superoxide dismutase (SOD1), (ii) mitochondrial Mn superoxide dismutase (SOD2), and (iii) Cu/Zn extracellular superoxide dismutase (SOD3) (D. Wu and Cederbaum 2003). They are responsible for  $O_2^{\cdot-}$ , the most catastrophic free radical, dismutation into  $H_2O_2$  (reactions I&II). Astoundingly, researchers found a link between increased ROS, disturbed antioxidants activities, and tumors. It was hypothesized that high expression of SOD2 leads to an accumulation of  $H_2O_2$  that will unlock the gates in front of tumor cell lines to gain invasive and metastatic properties, as shown in advanced stages of gastrointestinal (GI) cancer (Valko et al. 2006).

It should be noted that SOD enzymes work in conjunction with  $H_2O_2$ -removing enzymes, catalases, and glutathione peroxidases (Valko et al. 2006).

- **Glutathione peroxidase**

Glutathione peroxidases are primarily categorized into two forms: (i) selenium-independent glutathione-S-transferases (GST) and (ii) selenium-dependent glutathione-S-transferases (GPx). They use GSH as a cofactor that will be oxidized simultaneously to reduce peroxides ( $H_2O_2$ , ROOH) to water or alcohol (reaction III):



**Reaction III.**

- **Glutathione (GSH)**

L- $\gamma$ -glutamyl-L-cysteinyl glycine is a tripeptide that is abundant in eukaryotic cells (0.1-10 mM concentrations). More than 90 percent are present in the thiol reduced form, GSH. It is catalyzed by the consecutive action of  $\gamma$ -glutamylcysteine synthetase (glutamate-cysteine ligase) and GSH synthetase through the  $\gamma$ -glutamyl cycle or by oxidized glutathione (GSSG) reduction by glutathione disulfide reductase (GSSG reductase) in the presence of NADPH. GSH has multiple functions, including metabolism, catalysis, transport of cysteine moieties, and an antioxidant that reduces cellular components such as free radicals (Meister 1995). By donating an electron to reduce products, GSH becomes oxidized. Then NADPH, as an electron donor, will regulate GSH's turnover. Therefore, any misbalance in the ratio between oxidized and reduced GSH could indicate oxidative stress (D'Orazio et al. 2013).

- **Glutathione metabolism methods: inhibition vs induction**

In general, **L-buthionine-sulfoximine (BSO)** is used to reduce cysteine and irreversibly inhibit the first steps of GSH synthesis ( $\gamma$ -glutamylcysteine synthetase) and therefore selectively decreasing cellular levels of GSH. This will induce oxidative stress associated with elevated levels of 8-oxoGua, DNA SSBs, and gene deletions *in vitro* and *in vivo* (Ghetti et al. 2018; Fernandez et al. 2014). Several reports have indicated the effectiveness of BSO in inhibiting growth and inducing apoptosis of cancer cell lines when used in combination with irradiation and with/without other drugs, mainly dimethyl fumarate. **Dimethyl fumarate (DMF)** depletes pre-existing intracellular GSH rapidly by conjugating with it to be exported and/or metabolized by the cell. In addition, DMF has been shown to treat psoriasis and enhance the cytotoxicity of antitumor agents. Therefore, BSO/DMF combined treatment can completely deplete GSH and trigger intrinsic cell death in various cell lines, including CD44+ cancer stem-like cells and head and neck squamous cell carcinoma cells (HNSCC). Excitingly, BSO/DMSO showed efficiency at *in vivo* level too. Ninety-five percent of mean tumor volume decreased in mice after pretreatment and irradiating for five consecutive days (4Gy/day). This shows the potential of using such a combination with irradiation as a therapy to eliminate resistant tumor cells (Boivin et al. 2011).

On the contrary, the administration of compounds such as **N-acetyl-cysteine (NAC)**, which increases cellular cysteine levels, GSH synthetase substrates ( $\gamma$ -glutamylcysteine), or glutathione esters can increase cellular GSH levels *in vivo* and *in vitro* (Meister 1995). Specifically, NAC acts as a GSH precursor and/or free radicals' scavenger. Thus, it prevents and/or reduces the formation of 8-oxoGua lesions and photoproducts in embryonic cells, mice,

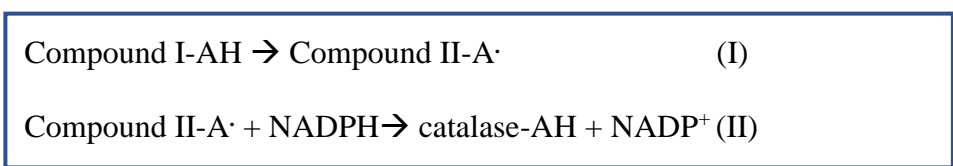
Atm deficient mice, UV-irradiated lung fibroblasts, and others (Reliene and Schiestl 2006). For instance, incubating skin fibroblasts for 4 hours with 5mM or 1 hour with 6mM NAC maximized GSH production and reduced ROS-induced DNA lesions at UVA physiological doses. Such a ROS downregulation is due to an inhibition in HMGB1 (High-mobility group box 1) release. This photoprotective effect is reduced with time. Nevertheless, it is ineffective in preventing UVB-induced-pyrimidine dimers formation. Therefore, NAC is postulated to be involved directly in repairing UV damages caused by ROS, and its supplementation is inexpensive, safe, FDA approved, and effective as dermatological therapy (Srinivas et al. 2019; Morley et al. 2003).

Additionally, it has been used to preserve lung function in idiopathic pulmonary fibrosis patients (Goodson et al. 2009).

- **Catalase**

Catalase is an iron-containing enzyme found in the peroxisome and competes with glutathione peroxidase on removing H<sub>2</sub>O<sub>2</sub>, resulting in water and O<sub>2</sub> (D. Wu and Cederbaum 2003). UVB is known to induce ROS at two distinct chronologic stages: immediately and few hours post-irradiation. Catalase overexpression could be involved in the latter stage. It was recorded to have a protective effect against UVB-induced apoptosis in cultured keratinocytes and reconstructed epidermis. Up to 50 percent of catalase's activity was found to be downregulated in XP patients due to a reduction in NADPH (H. R. Rezvani, Ged, et al. 2008). So, *what is the link between catalase and NADPH?*

Glutathione reductases and peroxidases need NADPH to eliminate H<sub>2</sub>O<sub>2</sub>. Also, it was discovered that each NADPH molecule tightly binds to each of the catalase's four subunits to protect and enhance the efficiency of the latter's activity. NADPH oxidation inhibits the formation of compound II, an inactive catalase form (Kirkman, Galiano, and Gaetani 1987) (Reaction IV):



Where Compound II is an oxidative inactive catalase, AH and A· are groups within the active center of catalase

Herein, we can conclude that the catalase and glutathione reductase/ peroxidase pathway act on H<sub>2</sub>O<sub>2</sub> NADPH-dependent reduction.

### 1.2.2. Non-enzymatic defense mechanism

- Nicotinamide (NIC)

**NIC**, also known as vitamin B<sub>3</sub> or niacinamide, has been used to treat different diseases (dermatitis, diarrhea, dementia, actinic keratosis, and melanoma) due to its effect on the intracellular functioning and metabolism. It produces nicotinamide adenine dinucleotide (NAD<sup>+</sup>), a pro-survival cofactor acting in the mitochondrial electron transport chain machinery to regulate cell survival and oxidative homeostasis. It is worth addressing that NIC has a dose-specific dual role, acting as an antioxidant at low concentrations and as an oxidative stressor after exceeding a certain threshold. At high concentrations (30 mM), NIC was shown to induce fibroblasts' apoptosis by elevating ROS, reducing GSH levels, inhibiting glucose-6-phosphate dehydrogenase (G6PD) enzyme (i.e., starving cells), and modulating the expression of antioxidant, anti-apoptotic, and pro-apoptotic genes (Hassan, Luo, and Jiang 2020). Meanwhile, at low concentrations, NIC has a photoprotective effect. Recently, scientists have proved that 50µM NIC protects melanocytes from UV-induced DNA damage (CPDs and 8-oxoGua). This may be by upregulating the expression of critical genes involved in NER and BER (*SIRT1*, *P53*, *DDB1*, *DDB2*, *OGG1*, *ERCC1*, *ERCC2*, *Nrf2*, and *CDK7*) in response to UVB-irradiation. Furthermore, a recent phase III clinical trial indicated that oral nicotinamide is non-cytotoxic and effective in reducing non-melanoma skin cancers and actinic keratosis incidences in high-risk patients (Chhabra et al. 2019).

- Crocin

Crocin is an active constituent present in saffron and traditional Chinese medicine that has been used to treat various diseases (neurodegenerative disorders, coronary artery diseases, respiratory diseases, and gastrointestinal diseases). Crocin is effective as an anti-carcinogenic, anti-inflammatory, and antioxidative agent. In addition, it could protect dermal fibroblasts from UVB damage by reducing ROS (directly or by increasing GPx expression), enhancing proliferation (by increasing the anti-apoptotic *Bcl-2* gene's expression), and upregulating ECM production (i.e., preventing aging) (Deng et al. 2018).

- Vitamin C

Vitamin C (L-ascorbic acid) is the most copious naturally occurring hydrophilic antioxidant incorporated in multiple dermatological cosmetics to protect and rejuvenate photoaged skin. Unfortunately, it cannot be synthesized by the body instead obtained from dietary sources (fruits, vegetables) or oral/topical supplementations. Topical vitamin C protects the skin from UVB-induced induced erythema, sunburns, photoproducts, and ROS. In other words, it



prevents photoaging and rejuvenates the skin. This occurs upon directly neutralizing ROS through donating it with electrons, inducing collagen synthesis (collagen I and III), and enhancing the activation of lipid-soluble vitamin E (Z. Liu et al. 2018; Farris 2005). This will reduce the membrane-bound  $\alpha$ -tocopherol from its oxidized states and ROS-mediated signaling cascades (JNK, NF $\kappa$ B). Several clinical studies proved such an enhancement of photoaged skin. A 3-month double-blind and randomized study showed that applying 10 percent of vitamin C improves the patients' skin wrinkles, tiredness, and hyperpigmentation brightness compared to control (Farris 2005). Also, vitamin C can prevent ROS-induced inflammation by suppressing transcription factor NF $\kappa$ B, thereby inhibiting the production of proinflammatory cytokines (TNF, Interleukin-1/6/8) (Z. Liu et al. 2018; Farris 2005).

- **Vitamin A (retinoids, carotenoids)**

Similar to vitamin E, vitamin A is a natural lipid-soluble antioxidant. It can be found as one of the two forms: (i) retinol and retinyl esters or (ii) as pro-vitamin carotenoids ( $\beta$ -carotene), and it is usually stored and metabolized in the liver. Vitamin A has many biological roles including, gene transcription, and immune responses, etc.... However, we will exclusively focus on its role in oxidative stress defense. Remarkably, several studies have shown that vitamin A supplementation could act as double hatted depending on its dose, administration mechanism, and the characteristics of the stressors. At low concentrations, Vitamin A acts as an anti-inflammatory and ROS scavenger that targets mainly peroxy and superoxide radicals. At higher concentrations, or upon strenuous exercises, it increases ROS production (superoxide) in parallel to decreasing the activity of the antioxidants, SOD and CAT. Due to this and vitamin A's lipophilic properties, lipid peroxidation and protein damages will occur (Petiz et al. 2017). For example, high concentrations of  $\beta$ -carotene act as prooxidants that boost the oxidative environmental stress's carcinogenic effect.  $\beta$ -carotene enhances lung cancer by increasing the partial pressure of oxygen in patients' lungs, increasing its oxidative metabolites (apocarotenoids) that will alter the retinoic acid level and signaling, and reducing the expression of tumor suppressor genes. Such events escalate further in smokers where  $\beta$ -carotene may interact with the carcinogenic components of cigarette smoke (Russell 2002). More experiments should be conducted to study deeper the role of vitamin A during stress.

- **Vitamin E ( $\alpha$ -tocopherol)**

Vitamin E acts as a ROS scavenger, protector against oxidative and UV-DNA damage (8-oxoGua, CPDs), and inhibitor of CPDs' production in melanocytes, HaCaT keratinocytes, and *in vivo* mouse skin. This could show a link between CPDs' production and ROS level. Vitamin

E may enhance CPDs' repair by inhibiting ROS levels and preventing the latter's drastic effect on different DNA repair enzymes (Delinasios et al. 2018).

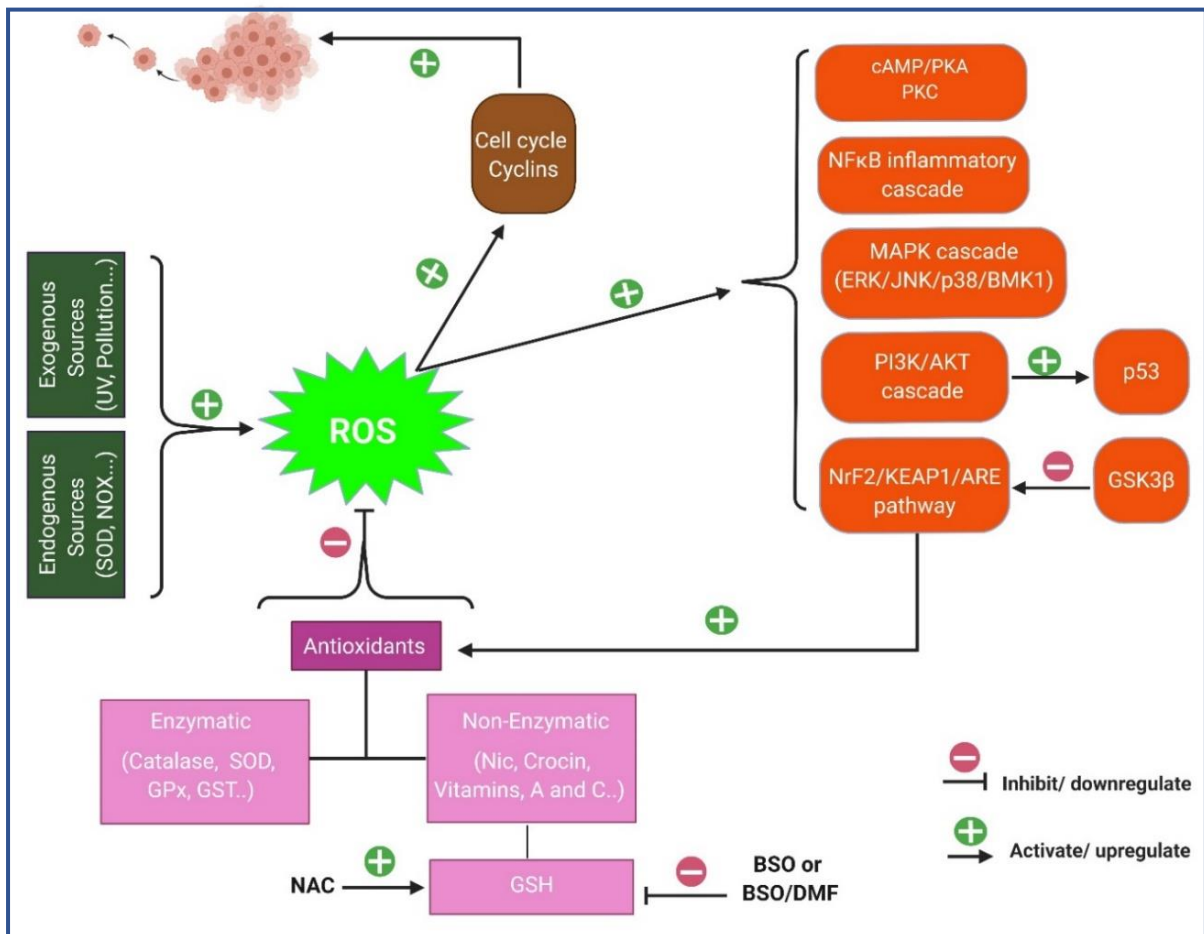
### 1.3. Pathologies linked to oxidative stress

Excessive ROS has been implicated in developing various diseases, including respiratory, digestive, cardiovascular, and neurodegenerative diseases. For example, increased mitochondrial H<sub>2</sub>O<sub>2</sub> was detected in insulin resistance and subsequent type 2 diabetes mellitus (T2DM) animal and human models. Similarly, high ROS contribute to the development of neurodegenerative diseases such as Alzheimer's disease (AD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), and spinocerebellar ataxia (SCA). Premature aging was found as a progressive step in AD. As Denham Harman suggested in the 1950s, aging is another field in which ROS is involved. Such an increase in the steady-state level of ROS in parallel to a decrease in antioxidants can oxidatively modify cellular proteins, lipids, and DNA; consequently, it contributes to cellular aging (Z. Liu et al. 2018; Finkel 2011). More importantly, oxidative stress plays a role in most carcinogenesis steps: (i) initiation by accumulating DNA mutations and altering the cellular energy and metabolism, (ii) promotion of cancerous cells' expansion by altering the cellular signaling cascades (i.e., enhances oncogenes and inhibit pro-apoptotic transcription factors), and (iii) progression (metastasis) by upregulating matrix metalloproteinases and downregulating the action of anti-proteases and angiogenesis (Z. Liu et al. 2018). These cancers may be internal (breast, lung, liver, colon, prostate, ovary, and brain) or skin cancers (NMSC and MSC) (S. K. Saha et al. 2017).

***Hence, why ROS has such a dramatic effect on cells? How can it target the cellular DNA, accumulate mutations, and trigger their escape from cellular repair mechanisms?***

ROS is involved in the regulation of different DNA repair pathways. For instance, it can inhibit OGG1's glycosylase activity, a factor involved in BER. This will lead to the accumulation of oxidative DNA lesions and single-strand breaks that may be converted into double-strand breaks (Srinivas et al. 2019). Such a link will be further discussed in detail throughout the manuscript.

## 1.4. Summary for ROS



**Figure 10. ROS: mechanisms of actions and alterations.** Either endogenous or exogenous processes can produce ROS. It will affect several signaling cascades (MAPK, NFκB, etc..) that could alter proliferation and cell survival and alter cell cycle cyclins to induce cancer initiation, promotion, and metastasis. In parallel, antioxidant signaling mechanisms, such as Nrf2, will be triggered as a defense mechanism. This will induce antioxidants activation. These antioxidants can be either enzymatic or non-enzymatic. They can be manipulated by pharmacological drugs (upregulated by NAC and downregulated by BSO or BSO/DMF).

## 2. Chapter Two: The Base Excision Repair Pathway (BER)

### 2.1. Overview of BER pathway

BER is a frontline repair pathway responsible for maintaining genome integrity, preventing premature aging, malignancy, and many other diseases assumingly occurring at the G1 phase of the cell cycle (Dianov and Hübscher 2013). As presented in figure 11, it is initiated by one of at least 11 distinct DNA glycosylases, depending on the type of base lesion (deaminated, methylated, or oxidized) (Krokan and Bjørås 2013). OGG1 and MYH are the most common nuclear glycosylases that we will focus on in this manuscript due to their role in recognizing and excising the most frequent oxidative DNA lesion, 8-oxoGua. The former recognizes the lesion directly; meanwhile, the latter recognizes the substrate A opposing 8-oxoGua (Krokan and Bjørås 2013). The arising baseless site (also called abasic site, apurinic/aprimidinic site, or AP site) is further processed by an AP endonuclease (APE1) that carries both an AP-endonuclease activity and a redox function required for activation of several transcription factors. It also protects against oxidative stress. APE1 cleaves the phosphodiester bond 5' to the AP site, generating a single-strand break containing a hydroxyl residue at the 3'-end and deoxyribose phosphate at the 5'-end (Krokan and Bjørås 2013; Wallace, Murphy, and Sweasy 2012). A DNA polymerase then fills single-strand breaks in, either through single nucleotide (short) or long-repair patch sub-pathways. The one nucleotide gap is filled in by the polymerase  $\beta$  (POL $\beta$ ), followed by strand sealing by ligase 3-XRCC1 complex. Meanwhile, long-patch BER occurs when DNA polymerase  $\delta/\epsilon$  initiates polymerization from the free 3'-OH adjacent to the deoxyribose phosphate (dRP) group by incorporating between 2 to 15 nucleotides displacing the flap strand, a strand containing the 5'-dRP. This occurs with the help of FEN1 nuclease and PCNA (figure 11) (Krokan and Bjørås 2013).

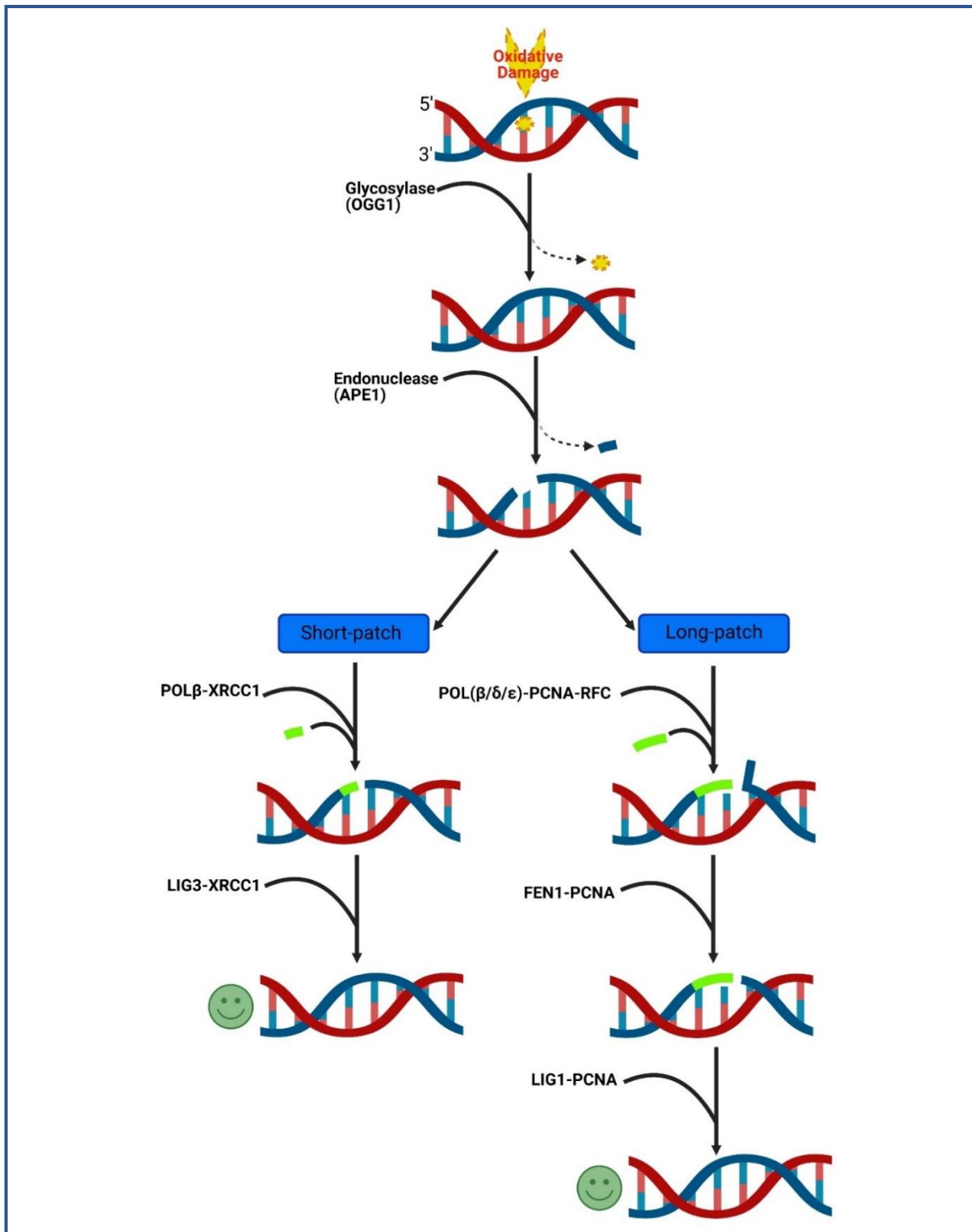


Figure 11. A simplified schematic representation of BER pathway. Exogenous or endogenous stresses induce a damaged base in the DNA (here, we took oxidative DNA damage as an example). Base damage is recognized by a lesion-dependent DNA glycosylase (OGG1) that will use its glycosylase activity for base excision leading to the formation of an abasic site. This abasic site will be recognized by 5'APE1 endonuclease as a rate-limiting enzymatic step. APE1 will cleave the site leaving behind a single-strand DNA break (SSB) with 3' OH and 5' deoxyribose phosphate (5'dRP) termini. This SSB will be recognized and repaired by either short-patch or long-patch sub-pathways: For the short-patch sub-pathway, POLB will synthesize a new nucleotide base for the LIG3-XRCC1 heterodimer to ligate the strand. However, for the long-patch sub-pathway, FEN1-PCNA will remove the generated 5'flap post-polymerization by POLδ/ε-PCNA-RFC. Then the strand will be sealed by LIG1 and its accessory protein PCNA. Note: XRCC1 acts as an accessory protein and coordinator for LIG3, PARP1, and POLβ.

## 2.2. BER factors roles and post-translational modifications

Different post-translational modifications (PTMs) have emerged as regulators of BER's localization, activity, and interactions (figure 12). These include acetylation, methylation, phosphorylation, SUMOylation, and ubiquitination (Carter and Parsons 2016).

**OGG1** consists of several isoforms, mainly OGG1- $\alpha$  and OGG1- $\beta$ , in the nucleus and mitochondria, respectively. It is a multifunctional glycosylase protein that is involved in DNA repair (cleavage of the glycosidic bond of a DNA base lesion and form DNA strand breaks) and in transcription regulation [interaction with NF $\kappa$ B and specificity protein 1 (Sp1)] (R. Wang et al. 2018). Following oxidative stress, p300 enzyme acetylates OGG1's lysines 338 and 341 (K338 and K341, respectively) to increase OGG1's turnover on AP sites that'll increase BER's activity (Carter and Parsons 2016). Also, tyrosine kinase c-Abl, PKC, and cyclin-dependent serine/threonine kinase 4 (Cdk4) phosphorylate OGG1's serine residues to stimulate OGG1's activity (Carter and Parsons 2016). On the other hand, ubiquitination by chaperone-dependent E3 ubiquitin-ligase triggers proteasomal degradation (Carter and Parsons 2016).

**MYH** encodes for at least 10 different protein glycosylases isoforms (three primary transcripts,  $\alpha$ ,  $\beta$ , and  $\gamma$ ) that are involved in DNA repair (excision of A bases opposing 8-oxoGua) at nuclear and mitochondrial levels (Markkanen, Dorn, and Hübscher 2013). *In vitro* study revealed that colorectal cancer was proposed due to MYH dephosphorylation (Carter and Parsons 2016; Markkanen, Dorn, and Hübscher 2013). Accordingly, PKC phosphorylates MYH's serine 524 (S524) to increase its DNA glycosylase activity and regulates repair by altering its binding to PCNA (Carter and Parsons 2016; Kundu et al. 2010). On the contrary to OGG1, MYH's ubiquitination by Mcl-1 ubiquitin ligase E3/ARF binding protein 1 (Mule/ARF-BP1) on at least one of five C-terminal lysine residues, between amino acids 475 and 535, stabilizes it, prevents its degradation, and influences subcellular localization and/or DNA binding (Carter and Parsons 2016).

**APE1** is a multifunctional protein that plays a role in BER and gene regulation (redox-dependent transcription activator and a co-repressor responding to intracellular calcium influx). Therefore, PTMs are needed to regulate and equilibrate the different roles of APE1 for cellular stability (Busso, Lake, and Izumi 2010). While the effect of acetylation appears to be minimal to APE1's DNA repair activity, it plays a role in APE1's transcriptional regulation and calcium-

dependent parathyroid hormone repression activities (Busso, Lake, and Izumi 2010). K6 and K7 lysine residues' acetylation by histone acetyltransferase (HAT) p300 enhances the gene repressor function of APE1 (Busso, Lake, and Izumi 2010). *In vivo* acetylation of K27, K31, K32, and K35 enhance APE1's interaction with RNA to modulate the latter's metabolism (Carter and Parsons 2016). SIRT1, a member of the HDAC deacetylase family, was shown to increase the APE1-XRCC1 interaction and regulate APE1's gene regulatory function (Busso, Lake, and Izumi 2010; Carter and Parsons 2016; Yamamori et al. 2010). Upon oxidative stress, APE1 deacetylation allows its de-attachment from genes' promoters for their expression inhibition, including parathyroid hormone gene and Y-box-binding protein 1 (YB-1); subsequently, it triggers its BER functional activity (Busso, Lake, and Izumi 2010; Yamamori et al. 2010). Another major modification is phosphorylation by serine/threonine casein kinases and protein kinase C, altering its endonuclease activity but enhancing its redox activity (Busso, Lake, and Izumi 2010; Choi, Joo, and Jeon 2016). Also, APE1's phosphorylation by cyclin-dependent kinase 5 (Cdk5)/p35 at threonine 233 (T233) inhibits its endonuclease activity and enhances its ubiquitination (Carter and Parsons 2016). APE1's ubiquitination by E3 ubiquitin ligase at multiple lysine residues near its N-terminus (K6, K7, K25, K25, K27, K31, K32, and K35). Upon stress, MDM2 is activated to mono-ubiquitinate APE1 post-T233 phosphorylation by CDK5. This will alter APE1's DNA repair and gene regulation functions which will trigger polyubiquitination, consequently degradation in the presence of oligomerized MDM2 (Carter and Parsons 2016; Busso, Wedgeworth, and Izumi 2011).

**POL $\beta$**  is a primary DNA polymerase that catalyzes the synthesis of new DNA nucleotides into the DNA strand. It is regulated at stability and activity levels by different modifications. For instance, POL $\beta$ 's lysine 72 (K72) is acetylated by p300 acetyltransferase as a regulatory step to inactivate its activity when not needed or upon shifting from short-patch to long-patch BER (Carter and Parsons 2016). Meanwhile, its arginine 137 (R137) is methylated by methyltransferases to inhibit its interaction with PCNA. This prevents POL $\beta$ 's involvement in PCNA-dependent processes, including long-patch BER. In contrast, an *in vitro* study showed that methylation of POL $\beta$  on arginines 83 and 152 (R83 and R152) by PRMT6 enhances the binding of the enzyme to DNA and increased processivity (Carter and Parsons 2016). However, methylation at Arg137 prevents its interaction with PCNA, thereby inhibiting its role (M. Bai et al. 2020). Ubiquitin proteasome pathway (UPP) regulates POL $\beta$ 's protein levels at steady and oxidative states. This is due to polyubiquitination or monoubiquitination within the 8-kDa N-terminal domain (K41, K61, and K8) containing the  $\beta$  lyase activity by the E3 ubiquitin

ligase CHIP and E3 ubiquitin ligase Mule/ARF-BP, respectively. It occurs when POL $\beta$  is free in the cytoplasm in the absence of DNA lesions. On the contrary, upon DNA damage, the interaction between XRCC1-LIG3 and POL $\beta$  on DNA stabilizes the polymerase to repair efficiently (Carter and Parsons 2016).

**POL $\epsilon$  and POL $\delta$**  are long-patch-DNA polymerases whose PTMs are rarely discussed in publications. However, phosphorylation S458 of POL  $\delta$ 's p68 subunit by protein kinase A (PKA) leads to a decreased binding affinity to PCNA; henceforth, decreased polymerase processivity (Carter and Parsons 2016).

**LIG3** is a DNA ligase that joins DNA strands together by catalyzing the formation of a phosphodiester bond. It has two isoforms that differ at their c-termini, 103-kDa ligase 3-alpha polypeptide expressed in all tissues and cells, and 96-kDa DNA ligase 3-beta polypeptide that is expressed only in the testis (Mackey et al. 1997). We will focus on the former's post-translational modifications since it is the most abundant form and due to the lack of information about the latter's modifications. LIG3-alpha is constitutively phosphorylated by casein kinase II (Cdk2) on its Ser123, in a cell cycle-dependent manner, from early S-phase into M-phase (Dong and Tomkinson 2006). Upon oxidative stress, it will be dephosphorylated in an ATM-dependent manner to repair DNA at S-phase (Dong and Tomkinson 2006). Notably, phosphorylated XRCC1 stabilizes nuclear LIG3 by forming a dimeric complex (Parsons et al. 2010). LIG3's activity in the mitochondria is independent of XRCC1 (Akbari et al. 2014). Lastly, rarely LIG3's ubiquitination is discussed; however, few had shown that CHIP and Iduna/RNF146 could polyubiquitylate and degrade it (Carter and Parsons 2016; Parsons et al. 2008).

**LIG1's** posttranslational modifications are not identified to date. It would be interesting to identify the different modifications it needs to be activated, inhibited, or degraded since it is the major DNA ligase involved in long-patch BER.

**XRCC1** is a scaffold protein that interacts with several enzymes involved in DNA single-strand break repair. XRCC1's phosphorylation on serine 518, threonine 519, and threonine 523 (S518, T519, and T523) by CK2 induces its interaction with the forkhead-associated domain (FHA) of aprataxin, a protein involved in DNA damage signaling and repair, to prevent genotoxicity promoting XRCC1's turnover and stability. Additional phosphorylation within its C-terminal



region promotes XRCC1-PNKP to repair DNA single-strand breaks efficiently. Post-stress (alkylation, oxidation, irradiation), XRCC1 has been reported to be phosphorylated on threonine 284 (T284) and serine 371 (S371) for single-strand and double-strand breaks repair, respectively. Like other proteins, polyubiquitination at the C-terminal by CHIP or E3 ubiquitin ligase Iduna/RNF146 leads to degradation, unless bound to POL $\beta$  or heat shock protein 90 (HSP90) (Carter and Parsons 2016).

**FEN1** is a long-patch BER endonuclease. Post-stress, its lysine residues are acetylated (K354, K355, K377, and K380) by p300, displaying reduced endonuclease activity and DNA binding. This regulates its enzymatic activity and prevents premature processing of Okazaki fragments. Reduction in its endonuclease activity was also observed upon phosphorylation by the Cdk1-cyclin A complex, particularly during the end of the S phase of the cell cycle. Intriguingly, a cross-talk between phosphorylation and other modifications has been reported. Upon oxidative stress, phosphorylation is inhibited while methylation is activated by methylating arginine 192 (R192) for efficient DNA damage repair. It also initiates a cascade of events leading to ubiquitination and degradation of FEN1. Phosphorylation of FEN1 at S187 promotes SUMOylation on lysine 168 (K168) that will trigger polyubiquitination mediated by the E3 ubiquitin ligase pre-mRNA processing factor 19 (PRP19) (Carter and Parsons 2016).

**PCNA** is a scaffold protein involved in DNA repair and replication. Multi-studies have analyzed PCNA's PTMs due to its role in DNA lesion repair during replication. During S-phase, SUMOylation at its Lys164 by Srs2 inhibits homologous recombination and prevents double-strand breaks (DSBs). Meanwhile, ubiquitination at K164 residue by RAD6–RAD18 complex activates the error-prone DNA damage tolerance pathway (TLS). Further ubiquitination, by RAD5 and the UBC13-MMS2 complex, at K63 triggers an alternative template switching mechanism from replicative to translesion synthesis DNA polymerases (ZHU et al. 2014, Spivak 2015).

**PARP1** is an active intermediate member of BER with a high affinity towards DNA SSBs and plays a role in cell cycle regulation. Hence, it is interesting to study its PTMs to target it. This will inhibit SSB repair, by which cancer cells' apoptosis will be triggered. PARP1's acetylation on different lysine residues (K498, K505, K508, K521, and K524) by p300 induces its interaction with the P50 subunit of NF $\kappa$ B to induce its transcriptional activity (Carter and Parsons 2016). However, phosphorylation on its serine 372 (S372) and threonine 373 (T373)

by extracellular signal-regulated kinases 1 and 2 (ERK1/2) are required for its accumulation on damaged DNA (Carter and Parsons 2016). It has been shown that SUMOylation on its lysines 203 and 486 (K203 and K486), in response to heat shock, induces its ubiquitinated clearance and controls its binding to intact and damaged DNA. In contrast, SUMOylating PARP1's lysine 482 (K482) controls its poly(ADP-ribosylation) of chromatin-associated proteins. Once lysine 486 (K486) is SUMOylated, acetylation is inhibited, thereby reducing its coactivator activity and regulating gene expression (Carter and Parsons 2016). As mentioned previously, ubiquitination occurs post-SUMOylation by E3 ubiquitin ligase RNF4. Iduna/RNF146 and RING finger domain protein (CHFR) are other proteins that induce poly(ADP-ribosyl)ated PARP1's ubiquitination and degradation due to elevated alkylated DNA damage and mitotic stress, respectively (Carter and Parsons 2016). This may allow the dissociation of activated PARP1 from damaged DNA sites to promote continuity of DNA repair cascade or prevent cancerous cells' survival.

Further studies should be done to fully understand the importance of post-translational modifications on the different BER factors, their interactions, roles. This could pave the way in front of personalized treatments against cancerous cells. Indeed, other types of modifications may be present in the BER enzymes. Therefore, their cellular consequences and importance should be studied too.

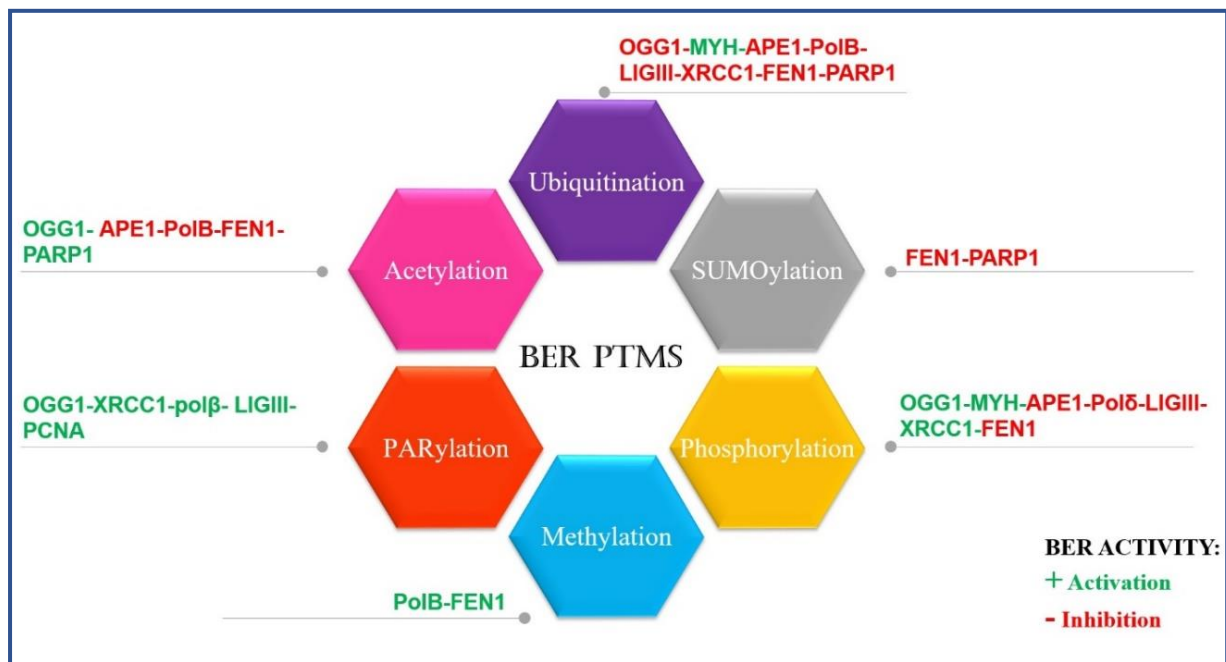


Figure 12. *Post-translational modifications (PTMs) of BER factors.* Several BER factors (main and accessory proteins) are subjected to different PTMs that interfere with their functions, including those involved in BER. These modifications can act as inhibitory (Red color) or excitatory (green color).

### 2.3. BER and human disorders: aging, oxidative stress, and ROS

BER pathway's dysregulation leads to DNA instability in several diseases associated with elevated oxidative stress, aging, and age-related neurodegenerations. Sliwinska et al. showed that *APE1*, *OGG1*, *MYH*, *PARP1*, and *NEIL1* genes are significantly downregulated in Alzheimer's disease patients (Sliwinska et al. 2017). This may explain why oxidative stress and elevated 8-oxoGua in brain cells are the main factors for its pathogenesis (Perry, Cash, and Smith 2002). High 8-oxoGua level and OGG1's loss contribute to Huntington's disease (Krokan and Bjørås 2013). Similarly, defective BER and excessive oxidative stress were reported in Parkinson's disease (Ciccione et al. 2013).

Interestingly, BER is linked to diabetes. Studies have demonstrated that chronic high glucose decreases OGG1's expression via Akt redox-dependent activation (Pang et al. 2012). In parallel, other experimental studies had shown that *Ogg1*<sup>-/-</sup> mice exhibit altered insulin levels, glucose tolerance, adiposity, hepatic steatosis (German et al. 2017). Besides, asthma, a complex chronic inflammatory lung disease, is mediated by multiple inflammatory mediators and ROS-induced oxidative stress. ROS elevates 8-oxoGua in Asthma patients' genome and body fluids (Ba et al. 2015). Therefore, ROS, 8-oxoGua, and BER are directly linked.

ROS are formed as by-products of proficient cellular metabolism and exposure to endogenous or exogenous chemical or physical stresses. Its main target in the DNA is guanine due to its lowest redox potential compared to other nucleic acid bases leading to around 10<sup>5</sup> 8-oxoGua DNA lesions daily per cell (Pang et al. 2012; Ba et al. 2015). Although 8-oxoGua does not induce local DNA structural change, ATP-dependent remodeling of the nucleosomal DNA facilitates lesion accessibility by OGG1. In addition, its Nrf2 binding site at the promoter region regulates the expression upon oxidative stress. This is done via modulating its PTMs (refer to part 2.2.). For example, phosphorylation mediates relocalization, and acetylation enhances its turnover (Boiteux, Coste, and Castaing 2017).

Another DNA glycosylase involved in oxidative stress is MYH. It excises adenine incorporated opposite to 8-oxoGua during replication. This restores G:C base pairs to maintain DNA replication integrity and fidelity (Jingwen Chen et al. 2019). Loss of MYH leads to the accumulation of oxidative DNA damage, including 8-oxoGua, in the liver, heart, kidney, etc.... In parallel, superoxide dismutase (SOD), an antioxidant associated with ROS activity, was downregulated in mouse *Myh*<sup>-/-</sup> kidney, lung, and hippocampus. This leads to high oxidative stress and numerous pathologies such as colorectal cancer, MYH-associated polyposis (MAP), and aging (Jingwen Chen et al. 2019). Chen et al. indicated that MYH deficiency induces

oxidative damage-response that is not efficient in the presence of excessive damage burden (Jingwen Chen et al. 2019).

#### 2.4.BER variants/mutations and cancer (skin and internal)

Studies showed a relationship between BER genes' alterations and cancer progression (H. Chen et al. 2019). 8-oxoGua, the most abundant consequence of ROS, is the substrate for both MYH and OGG1; consequently, a mutation in one or both glycosylases leads to dramatic consequences, especially when the mutation is at their C-terminal binding site. This mutation leads to protein's structural deformation and dysfunctionality (Rizzolo et al. 2018).

Studies have shown at least 30 mutations in the *MYH* gene predicted to truncate the protein, including nonsense, small insertions and deletions, and splice site variants (Wallace, Murphy, and Sweasy 2012). G:C to T:A transversions are considered a genetic signature of defective MYH protein activity in patients, including colorectal cancer patients (Ali et al. 2008). *MYH* single nucleotide polymorphism (SNP; rs3219487) with G/A and A/A genotypes (OR=9.27, 95% CI=2.39–32.1) was related to increased risk for liver and hepatocellular carcinoma compared to G/G genotype (Sakurada et al. 2018). G/A *MYH* genotype was also detected in breast and endometrial cancer patients (Out et al. 2012; Barnetson et al. 2007). Having the minor allele A in MYH decreases its transcription and activity in the carriers (Sakurada et al. 2018). Ty165Cys and Gly382Asp *MYH* variants lead to MYH-associated polyposis (MAP), an autosomal disorder with risks to develop colorectal, thyroid, and duodenal cancer. You et al. had shown that patients with a homozygous variant *MYH* genotype for rs3219472 have a high risk of developing bile duct cancer (You et al. 2013). Also, *MYH* Gln324His polymorphism seemed to increase the risk of lung (*His/His* genotype, OR= 3.03, 95% CI= 1.31–7.00) and colorectal (*Gln/His* and *His/His* genotypes, OR=4.08, 95% CI= 1.22–13.58) cancer incidences in the Japanese population (Miyaiishi et al. 2009). Additionally, an Italian study had identified p.Arg245His variant in male breast, colorectal and gastric cancers, p.Tyr179Cys, and p.Gly396Asp in breast and colorectal cancers, p.Gln338His in women breast cancer and p.Gly264Trpfs\*7 variant in male breast cancer (Rizzolo et al. 2018).

*OGGI* mutations also contribute to different cancer types, where studies have identified *OGGI* missense mutations in 3 out of 40 lung and kidney tumors (Chevallard et al. 1998). *OGGI* Gly308Glu acts as a low-penetrance allele that contributes to colorectal cancer, while SNP rs2304277 increases cancer risk (ovarian..) in *BRCA1* carriers due to lower *OGGI* gene expression levels, consequently, higher DNA damage, genomic instability, and telomeres shortening (Benitez-Buelga et al. 2016; Smith et al. 2013). This polymorphism was also linked

to urothelial bladder carcinoma (OR=3.55, 95% CI= 1.79-7.06) (Ahmed et al. 2018). *OGGI* Ser326Cys polymorphism (rs1052133) correlates with lower activity and risk of several cancers, including acute lymphoblastic leukemia, esophageal, bladder lung, prostate, gastric, and hepatocellular cancers (H. Chen et al. 2019; Peng et al. 2014; Smal et al. 2018). For example, a meta-analysis study showed that *OGGI* Ser326Cys polymorphism (Cys/Cys vs Ser/Ser homozygous genotype model) increased breast cancer risk (OR=1.14, 95% CI= 1.01-1.29) in the studied population (Moghaddam et al. 2018). Scientists also showed that *OGGI* Ser326Cys polymorphism plays a critical role in the pathogenicity of cervical carcinoma or precancerous cervical intraepithelial neoplasia (CIN III) lesions and participates in the infection process of high-risk human papillomavirus (HR-HPV) infection (H. Chen et al. 2019).

A double mutation in both 8-oxoGua glycosylases, *OGGI* and *MYH*, induces additional tumorigenesis. *Oggi*<sup>-/-</sup>*Myh*<sup>-/-</sup> double knockout mice exhibit a high frequency (65.7 percent) of lung and ovarian tumors and lymphomas (Wallace, Murphy, and Sweasy 2012; T.-H. Lee and Kang 2019; Xie et al. 2004; Tahara et al. 2018).

Other BER factors' SNP genetic variants were associated with increased cancer occurrence. For example, PARP-1 Ala762Ala and P53 Arg72Pro, are significantly associated with cervical cancer and HR-HPV infection, where P53 is considered part of the BER activation cascade (H. Chen et al. 2019). Wild-type P53 stimulates BER by interacting with APE1 and POL $\beta$  and stabilizing the latter's interaction with abasic DNA sites (J. Zhou et al. 2001). Additionally, *XRCC1* R399Q and *APE1* Asp148Glu were associated with an increased risk of breast cancer (T. Wang et al. 2018; Busso, Lake, and Izumi 2010). Also, the SNP combination of APE1 D148 and *XRCC1* R194W was linked to pancreatic cancer risk (Busso, Lake, and Izumi 2010). This position may be vital for APE1 and *XRCC1* interaction. Polymorphisms of *XRCC1* at codons 194, 280, and 399 are associated with a risk of several types of gastrointestinal cancers, breast cancer, and lung cancer. Also, T to C homozygous point mutation at nucleotide 889 (T889C) resulting in the substitution of Leucine by Serine amino acid 259 in POL $\beta$  have been identified to be involved in primary gastric cancer (Tan et al. 2015; Y. Seo and Kinsella 2009). This may be due to some of POL $\beta$ 's mutations that lead to DNA synthesis in low fidelity (Simonelli et al. 2016).

Hence, *could BER's role has a drastic effect on the acidic tumor microenvironment?*

This was well-illustrated by researchers who had found that inhibiting APE1/Ref1 and/or *XRCC1* (BER's factors) through drugs could inhibit cellular survival upon an accumulation of mutations that trigger apoptosis (Y. Seo and Kinsella 2009).

## 2.5. BER and cell cycle

Upon transcriptomic and proteomic data analysis of differently synchronized cell lines, researchers were able to elucidate the cell cycle regulation of different BER factors (figure 13). Short-patch BER expresses its genes equally in all the cell cycle phases (*POL $\beta$* , *APE1*...), with higher APE1's activity at G1-phase in the presence of ionizing radiation (measured by oligonucleotide incision assay); meanwhile, long-patch BER genes are extensively expressed in S-phase (Mjelle et al. 2015; Chaudhry 2007). 4 of the 11 genes encoding DNA glycosylases were cell cycle regulated. For example, *NTHL1* and uracil-DNA glycosylase (*UNG*) were shown to be overexpressed in the G1/S-phase. *UNG* showed upregulation at protein level too. In contrast, thymine-DNA glycosylase (*TDG*) peaks solely at G1-phase. *NEIL3* is upregulated at the S/G2-phase. During S-phase, *NEIL1* showed an upregulation only upon prolonged serum starvation of fibroblasts (for synchronization). Such upregulation of some glycosylases at the S-phase may be due to their defined role pre-/post-/ and during the replicative process. Other glycosylases (*OGG1*, *MYH*...) showed no evidence of cell cycle regulation. Downstream long-patch BER factors (*PARP1*, *PARP2*, *PCNA*, *FEN1*, *POLD1*, *POLD3*, *POLE*, and *POLE2*) were detected steadily regulated at S or G1/S-phase due to their additional roles as replication proteins. Common short and long-patch BER downstream factors, including *XRCC1* and *LIG1* were upregulated at S/G2 and S-phases, respectively. All the regulations mentioned above show that long-patch BER is implicated in proliferating cells, and arrest in G1 prevents the replication of damaged DNA while arrest in G2 prevents segregation of defective chromosomes (Mjelle et al. 2015; Chaudhry 2007).

Additionally, P53 can activate BER factors as APE1 and POL $\beta$  due to direct protein-protein interactions (Fitch 2003).

On a side note, BRG1, a chromatin remodeling factor, has also been proposed to regulate BER's polymerase activity (L. Zhang et al. 2009).

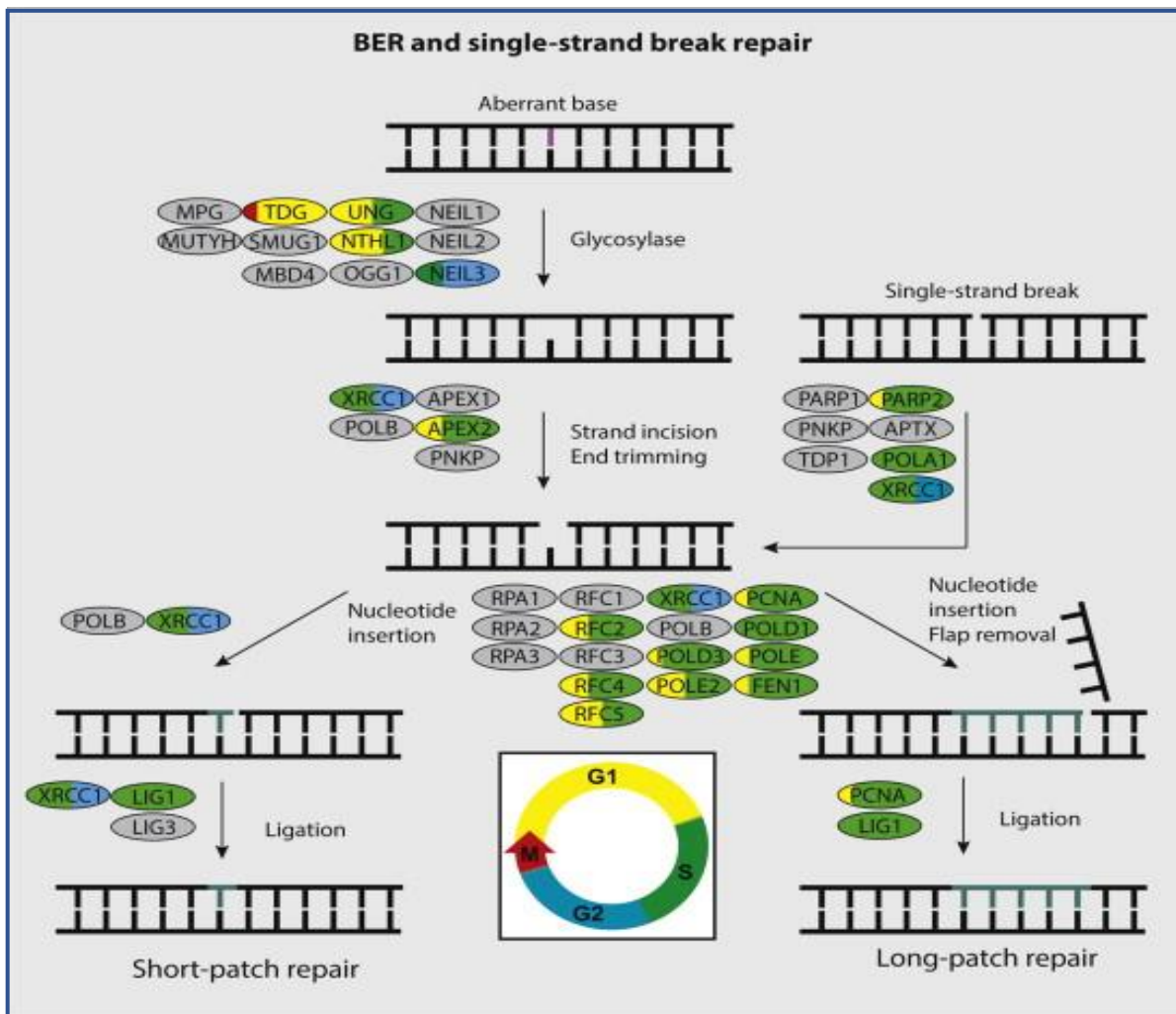


Figure 13. **Cell cycle regulated BER genes** (Mjelle et al. 2015). The genes are color-coded based on their level of expression in the different cell cycle phases. Only gene products shown to be cell cycle regulated in at least two transcriptome or translational studies are colored. Gray color-coded genes show that they were not found to be cell cycle regulated.

## 2.6. BER targeted treatment: The debased side of BER

Numerous *in vitro* and *in vivo* studies have been trying to find therapeutic drugs targeting or enhancing the different DNA repair pathways for treatments against carcinogenesis and metastasis. Despite the challenges and complications, DNA repair inhibitors that block a specific protein or multi-proteins' activities in a particular DNA repair pathway paved the way for personalized medicine that could help target chemo-resistant and sensitive cancer cells leading to their apoptosis (Kelley, Logsdon, and Fishel 2014).

BER pathway is a double-edged sword where its inhibition or overexpression may be cancerogenic. For example, studies found  $POL\beta$  overexpression at mRNA and protein levels in uterus, ovary, prostate, and stomach cancers due to genetic instability by facilitating cell survival to augment DNA repair capability and bypass various DNA damages causing apoptosis by commonly used chemotherapeutic drugs (Chan et al. 2007). Hence,  $POL\beta$  exhibits

dichotomous roles depending on its expression, either as an oncogene or tumor suppressor (Chan et al. 2007). Similarly, some reported OGG1's overexpression in ulcerative colitis-associated carcinogenesis and esophageal squamous cell carcinoma (Kumagai et al. 2018). This may reflect the persistence of cancer cells against stress, such as oxidative stress.

PARP1 has been the interest of scientists due to its dual role in regulating oncogenes and tumor suppressors' expression and DNA repair pathways, including BER. It has a high expression in some cancers as it activates OGG1's expression at the G1-phase. Some PARP1 inhibitors have already been in use (Olaparib) in BRCA-deficient cancers. In addition, iCDK4/6 (PD0332991, LEE011) was shown to be an efficient anti-cancer treatment for lung cancer cells since it impairs OGG1-dependent BER and sensitizes cancer cells to oxidative imbalance-induced death by decreasing PARP1 transcriptional expression. However, this dual treatment has its weak points. Tempka et al. suggested that for this treatment to be efficient, a functional link should exist between RB1 (retinoblastoma protein), PARP1, and OGG1. Unfortunately, RB1 is mutated in most retinoblastomas, osteosarcomas, small cell lung cancers, and other cancer types at lower frequencies. This may prevent efficient PARP1 targeting. Therefore, genomic and transcriptome profiles should be checked before administering PARP1 suppressive agents such as CDK4/6 inhibitors (Tempka et al. 2017).

8-oxoGua's repair is indispensable for cell survival. Inhibiting OGG1 by monotherapy or in combination with DNA damaging agents could also be interesting to target cancer cells where the loss of OGG1's function has been shown to sensitize cells to multiple chemotherapies and irradiation (IR) (Tahara et al. 2018). A study had suggested hydrazine and hydrazone as specific inhibitors of Schiff base formation during OGG1-mediated catalysis after screening a ~50 000-molecule Chembridge DIVERset library (Donley et al. 2015). In 2018, Tahara et al. screened almost 25975 potential compounds that could inhibit OGG1's activity. They found a new compound, SU0268, which has an acyl tetrahydroquinoline sulfonamide skeleton and is more potent than the previous inhibitors. The prior compounds exhibited delayed kinetics of inhibition, but SU0268 is a non-cytotoxic and specific compound that binds directly to the OGG1 enzyme in HEK293T and HeLa cells to inhibit its base excision activity (Tahara et al. 2018). Further studies are being applied in cellular and animal models of various disorders. TH5487 is another OGG1's DNA glycosylase incision activity inhibitor as it prevents OGG1 from binding to 8-oxoGua in DNA (Visnes et al. 2018). It also decreases proinflammatory gene expression, which may be a potentially helpful strategy for treating inflammation (Visnes et al. 2018). OGG1's downregulation has been shown to elevate the efficacy of bleomycin, an anticancer drug, to human lung adenocarcinoma cells (S. Liu, Wu, and Zhang 2010).



Unfortunately, OGG1 inhibitors have not reached clinical trials; however, different chemical screenings of pharmacological libraries are being done producing potential hits (Mechetin et al. 2020).

Scientists are focusing on a combinational treatment of OGG1 and MTH1 inhibitors to block the whole GO system triggering excessive oxidative stress triggering cancer cells' death. They had already identified an MTH1 inhibitor, crizotinib. Crizotinib is a chiral compound that can be: (R)-enantiomer, which blocks kinases (c-MET, ALK...) and (S)-enantiomer, which binds to and blocks MTH1. In addition, another MTH1 inhibitor, karonudib (TH1579), has been recently registered in two-phase one-clinical trials, and a possible phase two clinical trial was proposed (Mechetin et al. 2020).

Finally, POL $\beta$  inhibition has been shown to enhance the efficacy of some alkylating agents used in chemotherapies as mustard compounds, oxaliplatin, and temozolomide (S. Liu, Wu, and Zhang 2010). This is due to the DNA repair blockage. For instance, *in vitro* and *in vivo* studies showed that NSC666715 POL $\beta$  inhibitor enhances temozolomide sensitivity in colon cancer cells (S. Liu, Wu, and Zhang 2010).

### 2.7.BER and Drugs: Acetohexamide (ACETO)

*(experiments were done on ACETO but were not included in the manuscript)*

Interestingly, in 2014, Alli et al. published for the first time **acetohexamide** as a potential chemo-preventative and DNA repair enhancer agent against mutated BRCA1-associated malignancies. Due to the known role of BRCA1 in BER of oxidative DNA damage, its mutation limits BER's activity which will induce excessive oxidative DNA damage leading to genomic instability and high cancer risks. Such cancers are aggressive and prevalent. Therefore, they conducted high-throughput chemical screening, then transfected cells with GFP plasmid containing oxidized bases and identified ACETO (20 $\mu$ M) as a potential enhancer of BER's activity and inhibitor of basal and induced 8-oxoGua levels in BRCA1-mutated cells, with minimal cytotoxicity. Another DNA repair-activating agent showing similar results was benserazide. According to the U.S. National Library of Medicine TOXNET database, ACETO and benserazide are FDA-approved drugs lacking chemical, toxicological, and environmental threats. They may target directly or indirectly BER enzyme(s)/protein(s), inhibit BER's negative regulators, or they may activate BER's positive regulators. Hence, further studies must be investigated in addition to studying the structure-activity relationship deeply to avoid any unneeded adverse effects (Alli et al. 2014). For example, ACETO is used as an antidiabetic drug, i.e., it lowers blood glucose level by regulating insulin through targeting ATP-sensitive

potassium channels (Alli et al. 2014; Mazouzi et al. 2017). This may lead to hypoglycemia in cancer patients if left unmodified. These drugs may be effective for other DNA repair-deficient diseases or malignancies associated with oxidative stress or cancers (Alli et al. 2014). Another chemical screening study proved that ACETO plays a role other than quenching ROS and its effects; it can act as pyrimidine dimers repair enhancer (i.e., NER enhancer). It enhances the removal of pyrimidine dimers in NER-deficient cells. This may be through antagonizing MYH DNA glycosylase, thereby decreasing its binding to UV-DNA damage, such as CPDs. This synthetic viability concept could enhance pyrimidine photoproducts repair in an unknown NER-independent mechanism (Mazouzi et al. 2017). Therefore ACETO seems like an interesting drug to study as a potential treatment for DNA repair-deficient patients.

### 2.8. BER and other repair pathways

The body has developed a direct/indirect interaction network amongst the different DNA repair systems to provide the ultimate protection against the various stresses a human body could encounter. For example, during the repair process, BER factors are known to dynamically orchestrate with other DNA repair proteins to modulate the efficiency of their activity and to prevent accumulation of their DNA damage intermediates, i.e., apurinic/aprimidinic (AP) sites, single-strand, and double-strand breaks (table 1).

**Non-homologous End Joining (NHEJ), Homologous Recombination (HR), and BER:** NHEJ and HR repair double-strand breaks at G<sub>0</sub>-G<sub>1</sub> and S-G<sub>2</sub> phases, respectively. These double-strand breaks can occur as BER intermediates if close lesions are being repaired simultaneously. A lot of proteins from diverse DNA repair pathways are required for NHEJ, including NTHL1 and UNG. However, RAD52, HR factor, and OGG1 have reciprocal interactions. RAD52 enhances OGG1 turnover and 8-oxoGua incision. Conversely, OGG1 inhibits RAD52 catalytic activity. This may be because BER inhibits HR to induce NHEJ (Limpose, Corbett, and Doetsch 2017).

**Mismatch repair (MMR) and BER:** both repair pathways protect against oxidized DNA damage (8-oxoGua). MMR can recognize these lesions by MSH2/MSH6. Researchers demonstrated that MSH2/MSH6 heterodimer interacts with MYH to induce the latter's binding and glycosylase activity. This may be through PCNA acting as a coordinator between both repair systems (Cheadle and Sampson 2003).

**NER and BER:** several NER proteins have shown an interactive regulatory role towards BER. XPC, a GG-NER initiator, interacts with TDG (G:T mismatch glycosylase) directly and indirectly with OGG1 to enhance their turnover and activity. Probably, this is done upon an explicitly direct interaction between XPC and APE1 that will enhance the glycosylases excision from DNA (Limpose, Corbett, and Doetsch 2017; de Melo et al. 2016). Such a vital role of XPC in BER started after researchers found that fluorescently labeled XPC localizes to the nucleolus upon inducing oxidative DNA damage, 8-oxoGua (Limpose, Corbett, and Doetsch 2017). It may be because 8-oxoGua subverts the DNA helix's major groove cation binding and disrupts the bases' hydrophilicity (Menoni, Hoeijmakers, and Vermeulen 2012). This distortion is directly recognized by XPC that will activate both NER and BER pathways (Menoni, Hoeijmakers, and Vermeulen 2012). Similarly, such stress induces fluorescently labeled CSB, TC-NER factor, localization to the nucleolus and nucleoplasm where *CSB*<sup>-/-</sup> cells were found to be hypersensitive to ROS-inducing agents. CSB interacts with OGG1 indirectly to induce its turnover. Also, it interacts with NEIL1 and NEIL2 glycosylases. Such glycosylases excise oxidized DNA damage: FapyA and FapyG. Both share 8-oxoGua as an intermediate lesion. These lesions were found to increase in *CSB*<sup>-/-</sup> mice compared to control. This is due to the capacity of CSB in modulating the incision and APlyase activities and turnover of NEIL1 and NEIL2. In addition, it binds to APE1 directly to increase its endonuclease activity more than 4 folds. PARP1 and FEN1 are other BER proteins that bind with CSB.

XPG is another NER factor that stimulates NTHL1 glycosylase to recognize oxidized bases and thymine glycols during replication (Limpose, Corbett, and Doetsch 2017).

Understanding the nuances of regulation and interaction between the different DNA repair systems at the proteomic level could act as an effective targeted therapeutic and preventive approach against several disorders and carcinogenesis, including xeroderma pigmentosum (XP).

Table 1. A brief possible interaction of BER factors with other DNA repair proteins/enzymes. The green color identifies a positive interaction that could be direct or indirect. BER: Base excision repair, NER: Nucleotide excision repair, HR: Homologous recombination, and MMR: Mismatch repair.

		BER Factors							BER Accessory Proteins			
		OGG1	MYH	NEIL1	NEIL2	TDG	NTHL1	APE1	Polβ	FEN1	PARP1	XRCC1
NER FACTORS	XPC											
	XPG											
	XPA											
	CSB											
HR FACTORS	RAD51											
	RAD52											
MMR FACTORS	MSH2											
	MSH6											

### 3. Chapter Three: Nucleotide Excision Repair (NER)

#### 3.1. Overview of NER pathway

NER is a highly conserved nuclear repair pathway that recognizes a wide range of DNA mutations altering the DNA double-helical structure. They are bulky lesions including UV-induced photoproducts [CPDs and (6-4) PPs], environmental mutagens (benzo[*a*]pyrene, aromatic amines), some endogenous oxidative DNA lesions (cyclopurines), and intra-strand crosslinks. Their mode of recognition divides NER into two pathways. (i) Global-genome NER (GG-NER) recognizes the lesions across the entire genome (active, silent, and non-transcribed genes) directly by XPC-hRAD23B-centrin 2 (CENT2) complex post-kinking DNA duplex by DDB2 (XPE)-DDB1 complex (figure 14). In comparison, (ii) transcription-coupled NER (TC-NER) recognizes the lesions by CSB/CSA upon RNA polymerase II stalling in actively transcribed genes and ensures a rapid repair (figure 14). Prolonged transcription stalling induces severe cellular damages, including P53-dependent apoptosis (Arima et al. 2005).

After that, in both sub-pathways, TFIIH complex (XPB-XPB, p8, p52, p44...) will be recruited to unwind the DNA creating a ~20 to 30 nucleotide bubble. 5'-3' lesion incision follows this via XPA, XPF/ERCC1 (5' endonuclease), and XPG (3' endonuclease). Then the gap will be re-filled by synthesizing a new DNA sequence via polymerase, mainly POL $\beta$ , and its accessory proteins (PCNA, RCF, and RPA1). Finally, LIG3-XRCC1 or LIG1 ligates the sequence forming an intact DNA (figure 14) (Budden et al. 2016; Schärer 2013; Spivak 2015).

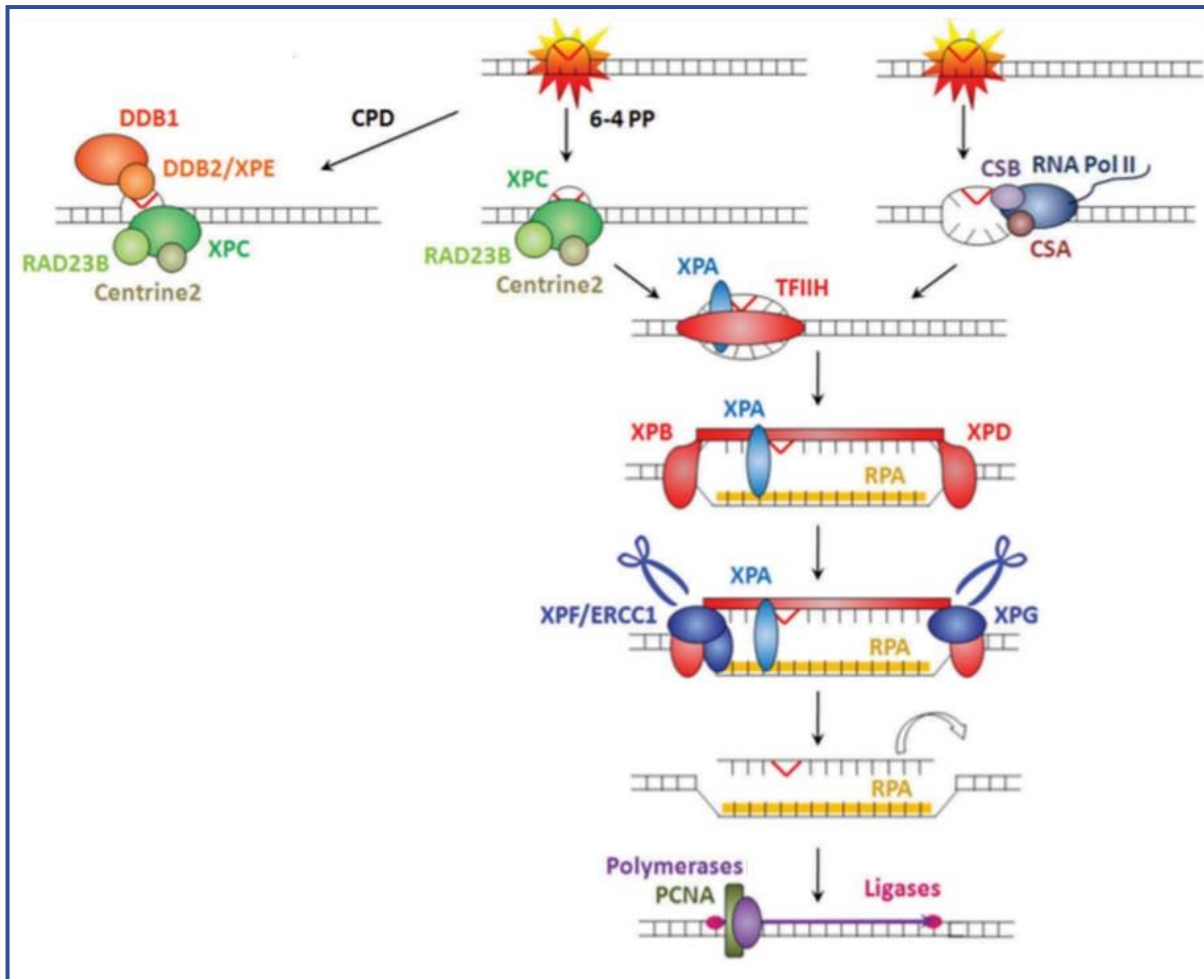


Figure 14. A modified schematic representation of NER pathway (Goncalves-Maia and Magnaldo 2017). NER is divided into GG-NER (top left) and TC-NER (top right). **GG-NER:** Endogenous or exogenous stress induces (6-4) PPs that will be recognized by XPC/Rad23B/centrine2 and CPDs identified by DDB1/DDB2 (XPE) then XPC/Rad23B/centrine2. This will initiate a cascade of repair events. TFIIH complex (includes XPB helicase and XPD 5'-3' helicase) will unwind the DNA, and RPA accessory protein will bind to and protect the DNA single-strand. Meanwhile, excision post-damage verification by XPA will occur, followed by the recruitment of ERCC1/XPF and XPG that will catalyze the ATP-dependent incision of 5' and 3' ends of the lesion, respectively. Synthesis of newly DNA strand will occur by DNA polymerase and its accessory proteins as PCNA, RPA, and RFC. Finally, the strand will be sealed by ligase. **TC-NER:** In transcriptionally active genes, RNA polymerase II (RNA Pol II)'s transcription process will be stalled due to bulky lesions triggering TC-NER and recruitment of Cockayne syndrome proteins A and B (CSA, CSB) that will initiate the repair process. The following steps of NER are then common with the GG-NER sub pathway.

### 3.1.1. Photoproducts: CPDs vs (6-4) PPs

CPDs and (6-4) PPs comprise 75 percent and 25 percent, respectively, of total UV-induced photoproducts recognized and repaired by NER (Yokoyama and Mizutani 2014). Their participation in mutagenesis and carcinogenesis arises from their capacity to severely distort the DNA structure, which means disturbing essential cellular processes (DNA replication, transcription, cellular survival, function, cell cycle, etc..) (Lo et al. 2005). This has provoked the researchers to deeply study their repair rate, quantity, cell signaling responses, and differences amongst different models (*in vitro* and *in vivo*) (Lima-Bessa et al. 2008).

**CPDs** are highly mutagenic and are produced in substantial quantities by UVA and UVB-irradiations. They correspond to a dimerization between two adjacent pyrimidines (thymine, cytosine, 5-methyl cytosine, or 5-hydroxymethylcytosine) at carbons 5 and 6, forming a four-membered ring structure (S. Kim, Jin, and Pfeifer 2013; Lo et al. 2005). Once their cytosine/5-methylcytosine is deaminated, CPDs bypass repair triggering mutagenic events, initiating cytokines, inducing photo immunosuppression, and initiating skin cancer. Indeed, the most common mutation post-UV is G:C to T:A transversion that has been described in a lot of genes, including *P53* in melanoma and non-melanoma skin cancers (Delinasios et al. 2018; S. Kim, Jin, and Pfeifer 2013).

Experiments showed that CPDs' repair reduces UV-induced apoptosis, erythema, and hyperplasia *in vivo* and *in vitro*. Contradictory, an *in vitro* experiment treating cells with UVB, CPD/(6-4) PP photolyases, and Annexin V showed that the repair of minor DNA lesions, (6-4) PPs, reduces apoptosis by 70 percent. In comparison, CPDs repair reduces it by 40 percent. Such findings indicate that (6-4) PPs are more apoptotic inducers than CPDs and that the latter are more involved in cell cycle arrest in the absence of increased apoptosis. In other words, CPDs affect repair by halting the cell cycle, which may increase their mutation accumulation, while (6-4) PPs induce apoptosis to eliminate damaged cells, which reduces their mutation accumulation. Such a contradiction amongst publications may depend on *P53*'s studied cells/animals status due to its vital role in triggering cellular death. In this study, *P53* mutated XP cells were used. This may affect the contribution of CPDs in inducing apoptosis. Other studies showed that CPDs decrease UV-induced apoptosis by inducing *P53* mutations and provoking skin carcinogenesis in mice models. However, when *P53* is wild-type, CPD lesions induce apoptosis and cell cycle arrest.

It is worth mentioning that **(6-4) PPs** are *P53*-independent and less mutagenic due to their rapid repair and 5 to 10 folds less frequency than CPDs (Budden et al. 2016; S. Kim, Jin, and Pfeifer 2013). While CPDs arise in nucleosome cores, (6-4) PPs are formed in linker DNA consisting of a non-cyclic bond between two adjacent pyrimidines at carbons 6 and 4 through Paterno-Büchi reaction (Lo et al. 2005; Puumalainen et al. 2016). (6-4) PPs' strong absorption to a 320 nm UV-wavelength leads to an electrocyclization and the formation of dewar isomers (Douki, Koschembahr, and Cadet 2017).

### 3.1.2. NER factors roles and post-translational modifications

Like BER, NER is regulated by various post-translational modifications that could act simultaneously in a coordinated manner (Figure 15).

**XPC** is the initiator of GG-NER; hence, its PTM regulation plays a vital role in protecting cells against DNA damage. For instance, ubiquitination plays an essential role in XPC's turnover. After irradiation, ubiquitination by CRL4<sup>DDB2</sup> E3 ligase increases the DNA binding affinity of XPC, while ubiquitination by RNF111 and its cognate E2-UBC13 releases XPC from the DNA damage site. Through co-immunoprecipitation, researchers found OTUD4 deubiquitinase responsible for removing the ubiquitin moiety from XPC for its turnover (Lubin et al. 2014). Ubiquitination by RNF111 relies on DDB2- and XPA-dependent SUMOylation for XPC's stability (van Cuijk et al. 2015). Such a SUMOylation (Small ubiquitin-related modification) has an opposite effect once created on Lysine (Lys 655) (Park and Kang 2016). Another critical PTM is phosphorylation at various sites, including serine (Ser 61, 94, 397, 399, 883, 884, and 892) and threonine (T169) (Shah et al. 2018). The function of such different phosphorylated sites is still inconclusive. However, Shah et al. identified that phosphorylation at Ser94 and Ser892 regulate ubiquitylated XPC's recruitment to the damage site (Shah et al. 2018). Also, XPC can be PARylated to be more effective in recognizing and binding to DNA lesions (Rechkunova, Maltseva, and Lavrik 2019).

**XPE (DNA Damage Binding Factor 2, DDB2)** recognizes DNA damage sites and acts as a ubiquitination inducing-protein ligase (Krzyszinski et al. 2014). It is PARylated and regulated by XPC for stabilization while ubiquitinated for degradation (Park and Kang 2016, Matsumoto et al. 2015). UVR-induced SUMOylation of DDB2 enhances NER at recognition and processing steps (C. Han et al. 2017).

**CSB (ERCC6)** and CSA initiate TC-NER post-RNA stalling. CSB is degraded by CSA-dependent ubiquitination (Spivak 2015). However, for an efficient function in TC-NER initiation, several PTMs are needed: Phosphorylation is required for CSB nuclear localization, then SUMOylation and PARylation are required for efficient bulky and oxidative DNA damage repair, respectively (Park and Kang 2016).

**TFIIH** is a hetero decameric protein consisting of XPB, XPD, p62, p52, p44, p34, p8, Cdk7, Cyclin H, and Mat1. In addition to its role in NER, it is involved in transcription, chromatin



remodeling, and ubiquitination (Krzyszinski et al. 2014; Sandoz et al. 2019). Phosphorylation of XPB and ubiquitination of XPD inhibits NER by inactivating the former proteins (Park and Kang 2016; Rechkunova, Maltseva, and Lavrik 2019).

**XPG (ERCC5)** nuclease plays a role in assembling the preincision NER complex and 3' incision to the damaged site in the DNA. It is acetylated to be stabilized at the DNA damage site while ubiquitylated for degradation (Park and Kang 2016).

**XPA** is recruited to the DNA damage site by TFIIH complex to verify the lesion and the assembly of NER incision complex. It is stabilized and activated by phosphorylation stabilizes it. Meanwhile, acetylation and ubiquitination degrade XPA and inhibit its activity in lesion incision with ERCC1 (Rechkunova, Maltseva, and Lavrik 2019).

**ERCC1** is involved in the incision step of NER. It is polyubiquitinated at its C-terminal to form a complex with XPF (Borsos, Majoros, and Pankotai 2020).

**DNA Polymerase  $\beta$  and  $\delta$**  are previously mentioned in section 2.2.

**XRCC1's** post-translational modifications are previously mentioned in section 2.2.

**PCNA's** post-translational modifications are previously mentioned in section 2.2.

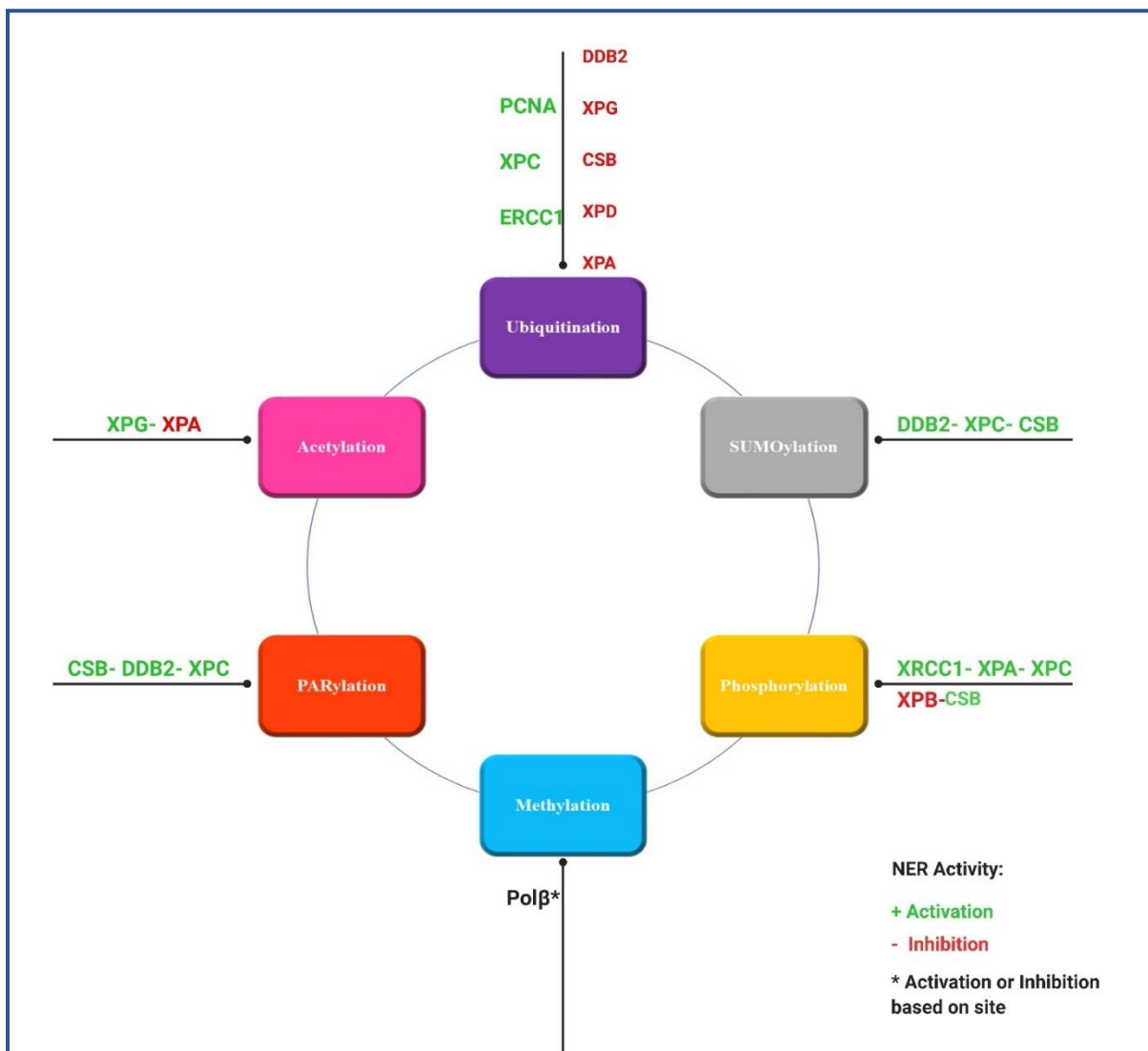


Figure 15. **Post-translational modifications (PTMs) of NER factors.** Several NER factors (main and accessory proteins) are subjected to different forms of posttranslational modifications that interfere with their function, including that involved in NER. These modifications can act as inhibitory (Red color) or excitatory (green color).

### 3.2. NER and cell cycle

In general, NER factors are linked to cell cycle checkpoints for regulating apoptosis, cell cycle arrest, and DNA repair and augmenting genomic stability and cell survival (Lu Liu et al. 2016). Although downstream genes (polymerases, ligases...) are upregulated at transcriptional and/or translational level in the G1/S-phase, the genes involved in the initial steps (XPC, CSA, CSB, XPA, XPB, XPD, XPE, XPG...) are expressed independently of the cell cycle (Mjelle et al. 2015). For example, XPF is not regulated at the transcriptional level rather upregulated at the translational level in G1/S/M phases (Mjelle et al. 2015). Although XPA's expression is independent of the cell cycle, it is exported to the nucleus to induce P53-independent NER due to UV in the S-phase (Mjelle et al. 2015).

### ***What about the regulation of the cell cycle by NER?***

DNA damage response (DDR) is a signaling pathway that is driven mainly by protein phosphorylation and contains sensors, transducers, and effectors that will ameliorate the outcome of genotoxic stresses (intrinsic and extrinsic) to maintain genomic stability (Maréchal and Zou 2013). Two primary transducers of the DDR signaling pathway and cell cycle are ATM (ataxia-telangiectasia mutated) and ATR (ATM- and Rad3- related) kinases. Even though both kinases belong to PIKK (phosphoinositide-3-kinase-related-protein kinase) family and are activated post-DNA damage to initiate DNA repair and checkpoint arrest, they differ in their damage selectivity (Maréchal and Zou 2013; A. Ray et al. 2016).

Briefly, ATR is activated by UV-induced single-strand DNA gaps in the G1-phase and by replication stalling due to bulky lesions [CPDs and (6-4) PPs] in the S-phase. This will trigger the phosphorylation of CHK1 and Cdc25 phosphatase causing cell cycle arrest and the activation of the DDR pathway, which includes a complex of proteins as ATRIP-ATR complex, TopBP1, MRE11, Rad50, and Rad17.

If replication halting persists, DNA double-strand gaps will be formed, inducing the activation and recruitment of ATM. ATM will phosphorylate Chk2 phosphatase that will phosphorylate Cdc25 for cell cycle arrest. In the presence of DNA double-strand breaks, ATR and ATM phosphorylate histone H2AX ( $\gamma$ H2AX) and BRCA1, double-strand breaks, and DNA repair biomarkers, respectively (A. Ray et al. 2016). Interestingly, Ray et al. proved by single-cell analysis that ATR/ATM are phosphorylated and recruited to the UV-induced DNA damage sites in DDB2-XPC- and XPA- dependent manners during the G1-phase and not S-phase (Budden et al. 2016; A. Ray et al. 2016). For instance, no defect in S-phase was detected in XP-C fibroblasts despite the accumulation of mutations and single-strand DNA breaks due to the dysregulated DNA damage repair (A. Ray et al. 2016). Besides, in the presence of cisplatin (alkylating agent), XP-C cells were identified lacking caspase-2 and caspase-3 activation, thereby delayed or diminished apoptosis (Budden et al. 2016). The relation between XPC and apoptosis was also resembled in DDIT3-RPS3A-XPC regulated apoptotic pathway (Lubin et al. 2014).

These may be some of the reasons explaining the 10000-fold increase in skin cancer incidences in XP- patients (A. Ray et al. 2016). In conclusion, NER, GG-NER particularly, is tightly linked to the cell cycle and checkpoint pathway by regulating the upstream proteins (ATR/ATM).

Additionally, Krzeszinski et al. showed that XPC triggers post-ubiquitination MDM2-dependent P53 proteolysis via Rad23 to reset cells back to their quiescent state post-repair (Krzeszinski et al. 2014). In parallel, P53 upregulates UV-induced DDB2 (p48) and XPC

expressions, stimulates nuclear import of XPA, and promotes histone modification to facilitate XPB binding to DNA lesions (Krzyszinski et al. 2014; Fitch 2003).

### 3.3. NER and human disorders

**Cockayne Syndrome (CS)** is mainly due to mutations either in the *CSA (ERCC8)* or *CSB (ERCC6)* genes (figure 16). Recently, it has been found that mutations in *XPF (ERCC4)* or *ERCC1* may also play a role in such a disorder. This leads to TC-NER deficiency. Some of its main characteristics are photosensitivity, neurological disorders, microcephaly, hypertension, and progeria with shortened life span. Surprisingly, there have been no reports of carcinogenesis in CS-diagnosed patients (Spivak and Hanawalt 2015).

**Cerebro oculofacial-skeletal syndrome (COFS)** is due to mutations in *CSB*, *ERCC1*, *XPD*, or *XPG*. It shares some characteristics with CS, including photosensitivity. Some of its additional traits are hypotonia, poor vision, and abnormal kidneys and heart. The severity of its symptoms leads to a short life span or lethality during infancy (Spivak and Hanawalt 2015).

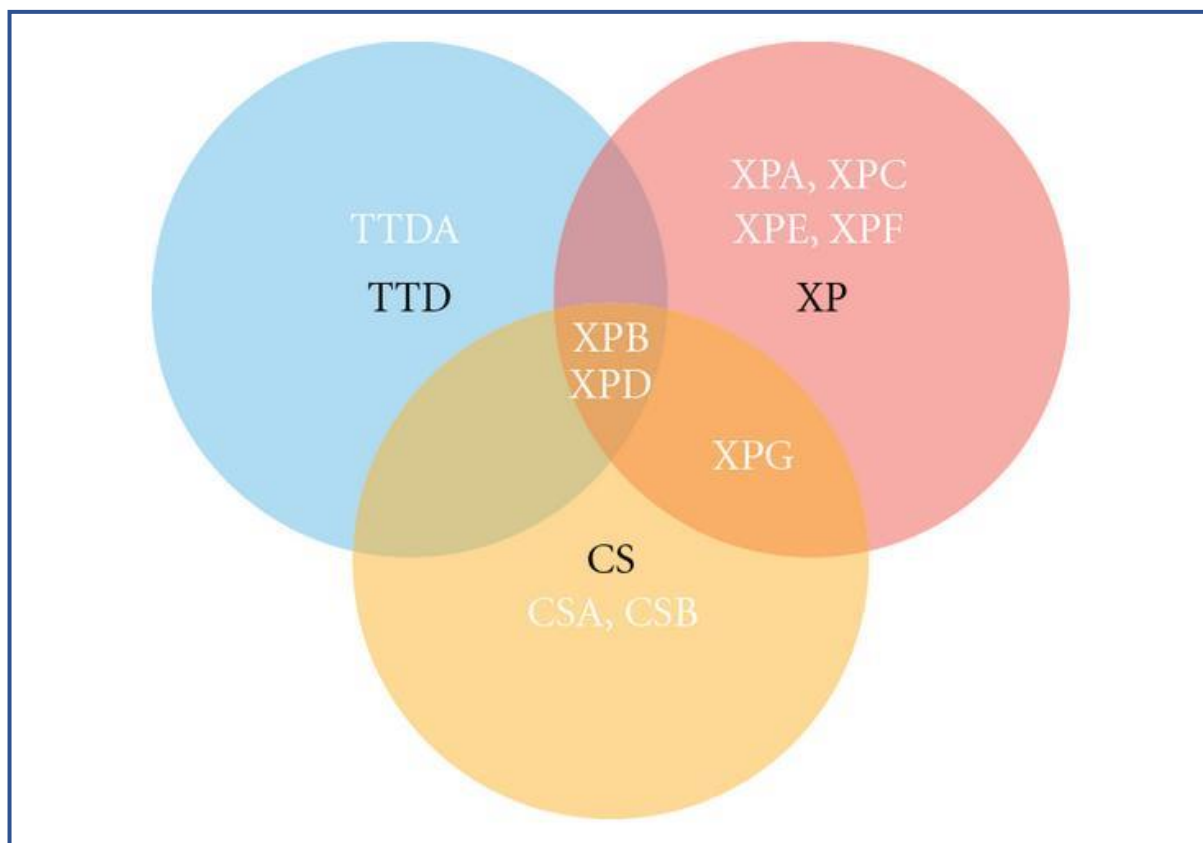
**De Sanctis-Cacchione (DSC)** syndrome was initially known as “xerodermic idiocy” due to similar symptoms to xeroderma pigmentosum patients. It is due to mutations in *XP*, mainly *XPA* and *CSB* genes. DSC’s clinical symptoms are mental deficiency, progressive neurologic deterioration, dwarfism, and gonadal hypoplasia (Spivak and Hanawalt 2015).

**Trichothiodystrophy (TTD)** is the result of mutations in some of TFIID genes: *XPD (ERCC2)*, *XPC (ERCC3)*, and *TTDA* (figure 16). Its main characteristics are sun sensitivity, brittle and abnormal hair and nails, reduced fertility, and premature aging (Spivak and Hanawalt 2015).

**UV-sensitive syndrome (UV<sup>SS</sup>)** is due to mutations in TC-NER *CSA*, *CSB*, or *UVSSA* genes. UV<sup>SS</sup> patients suffer from photosensitivity and hyperpigmentation. Due to its mild symptoms and the fully functional GG-NER and MMR, they do not develop cancer and are rarely diagnosed with a genetic disorder (Spivak and Hanawalt 2015).

**Xeroderma Pigmentosum (XP)** is a rare skin genodermatosis that is characterized by skin pigmentation, photosensitivity, high cancer incidences in photo-exposed areas (eyes, ears, skin, tip of the tongue), and internal cancer (glioma, lung, breast, leukemia, uterus, prostate). XP

patients have a 10000-fold more skin cancer risk (BCC, SCC, and melanoma) with an earlier onset of NMSC compared to the general population (Ming et al. 2011). In addition, almost 30 percent of the XP patients develop neurological disorders (deafness, ataxia, microcephaly, walking difficulties, intellectual deficiency, and progressive cognitive impairment). Such heterogeneity in the clinical symptoms is due to the mutations in one of the seven XP complementation groups (A to G) or XP variant (XPV) (figure 16). Such proteins are usually implicated in NER, except for XPV, which is involved in DNA translesion. A recent publication mentioned that 50 percent of the worlds’ XP patients suffer from mutations in XPC protein (Spivak and Hanawalt 2015). Therefore, they lack an effective GG-NER (Spivak and Hanawalt 2015; Sarasin et al. 2020).



*Figure 16. The link between NER mutated genes and three different disorders (Xeroderma Pigmentosum “XP”, Trichothiodystrophy “TTD”, and Cockayne syndrome “CS”) (Le May, Egly, and Coin 2010). Mutations in TTDA result in TTD disorder, while mutations in CSA and CSB lead to CS syndrome. Mutations in XPA, XPC, XPE, or XPF lead to XP syndrome. Interestingly, mutations in some NER genes could coincide amongst the disorders: XPG mutation is common between XP and CS, while XPB and XPD mutations are common amongst the three syndromes.*

## 4. Chapter Four: XPC the Bridge Between BER and NER

XPC has been identified to interact with BER (discussed previously) and play a significant role in GG-NER. XPC's impairment leads to XPC disorder which is the most common XP genodermatosis. In the absence of any current treatment, studying this protein thoroughly could provide better insight for further understanding of the disease at mechanistic standpoint. This could pave the way for potential treatments.

### 4.1. XPC expression and interactions

*XPC* gene contains around 16 exons and 15 introns located on the short arm of chromosome 3 at position 25 (3p25) (Zebian et al. 2019; Bensenouci et al. 2016). It is translated into 940 amino acid protein (125 KDa) which usually forms a heterotrimeric complex with RAD23B and CETN2 for stabilization and proper folding (Puumalainen et al. 2016). XPC harbors different domains for binding to both the DNA and various protein partners (figure 17): (i) a C-terminal segment (492-940 residues): the 847-863 residues form  $\alpha$ -helix with EF-hand calcium-binding protein centrin-2 (CETN2) while the 816-940 residues and part of the amino-terminal (334 residues) interact with TFIIH (P62 and XPB) (Puumalainen et al. 2016; Bunick et al. 2006), (ii) N-terminal that binds with XPA, P62, and glycosylases (OGG1) at the TGD domain (154-331 residues) (Puumalainen et al. 2016). Other identified interacting domains are TGD (496-637 residues) that interacts with DNA, RAD23B, and DDB2 and the three  $\beta$ -hairpin domains (BHD1-3) (Puumalainen et al. 2016). XPC interacts with several other proteins involved in DNA synthesis, transcription [DNA topoisomerase 2-beta (TOP2B)], proteolysis, posttranslational modifications [the OTU deubiquitinase 4 (OTUD4), USP7,11 deubiquitinase (for Ubiquitin-Specific-processing Protease 7,11)], signal transduction (SMAD1), pluripotency ( Oct4-Sox2 activator), and metabolism (Puumalainen et al. 2016, Lubin et al. 2014).

Post-stress, XPC scans through its BHD1-BHD2 domain the DNA double helix for unpaired bases. Once it senses damage, it increases the efficiency of its binding to DNA through BHD3, which inserts its  $\beta$ -hairpin finger onto the DNA lesion site. In parallel, it flips out the unpaired bases opposing the bulky lesion. Then RAD23B is released to initiate the cascade of GG-NER events (Puumalainen et al. 2016).

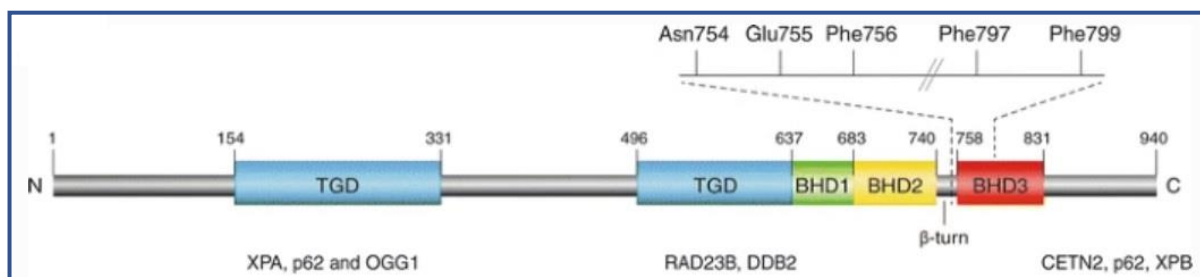


Figure 17. A **modified schematic representation of the human XPC protein** (Puumalainen et al. 2016). Transglutaminase homology domain (TGD) in blue, BHD1, BHD2, and BHD3 are  $\beta$ -hairpin domains in green, yellow, and red. The left side represents N-terminus, while the right side represents C-terminus.

#### 4.2. XPC's regulation

XPC is regulated positively through the MAPK signaling pathway by interacting with mitogen-activated protein kinase kinase kinase 5 (MAP3K5) and PTEN while regulated negatively by protein tyrosine phosphatase type IVA, member 2 (PRL2) (Lubin et al. 2014).

In the presence of AKT, PTEN tumor suppressor and p38 have been reported to positively regulate XPC in UVB-induced keratinocytes where their loss impair XPC, triggering the predisposition for human skin cancer initiation and progression (papilloma, squamous cell carcinoma, actinic keratosis) (Budden et al. 2016; Ming et al. 2011).

At steady-state, E2F4-p130 downregulates XPC gene expression. Such repression is inverted by SIRT1 (sirtuin-1 NAD-dependent deacetylase), ARF (alternative reading frame), and BRCA1 (Puumalainen et al. 2016). Another study showed that P53 positively regulates the basal level of XPC and DDB to maintain a critical cellular level that is ready for action and to prevent excessive loss of XPC and DDB. P53 also triggers the latter proteins post-UV response, reaching the highest levels post-24 hours (Fitch 2003). Of note, XPF is suggested to stimulate the UV-induced XPC expression (Q.-E. Wang et al. 2007). P38 also promotes GG-NER by stabilizing DDB2 (Ming et al. 2011). Furthermore, XPC was shown to be regulated during the cell cycle. Retinoblastoma protein (RB) stabilizes XPC for DNA repair during the G1-phase. Phosphorylation of RB inhibits RB-stabilized XPC and DNA repair activity for G1/S transition and DNA replication to proceed (Nemzow et al. 2015).

Lastly, several NER factors are transcriptionally regulated upon attenuated HIF-1 $\alpha$ , such as XPC, XPD, XPB, and XPG (Mahfouf et al. 2019). This upregulates UVB-induced NER and regulates the cell cycle (Mahfouf et al. 2019).

### 4.3. XPC's role

#### 4.3.1. Canonical role

At the basal level, XPC protein shuttles between the cytoplasm and the nucleus via nuclear export signals (Puumalainen et al. 2016).

In response to UV stress, CPDs and (6-4) PPs bulky lesions are formed in a ratio of 3:1 (Puumalainen et al. 2016). Phosphorylated p300 acetylates histones (heterochromatin regions) for DNA recognition by XPC (Lubin et al. 2014). DDB2 (p48) and DDB2 dependent-BRG1, INO80, PRKACB, PRKACA, and Snf5 (chromatin remodeling factors) recognize CPDs and relax the chromatin triggering XPC's stabilization, activation, higher recruitment from the cytoplasm into the nucleus, and binding to the chromatin. P53 can also assist in double-checking whether CPDs were repaired effectively (Fitch 2003). However, XPC recognizes (6-4) PPs independent of DDB (L. Zhang et al. 2009; Puumalainen et al. 2016; Lubin et al. 2014; A. Ray et al. 2013).

Afterward, XPC binds to the ssDNA opposing the DNA damage (A. Ray et al. 2013). This initiates GG-NER, where the TFIIH complex is recruited to open the DNA strand and trigger XPA's recruitment that will be stabilized by XPG (3' endonuclease). However, XPC and XPG cannot exist simultaneously at the CPD site. So, XPA-RPA will disengage XPC to be degraded for the progression of the cascade of repair (Koch et al. 2016). Meanwhile, (6-4) PPs induce pronounced helical DNA distortion accommodating the congregation of all the repair machinery, including XPC and XPG (Q.-E. Wang et al. 2007). Succinctly, a set of chronological events will be activated after recognizing lesions by XPC and its protein-protein interactions with some NER factors leading to the complete repair.

The medical relevance and phenotypes of mutated XPC extend beyond its GG-NER function as several studies implicated, suggesting XPC as a multifunctional protein.

#### 4.3.2. Other roles

The prominence of XPC is elucidated in its recognition of a wide range of substrates, including multiple repair factors other than NER that result in pleiotropy. More than 49 potential interactors are involved in DNA synthesis, DNA repair, transcriptional regulation, protein degradation, signal transduction, redox homeostasis, cellular metabolism, etc.... (figure 18). Therefore, there is a complex heterogeneity in the clinical phenotype of XP-C patients.



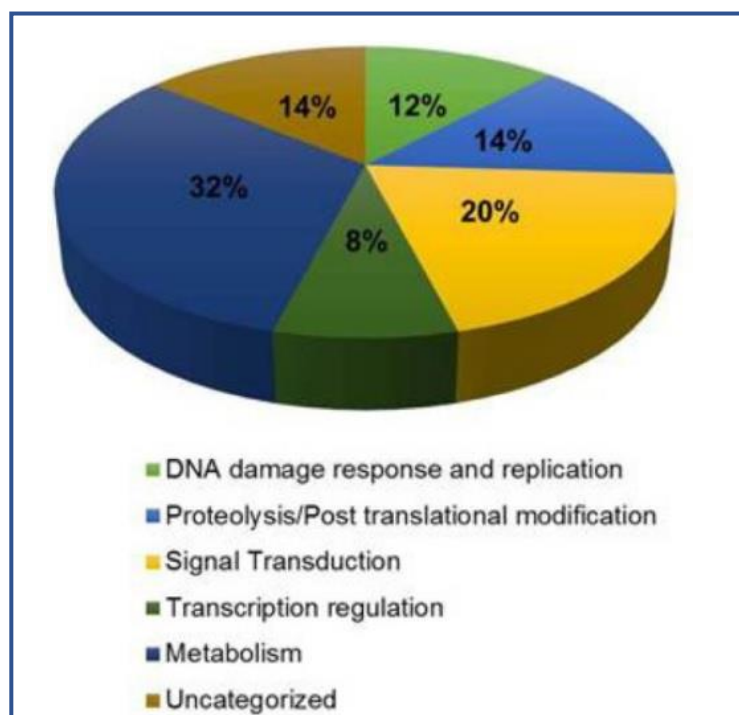


Figure 18. A pie chart categorizing interactors/roles of XPC (Lubin et al. 2014). This analysis was done by the yeast two-hybrid screening.

- Regulation of the immune response

XPC regulates the immune response by upregulating the expression of cytokines [interleukin-6 and interferon (IFN)-related genes (IFN $\alpha$ , IFN $\beta$ , and IFN $\gamma$ )] (Bidon et al. 2018). In addition, researchers presented a direct link between the upregulation of XPC's expression in melanoma and the increase in immune response related-transcripts expression leading to a better prognosis (Budden et al. 2016).

- Regulation of the gene expression: transcription

Le may et al. showed that NER, including XPC, plays a role in preparing the adequate environment (DNA demethylation, histone posttranslational modifications...) for synthesizing the primary transcript (Zebian et al. 2019).

XPC complex (XPC-RAD23B-CETN2) has been identified as an essential factor of the multi-subunit stem cell coactivator complex (SCC). It is involved in the chromatin reorganization, cell division, reprogramming, self-renewal of stem cells, and modulating the expression and pluripotency of dental pulp cells (DPCs) by binding to enhancers and regulating Oct-4/Sox2/c-Myc, key transcriptional activators of DNA reprogramming markers. This regulation may be via triggering *Nanog*'s expression. The DPCs importance had been shown through their medical efficiency in various degenerative diseases as Alzheimer's disease, diabetes mellitus, spinal cord injuries, and myocardial infarction. Therefore, their regulation by XPC could be

the key for the potential usage of DPCs in therapies where their limited progenitors, pluripotency, and cell division could be manipulated (Lu Liu et al. 2016).

Additionally, it interacts with hSNF5, SWI/SNF ATP-dependent chromatin remodeling complex. XPC acts as a coactivator and epigenetics remodeler of RNA polymerase II-mediated transcription (Puumalainen et al. 2016).

XPC and E2F1 interact with and activate histone acetyltransferase (HAT) lysine acetyltransferase 2A (KAT2A) to induce gene promoters positively. Furthermore, XPC acts as a positive linker between E2F1 transcription factor and ATAC coactivator and as an activator of DNA damage-inducible transcript 3 (DDIT3) and SET1 methyltransferase that will methylate histone H3 at lysine 4 (H3K4) for chromatin remodeling (Bidon et al. 2018, 1; Lubin et al. 2014). This stimulates transcription. Therefore, XP-C cells show a reduced transcript level of coactivator genes (Puumalainen et al. 2016).

Another evidence is that XPC is known to recruit itself and other NER factors (CSB, XPA/RPA, XPG/XPF/Gadd45 $\alpha$ ) to the retinoic acid receptor beta2 (*RAR $\beta$ 2*) in the presence and absence of UV to induce the promoter's transactivation and chromatin remodeling (Zebian et al. 2019; Nemzow et al. 2015).

- **Regulation of cell cycle**

In the absence of adequate DNA repair, XPC triggers damage-induced apoptosis via downregulating anti-apoptotic casp-2S to avoid the division and progression of mutation-prone cells. Accordingly, XP-C cells exhibit a caspase-3 (apoptotic) inhibition, caspase-2 (anti-apoptotic) upregulation, hypersensitivity to genotoxic stress (UVB...), and faster stress induced-tumor growth rate (Budden et al. 2016; Nemzow et al. 2015). For instance, some researchers reported a more apoptotic resistance in *xpc*<sup>-/-</sup> mouse skin compared to control post-UVB (H. R. Rezvani, Ged, et al. 2008).

- **Regulation of proteolysis**

XPC is implicated in the ubiquitination and degradation of some proteins. For example, XPC degrades hUfd2 through the ubiquitin fusion degradation (UFD) pathway. It also induces P53 degradation via MDM2 (Nemzow et al. 2015).

XPC is expected to have proteolytic substrates other than P53. Identifying these substrates could contribute to better understanding the cellular signaling processes during disease progression (Zebian et al. 2019). **An interesting project (*How a sun protection complex moonlights in proteolysis, UT Health San Antonio, funded by NIH-General Medical Sciences*) is in progress to detect such substrates.**

- Regulation of other repair pathways

For activity regulation, XPC interacts with: (i) mismatch repair protein (MSH2), (ii) homologues recombination repair proteins (ATM, ATR, and Rad51) (discussed in section 3.2., NER and cell cycle), (iii) Non-homologues recombination repair proteins (PKCs, XRCC4, XRCC6, and LIG IV), and (iv) BER proteins [glycosylases (OGG1, MPG, TDG, SMUG1) and APE1] (Zebian et al. 2019). **In our studies, we will focus only on the link between XPC and BER.** In this respect, scientists had already identified OGG1's interaction domain in XPC (in the region of codon 334) and that XPC mutations delay activation of APE1 and inhibit OGG1's and MYH's activation and expression (Qiao, Ansari, et al. 2011).

- Regulation of redox homeostasis

Regulation of redox homeostasis has been linked to XPC due to the high sensitivity and detrimental effects of the oxidizing agents on XP-C cells. For example, D'Errico et al. and Kassam et al. demonstrated XP-C fibroblasts and keratinocytes sensitivity towards KBrO<sub>3</sub> and methylene blue, thereby accumulating mutations including oxidized lesions (Kumar et al. 2020). Also, Melis et al. reported slow but higher somatic mutagenesis in *xpc*<sup>-/-</sup> mice compared to control when exposed to oxidative stressors [diethylhexyl phthalate (DEHP) or paraquat] (Melis et al. 2013). This suggests a slow accumulation of oxidative stress and oxidative DNA damage in the absence of XPC. Another example is the increase in NER factors' level (including XPC) in the presence of oxidative stress in parallel to the glutathione-dependent regulation of NER and XPC-dependent regulation of glutathione in the presence of prooxidant (discussed in enzymatic defense mechanism section 1.2.1) (Melis et al. 2011). Liu et al. found that UV and arsenic trioxide trigger XPC that will suppress superoxide production, induce glutathione production, regulate the cell cycle, and modulate ROS scavenging systems (GSH, catalase, and SOD) to induce redox balance (S.-Y. Liu et al. 2010). For instance, 35 and 40 percent lower catalase activity were detected in XP-C keratinocytes and fibroblasts, respectively, compared to control (H. R. Rezvani, Ged, et al. 2008).

In parallel, Melis et al. reported that, in the presence of DEHP or paraquat oxidative stressors, *xpc*<sup>-/-</sup> mice showed an increase in mutations and liver lipofuscin, a granular pigment acting as *in vivo* oxidative stress biomarker (Zebian et al. 2019). Another oxidative stress biomarker is hyperoxidized peroxiredoxin (Prx-SO<sub>2</sub>). XP-C cells exhibit sensitivity towards these stresses and others such as IR, UV, and H<sub>2</sub>O<sub>2</sub> (S.-Y. Liu et al. 2010).

In the absence of XPC, DNA damage will trigger DNA-dependent protein kinase (DNA-PK), which subsequently activates AKT1 and NOX1, inducing ROS and further DNA mutations

(Melis et al. 2011). This NOX-mediated ROS has been detected to contribute to altered bioenergetics (hyperpigmentation...) and metabolic alterations in XP-C keratinocytes leading to photosensitivity and carcinogenesis (Kasraian et al. 2019).

#### 4.4. XPC disorder

Briefly, as discussed throughout the manuscript, a mutation in XPC at gene/protein level leads to functional dysregulation, consequently, XPC disease (figure 19). This genodermatotic disease differs in severity amongst patients based on the type of mutation and genetic background (Kasraian et al. 2019). For instance, patients with homozygous XPC mutations exhibit different pigmentation abnormalities (extensive, moderate, and none) and different repair capacities (between 13 and 40 percent) (Kasraian et al. 2019). Furthermore, K655A XPC mutation affects its degradation, not recruitment nor activity. This prevents XPG recruitment and compromises NER efficiency (Q.-E. Wang et al. 2007).

A study revealed that 49 and 59 percent of invasive immunocompetent and immunosuppressive cutaneous squamous cell carcinoma, respectively, in non-XP patients had absent XPC protein. Therefore, the absence of XPC plays a significant role in promoting malignancy and carcinogenesis (J. W. Lee et al. 2020).



*Figure 19. Clinical features of a Mahori XP-C patient at 6- and 8-years-old (Cartault et al. 2011). When he was 6-years-old, skin damage and pigmentations were detected. As the patient got older, the symptoms became more severe. After two years (8-years-old), he had a running nose, tumors at different body sites, including the tip of the tongue, and pigmentation and skin damage.*

##### 4.4.1. Clinical features

Due to the multiple roles of XPC, patients suffer from inter-individual heterogeneity in the clinical symptoms. Nevertheless, some of the main clinical features are (Krzyszynski et al. 2014; Lehmann, McGibbon, and Stefanini 2011; Yurchenko et al. 2020):

- Photosensitivity
- Dry skin

- Pigmentation, poikiloderma, and lentiginosis
- Premature skin aging
- Skin cancer in photo-exposed areas (SCC, BCC, Melanoma)
- Early incidences of internal cancers (leukemia, lung, thyroid, sarcoma, etc..) in photo-protected areas (median age of 24 years)
- Other symptoms (autism, hypoglycemia...)

#### 4.4.2. Clinical treatments

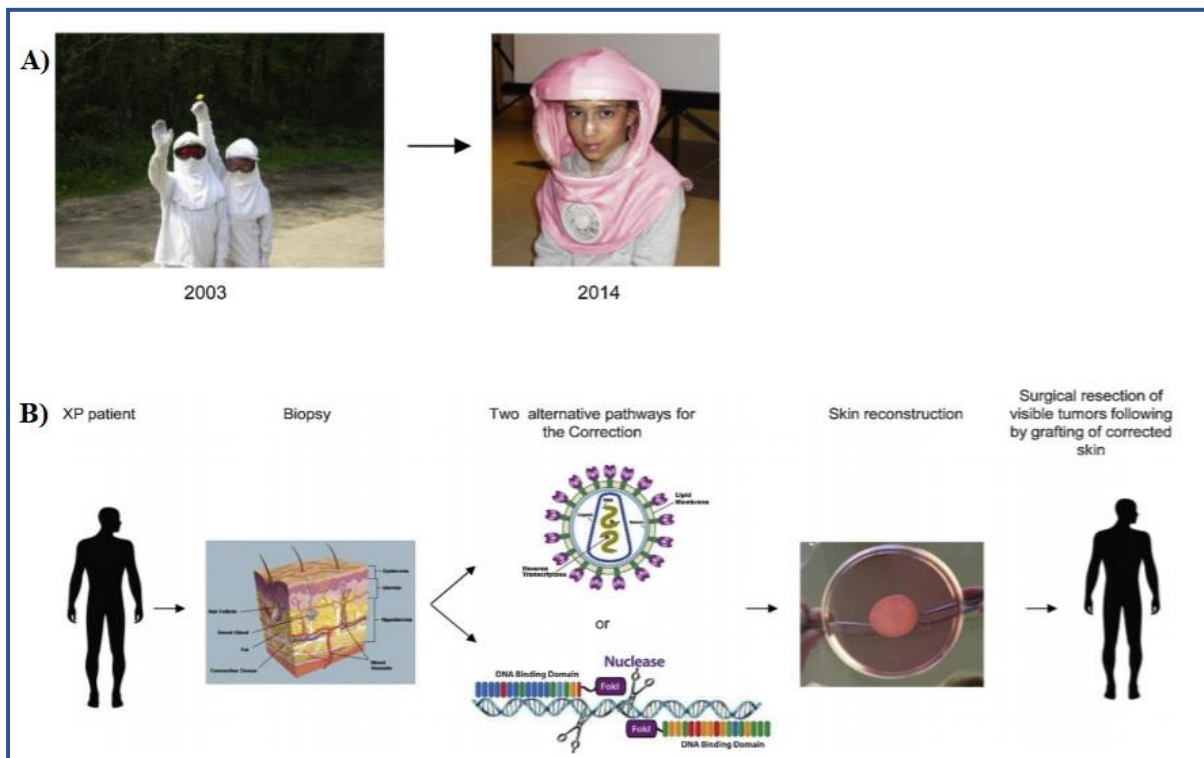
Unfortunately, no treatment has been found targeting mutated *XPC* directly; instead, precautions must be considered. For instance, protective suits along with eye protection [figure 20 (A)], photoprotection (sunscreens, antioxidant creams, serums, food, and supplements), and therapies are used to limit the severeness of the clinical symptoms (H. R. Rezvani, Ged, et al. 2008; Delinasios et al. 2018). Therefore, an early diagnosis is essential to limit the severity of the clinical symptoms.

Some of the available/in progress palliative treatments of symptoms include:

- Surgical tumor removal
- Retinoids (vitamin A) (discussed in section 1.2.2.)
- Enzymatic therapy with liposomes containing bacteriophage T4 endonuclease V (T4N5) reduces skin cancer and actinic keratoses and increases the rate of repair of UV-induced DNA damage in XP cells (Yarosh et al. 1996; Gache et al. 2013).
- *XPC* gene therapy [under clinical trials, figure 20 (B)]: engineered nucleases (meganucleases and TALE nucleases) correct mutations in XP-C cells (as TG deletion of exon 9, the most common *XPC* mutation), generating double-strand DNA break to promote *XPC* locus correction and *XPC* protein efficiency by homologues recombination. For activity optimization, it should be used in parallel with demethylation treatment (5aza-dC) (Dupuy and Sarasin 2015). However, global genome demethylation induces cytotoxicity. Henceforth, TALEN<sup>TM</sup> (Transcription Activator Like Effector) modified nuclease is a better selection as gene therapy. Such an approach is considered better than retroviral vector transfection as it showed its efficiency in XP-C fibroblasts by reversing the genotype without under- or over-expressing the gene uncontrollably. Researchers are always trying to design other new vectors with better efficiency and safety (Dupuy and Sarasin 2015). For example, two of the most recent and deliberated strategies are CRISPR/cas9, CRISPR/pass (Dupuy and Sarasin 2015). CRISPR/pass has been shown to convert A to G in XP-C patients

successfully (nonsense *XPC* mutated, GM14867), which restores *XPC*'s function independently of HR (C. Lee et al. 2019). CRISPR/cas restores genes by reading through premature termination codons (nonsense mutations) (figure 21) (C. Lee et al. 2019).

Other new therapeutic strategies are expected to be developed to repair mutated *XPC* selectively. This could be the solution for most monogenic pathologies, especially *XPC*, as monoallelic corrections of few keratinocytes might be clinically efficient.



**Figure 20. *XP-C patients' precautions and proposed treatments* (Dupuy and Sarasin 2015). A) *XP-C patients must wear protective suits during their whole life. Hence, scientists are trying to provide complete protection suits (as shown between 2003 and 2014) for better UV protection, ventilation, and suitability for everyday life as much as possible.* B) *XP-C skin gene therapy. A skin biopsy is extracted from XP-C patients to culture epidermal cells ex-vivo. This is followed by gene correction in keratinocytes and fibroblasts (including stem cells) via the modified virus vector or by repairing via nucleases and homologues recombination repair activation. After modifications, cells are cultured in vitro to form reconstructed skins that will be autografted.***

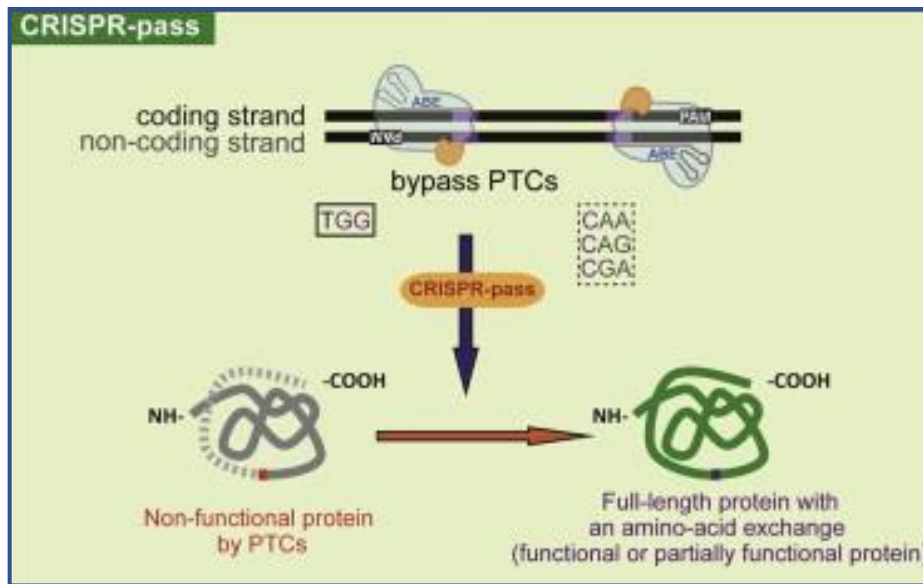


Figure 21. **CRISPR/pass mechanism of action** (C. Lee et al. 2019). CRISPR/pass is an Adenine base editor that bypasses premature termination codons (PTCs) by converting adenine (A) to guanine (G) or thymine (T) to cytosine (C). Such a process will convert a non-functional protein into a full-length protein with partial or complete function.

#### 4.5. XPC mutations/polymorphic variants and cancer

Researchers identified altered *XPC* gene expression (mutated or deleted) in NMSC patients without variations in photosensitivity and in invasive SCCs patients (I. Kim and He 2014; Feraudy et al. 2010). This loss of expression is P53-independent and could be due to (i) mutagenesis, (ii) promoter methylation, or (iii) *XPC* region vulnerability for inactivation during UV-induced carcinogenesis (Feraudy et al. 2010). Kgokolo et al. presented in a study that South African XP-C patients shared SCC/BCC as a clinical profile (Kgokolo et al. 2019). However, BBC occurs in some XP-C patients (17 years) at a higher median age compared to other XP patients (9 years) (Ben Rekaya et al. 2013). As a result, mutations (C > T or CC > TT transitions at dipyrimidine sites) will accumulate, leading to the double-strand breaks and upregulation of their repair pathway (HR) (Budden et al. 2016). Other studies revealed that some *XPC* polymorphisms and mutations increase the risk of melanoma (Budden et al. 2016; Zebian et al. 2019). For example, high UV-induced-mutational loads and *XPC* mutations were detected in the melanoma genome promoters compared to normal melanocytes (Budden et al. 2016). Conclusively, *XPC* plays a significant role as a tumor initiation- and progression- suppressor.

Since SNPs can lead to protein variations, studying *XPC* SNPs (point mutations) could genotypically and phenotypically explain some clinical outcomes such as an increased predisposition to internal (bladder, ovarian, breast, colorectal, and lung) and skin cancers. Such cancer types result from an accumulation of oxidative DNA damage and bulky DNA damage, respectively.

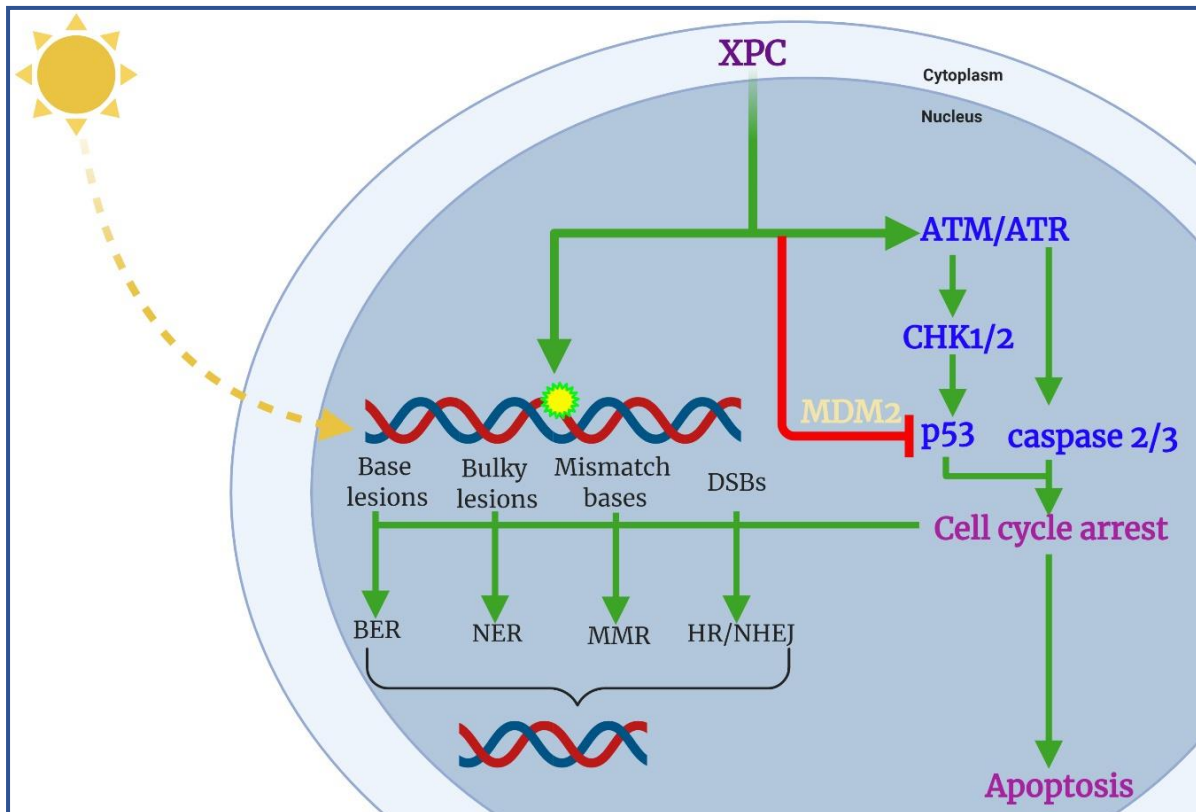
Until the moment, between 46 and 60 mutations [deletions (~37 percent), substitutions (~35 percent), splicing (19.5 percent), and insertions (8.6 percent)] have been described in *XPC* (Ben Rekaya et al. 2013; Doubaj et al. 2017). 25 percent of the *XPC* mutations were shown to be located in the exon 9 as: c.1211delG, c.1103\_1104delAA, c.1292\_1293delAA, c.1421dupA, c.1627\_1872del246, c.1243C>T, c.658C>T (Doubaj et al. 2017; Soufir et al. 2010). Other identified mutations are c.652delT (p. Phe218SerfsX41), c.2092\_2093insGTG (p. Val696\_Val697insVal), and c.2287delC (p. Leu763CysfsX4) at exons 6, 9, and 13, respectively (Soufir et al. 2010). Mutations at splice sites were also identified in introns 2 and 5. Furthermore, c.1643\_1644delTG (p. Val548AlafsX25) is the most common mutation amongst patients (1/250 estimated frequency in Moroccan patients), leading to a premature stop codon. Another identified frameshift mutation was c.1644\_1645insCATG (p. G550Afs\*25). It induces a stop codon, thereby affecting the domain of interaction with RAD23B (Ben Rekaya et al. 2013; Doubaj et al. 2017).

C/A polymorphism in intron 11 has been shown to increase the susceptibility to sporadic colorectal and bladder cancer, while CG polymorphism has been associated with gastric cancer (Zebian et al. 2019; Melis et al. 2011; Ben Rekaya et al. 2013). Ben Rekaya et al. identified homozygous G to T substitution mutation (g.18810G>T ; c.850G>T) in a Tunisien patient. It leads to a premature stop codon (p284X) and carcinogenesis (BCC, melanoma, and thyroid cancers) (Ben Rekaya et al. 2013). C.905T>C (rs121965091) increases bladder cancer risk. A>C transversion (Lys939Gln, rs2228001) in exon 15 increases the risk of melanoma, prostate, lung, bladder, digestive, thyroid, and colorectal cancers despite having a proficient NER activity. C/T polymorphism (Ala499Val, rs2228000) increases the risk of melanoma, leukemia, breast, bladder, and head and neck cancers; while the poly (AT) insertion/deletion polymorphism (PAT<sup>-/+</sup>, PAT<sup>+/+</sup>) in intron 9 is associated with SCC, melanoma, head and neck, gastric, urinary, bladder, prostate, and lung cancer (Zebian et al. 2019; Melis et al. 2011; Yoshino et al. 2016). C/A polymorphism (rs2733533) is significantly associated with sporadic colorectal, bladder, prostate, and lung cancers, while 499CT/TT genotype increases the risk of lung cancer (Ben Rekaya et al. 2013; Qiu et al. 2008; Yoshino et al. 2016). Finally, *XPC* 499 Val/Val genotype is associated with head and neck cancer (Qiu et al. 2008).



**Remarkably, as shown above, most of the distinct listed and unlisted *XPC* mutations and variant polymorphisms are the hallmarks of skin and internal cancers. Moreover, the latter are classified as common clinical symptoms of BER deficiency and oxidative stress (segment 2.4). This shows the profound correlation between both DNA repair-inducing players (*XPC* and BER).**

## 5. Representative Summary



*Figure 22. Schematic summary of the role of XPC post-UV irradiation. UV irradiation triggers different types of DNA lesions. Once activated, XPC will be present exclusively in the nucleus to recognize bulky lesions initiating GG-NER and boosting the efficiency of other repair systems in mending their respective target lesions. In parallel, XPC activates the cell cycle regulatory pathway by activating the ATM/ATR pathway to induce cell cycle arrest and apoptosis when needed.*

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*Materials and  
Methods*

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## Materials and Methods

Since our project is divided into two parts, both share similar experimental strategies but differ in UVB dosage ( $J/cm^2$ ) and the cell types.

Moving forward in the materials and methods, we identified the part of the project when we wanted to mention specific details presented in one part but not the other as follows:

Part One “[Deciphering the Role of XPC in BER and Oxidative Stress](#)” or Part Two “[Ameliorating the DNA repair of XP-C cells by modulating their redox state via pharmacological treatments](#)”.

Not specifying the part of the project means that both share the same strategy and information.

### 1. Cell culture and treatments

#### 1.1. Cell culture

##### (i) Part One “Deciphering the Role of XPC in BER and Oxidative Stress”

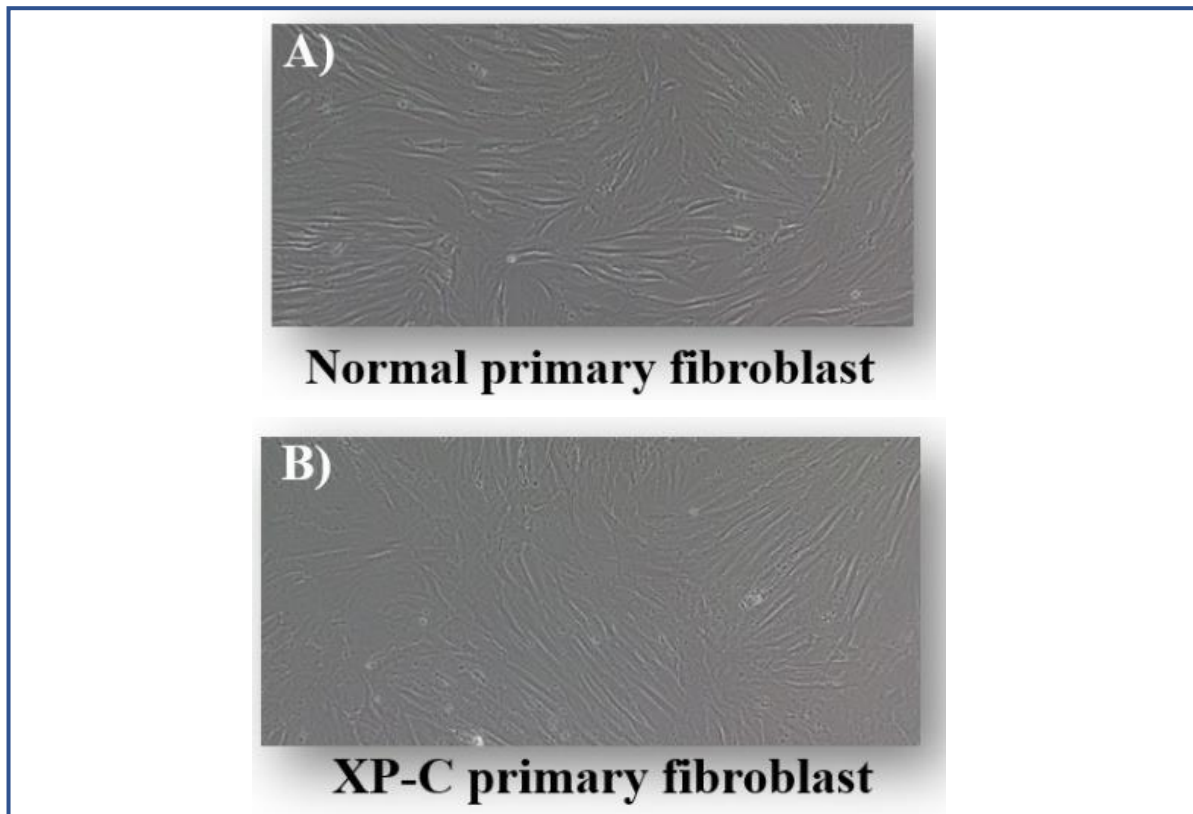
For the *first objective*, we studied three different XP-C and three different normal (N=3) primary fibroblasts.

Our collaborators in Bordeaux, France, extracted the XP-C fibroblasts (XP-C1, XP-C2, and XP-C3) from punch biopsies obtained from photo-protected body sites in three unrelated young patients whose parents gave the consent for the procedure. Afterward, they sequenced the XP-C mutations (table 2, figure 23).

*Table 2. Main characteristics of the XP-C patients involved in the study. After having the parents' consent, the three selected patients were diagnosed and followed up regularly at the Dermatology Department, Bordeaux hospital, to examine precancerous lesions. The three different XPC mutations (XP-C1, XP-C2, and XP-C3) derived from different patients were identified by Sanger sequencing: XP-C1: homozygous deletion (c.1643\_1644del) resulting in premature stop codons in both alleles, XP-C2: deletion at the splice site of allele 1 (c.413\_3delC) and deletion at allele 2 (c.1086del) resulting in a premature stop codon, and XP-C3: non-sense mutation in allele 1 (c.1243C>T) and deletion mutation resulting in a premature stop codon in allele 2 (c.2287del).*

Patient id	Geographic origin	Main clinical features	XPC gene defect (NM_004628.4)	Age at diagnosis
XP-C1	Morocco	Early skin photosensitivity	c.1643_1644del, p. (Val548Alafs*25) homozygous	2 y
XP-C2	Portugal	Moderate skin photosensitivity - multiple lentigines	[c.413-3delC, p.?], [c.1086del, p. (Ser363Alafs*163)] compound heterozygous	14 y
XP-C3	West Indies/Chile	Skin photosensitivity - multiple lentigines - precancerous lesions	[c.1243C>T, p. (Arg415*)], [c.2287del, p.(Leu763Cys*4)] compound heterozygous	6 y

These XP-C patients were clinically diagnosed with classical XPC disorder lacking neurologic and extracutaneous clinical symptoms. This is compatible with our aim in explaining the reason for the development of malignancy features in photo-protected areas in some XP-C patients. They were compared to normal primary fibroblasts extracted by our team, CIBEST, from skin biopsies obtained from young mammary hypertrophy patients at CHU (Centre Hospitalier Universitaire), Grenoble.



*Figure 23. Morphological images of normal and XP-C primary fibroblasts. Both fibroblasts share a similar morphological structure.*

Primary fibroblasts were cultured in DMEM medium (DMEM, high glucose, GlutaMAX™ Supplement, + Pyruvate, ThermoFisher Scientific) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in falcon flasks (75 cm<sup>2</sup>) at 37°C in 5% CO<sub>2</sub> incubator.

Cells were collected at 80 percent of confluency post-trypsinization with trypsin-ethylenediaminetetraacetic acid (EDTA) (11590626, Thermo Fisher Scientific) for passage and further experiments.

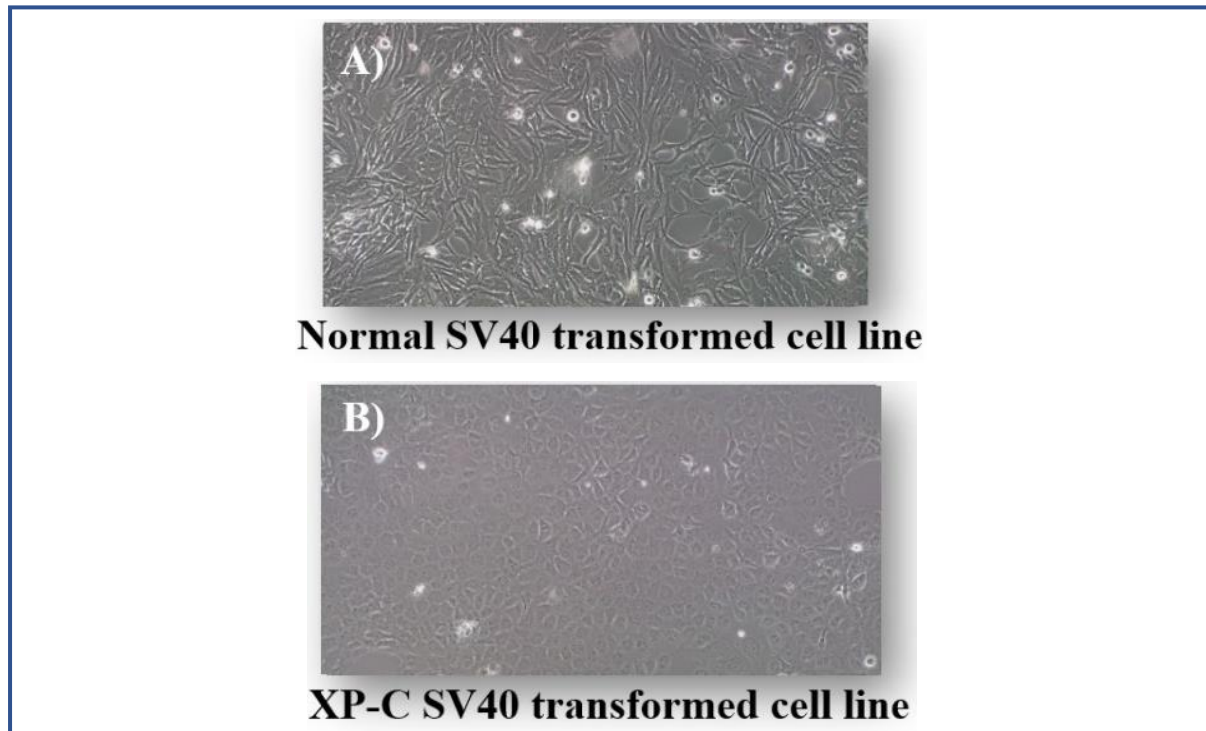
**(i) Part Two “Ameliorating the DNA repair of XP-C cells by modulating their redox state via pharmacological treatments”**

SV40-transformed normal (AG10076, Normal) and *XPC* mutated (GM15983, XP4PA-SV-EB, XP-C) fibroblast cell lines were purchased from Coriell Institute for Medical Research, USA (figure 24, table 3).

*Table 3. Main characteristics of the XP-C transformed cell line involved in the study. It was received from Coriell Institute as an immortalized fibroblast by Simian Virus 40 (SV40) (Qiao, Scott, et al. 2011).*

	<b>XP-C gene defect</b>
<b>XP-C</b>	2 bp frameshift at codon 431 p. (Val431fsX)

Similar to the previously used primary fibroblasts (part one), both cell lines were cultured in DMEM medium. They were harvested at 80 percent of confluency by trypsinization for passage and experiments.



*Figure 24. Morphological images of normal and XP-C SV40 transformed fibroblasts. Both fibroblasts do not share similar morphology.*

## Workflow

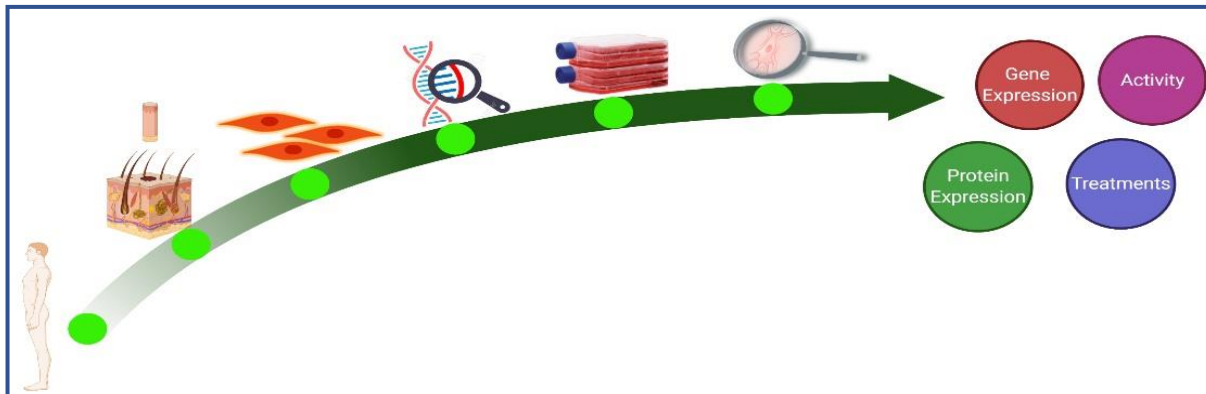


Figure 25. A summary of the thesis workflow. After a biopsy had been punched from patients/controls, fibroblasts were isolated and sequenced, followed by their culture in incubators. After reaching 80% of confluency, cells were collected and characterized at expression and activity levels. Afterward, cells were cultured and collected at 80 percent of confluency to study the effect of XPC on BER's expression and activity levels with/without irradiation and treatments.

### 1.2. Cell treatments

#### 1.2.1. Drugs' pretreatments

#### Part Two “Ameliorating the DNA repair of XP-C cells by modulating their redox state via pharmacological treatments”:

As shown in figure 26, cell lines were seeded for 24 hours then treated with the appropriate concentrations of one of the following treatments:

- ✓ for 24 hours: Nicotinamide (NIC, N0636-100G, Sigma Aldrich) and N-acetylcysteine (NAC, A9165-5G, Sigma Aldrich)
- ✓ or for 4 hours with L-buthionine sulfoximine/ dimethyl fumarate (BSO/DMF, B2515-1G/ 242926, Sigma Aldrich). This was followed by UVB-irradiation.

Selected controls were cell lines without treatments and irradiation.

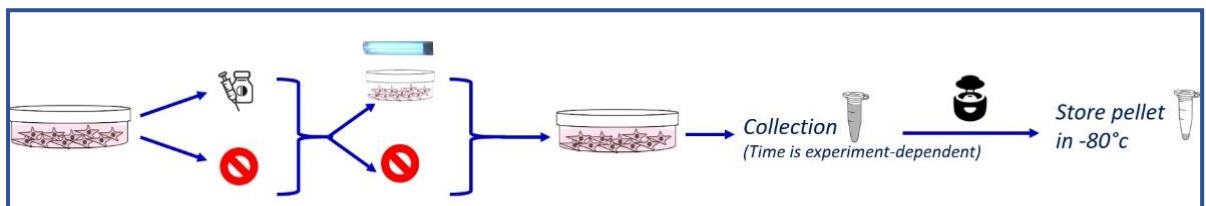


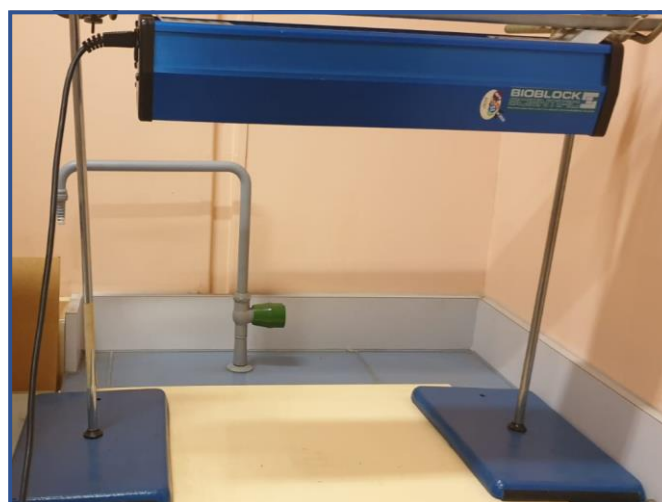
Figure 26. Cellular treatments and collection. Cells were cultured in 100mm petri dishes until 80 percent of confluency, where they will be treated with selected drugs or not. Then, after a chosen incubation time, cells were treated with UVB ( $0.01 \text{ J/cm}^2$ ) (untreated cells were considered as control) to be then collected after a specific time (based on the experiment type).

The drugs' concentrations were selected based on the following criteria:

- (i) Literature: several articles worked at the same concentrations: for example NIC 50 $\mu$ M for 24 hours (Thompson et al. 2015, Malesu et al. 2019), NAC 1mM for 24 hours (Zhang et al. 2011), BSO/DMF 100 $\mu$ M for 4 hours (Boivin et al. 2011)
- (ii) Dose response curves (some of the tested concentrations are shown later in the results and discussion section)
- (iii) We did not want to choose a high dose that could have side effects or force any pathway or role. Therefore, concentrations that could be reached *in vivo* have been selected.

### 1.2.2. UVB-irradiation

For each experiment, we measured the mean of one-minute irradiation's doses (J/cm<sup>2</sup>) at 5 different zones at the surface via a dosimeter before irradiating our cells. This allowed us to calculate the needed time to obtain the targeted UVB dose (figure 27).



*Figure 27. Bioblock Scientific with UVB lamp (312nm, 15W) used by our laboratory, CIBEST/CEA. Before irradiation, we measured 5 zones at the surface with a dosimeter and calculated the mean. This mean allowed us to know the incubated time corresponding to the needed UVB dose. After that, the plate/dish was located at the center of the surface each time for homogeneity.*

After that, we irradiated our cells with the UVB lamp (312nm, 15W) as follows:

We treated our studied cells with different doses of **UVB lamp** (312nm, 15W) (figure 27) to check their photosensitivity (24 hours post-UVB) and to select the most suitable dose for the rest of our experiments.



As discussed later, we used the following UVB doses:

- (i) **Part One “Deciphering the Role of XPC in BER and Oxidative Stress”**: 0.05 J/cm<sup>2</sup>. It resembles more than 50 percent of cellular survival. Untreated cells were used as control.
- (ii) **Part Two “Ameliorating the DNA repair of XP-C cells by modulating their redox state via pharmacological treatments”**: 0.01 J/cm<sup>2</sup>. It resembles the most suitable dose to be selected with more than 50 percent of cellular survival.

### 1.2.3. Solar simulation

Done only for one experiment: **Part One “Deciphering the Role of XPC in BER and Oxidative Stress”**

We treated our primary fibroblasts with LS1000 Solar Simulator (Solar Light Company, Glenside, PA) (figure 28), which emits UVA+UVB radiations at 290–400 nm range, at different doses to check the cellular photosensitivity 24 hours post-UV by MTT.



Figure 28. **LS1000 Solar Simulator** used by our laboratory, **CIBEST/CEA**. The photo was taken from the original website for purchasing this model. (“6" (15.25 Cm) 1000W PV Cell Testing Solar Simulator Kit Model LS1000-6R-002 - Solarlight” n.d.).

### 1.3. Immunocytochemistry (Immunofluorescence) and associated microscopy

**Part One “Deciphering the Role of XPC in BER and Oxidative Stress”**: (6-4) PPs repair efficiency was detected by immunocytochemistry. Briefly, primary fibroblasts were exposed to 0.03 J/cm<sup>2</sup> UVB-irradiation. Afterward, they were fixed at 0- and 24-hours post-UVB irradiation with 4 percent paraformaldehyde (15 minutes, room temperature) and were

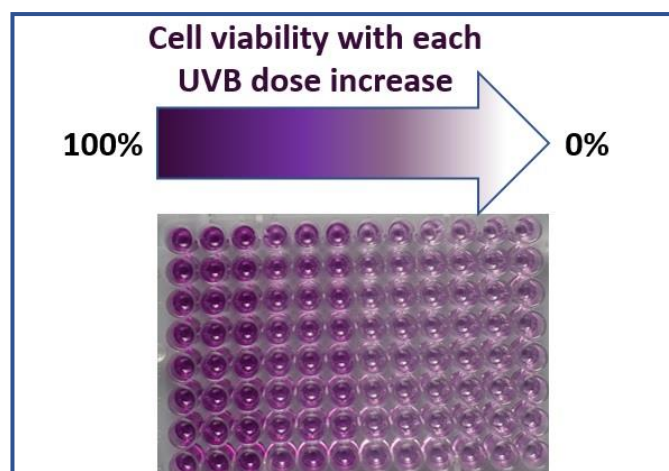
permeabilized with 0.2 percent triton X-100 (5 minutes, room temperature). Then, cells were washed with PBS-1X (12037539, Thermo Fisher Scientific) and their DNA were denatured with 2M HCL (30 minutes, room temperature) to then be blocked with 3 percent fetal bovine serum (FBS) in PBS-1X. Next, cells were incubated with primary anti-pyrimidine (6-4) pyrimidone photoproducts (64M-2, Cosmo Bio) diluted in 1 percent FBS. After washing three times with PBS-1X, cells were incubated with secondary antibodies (Alexa Fluor 488 goat anti-mouse, Invitrogen) diluted in 1 percent FBS. Finally, nuclear DNA was counter-stained with Hoechst (Sigma-Aldrich). Cell images were captured by the Cell-insight NXT high content screening platform at 10X magnification. Data were normalized against non-irradiated samples.

#### 1.4. Short-term cytotoxicity (MTT)

In both project parts, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, M5655-1G, Sigma Aldrich) colorimetric assay was selected for drug's concentration selection (dose response curve) and to evaluate cell viability 24 hours post-UVB irradiation.

It is based on the measurement of the cellular metabolic activity that is directly linked to cellular viability. This is due to reducing the yellow MTT into formazan, the insoluble purple pigmentation product, by NADPH-dependent mitochondrial oxidoreductase.

5mg/mL of MTT were added to each well of cells followed by a 2 hours-incubation at 37°C. Then the supernatant was discarded, and DMSO was added to solubilize formazan crystals. The intensity of the purple color was measured at 560 nm spectrophotometrically (Spectramax M2; from Molecular Devices) (figure 29).



*Figure 29. Cell viability versus different UVB doses ( $J/cm^2$ ). We exposed each 3 columns of the plate to different UVB doses (0, 0.01, 0.02, 0.04  $J/cm^2$ ) and quantified the cellular viability based on the darkness of the purple pigmentation. The darker the pigmentation color, the higher is the viability.*

All data were normalized by comparing the yield of MTT conversion in non-irradiated control samples set at 100 percent viability.

## 2. Transcriptional and translational genes' expressions

### 2.1. Gene expression by RT-qPCR

Four hours post-UVB irradiation, cell pellets were collected, and the total RNA was extracted by the GenElute™ Mammalian Total RNA Miniprep Kit (RTN70-1KT, SIGMA ALDRICH). RNA's integrity was double-checked by: (1) nanodrop and (2) agarose gel. Then, reverse transcription was performed using superscript III reverse transcriptase (18080093, Thermo Fisher Scientific). This was followed by cDNA dosage using the nanodrop.

Real-time quantitative PCR (RT-qPCR) was carried out on the cDNA using Mesa Blue Master Mix Plus (RT-SY2X-03+WOULRB, Eurogentec). After adding each of the primers used (table 4), the expression of each gene was detected by qPCR machine (CFX96 thermal cycler C1000-touch, Bio-Rad) and was normalized to GAPDH (figure 30).



Figure 30. *The CFX96 thermal cycler C1000-touch used by our laboratory, CIBEST/CEA. It monitors the amplification of targeted short DNA fragments in real-time.*

*Note: GAPDH was selected as the most suitable housekeeping gene for normalization after comparing it with other housekeeping genes (CycloA and CycloB). This was done through **the BEST KEEPER program, which selects the most stable and suitable housekeeping gene** using repeated pair-wise correlation analysis.*

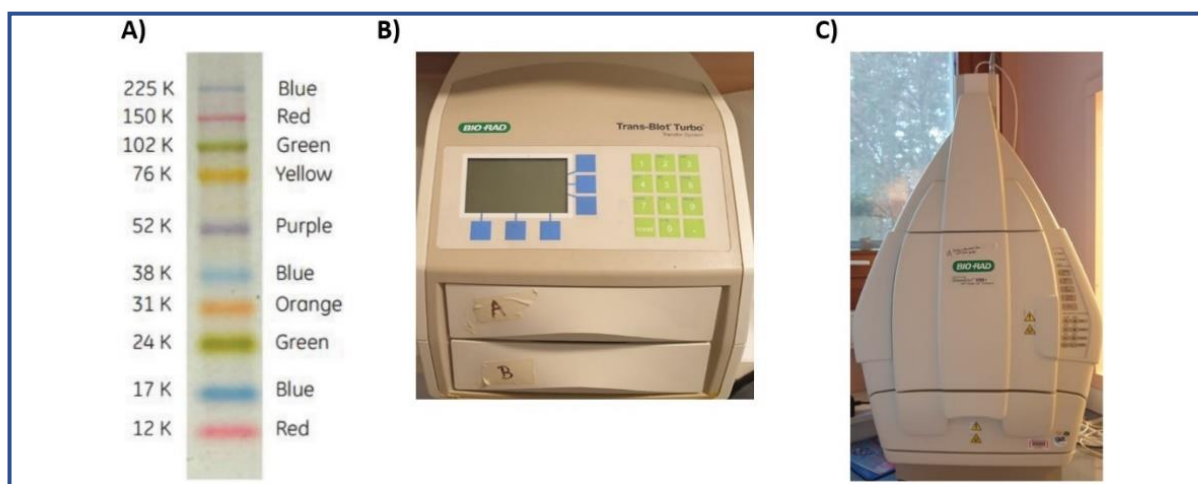
Table 4. Oligonucleotides' sequences that were used in RT-qPCR experiments as gene primers. Their efficiency was tested (E~99%) before moving forward with the experiments.

<b>Genes</b>		<b>Primers</b>	<b>Forward</b>	<b>Reverse</b>
<b>NER</b>		<i>XPC</i>	CCA-TGA-GGA-CAC-ACA-CAA-GG	TCC-AAT-GAA-CCA-CTT-CAC-CA
<b>BER</b>		<i>OGG1</i>	TGG-AAG-AAC-AGG-GCG-GGC-TA	ATG-GAC-ATC-CAC-GGG-CAC-AG
		<i>MYH</i>	CCA-GAG-AGT-GGA-GCA-GGA-AC	TTT-CTG-GGG-AAG-TTG-ACC-AC
		<i>APE1</i>	GCT-GCC-TGG-ACT-CTC-TCA-TC	GCT-GTT-ACC-AGC-ACA-AAC-GA
		<i>LIG3</i>	GCT-CAG-CAG-GAG-ATG-GTT-TC	TCT-AGG-TCC-CGT-GCC-ATA-TC
		<i>XRCC1</i>	CAG-CCC-TAC-AGC-AAG-GAC-TC	GCT-GTG-ACT-GGG-GAT-GTC-TT
		<i>POLβ</i>	GAG-AAG-AAC-GTG-AGC-CAA-GC	CGT-ATC-ATC-CTG-CCG-AAT-CT
		<i>LIG1</i>	AGG-AGT-GGA-ATG-GAG-TGG-TG	AGG-TGT-CAG-AGA-GGG-AAG-CA
		<i>FEN1</i>	ACC-AAG-CTT-TAG-CCG-CCG-AG	GGC-ATC-AAT-GGC-CAC-CTT-ACG
<b>Cell cycle</b>		<i>P53</i>	GTT-CCG-AGA-GCT-GAA-TGA-GG	TCT-GAG-TCA-GGC-CCT-TCT-GT
		<i>Gadd45a</i>	AGG-AAG-TGC-TCA-GCA-AAG-CC	GCA-CAA-CAC-CAC-GTT-ATC-GG
<b>Antioxidants</b>		<i>Nrf2</i>	CAG-TCA-GCG-ACG-GAA-AGA-GT	ACC-TGG-GAG-TAG-TTG-GCA-GA
		<i>SOD1</i>	AGG-GCA-TCA-ATT-TCG-AG	ACA-TTG-CCC-AAG-TCT-CCA-AC
		<i>SOD2</i>	TCC-ACT-GCA-AGG-AAC-AAC-AG	TCT-TCC-TGG-GAT-CAT-TAG-GG
		<i>Gpx1</i>	CCA-GTC-GGT-GTA-TGC-CTT-CT	CTC-TTC-GTT-CTT-GGC-GTT-CT
<b>House keeping</b>		<i>GAPDH</i>	GAG-TCA-ACG-GAT-TTG-GTC-GT	TTG-ATT-TTG-GAG-GGA-TCT-CG

## 2.2. Western blot

Four hours post-UVB irradiation, cell pellets were collected, and the total proteins were extracted and solubilized effectively upon cell lysis by RIPA lysis buffer (R0278-50mL, Sigma Aldrich). This was followed by total protein dosage (MicroBC Assay Protein Quantification kit, UP75860A, Interchim) and storage in  $-80^{\circ}\text{C}$ . On the experiment day, samples were prepared by adding 1X lamellae blue and ribonuclease-free water, heated at  $90^{\circ}\text{C}$  for 10 minutes, and equally loaded in Bio-Rad 4-20% mini-protein gels (2553103). Then, blotting and blockage of Bio-Rad membrane (2553047) by 5 percent lyophilized milk were done (figure 31). After that, membranes were incubated with primary antibodies at  $4^{\circ}\text{C}$  overnight (table 5). The next day, membranes were washed several times and incubated for one hour with a secondary antibody (table 5). This was followed by washing and revealing. The membranes were visualized through Bio-Rad Molecular Imager® Chemi Doc™ XRS+ using Image Lab™ software (figure 31). The analysis was done by normalizing the target protein's expression to the stain-free total protein extract via Image Lab program

Figure 31 shows the Amersham™ ECL™ Rainbow™ Marker full range ladder used to determine the molecular weights of the targeted bands on the membrane.



*Figure 31. Western blot instruments used by our laboratory, CIBEST/CEA. Panel A) Amersham™ ECL™ Rainbow™ Marker-Full Range ladder (RPN800E, Sigma Aldrich). Panel B) the BIO-RAD Transfer-Blot Turbo™ transfer system used to blot our gels into membranes. Panel C) BIO-RAD ChemiDoc™ XRS (HELA commercial nuclear protein extract was used as positive control)*

Table 5. Antibodies that were used in western blot experiments.

Antibodies	Type	Dilution	Supplier	Reference
<b>NER</b>	XPC	1/1000, 1/500	Santa Cruz	sc-74410
<b>BER</b>	OGG1	1/50000	Abcam	ab124741
	MYH	1/250	Novus Biologicals	NB600-1032
	APE1	1/1000	Sigma	HPA002564
	LIG3	1/3000	Novus Biologicals	NBP1 41190
	XRCC1	1/500	Abcam	ab1838
	FEN1	1/10000	Abcam	ab109132
	PARP1	1/3000	Cell Signaling Technology	9542
<b>Cell Cycle</b>	P53	1/5000	Sigma	p6874
<b>Cytoprotection against Oxidative Stress</b>	Nrf2	1/1000	Abcam	ab62352
<b>Positive control</b>	GAPDH	1/5000	Santa Cruz	sc-137179
<b>Secondary</b>	Mouse	1/10000	Amersham Biosciences	NA931
	Rabbit	1/10000	Amersham Biosciences	NA934

### 3. Detection of DNA lesions

#### 3.1. HPLC-MS/MS: detection of bulky lesions

For this experiment, after cell collection at different time points, we needed to do DNA extraction and enzymatic digestion as follows:

Similar to Mouret et al., cell pellet was lysed by triton X-100 then the nuclei were isolated by centrifugation and solubilized by SDS. To precipitate DNA solely, successive treatments with RNases and proteinase were done, followed by the addition of sodium iodide and 2-isopropanol. Finally, the resulting DNA pellet was solubilized with 0.1 mM deferoxamine mesylate solution ready for digestion (Mouret et al. 2006).

Two successive digestions were done as follows: (i) phosphodiesterase II (0.1 U/ $\mu$ L), DNase II (10 U/ $\mu$ L), nuclease P1 (0.2 U/ $\mu$ L), and MNSPDE buffer (200 mM succinic acid, 100 mM CaCl<sub>2</sub>, pH 6) were added to the DNA for 2 hours at 37°C. Then, (ii) phosphodiesterase I and alkaline phosphatase (2 units, pH 8) were added for another 2 hours at 37°C. Finally, HCL

(0.1M) was added, followed by sample centrifugation (5000g, 5 minutes), transfer in HPLC vial, and storage at -20°C (Mouret et al. 2006).

For injection in the HPLC-MS/MS, samples were freeze-dried overnight then the remaining residues were solubilized in triethylammonium acetate solution (20 mM). We used the transitions mentioned in table 6 to detect the different bipyrimidine photoproducts (Mouret et al. 2006):

**Table 6. The transitions used for our analysis were based on specific chromatographic conditions and mass spectrometry features** (Mouret et al. 2006). Standard nucleosides were quantified at 270 nm. For identified CPDs: T<>T (TT cyclobutane dimer), C<>C (CC cyclobutane dimer), T<>C (TC cyclobutane dimer), C<>T (CT cyclobutane dimer). For identified 6-4 PPs : TT (TT (6-4) photoproduct), TC (TC (6-4) photoproduct).

Photoproducts	Transitions
T<>T	545→447
C<>C	517→195
T<>C	531→195
C<>T	531→195
TT	545→432
TC	530→195

### 3.2. Comet Assay ± Fpg: detection of oxidized purines (8-oxoGua)

In our experiments, we did both: the classical and modified Fpg-comet assay. It is usually done to measure the induced DNA lesions of cellular extracts and their repair at various kinetic points.

After cell collection (as shown in part 1.2), cell pellets were suspended in freezing buffer [Sucrose (85.5 g/L), sodium citrate (11.76 g/L), DMSO (50 mL/L), pH 7.6 (adjusted via citric acid 0.1 M)] (2\*10<sup>5</sup>cells/100µL) and stored at -80°C until use.

Before the day of the experiment, slides were also prepared by covering them with normal agarose (A9539-250G, Sigma Aldrich). On the experimental day, cells were diluted with 0.6 percent of low-melting agarose (A9414-5G, Sigma Aldrich), deposited on the previously prepared slides, and covered by coverslips. This was followed by incubating slides with lysis buffer for one hour and washing 3 times with neutralizing buffer. After that, Fpg (5U/slide and 2.5U/slide for [parts one and two](#), respectively) + Fpg buffer (+FPG) and Fpg buffer (-FPG) were prepared and added to the slides for a humidified incubation at 37°C for 40 minutes. The reaction was stopped by incubating slides on ice for 10 minutes. Afterward, slides were incubated in cold buffer for 30 minutes followed by DNA electrophoresis (25 V, 530 mA) at 4°C for further 30 minutes. Then, slides were washed by neutralizing buffer, dried, and 50µL

1X-Gel Red was added to each one for the next day's reading. Slides were read using a 10X objective microscope and Comet Assay IV software (Perceptive Instruments, Suffolk, UK) by randomly selecting 50 nuclei (DNA). The extent of damage was evaluated by the mean tail intensity percentage of DNA, and normalization was done as a ratio of irradiated/non-irradiated at each condition. Each experiment was repeated 3 different times and contained triplicate for each condition.

Two positive controls were used in the experiments: Hydrogen peroxide ( $\text{H}_2\text{O}_2$ , 400 $\mu\text{M}$ ) and UVA + Riboflavin (10218153, AlfaAesar) (10 J/cm<sup>2</sup>+0.1mM)

*Note: The positive control concentration was selected after checking different concentrations and selecting the best one for the experiment.*

#### 4. Studying oxidative stress

##### **Part Two “Ameliorating the DNA repair of XP-C cells by modulating their redox state via pharmacological treatments”:**

##### 4.1. ROS assay

Intracellular ROS was quantified using DHR123 (Dihydrorhodamine 123, Sigma Aldrich). First, cells were plated in dark 96-well plates for 24 hours, then treated with different drugs, as shown in part 1.2. After that, cells were rinsed with 1X-PBS and incubated with diluted DHR123 (1/100 PBS) for 30 minutes. This was followed by irradiation with UVB (0.02 J/cm<sup>2</sup>), adding cell medium, and measuring the fluorescence intensity ( $\lambda_{exc}/\lambda_{em}$  480/530 nm) at different kinetic points (0-, 0.5-, 1-, 2-, 24-, and 48-hours post-irradiation).

$\text{H}_2\text{O}_2$  (500 $\mu\text{M}$ ) was used as a positive control.

##### 4.2. Glutathione assay

We collected cells post-UVB irradiation (0.01 J/cm<sup>2</sup>) using a rubber policeman and experimented as suggested by the supplier (703002, Cayman). Cell pellets were suspended in 1X 2-(N-morpholino) ethanesulfonic acid (MES, 703010, Sigma Aldrich) cold buffer then lysed using liquid nitrogen (freezing-thawing 5 times). This was followed by centrifugation at 10,000 g for 15 minutes at 4°C. The supernatant was then deproteinated as follows:

1mL of metaphosphoric acid (MPA) (5g/50mL) was added to the sample, followed by vortexing and 5 minutes incubation at room temperature. Then, samples were centrifuged (2000 g, 2 minutes), and the supernatant was carefully collected and stored at -20°C. On the experiment day, triethanolamine (TEAM, 4M, T58300, Sigma Aldrich) was prepared, and 50 $\mu\text{L}$  was added to the samples to increase the pH. Next, we designed a plate of standard and



sample wells and added freshly prepared cocktail solution (MES buffer, reconstituted Cofactor Mixture, reconstituted Enzyme Mixture, water, and reconstituted DTNB). Then GSH concentration was quantified by the endpoint method (405-414 nm) after 25 minutes.

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# *Results & Discussion*

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## Part One “Deciphering the Role of XPC in BER and Oxidative Stress”

**X**PC has been linked to BER and redox homeostasis by various researchers to explain the clinical heterogeneity amongst XP-C patients (high skin and internal cancers). XP-C cell lines showed reduced excision of oxidized DNA damage (8-oxoGua, 8-oxoA, TG) and sensitivity to oxidants (Kumar et al. 2020).

In our study, we tried to scan such a link in a detailed manner to better understand the biological role of XPC in UVB-induced-oxidized DNA repair. For that, we used three distinct XP-C primary fibroblasts. We studied the effect of such mutations on the stimulated expression (gene, protein) and activity of the BER pathway, the main pathway responsible for repairing oxidized bases, compared to three normal primary fibroblasts. However, before going deeper in the research, we devoted the first part of our study to characterize these primary fibroblasts and to optimize their experimental conditions.

*This work was published in Frontiers in Genetics Journal (<https://doi.org/10.3389/fgene.2020.561687>) (refer to Annex).*

# 1. Chapter One: Characterization of Primary Fibroblasts

## 1.1. Impaired XPC gene and protein expression in XP-C fibroblasts

To verify that the three different mutated primary fibroblasts (materials and methods, table 2) lack XPC, we analyzed the gene expression at mRNA and protein basal levels through RT-qPCR and western blot, respectively.

As expected, XP-C1, XP-C2, and XP-C3 cells showed a significant 8, 4, and 3-fold downregulation ( $p < 0.0001$ , \*\*\*\*), respectively, in *XPC*'s mRNA level compared to control cells (figure 32). Similarly, Khan et al. and Kuschal et al. showed that XP-C cells extracted from patients had less than 25 percent of the *XPC* mRNA level present in normal control (Khan et al. 2006; Kuschal et al. 2013).

Patients with low *XPC* mRNA level have a poorer cancer prognosis compared to those with higher expression level. Notably, this was shown in adenocarcinoma patients and mice with lung cancer (Y.-H. Wu et al. 2010; Hollander et al. 2005). Hence, it may not be a surprise for XP-C patients to develop cancer frequently where reduced *XPC* mRNA level may act as a prognostic to carcinogenesis (Y.-H. Wu et al. 2010).

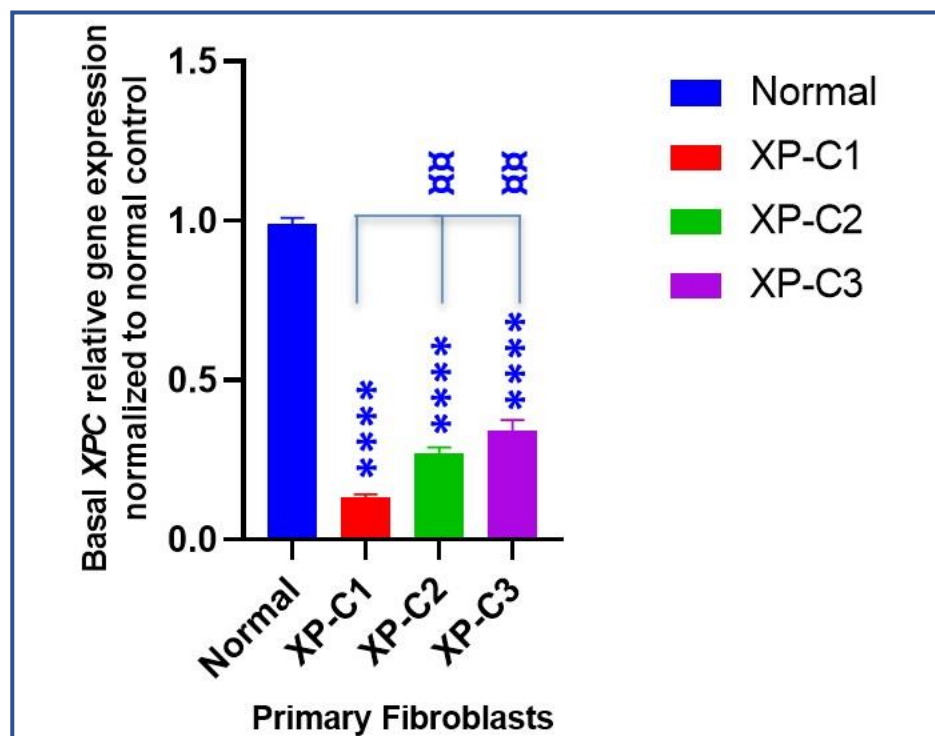
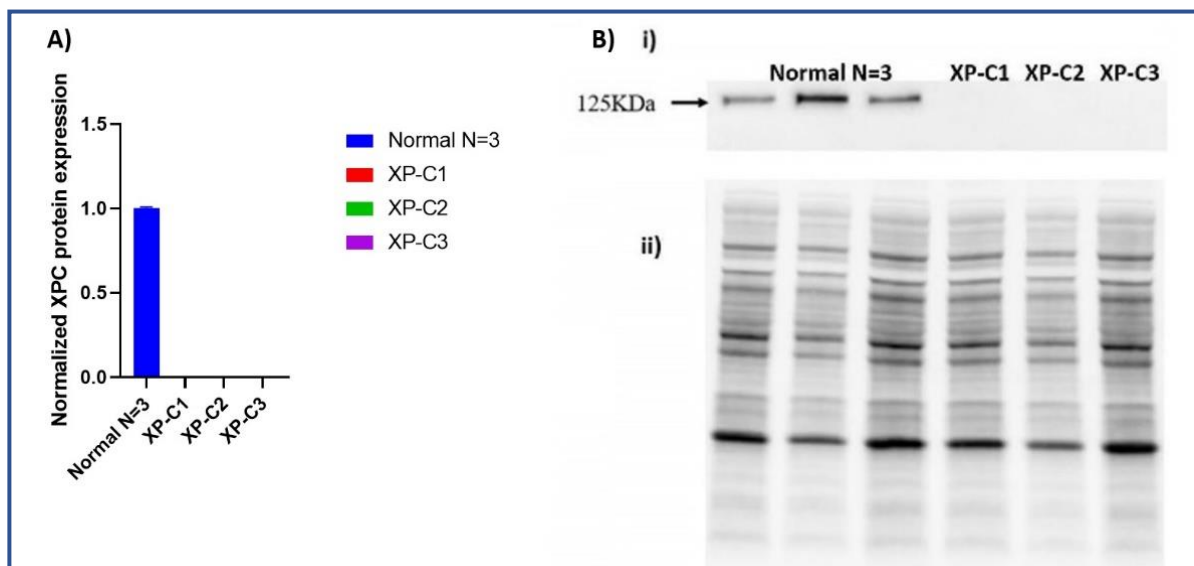


Figure 32. Lower *XPC* mRNA level in XP-C fibroblasts compared to normal control, at basal level. RT-qPCR showed that XP-C1, XP-C2, and XP-C3 had a significantly lower *XPC* mRNA expression ( $p < 0.0001$ , \*\*\*\*) compared to the normal control ( $N=3$ ). XP-C2 and XP-C3 showed significantly higher *XPC* mRNA expression than XP-C1 ( $p < 0.01$ , ȳȳ). The data were normalized relative to the *GAPDH* mRNA levels, where *GAPDH* was used as an endogenous control. Unpaired t-test was used to compare *XPC* mRNA level between normal and each

XP-C fibroblast ( $p < 0.05$ , \*), while paired *t*-test was used to compare XPC mRNA level between XP-C fibroblasts ( $p < 0.05$ ,  $\boxtimes$ ). The results are the mean  $\pm$  SEM from three independent experiments,  $n=3$ .

Of note, XP-C2 and XP-C3 had significantly higher XPC mRNA level compared to XP-C1 ( $p < 0.01$ ,  $\boxtimes\boxtimes$ ). This could be due to the difference in mutations amongst the studied fibroblasts. Their premature termination codons' positions could affect the susceptibility to nonsense-mediated decay in degrading transcripts (table 2).

Nevertheless, as shown in figure 33, all three low mRNA levels failed to be translated into full or truncated detectable XPC proteins. XP-C1's frameshift mutation (c. 1643-1644del) on exon 9 is most prevalent in Mediterranean countries such as Morocco, Algeria, Tunisia, Egypt, and Italy (Senhaji et al. 2013; N et al. 2018). It leads to mRNA harboring premature termination codon (PTC). This will trigger nonsense-mediated RNA decay (NMD) pathway that leads to translation termination (N et al. 2018; H. R. Rezvani, C. Ged, et al. 2008). The other studied mutations [(c.413-3delC, c.1086del) in XP-C2 and (c.1243C>T, c.2287delC) in XP-C3] also lead to PTC and proteins' absence. C.1243C>T and c.2287delC had been already identified on exons 9 and 13, respectively, in French families (Soufir et al. 2010).

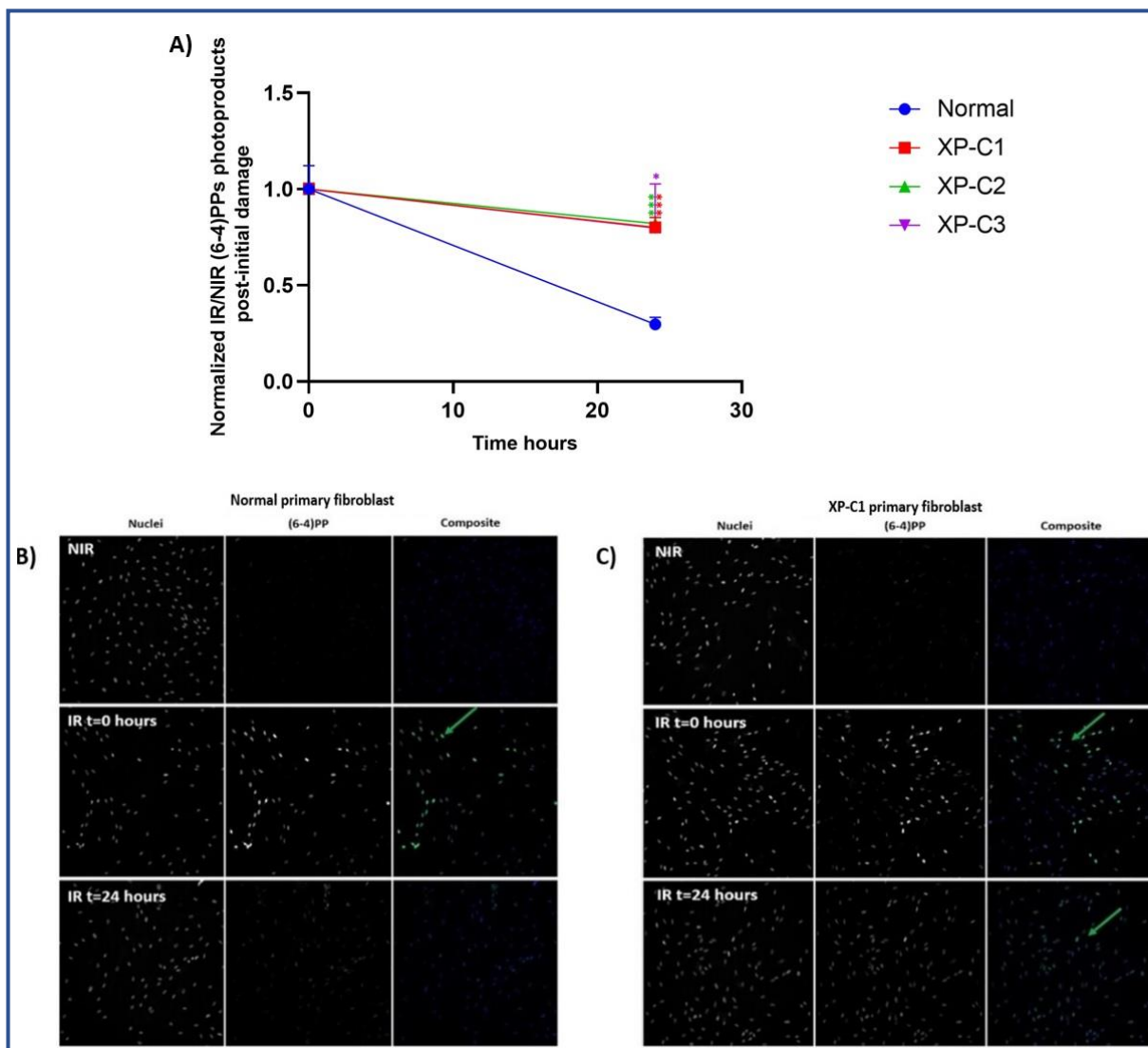


**Figure 33. Absence of XPC protein in XP-C primary fibroblasts compared to normal control, at basal level.** A) XPC protein was absent in the three XP-C fibroblasts (XP-C1, XP-C2, XP-C3) while detectable in the normal control, at MW=125KDa. B) represents the western blot membrane images (i) of the XPC band in normal vs XP-C cells upon hybridization with anti-XPC, and (ii) shows the total protein membrane that was used for normalization. The results correspond to the mean  $\pm$  SEM from three independent experiments,  $n=3$ .

### 1.2. Impaired NER capacities in XP-C primary fibroblasts compared to control cells

We wanted to check further whether the three XP-C primary fibroblasts used in our study have a moderate, mild, or deficient GG-NER activity. Therefore, we selectively studied the repair of (6-4) PPs recognized more specifically than CPDs by XPC (refer to canonical roles, 4.3.1).

As shown in figure 34, the UVB-induced (6-4) PPs persisted after 24 hours in the three XP-C mutated fibroblasts where they were significantly higher compared to the normal control (XP-C1:  $p < 0.001$ , \*\*\*; XP-C2:  $p < 0.001$ , \*\*\*; and XP-C3:  $p < 0.05$ , \*). Thus, after 24 hours, the repair was almost 20 percent in the XP-C cells compared to 70 percent in the normal cells. This agrees with other studies, which showed that (6-4) PPs are repaired efficiently after 24 hours in normal primary fibroblasts but are less repaired (10-20 percent) in XP-C fibroblasts (Chavanne et al. 2000).



**Figure 34. Deficient (6-4) PPs repair in XP-C primary fibroblasts compared to normal control, post-UVB irradiation.** Panel A) XP-C1, XP-C2, and XP-C3 showed a significantly slower repair and more persistence of lesion 24 hours after irradiation ( $p < 0.001$ , \*\*\*;  $p < 0.001$ , \*\*\*;  $p < 0.05$ , \* respectively). We used immunocytochemistry to monitor the repair of (6-4) PPs at 0 and 24 hours post-UVB ( $0.03 \text{ J/cm}^2$ ). For that, we stained the nuclei with Hoechst and the (6-4) PPs with green fluorescently labeled primary antibody, then we merged both fluorescence and quantified the fluorescence signal. Normalization was done relative to non-irradiated values. An absence of primary antibody was used as the negative control. Unpaired  $t$ -test was used to compare the normalized lesion ratio between normal and each XP-C fibroblast at each UVB dose (0 and 24 hours) ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from two independent experiments,  $n=2$  (each experiment was done as a triplicate). IR= irradiated, NIR= non-irradiated. Panels B and C) represent the immunocytochemistry images of

normal and XP-C1 primary fibroblasts, respectively. The green color represents the presence of (6-4) PPs in the nuclei. Nuclei were visualized by Hoechst staining. 0.03 J/cm<sup>2</sup> was used to avoid over-expressed fluorescence and cellular over-staining.

Note: As we proceeded with the characterization and results validation, we compared normal and XP-C1 fibroblasts. As presented in table 2, XP-C1 harbors homozygous frameshift mutation (c. 1643-1644del), classified as the most common and studied mutation.

To confirm the results obtained by immunofluorescence, we did HPLC-MS/MS to monitor the kinetics of repair in XP-C1 versus normal fibroblast between 0 and 48 hours post-UVB irradiation (0.05 J/cm<sup>2</sup>). As shown in figure 35, (6-4) PPs were completely repaired at 24 hours in the normal fibroblasts but persisted, almost unrepaired, in XP-C1 fibroblasts for more than 48 hours. Similarly, more than 20 percent of CPDs were repaired at 48 hours in the normal fibroblasts but persisted, almost unrepaired, in XP-C1 fibroblasts.

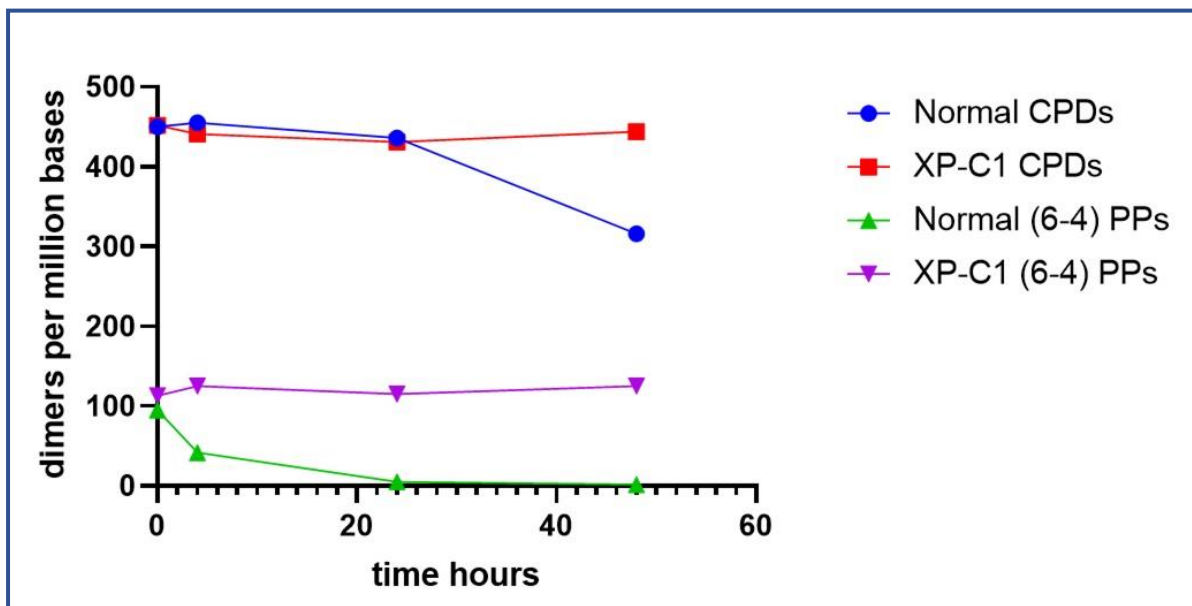


Figure 35. Kinetics of bulky lesions' [CPDs and (6-4) PPs] repair in XP-C1 versus normal fibroblast post-UVB irradiation. We collected cells at different kinetic points (0, 4, 24, and 48 hours) post-UVB (0.05 J/cm<sup>2</sup>) and monitored the repair of both types of lesions: CPDs and (6-4) PPs by HPLC-MS/MS. (preliminary result, n=1)

HPLC-MS/MS provided us the privilege to monitor how each type of (6-4) PPs and CPDs were repaired (supplementary figures 1 and 2). We showed that, in normal fibroblast, the (6-4) PPs and CPDs were repaired at 24 and 48 hours post-UVB, respectively, while they persisted in XP-C cells.

Therefore, XP-C fibroblasts failed to repair the studied bulky lesions.

### 1.3. Similar UVB-induced photosensitivity between XP-C and normal primary fibroblasts

Photosensitivity represents the exaggerated skin response to UVA/UVB light. This response is present in numerous diseases like xeroderma pigmentosum and is translated clinically as skin burning, redness, freckles, and carcinogenesis. Hence, *do our studied XP-C primary fibroblasts have photosensitivity?*

As shown in figure 36, 24 hours post-UVB irradiation, the MTT cytotoxicity test revealed that normal and XP-C primary fibroblasts share similar photosensitivity. This may be due to the fully functional TC-NER in XP-C cells that prevents P53-dependent apoptotic pathways in the absence of RNA synthesis arrest (Qiao, Scott, et al. 2011). For example, Queille et al. showed that XP-D and XP-A cells are more sensitive to UV-induced apoptosis compared to XP-C and normal cells due to their TC-NER deficiency where bulky lesions at DNA transcribed strands trigger apoptosis (Queille et al. 2001). Also, it was shown that *xpc*<sup>-/-</sup> mice and mouse embryonic fibroblasts (MEF) are moderately UVB-photosensitive (Boonstra et al. 2001; de Waard et al. 2008). In parallel, Khan et al. and Sethi et al. showed that XP-C patients do not have acute photosensitivity and severe sunburns at low UV doses (Khan et al. 2009; Sethi et al. 2013). Interestingly, XP-C fibroblasts did not show any variation in viability compared to control cells when exposed to other stress types, including oxidative agents (H<sub>2</sub>O<sub>2</sub>, methylene blue..) (de Melo et al. 2016). Therefore, XPC could be considered as a non-vital protein for cell viability.

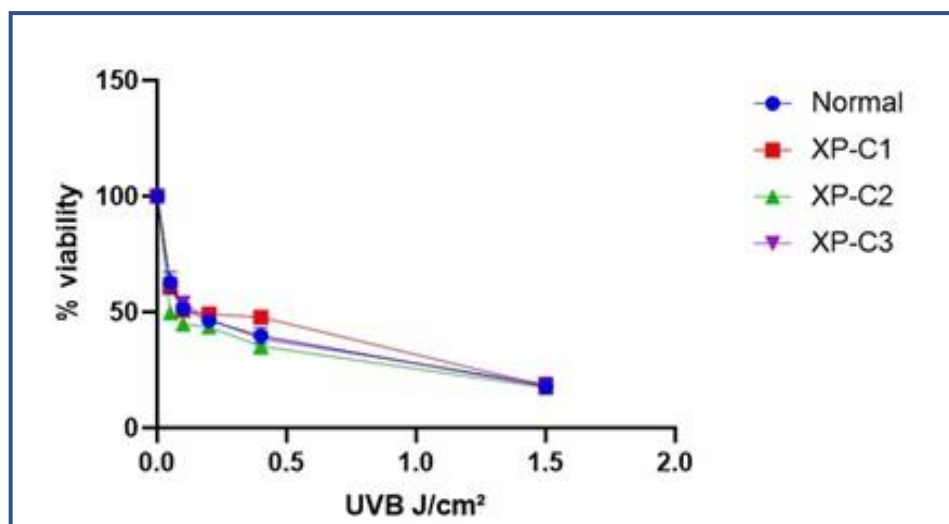


Figure 36. **Similar photosensitivity between normal and XP-C primary fibroblasts.** We measured the percentage of cellular viability 24 hours post-UVB irradiation by a short-term cytotoxicity test (MTT). Then we normalized each sample by its untreated value (100% viability). Unpaired *t*-test was used to compare photosensitivity between normal and each XP-C fibroblast at each UVB dose. The results are the mean  $\pm$  SEM from three independent experiments, *n*=3.



This experiment allowed us to choose a UVB dose for our experiments selectively. Based on the regression analysis (table 7), we should select a UVB dose as follows: <math><0.15 \text{ J/cm}^2</math> for normal and XP-C3 cells, <math><0.18 \text{ J/cm}^2</math> for XP-C1 cell, and <math><0.06 \text{ J/cm}^2</math> for XP-C2 cell. Therefore, we decided to use **0.05 J/cm<sup>2</sup>**, a dose that kills less than 40 percent of normal, XP-C1, and XP-C3 fibroblasts and almost 50 percent of XP-C2 primary fibroblast. Such a dose seems adequate for our aims as it will not kill more than 50 percent of the cells but could induce a detectable and monitored effect.

*Table 7. Measurement of LD<sub>50</sub> for normal and XP-C primary fibroblasts post-UVB irradiation. LD<sub>50</sub> represents the UVB dose that will reduce 50% of the in vitro cell survival. It is calculated after the short-cytotoxicity test that represents cellular viability vs UVB doses. The results represent the average of three different experiments, n=3.*

Fibroblasts	LD <sub>50</sub> J/cm <sup>2</sup>	LD <sub>50</sub> for all together J/cm <sup>2</sup>
Normal N=3	0.15	0.14
XP-C1	0.18	
XP-C2	0.06	
XP-C3	0.15	

#### 1.4.Higher ROS level in XP-C1 fibroblasts

To show whether UVB affects XP-C cells at levels other than cellular viability, we selected XP-C1 and normal fibroblasts and treated them with DHR123 followed by UVB-irradiation (0.02 J/cm<sup>2</sup>) and monitored the kinetic of ROS fluorescence at 0, 0.5, 1, 2, 3, 24, and 48 hours. Higher ROS level was observed in XP-C1 compared to normal, particularly 48 hours post-UVB ( $p < 0.05$ , \*) where ROS was decreasing slower in the absence of XPC compared to its presence (figure 37). Furthermore, high ROS levels were also detected in keratinocytes upon XPC silencing (Hamid Reza Rezvani et al. 2011). This could be one of the main reasons behind XP-C patients' physiopathology.

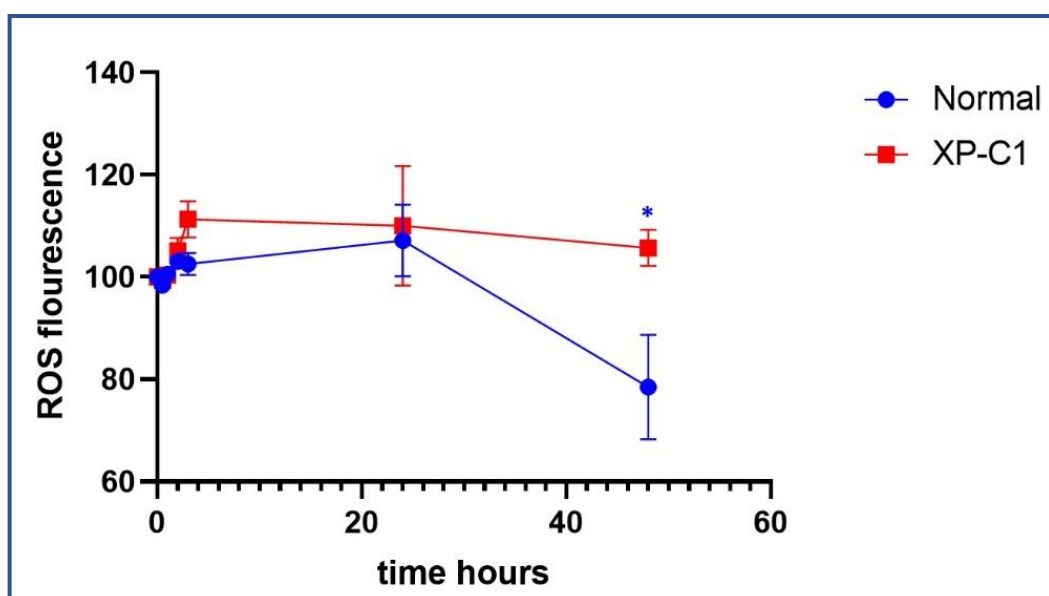


Figure 37. **Kinetics of ROS level in XP-C1 and normal primary fibroblasts.** We treated cells with DHR123 (1/1000) followed by UVB and monitored fluorescence kinetics at different kinetic time points. Then we normalized each sample by its untreated value (100% viability). H<sub>2</sub>O<sub>2</sub> was used as a positive control. Unpaired *t*-test was used to compare the ROS fluorescence between normal and XP-C1 fibroblast at each kinetic time point (hours) ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from three independent experiments,  $n=3$ .

Therefore, we suggest that the higher persistent ROS level in XP-C1 in parallel to the acute UVB photosensitivity could play a major role in the accumulation of mutations and the slow initiation and progression of multiple of tumorigenesis in absence of detectable cell death.

### 1.5.Higher photoresistance in XP-C1 fibroblasts to solar simulation

After checking the effect of UVB on XP-C cells' viability, we wanted to check whether it was the case with UVA+UVB. This will allow us to detect any synergistic, antagonistic, or independent effect of UV-induced lesions and oxidative stress on the cells. Furthermore, it will support the link between *XPC* mutations, cell viability, and oxidative stress.

When we monitored the effect of the solar simulator, which mimics the sun's radiation, on XP-C1 and normal primary fibroblasts, we detected more photoresistance from the former cells than the normal control (figure 38). XP-C1 fibroblast had significantly higher viability compared to control at 1 ( $p<0.05$ , \*) and 2 ( $p<0.01$ , \*\*) MED. Since solar simulator contains UVA+UVB and UVA is known to produce more oxidative stress than UVB, this may further confirm our hypothesis and show resistance of XP-C fibroblasts to high oxidative stress as suggested by de Melo et al. (de Melo et al. 2016).

XP-C cells accumulate mutations and macromolecular damages, which provokes them to transform later and induce metastasis; meanwhile, once the damage exceeds the capacities of DNA repair pathways, it triggers death in normal cells. In addition, XP-C cells have high NOX1 levels, which play a role in P53 dysregulation via SIRT1 and induce apoptosis suppression (Puca et al. 2010; Hamid Reza Rezvani et al. 2011). On the other hand, Naik et al. had shown that UV induces programmed cell death in primary keratinocytes and fibroblasts via neutralizing the anti-apoptotic Bcl-2 family that regulates P53-dependent and P53-independent pathways against UV (Naik et al. 2007).

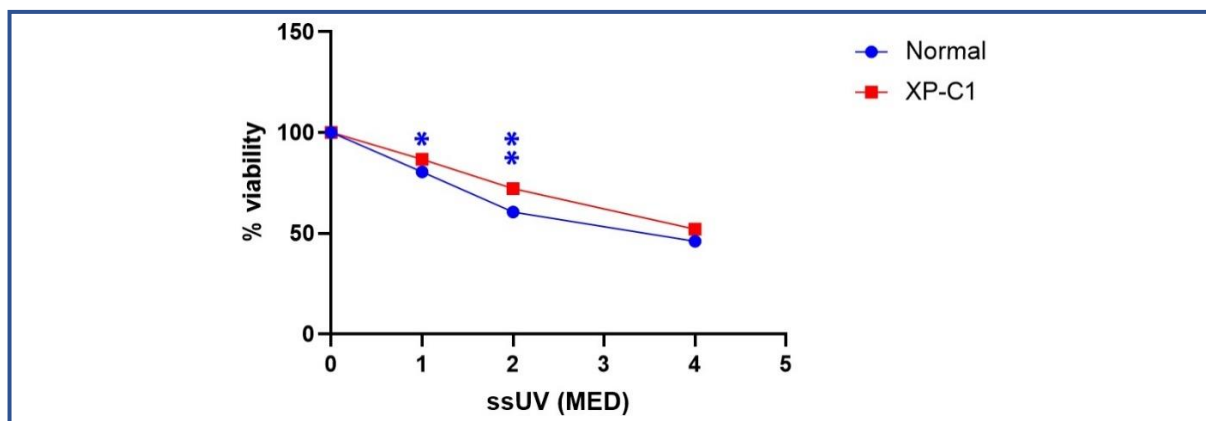


Figure 38. **Higher photo-resistance in XP-C1 primary fibroblast compared to normal control.** We measured the percentage of cellular viability 24 hours post-solar simulator (UVA+UVB) irradiation by short-term cytotoxicity test (MTT). Then we normalized each sample by its untreated value (100% viability). Unpaired *t*-test was used to compare normal and each XP-C fibroblast photosensitivity at each UV dose ( $p<0.05$ , \*). The results are the mean  $\pm$  SEM. ssUV= solar simulator UV; MED=minimal erythema dose.

Therefore, our studied XP-C cells show persistence to high UV-induced oxidative stress (ssUV, UVB), which may trigger mutagenesis and tumorigenesis.

## Briefing of the characterization

The three different XP-C fibroblasts had impaired XPC gene expression compared to the normal fibroblasts. This led to:

- Similar UVB-photosensitivity
- High ROS level and resistance to oxidative stress
- Deficiency in bulky lesions' [CPDs, (6-4) PPs] repair

Such cellular profiling could explain why the absence of XPC triggers a variety of cancers. For instance, Hollander et al. showed that one hundred percent of the studied *xpc*<sup>-/-</sup> mice developed lung cancer (Hollander et al. 2005). This paved the way for checking whether the XPC-BER interplay is the primary key in such an event and whether BER could endure the consequences of high ROS levels in XP-C cells.

## 2. Chapter Two: Effect of XP-C Mutations on BER's Expression and Excision Activity

Studies have shown a complexity in the NER and BER pathways due to their interplay. One of the critical players in such crosstalk is XPC: it interacts and/or stimulates SMUG1, TDG, OGG1, and APE1, but *does it affect the rest of the BER factors?*

### 2.1. Effect of XP-C on BER's mRNA expression post-UVB

For that, we explored the UVB-stimulated gene expression ratio [irradiated (IR)/non-irradiated (NIR)] of the main BER factors starting with the incision/excision step (*OGG1*, *MYH*, *APE1*), then the synthesis step (*POLβ*), and ending with the ligase step (*LIG3*). BER is known to repair bases (oxidized, alkylated, deaminated) and single-strand DNA breaks. This could show us how cells adapt to UVB stress. We mainly focused on the short-patch BER pathway because 8-oxoGua is primarily repaired; nevertheless, this does not exclude its repair by the long-patch BER pathway (Marsin et al. 2003).

As discussed earlier in section 2.1. (overview of BER pathway), OGG1, MYH, and APE1 are common BER initiation factors in both sub-pathways (short-patch and long-patch). OGG1 and MYH are responsible for the excision of oxidized purines (8-oxoGua) and adenine opposing the oxidized purines, respectively, while APE1 plays a role as an endonuclease and redox regulator. POLβ and LIG3 had also been shown to be vital for oxidative DNA damage repair (Akbari et al. 2014). POLβ adds newly synthesized nucleotide to 3' end of the previously arising abasic site and removes the 5' sugar-phosphate due to its polymerase and 5'-deoxyribose-5-phosphate lyase (dRP lyase) activities, respectively. On the other hand, LIG3 seals the nick of the DNA strand. Furthermore, we were inquisitive about considering other genes that could affect the expression and/or efficiency of previously mentioned factors. For that, we checked the gene expression of the accessory genes: *XRCCI*, *FEN1*, *PARP1*, and stress-inducible genes: *P53* and *GADD45a*.

#### 2.1.1. BER factors

We showed a significant global inhibition of BER mRNAs' stimulated expression post-UVB in the presence of the three different XP-C mutations compared to the normal control (figure 39).

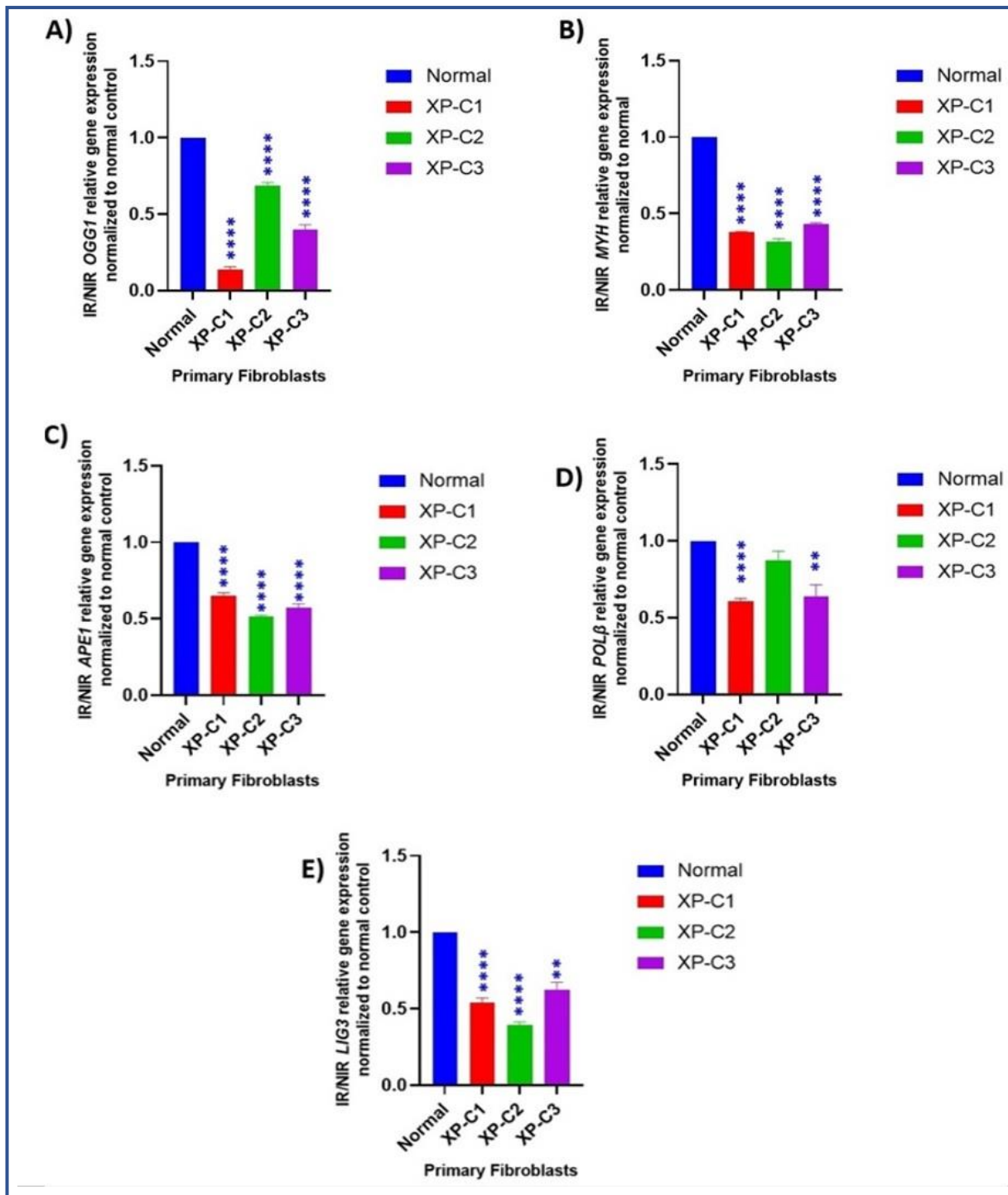
*OGG1*, *MYH*, *APE1*, and *LIG3* showed a significant downregulation ( $p < 0.01$ ) in all the three XP-C primary fibroblasts, while *POLβ* showed a downregulation ( $p < 0.01$ ) in XP-C1 and XP-C3 but not XP-C2. This allows us to hypothesize that XPC deficiency hinders BER at the

transcriptional level. De Melo et al. had already suggested an inhibition of *OGG1* and *APE1*'s mRNA expression following H<sub>2</sub>O<sub>2</sub> treatment in SV-40 immortalized XP-C fibroblasts (de Melo et al. 2016). To our knowledge, there is no other research discussing the effect of *XPC* mutation on the rest of the BER transcripts. However, several studies reported a link between a downregulation in their expression level and cancer, which may explain the cancer risk heterogeneity in XP-C patients. For example, *MYH* mRNA and protein expressions were downregulated in gastric cancer (Shinmura et al. 2011). Similarly, *POLβ* mRNA and protein expressions were downregulated in breast cancer. Likewise, *LIG3* mRNA was shown to be downregulated in dermal fibroblasts isolated from nevoid basal cell carcinoma patients (Abdel-Fatah et al. 2014; Charazac et al. 2020).

Besides the direct interactions between *XPC* and BER factors, ***could such an impaired BER factors' mRNA expression be due to the role of XPC in inducing BER's expression through specific signaling mechanisms or promoters directly and/or indirectly?***

Apart from *XPC*'s role in DNA repair, it plays a role in transcription regulation. For example, *XPC* deficiency has been shown to reduce *BRCA1* expression via upregulating its repressor, Pit-1 (H. Wang et al. 2019). In its turn, *BRCA1* was reported to stimulate the expression of some BER factors and enhance their activity, including *OGG1*, *XRCC1*, and *APE1* (T. Saha et al. 2010, 1).

Therefore, we propose a direct and indirect role of *XPC* in stimulating BER's expression post-stress (UVB).



**Figure 39. Downregulated BER-associated gene transcription in XP-C fibroblasts compared to normal control, post-UVB irradiation.** After total RNA extraction and reverse transcription, we did RT-qPCR to study the BER gene transcription 4 hours post-UVB (0.05 J/cm<sup>2</sup>). Panels A), B), and C) show that XP-C fibroblasts had a significant downregulation of normalized IR/NIR mRNA expression ( $p < 0.0001$ , \*\*\*\*) of OGG1, MYH, and APE1, respectively. Panel D) shows a significant downregulation of normalized IR/NIR POLB mRNA expression in XP-C1 ( $p < 0.0001$ , \*\*\*\*) and XP-C3 ( $p < 0.01$ , \*\*) compared to control. Panel E) shows a significant downregulation of normalized IR/NIR LIG3 mRNA expression in XP-C1 ( $p < 0.0001$ , \*\*\*\*), XP-C2 ( $p < 0.0001$ , \*\*\*\*), and XP-C3 ( $p < 0.01$ , \*\*) compared to control. Normalization was done relative to non-irradiated control. Unpaired t-test was used to compare the normalized gene expression ratio between normal and each XP-C fibroblast ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from three independent experiments,  $n=3$ . Normal ( $N=3$ ). IR= irradiated, NIR= non-irradiated

### 2.1.2. BER accessory factors

Studying the gene expression at the transcriptional level is vital for the characterization and understanding of the molecular basis underlying the phenotypic changes. Hence, we further screened BER's stimulated expression (IR/NIR) and studied *XRCC1*, *FEN1*, and *PARP1* (figure 40). Although *XRCC1* lacks an enzymatic activity, it is vital to coordinate an efficient DNA repair by interacting with several BER factors (Marsin et al. 2003). It interacts with *OGG1* and *APE1* to stimulate their excision and glycosylase activities, respectively (Marsin et al. 2003). It also interacts with *LIG3* for the latter's stabilization and with *POLB* (Marsin et al. 2003). *FEN1* is an endonuclease involved in long-patch BER of oxidized abasic sites as 2-deoxyribonolactone (P. Liu et al. 2008). It cleaves within the apurinic/apyrimidinic (AP) site-terminated flap that will be then sealed by *LIG1* (Ranalli, Tom, and Bambara 2002). *FEN1* interacts with *APE1* (Ranalli, Tom, and Bambara 2002).

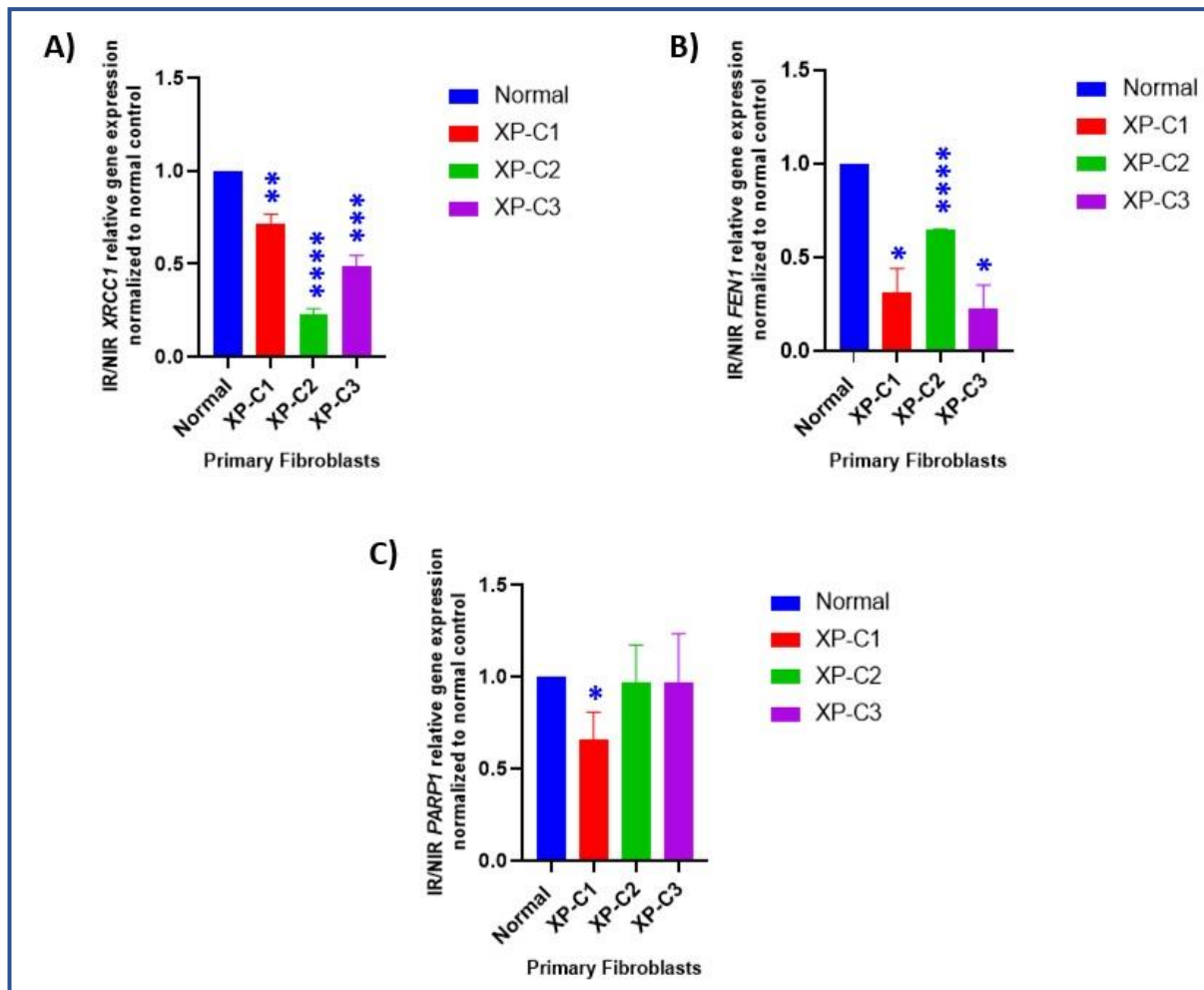
Furthermore, *PARP1* is involved in several mechanisms, including DNA repair. *XRCC1*'s recruitment to DNA damage is *PARP1*-dependent (Ray Chaudhuri and Nussenzweig 2017). *PARP1* also interacts with *POLB*, *APE1*, and *XPC* (Abdel-Fatah et al. 2014; Lili Liu et al. 2017, 1; Robu et al. 2017). Such proteins have a direct impact on the activity and efficacy of BER proteins. However, ***does XPC has the same impact on their mRNA expression as it did on the previously studied BER factors?*** Answering this question could contribute to understanding the disease's molecular mechanism and regulatory networks for mRNA and targeted therapies, especially RNA-based therapy.

As shown in figure 40, we found that *XRCC1* and *FEN1* were significantly inhibited in all XP-C fibroblasts compared to normal cells ( $p < 0.05$ ). Additionally, *PARP1* was significantly inhibited in XP-C1 ( $p < 0.05$ , \*) but not in XP-C2 and XP-C3. It was demonstrated that *XRCC1* and *FEN1* deficiencies induce genetic instability and cellular transformation post-genotoxic stress (Brem and Hall 2005; H. Sun et al. 2017). As *PARP1* plays a role in both, NER and BER, its downregulation could augment the accumulation of CPDs and oxidative DNA lesions post-UVB irradiation, thereby triggering photocarcinogenesis (Hegedűs et al. 2019). It is also possible that the downregulation of *LIG3*, *APE1*, and *POLB* in the absence of *XPC* could influence the activation and expression of *XRCC1*, *FEN1*, and *PARP1*. This confirms the direct interaction between the BER factors and their accessory proteins. For instance, *PARP1* needs *APE1* to become activated and recruit downstream BER factors at the AP site (Lili Liu et al. 2017).



In addition, it was reported that XP-C cells show low apoptosis due to low PARP1 levels post-methylene blue treatment (de Melo et al. 2016). This is coherent with our photosensitivity study (figure 36).

It is important to address that it would have been interesting to check the efficacy of the BER factors at the translational level and posttranslational modifications as the latter play a major role in their efficiency and activity.



**Figure 40. Downregulated BER-associated accessory gene transcription in XP-C fibroblasts compared to normal control, post-UVB irradiation.** After total RNA extraction and reverse transcription, we did RT-qPCR to study the gene transcription 4 hours post-UVB (0.05 J/cm<sup>2</sup>). Panel A) shows a downregulation in normalized IR/NIR mRNA expression of XRCC1 in XP-C1 ( $p < 0.01$ , \*\*), XP-C2 ( $p < 0.0001$ , \*\*\*\*), and XP-C3 ( $p < 0.001$ , \*\*\*) compared to control. Panel B) shows a downregulation in normalized IR/NIR mRNA expression of FEN1 in XP-C1 ( $p < 0.05$ , \*), XP-C2 ( $p < 0.0001$ , \*\*\*\*), and XP-C3 ( $p < 0.05$ , \*) compared to control. Panel C) shows a significant downregulation in normalized IR/NIR mRNA expression of PARP1 in XP-C1 ( $p < 0.05$ , \*) but not XP-C2 and XP-C3. Normalization was done relative to non-irradiated control. Unpaired *t*-test was used to compare normalized gene expression ratio between normal and each XP-C fibroblast ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from three independent experiments,  $n = 3$ . Normal ( $N = 3$ ). IR= irradiated, NIR= non-irradiated

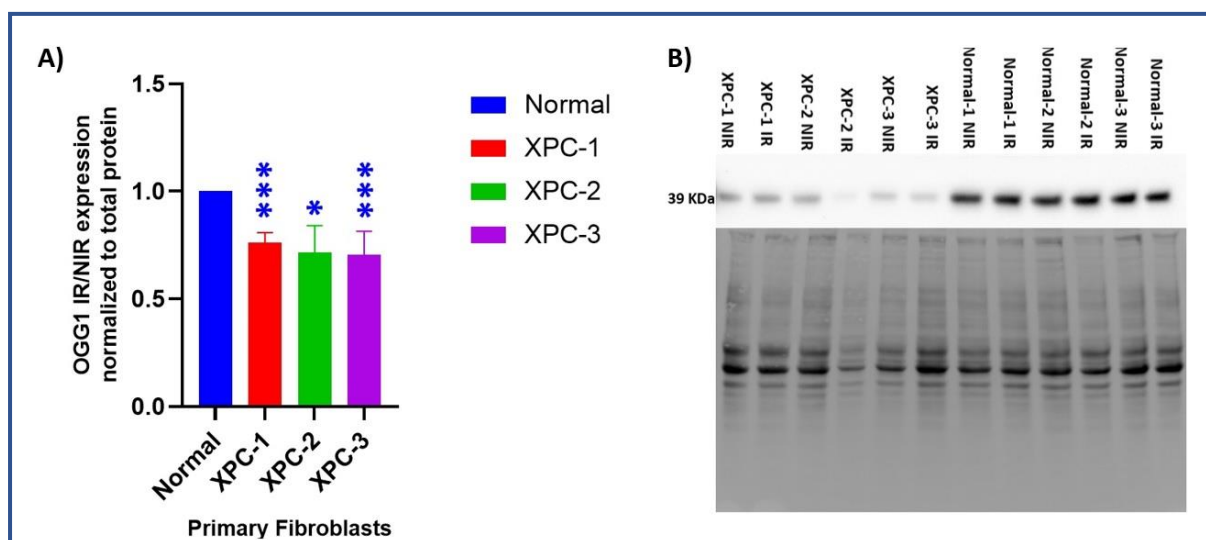
## 2.2. Effect of XPC mutations on BER's protein expression post-UVB

After screening the fibroblasts at the transcriptional level, we wanted to selectively study the stimulated translational level of OGG1, MYH, and APE1 post-UVB. This may further elucidate the regulations of the molecular interaction network of the disease, which in turn can pave the way in front of methods for prevention, diagnosis, and treatment. Therefore, OGG1, MYH, and APE1 were selected for western blot analysis due to their essential role in initiating oxidative DNA lesions' repair.

As presented in figure 41, OGG1 stimulated protein expression (IR/NIR) was downregulated in XP-C1 ( $p < 0.001$ , \*\*\*), XP-C2 ( $p < 0.05$ , \*), and XP-C3 ( $p < 0.001$ , \*\*\*) compared to normal. Several studies support our finding. This indicates the involvement of XPC in stimulating the initiation of BER post-stress.

De Melo et al. showed that OGG1 protein level decreases in the absence of XPC. Once cells are complemented with XPC, the level of OGG1 protein augments (de Melo et al. 2016). A downregulated OGG1 protein in the absence of XPC could lead to a lower concentration than the UV-induced lesion substrates. This induces steady phase activity and lower efficiency in lesions' excision, particularly, in the presence of high ROS level and oxidative DNA damage (as discussed before).

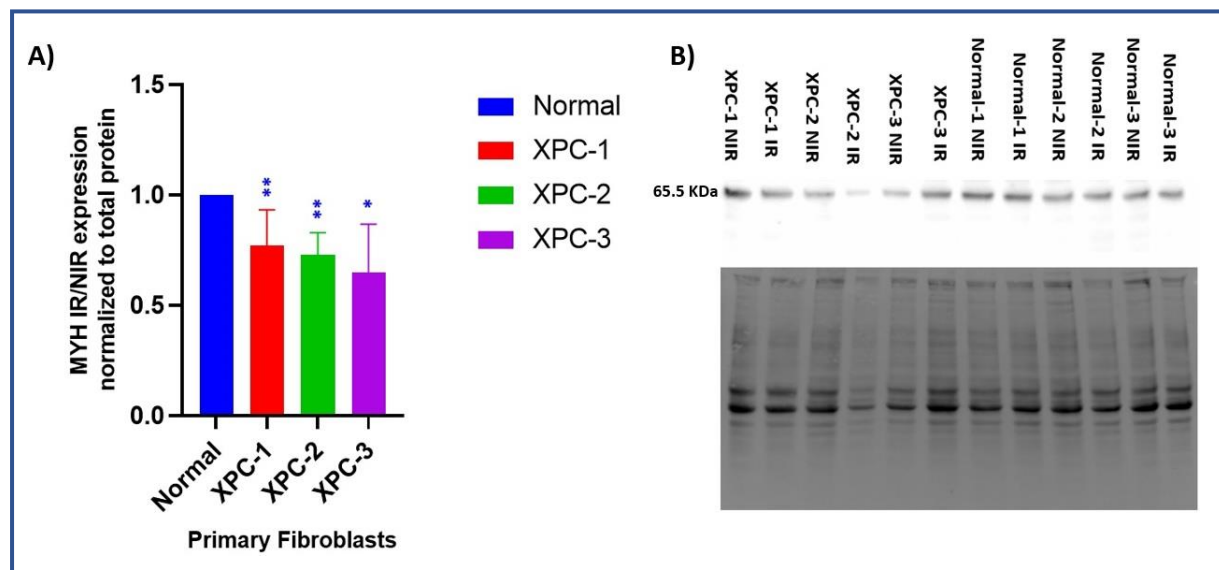
This contributes to a higher oxidative DNA repair efficacy and elucidates the role of XPC in BER.



**Figure 41. Downregulated BER-associated OGG1 protein level in XP-C fibroblasts compared to normal control, post-UVB irradiation.** After total protein extraction and quantification, a western blot was done to check the expression of OGG1 protein in XP-C vs normal fibroblasts 4 hours post-UVB ( $0.05 \text{ J/cm}^2$ ). Panel A shows a downregulation of normalized OGG1 IR/NIR protein level in XP-C1 ( $p < 0.001$ , \*\*\*), XP-C2 ( $p < 0.05$ , \*), and XP-C3 ( $p < 0.001$ , \*\*\*) compared to control. Panel B) represents the western blot membrane image of the OGG1

protein band and total protein. Normalization was done by the total protein then ratio was done relative to non-irradiated samples. Unpaired *t*-test was used to compare the normalized protein expression ratio between normal and each XP-C fibroblast ( $p < 0.05$ , \*). Values shown are the mean  $\pm$  SEM from three independent experiments,  $n = 3$ . Normal ( $N = 3$ ). IR = irradiated, NIR = Non-Irradiated.

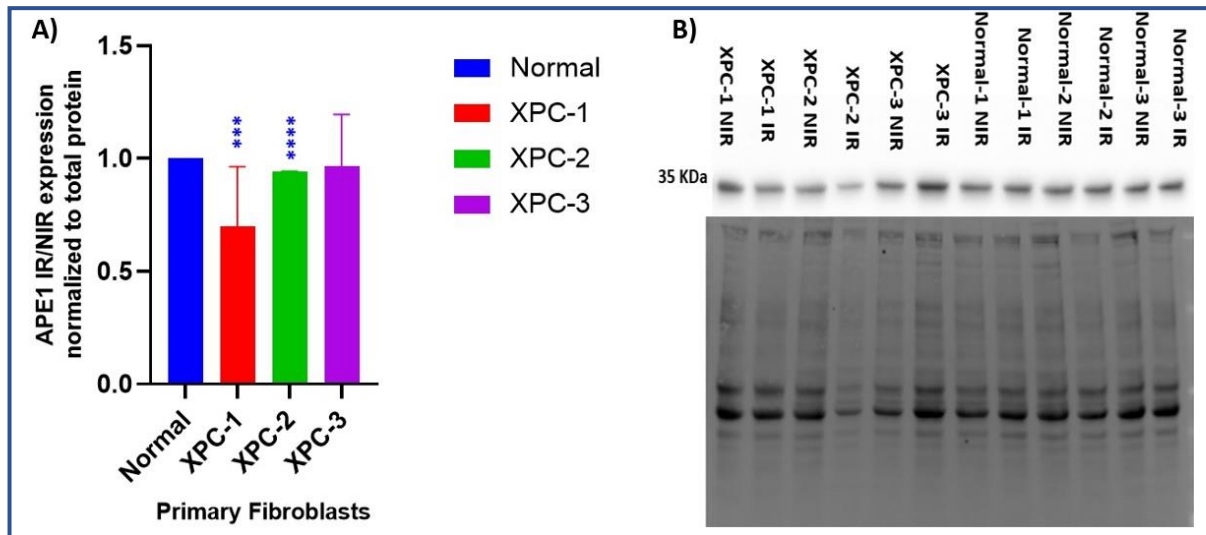
Furthermore, MYH IR/NIR protein expression was downregulated in XP-C1 ( $p < 0.01$ , \*\*), XP-C2 ( $p < 0.01$ , \*\*), and XP-C3 ( $p < 0.05$ , \*) (figure 42). Like OGG1, a low MYH protein level could affect its ability to repair high DNA lesions, contributing to XP-C pathogenesis. If both OGG1 and MYH fail to repair all the lesions, then G:C to T:A transversions will increase, inducing carcinogenesis (figure 2).



**Figure 42. Downregulated BER-associated MYH protein level in XP-C fibroblasts compared to normal control, post-UVB irradiation.** After total protein extraction and quantification, a western blot was done to check the expression of MYH protein in XP-C vs normal fibroblasts 4 hours post-UVB ( $0.05 \text{ J/cm}^2$ ). Panel A) shows a downregulation of normalized MYH IR/NIR protein level in XP-C1 ( $p < 0.01$ , \*\*), XP-C2 ( $p < 0.01$ , \*\*), and XP-C3 ( $p < 0.05$ , \*) compared to control. Panel B) represents the western blot membrane image of the OGG1 protein band and total protein. Normalization was done by the total protein then the ratio was done relative to non-irradiated samples. Unpaired *t*-test was used to compare the normalized protein expression ratio between normal and each XP-C fibroblast ( $p < 0.05$ , \*). Values shown are the mean  $\pm$  SEM from three independent experiments,  $n = 3$ . Normal ( $N = 3$ ). IR = irradiated, NIR = Non-Irradiated.

However, APE1 IR/NIR protein expression was only significantly downregulated in XP-C2 ( $p < 0.0001$ , \*\*\*\*) compared to normal control (figure 43). These results show downregulation in stimulation and initiation of BER in response to UVB stress. De Melo et al. showed that APE1 protein level also decreased in the absence of XPC but not significantly (de Melo et al. 2016). Moreover, APE1 is a multifunctional protein that plays a role in DNA repair and redox regulation. Therefore, its protein downregulation could explain the high oxidative stress in XP-C cells (Hamid Reza Rezvani et al. 2011). Another reason could be OGG1 and/or MYH downregulation. The downregulated OGG1 and MYH glycosylases may be insufficient to

repair 8-oxoGua efficiently and A:8-oxoG mispair, respectively. This might contribute to the high oxidized purines in a time-dependent manner and BER's dysregulation. Therefore, the downregulation in OGG1, MYH, and APE1 expression could propose the interconnection between them and the activation coordination to stimulate BER cascade.



**Figure 43. Downregulated BER-associated APE1 protein level in XP-C fibroblasts compared to normal control, post-UVB irradiation.** After total protein extraction and quantification, a western blot was done to check the expression of APE1 protein in XP-C vs normal fibroblasts 4 hours post-UVB (0.05 J/cm<sup>2</sup>). Panel A) shows downregulation of normalized APE1 IR/NIR protein level in XP-C1 ( $p < 0.001$ , \*\*\*) and XP-C2 ( $p < 0.0001$ , \*\*\*\*) but not in XP-C3 compared to control. Panel B) represents the western blot membrane image of the OGG1 protein band and total protein. Normalization was done by the total protein then ratio was done relative to non-irradiated samples. Unpaired *t*-test was used to compare the normalized protein expression ratio between normal and each XP-C fibroblast ( $p < 0.05$ , \*). Values shown are the mean  $\pm$  SEM from three independent experiments,  $n = 3$ . Normal ( $N = 3$ ). IR= irradiated, NIR= Non-Irradiated.

### 2.3. Effect of XP-C on BER's activity post-UVB

Undeniably, XP-C is an interesting model due to its intricacy, and studying the gene expression of BER gave us an idea about the tight connection and crosslink between the two. This connection is presented at the phenotypic level where XP-C patients have a high risk of internal cancer linked to oxidative stress and dysfunctional BER.

However, ***how profound is the effect of dysregulated XPC on BER's activity?***

#### *Comet $\pm$ FPG assay*

For that, we selected comet assay due to its advantages as accuracy, sensitivity, reliability, and the need for a small number of cells per sample. The latter was one of the main reasons for selecting comet assay over HPLC-MS/MS, which needs more than 2 million cells per sample. Collecting a high cell number is not practical when working with primary fibroblasts, especially when we studied six different primary fibroblasts with/without irradiation at different kinetic points.

We used modified comet assay ( $\pm$ FPG) where FPG glycosylase (DNA-formamidopyrimidine glycosylase) excises FPG-sensitive sites (oxidized purines: 8-oxoGua, Fapy) into abasic sites then DNA single-strands. We can quantify the oxidized purines by subtracting the value without FPG (-FPG) from values with FPG (+FPG) for each sample at each time point.

Figure 44 represents an example of the intact DNA (head of the comet) and damaged DNA (tail of the comet)  $\pm$  UVB  $\pm$  FPG in normal and XP-C1 fibroblasts.

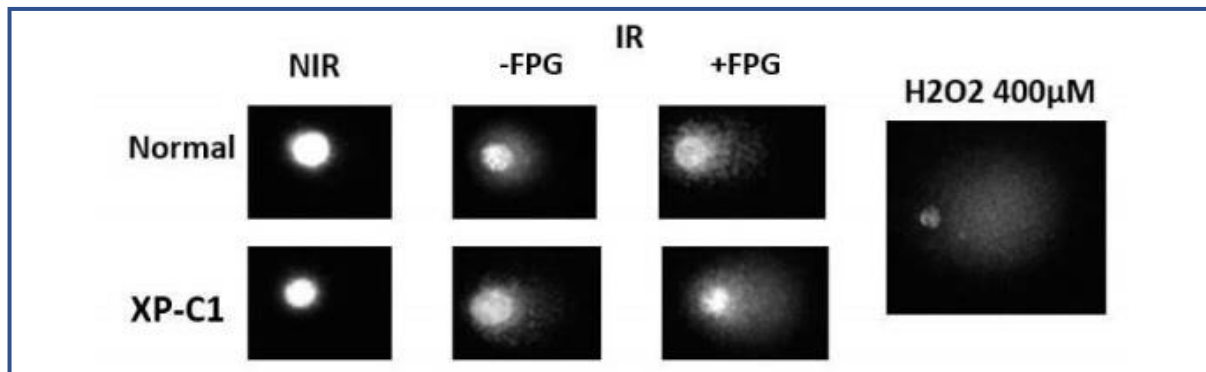


Figure 44. The undamaged (comet head) and damaged (comet tail) DNA  $\pm$  FPG in normal and XP-C1 fibroblasts and positive control  $H_2O_2$ . The tail intensity and its length are proportional to the DNA damage.

Figure 45 shows a significantly higher DNA damage ( $\pm$  FPG) in XP-C fibroblasts compared to control, post-UVB. It persisted until 24 hours. This indicates that UVB induces more DNA lesions instantaneously in the absence of XPC compared to control. These lesions include oxidized purines.

#### In the absence of FPG (-FPG)

DNA strand breaks (alkali-sensitive and abasic sites) were significantly higher in XP-C1 and XP-C2 fibroblasts ( $p < 0.05$ , \*) compared to normal control at 0, 2, and 24 hours post-UVB (figure 45). Similarly, Berra et al. showed that photosensitized methylene blue induced higher DNA damage in XP-C cells than in control (Berra et al. 2013). Also, Zhou et al. indicated through measuring alkaline comet assay that XPC protected primary murine type II pneumocytes from cigarette smoke-induced DNA damage, including bulky lesions and oxidative purines (H. Zhou et al. 2019).

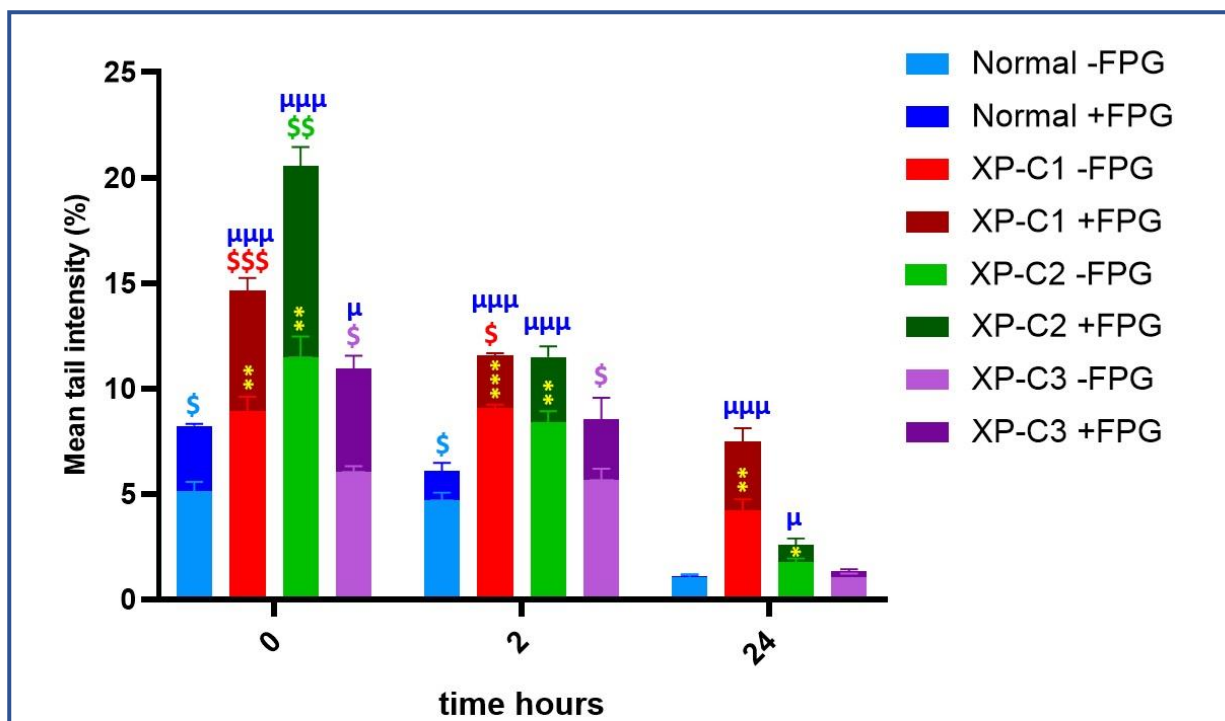
These induced lesions represent a crucial initial event in carcinogenesis.

This indicates that, in addition to dysregulated GG-NER, XP-C fibroblasts may have other impaired DNA repair pathways that fail to repair UVB-induced genomic lesions. Therefore, we used a modified comet assay (+FPG) to selectively study the link between XPC and BER.

### In the presence of FPG

As presented in figure 45, we detected a significantly increased % mean tail intensity ( $p < 0.05$ , \$) in all fibroblasts in the presence of FPG compared to its absence, at 0 hours post-UVB. After 2 hours, the high level persisted in all fibroblasts except for XP-C2. This indicates that UVB induces oxidized purines in all the fibroblasts. But *is it more potent in XP-C cells?*

At 0, 2, and 24 hours, XP-C1 and XP-C2 had significantly higher % mean tail intensity ( $p < 0.05$ ,  $\mu$ ) compared to normal control. However, XP-C3 showed a significantly higher % mean tail intensity ( $p < 0.05$ ,  $\mu$ ) 0 hours post-UVB (figure 45). This indicates that oxidized purines are more prominent and persistent in XP-C cells.



**Figure 45. Downregulated BER excision repair capacities in XP-C primary fibroblast compared to normal fibroblasts, post-UVB irradiation.** To follow-up the kinetics of repair of UV-induced lesions [single-strand breaks (SSB), alkali-labile sites (ALS) and oxidative purines (including 8-oxoGua)] in XP-C vs normal fibroblasts, we did Comet  $\pm$  FPG 0, 2, and 24 hours post-UVB (0.05 J/cm<sup>2</sup>). This graph represents the % mean tail intensity for each IR/NIR sample with FPG (oxidized purines, dark-colored) and without FPG (single-strand breaks, double-strand breaks, and alkali-labile sites). Although all primary fibroblasts could repair the DNA damage ( $\pm$  FPG) over time, XP-C cells showed a downregulated and slower repair than the normal control. In addition, more oxidized purines were induced at time= 0 hours in the absence of XPC. Paired t-test was done to compare each sample with 2 conditions (FPG-ve or FPG+ve), while an unpaired t-test was done to compare different samples within the same condition. The results are the mean  $\pm$  SEM from three independent experiments, n=3. Normal (N=3).

\$ Sample significantly ( $p < 0.05$ ) higher in its tail intensity with the presence of FPG (+FPG) compared to its absence (-FPG)

\* XP-C fibroblast significantly ( $p < 0.05$ ) higher in its tail intensity compared to normal fibroblast, FPG-ve condition.

$\mu$  XP-C fibroblast significantly ( $p < 0.05$ ) higher in its tail intensity compared to normal fibroblast, at FPG+ve condition.

-FPG= FPG alkaline buffer without the enzyme; +FPG= FPG alkaline buffer and FPG enzyme; IR= irradiated; NIR= Non-Irradiated

This is well-illustrated in figure 46. It represents oxidized purines kinetic repair in each sample post-UVB. At 0 and 2 hours, significantly higher oxidized purines ( $p < 0.05$ , \*) were detected in XP-C cells compared to normal fibroblasts, while at 24 hours, oxidized purines were significantly higher in XP-C1 and XP-C2 but not XP-C3 compared to normal control.

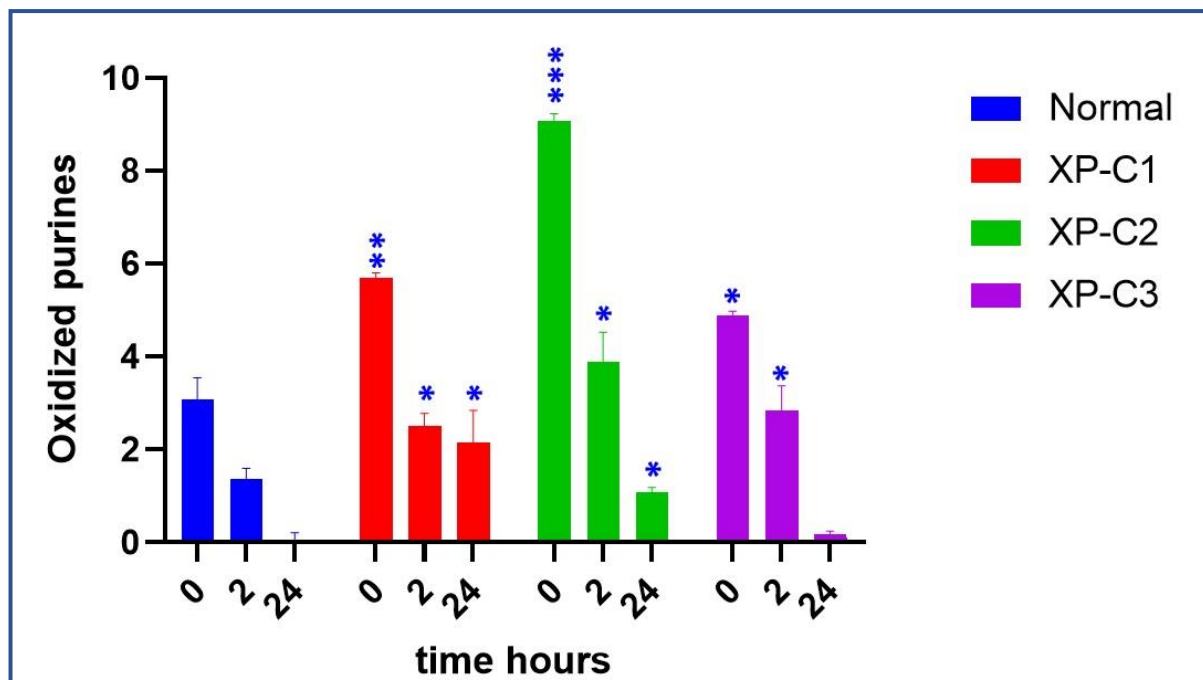


Figure 46. A simple graphical representation of BER excision repair in XP-C primary fibroblasts compared to normal fibroblasts, post-UVB irradiation. It displays the oxidized purines (8-oxoGua and Fapy) repaired over time in XP-C vs normal fibroblasts (N=3). Such DNA damage can be quantified by subtracting the values without FPG (-FPG) from values with FPG (+FPG) for each sample at each time point. Oxidized purines were significantly higher at each time point in all the XP-C fibroblasts than normal, except for XP-C3 24 hours post-UVB ( $p < 0.05$ ). Unpaired t-test was done to compare different samples within the same condition ( $p < 0.05$ , \*). Shown values correspond to the mean  $\pm$  SEM from three independent experiments (n=3). Normal (N=3).

\* XP-C fibroblast significantly ( $p < 0.05$ ) higher in its oxidized purines compared to normal fibroblast.

Therefore, XP-C cells have higher induced oxidized purines. This may be due to the higher oxidative stress and ROS in XP-C cells compared to normal control. Several studies showed that UVB generates excessive quantities of ROS that could induce DNA oxidation *in vivo* and *in vitro* (H. R. Rezvani, C. Ged, et al. 2008). In parallel, Menoni et al. detected through live cellular imaging that XPC is recruited to laser micro-irradiation induced 8-oxoGua (D'Augustin et al. 2020). Furthermore, XP-C cells are sensitive to oxidants as photoactivated methylene blue and KBrO<sub>3</sub> (Berra et al. 2013; Mariarosaria D'Errico et al. 2006).

As shown in figure 46, oxidized purines were repaired slower in XP-C cells than normal cells. This is discordant with Berra et al. Their results showed that FPG-sensitive sites were slightly higher in XP-C cells but repaired efficiently at 24 hours (Berra et al. 2013). Our results may differ due to different treatments and cell types (primary fibroblasts vs SV40-transformed skin fibroblasts) with different genetic backgrounds. Meanwhile, D'Errico et al. showed results that agree with ours. Their results showed that XP-C primary fibroblasts and keratinocytes have a downregulated KBrO<sub>3</sub>-induced 8-oxoGua repair (Mariasosaria D'Errico et al. 2006). A supporting result showed, by HPLC-ED, that *xpc*<sup>-/-</sup> mouse embryo fibroblasts displayed a reduced 8-oxoGua removal compared to control following treatment with an oxidizing agent, KBrO<sub>3</sub> (Kumar et al. 2020).

Accumulation of oxidized purines contributes to carcinogenesis (skin and internal) in XP-C patients. Multiple studies emphasized an interplay and interaction between NER and BER in oxidative DNA damage and bulky lesions repair (Kumar et al. 2020). High ROS levels and lack of XPC can inactivate OGG1's enzymatic activity by oxidizing its cysteine residue and halting its turnover, respectively. This results in G:C to T:A transversions mutations and carcinogenesis if intermediates are not repaired by MYH (Mariasosaria D'Errico et al. 2006; Hao et al. 2018). MYH low mRNA and protein levels were also linked to impaired repair of elevated A:8-oxoGua (Markkanen, Dorn, and Hübscher 2013). A low MYH activity or gene expression has been linked to gastric cancers, while reduced OGG1 activity has been linked to high breast cancer risk incidences (Shinmura et al. 2011; Yuzefovych et al. 2016). Mentioned internal cancers are also present in XP-C patients, revealing the impact of *XPC* mutations on BER and the consequences (Giglia et al. 1998; X. Bai et al. 2012). Indeed, other factors may play a role in such events. This may include the protein oxidation, in the presence of high ROS level, that will directly affect BER factors, thereby reducing their ability to repair oxidized purines efficiently and rapidly (Kumar et al. 2020).

Henceforth, ***does lack of XPC affect OGG1 activity or ROS level?*** We would say both based on our experiments (gene expression and activity, ROS level quantification) and other experiments.

Other severe clinical symptoms induced by oxidation, including neurological disorder, are rarely found in these patients due to the capacity of BER to repair oxidized lesions, although at a slower pace compared to normal control. For instance, 24 hours post-UVB, more than half of the oxidized purines were repaired in XP-C cells. This could be explained by the following



scenarios: (i) XPC deficiency downregulates but does not inhibit BER's activity and efficiency. OGG1's turnover is slower, so it will take more time to recognize consecutive lesions, and/or (ii) other backup-glycosylases and repair systems (lower kinetics) will step up once BER/OGG1 is incompetent.

### 3. Chapter Three: Effect of XP-C on Some Genes Linked to Cell Cycle Regulation

As mentioned previously (NER and cell cycle, section 3.2), XPC is involved in cell cycle regulation. Therefore, it would be interesting to check whether P53 and GADD45a's gene expressions are cluttered in the absence of XPC. This could provide us with an idea about the cellular behavior post-UVB.

Figure 47 shows that *P53*'s mRNA level was downregulated significantly in XP-C1 ( $p < 0.01$ , \*\*) and XP-C3 ( $p < 0.05$ , \*) and XP-C2 compared to control cells ( $N=3$ ).

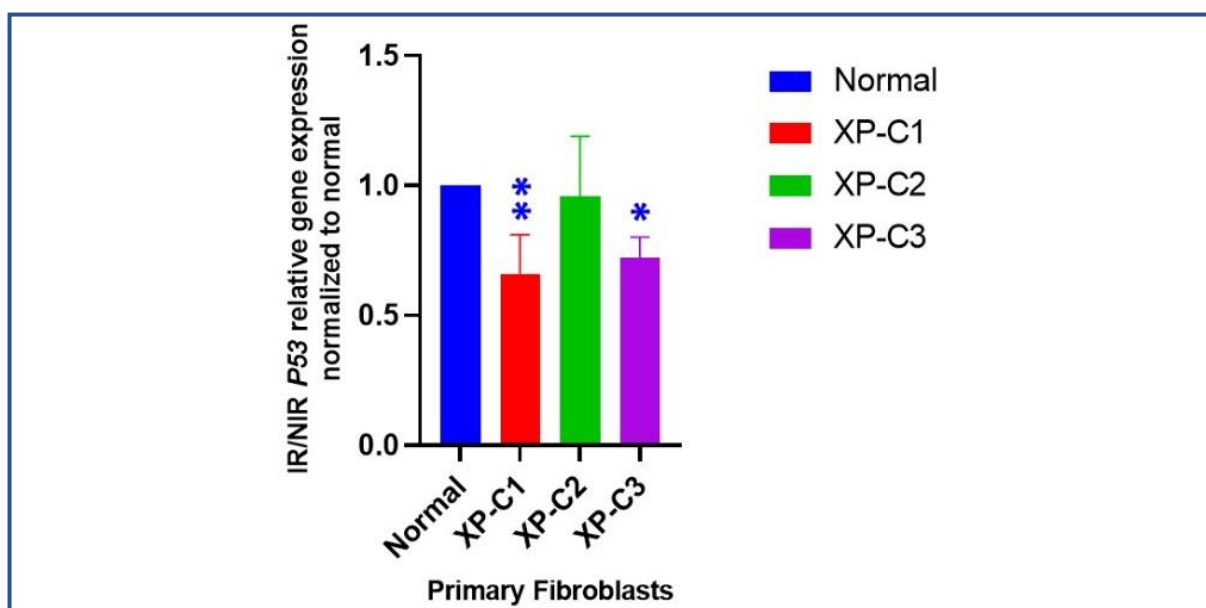
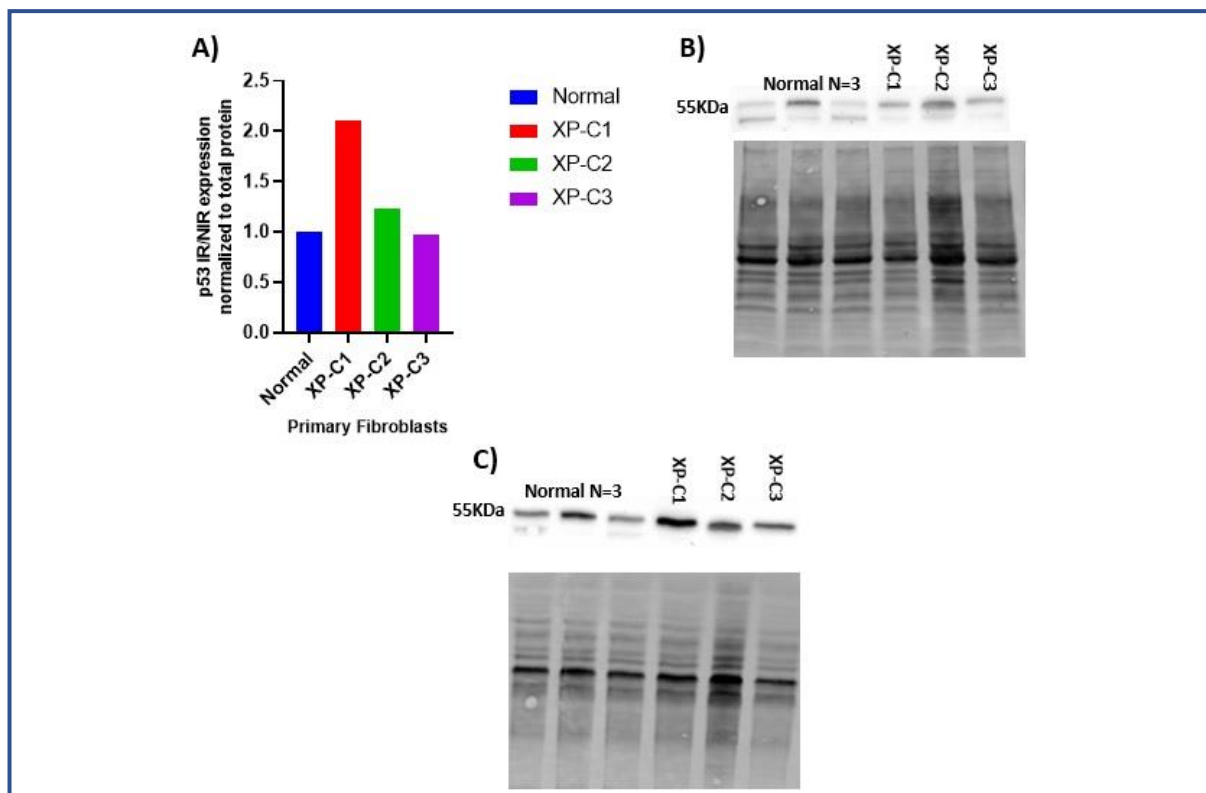


Figure 47. **Different P53 mRNA level between XP-C and normal fibroblasts.** After total RNA extraction and reverse transcription, we did RT-qPCR to study the gene transcription 4 hours post-UVB (0.05 J/cm<sup>2</sup>). Normalized IR/NIR P53's mRNA expression was downregulated in XP-C1 ( $p < 0.01$ , \*\*) and XP-C3 ( $p < 0.05$ , \*) but not XP-C2 compared to control cells ( $N=3$ ). Normalization was done relative to non-irradiated control. Unpaired t-test was used to compare normalized gene expression ratio between normal and each XP-C fibroblast ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM ( $n=3$ ). Normal ( $N=3$ ). IR= irradiated, NIR= non-irradiated

However, unlike some articles, figure 48 proposes a potentially higher P53 UV-induced-expression in XP-C1 and XP-C2 than normal cells (preliminary result). Under normal conditions, P53 level is low and increases upon genotoxic stress, including UV, for a short time to trigger cell cycle arrest for DNA repair or apoptosis. Nevertheless, it was addressed that once the P53 is mutated, it may function differently. Mutated P53 is more stable and triggers transcriptional dysregulation and uncontrolled tumor growth (HUANG et al. 2014). For instance, a high P53 protein level was detected in various cancer types, including colorectal, breast, endometrial, and esophageal squamous cell carcinomas (HUANG et al. 2014). In

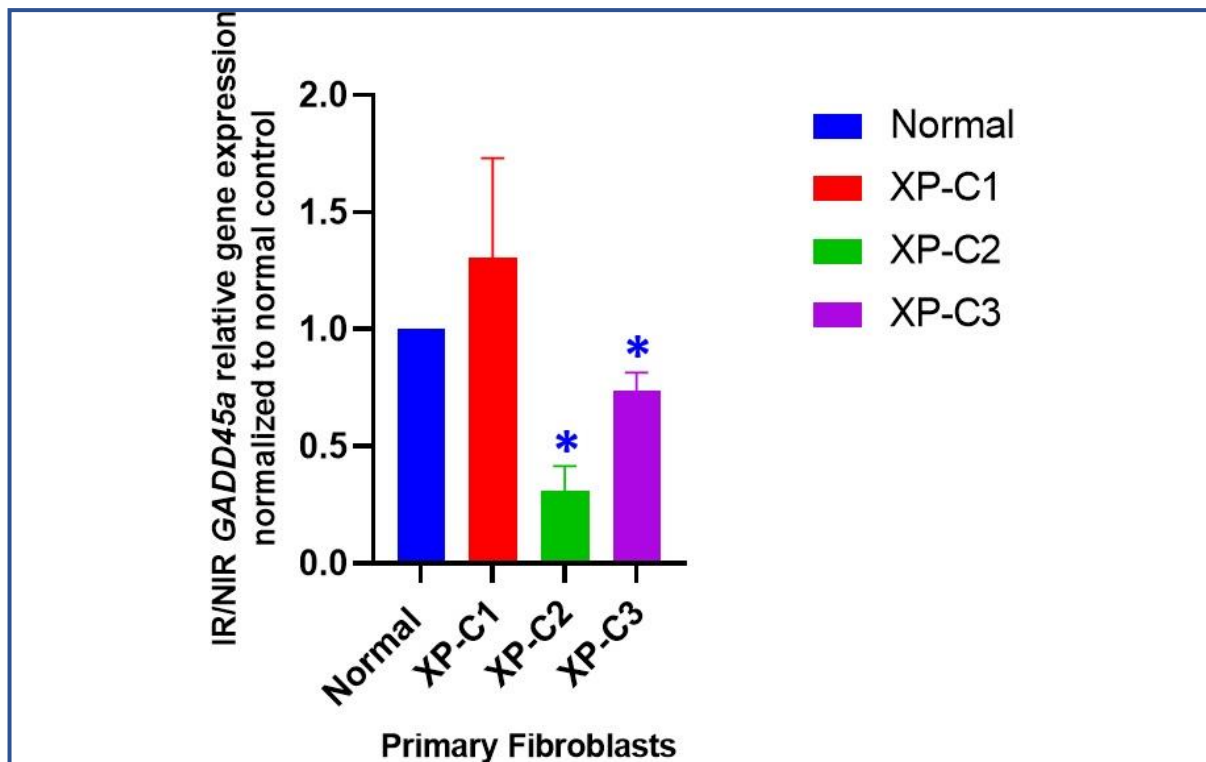
parallel, P53 is known to be mutated and dysregulated in XP patients, mainly due to G:C to T:A transversions and C to T and CC to TT UV signature mutations, which could play a significant role in carcinogenesis (Khan et al. 2006). Such mutations might have been induced in our cells due to ROS accumulation and oxidative DNA damage triggering mutagenesis. This anticipates in the dysregulation of P53's gene expression. Krzeszinski et al. showed in their study that P53 regulates UV-induced XPC expression. Once the XPC level becomes sufficient for an efficient DNA repair, it downregulates P53 for cells' return to their resting state. This may be through regulating P53's turnover via facilitating MDM2-Rad23-dependent degradation of polyubiquitylated P53 (Krzeszinski et al. 2014). Our preliminary result supports this scenario (figure 48). This protein accumulation in XP-C cells may indicate a failure in ubiquitylated-P53 proteolysis that negatively affect its mRNA expression.

Therefore, we suggest that the observed high P53 level could be a mutated and dysfunctional P53. Indeed, further experiments are required to confirm such a hypothesis such as repeating the western blot and checking the activity and the regulations post-translationally of P53.



**Figure 48. Different BER-associated P53 protein level in XP-C fibroblasts compared to normal control, post-UVB irradiation.** After total protein extraction and quantification, a western blot was done to check the expression of P53 in XP-C vs normal fibroblasts 4 hours post-UVB (0.05 J/cm<sup>2</sup>). Panel A) shows a non-significant upregulation of normalized P53 IR/NIR protein level in XP-C1 and XP-C2 but not XP-C3 compared to control. Panel B) represents the P53 band and the total protein of normal and XP-C fibroblasts at basal level. Panel C) represents the P53 band and the total protein of normal and XP-C fibroblasts at UVB level. Normalization was done by the total protein then ratio was done relative to non-irradiated samples. Values shown are from one experiment, n=1. Normal (N=3). IR= irradiated, NIR= Non-Irradiated. (preliminary result)

Such a dysregulated P53 affects its interactions and regulations of several signaling cascades post-genotoxic stress (such as UV). GADD45a (growth arrest and DNA damage-inducible45 alpha) is known to protect cells from UV-induced carcinogenesis in a P53-dependent and P53-independent manner to induce positive feedback on the P53 signaling pathway (Hildesheim et al. 2002). Our transcriptional analysis (figure 49) showed that *GADD45a* mRNA level was downregulated significantly ( $p < 0.05$ , \*) in XP-C2 and XP-C3 compared to normal cells. Hildesheim et al. showed that *GADD45a* knockout reduces UV-induced MAPK/P53 dependent apoptosis of keratinocytes. Herein, GADD45a prolongates the activation of apoptosis and/or cell cycle arrest pathways (p38/JNK MAPK and P53 pathways, G1/G2 arrest) and orchestrates DNA repair by interacting with NER (XPC, XPE) and BER (PCNA, APE1) to prevent genomic instability. Inhibition of P53 and GADD45a contributes to carcinoma progression and metastasis, especially in lung adenocarcinoma patients (Hildesheim et al. 2002, 53; Jung et al. 2013; Y.-H. Wu et al. 2010).



**Figure 49. Different GADD45a mRNA level between XP-C and normal fibroblasts.** After total RNA extraction and reverse transcription, we did RT-qPCR to study the gene transcription 4 hours post-UVB (0.05 J/cm<sup>2</sup>). Normalized IR/NIR gene expression of GADD45a was downregulated in XP-C2 and XP-C3 ( $p < 0.05$ , \*) but not XP-C1 compared to control cells. Normalization was done relative to non-irradiated control. Unpaired t-test was used to compare normalized gene expression ratio between normal and each XP-C fibroblast ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM. Normal (N=3). IR= irradiated, NIR= non-irradiated

The dysregulation of P53 and GADD45a might explain the absence of extreme photosensitivity for the studied XP-C fibroblasts and the high cancer risk due to mutagenesis in XP-C patients. Nevertheless, we should consider other *GADD45* genes that are usually highly expressed in dermal fibroblasts (like *GADD45g*) and could compensate for the downregulation in *GADD45a* to activate G1 and G2 checkpoints (Hildesheim et al. 2002).

It should be noted that studying GADD45a protein's expression level and cell cycle (P53 and GADD45a's activities) could have been interesting primarily due to the high mutated P53 recorded level in XP patients. Therefore, these studies may be considered as part of this project's perspectives.

# Conclusion and Perspective

The three studied XP-C primary fibroblasts:

- ❖ Had low *XPC* mRNA and no XPC protein
- ❖ Inhibited NER activity
- ❖ Downregulated BER at gene level (mRNA and protein) and at activity (excision) level

XP-C1 had higher ROS level compared to normal control.

**In summary, the synergic effects of amassed oxidative DNA damage and impaired BER could explain the heterogeneity in the clinical spectrum of XP-C patients.**

Could such a DNA repair impairment be adjusted by drug treatments?

To answer this question, we studied the effect of different antioxidants/oxidants on NER and BER pathways in the presence and absence of *XPC* mutation by ameliorating their redox state (PART TWO).

Indeed, it would be interesting to try later the same experiments on keratinocytes and to use UVA or solar simulator to study the same objectives on fibroblasts, keratinocytes, and 3D-reconstructed skin models. Another future perspective is to check the protein expression of the different tested factors at 24 hours post-UVB. Perhaps, a better variation could be detected (at level of p53 for example).

# Schematic Summary

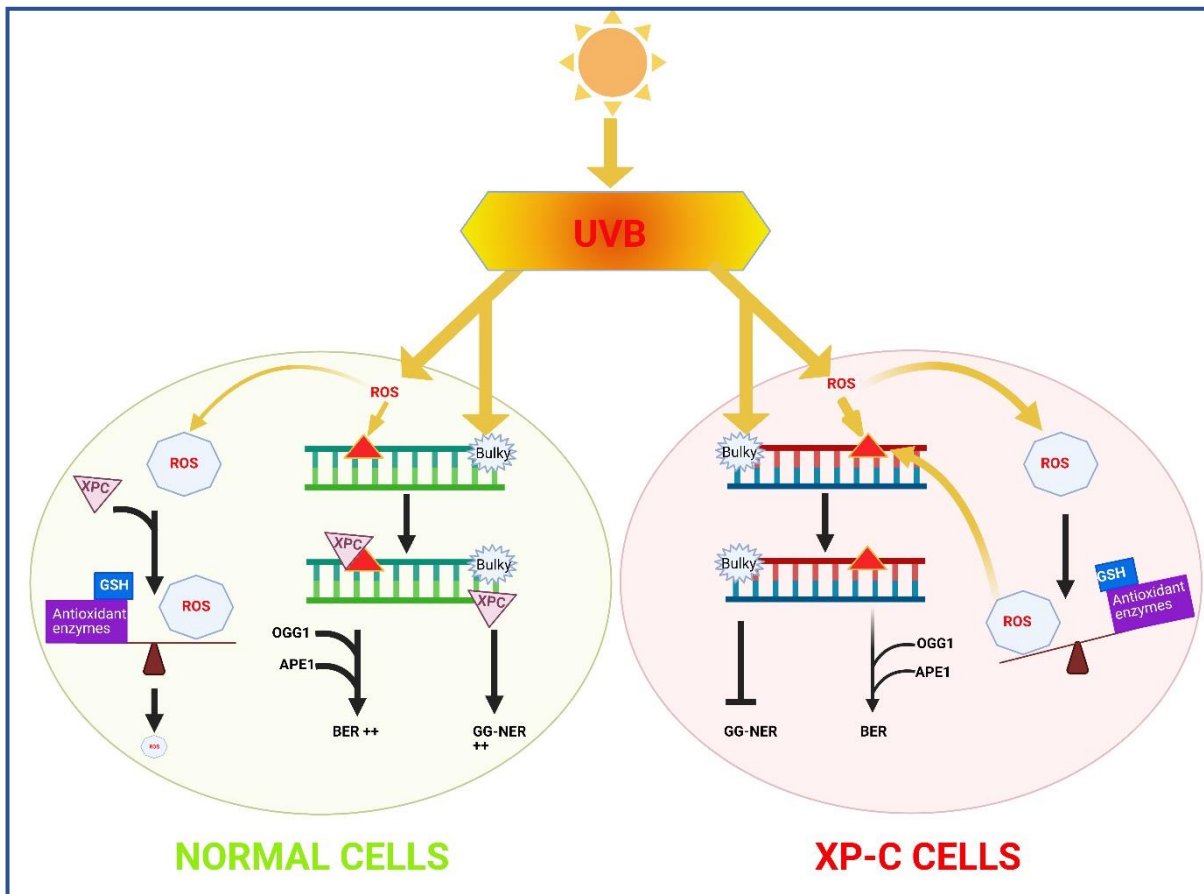


Figure 50. *The difference between normal and XP-C in response to UVB stress. UVB induces direct DNA damage (bulky lesions) and indirect ROS-induced DNA damage (oxidative DNA damage) that are usually recognized by XPC and repaired by NER and BER, respectively. XPC also plays a role in maintaining redox homeostasis and preventing the increase of free ROS. However, in the absence of XPC, bulky lesions will accumulate, BER will be less efficient, and unbalanced redox homeostasis will occur. All these events participate in XP-C patients' clinical phenotype—red rectangle=oxidized purines.*

## Part Two “Ameliorating the DNA repair of XP-C cells by modulating their redox state via pharmacological treatments”

**S**tudying the effect of *XPC* mutations on BER’s gene expression and activity in XP-C cells derived from XP-C patients (part one) imposes a critical question: *Is this effect reversible? Could treatments with antioxidants manipulate the deficient NER and impaired BER in XP-C cells? Could this be done mainly through balancing the cellular redox state with glutathione?*

Such a question is fascinating, mainly because XPC was previously linked to glutathione production (S.-Y. Liu et al. 2010).

Xeroderma pigmentosum C disorder (XPC) is a multisystem disease with a wide range of heterogeneous clinical symptoms. Unfortunately, diagnosis is usually made after the disease has already been initiated. Hence, finding a treatment has been challenging and focuses on numerous researchers, oncologists, and dermatologists. Succeeding in developing a preventive and/or protective therapy could be a major turning point for it and other DNA repair-deficient and skin diseases.

As mentioned previously in the introduction (XPC disorder, section 4.2.2.), several drugs have arisen to limit XPC disorder’s symptoms, including retinoids, vismodegib, and surgery for skin tumors. Potential treatments are also in progress, such as gene therapy. Gene therapy is based on transferring specific genetic material into the cells via viral vectors or nonviral administrations to reverse or halt disorders. Multiple clinical trials targeting T-cells to reduce solid tumors such as melanoma have been conducted (Gorell et al. 2014). Researchers had also tried to correct the DNA repair in XP-C keratinocytes. Gene therapy still has many challenges to overcome, such as the effectiveness, safety, and durability of delivered vectors (Gorell et al. 2014). Hence, trying to scan for therapy for xeroderma pigmentosum from a different aspect could also be interesting. Activating or inhibiting different cellular signaling pathways could interfere with halting the clinical phenotypes of xeroderma pigmentosum disorder. As XPC has been directly linked to BER and redox homeostasis (proven in part one and by other researchers), studying the efficacy of antioxidant drugs on this disease (part two) brings us a step closer towards exploring new treatments and profiling the disease further at genotypic and phenotypic sides.



We divided part two of the project into three chapters. Each chapter discusses the role and effect of one of the three different types of drugs used:

- Nicotinamide (NIC)
- N-acetylcysteine (NAC)
- Buthionine sulfoximine/Dimethylfumurate (BSO/DMF)

Studies were done at basal and UVB levels to monitor the detailed effect of each treatment as a preventive, protective, and therapeutic (stimulator).

*Part of this project will be the subject of an article in preparation.*

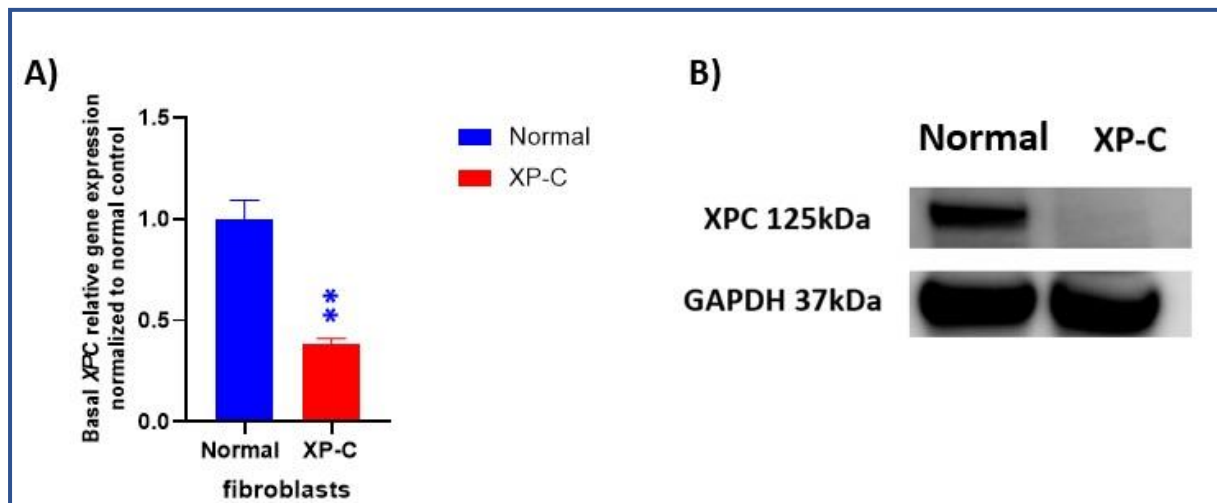
For this part of the project, we used SV40 immortalized fibroblasts instead of primary cells for multiple reasons: (i) we have many conditions. For each condition, we need a high cell number for most of the experiments. This is not practical when using primary fibroblasts as they have a slower growth rate and limited self-renewal, (ii) it is challenging to have primary fibroblasts from the same source that could be sufficient for our objectives. However, it is essential to address that repeating the experiments on primary fibroblasts is one of the main perspectives for the future since they are more relevant in representing *in vivo* models and patients.

## 1. Chapter One: Characterization of Cell lines

Similarly to part one, we characterized the two SV40 transformed fibroblasts [normal (AG10076) and XP-C (GM15983)] at gene and activity levels before launching the experiments.

### 1.1. Impaired XPC gene and protein expression in XP-C cell line

As expected, *XPC* mRNA level was significantly downregulated in XP-C cells ( $p < 0.01$ , \*\*) at basal level compared to normal control (figure 51). This shows that the *XPC* mutation inhibited *XPC*'s expression at mRNA level. In normal cases, UVB triggers the upregulation of *XPC*'s expression and efficacy to neutralize the formation of DNA lesions. Once the *XPC* gene is mutated, the expression will be faltered. The low *XPC* mutated mRNA level in XP-C cell line was not stable to be translated into protein. This was similar to the observations of part one. In agreement with Qiao et al., there was a complete absence of XPC protein compared to normal (Qiao, Scott, et al. 2011).

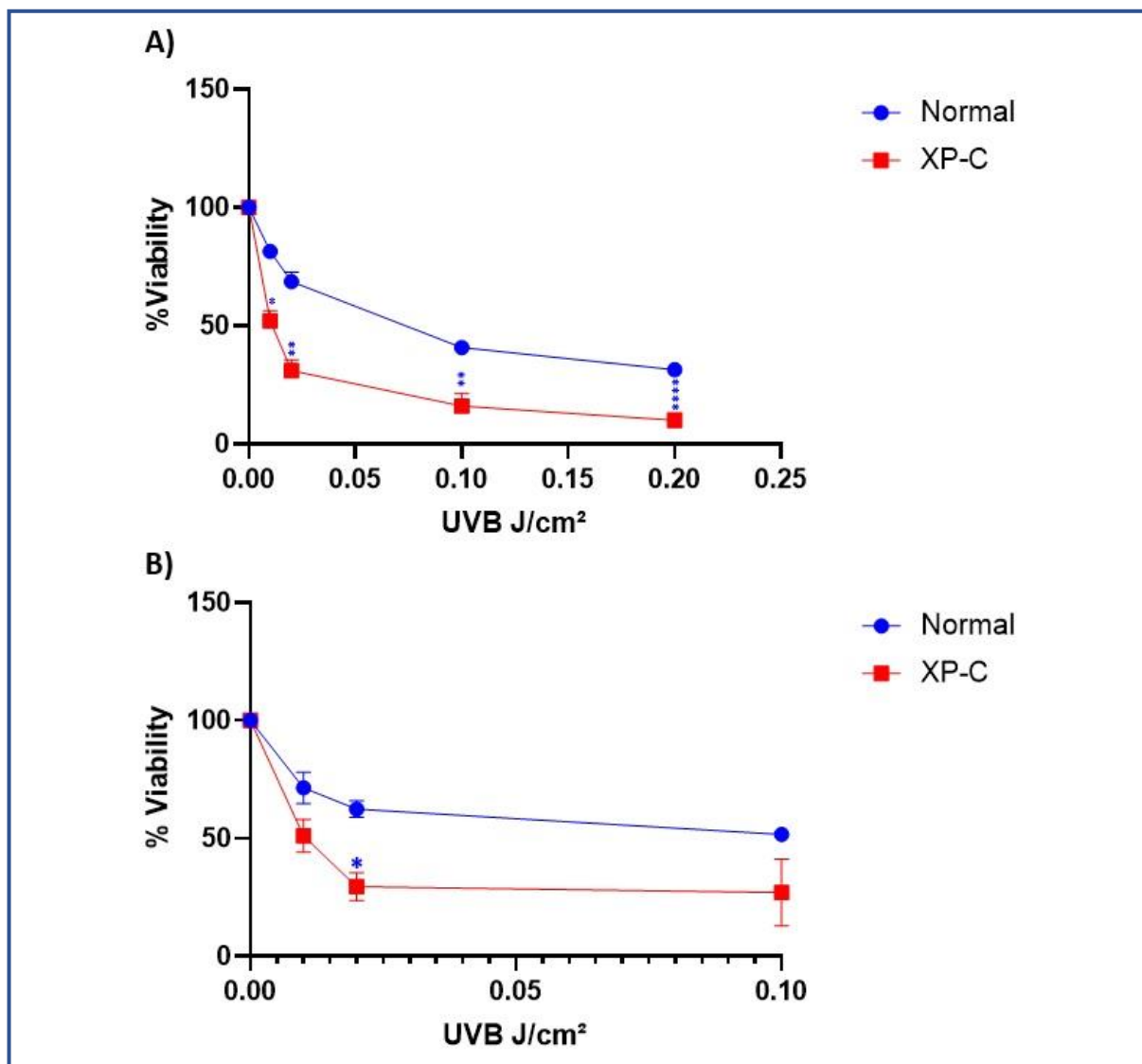


**Figure 51. Impaired XPC mRNA and protein expressions in XP-C cells compared to normal control, at basal level.** Panel A) RT-qPCR showed that XP-C had a significantly lower XPC mRNA expression ( $p < 0.01$ , \*\*) than the control. The data were normalized relative to the GAPDH mRNA levels, where GAPDH was used as an endogenous control. Unpaired *t*-test was used to compare XPC mRNA level between the normal and XP-C fibroblasts ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from two independent experiments,  $n=2$ . Panel B) shows the XPC protein band (125 KDa, 1/500) in normal but not XP-C cells. GAPDH (37KDa, 1/5000) was used as the loading control.

### 1.2.Higher UVB-induced photosensitivity in XP-C cells compared to control

On the contrary to part one, XP-C cells are significantly ( $p<0.05$ ) more photosensitive compared to control (figure 52).

Due to the high photosensitivity of XP-C cells, a lower UVB dose ( $0.01 \text{ J/cm}^2$ ) was selected for the experiments compared to that used in part one ( $0.05 \text{ J/cm}^2$ ). Thus, it provides the necessary damage to monitor an effect but is not cytotoxic nor lethal (52 and 81.4 percent of viability in XP-C and normal cells, respectively).



**Figure 52. Higher photosensitivity in XP-C cells compared to normal control.** We measured the percentage of cellular viability 24 hours post-UVB irradiation panel A) by short-term cytotoxicity test (MTT) were XP-C had lower % viability compared to normal control at 0.01 ( $p<0.05$ , \*), 0.02 ( $p<0.01$ , \*\*), 0.1 ( $p<0.01$ , \*\*), and 0.2 ( $p>0.0001$ , \*\*\*\*)  $\text{J/cm}^2$  of UVB doses and panel B) by trypan blue were XP-C had lower % viability compared to normal control ( $p<0.05$ , \*) at 0.02  $\text{J/cm}^2$  of UVB. We normalized each sample by its untreated value (100% viability). Unpaired *t*-test was used to compare normal and each XP-C fibroblast photosensitivity at each UVB dose ( $p<0.05$ , \*). The results are the mean  $\pm$  SEM from three independent experiments,  $n=3$ , in MTT and two independent experiments,  $n=2$ , in trypan blue manipulations.

In the beginning, we were worried that such a low dose could be lower than the effects' limit of detection (LOD). So, we did HPLC-MS/MS and verified that it is sufficient to induce a high amount of DNA damage (table 8).

*Table 8. Level of dimeric photoproducts [CPDs and (6-4) PPs] per million normal DNA bases. Dewar isomers were not detected due to the low UVB dose used (0.01 J/cm<sup>2</sup>). The low value of the standard deviation represents the reproducibility of both the experimental and analytical protocols.*

Sample	TT CPD	TT 64PP	TT Dewar	TC CPD	TC 64PP	TC Dewar	CT CPD	CC CPD	total CPD
sample 1	126.4	10.7	0.0	36.0	53.6	0.0	38.7	10.3	201.0
sample 2	139.3	11.4	0.0	43.7	68.2	0.0	34.9	6.6	217.9
sample 3	128.4	11.6	0.0	48.7	67.6	0.0	49.9	4.9	227.0
mean	131.4	11.2	0.0	42.8	63.1	0.0	41.2	7.3	215.3
std dev	7.0	0.5	0.0	6.4	8.3	0.0	7.8	2.8	13.2

### 1.3. Impaired NER capacities in XP-C cells compared to control

We did HPLC-MS/MS to examine the repair of different bulky photoproducts by XP-C and normal cells. As expected, XP-C cells had lower photoproducts' repair efficiency compared to normal control (figures 53 and 54). Studying the repair capacities by HPLC-MS/MS instead of immunocytochemistry provided us the privilege to monitor each type of CPD and (6-4) PP lesions.

Figure 53 shows that UVB-induced TT and TC (6-4) PPs were repaired slower and more persistent in XP-C cells. Almost 60 percent of the (6-4) PPs were detected at 48 hours. On the contrary, both (6-4) PPs were efficiently repaired 24 hours post-UVB in normal cells. Thus, independently of the UV dose, normal fibroblasts can repair efficiently (6-4) PPs within 24 hours (Courdavault et al. 2004).

Perhaps we did not detect CT and CC (6-4) PPs because they are less frequent. The frequency of the (6-4) PPs with the different dinucleotides is as follows: TC>TT>CC and CT (Khoe, Chung, and Murray 2018).

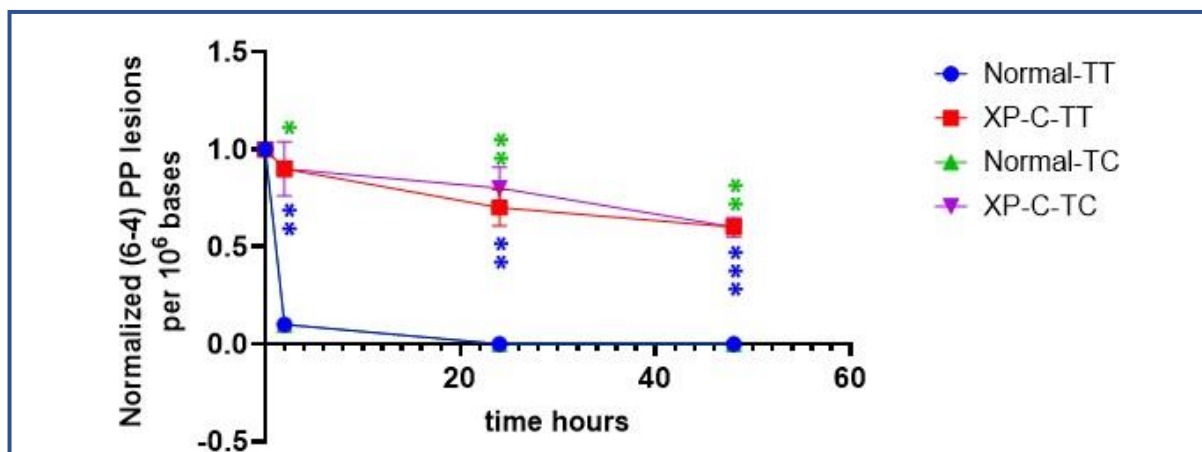


Figure 53. **Deficient (6-4) PPs lesions repair in XP-C cells compared to normal control.** We monitored repair kinetics for (6-4) PPs bulky photoproducts at different hours post-UVB by HPLC-MS/MS. TT and TC (6-4) PPs were significantly higher ( $p < 0.05$ ) from 0 until 48 hours in XP-C cells compared to control. Normalization was done relative to irradiated values at 0 hours. Unpaired t-test was used to compare the normalized lesion ratio between normal and each XP-C fibroblast at each time point ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from two independent experiments,  $n=2$ .

Figure 54 shows the kinetics of repair for TT (the hallmark of UV-induced DNA damage), TC, CT, and CC CPDs (Douki, Koschembahr, and Cadet 2017).

Higher CPDs with higher persistence and slower repair were detected in the XP-C cells compared to normal control. At 48 hours, more than 60 percent of CPDs were repaired in normal control, while less than 25 percent were repaired in XP-C cells. The high level was significant in CT dinucleotides [figure 54 (D)], while the other types lack significance due to a high SEM. It is worth mentioning that the increase in CPDs at 2 hours post-UVB may be due to dark CPDs' formation. This phenomenon has been detected in human keratinocytes and melanocytes (Delinasios et al. 2018). *In vivo* studies revealed that dark CPDs have faster repair than the other CPDs (light CPDs, ~72 hours). The higher cytosine-containing nucleobases in dark CPDs could contribute to more rapid repair (Delinasios et al. 2018).

We would like to address that if the experiment was done as a triplicate, significantly higher CPDs lesions at different kinetic points in XP-C cells could be detected compared to the normal control.

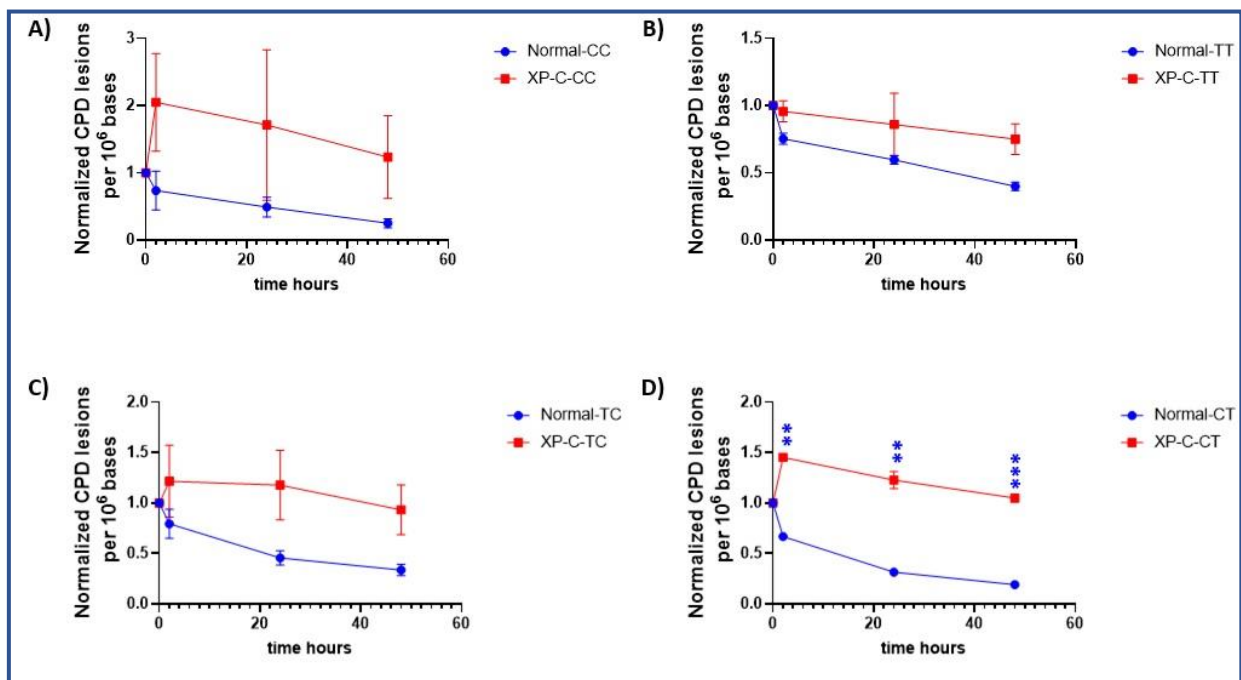


Figure 54. **Deficient CPDs lesions repair in XP-C cells compared to normal control.** We monitored repair kinetics for the different types of CPDs bulky photoproducts at different hours post-UVB by HPLC-MS/MS. Panel A) shows no significant difference in CC CPDs repair between XP-C and normal cells. Panel B) shows no significant difference in TT CPDs repair between XP-C and normal cells. Panel C) shows no significant difference

*in TC CPDs repair between XP-C and normal cells. Panel D) shows a significantly higher CT CPDs at 2 ( $p < 0.01$ , \*\*), 24 ( $p < 0.01$ , \*\*), and 48 ( $p < 0.001$ , \*\*\*) hours post-UVB irradiation. Normalization was done relative to irradiated values at 0 hours. Unpaired *t*-test was used to compare the normalized lesion ratio between normal and each XP-C fibroblast at each time point ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from two independent experiments,  $n=2$ .*

### **Briefing of the characterization**

The XP-C cell line had:

- Low *XPC* mRNA level and no XPC protein at basal level
- High UVB-photosensitivity
- Deficiency in bulky lesions' [CPDs, (6-4) PPs] repair

The XP-C cells used have GG-NER deficiency due to the absence of XPC protein, the inducer of GG-NER. However, the slow-paced detected repair could be due to the active TC-NER usually not being affected in XP-C patients.

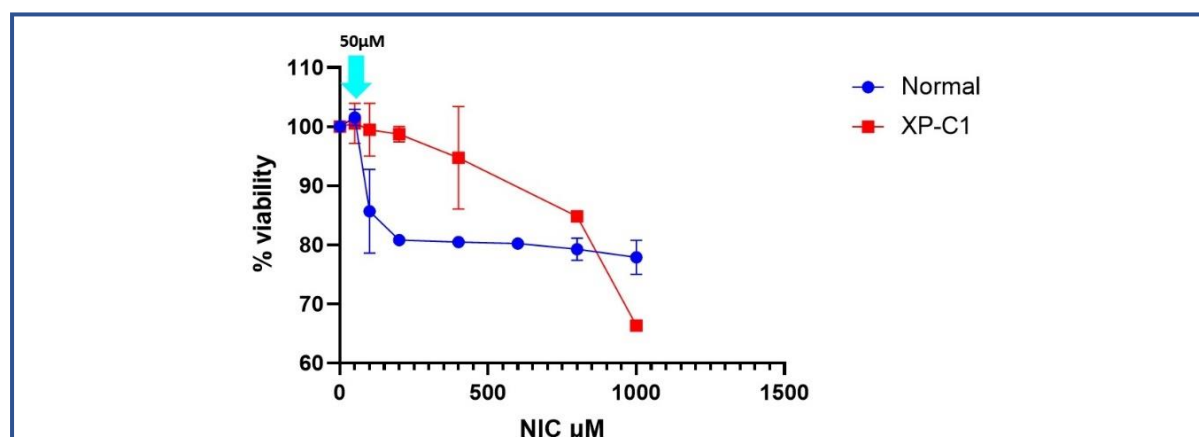
## 2. Chapter Two: The Effect of Nicotinamide (NIC) on UVB-Induced Oxidative Stress and DNA Repair in XP-C and Normal Cells

NAD-dependent pathways were suggested to play a vital role in impeding the initiation and promotion of skin carcinogenesis, the principal clinical phenotype in XP-C patients (Benavente and Jacobson 2008). In parallel, NIC is known as NAD<sup>+</sup> source. Studies had demonstrated that NIC can enhance the repair of CPDs and 8-oxoGua *in vitro* (normal melanocytes, lymphocytes, HaCat keratinocytes) and *ex vivo* human skin (Chhabra et al. 2019; Thompson, Halliday, and Damian 2015; Malesu et al. 2020). Herein, we were interested in validating whether such a treatment could enhance the redox state and deficient DNA repair (NER and BER) in XP-C cells.

### 2.1.Characterization of NIC-treated normal and XP-C cells

#### 2.1.1. NIC dose selection

We did a dose-response curve to select the optimal dose of NIC for our experiments. Figure 55 shows that 50 $\mu$ M of NIC treatment for 24 hours is the most suitable dose to work within normal and XP-C cells. It is the optimal dose with the highest concentration to be used before cytotoxicity. Other reasons for such a dose selection: (i) it is achievable *in vivo*, as a dose representing supplementation with 500mg tablet, and used *in vitro* in several articles (Malesu et al. 2020; Thompson, Halliday, and Damian 2015) and (ii) higher concentrations were shown to act as proapoptotic by inactivating SIRT1 and inducing P53. This helps in inhibiting the progression of tumors (Malesu et al. 2020). Nevertheless, this also means that they are cytotoxic and may alter the natural cellular behavior. This is not our primary concern in this project.

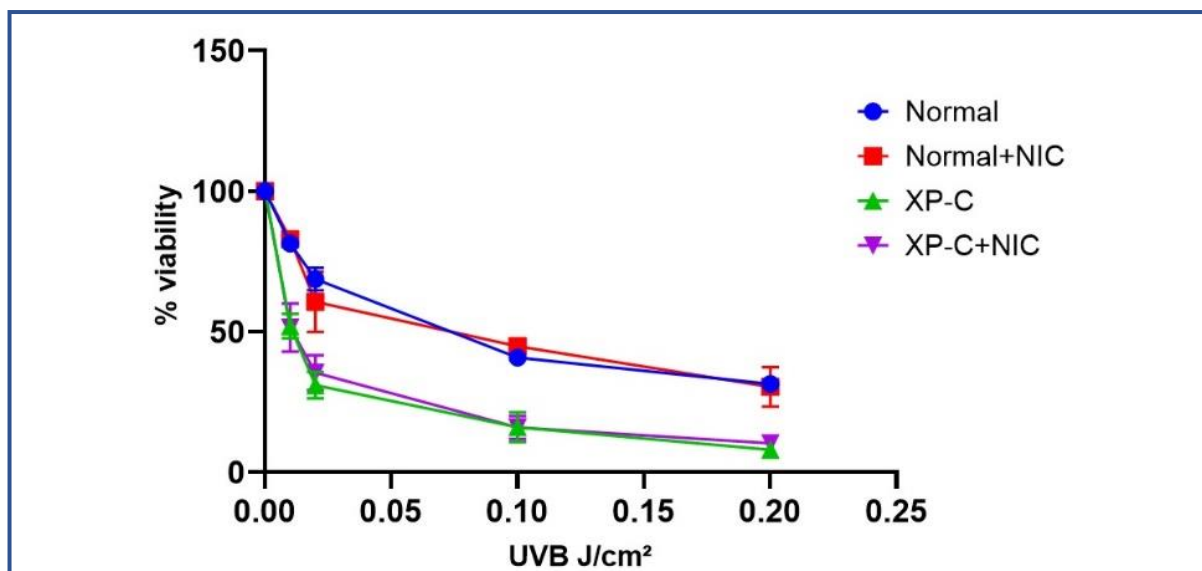


*Figure 55. NIC dose-response curve. Normal and XP-C cells were tested with different doses of NIC for 24 hours, followed by MTT test to monitor the cellular viability. 50 $\mu$ M is the most suitable dose with the least cytotoxicity. The results are the mean  $\pm$  SD.*

### 2.1.2. Effect of NIC on XP-C and normal cells' UVB-induced photosensitivity

We treated XP-C and normal cells with/without NIC for 24 hours, then we UVB-induced cells at different doses and checked their viability after 24 hours. This will allow us to monitor whether NIC could alter the cell response (viability, cell death) against UVB.

As shown in figure 56, each cell line showed no difference with and without NIC at different UVB doses. This indicates that NIC does not affect the cell viability. Similarly, several results showed no effect of 50 $\mu$ M NIC on keratinocytes, melanoma, and melanocytes' viability and proliferation (Thompson, Halliday, and Damian 2015; Malesu et al. 2020). It should be addressed that depletion of NAD<sup>+</sup> due to NIC restriction could affect cell viability (Benavente and Jacobson 2008). Both cell lines were not deprived of NIC instead supplemented with a non-cytotoxic dose. Also, our cell culture medium (DMEM Glutamax) contains 4mg/L of NIC, and we did not allow our cells to have high population doublings to prevent any stress other than the ones we induce. Therefore, 50  $\mu$ M NIC is safe to be used mainly in high cancer risk XP-C patients.



*Figure 56. Similar UVB-induced photosensitivity in each cell line (normal and XP-C), with and without NIC. We treated cells with 50  $\mu$ M NIC for 24 hours, then we measured the percentage of cellular viability 24 hours post-UVB irradiation by a short-term cytotoxicity test (MTT). For each condition, the sample was normalized by its unirradiated value (100% viability). Paired t-test was used to compare photosensitivity for each condition at each UVB dose ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from three independent experiments,  $n = 3$ .*



## 2.2.Effect of NIC on XP-C and normal cells' redox state

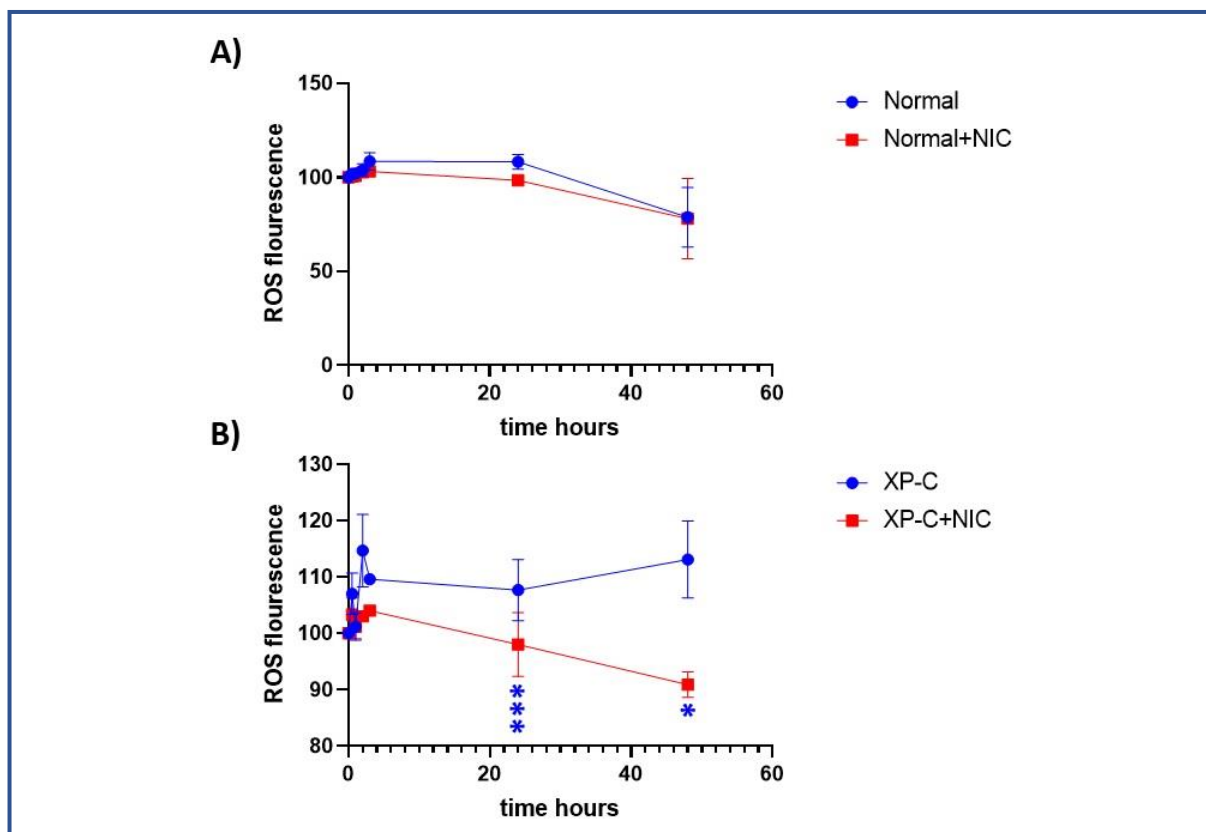
### 2.2.1. Effect of NIC on XP-C and normal cells' ROS and glutathione levels

- Effect on UVB-induced ROS

In part one, we showed that *XPC* mutation upregulates ROS level that persisted 48 hours post-UVB in XP-C1 compared to normal primary fibroblast ( $p < 0.05$ , \*). However, NIC had already been addressed to exert an antioxidant effect by downregulating ROS level in primary fibroblasts (Kwak et al. 2015). Therefore, we wanted to check whether it was the case with our cells.

As shown in figure 57 (A), NIC did not affect ROS level in normal cells. However, ROS level dramatically decreased at 24 hours ( $p < 0.001$ , \*\*\*) and 48 hours ( $p < 0.05$ , \*) post-UVB irradiation in XP-C cells in presence of NIC pretreatment [figure 57 (B)]. This shows that NIC was able to protect XP-C cells from ROS accumulation and inhibited their level. This could be mediated via enhancing the antioxidant defense system in the cells. For example, NIC decreases NOX-mediated ROS via increasing antioxidants and NAD<sup>+</sup> levels, thereby halts premature skin aging and neoplastic transformation in skin cells (Fania et al. 2019).

The absence of such an effect on normal cells could be due to the rapid response of normal cells in adapting to the induced stress. Besides, we used DHR123 to detect the ROS level. Therefore, it may not be sensitive to detect the small changes as accurately as flow cytometry.



**Figure 57. Effect of NIC on ROS level in normal and XP-C cells, post-UVB irradiation.** Panel A) No effect of NIC on ROS level in normal cells. Panel B) NIC downregulated ROS level in XP-C cells significantly at 24 and 48 hours ( $p < 0.001$ , \*\*\*;  $p < 0.05$ , \*, respectively). We treated cells with 1/1000 DHR123 followed by UVB ( $0.02 \text{ J/cm}^2$ ) and monitored the kinetics of fluorescence at different kinetic time points. Then we normalized each sample by its untreated value (100% viability).  $\text{H}_2\text{O}_2$  was used as a positive control. Paired t-test was used to compare the ROS fluorescence between NIC treated and untreated samples in normal and XP-C1 fibroblast, respectively, at each kinetic time point (hours) ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from three independent experiments,  $n=3$ .

Note:  $0.02 \text{ J/cm}^2$  was used to detect the ROS fluorescence more accurately as DHR123 is not a very sensitive experiment.

- Effect on glutathione level 24 hours post-UVB

Briefly, glutathione ( $\gamma$ -L-glutamyl-L-cysteinyl-glycine, GSH) is the cells' guardian against oxidative damage. It maintains redox homeostasis by: (i) reducing free or ROS conjugated to DNA as well as to other biomolecules (methylglyoxal and 4-hydroxynonenal) and (ii) acting as the electron donor and cofactor of glutathione peroxidase in the reduction of peroxides into water (reaction III) (Gaucher et al. 2018).

So, *how could NIC influence such an interesting tripeptide?*

It was shown that NIC increases NADPH ( $\text{NAD}^+ \rightarrow \text{NADH} \rightarrow \text{NADPH}$ ) and GSH levels in mice (Ghosh et al. 2012). NADPH is usually used for GSH turnover (figure 58). Its low cellular level was detected in XP cells associated with diminished catalase activity (Parlanti et al. 2015). In parallel, GSH depletion leads to oxidative stress, oxidative damage, and tumorigenesis (figure 58).

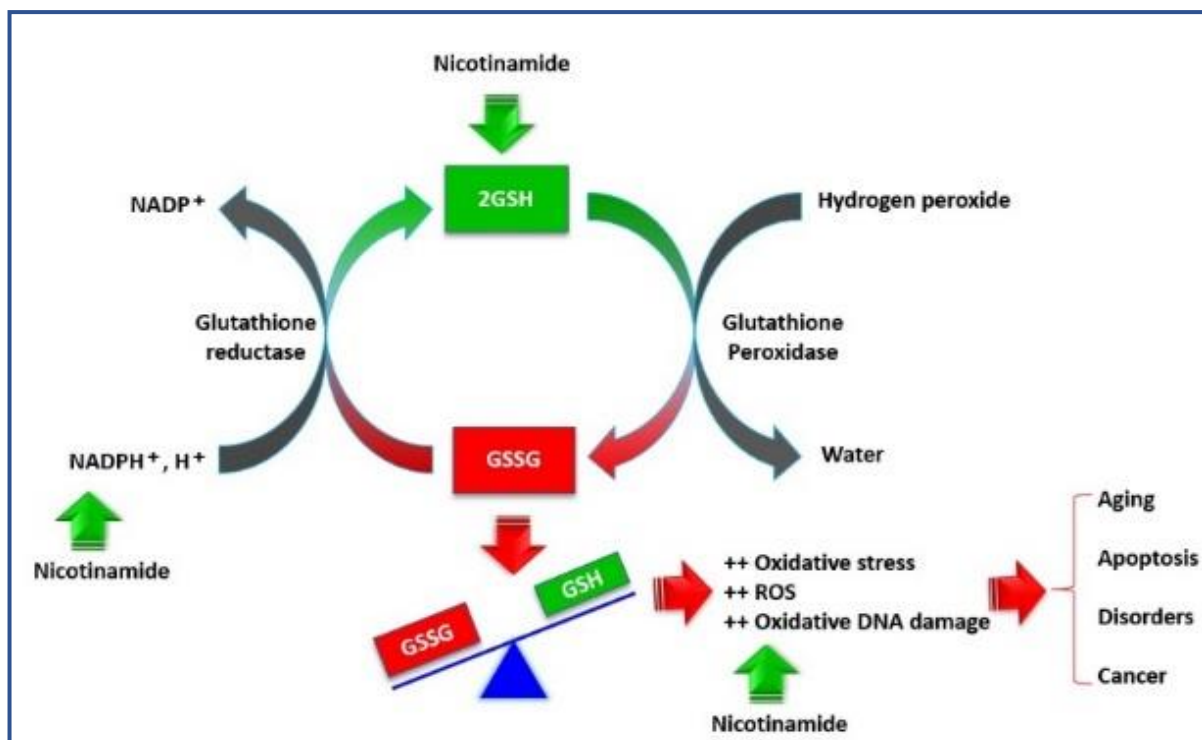


Figure 58. **The turnover of glutathione (GSH).** GSSG and NAPH+H+ produce NADP+ (side product) and 2GSH. These GSH reduce radicals like hydrogen peroxide through glutathione peroxidase to produce oxidized GSSG and water. A misbalance in GSH:GSSG ratio towards GSSG leads to oxidative stress and damage contributing to aging, diseases, and carcinogenesis.

So, it could be interesting to have an upregulation of GSH (directly or via NADPH upregulation) in our studied cells in the presence of NIC. This could play a role in reversing XP-C patients' phenotype. Figure 59 shows that NIC increased the UVB-induced GSH level at 24 hours post-UVB in both cell lines (normal and XP-C). A high GSH level upregulates GSH-dependent antioxidants and their role as ROS scavengers to protect oxidant-sensitive cells like our XP-C cells.

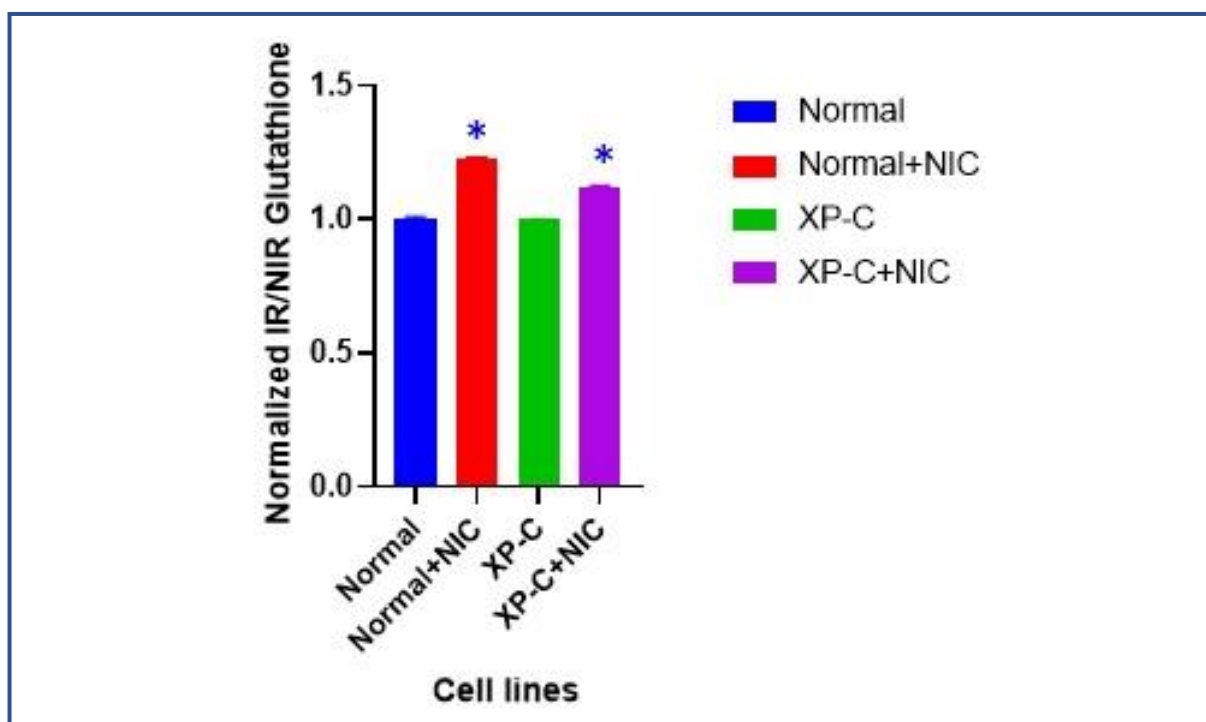


Figure 59. **Effect of NIC on the UVB-induced IR/NIR glutathione (GSH) level in normal and XP-C cells.** After NIC treatment and UVB-irradiation for 24 hours, we extracted cells and measured glutathione level calorimetrically using a kit from CAYMAN. This treatment showed a significant upregulation ( $p < 0.05$ , \*) in glutathione level in each cell compared to its untreated sample. For each condition, the sample was normalized by its untreated value. Paired  $t$ -test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SD.

Therefore, NIC influences several pathways to protect the cells against UV stress, including antioxidant/oxidant defense mechanisms.

### 2.2.2. Effect of NIC on XP-C and normal cells' oxidative stress-linked genes' expression

To screen the effect of NIC as a protective pretreatment in XP-C cells against oxidative stress, we studied the mRNA expression of some detoxificant genes. The studied antioxidants could be upregulated to protect XP-C cells from UVB-induced ROS and DNA damage in GSH dependent or independent manner.

As previously mentioned, an antioxidant defense system is present to protect cells against ROS and oxidative damage. Superoxide dismutases (SOD1, SOD2) and glutathione peroxidase (GPx1) are vital components of such a protective system.

At basal level, NIC upregulated SOD2 ( $p < 0.05$ , \*) in normal cells but had no significant effect on XP-C cells [figure 60 (A) and (B)]. Meanwhile, post-UVB, NIC upregulated SOD1 and SOD2 ( $p < 0.05$ , \*) in normal cells and SOD1 ( $p < 0.01$ , \*\*) in XP-C cells [figure 60 (C) and (D)].

This shows that NIC influences the antioxidant defense, particularly post-UVB irradiation.

Harlan et al. showed that NIC enhances oxidative stress resistance via redox reactions and NAD<sup>+</sup> dependent pathways in SOD1 mutated astrocytes (Harlan et al. 2016). Therefore, the effect of NIC could be via multiple pathways to protect against oxidative stress, including the suggested one.

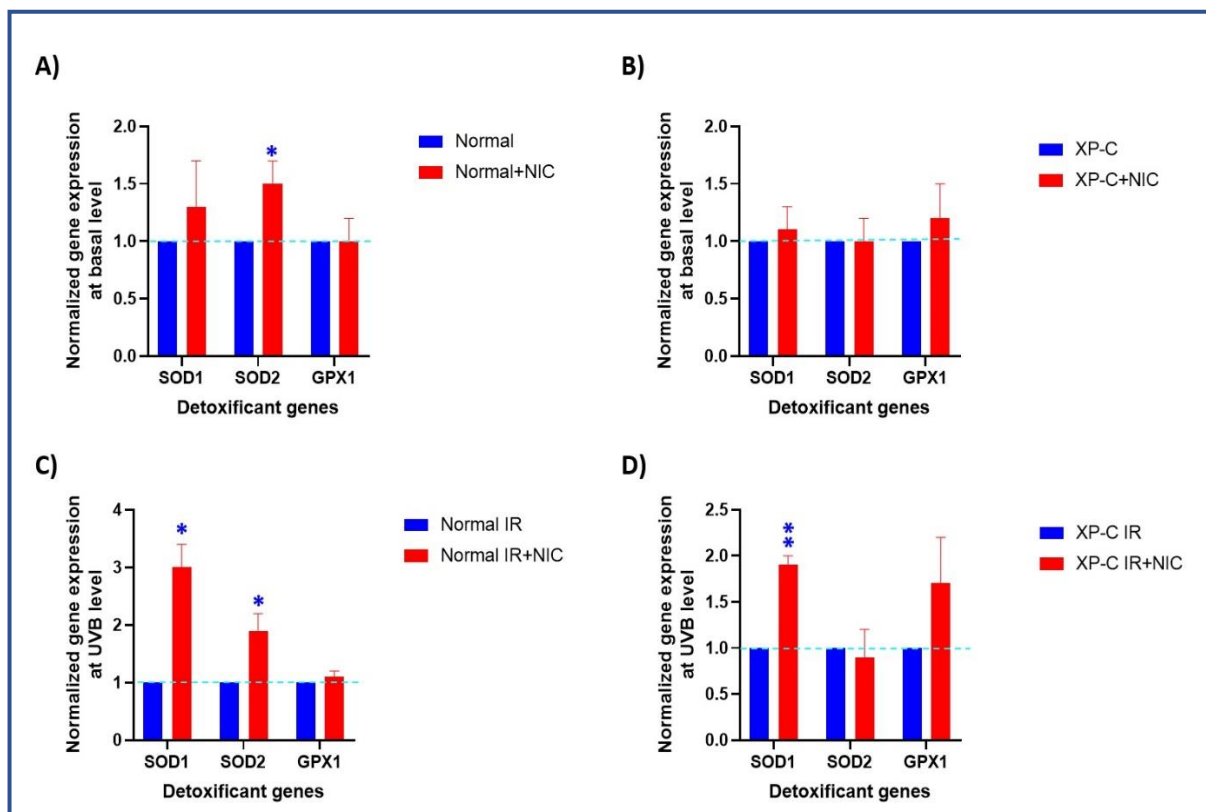


Figure 60. **Effect of NIC on detoxificants' mRNA expression at basal and UVB levels in normal and XP-C cells.** Panel A) shows an upregulation in SOD2 expression upon NIC treatment ( $p < 0.05$ , \*) in normal cells at basal level. Panel B) shows no difference in expression upon NIC treatment ( $p < 0.05$ , \*) in XP-C cells at the basal level. Panel C) shows an upregulation of SOD1 and SOD2 upon NIC treatment ( $p < 0.05$ , \*) in normal cells post-UVB irradiation. Panel D) shows an upregulation in SOD1 ( $p < 0.01$ , \*\*) in XP-C cells post-UVB irradiation. For each condition, the sample was normalized by its untreated value (100% viability). Paired t-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from three independent experiments,  $n=3$ . IR=irradiated with UVB (0.01 J/cm<sup>2</sup>).

We did not detect a specific enhancement of certain detoxification genes rather a general upregulation of the antioxidant defense system. Therefore, we propose Nrf2 as a suggested pathway for an interplay with NIC to protect cells.

Nrf2 can mediate protection from oxidative stress by enhancing the expression of antioxidants, as GPx1, and increasing the synthesis of GSH (Schäfer et al. 2010). It is also involved in cellular signaling against oxidative stress.

We did not detect any significant variation in *Nrf2*'s expression in both cell lines upon adding NIC (figure 61).

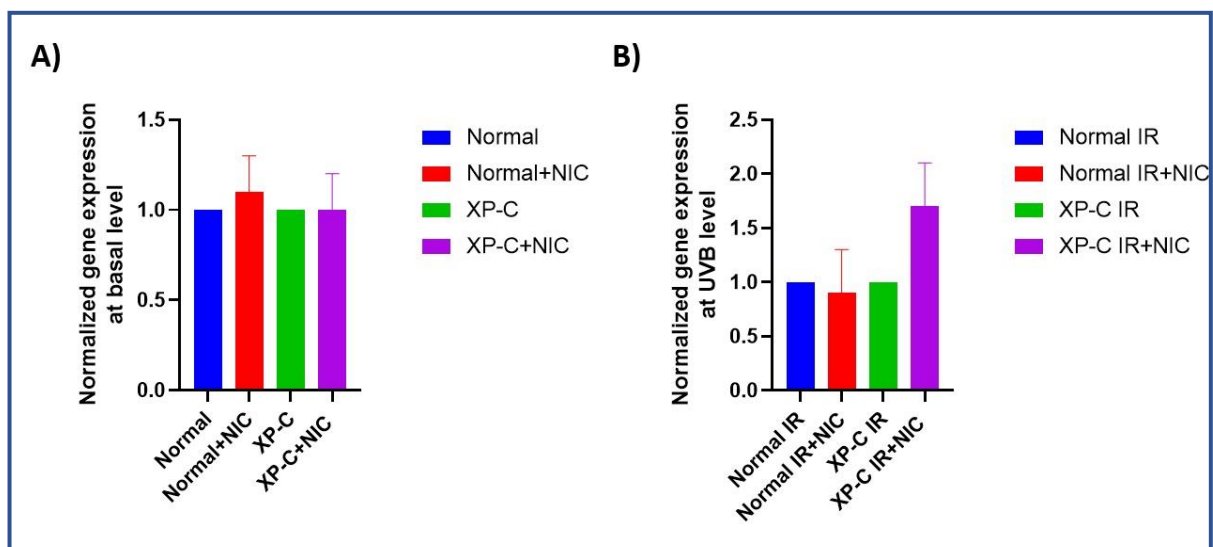
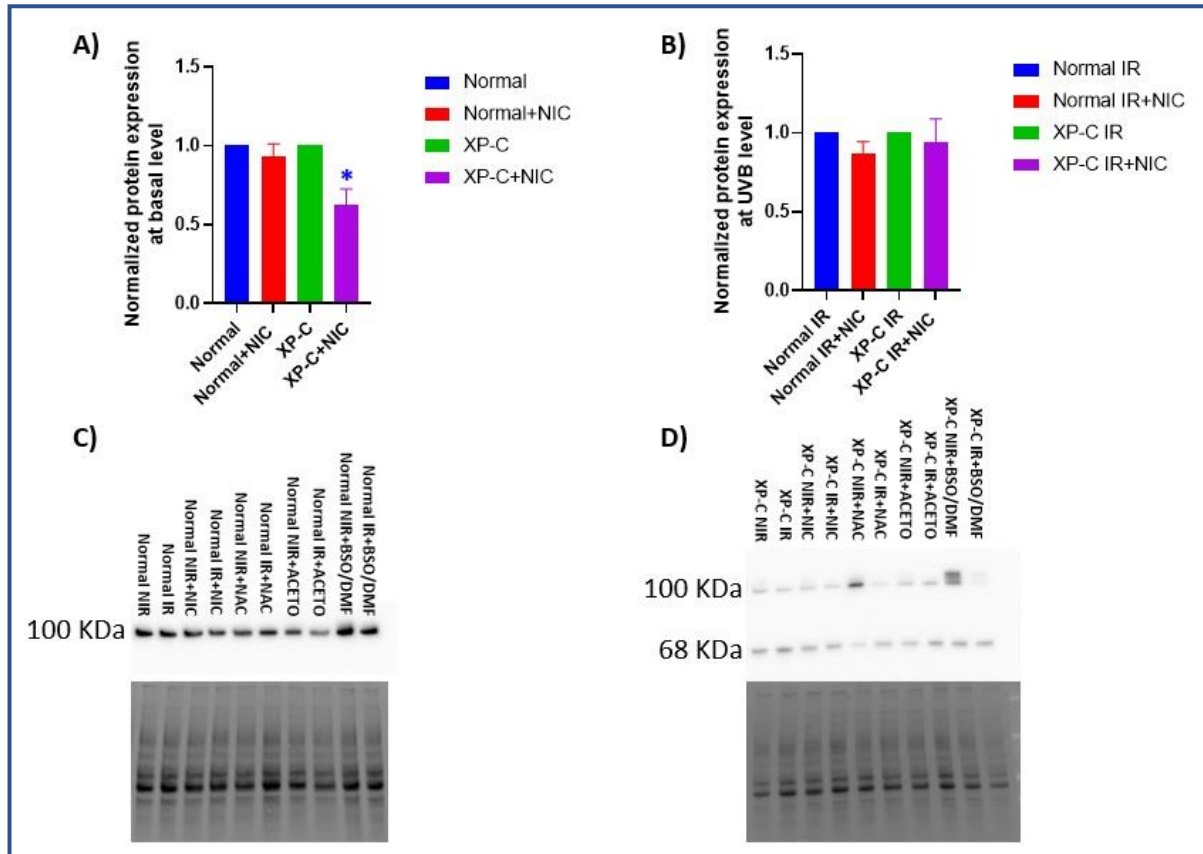


Figure 61. **Effect of NIC on Nrf2 mRNA expression at basal and UVB levels in normal and XP-C cells.** The sample was normalized by its untreated value (100% viability). Paired t-test was used to compare the expression for each condition. The results are the mean  $\pm$  SEM from three independent experiments,  $n=3$ . IR=irradiated with UVB (0.01 J/cm<sup>2</sup>).

Surprisingly, figure 62 shows that NIC downregulated Nrf2 protein in XP-C cells at the basal level. No further changes were detected in other conditions. Thus, our result may indicate that NIC prevented any genotoxicity at the basal level in XP-C cells. This leads to a downregulation in Nrf2 protein expression as Nrf2 is usually stimulated post-oxidative stress.

Since the Nrf2 gene expression was not enhanced in the presence of NIC, we suspect that the effect of NIC is via other pathways, by enhancing Nrf2's activity, or by downstream factors. Perhaps upregulating Nrf2's gene expression requires NIC treatment pre- and post-UVB.



**Figure 62. Effect of NIC on Nrf2 protein expression at basal and UVB levels in normal and XP-C cells.** Panel A) shows that NIC downregulates Nrf2 in XP-C cells at the basal level. Panel B) shows no significant effect of NIC on Nrf2 in both cells at UVB level. Panels C) and D) show the Nrf2 bands in normal and XP-C cells, respectively, treated with different conditions (treatments and irradiation). The total protein was used for normalization. Paired t-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SD from three independent experiments,  $n=3$ . IR=irradiated with UVB ( $0.01 \text{ J/cm}^2$ ).

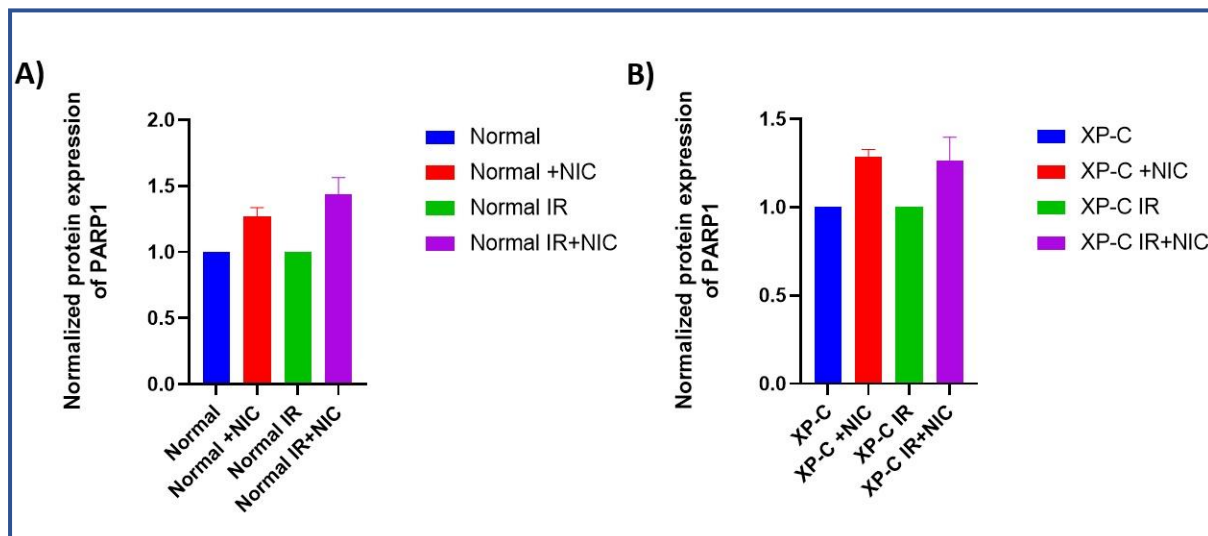
### 2.3. Effect of NIC on XP-C and normal cells' PARP1 protein expression

#### PARP1

Moving forward, PARP1 regulates around 60 to 70 percent of genes involved in the cell cycle, cell metabolism, and transcription (Chaitanya, Alexander, and Babu 2010). A dysregulation in PARP1 increased the risk of skin diseases, indicating a role of PARP1 in UV-induced DNA damage repair (Chaitanya, Alexander, and Babu 2010). P53 interacts with PARP1, where the former uses  $\text{NAD}^+$  to PARylate proteins, including those involved in DNA repair and DNA damage response (Pfister, Yoh, and Prives 2014).

Our preliminary results show that NIC may enhance PARP1 protein expression at basal and UVB levels in both cells, normal and XP-C [figure 63 (A) and (B) and figure 64]. PARP1 is

involved in single-strand break repair, an intermediate part of the BER pathway. Herein, NIC could enhance PARP1 and ATP levels to augment BER's efficacy (Ray Chaudhuri and Nussenzweig 2017). In addition, an *in vivo* study showed that PARP1-deficient cells lack efficient oxidative DNA damage repair. This suggests that PARP1 may play a role in some of the BER steps to enhance the excision of oxidative purines (Marsin et al. 2003). However, several studies showed that NIC could inhibit PARP1's activity to prevent ATP and NAD<sup>+</sup> depletion at high DNA damage (Salech et al. 2020). This may not be the case in our study as the DNA damage caused by UVB at 0.01 J/cm<sup>2</sup> is not severe.



**Figure 63. Effect of NIC on PARP1 protein expression at basal and UVB levels in normal and XP-C cells.** Panels A) shows no significant effect of NIC on PARP1 in normal cells at basal and UVB levels. Panel B) shows no significant effect of NIC on PARP1 in XP-C cells at basal and UVB levels. The total protein was used for normalization. Paired t-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SD from two independent experiments,  $n=2$ . IR=irradiated with UVB (0.01 J/cm<sup>2</sup>). (preliminary result)

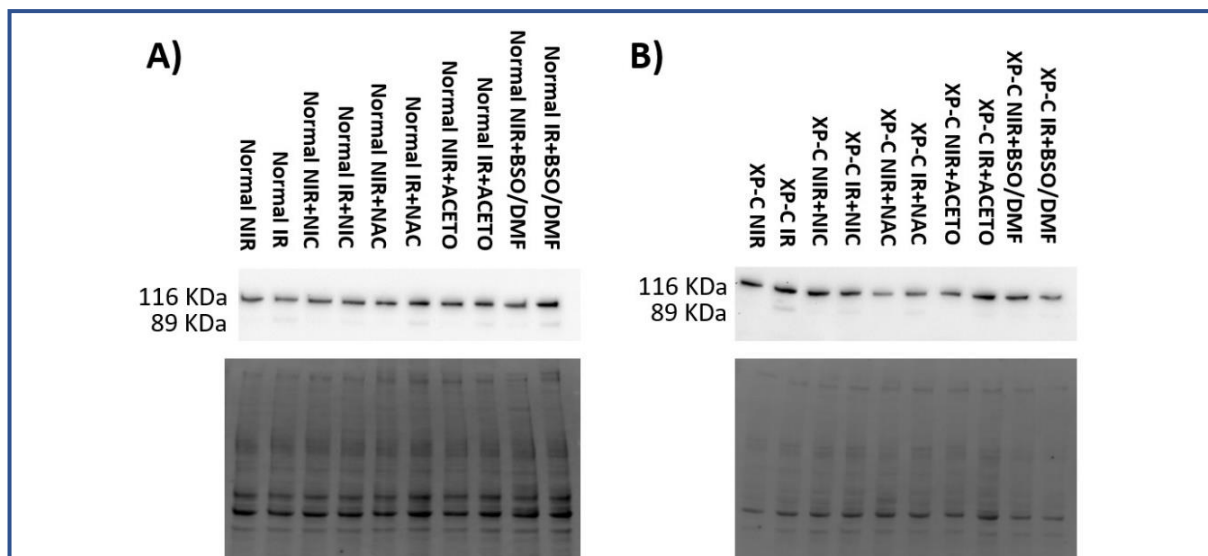


Figure 64. **Band images showing the effect of NIC on PARP1 protein expression at basal and UVB levels in normal and XP-C cells.** Panels A) and B) show the cleaved (89 KDa) and complete (116 KDa) PARP1 bands in normal and XP-C cells, respectively, treated with different conditions (treatments and irradiation). The total protein was used for normalization. The results are the mean  $\pm$  SD from two independent experiments,  $n=2$ . IR=irradiated with UVB (0.01 J/cm<sup>2</sup>).

As previously mentioned, the bands referring to ACETO are due to experiments done on this pretreatment in normal and XP-C cells.

### Cleaved PARP1 “apoptosis hallmark”

On the other hand, monitoring PARP1’s cleavage provides us with some knowledge about the apoptotic state of our cells in the presence and absence of NIC and UVB stress.

As shown in figures 64 and 65 [(A) and (B)], no cleaved PARP1 was present in cells at the basal level due to the lack of cytotoxic stress at the resting state.

UVB increased the level of cleaved PARP1 as a cellular defense mechanism against mutagenesis to prevent carcinogenesis.

NIC significantly downregulated cleaved PARP1’s expression in XP-C cells ( $p<0.05$ , \*). In the presence of high-stress levels, suicidal proteases (caspases, granzymes, etc..) cleave PARP1 to induce cell death (Chaitanya, Alexander, and Babu 2010). This shows that NIC protects XP-C cells from cytotoxic damage.

Notably, cleaved PARP1 had been implicated in several diseases as Alzheimer and Parkinson and internal cancers as brain tumor. Such pathologies had been linked to a deficiency in BER’s expression and/or function due to the accumulation of high oxidative DNA damage (Chaitanya, Alexander, and Babu 2010). Therefore, we can speculate that lowering cleaved PARP1’s expression might indicate enhanced oxidative DNA damage repair in the presence of NIC.

Further experiments were done later to prove such a hypothesis.

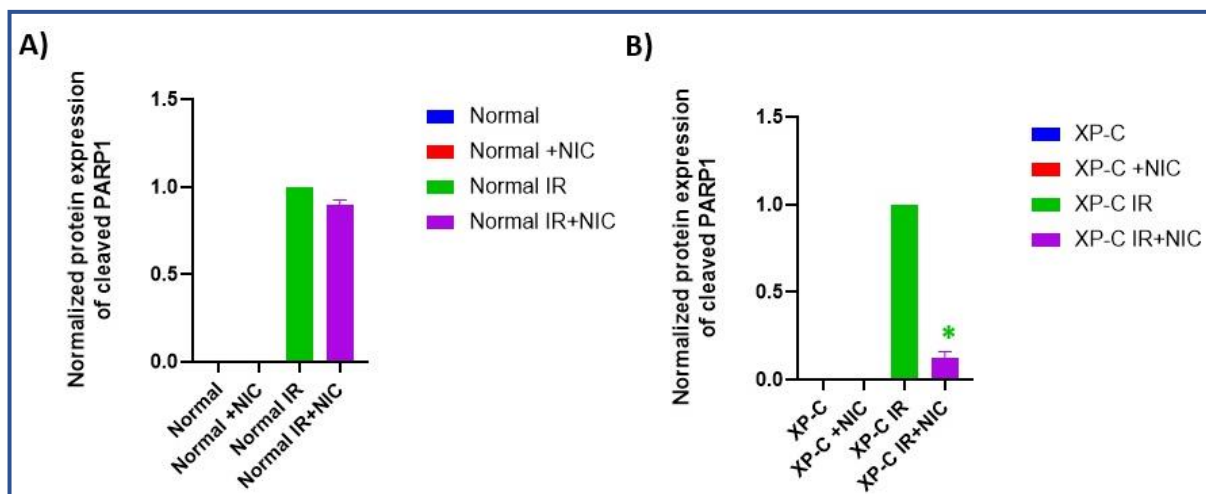


Figure 65. **Effect of NIC on cleaved PARP1 protein expression at basal and UVB levels in normal and XP-C cells.** Panel A) shows, in normal cells, the absence of cleaved PARP1 in the presence and absence of NIC at basal level while no significant effect was detected in the presence and absence of NIC at UVB level. Panel B) shows, in XP-C cells, an absence of cleaved PARP1 in the presence and absence of NIC at basal level while NIC



downregulated the cleaved PARP1 significantly ( $p < 0.05$ , \*) at UVB level. Paired *t*-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SD from two independent experiments,  $n = 2$ . IR=irradiated with UVB (0.01 J/cm<sup>2</sup>). (preliminary result)

#### 2.4. Effect of NIC on XP-C and normal cells' P53 gene expression

After checking cleaved PARP1's status, we were interested in P53's gene expression. In addition to its role in cell cycle regulation, P53 has been linked to NER and BER to protect cells from genotoxicity. Therefore, it is speculated that P53's downregulation could affect the efficiency of DNA repair activity (Y. R. Seo and Jung 2004). Consequently, we wanted to check whether NIC could enhance P53's expression as a substitute method to enhance the background DNA repair pathway, particularly BER, and whether it can downregulate apoptosis in cells.

NIC did not affect P53 in both cell lines at basal and UVB levels (figure 66).

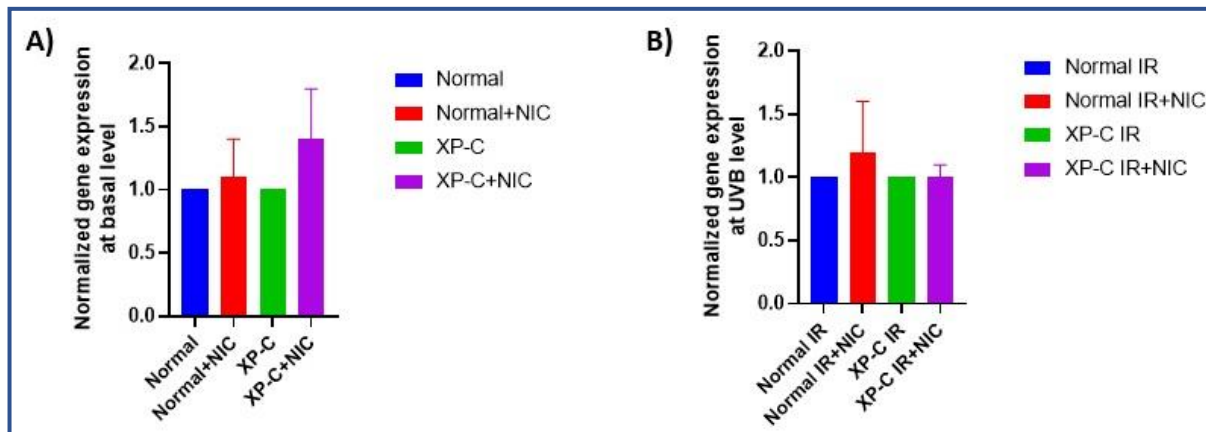


Figure 66. **Effect of NIC on P53 mRNA expression at basal and UVB levels in normal and XP-C cells.** Panel A) shows no effect of NIC on P53 in normal and XP-C cells, at the basal level. Panel B) shows no effect of NIC on P53 in normal and XP-C cells, at UVB level. For each condition, the sample was normalized by its untreated value (100% viability). Paired *t*-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from three independent experiments,  $n = 3$ . IR=irradiated with UVB (0.01 J/cm<sup>2</sup>).

Similarly, no effect of NIC was detected on P53 protein in both cell lines at basal and UVB levels [figure 67 (A) and (B)].

However, NIC was shown to enhance P53's protein expression post-stress (chemotherapy) to induce P53-dependent pathways, including apoptosis (Audrito et al. 2011). Despite that we could not see such an increase in our result, it cannot be excluded. Perhaps the selected time was too early to detect variations, and we should have studied P53's expression 24 hours post-UVB. P53's upregulation might also be an indication for P53-dependent DNA repair enhancement (section 2.6.).

P53's activity and its downstream factors (as p21) should have been tested to verify which possibility occurred in our cells.

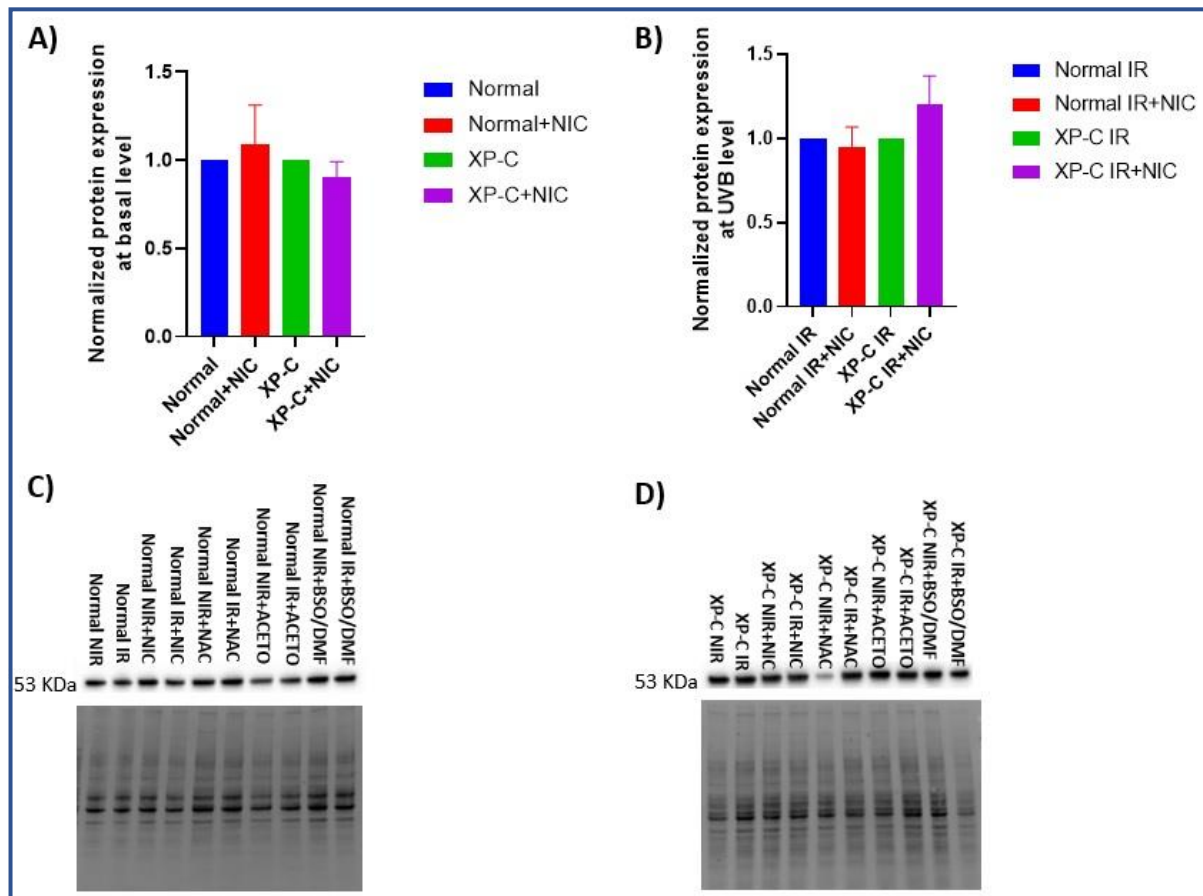


Figure 67. Effect of NIC on P53 protein expression at basal and UVB levels in normal and XP-C cells. Panel A) shows no effect of NIC on P53 in normal and XP-C cells at the basal level. Panel B) shows no effect of NIC on P53 in normal and XP-C cells at UVB level. Panel C) shows the P53 band (53 KDa) in normal cells with different treatments at basal and UVB levels and the total protein used for normalization. Panel D) shows the P53 band (53 KDa) in XP-C cells with different treatments at basal and UVB levels and the total protein used for normalization. For each condition, the sample was normalized by its untreated value (100% viability). Paired *t*-test was used to compare the expression for each condition. The results are the mean  $\pm$  SD from three independent experiments,  $n=3$ . IR=irradiated with UVB (0.01 J/cm<sup>2</sup>).

## 2.5. Effect of NIC on XP-C and normal cells' BER gene expression

After checking the oxidative status in cells after NIC treatment, we studied the detailed impact on BER's expression and activity where BER is directly involved in repairing oxidative stress byproducts 8-oxoGua. This will allow us to check whether NIC regulates the oxidative stress level and/or its consequences (DNA repair).

### 2.5.1. Effect of NIC on BER's mRNA level

As presented in figure 68, we studied the mRNA expression of BER factors at basal and UVB levels in normal and XP-C cells with and without NIC treatment. At the basal level, NIC significantly upregulated *OGG1* and *MYH* mRNA levels in normal cells ( $p<0.05$ , \*) [figure 68

(A)] and *APE1* and *LIG3* mRNA levels in XP-C cells ( $p < 0.05$ , \*) [figure 68 (B)] compared to their untreated controls. This may indicate a preventative and protective role of NIC by increasing the expression of oxidative DNA repair genes pre-stress. Benavente et al. showed that  $\text{NAD}^+$  depletion increases ROS and oxidative DNA damage in keratinocytes and NIC supplementation reverses the genotoxicity (Benavente and Jacobson 2008). This could be the case in figure 68 (A) and (B), where NIC supplementation prevented any spontaneous DNA damage at the basal level by enhancing some BER factors' expression.

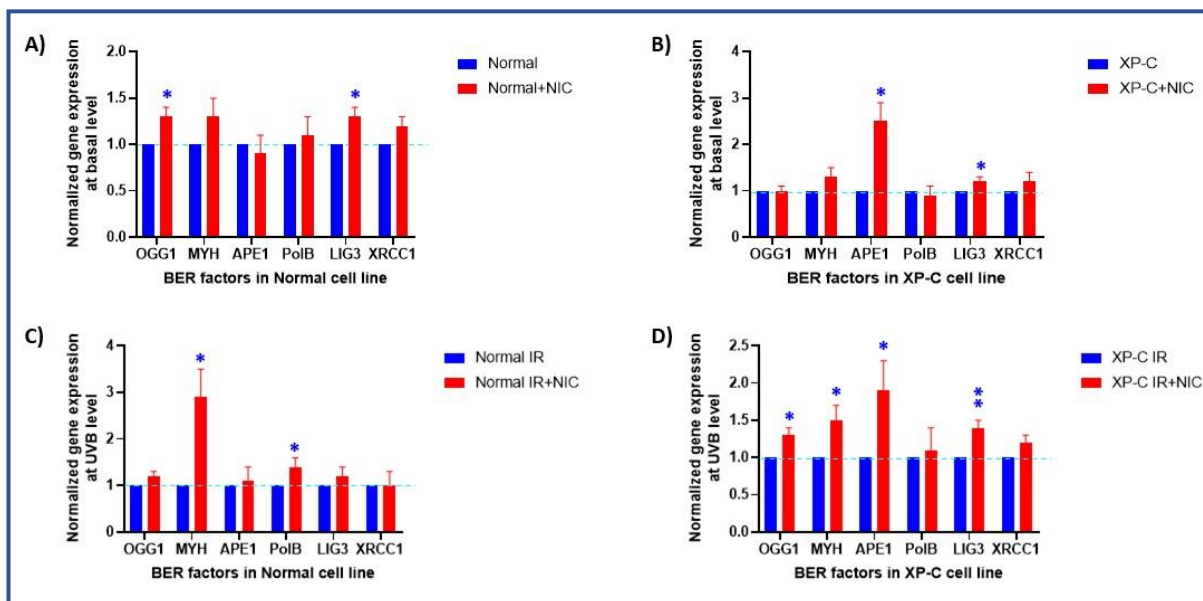


Figure 68. Effect of NIC on BER mRNA expression at basal and UVB levels in normal and XP-C cells. Panel A) shows an upregulation in *OGG1* and *LIG3* expression upon NIC treatment ( $p < 0.05$ , \*) in normal cells at the basal level. Panel B) shows an upregulation in *APE1* and *LIG3* expression upon NIC treatment ( $p < 0.05$ , \*) in XP-C cells at the basal level. Panel C) shows an upregulation of *MYH* and *POLB* upon NIC treatment ( $p < 0.05$ , \*) in normal cells post-UVB irradiation. Panel D) shows an upregulation in *OGG1* ( $p < 0.05$ , \*), *MYH* ( $p < 0.05$ , \*), *APE1* ( $p < 0.05$ , \*), and *LIG3* ( $p < 0.05$ , \*) in XP-C cells post-UVB irradiation. For each condition, the sample was normalized by its untreated value (100% viability). Paired t-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from three independent experiments,  $n = 3$ . IR=irradiated with UVB (0.01 J/cm<sup>2</sup>).

Post-UVB, *MYH* and *POLB* were significantly upregulated ( $p < 0.05$ , \*) in normal cells in presence of NIC compared to its absence [figure 68 (C)] while an upregulation of *OGG1* ( $p < 0.05$ , \*), *MYH* ( $p < 0.05$ , \*), *APE1* ( $p < 0.05$ , \*), and *LIG3* ( $p < 0.01$ , \*\*) were observed in XP-C cells in the presence of NIC compared to its absence [figure 68 (D)]. Chhabra et al. showed that NIC protects the melanocytes from UV-induced DNA damage by upregulating the mRNA expression of genes involved in NER and BER pathways. Their results showed that damage-specific DNA binding subunits (*DDB1* and *DDB2*), excision repair cross-complementation groups (*ERCC1* and *ERCC2*), and cyclin-dependent kinase 7 (*CDK7*; TFIIH subunit) mRNA expressions were enhanced in the presence of NIC+UVB compared to UVB (Chhabra

et al. 2019). This confirms that NIC could enhance DNA repair by inducing the expression of NER factors. Their results also indicated that NIC enhanced *OGG1* mRNA expression in the presence of UVB (Chhabra et al. 2019). Such an interesting study provided a new insight towards protection against DNA damage and melanoma.

Therefore, NIC protects the cells from UV-induced DNA damage by stimulating the expression of BER genes before and after stress.

Interestingly, BER's gene expression stimulation was induced more prominently in XP-C cells compared to normal control. This may be due to the higher sensitivity of XP-C cells to UVB-induced ROS and the low BER activity and expression, as proven in part one. Therefore, NIC's protective effect was more prominent due to increasing NAD<sup>+</sup> level and more BER gene expression induction to compensate and protect from oxidative DNA damage. Furthermore, we suggest that such a detected enhancement of BER's mRNA expression is XPC-independent. To check our hypothesis and whether NIC solely affects BER's transcript level or also its protein level, activity, and cellular ROS level, further experiments were done.

#### 2.5.2. Effect of NIC on XP-C and normal cells' UVB-induced BER protein expression

We did a western blot to examine whether NIC influenced the translational level of BER factors similar to the transcriptional level. However, we were selective in choosing those we already studied in part one and are known to be at the initiation step of BER (OGG1, MYH, and APE1) as they were proven to be downregulated in XP-C primary fibroblasts (part one). This could reveal how NIC may act as a potential preventative therapy in XP-C patients.

As presented in figures 69 and 70, NIC increased MYH's protein expression in normal cells at the basal level ( $p < 0.05$ , \*). However, no effect of NIC was observed post-UVB irradiation except for a downregulation of OGG1's protein expression ( $p < 0.05$ , \*) in XP-C cells.

Interestingly, OGG1 is known to be at the early steps of BER. Therefore, its downregulation could reveal that cells had already started their DNA repair and recovery from UVB-induced stress, which negatively affected the studied protein. In fact, this could prevent the excessive expression of BER proteins that are usually indicative of carcinogenesis.

Therefore, the preventative effect of NIC may be through (i) enhancing BER's turnover (OGG1), (ii) increasing the stimulation and activity of BER via ATP upregulation and/or (iii) reducing the amount of induced oxidative stress. (section 2.2.).

Another explanation for the observed protein expressions' level could be the selected time. Perhaps, we should have taken another kinetic point to give proteins more time to be stimulated and expressed after stress.

Therefore, post-UVB, NIC may have succeeded in boosting cellular defense against UVB and its damages which is convenient with its suggestive role as a preventative method against stress.

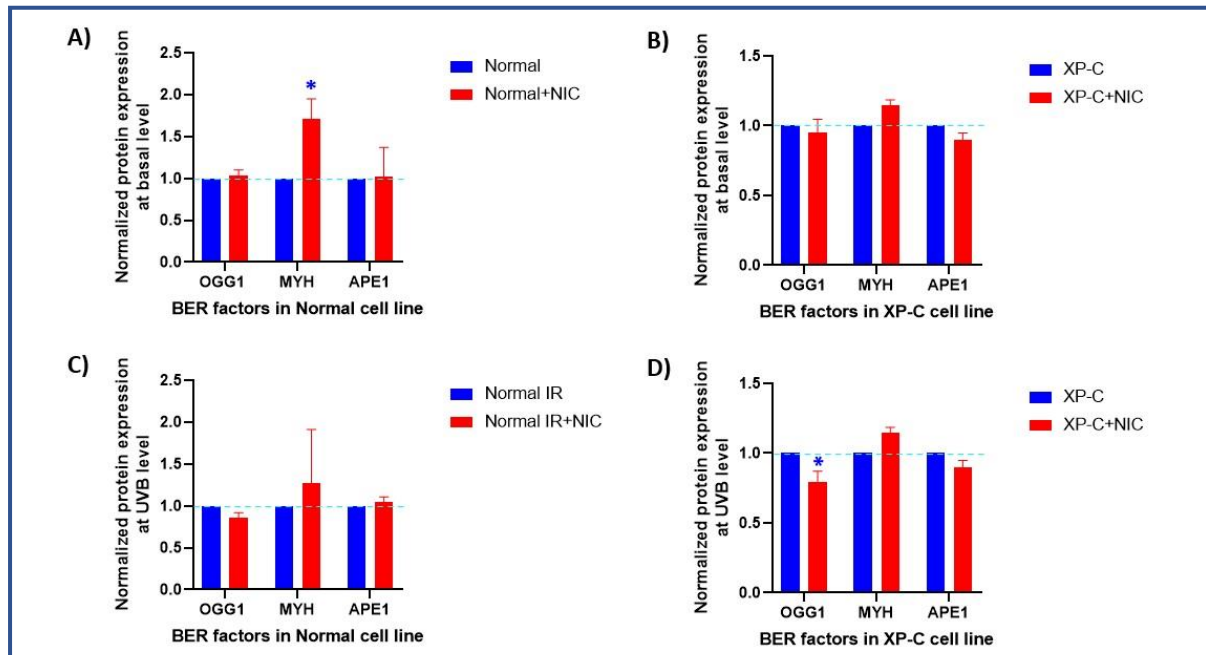
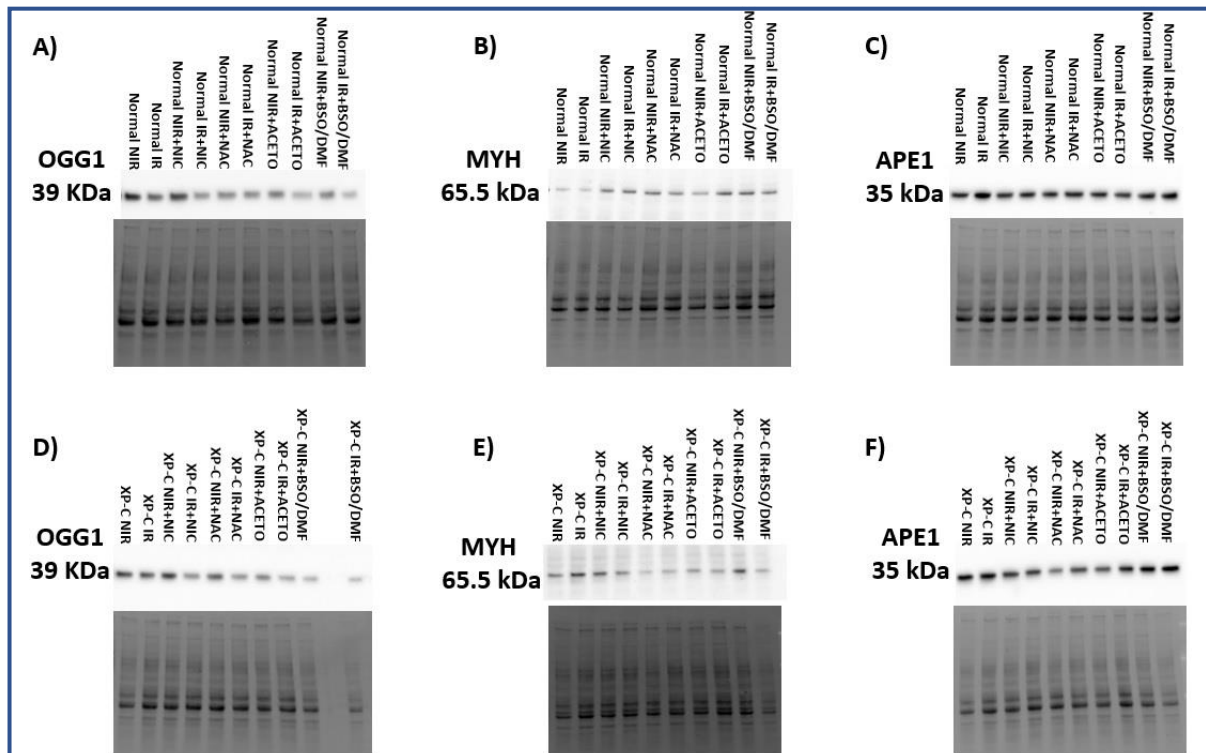


Figure 69. Effect of NIC on BER protein expression at basal and UVB levels in normal and XP-C cells. Panel A) shows that NIC upregulated MYH ( $p < 0.05$ , \*) in normal cells at the basal level. Panel B) that NIC has no effect on BER proteins in XP-C cells at the basal level. Panel C) shows that NIC has no impact on BER factors in normal cells post-UVB irradiation. Panel D) shows that NIC downregulated OGG1 ( $p < 0.05$ , \*) in XP-C cells post-UVB irradiation. For each condition, the sample was normalized by its untreated value (100% viability). Paired t-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SD from three independent experiments,  $n=3$ . IR=irradiated with UVB (0.01 J/cm<sup>2</sup>).



*Figure 70. Effect of different treatments (NIC, NAC, ACETO, BSO/DMF) on BER protein expression at basal and UVB levels in normal and XP-C cells. Panels A), B), and C) show OGG1, MYH, and APE1 bands in normal cells, respectively, at different conditions and their respective total protein membranes used for normalization. Panels D), E), and F) show OGG1, MYH, and APE1 bands in XP-C cells, respectively, at different conditions and their respective total protein membranes used for normalization. NIC=Nicotinamide, NAC=N-acetylcysteine, ACETO=Acetohexamide (done but not included in manuscript), BSO/DMF=Buthionine sulfoximine/Dimethylfumurate, NIR=Non-irradiated, IR=irradiated with UVB (0.01 J/cm<sup>2</sup>)*

## 2.6.Effect of NIC on XP-C and normal cells' UVB-induced BER and NER activity

It has been demonstrated that NIC protects against CPDs and oxidative damage at the DNA, proteins, and lipids levels (Kamat and Devasagayam 1999; Thompson et al. 2014). We also showed some effect on BER's expression. Therefore, we intended to check whether NIC pretreatment could protect our studied cells from induced bulky lesions [(6-4) PPs and CPDs] and oxidative DNA damage and improve their repair.

### 2.6.1. Monitoring NER activity

Using HPLC-MS/MS we examined the kinetic repair of (6-4) PPs (figure 71) and CPDs (figure 72) in XP-C and normal cells with/without NIC (50μM).

NIC showed no effect on the repair of (6-4) PPs in normal cells [figure 71 (A)]. This may indicate that DNA repair is fully active in the normal cells and repaired these photoproducts rapidly with no need to use the extra ATP that is usually provided in the presence of NIC via the following reaction:  $\text{NAD}^+ + \text{ADP} \rightarrow \text{NADH} + \text{ATP}$  to improve DNA repair. However, we were able to detect an improvement in the repair of TT (6-4) PPs in the presence of NIC in GG-NER deficient XP-C cells [figure 71 (B)].

The high TT compared to TC in XP-C cells was consistent with what was previously reported about UVB (figure 6). Hu et al. showed that TC (6-4) PPs are repaired more rapidly than TT (6-4) PPs in human fibroblasts (Hu et al. 2017).

It should be stressed that we tried to quantify the Dewar isomers as they are mutagenic photoisomerization products of excited (6-4) PPs where the former induce mutations at the 3' end of TT sequences while the latter induce mutations at the end of 3' TC doublets. This could strongly impact the DNA structure. We did not detect these lesions at 0.01 J/cm<sup>2</sup>. Perhaps UVA and UVB are needed to induce such a lesion. First, UVB will lead to the (6-4) PPs formation; then UVA photons will be absorbed by them, leading to their excitation (Douki 2016).

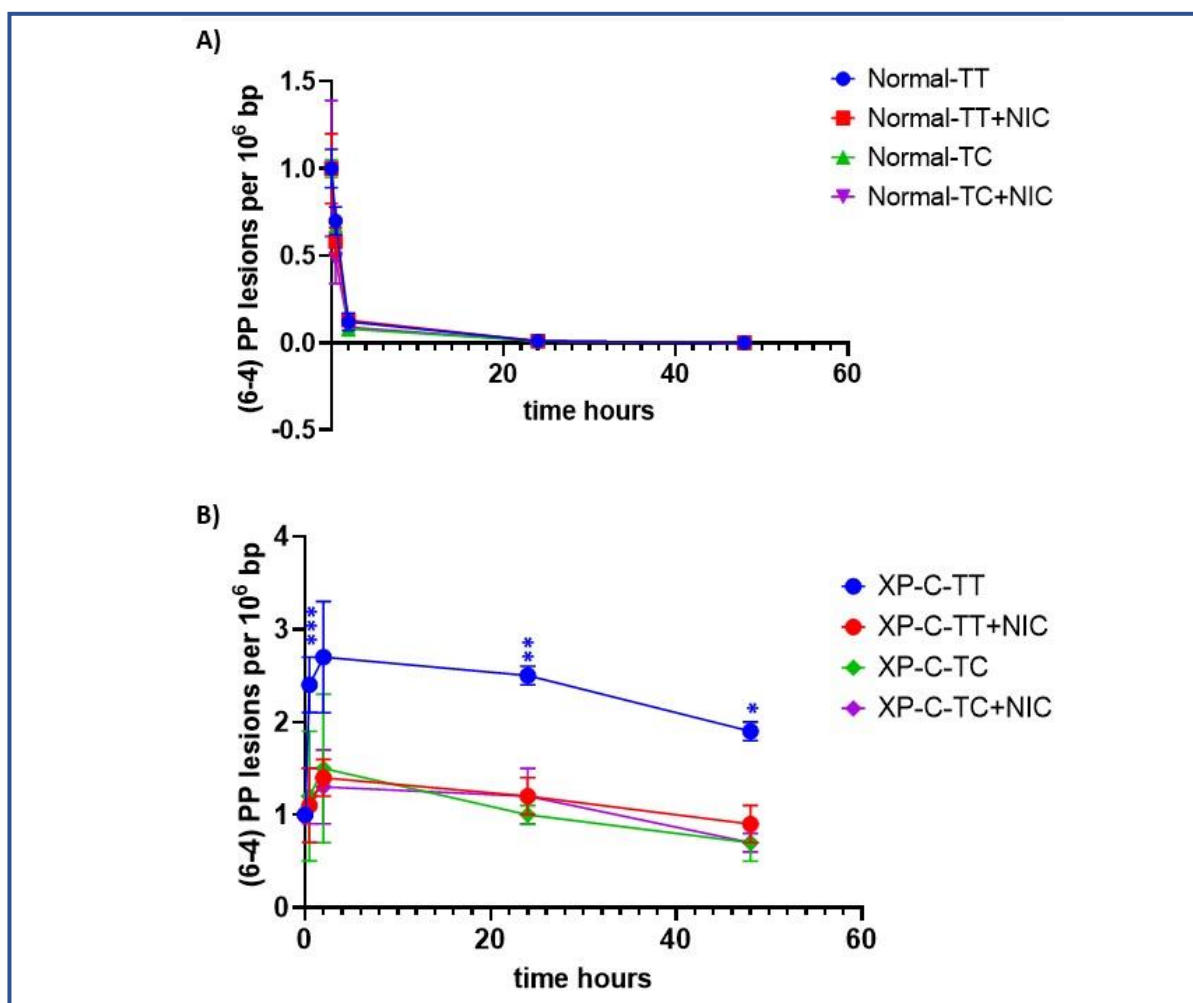


Figure 71. Effect of NIC on UVB-induced-(6-4) PPs kinetic repair in normal and XP-C cells. Panel A) shows no significant effect of NIC in repairing TT and TC (6-4) PPs in normal cells. Panel B) shows no significant effect of NIC in TC (6-4) PPs in XP-C cells. However, significantly higher TT (6-4) PPs at 0.5 ( $p < 0.001$ , \*\*\*), 24 ( $p < 0.01$ , \*\*), and 48 ( $p < 0.05$ , \*) hours in XP-C cells in presence of NIC compared to its absence. For each condition, the sample was normalized by its irradiated value at 0 hours (100% viability). Paired *t*-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SD from two independent experiments,  $n=2$ .

Moving forward, TT and TC CPDs are the most dominant UVB-induced CPD lesions. Figure 72 shows that NIC did not significantly affect the repair of CPDs in normal cells or TC CPDs in XP-C cells. Interestingly, this treatment successfully improved the repair of TT CPDs in the absence of XPC. Such a conclusion was similar to that shown in (6-4) PPs repair. However, it should be emphasized that NIC reduced the induced lesions promptly post-UVB in normal cells. Similarly, Surjana et al. showed that NIC enhanced CPDs repair and reduced their level post-solar simulation in keratinocytes (Thompson, Halliday, and Damian 2015).

These results are consistent with the proposed role of NIC as a non-toxic chemopreventive agent against skin cancer.

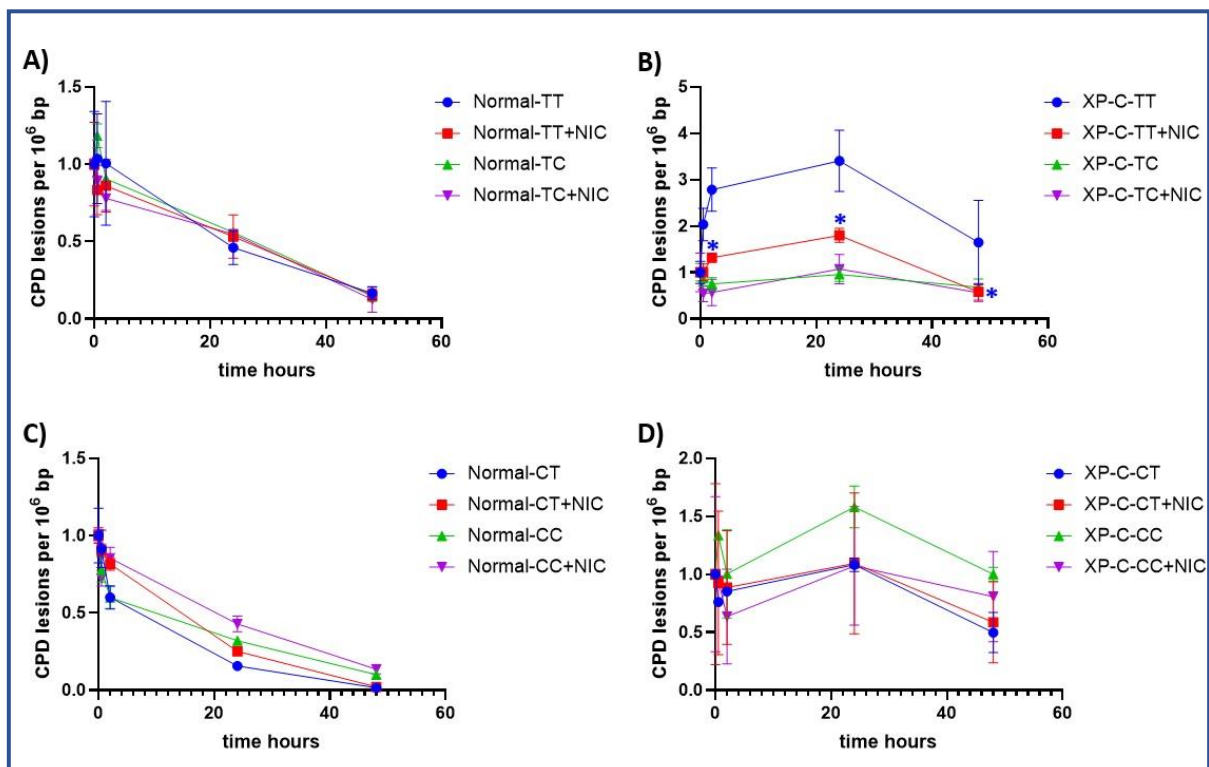


Figure 72. *Effect of NIC on UVB-induced-CPDs kinetic repair in normal and XP-C cells.* Panels A) shows no effect of NIC on TT and TC CPDs repair in normal cells. Panel B) shows no effect of NIC on TC CPDs repair but was able to downregulate TT CPDs at 2, 24, and 48 hours significantly ( $p < 0.05$ , \*) in XP-C cells. Panel C) shows no effect of NIC on the repair of CT and CC CPDs in normal cells. Panel D) shows no effect of NIC on CT and CC CPDs repair in XP-C cells. For each condition, the sample was normalized by its irradiated value at 0 hours (100% viability). Paired *t*-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SD from two independent experiments,  $n=2$ .

### 2.6.2. Monitoring BER activity

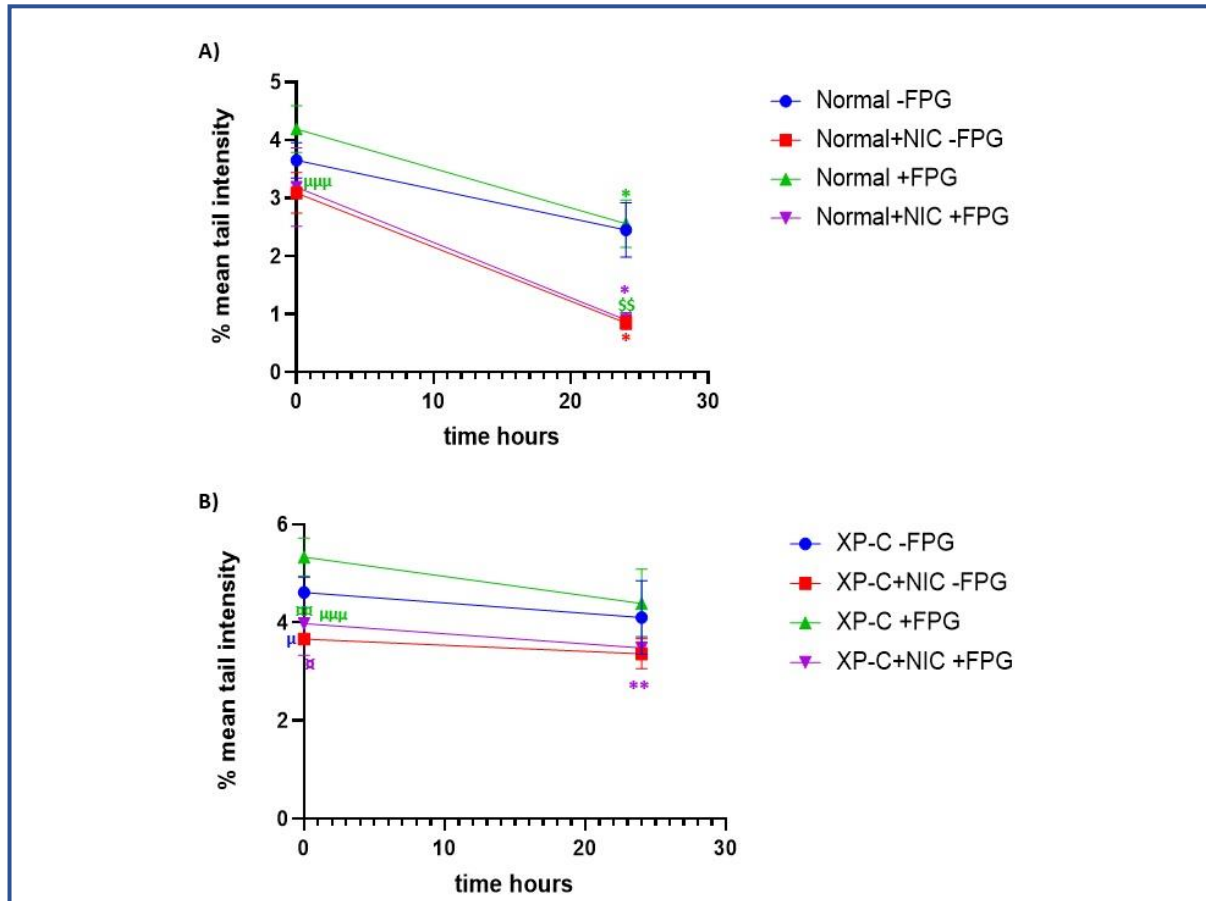
As previously mentioned, studies showed that NIC enhances the repair of 8-oxoGua. Therefore, we sought to check whether it is the case in our study. It was shown that UVB induces 8-oxoGua in murine and human epidermal keratinocytes (Thompson, Halliday, and Damian 2015). Hence, we used the modified comet  $\pm$ FPG assay to check the effect of NIC on UVB-induced oxidized lesions' excision in normal and XP-C cells at 0 and 24 hours.

We already verified a lower kinetic repair of oxidized purines, including 8-oxoGua, in XP-C primary fibroblasts compared to normal (part one). This seems to be the case in the current studied XP- C cells (figure 73).

NIC pretreatment reduced the quantity of initiated oxidized purines in XP-C and normal cell lines ( $P < 0.001$ ,  $\mu\mu\mu$ ). It also enhanced their repair at 24 hours compared to their amount promptly post-UVB ( $p < 0.05$ , \*). This indicates that NIC pretreatment could protect against oxidative DNA damage and could enhance their repair. This is done via enhancing BER's mRNA expression (as shown previously), preventing UV-induced ATP depletion, improving cellular energy for better DNA repair, and/or reducing produced ROS levels (section 2.8).



Similarly, Surjana et al. showed that *ex vivo* skin pretreated with NIC (50 $\mu$ M for 24 hours) before solar-simulated UV (4 J/cm<sup>2</sup>) had lower induced epidermal 8-oxoGua and higher repair compared to untreated control (Thompson, Halliday, and Damian 2015).



**Figure 73. Effect of NIC on alkaline and oxidized purines repair in normal and XP-C cells, post-UVB irradiation.** Panel A) shows that NIC decreased the UVB-induced oxidized purines at 0 hours and 24 hours (+FPG,  $p < 0.001$ ,  $\mu\mu\mu$  and  $p < 0.01$ , \$, respectively) in normal cells. Panel B) shows that NIC decreased the UVB-induced oxidized purines at 0 hours (+FPG,  $p < 0.001$ ,  $\mu\mu\mu$ ). For each condition, the sample was normalized by its unirradiated value (100% viability). Paired *t*-test was used to compare the expression for each condition. The results are the mean  $\pm$  SEM from three independent experiments,  $n = 3$ . \*  $p < 0.05$  between -FPG and +FPG for each treatment at 0 and 24 hours. \$  $p < 0.05$  between the NIC and untreated samples at 24 hours +FPG.  $\mu$   $p < 0.05$  between the NIC and untreated samples at 0 hours +FPG. ✕  $p < 0.05$  between -FPG and +FPG for NIC and untreated samples at 0 hours.

Therefore, NIC pretreatment could decrease incidences of non-melanoma skin cancer and basal cell carcinoma in XP-C patients by reducing the DNA damage (Thompson, Halliday, and Damian 2015). More importantly, it could also play a role in halting internal cancer and preventing macromolecular cellular damage.

# Conclusion and Perspective

## Nicotinamide (50 $\mu$ M):

- ❖ Upregulated some BER gene expressions:
  - ✓ mRNA level:  
Basal: *OGG1* and *LIG3* in normal cells while *APE1* and *LIG3* in XP-C cells  
UVB: *MYH* and *POLB* in normal cells while *OGG1*, *MYH*, *APE1* and *LIG3* in XP-C cells
  - ✓ Protein level:  
Basal: MYH in normal cells
  
- ❖ Downregulated some BER protein expressions:
  - ✓ OGG1 at UVB level in XP-C cells
  
- ❖ Enhanced TT (6-4) PP and TT CPDs NER repair in XP-C cells
- ❖ Enhanced oxidized purines repair by BER in XP-C and normal cells
- ❖ Upregulated Glutathione level
- ❖ Downregulated ROS level in XP-C cells

In summary, NIC is a non-toxic therapeutic agent. It could act as a preventive treatment to protect XP-C cells against UV-induced DNA lesions and oxidative stress. This could prevent the initiation of dramatic cascade of events leading to the severe clinical symptoms in XP-C patients.

### 3. Chapter Three: The effect of N-acetylcysteine (NAC) on UVB-Induced Oxidative Stress and DNA Repair in XP-C and Normal Cells

After checking NIC pretreatment, we were interested in studying a more specific antioxidant treatment, NAC. NAC has been used a lot in research due to its significant protective and detoxifying role, particularly at the level of oxidative stress.

#### 3.1.Characterization of NAC-treated normal and XP-C cells

##### 3.1.1. NAC dose selection

We did a dose-response curve to select the most suitable NAC dose for our experiments. Figure 74 shows that 1mM of NAC treatment for 24 hours is the most suitable dose for the experiments. It is the highest concentration to use before cytotoxicity. Such a dose had already been used in research (Zhang et al. 2011).

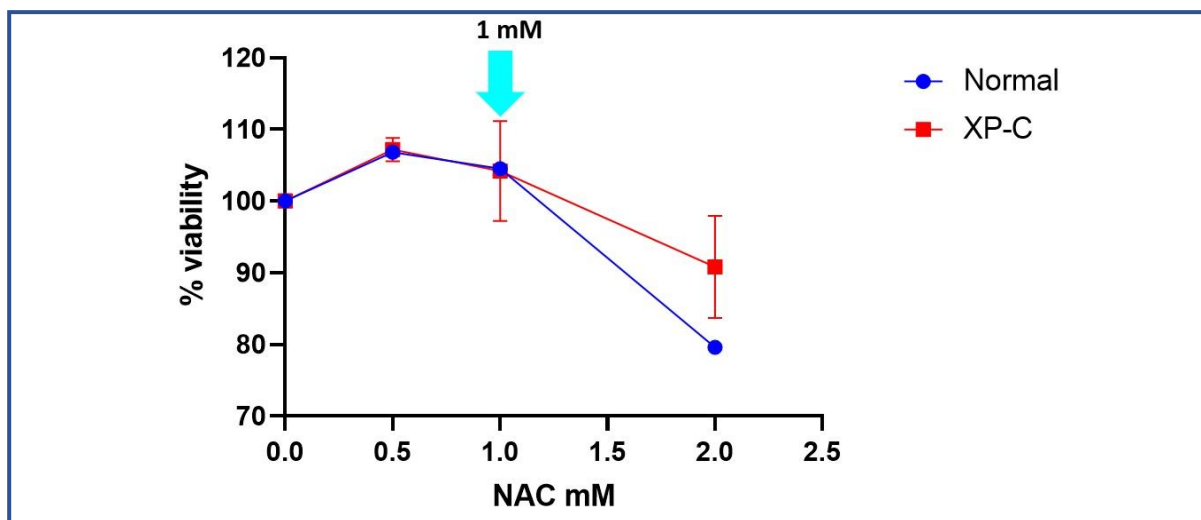


Figure 74. NAC dose-response curve. Normal and XP-C cells were tested with different doses of NAC for 24 hours followed by MTT test to monitor the cellular viability. 1mM is the most suitable dose with least cytotoxicity. The results are the mean  $\pm$  SD.

### 3.1.2. Effect of NAC on XP-C and normal cells' UVB-induced photosensitivity

Before studying NAC's effect on DNA repair, we wanted to check whether it affects cellular viability post-UVB. As shown in figure 75, no difference in photosensitivity between treated and untreated samples in both cell lines, normal and XP-C. Perhaps higher NAC doses or a pre- and post-UVB treatment are needed to obtain adequate protection. Then, NAC pretreatment does not interfere with cellular viability as they are not cytotoxic at low doses. A similar observation was illustrated by Mitsopoulos et al. They showed that NAC pretreatment did not affect cell viability post-oxidative stress (Paraquat) (Mitsopoulos and Surtres 2011). However, high NAC doses were recorded as cytotoxic at variable concentrations depending on the cell type. For instance, NAC concentrations below 10 mM did not affect A547 cells' viability, while higher doses (50 mM) decreased cell viability after 24 hours. Such a high dose was detected as non-toxic for aortic endothelial cells (Mitsopoulos and Surtres 2011).

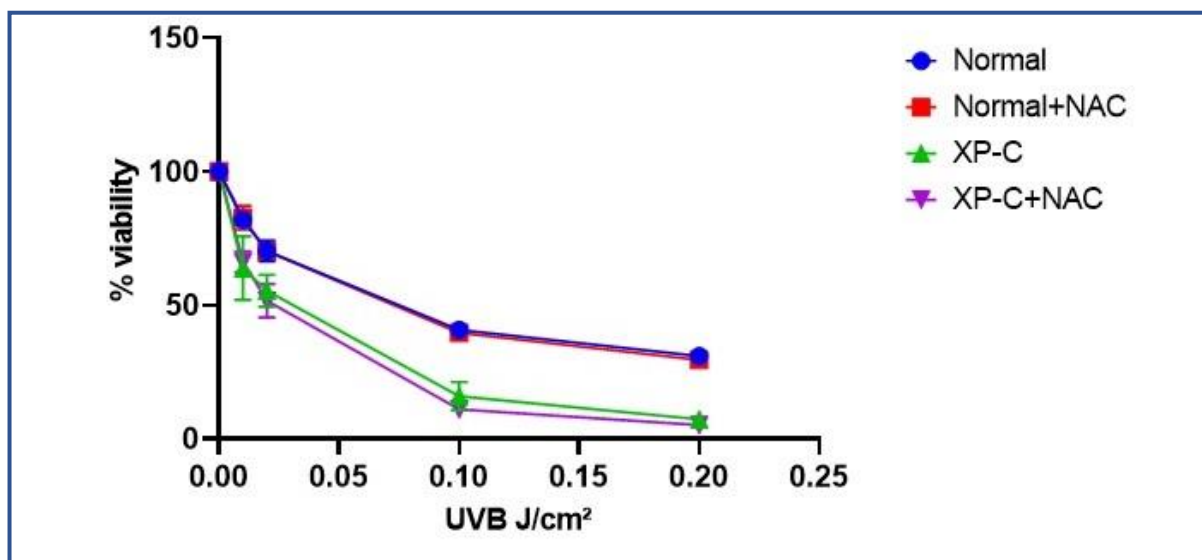


Figure 75. *Similar UVB-induced photosensitivity in each cell line (normal and XP-C), with and without NAC. We treated cells with 1 mM NAC for 24 hours then we measured the percentage of cellular viability 24 hours post-UVB irradiation by short-term cytotoxicity test (MTT). For each condition, the sample was normalized by its unirradiated value (100% viability). Paired t-test was used to compare photosensitivity for each condition at each UVB dose ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from three independent experiments,  $n=3$ .*

### 3.2. Effect of NAC on XP-C and normal cells' redox state

#### 3.2.1. Effect of NAC on XP-C and normal cells' ROS and glutathione levels

- Effect on UVB-induced ROS

As mentioned earlier, NAC is an efficient thiol-containing ROS scavenger. The dose and treatment duration used in our experiment proved efficient in reducing ROS in normal and XP-C cells (figure 76).

Figure 76 (A) shows that NAC protected normal cells from the UVB-induced ROS at early stages where ROS fluorescence was significantly lower at 0.5, 1, and 2 hours post-UVB ( $p < 0.05$ , \*;  $p < 0.001$ , \*\*\*;  $p < 0.05$ , \*, and  $p < 0.01$ , \*\*, respectively). Figure 76 (B) showed that ROS level was downregulated in presence of NAC at 0.5, 2, 3, and 24 hours post-UVB irradiation ( $p < 0.01$ , \*\*;  $p < 0.05$ , \*;  $p < 0.05$ , \*; and  $p < 0.05$ , \*, respectively). NAC was able to downregulate the UVB-induced ROS levels in XP-C cells and decrease their persistence and accumulation throughout time. Therefore, NAC pretreatment protected both cell types from oxidative stress. Similarly, NAC scavenged UVB-induced and  $H_2O_2$ -induced ROS in HaCaT and H9c2 cells, respectively (X. Liu et al. 2019; Zhu et al. 2017).

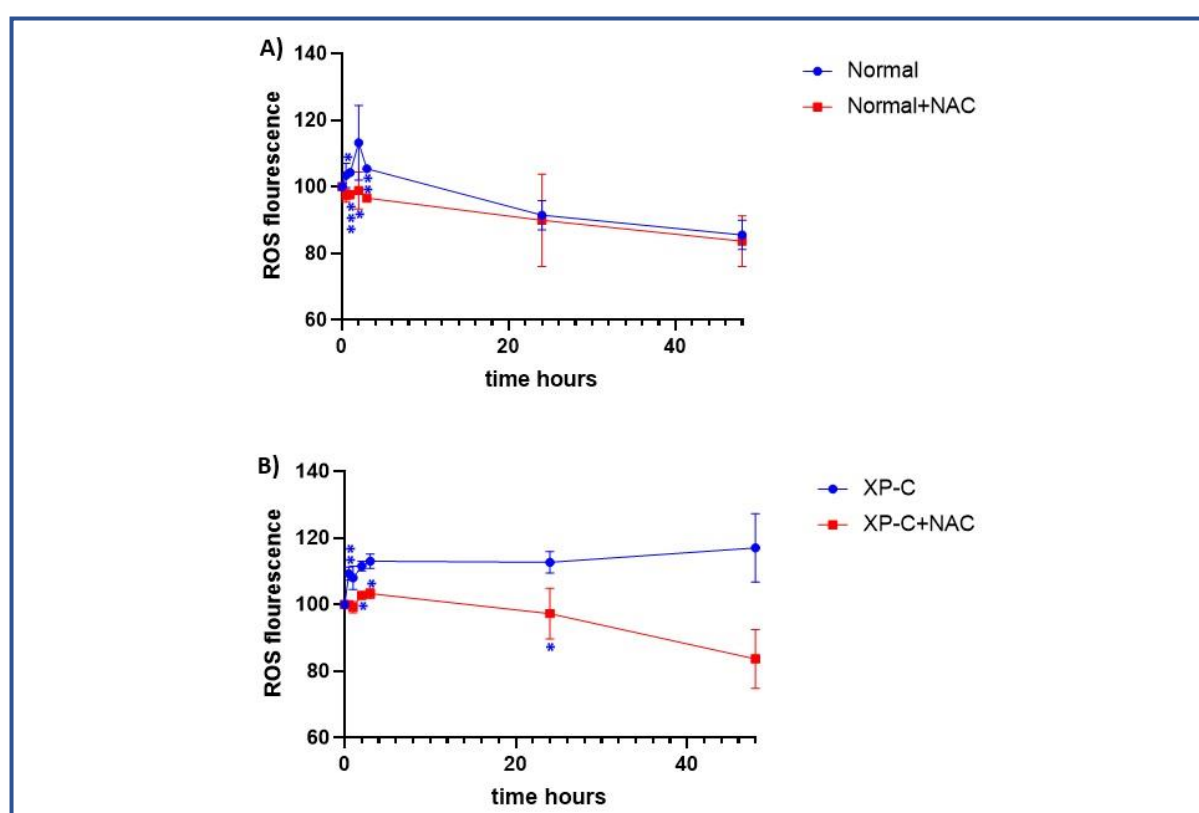


Figure 76. Effect of NAC on ROS level in normal and XP-C cells, post-UVB irradiation. Panel A) NAC downregulated ROS level at 0.5, 1, and 2 hours post-UVB ( $p < 0.05$ , \*;  $p < 0.001$ , \*\*\*;  $p < 0.05$ , \*, and  $p < 0.01$ , \*\*, respectively) in normal cells. Panel B) NAC downregulated ROS level at 0.5, 2, 3, and 24 hours post-UVB irradiation ( $p < 0.01$ , \*\*;  $p < 0.05$ , \*;  $p < 0.05$ , \*; and  $p < 0.05$ , \*, respectively) in XP-C cells. We treated cells with

1/1000 DHR123 followed by UVB (0.02 J/cm<sup>2</sup>) and monitored the kinetics of fluorescence at different kinetic time points. Then we normalized each sample by its untreated value (100% viability). H<sub>2</sub>O<sub>2</sub> was used as a positive control. Paired *t*-test was used to compare the ROS fluorescence between NAC treated and untreated samples in normal and XP-C1 fibroblast, respectively, at each kinetic time point (hours) ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from three independent experiments,  $n = 3$ .

- Effect on glutathione level 24 hours post-UVB

To show whether NAC pretreatment protected cells by halting ROS levels, neutralizing them, and/or improving oxidative DNA repair, we measured GSH concentration in cells 24 hours post-UVB.

Our studied XP-C cells had lower GSH level compared to normal (figure 77). A reduced *XPC* mRNA expression was detected in keratinocytes upon GSH downregulation, demonstrating that *XPC*'s transcription requires redox homeostasis (W. Han et al. 2012). Additionally to PTC, this could contribute to the lower mRNA *XPC* levels in XP-C cells compared to control (figure 51). Further, silencing *XPC* using siRNA decreased GSH and increased catalase and superoxide dismutase activities in glioma cells post-oxidant treatment (arsenic trioxide) (S.-Y. Liu et al. 2010). This shows a direct link between glutathione and *XPC*.

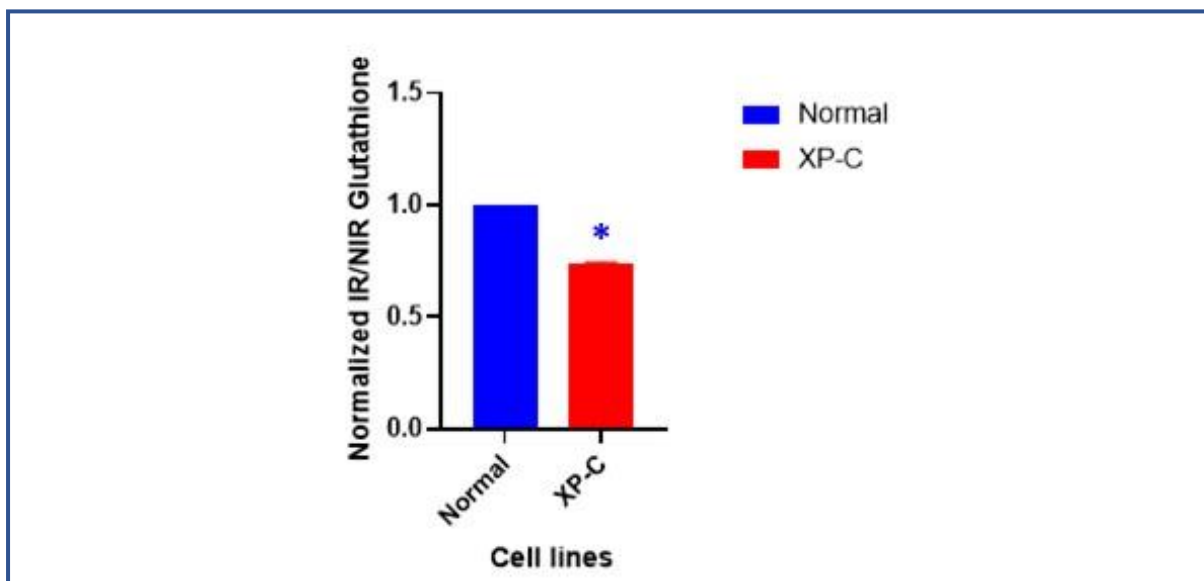
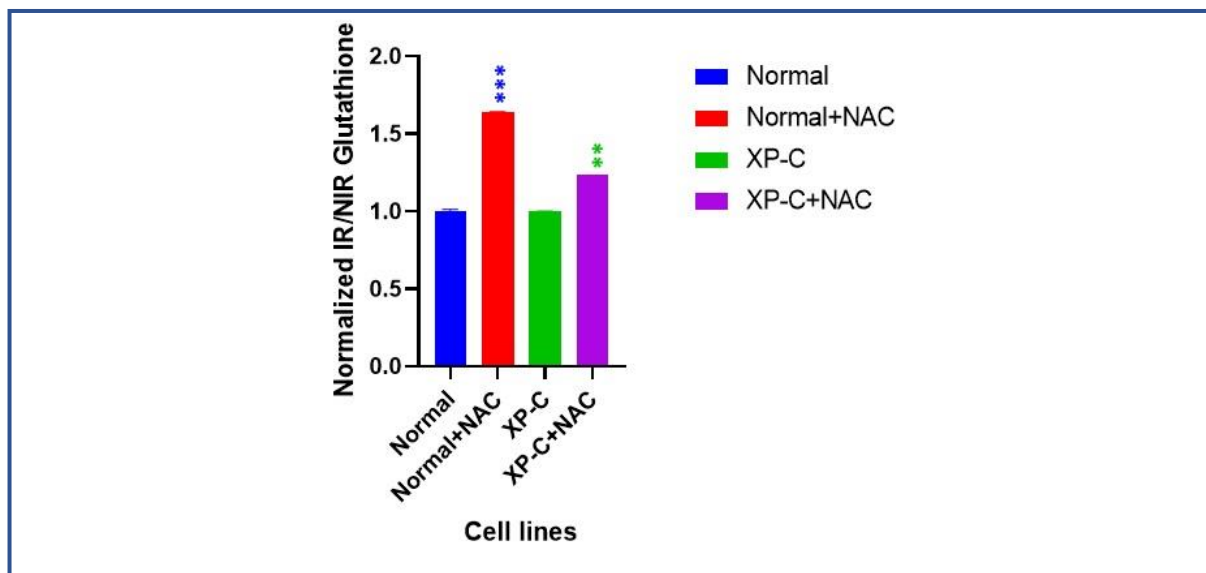


Figure 77. **The induced IR/NIR glutathione (GSH) level in normal and XP-C cells.** After UVB-irradiation for 24 hours, we extracted cells and measured glutathione levels calorimetrically using a CAYMAN kit. This treatment showed significant downregulation in glutathione level in XP-C ( $p < 0.05$ , \*) compared to the normal cells. XP-C and normal samples were normalized by normal IR/NIR value. Paired *t*-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SD.

**So, will supplementation with NAC improve GSH downregulation?**

As expected, NAC pretreatment significantly enhanced the induced GSH level in normal ( $p < 0.001$ , \*\*\*) and XP-C ( $p < 0.01$ , \*\*) cells (figure 78). This could explain the results previously stated where NAC was reported to efficiently downregulate UV-induced ROS and prevent GSH depletion in human skin (Goodson et al. 2009).

A clinical study done in 2009 showed that melanocytes extracted from melanoma patients had reduced antioxidants and increased ROS. So then, melanoma and UV-induced oxidative stress are tightly linked. In 1989, almost 16 percent of xeroderma pigmentosum Japanese patients were recorded to have melanoma (Takebe, Nishigori, and Tatsumi 1989). Therefore, pretreatment with NAC orally can prevent GSH depletion and reduce triggered oxidative stress in patients' nevi in the presence of acute UV exposure. This may suggest NAC as a preventive and protective treatment against melanoma risk (Goodson et al. 2009).



**Figure 78. Effect of NAC on the UVB-induced IR/NIR glutathione (GSH) level in normal and XP-C cells, with and without NAC.** After NAC pretreatment and UVB-irradiation for 24 hours, we extracted cells and measured glutathione levels calorimetrically using a CAYMAN kit. This treatment showed a significant upregulation in glutathione level in normal ( $p < 0.001$ , \*\*\*) and XP-C ( $p < 0.01$ , \*\*) cells compared to their untreated sample. For each condition, the sample was normalized by its untreated value. Paired *t*-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SD.

### 3.2.2. Effect of NAC on XP-C and normal cells' oxidative stress-linked genes' expression

One way to start monitoring the antioxidant defense status of cells is by checking their gene expression. Similarly to NIC, we studied the effect of NAC pretreatment on several antioxidants involved in halting oxidative stress in the nucleus and mitochondria.

NAC pretreatment abolished *GPx1* and *SOD2* expressions in normal and XP-C cells, respectively, at basal level [figure 79 (A) and (B)]. Perhaps this is the cellular response to a resting state.

After UVB-irradiation, *SOD2* was significantly upregulated by NAC in normal cells [figure 79 (C)]. This may indicate the presence of superoxide byproducts that need to be neutralized by the enzyme. Meanwhile, *GPx1* was significantly upregulated by NAC in XP-C cells which may indicate the presence of hydrogen peroxide that needs to be neutralized by this enzyme and the efficiency of NAC's role in boosting GSH level for GPx1's role enhancement. Furthermore, our result may suggest that SOD and GPx1 are implicated in preventing UVB-induced damage. Previously, NAC pretreatment had shown an augmentation of *SOD2* gene expression in chicken embryo post-oxidative stress (cadmium) (Doi et al. 2011). Thus, despite that NAC acts as a ROS scavenger and GSH precursor, it could improve the antioxidant defense system. Of course, we are well aware that most of the changes could be post-translational and could have been interesting to be monitored.

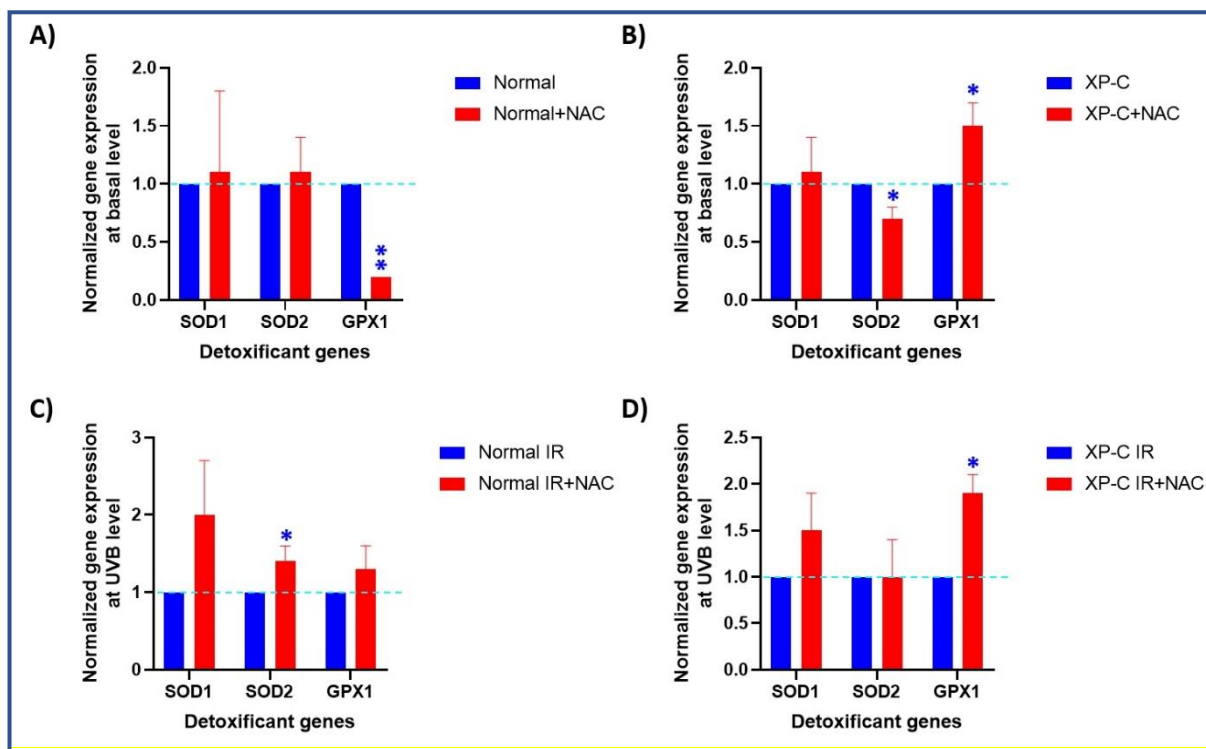




Figure 79. **Effect of NAC on the detoxificants' mRNA expression at basal and UVB levels in normal and XP-C cells.** Panel A) shows a downregulation in GPX1 expression ( $p < 0.01$ , \*\*) upon NAC treatment in normal cells at basal level. Panel B) shows downregulation in SOD2 ( $p < 0.05$ , \*) but an upregulation in GPX1 ( $p < 0.05$ , \*) expression upon NAC treatment in XP-C cells, at basal level. Panel C) shows an upregulation of SOD2 upon NAC treatment ( $p < 0.05$ , \*) in normal cells post-UVB irradiation. Panel D) shows an upregulation in GPX1 ( $p < 0.05$ , \*) in XP-C cells, post-UVB irradiation. For each condition, the sample was normalized by its untreated value (100% viability). Paired t-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from three independent experiments,  $n=3$ . IR=irradiated with UVB (0.01 J/cm<sup>2</sup>).

As both, SOD and GPx1, were shown to be affected by NAC, we suspected that it might influence an upstream gene, Nrf2. However, figure 80 shows that NAC could only enhance Nrf2's expression in XP-C at UVB level and not at the basal level. This indicates that Nrf2 is triggered post-stress efficiently by NAC pretreatment.

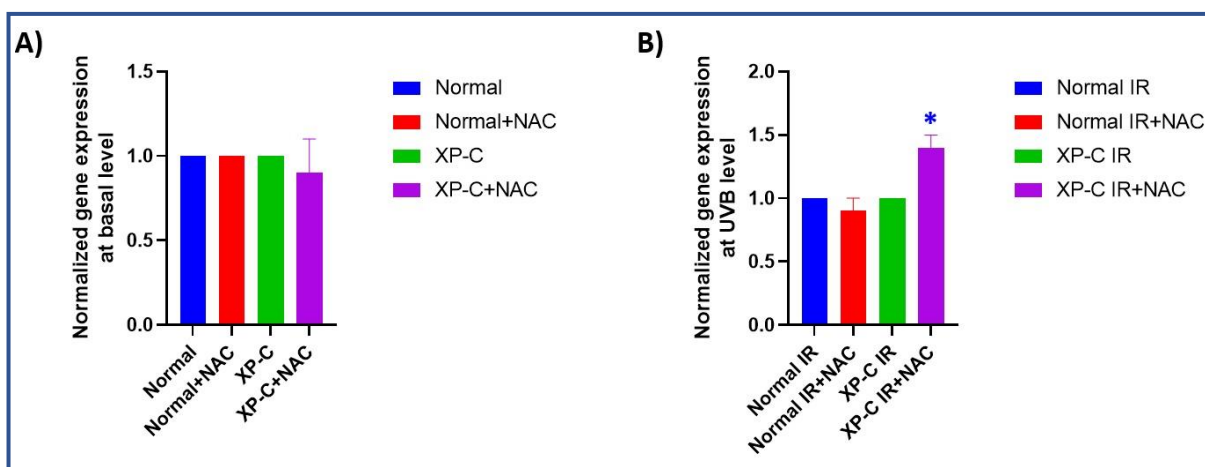


Figure 80. **Effect of NAC on the Nrf2 mRNA expression at basal and UVB levels in normal and XP-C cells.** NAC upregulated Nrf2 significantly ( $p < 0.05$ , \*) in XP-C cells at UVB level. The sample was normalized by its untreated value (100% viability). Paired t-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from three independent experiments,  $n=3$ . IR=irradiated with UVB (0.01 J/cm<sup>2</sup>).

Nrf2 showed a downregulation at the basal level in normal cells but an upregulation in XP-C cells [figure 81 (A) and figure 62 (C) and (D)]. The attenuated oxidative stress could explain the downregulated expression. Nevertheless, the upregulation in XP-C cells is spectacular. Perhaps this is due to the different cell lines used, and using the same cell line with/without XPC's expression could have more relevant results. Jannatifar et al. previously demonstrated that NAC pretreatment increases Nrf2 gene expression in Asthenoteratozoospermia Men (Jannatifar et al. 2020). Another study showed that NAC supplementation induces Nrf2 and OGG1 as Nrf2 regulates the latter's expression (K. C. Kim et al. 2017).

This demonstrates the role of NAC not only in increasing GSH level per se rather also in regulating signaling pathways, including the Nrf2-dependent antioxidant signaling pathway.

However, no effect was detected at the UVB level. Maybe 4 hours post-UVB irradiation is more than the time needed to detect such an early response to stress, or 0.01 J/cm<sup>2</sup> is not enough dose to detect notable changes.

Therefore, our previous and following results may indicate that NAC rapidly increases repair and defense rates against oxidative stress and damage.

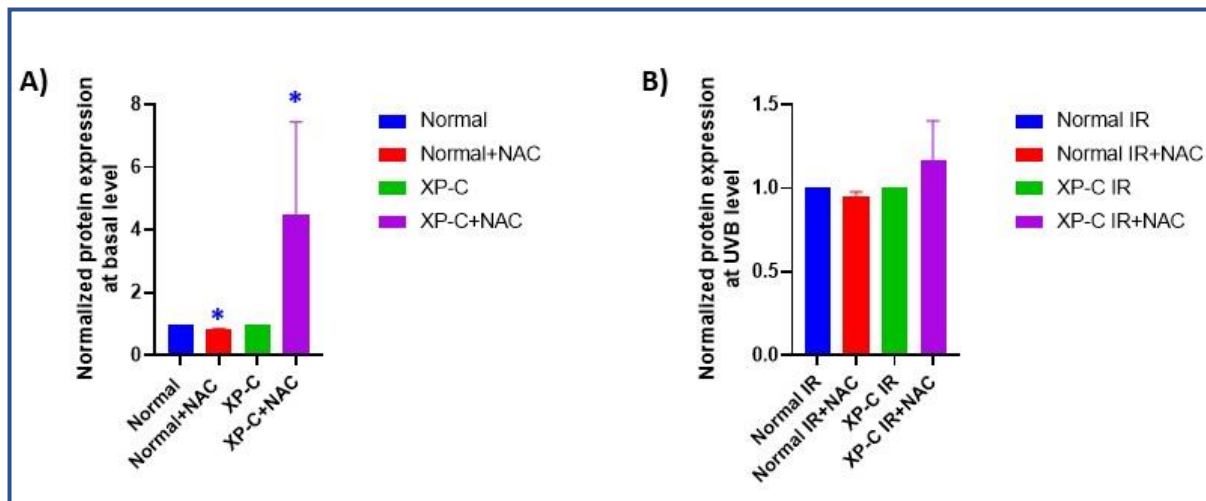


Figure 81. **Effect of NAC on the Nrf2 protein expression at basal and UVB levels in normal and XP-C cells.** In the presence of NAC, panel A) shows a significant downregulation of Nrf2 ( $p < 0.05$ , \*) in normal cells but an upregulation of Nrf2 ( $p < 0.05$ , \*) in XP-C cells, at the basal level. On the other hand, Panel B) shows no effect of NAC on Nrf2 in normal and XP-C cells at UVB level. For each condition, the sample was normalized by its untreated value (100% viability). Paired t-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SD from two independent experiments,  $n = 2$ . IR=irradiated with UVB (0.01 J/cm<sup>2</sup>). (preliminary result)

### 3.3. Effect of NAC on XP-C and normal cells' PARP1 protein expression

#### PARP1

Due to the importance of PARP1's role in DNA repair, cell survival, and signaling, we monitored its gene expression (similarly to above, section 2.3.). No significant change in PARP1 was detected in both cell lines in the presence of NAC at basal and UVB levels [figure 82 (A) and (B) and figure 64 (C) and (D)]. An *in vivo* study showed that NAC supplementation attenuated PARP1 gene expression in mice (G. Wang et al. 2019). This is proposed due to lower oxidative DNA damage and single-strand DNA damage; thereby, PARP1 activation is redundant.

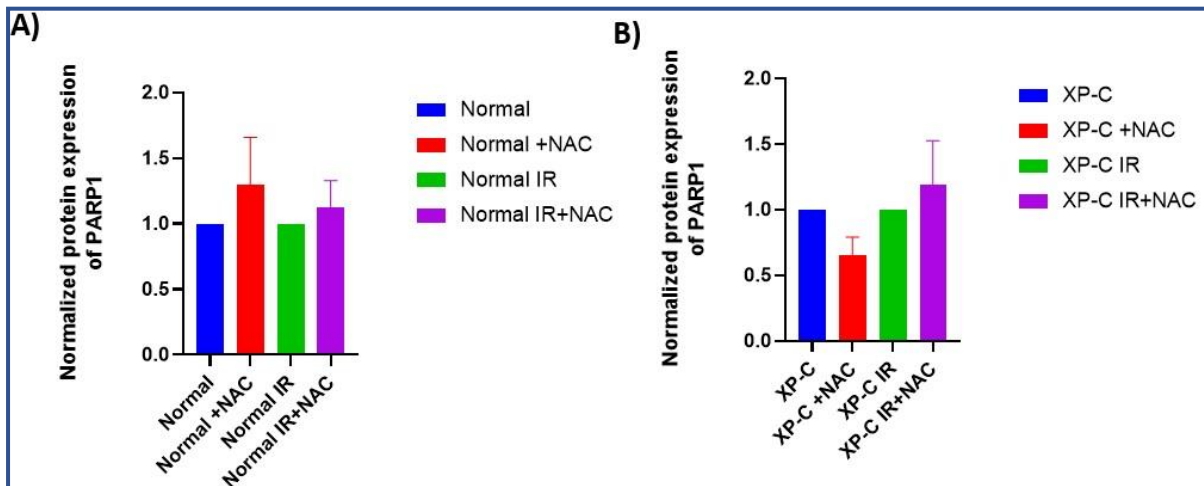


Figure 82. Effect of NAC on the PARP1 protein expression at basal and UVB levels in normal and XP-C cells. Panel A) shows no significant effect of NAC on PARP1 in normal cells at basal and UVB levels. Panel B) shows no significant effect of NAC on PARP1 in XP-C cells at basal and UVB levels. The total protein was used for normalization. Paired t-test was used to compare the expression for each condition. The results are the mean  $\pm$  SD from two independent experiments,  $n=2$ . IR=irradiated with UVB ( $0.01 \text{ J/cm}^2$ ). (preliminary result)

### Cleaved PARP1 “apoptosis hallmark”

NAC showed a similar role to NIC in downregulating cleaved PARP1 in XP-C cells post-UVB ( $p < 0.01$ , \*\*) compared to the untreated control (figure 83). No change was detected at the level of normal cells (figure 83). Therefore, the downregulated cleaved PARP1 level in XP-C cells may indicate lower cell death due to the successful protection of NAC to cells from UVB-induced cytotoxicity and lethality. NAC had already shown protection against apoptosis in brain cells by reducing oxidative stress and enhancing cell signaling as the modulating group I metabotropic glutamate receptor with a neuroprotective role (L. Sun et al. 2012).

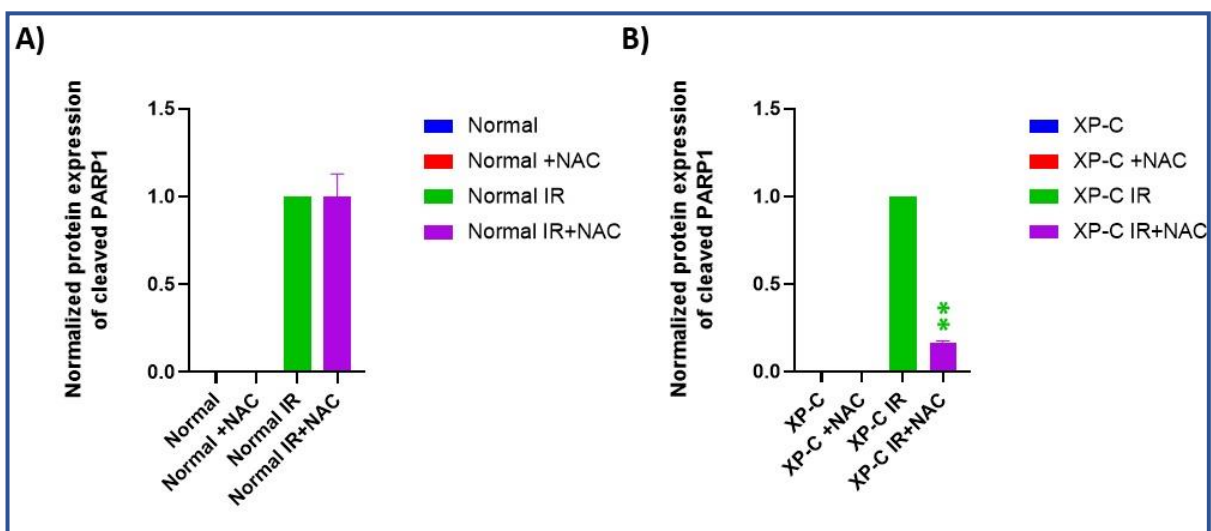


Figure 83. Effect of NAC on cleaved PARP1 protein expression at basal and UVB levels in normal and XP-C cells. Panel A) shows, in normal cells, the absence of cleaved PARP1 in the presence and absence of NAC at the basal level, while no significant effect was detected in the presence and absence of NAC at UVB level. Panel B)

shows, in XP-C cells, the lack of cleaved PARP1 in the presence and absence of NAC at basal level while NAC downregulated the cleaved PARP1 significantly ( $p < 0.01$ , \*\*) at UVB level. Paired *t*-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SD from two independent experiments,  $n=2$ . IR=irradiated with UVB ( $0.01 \text{ J/cm}^2$ ). (preliminary result)

### 3.4. Effect of NAC on XP-C and normal cells' P53 gene expression

P53 is a highly complex protein involved in several cell signaling pathways other than apoptosis, including DNA repair. Therefore, it is interesting to check whether NAC treatment interferes with P53's expression.

Figure 84 showed no significant effect of NAC on P53 in both cells, normal and XP-C, at basal and UVB levels.

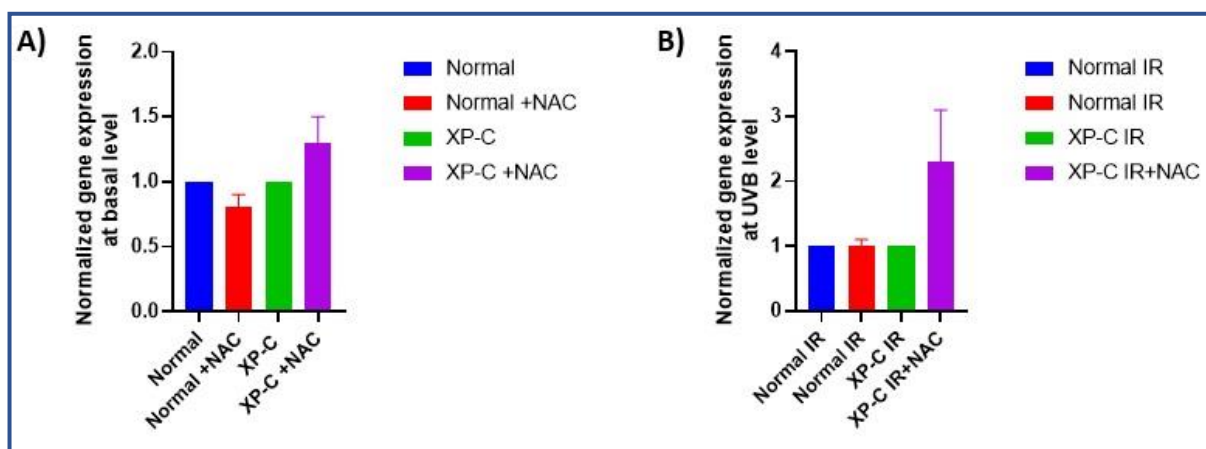


Figure 84. Effect of NAC on the P53 mRNA expression at basal and UVB levels in normal and XP-C cells. Panel A) shows no effect of NAC on P53 in normal and XP-C cells, at the basal level. Panel B) shows no effect of NAC on P53 in normal and XP-C cells at UVB level. For each condition, the sample was normalized by its untreated value (100% viability). Paired *t*-test was used to compare the expression for each condition. The results are the mean  $\pm$  SEM from three independent experiments,  $n=3$ . IR=irradiated with UVB ( $0.01 \text{ J/cm}^2$ ).

As it is speculated that P53 is mainly regulated at translational and post-translational levels, we checked its protein expression as P53 is known to be UVB-induced to regulate genes through the stress-signaling pathway (Amundson et al. 2005).

Similar to NIC pretreatment, the only detected variation was in XP-C cells at the basal level where P53 was downregulated significantly ( $p < 0.01$ , \*\*) (figure 85). This could show that cells at rest lack stress where P53 is not needed to trigger apoptosis or stabilize POLB for DNA repair (J. Zhou et al. 2001). Notably, APE1 was shown to be downregulated in XP-C cells at the basal level [figure 87 (B)]. APE1 usually interacts with and activates P53 *in vitro* and *in vivo* (J. Zhou et al. 2001). Therefore, this could be the case in our experiment.

Another interesting proposed theory is that in XP-C cells, P53 usually is trapped by MDM2 and not degraded, disrupting its turnover and efficacy (Krzyszinski et al. 2014). This was

observed in figure 48 due to a high P53 level in XP-C fibroblasts compared to control at basal level. Therefore, NAC may have enhanced P53's turnover and degradation at a resting state independently of XPC.

Such proposed theories may contradict with the role of P53 in apoptosis to prevent carcinogenesis. However, we should consider that we are discussing the cellular resting state where P53 maybe not be needed, the complexity of such a protein and that our result needs further validations, particularly at the interactome level.

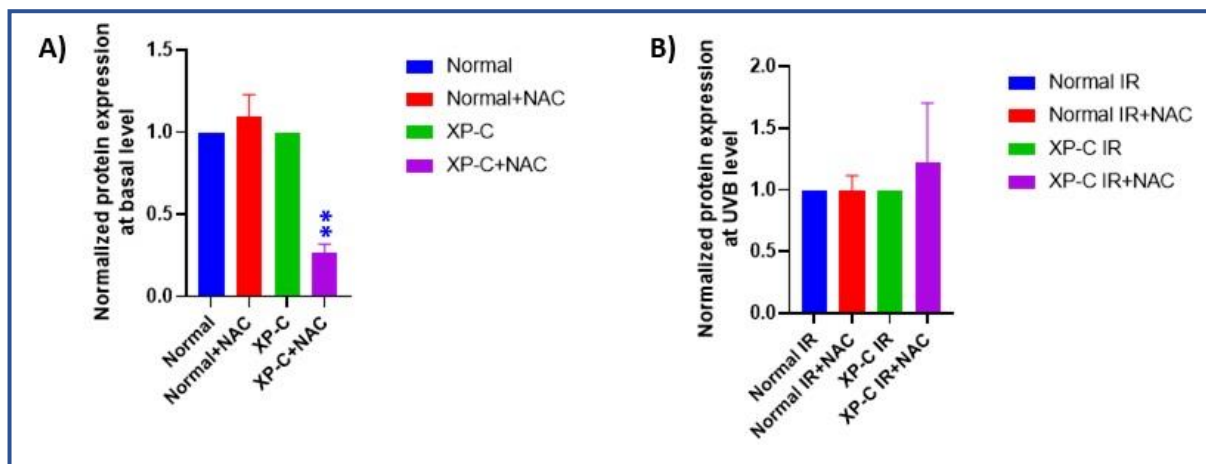


Figure 85. Effect of NAC on the P53 protein expression at basal and UVB levels in normal and XP-C cells. Panel A) shows a downregulation in P53 ( $p < 0.01$ , \*\*) in the presence of NAC in XP-C cells, at the basal level. Panel B) shows no effect of NAC on P53 in normal and XP-C cells at UVB level. For each condition, the sample was normalized by its untreated value (100% viability). Paired t-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SD from three independent experiments,  $n = 3$ . IR=irradiated with UVB ( $0.01 \text{ J/cm}^2$ ).

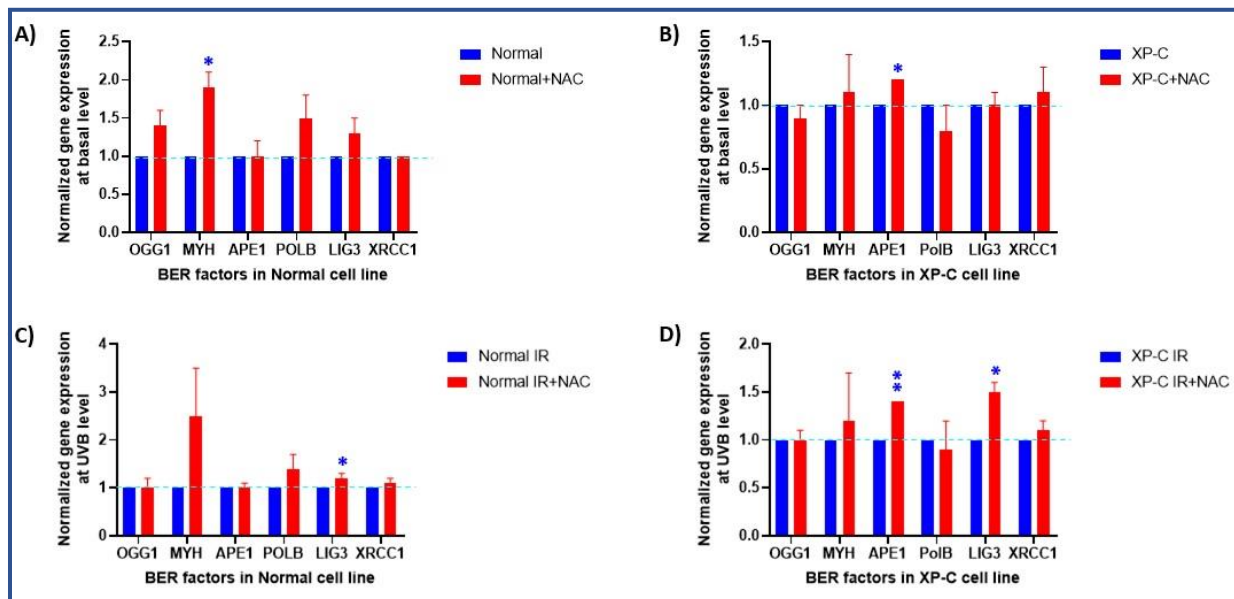
### 3.5.Effect of NAC on XP-C and normal cells' BER gene expression

Several studies showed that NAC downregulates oxidative DNA damage, particularly 8-oxoGua. To establish whether this occurs via stimulating BER or reducing ROS, we started by examining the BER mRNA and protein expression.

#### 3.5.1. Effect of NAC on XP-C and normal cells' BER mRNA level

At the basal level, NAC induced an upregulation in *MYH* and *APE1*'s mRNA expressions ( $p < 0.05$ , \*) in normal and XP-C cells, respectively [figure 86 (A) and (B)]. Post-UVB irradiation, figure 86 (C) and (D) shows that NAC induced an upregulation of *LIG3* ( $p < 0.05$ , \*) in normal cells and an upregulation of *APE1* and *LIG3* ( $p < 0.01$ , \*\*;  $p < 0.05$ , \*, respectively) in XP-C cells. This may indicate a positive effect of NAC on BER's transcriptional expression, particularly in steps following the initiation of the pathway.

However, to know further about the effect of NAC on BER's gene expression, we selected OGG1, MYH, and APE1 and checked their expression at the translational level.



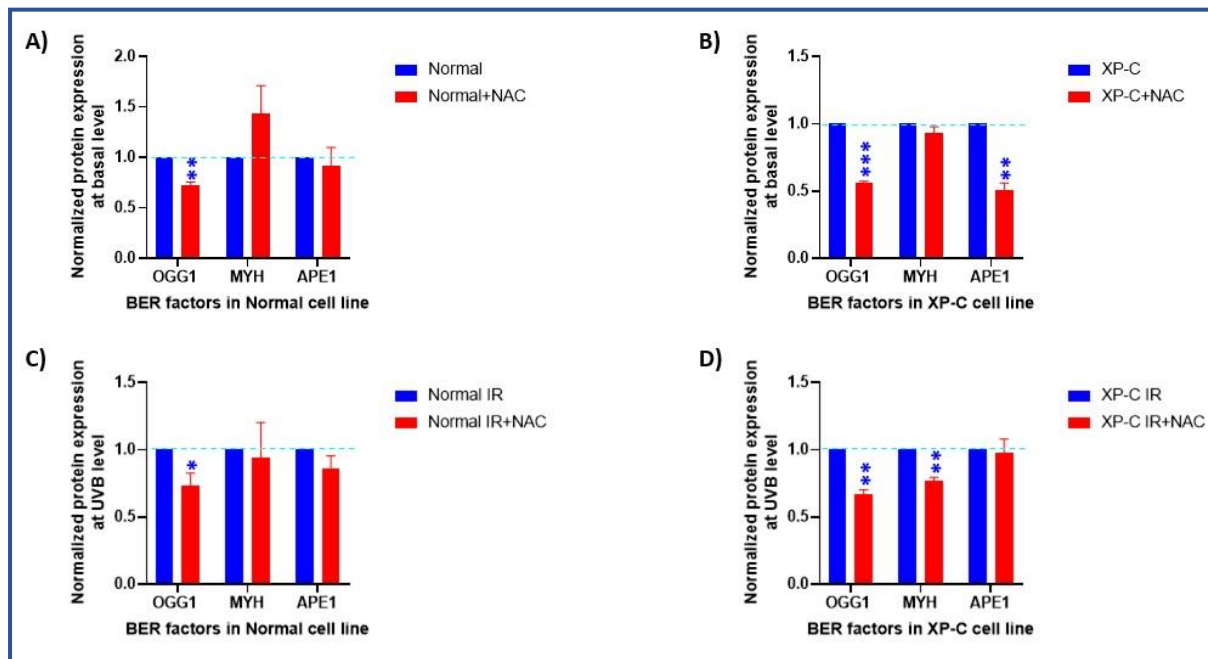
**Figure 86. Effect of NAC on BER mRNA expression at basal and UVB levels in normal and XP-C cells.** Panel A) shows that NAC increased MYH mRNA expressions at the basal level ( $p < 0.05$ , \*) in normal cells. Panel B) shows that NAC increased APE1 mRNA expression ( $p < 0.05$ , \*) at the basal level in XP-C cells. Panel C) shows that NAC increased LIG3 mRNA expressions at UVB level ( $p < 0.05$ , \*) in normal cells. Panel D) shows that NAC increased APE1 ( $p < 0.01$ , \*\*) and LIG3 ( $p < 0.05$ , \*) mRNA expressions at UVB level in XP-C cells. For each condition, the sample was normalized by its untreated value (100% viability). Paired t-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from three independent experiments,  $n = 3$ . IR=irradiated with UVB ( $0.01 \text{ J/cm}^2$ ).

### 3.5.2. Effect of NAC on XP-C and normal cells' BER protein expression

UVB produces ROS and oxidative damages [lipid, protein, and DNA (8-oxoGua)]. Such DNA damage requires the activation of BER factors. Figure 87 shows that some of these BER factors were downregulated at basal and UVB levels. Figure 87 [(A) and (C)] shows that OGG1 is downregulated at basal ( $p < 0.01$ , \*\*) and UVB ( $p < 0.05$ , \*) levels in normal cells, respectively. Also, NAC downregulated OGG1 ( $p < 0.001$ , \*\*\*) and APE1 ( $p < 0.01$ , \*\*) at basal level and OGG1 and MYH ( $p < 0.01$ , \*\*) at UVB level in XP-C cells.

On the other hand, it was reported that NAC enhances OGG1's expression (K. C. Kim et al. 2017). This may be due to the different stress, treatment conditions, and cells between our experiments. They pretreated HaCat keratinocytes with 1mM of NAC for 30 minutes before exposing them to non-thermal dielectric barrier discharge (DBD) plasma (K. C. Kim et al. 2017). Perhaps our result indicates that pretreatment of cells with NAC enhanced BER for a rapid repair before cell collection (4 hours). It may have also reduced ROS and the oxidative

DNA damage efficiently, thereby inhibiting the initiation and stimulation of BER. However, a background expression (IR/NIR) is still active.



**Figure 87. Effect of NAC on BER protein expression at basal and UVB levels in normal and XP-C cells.** Panel A) shows that NAC downregulated OGG1 ( $p < 0.01$ , \*\*) at basal level in normal cells. Panel B) shows that NAC downregulates OGG1 ( $p < 0.001$ , \*\*\*) and APE1 ( $p < 0.01$ , \*\*) at basal level in XP-C cells. Panel C) shows that NAC downregulates OGG1 ( $p < 0.05$ , \*) in normal cells at UVB level. Panel D) shows that NAC downregulates OGG1 and MYH ( $p < 0.01$ , \*\*) in XP-C cells at UVB level. For each condition, the sample was normalized by its untreated value (100% viability). Paired *t*-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SD from three independent experiments,  $n = 3$ . IR=irradiated with UVB (0.01 J/cm<sup>2</sup>).

### 3.6. Effect of NAC on XP-C and normal cells' UVB-induced BER and NER activity

NAC is an exciting drug that has an impressive array of cellular regulatory and protective effects. This is due to its nucleophilicity and multiple roles as ROS scavenging, cell signaling and DNA repair modulation, and inhibition of genotoxicity and carcinogenesis (De Flora et al. 2001). Herein, it is highly expected to enhance repair in normal and XP-C cells.

#### 3.6.1. Monitoring NER activity

Figures 88 and 89 represent the kinetic repair of UVB-induced (6-4) PPs and CPDs, respectively, in the presence and absence of NAC pretreatment.

No effect of NAC on (6-4) PPs in normal and XP-C cells [figure 88 (A) and (B)].

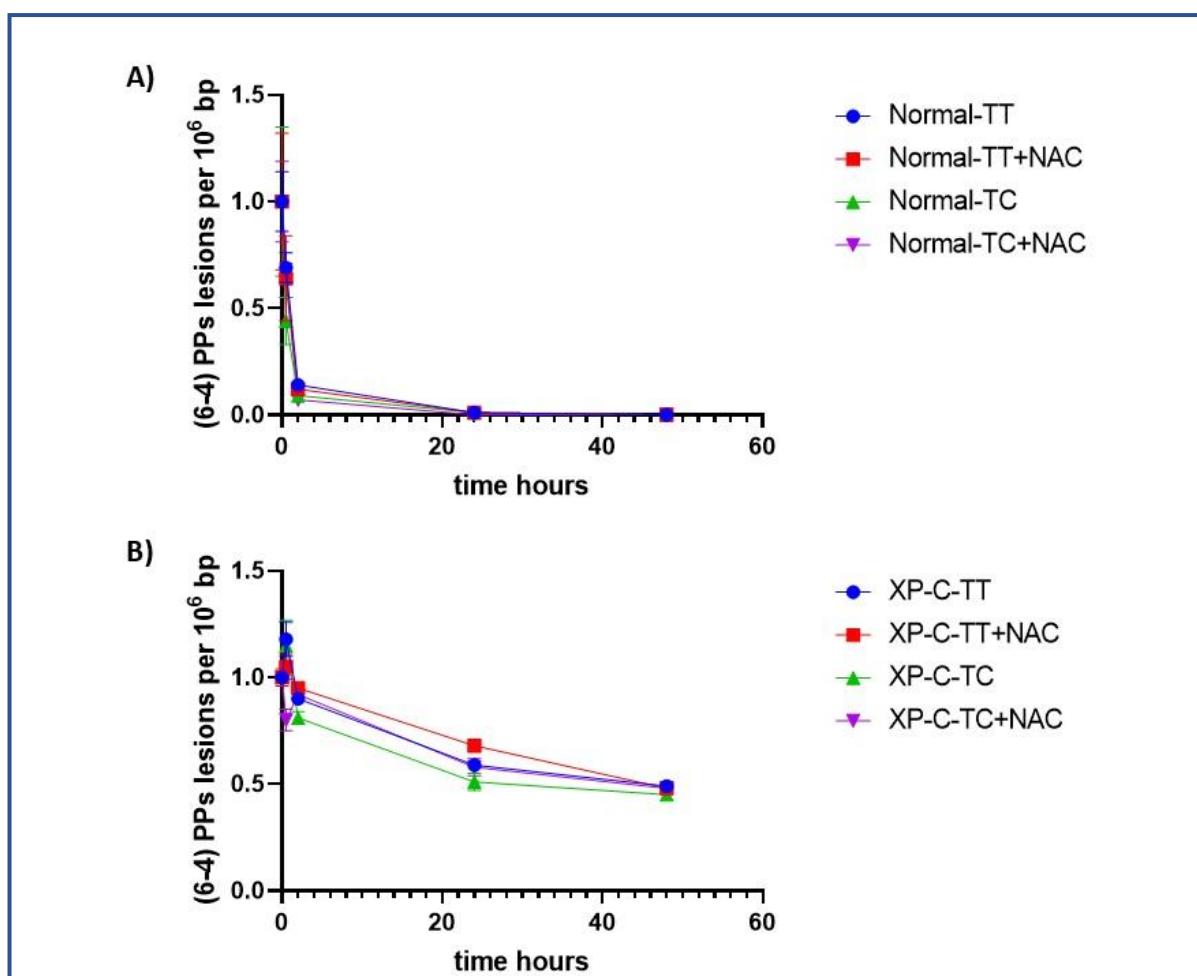


Figure 88. Effect of NAC on UVB-induced (6-4) PPs kinetic repair in normal and XP-C cells. Panels A) shows no difference in (6-4) PPs repair in the presence and absence of NAC in normal cells. Panel B) shows no difference in (6-4) PPs repair in the presence and absence of NAC in XP-C cells. For each condition, the sample was normalized by its irradiated value at 0 hours (100% viability). Paired t-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SD from two independent experiments,  $n=2$ .

However, a potential of enhanced CPDs repair was observed in both cells (figure 89). For instance, TU and UT CPD lesions were significantly lower ( $p < 0.05$ , \*) in normal cells in the presence of NAC at 24 and 2 hours post-UVB, respectively. Also, TT, UT, and UU CPDs were significantly lower ( $p < 0.05$ , \*) at 0.5 and 2 hours, respectively, in XP-C cells. Unfortunately, such an improvement was moderate due to the high SEMs and/or that pretreatment with such a dose could not be efficient to induce such a detected shift in repair.

It should be stated that the observed U residues result from the deamination of C in CPD lesions. Therefore, they are highly mutagenic and mimic T during replication (Douki, Koschembahr, and Cadet 2017).



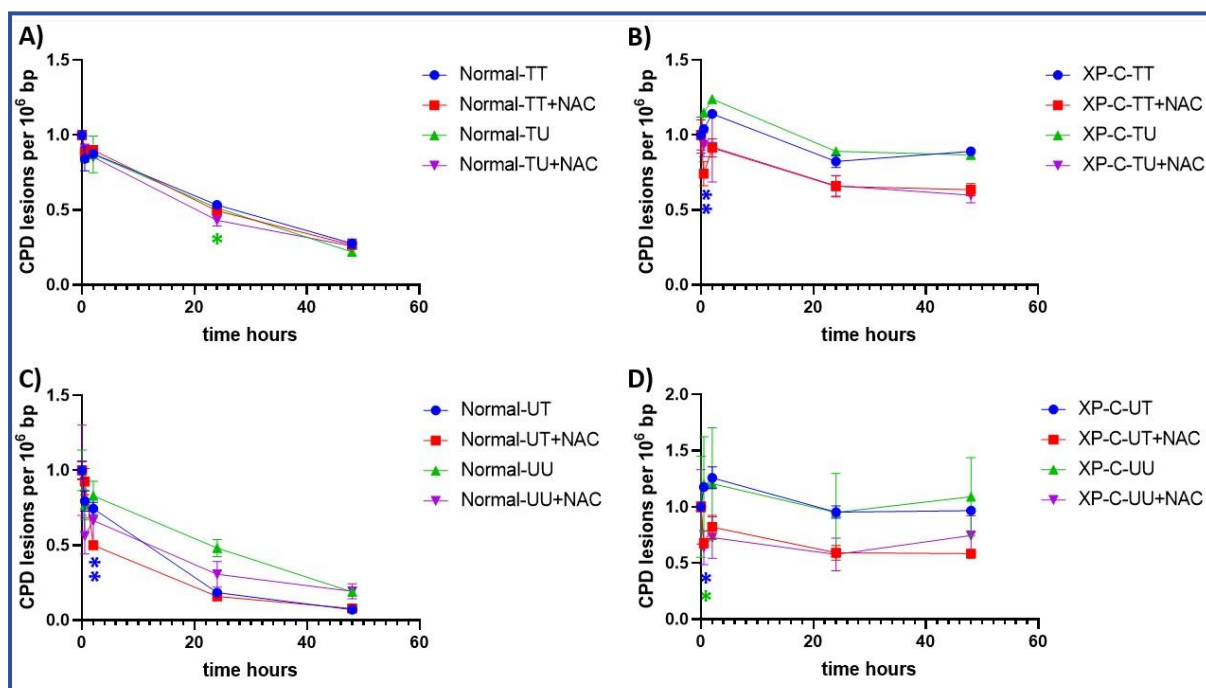


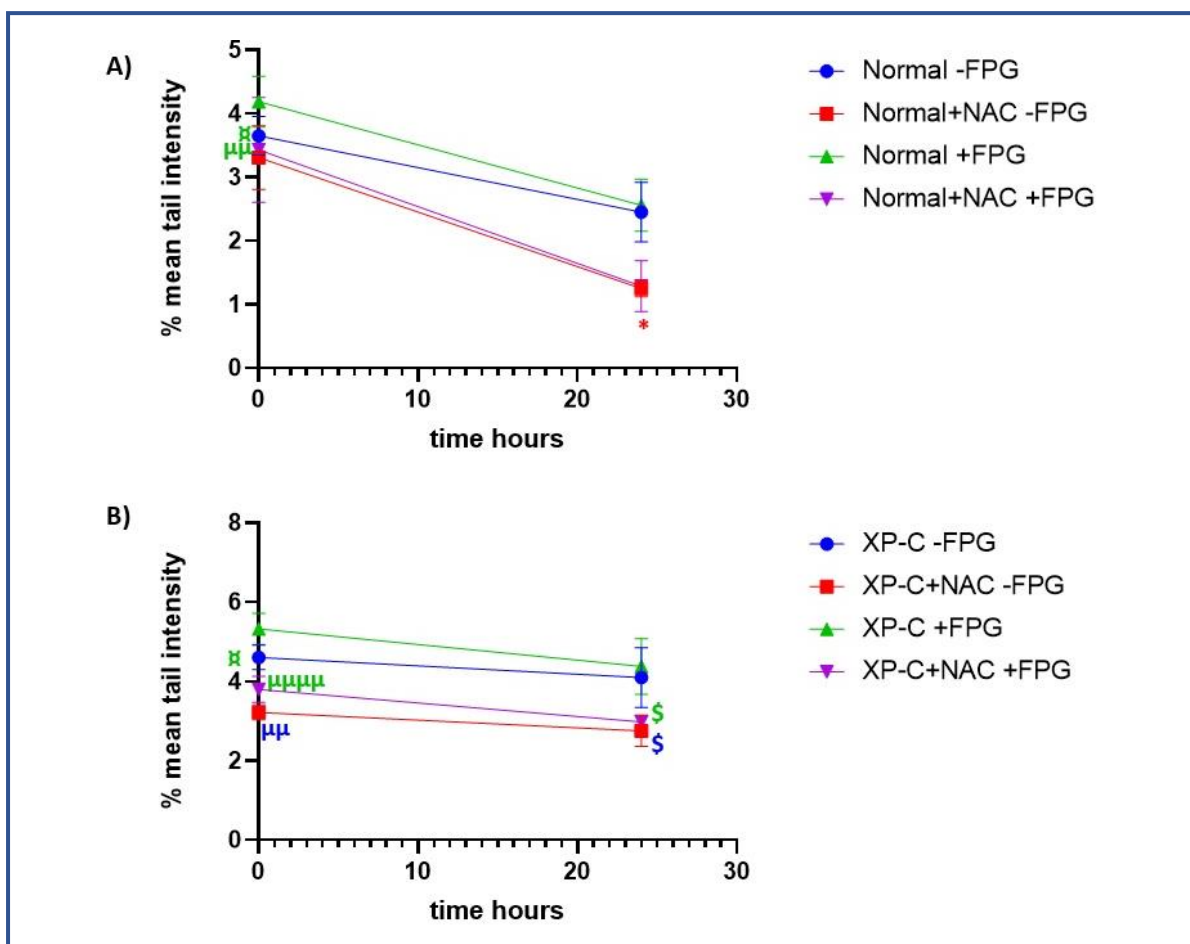
Figure 89. *Effect of NAC on UVB-induced CPDs kinetic repair in normal and XP-C cells.* Panels A) shows that NAC improved TU CPDs ( $p < 0.05$ , \*) repair at 24 hours in normal cells. Panel B) shows that NAC improved repair of TT CPDs ( $p < 0.01$ , \*\*) at 0.5 hours in XP-C cells. Panel C) shows that NAC improved repair of UT CPDs ( $p < 0.01$ , \*\*) at 2 hours in normal cells. Panel D) shows that NAC downregulated UT and UU CPDs at 0.5 hours ( $p < 0.05$ , \*), respectively, in XP-C cells. For each condition, the sample was normalized by its irradiated value at 0 hours (100% viability). Paired *t*-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from two independent experiments,  $n = 2$ .

Maybe the enhanced repair detected in CPDs but not (6-4) PPs in the presence of NAC could not be linked to the distorted DNA duplex rather due to the quantity of lesions induced subsequently to UVB. Another reason could be the need to repeat the experiment as a triplicate due to the high SEM and variations between the samples in experiments. NAC could have enhanced (6-4) PPs repair in normal cells. Still, it was not detected due to its efficiency in rapidly repairing such a bulky lesion with/without treatment (24 hours).

### 3.6.2. Monitoring BER activity

NAC pretreatment seemed to protect the cells from induced DNA damage, mainly oxidized purines ( $p < 0.05$ ,  $\mu$ ), and enhance the repair. XP-C cells had significantly lower single-strand breaks and oxidized purines 24 hours post-UVB in the presence of NAC compared to its absence [figure 90 (A) and (B)].

Such a result is due to the role of NAC as a ROS scavenger. As previously shown, NAC enhances GSH level, thereby balancing the ratio of oxidants/antioxidants to the latter's favor. This prevents UVB-induced ROS from targeting the DNA-forming lesions. Thereby, less BER is required to remove UVB-induced DNA damage.



**Figure 90. Effect of NAC on alkaline and oxidized purines repair in normal and XP-C cells, post-UVB irradiation.** Panel A) shows that NAC decreased the UVB-induced oxidized purines at 0 hours (+FPG,  $p < 0.01$ ,  $\mu\mu$ ) in normal cells. Panel B) shows that NAC decreased the UVB-induced oxidized purines at 0 and 24 hours (+FPG,  $p < 0.0001$ ,  $\mu\mu\mu\mu$ ;  $p < 0.05$ , \$, respectively). For each condition, the sample was normalized by its unirradiated value (100% viability). Paired t-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from three independent experiments,  $n=3$ . \*  $p < 0.05$  between -FPG and +FPG for each treatment at 0 and 24 hours. \$  $p < 0.05$  between the NAC and untreated samples at 24 hours +FPG.  $\mu$   $p < 0.05$  between the NAC and untreated samples at 0 hours +FPG.  $\mu\mu$   $p < 0.05$  between -FPG and +FPG for NAC and untreated samples at 0 hours.

# Conclusion and Perspective

## N-acetylcysteine (1mM):

- ❖ Effect on some BER gene expressions:
  - ✓ Upregulated mRNA level:  
Basal: *MYH* and *APE1* in normal and XP-C cells
  - ✓ Upregulated Protein level:  
UVB: APE1 and LIG3 in XP-C cells
  - ✓ Downregulated Protein level:  
Basal: OGG1 in normal and XP-C cells, APE1 in XP-C cells  
UVB: OGG1 and MYH in XP-C cells
  
- ❖ Enhanced moderately CPDs NER repair in XP-C cells
- ❖ Enhanced oxidized purines repair by BER and reduced their induction in XP-C and normal cells
- ❖ Upregulated Glutathione level
- ❖ Downregulated ROS level

In summary, NAC is non-toxic therapeutic agent. It could act as a preventative treatment to protect XP-C cells against UV-induced DNA lesions, oxidized purines, and oxidative stress. This could prevent the initiation of dramatic cascade of events leading to the severe clinical symptoms in XP-C patients.

To strengthen further our conclusion about NAC, we repeated the same experiments with BSO/DMF (chapter four) which should trigger opposite effects.

It would be interesting to try later the same experiments on keratinocytes and 3D-skin models. Perhaps, checking the detailed signaling mechanism will be ideal in understanding furthermore the efficacy of not only the therapeutic effect per se rather also the protective effect of NAC.

## 4. Chapter Four: The Effect of Buthionine sulfoximine/Dimethylfumurate (BSO/DMF) on UVB-Induced Oxidative Stress and DNA Repair in XP-C and Normal Cells

In this chapter, we used BSO (100 $\mu$ M) and DMF (100 $\mu$ M) to support the results of NAC treatment.

### 4.1.Characterization of BSO/DMF-treated normal and XP-C cells

#### 4.1.1. BSO/DMF dose selection

Dose selection was based on a dose-response curve to check the % viability and choose the most suitable and maximum concentration of BSO/DMF (1:1) before inducing cellular cytotoxicity. As shown in figure 91, 100  $\mu$ M was the best dose for pretreatment 4 hours before UVB-irradiation. This dose had also been used by different researchers (Boivin et al. 2011).

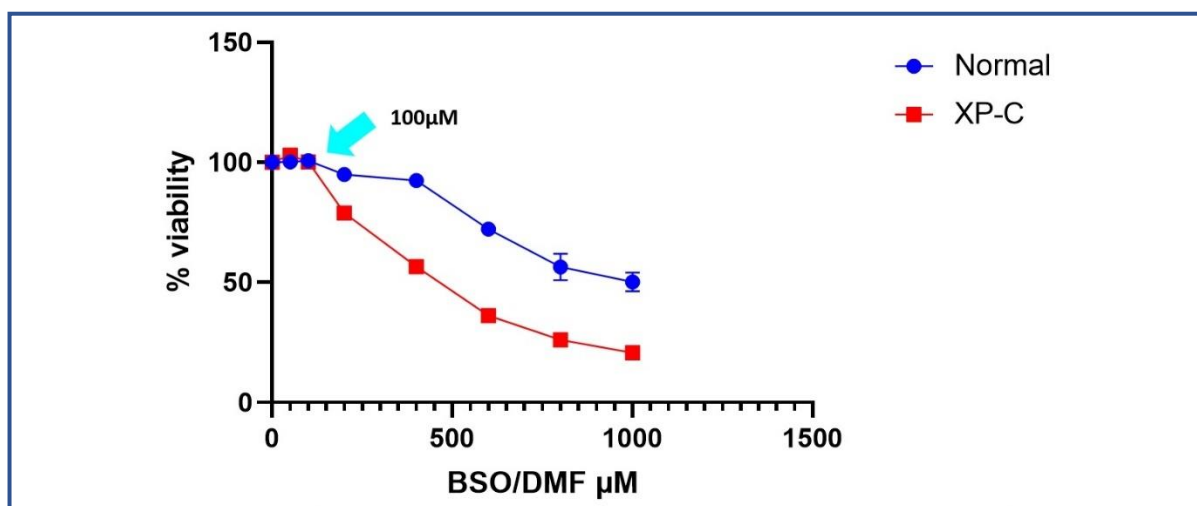


Figure 91. **BSO/DMF dose-response curve.** Normal cells were tested with different doses of BSO/DMF for 4 hours followed by MTT test to monitor the cellular viability. 100 $\mu$ M is the most suitable dose with the least cytotoxicity. The results are the mean  $\pm$ SD.

BSO acts as a GSH biosynthesis inhibitor, while DMF acts as GSH depleting agent. Using a combination of both drugs guaranteed the total depletion of GSH to study such an effect on normal and XP-C cells. The results could be dramatic due to the prominent ROS level, thereby confirming the antioxidant role of NAC in enhancing repair and neutralizing oxidative stress as shown in previously illustrated experiments.

#### 4.1.2. Effect of BSO/DMF on XP-C and normal cells' UVB-induced photosensitivity

Similar to previously tested drugs, we wanted to check whether BSO/DMF could affect the photosensitivity of cells to UVB. Figure 92 shows that BSO/DMF significantly decreased the cellular viability of normal cells at 0.01 and 0.1 J/cm<sup>2</sup> ( $p < 0.01$ , \*\*;  $p < 0.05$ , \*, respectively) and of XP-C cells at 0.02, 0.1, and 0.2 J/cm<sup>2</sup> ( $p < 0.05$ , \*;  $p < 0.01$ , \*\*, respectively). Similarly, Boivin et al. showed that the combination of BSO/DMF decreases cell survival more than each drug solely due to a depletion of GSH for more than 4 hours (Boivin et al. 2011).

Briefly, GSH has been proposed as essential for cell viability and a photoprotective mediator in the skin. Thereby, its depletion increases the UV-induced photosensitivity of normal and XP-C cells.

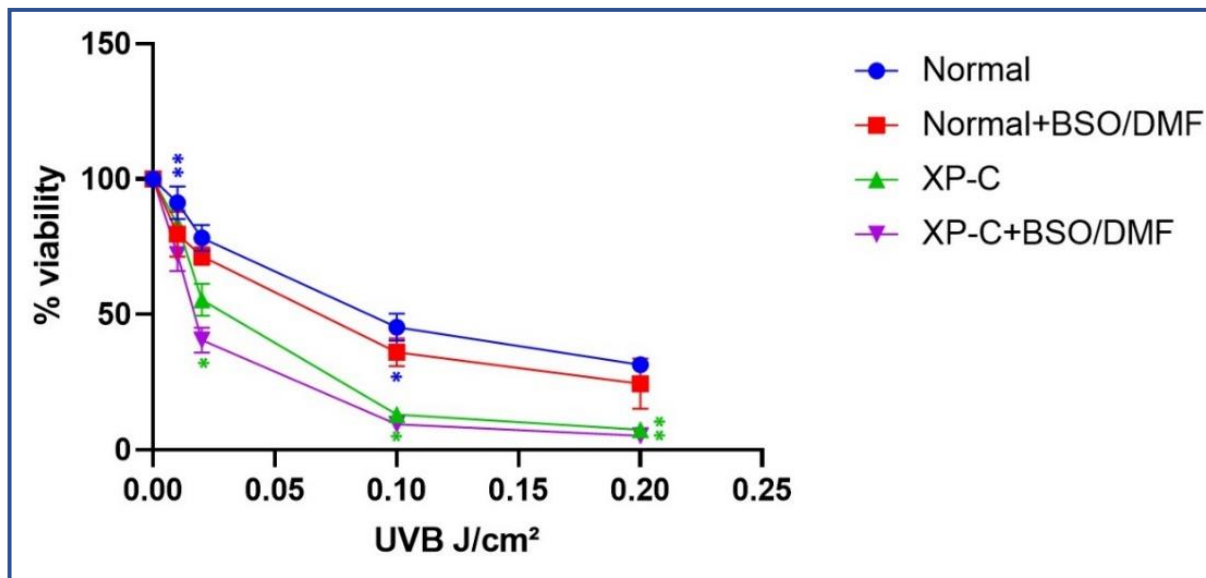


Figure 92. **Higher photosensitivity in each cell line (normal and XP-C) in presence of BSO/DMF compared to its untreated control.** We treated cells with BSO (100 $\mu$ M) and DMF (100 $\mu$ M) for 4 hours then we measured the percentage of cellular viability 24 hours post-UVB irradiation by short-term cytotoxicity test (MTT). For each condition, the sample was normalized by its unirradiated value (100% viability). Paired *t*-test was used to compare photosensitivity for each condition at each UVB dose ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from three independent experiments,  $n=3$ .

## 4.2. Effect of BSO/DMF on XP-C and normal cells' redox state

### 4.2.1. Effect of BSO/DMF on XP-C and normal cells' ROS and glutathione levels

- Effect on UVB-induced ROS

On the contrary to NAC, BSO/DMF increased the level of ROS dramatically in normal and XP-C cells. Figure 93 (A) shows that BSO/DMF upregulated ROS level at 0.5, 1, 3, 24, and 48 hours post-UVB irradiation in normal cells. In parallel, figure 93 (B) shows that BSO/DMF upregulated ROS levels at 2,3 and 48 hours post-UVB irradiation in XP-C cells. This indicates that GSH is vital for protection against ROS and that the dramatic effects of *XPC* mutations could be partly linked to a downregulated GSH level. Such a result was well demonstrated in several studies. For instance, Bohl et al. used BSO to induce severe ROS in breast cancer cells to induce apoptosis (Bohl et al. 2012).

Thereby, supplementing cells with GSH could enhance and reverse some of the XP-C's phenotype, including those linked to oxidative DNA damage repair and antioxidant defense pathways.

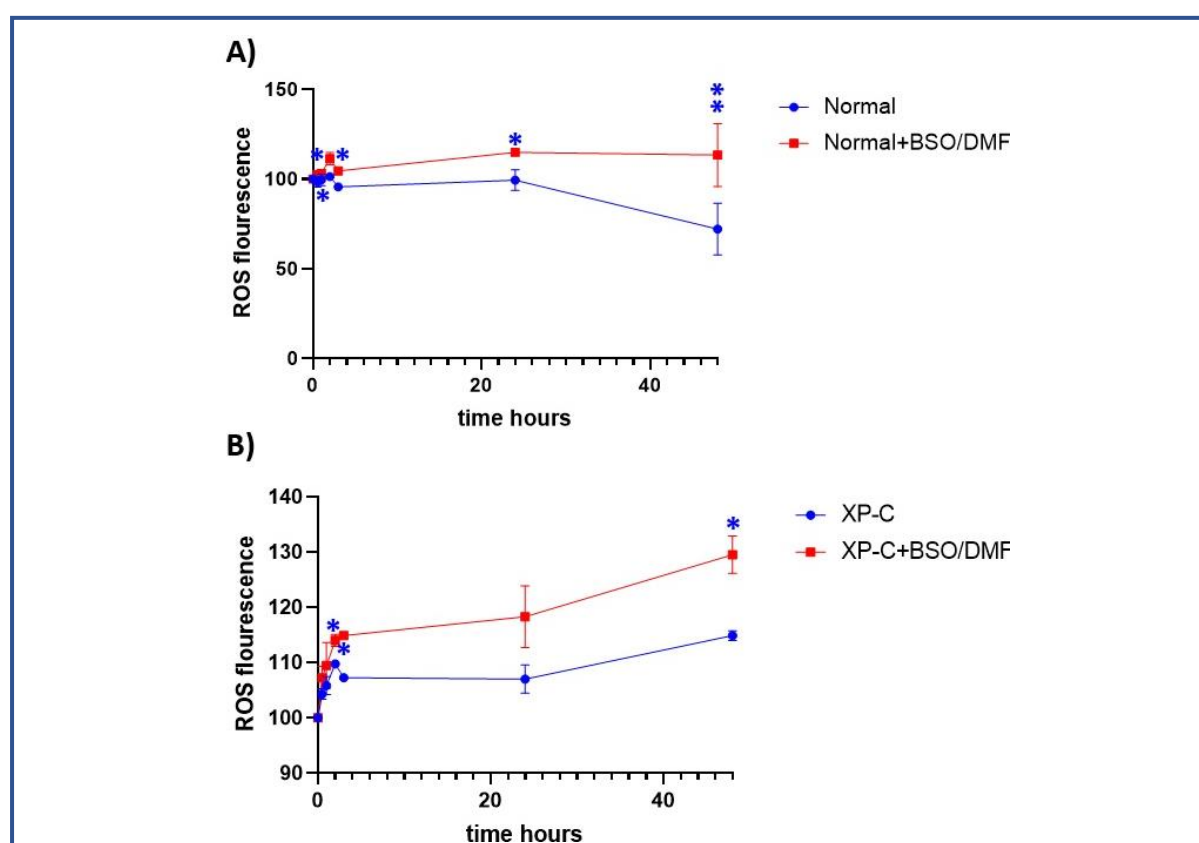


Figure 93. **Effect of BSO/DMF on ROS level in normal and XP-C cells, post-UVB irradiation.** Panel A) BSO/DMF upregulated ROS level at 0.5, 1, 3, 24 and 48 hours post-UVB ( $p < 0.05$ , \*,  $p < 0.01$ , \*\*, respectively) in normal cells. Panel B) BSO/DMF upregulated ROS level at 2, 3, and 48 hours post-UVB irradiation ( $p < 0.05$ ) in XP-C cells. We treated cells with 1/1000 DHR123 followed by UVB ( $0.02 \text{ J/cm}^2$ ) and monitored fluorescence kinetics at different kinetic time points. Then we normalized each sample by its untreated value (100% viability).  $\text{H}_2\text{O}_2$  was used as a positive control. Paired *t*-test was used to compare the ROS fluorescence between NAC treated

and untreated samples in normal and XP-C1 fibroblast, respectively, at each kinetic time point (hours) ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from three independent experiments,  $n=3$ .

- Effect on glutathione level 24 hours post-UVB

Studying the level of cellular glutathione in the presence of BSO/DMF confirmed the efficiency of the treatment. As shown in figure 94, BSO/DMF significantly abolished the GSH level in normal ( $p < 0.01$ , \*\*) and XP-C ( $p < 0.001$ , \*\*\*) cells. This complements our previously illustrated results in the presence of BSO/DMF treatment.

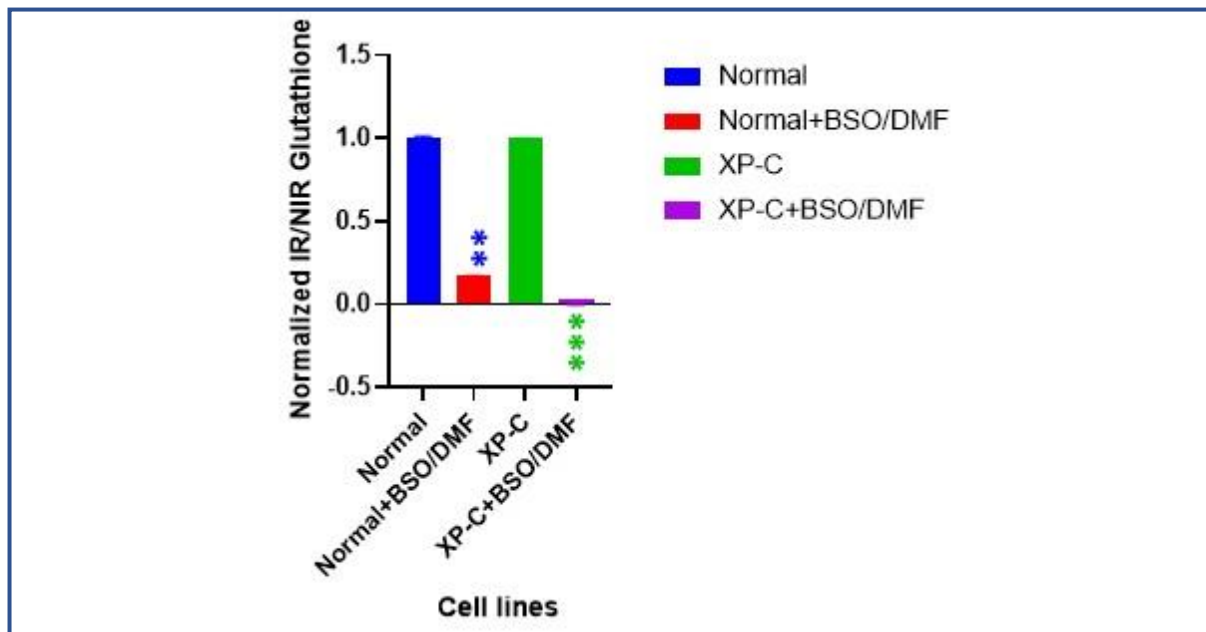


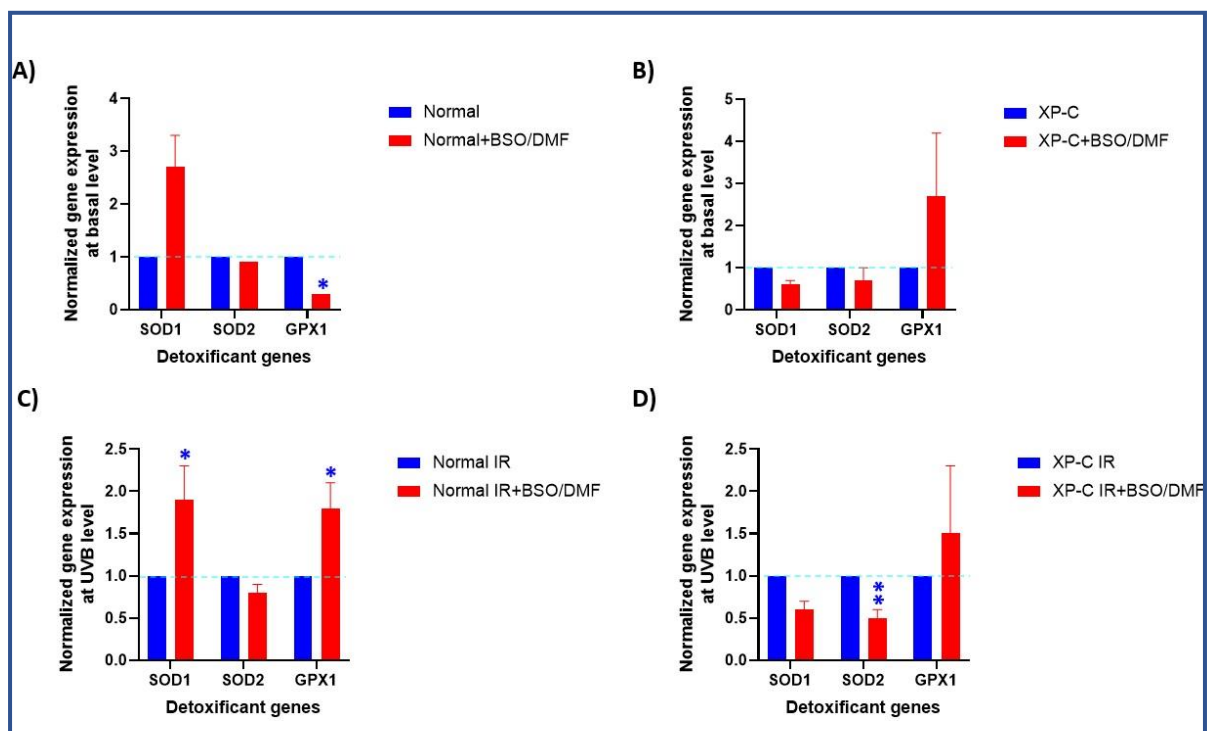
Figure 94. Effect of BSO/DMF on UVB-induced IR/NIR glutathione (GSH) level in normal and XP-C cells. After UVB-irradiation for 24 hours, we extracted cells and measured glutathione levels calorimetrically using a CAYMAN kit. This treatment showed significant downregulation in glutathione level in normal ( $p < 0.01$ , \*\*) and XP-C ( $p < 0.001$ , \*\*) cells. XP-C and normal samples were normalized by normal IR/NIR value. Paired t-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SD.

#### 4.2.2. Effect of BSO/DMF on XP-C and normal cells' oxidative stress-linked genes' expression

Checking the mRNA expression of some genes involved in the oxidative stress defense pathway showed that *GPx1* was downregulated significantly ( $p < 0.05$ , \*) in normal cells at basal level in the presence of BSO/DMF pretreatment [figure 95 (A)]. However, post-UVB, BSO/DMF induced higher expression of *GPx1* and *SOD1* ( $p < 0.05$ , \*) compared to untreated control [figure 95 (C)]. This may show that BSO/DMF triggers oxidative stress that will stimulate the antioxidants defense mechanism. A depletion in GSH may not inhibit antioxidants expression rather activity. This could lead to depletion of cellular resources as ATP and  $NAD^+$ , which are necessary for normal cellular activity and protection. XP-C cells did not show any

effect significant effect except for a downregulation in *SOD2* ( $p < 0.01$ , \*\*) after BSO/DMF treatment at UVB level [figure 95 (D)].

Similarly, Krifka et al. showed that the gene expression of GPx1 increased in the presence of BSO in macrophages due to high  $H_2O_2$  induced levels due to GSH depletion (Krifka et al. 2012). Treating cells with an additional oxidative stress source (2-hydroxyethyl methacrylate) leads to ROS levels that exceed the capacity of the antioxidant defense system. To compensate, catalase was overexpressed (Krifka et al. 2012). Unfortunately, our XP-C cells are known to lack catalase activity (Vuillaume et al. 1986). This could cause extreme ROS levels that may have caused negative feedback on SOD enzymes' expression in XP-C but not normal cells.



**Figure 95. Effect of BSO/DMF on the detoxificants' mRNA expression at basal and UVB levels in normal and XP-C cells.** Panel A) shows downregulation in *GPX1* expression ( $p < 0.05$ , \*) upon BSO/DMF treatment in normal cells at the basal level. Panel B) shows no significant effect upon BSO/DMF treatment in XP-C cells at the basal level. Panel C) shows an upregulation of *SOD1* and *GPX1* ( $p < 0.05$ , \*) upon BSO/DMF treatment in normal cells post-UVB irradiation. Panel D) shows a downregulation in *SOD2* ( $p < 0.01$ , \*\*) in XP-C cells post-UVB irradiation. For each condition, the sample was normalized by its untreated value (100% viability). Paired *t*-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from three independent experiments,  $n=3$ . IR=irradiated with UVB (0.01 J/cm<sup>2</sup>).

BSO/DMF seems to significantly impact different pathways linked to cell survival, signaling, and DNA repair. One of these pathways is the antioxidant defense pathway. For that, we studied the expression of an upstream factor, *Nrf2*, at the mRNA and protein levels. As a result, *Nrf2* increased in the presence of BSO/DMF at UVB level in normal cells ( $p < 0.05$ , \*) (figure 96).



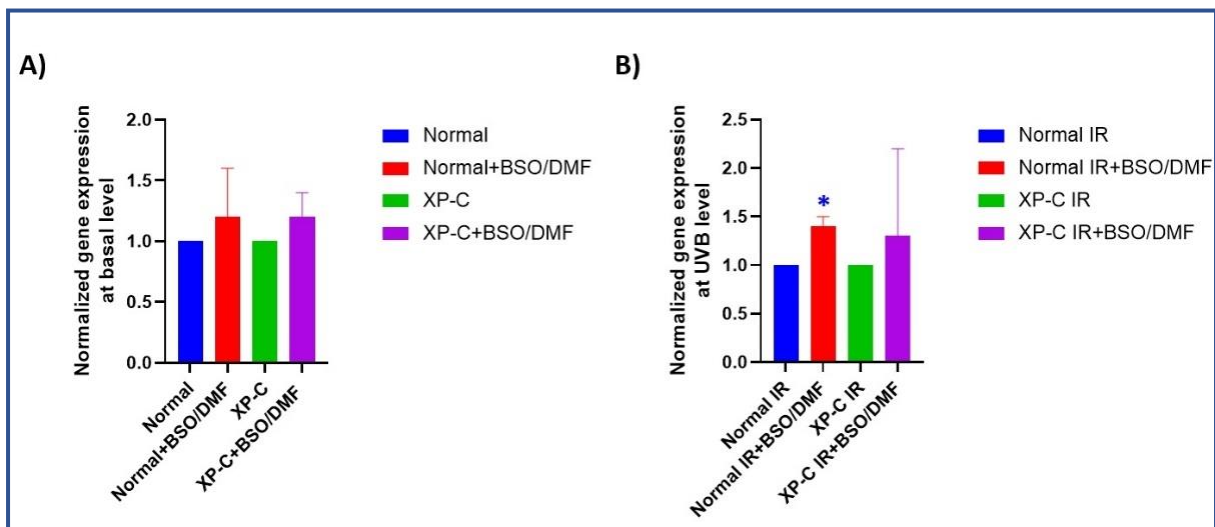


Figure 96. *Effect of BSO/DMF on the Nrf2 mRNA expression at basal and UVB levels in normal and XP-C cells.* NAC upregulated Nrf2 significantly ( $p < 0.05$ , \*) in normal cells, at UVB level. The sample was normalized by its untreated value (100% viability). Paired *t*-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from three independent experiments,  $n=3$ . IR=irradiated with UVB (0.01 J/cm<sup>2</sup>).

Our preliminary results in figure 97 suggest that BSO/DMF triggers Nrf2 at basal and UVB levels in normal cells. However, Nrf2 is almost inhibited in XP-C cells at the UVB level (figure 97). Similar to our studied normal cells, BSO had been shown to activate the Nrf2 pathway in murine embryonic fibroblasts to upregulate the expression of antioxidant genes as a protection mechanism against oxidative stress (H.-R. Lee et al. 2008). Maybe this was not the case in XP-C cells because they are susceptible to stress and were not BSO-resistant. Therefore, it is interesting to check whether Nrf2 is deficient in the studied XP-C cells. Maybe BSO/DMF and the absence of XPC leading to oxidative stress and high NOX1 contribute to such a dramatic inhibition in Nrf2. This could be via the P53-dependent pathway (SIRT1...).

It should be noted that high levels of NOX were detected in Nrf2 deficient cells and XP-C cells are known to have high NOX1 level (Kovac et al. 2015).

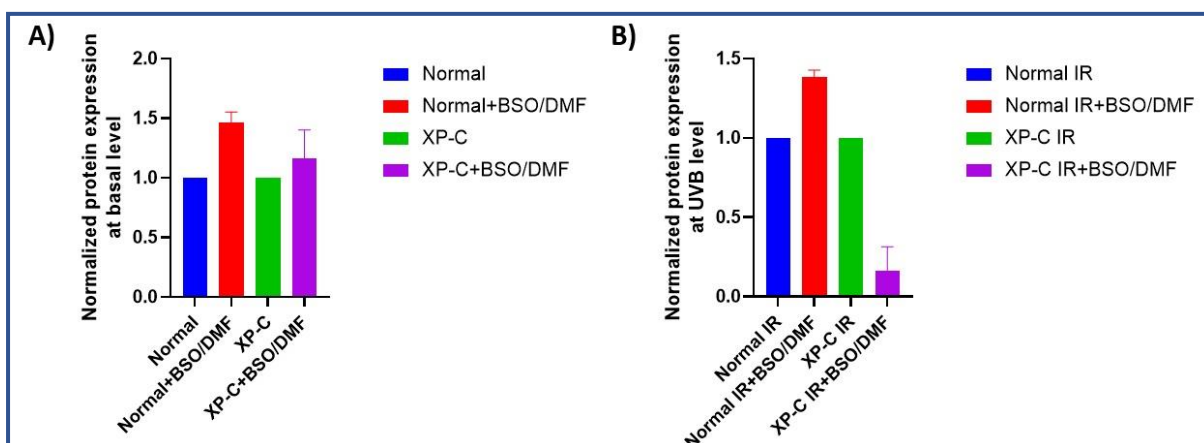


Figure 97. **Effect of BSO/DMF on the Nrf2 protein expression at basal and UVB levels in normal and XP-C cells.** Panel A) shows a difference in PARP1 in the presence and absence of BSO/DMF in normal and XP-C cells, at the basal level. Panel B) shows a difference in PARP1 in the presence and absence of BSO/DMF in normal and XP-C cells, at UVB level. For each condition, the sample was normalized by its untreated value (100% viability). Paired t-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$ SD from two independent experiments,  $n=3$ . IR=irradiated with UVB ( $0.01 \text{ J/cm}^2$ ) (preliminary result).

#### 4.3.Effect of BSO/DMF on XP-C and normal cells' PARP1 protein expression

##### PARP1

PARP1 induces cell survival via the HIF1 $\alpha$ -dependent pathway in the presence of oxidative stress (Pietrzak et al. 2018). It is also involved in DNA repair and other signaling pathways, as previously mentioned.

Our preliminary results in figure 98 suggest that BSO/DMF enhances PARP1's expression at UVB level in normal cells and at basal and UVB levels in XP-C cells. Answering whether this is linked to cell viability or other signaling pathways is tricky and needs further experiments. Nevertheless, a recent publication showed that BSO treatment enhanced PARP1's expression in the presence of oxidative stress (Yıldızhan and Nazıroğlu 2020). Excessive activated PARP1 could deplete cells from ATP in an attempt to repair damaged DNA. This could trigger cell death.

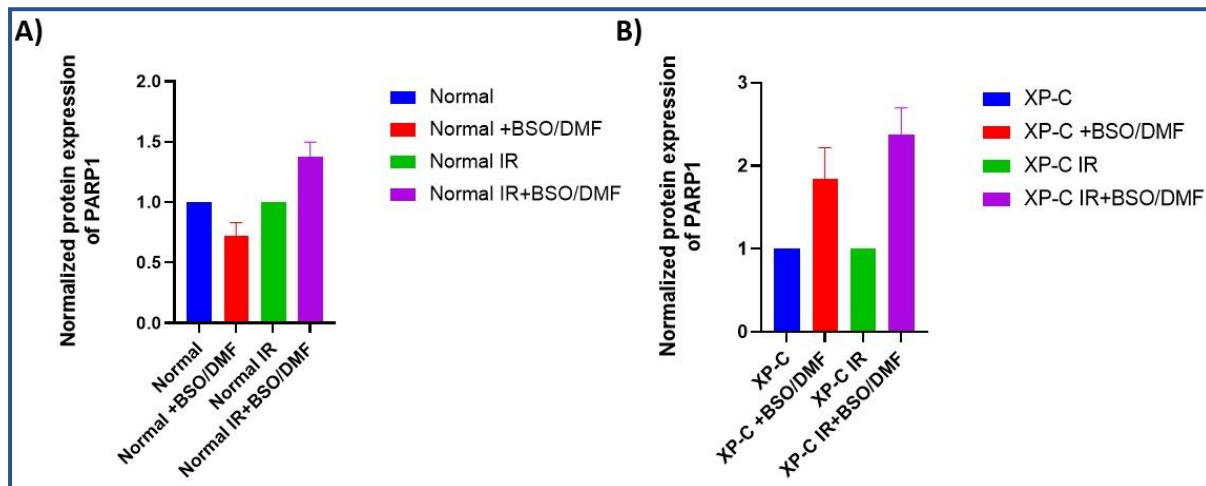
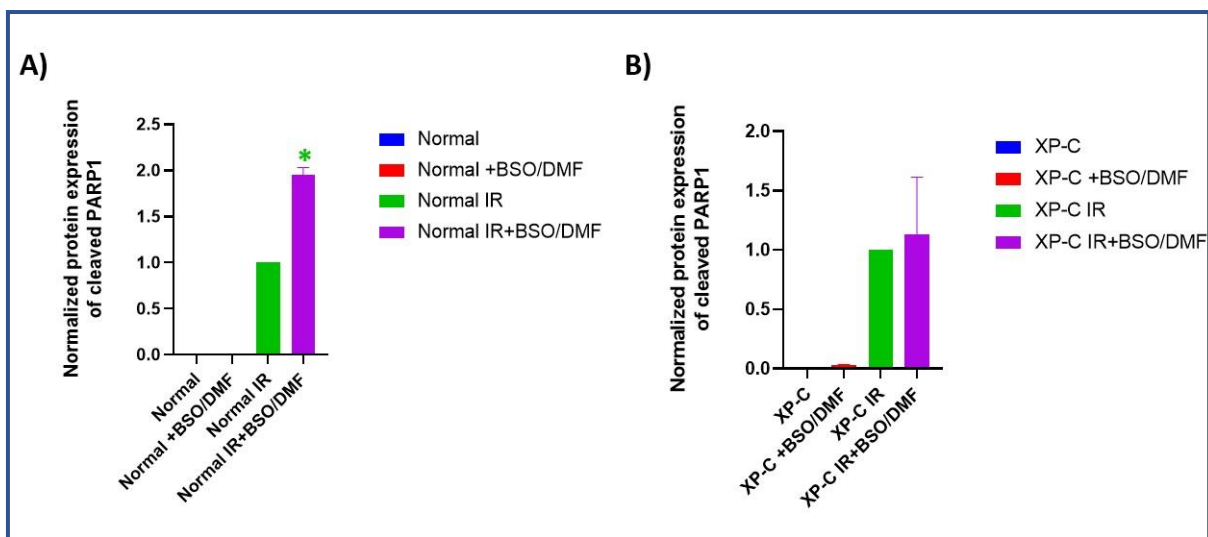


Figure 98. **Effect of BSO/DMF on the PARP1 protein expression at basal and UVB levels in normal and XP-C cells.** Panels A) shows no significant effect of BSO/DMF on PARP1 in normal cells at basal and UVB levels. Panel B) shows no significant effect of BSO/DMF on PARP1 in XP-C cells at basal and UVB levels. The total protein was used for normalization. Paired t-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$ SD from two independent experiments,  $n=2$ . IR=irradiated with UVB ( $0.01 \text{ J/cm}^2$ ). (preliminary result)

## Cleaved PARP1 “apoptosis hallmark”

Normal cells showed an upregulated cleaved PARP1 shown in figure 99 (A) and (B) post-UVB in the presence of BSO/DMF ( $p < 0.05$ , \*). This could indicate the presence of cell apoptosis. Similarly, the cleaved PARP1 in XP-C cells at basal level post-BSO/DMF implies how dramatic the cellular changes were against the highly induced oxidative stress. Consistently, BSO pretreatment had been shown to trigger late apoptosis and necrosis in macrophages (Krifka et al. 2012).



*Figure 99. Effect of BSO/DMF on cleaved PARP1 protein expression at basal and UVB levels in normal and XP-C cells. Panel A) shows, in normal cells, the absence of cleaved PARP1 in the presence and absence of BSO/DMF, at the basal level while BSO/DMF pretreatment significantly upregulated the cleaved PARP1 ( $p < 0.05$ , \*) at UVB level. Panel B) shows, in XP-C cells, absence of cleaved PARP1 in presence and absence of BSO/DMF at basal level while no effect of BSO/DMF was shown on the cleaved PARP1 at UVB level. Paired t-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SD from two independent experiments,  $n=2$ . IR=irradiated with UVB ( $0.01 \text{ J/cm}^2$ ). (preliminary result)*

### 4.4. Effect of BSO/DMF on XP-C and normal cells' P53 gene expression

Another factor linked to cell death is P53. GSH depletion has been shown to play a critical role in triggering apoptosis. Furthermore, an activation and translocation of NF $\kappa$ B and cytochrome C release were detected in cells 3 hours after BSO treatment as hallmarks of apoptosis induction (Armstrong et al. 2002). Therefore, to check whether we had a stimulation of apoptosis in our cells treated with BSO/DMF we monitored the gene expression of P53.

As presented in figure 100, P53 was significantly reduced at basal level in normal cells ( $p < 0.01$ , \*\*) and UVB level in XP-C cells ( $p < 0.05$ , \*).

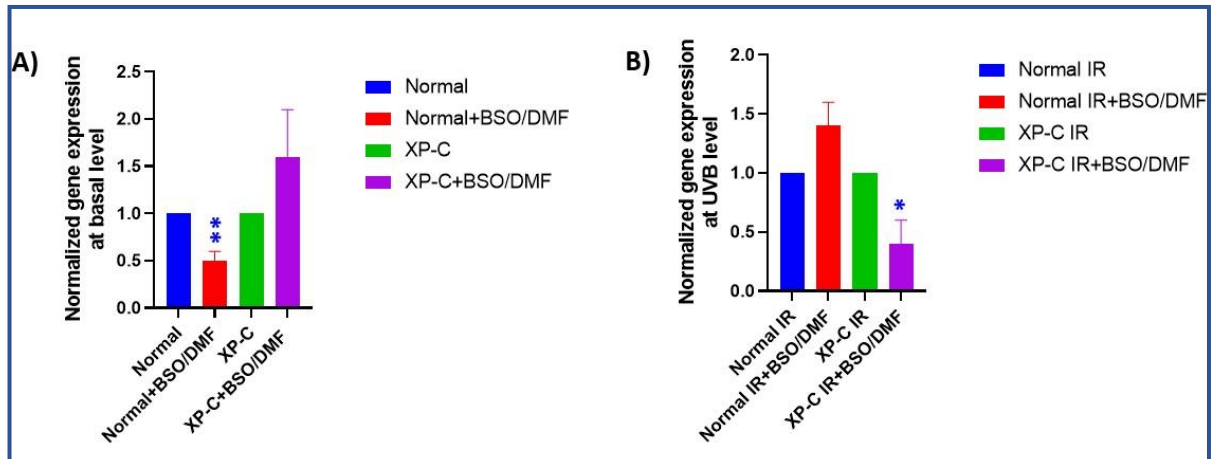


Figure 100. *Effect of BSO/DMF on the P53 mRNA expression at basal and UVB levels in normal and XP-C cells. Panel A) shows that BSO/DMF downregulated P53 ( $p < 0.01$ ; \*\*) in normal cells, at the basal level. Panel B) shows that BSO/DMF downregulated P53 ( $p < 0.05$ , \*) in XP-C cells, at UVB level. For each condition, the sample was normalized by its untreated value (100% viability). Paired *t*-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from three independent experiments,  $n = 3$ . IR=irradiated with UVB ( $0.01 \text{ J/cm}^2$ ).*

Such a result reflected P53's protein expression in normal cells [figure 101 (A)]. It was significantly downregulated in the presence of BSO/DMF compared to its absence at the basal level ( $p < 0.05$ , \*). However, no change was detected at the UVB level. This was consistent with Armstrong et al. They showed that P53's expression did not vary post-redox modulation in the presence of BSO (Armstrong et al. 2002). However, irreversible apoptosis will be induced after 48-72 hours of BSO treatment (Armstrong et al. 2002). This was not the case in our study, as we incubated the cells only for 4 hours in BSO/DMF.

Interestingly, P53 was upregulated ( $p < 0.05$ , \*) in XP-C cells at UVB level [figures 101 (B) and 67 (C) and (D)]. This proposes a different possible signaling pathway induced in normal versus XP-C cells to decrease cell viability and induce apoptosis. Remarkably, we previously mentioned that XPC plays a role in P53's turnover. Herein, in the absence of XPC and high stress (UVB+BSO/DMF), cells accumulate P53 that could be inactive despite its high expression level.

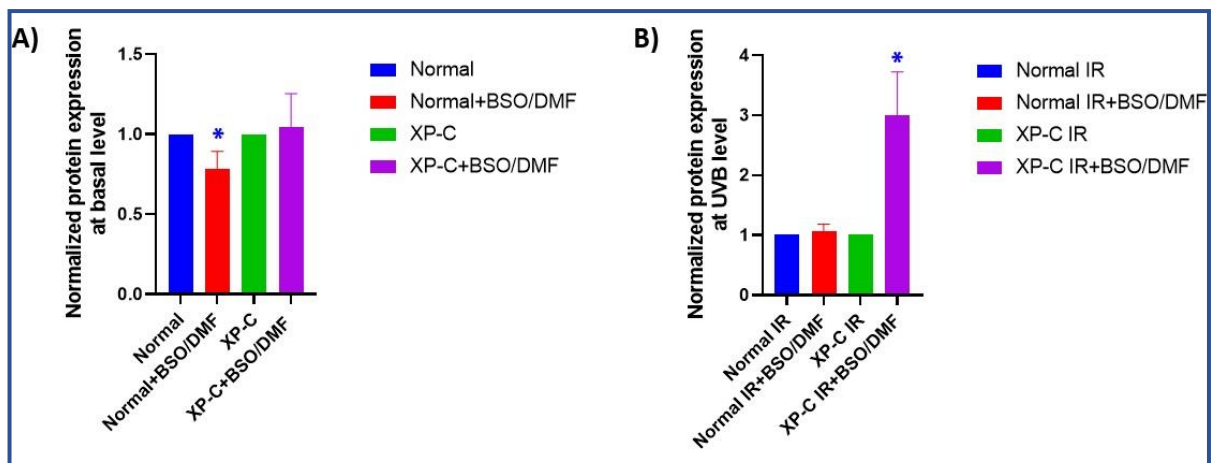


Figure 101. *Effect of BSO/DMF on the P53 protein expression at basal and UVB levels in normal and XP-C cells.* Panel A) shows a downregulation in P53 ( $p < 0.05$ , \*) in the presence of BSO/DMF in normal cells at the basal level. Panel B) shows that BSO/DMF upregulated P53 in XP-C cells at UVB level. For each condition, the sample was normalized by its untreated value (100% viability). Paired *t*-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SD from three independent experiments,  $n=3$ . IR=irradiated with UVB (0.01 J/cm<sup>2</sup>).

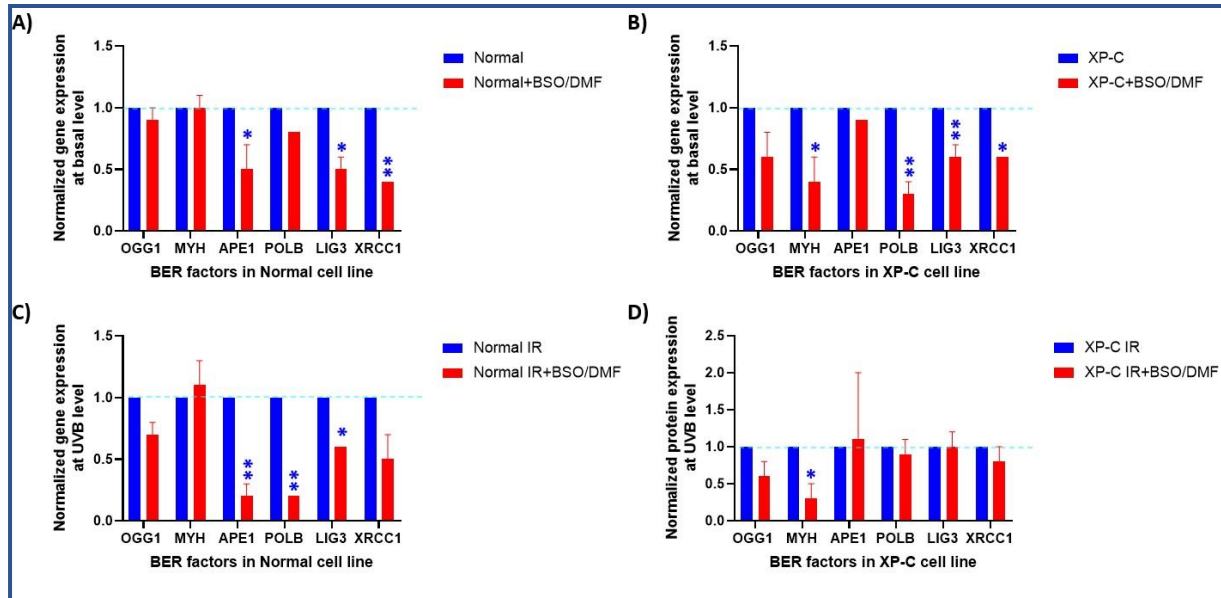
#### 4.5. Effect of BSO/DMF on XP-C and normal cells' BER's gene expression

##### 4.5.1. Effect of BSO/DMF on XP-C and normal cells' BER mRNA expression

Figure 102 shows a global downregulation in BER factors at transcriptional level after pretreatment with BSO/DMF at basal and UVB levels. In normal cells, figure 102 (A) and (C) shows that BSO/DMF downregulated *APE1* ( $p < 0.05$ , \*), *LIG3* ( $p < 0.05$ , \*), and *XRCC1* ( $p < 0.01$ , \*\*) at basal level while *APE1* ( $p < 0.01$ , \*\*), *POLB* ( $p < 0.01$ , \*\*), and *LIG3* ( $p < 0.05$ , \*) at UVB level. Similarly, figure 101 (B) and (D) shows a significant downregulation of *MYH* ( $p < 0.05$ , \*), *POLB* ( $p < 0.01$ , \*\*), *LIG3* ( $p < 0.01$ , \*\*), and *XRCC1* ( $p < 0.05$ , \*) at basal level and *MYH* ( $p < 0.05$ , \*) at UVB level in XP-C cells post-BSO/DMF treatment.

Indeed, the reason for such a downregulation should oppose that found in the presence of NAC. BSO/DMF depletes cells from GSH. This will induce high oxidative stress triggering signaling cascades' dysregulation and cellular molecules' oxidation. Maybe the induction of BER factors' promoters was inhibited. In fact, such a hypothesis was proposed for NER where GSH depletion downregulations its expression and capacity. XPC's expression has been identified as GSH-dependent (W. Han et al. 2012). Therefore XPC's expression may have been altered in normal cells too. Of notice, variations in the redox homeostasis may result in alterations in gene expression profile. A downregulation in BER mRNA expression had been recorded in neurological diseases with high oxidative stress, such as Alzheimer's disease (AD). Forestier et al. showed that an overall downregulation of BER-associated genes (*OGG1*, *MYH*, *APE1*, *XRCC1*, etc....) were detected in neuroblastoma cell line secreting high amyloid- $\beta$  protein, AD's pathological hallmark that leads to elevated oxidative stress (Forestier et al. 2012).

Therefore, the downregulation of BER mRNA expression in both cells could be due to (i) the highly induced total oxidative stress and (ii) downregulated *XPC* expression that had been addressed previously to interfere in BER's expression (part one).



**Figure 102. Effect of BSO/DMF on BER mRNA expression at basal and UVB levels in normal and XP-C cells.** Panel A) shows that BSO/DMF downregulated *APE1* ( $p < 0.05$ , \*), *LIG3* ( $p < 0.05$ , \*) and *XRCC1* ( $p < 0.01$ , \*\*) at basal level in normal cells. Panel B) shows that BSO/DMF downregulated *MYH* ( $p < 0.05$ , \*), *POLB* ( $p < 0.01$ , \*\*), *LIG3* ( $p < 0.01$ , \*\*) and *XRCC1* ( $p < 0.05$ , \*) at the basal level in XP-C cells. Panel C) shows that BSO/DMF downregulated *APE1* ( $p < 0.01$ , \*\*), *POLB* ( $p < 0.01$ , \*) and *LIG3* ( $p < 0.05$ , \*) at UVB level in normal cells. Panel D) shows that BSO/DMF downregulated *MYH* ( $p < 0.05$ , \*) at UVB level in XP-C cells. For each condition, the sample was normalized by its untreated value (100% viability). Paired *t*-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SEM from three independent experiments,  $n = 3$ . IR=irradiated with UVB (0.01 J/cm<sup>2</sup>).

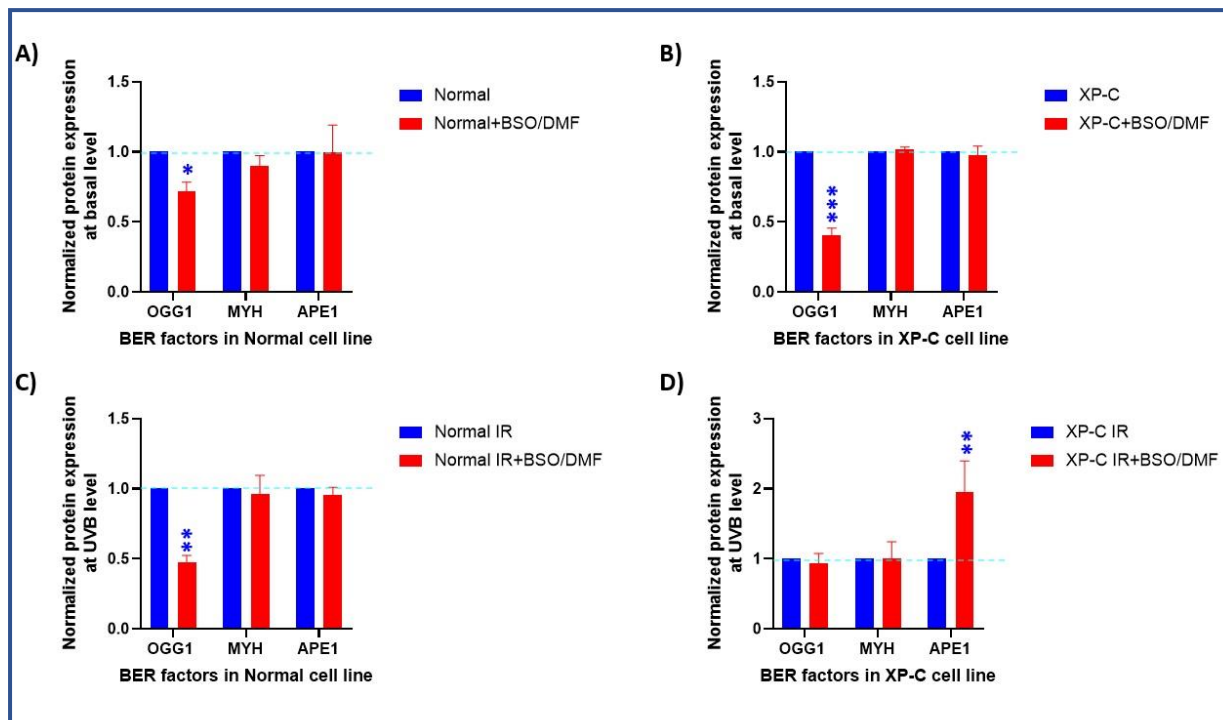
#### 4.5.2. Effect of BSO/DMF on XP-C and normal cells' BER protein expression

We sought to check the effect of BSO/DMF on the translation of BER factors' transcripts. So we did western blot on the three main key proteins in initiating BER and repairing oxidative DNA damage: OGG1, MYH, and APE1.

Figure 103 [(A) and (C)] shows that BSO/DMF downregulated OGG1's expression ( $p < 0.05$ , \*) at basal and UVB levels in normal cells. Similarly, OGG1 was downregulated ( $p < 0.001$ , \*\*\*) upon BSO/DMF in XP-C cells at basal level [figure 103 (B)].

Previously, Dusinka et al. had proposed that glutathione s-transferases (GSTs) might be involved in DNA damage signaling (MAPK kinase). Their activity is induced by ROS, which can influence DNA stability and oxidative DNA damage repair. As they require GSH to reduce substrates, a deficiency in this tripeptide downregulates GSTs activity. Such activity had been correlated with BER capacity (Dusinska et al. 2012). Hence, their level could have been abolished, leading to lower activation of BER.

Interestingly, APE1 at UVB level was significantly upregulated ( $p < 0.01$ , \*\*) at the protein level in XP-C cells in the presence of BSO/DMF pretreatment [figure 103 (D)]. APE1 has been identified as a DNA repair protein and plays a role in transcription and redox regulations. In part one, we showed that XP-C cells have higher oxidative DNA damage and oxidative stress than control. BSO/DMF worsens the scenario dramatically. It was suggested that APE1's DNA repair and redox regulatory functions are independent. *In vivo* and *in vitro* studies demonstrated that acute and chronic ROS levels induce APE1 protein expression rapidly. Hence, we suggest that APE1 was upregulated based on its dual role to protect cells as much as possible from genome instability by (i) activating some transcription factors (P53, p21, NFκB...) for cell cycle and proliferation regulation, (ii) antioxidant response, and (iii) other DNA repair pathways regulation where a crosslink between APE1 and DNA repair genes from other pathways had already been demonstrated (GADD45a, BRCA1...) (Tell et al. 2009).



**Figure 103. Effect of BSO/DMF on BER protein expression at basal and UVB levels in normal and XP-C cells.** Panels A) and C) show that BSO/DMF downregulated OGG1 at basal ( $p < 0.05$ , \*) and UVB ( $p < 0.01$ , \*\*) levels in normal cells. Panel B) shows that BSO/DMF downregulated OGG1 ( $p < 0.001$ , \*\*\*) at the basal level in XP-C cells. Panel D) shows that BSO/DMF upregulated APE1 ( $p < 0.01$ , \*\*) at UVB level in XP-C cells. For each condition, the sample was normalized by its untreated value (100% viability). Paired t-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SD from three independent experiments,  $n = 3$ . IR=irradiated with UVB ( $0.01 \text{ J/cm}^2$ ).

## 4.6. Effect of BSO/DMF on XP-C and normal cells' UVB-induced BER and NER activity

### 4.6.1. Monitoring NER activity

Treatment of keratinocytes with BSO and UVA leads to increased oxidative stress triggering protein oxidation, consequently inhibiting NER (Karran and Brem 2016). Similarly, BSO/DMF treatment affected the NER repair in normal and XP-C cells (figure 104). However, it should be stressed that a moderate effect was visualized due to the high SD.

It is highly recommended to repeat the experiment due to the high potentials of the results.

Figure 104 [(A) and (C)] shows that in normal cells: BSO/DMF significantly increased TT and TC (6-4) PPs ( $p < 0.05$ , \*) at 2 hours. In parallel, figure 105 shows that BSO/DMF upregulated TC CPDs ( $p < 0.01$ , \*\*) at 24 hours, CT CPDs at 0.5 and 24 hours ( $p < 0.05$ , \*;  $p < 0.01$ , \*\*, respectively) and CC CPDs were significantly higher at 24 and 48 hours ( $p < 0.05$ , \*). Meanwhile, in XP-C cells, BSO/DMF upregulated TT (6-4) PPs at 2 and 24 hours ( $p < 0.05$ , \*) [Figure 104 (B)]. TC and CC CPDs were also significantly higher at 24 hours ( $p < 0.05$ , \*) compared to control [figure 105 (B) and (D)].

Therefore, BSO/DMF increases the UVB-induced bulky lesions and dysregulates their repair by boosting their persistence. This is due to the high ROS level in the absence of GSH that could enhance proteins' oxidation and their inactivation in normal cells. On the other hand, NER is already diminished in XP-C cells, and adding BSO/DMF worsens the situation.

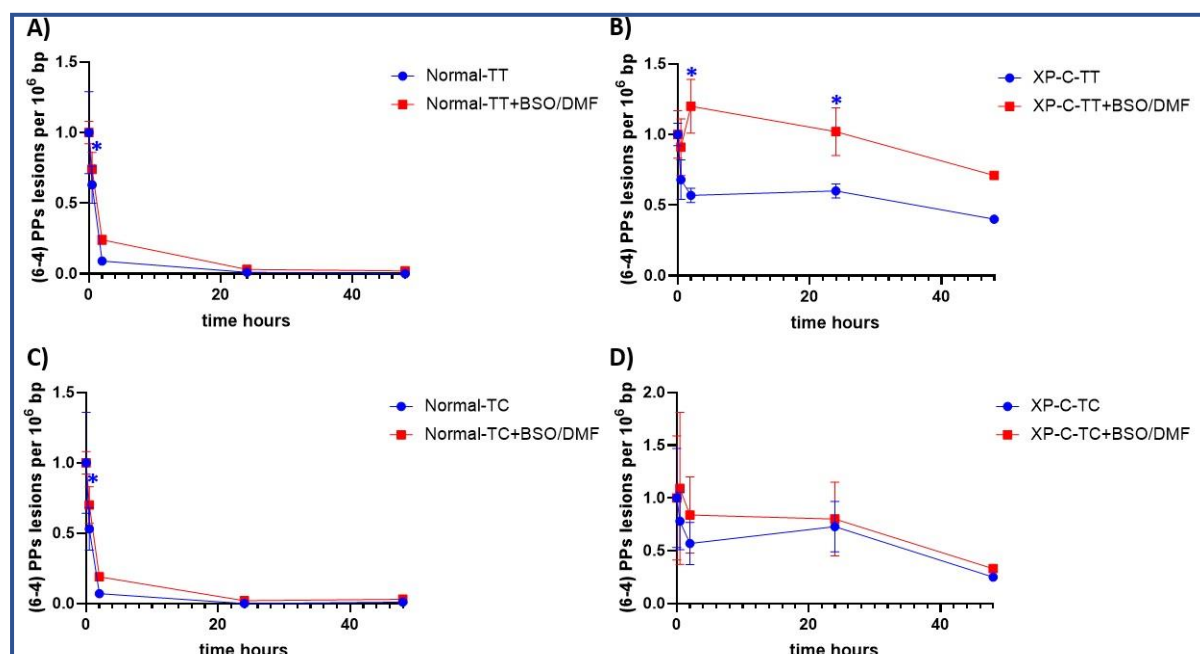
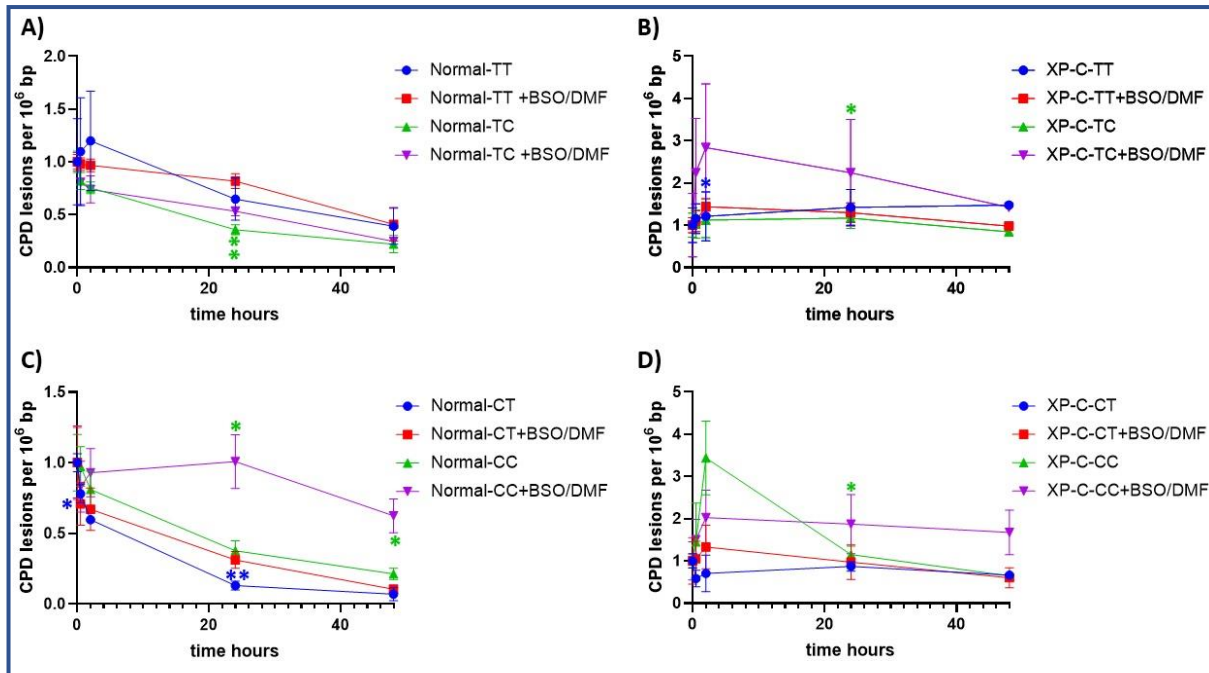


Figure 104. Effect of BSO/DMF on UVB-induced (6-4) PPs kinetic repair in normal and XP-C cells. Panels A) and C) show that normal cells had a downregulated TT and TC (6-4) PPs ( $p < 0.05$ , \*) in the presence of BSO/DMF, respectively, at 2 hours post-UVB irradiation. Panel B) shows that BSO/DMF induced downregulated TT (6-4) PPs at 2 ( $p < 0.05$ , \*) and 24 ( $p < 0.05$ , \*) hours post-UVB irradiation in XP-C cells. Panel D) shows No significant effect of BSO/DMF on TC (6-4) PPs' repair in XP-C cells. For each condition, the sample was



normalized by its irradiated value at 0 hours (100% viability). Paired *t*-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SD from two independent experiments,  $n = 2$ .

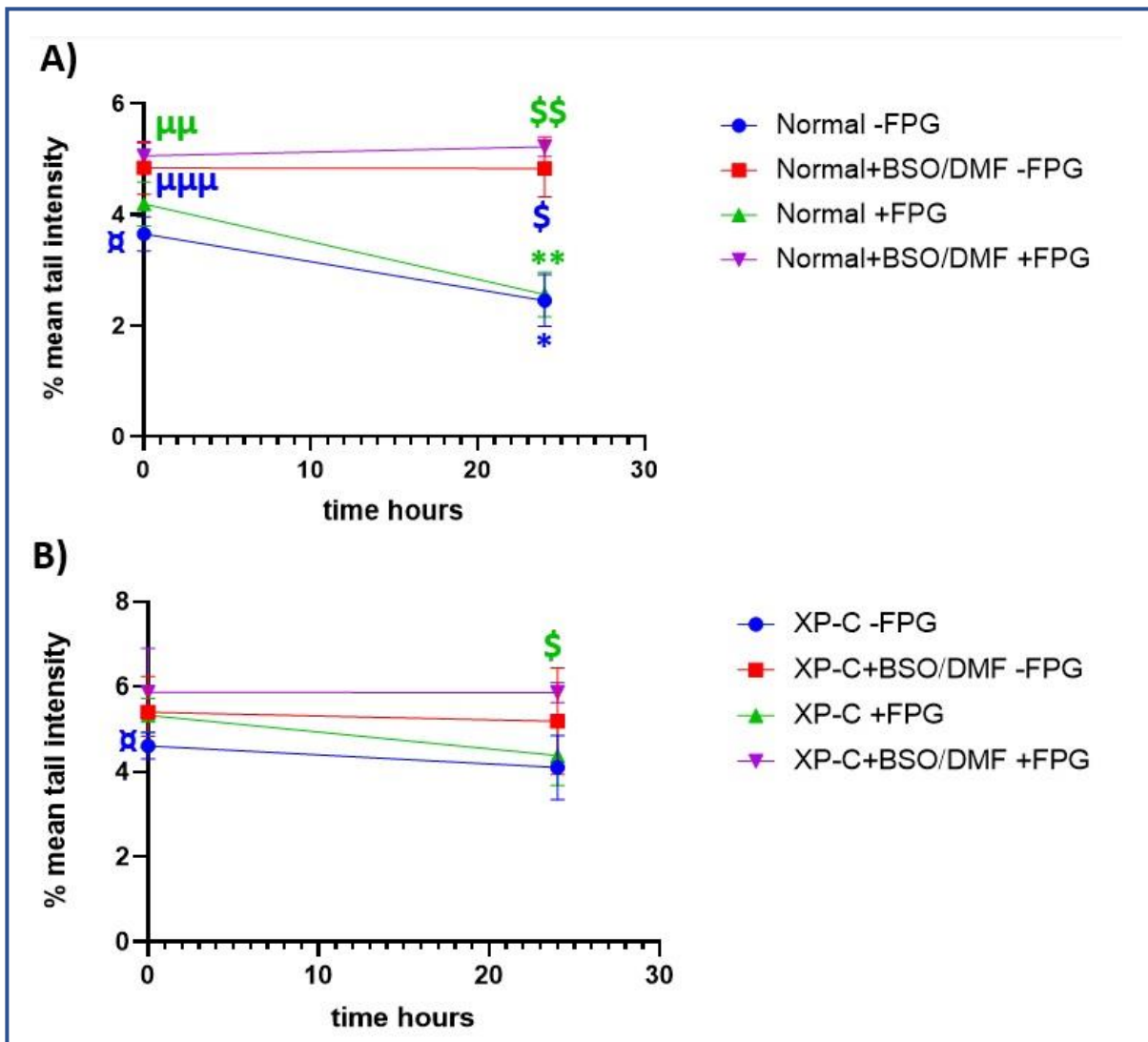


**Figure 105. Effect of BSO/DMF on UVB-induced CPDs kinetic repair in normal and XP-C cells.** Panels A) shows that BSO/DMF downregulated TC CPDs ( $p < 0.01$ , \*\*) repair at 24 hours post-UVB irradiation in normal cells. Panel B) shows that BSO/DMF downregulated TT and TC CPDs repair ( $p < 0.05$ , \*) at 2 and 24 hours, respectively, post-UVB irradiation in XP-C cells. Panel C) shows that BSO/DMF downregulated CC CPDs at 24 and 48 hours ( $p < 0.05$ , \*) and CT at 0.5 ( $p < 0.05$ , \*) and 24 hours ( $p < 0.01$ , \*\*) post-UVB irradiation in normal cells. Panel D) shows that BSO/DMF downregulated CC CPDs repair ( $p < 0.05$ , \*) at 24 hours post-UVB irradiation in XP-C cells. For each condition, the sample was normalized by its irradiated value at 0 hours (100% viability). Paired *t*-test was used to compare the expression for each condition ( $p < 0.05$ , \*). The results are the mean  $\pm$  SD from two independent experiments,  $n = 2$ .

#### 4.6.2. Monitoring BER activity

Figure 106 illustrates that BSO/DMF increased the oxidized purines and single-strand breaks significantly in normal cell-lines compared to its absence ( $p < 0.01$ ,  $\mu\mu$ ;  $p < 0.001$ ,  $\mu\mu\mu$ , respectively). Such treatment also impacted the repair efficacy in normal and XP-C cells. At 24 hours post-UVB, normal cells failed to repair the damage where significantly higher oxidized lesions ( $p < 0.01$ , \$\$) and single-strand DNA damage ( $p < 0.05$ , \*) persisted. Similarly, oxidized purines persisted at a higher level at 24 hours in XP-C cells ( $p < 0.05$ , \$).

This may show that GSH depletion impacted the efficacy of BER activity in both cell lines, where it induced further oxidized purines reaching a plateau that is not repaired. Therefore GSH plays an essential role in protecting cells against oxidative DNA damage independently of XPC protein.



**Figure 106. Effect of BSO/DMF on alkaline and oxidized purines repair in normal and XP-C cells, post-UVB irradiation.** Panel A) shows that BSO/DMF increased the UVB-induced single-strand DNA damages and oxidized purines at 0 hours ( $p < 0.001$ ,  $\mu\mu\mu$ ;  $p < 0.01$ ,  $\mu\mu$ , respectively) in normal cells. Both DNA damages persisted after 24 hours significantly ( $p < 0.05$ , \$). Panel B) shows that BSO/DMF stimulated oxidized purines persistence at 24 hours post-UVB in XP-C cells ( $p < 0.05$ , \$). For each condition, the sample was normalized by its unirradiated value (100% viability). Paired *t*-test was used to compare the expression for each condition. The results are the mean  $\pm$  SEM from three independent experiments,  $n=3$ . \*  $p < 0.05$  between -FPG and +FPG for each treatment at 0 and 24 hours. \$  $p < 0.05$  between the BSO/DMF and untreated samples at 24 hours +FPG.  $\mu$   $p < 0.05$  between the BSO/DMF and untreated samples at 0 hours +FPG. ✕  $p < 0.05$  between -FPG and +FPG for each of BSO/DMF and untreated samples at 0 hours.

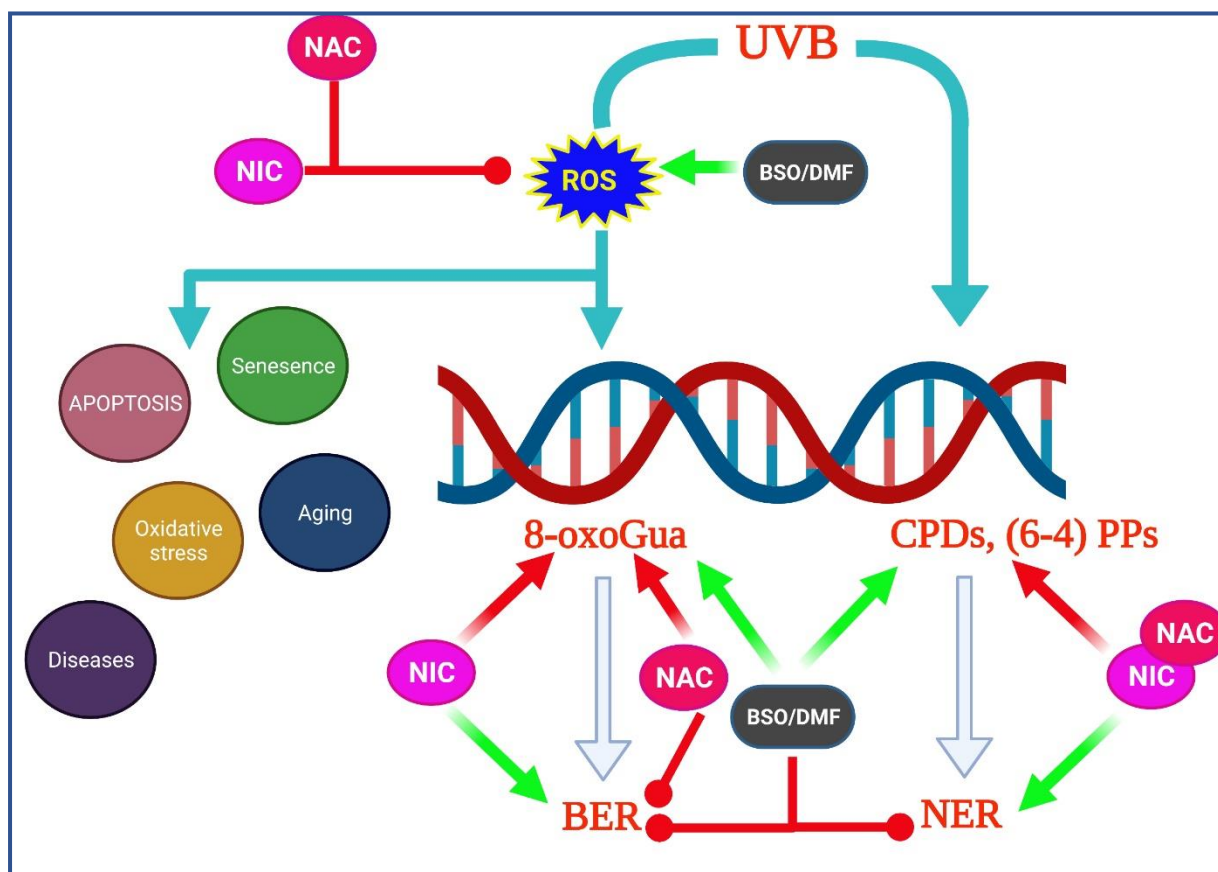
# Conclusion and Perspective

## **BSO/DMF (100 $\mu$ M/100 $\mu$ M):**

- ❖ Increased photosensitivity
- ❖ Effect on some BER gene expressions:
  - ✓ downregulated mRNA level:
    - Basal: *APE1*, *LIG3*, and *XRCC1* in normal cells and *MYH*, *POLB*, *LIG3*, and *XRCC1* in XP-C cells
    - UVB: *APE1*, *POLB*, and *LIG3* in normal cells and *MYH* in XP-C cells
  - ✓ downregulated protein level:
    - Basal: OGG1 in normal and XP-C cells
    - UVB: OGG1 in normal cells
  - ✓ Upregulated Protein level:
    - UVB: APE1 in XP-C cells
- ❖ Dysregulated NER and BER activity
- ❖ Depleted Glutathione level
- ❖ Upregulated ROS level

In summary, BSO/DMF is cytotoxic. It increases the UV-induced oxidative DNA damage in cells. This proves the importance of GSH in DNA repair and other signaling pathways including cell survival.

## Schematic Summary



**Figure 107. Effect of NIC, NAC, and BSO/DMF on cells post-UVB.** UVB induces bulky lesions (CPDs, (6-4) PPs) that are usually repaired by NER in the absence of any drug. Such a repair is impaired in XP-C patients triggering mutagenesis. Furthermore, UVB initiates ROS-induced oxidative damage, including oxidized purines (8-oxoGua) that BER usually repairs. As illustrated before, BER is downregulated in XP-C patients triggering mutagenesis and oxidative stress. So, NIC has been shown to decrease ROS, 8-oxoguanine, and bulky lesions. In parallel, it enhances NER activity and BER's expression and activity. NAC decreased ROS, 8-oxoguanine and bulky lesions while enhancing NER.

Interestingly, it inhibited BER's expression. That is probably due to the dramatic downregulation in oxidative stress as a ROS scavenger. Lastly, BSO/DMF induced ROS, 8-oxoGua and bulky lesions. In parallel, it inhibited BER and NER. This could enhance senescence and apoptosis. Therefore, the drugs used are potentially protective measurements pre-UVB (NIC, NAC) and potential use in cancer treatment (BSO/DMF). Nevertheless, more studies are needed at proteomic and posttranslational levels, in addition to studies in vivo, before confirming such encouraging results.

**X**eroderma pigmentosum C (XPC) protein plays a major role in initiating GG-NER pathway to excise DNA helix distorting lesions. More than forty-five *XPC* inactivating mutations were detected, leading to the impairment of the protein product and the accumulation of the bulky DNA lesions (Ben Rekaya et al. 2013). This could increase skin sensitivity to sun radiation leading to mutagenesis, morbidity, skin carcinogenesis, and early mortality. Nevertheless, XP-C patients' phenotype spectrum is heterogeneous and broad including internal tumorigenesis (thyroid, hematological, gynecological, spinal cord, and brain cancers) and rarely neurological abnormalities (Uribe-Bojanini, Hernandez-Quiceno, and Cock-Rada 2017; Yurchenko et al. 2020; Zebian et al. 2019). Herein, researchers revealed other versatile roles in other DNA repair pathways, cell cycle regulation, and redox homeostasis (Melis et al. 2011).

As oxidative stress induces oxidative DNA damage that BER usually repairs, we thought to selectively check the **link between XPC, base excision repair, and oxidative stress** from a mechanistic standpoint. This could show whether XPC acts directly on BER and/or indirectly via regulating the cellular redox state. For that, we split the project into two parts: **part one** discusses the effect of XPC protein on BER and ROS. Meanwhile, **part two** checks whether modulating the redox state in XP-C cells by different treatments could reverse their phenotype and ameliorate the background DNA repair.

- **Part One “Deciphering the Role of XPC in BER and Oxidative Stress”**

Evidence had already discussed a potential role of XPC in BER due to its interactions with various DNA glycosylases and regulation of their activities. Nevertheless, we were further interested in checking the effect of XPC on stimulating the global BER pathway at both gene and activity levels. For that, we treated the cells with ultraviolet radiation B (UVB) before the experimental setup. UVB allows us to induce both (i) NER substrates [bulky lesions, CPDs and (6-4) PPs] and (ii) BER substrates (oxidative DNA damage, 8-oxoGua) due to an elevated ROS level (Budden and Bowden 2013; Deng et al. 2018).

First, we showed that the different XP-C cells, derived from different XP-C patients, had the standard characteristics: impaired XPC basal gene expression and reduced GG-NER of UVB-

induced bulky lesions. TC-NER is known to be faster in repair rate than GG-NER (Soufir et al. 2010). Hence, the lesions repaired by TC-NER could not have been detected. Therefore, what we observe in our experiments could be solely due to the deficiency of GG-NER or NER inactivation in the presence of high oxidative stress.

Langie et al. showed that exposure of epithelial cells to oxidant ( $H_2O_2$ ) impeded an effective NER activity (Langie et al. 2007). Therefore, we may link the results of the ensuing experiments with the lack of XPC and NER deficiency.

Our RT-qPCR experiments revealed that the absence of XPC contributed to the downregulation of UVB-stimulated BER factors, including *OGG1*, *MYH*, *APE1*, *LIG3*, *XRCC1*, and *POL $\beta$* . Inhibited *OGG1* and delayed *APE1* mRNA expression were also shown in XP-C deficient cells following oxidant treatment ( $H_2O_2$ ) (de Melo et al. 2016). This indicates that XPC may play a direct/indirect role in stimulating the mRNA expression of BER genes due to its role as a transcriptional regulator. For example, XPC has been shown to regulate P53, and our results indicated a dysregulation in its expression (Krzyszinski et al. 2014). In parallel, P53 is one of the transcription factors regulating *APE1* and *MYH*'s expressions (de Melo et al. 2016; Oka et al. 2014). Therefore, XPC may also be involved in their regulation. Further investigations are required to confirm such a hypothesis.

Moving forward, *OGG1* and *MYH* DNA glycosylases excise 8-oxoGua and adenine facing 8-oxoGua, respectively, and *APE1* is known as an endonuclease and redox regulator. Therefore, we studied these factors at the protein level and showed that they are downregulated in the absence of XPC. This shows that the lack of XPC affects the BER's expression in the presence of stress. Such a decrease in the BER initiation factors' expression delayed the excision efficacy of UVB-induced oxidized purines. Using modified comet assay ( $\pm$ FPG), we monitored the excision capacity of XP-C cells compared to normal at different kinetic points, which could reflect the cellular repair efficiency of the UVB-induced oxidized purines. Oxidized purines persisted for more than 24 hours in the XP-C cells compared to normal control. At standard conditions, half of the oxidized purines were shown to be repaired in 2 hours. Such oxidized purines involve 8-oxoGua and Fapy, where 8-oxoGua is the most common type of oxidative DNA damage (Fortini et al. 2003). Accordantly, D'Errico et al. showed that 8-oxoGua is fully repaired 7 hours post-oxidative stress (KBrO<sub>3</sub>) in keratinocytes and fibroblasts (M. D'Errico et al. 2007). This could be due to the rapid recruitment of XPC to the site of oxidative DNA damage, 8-oxoGua. In parallel, XP-C cells showed an impairment in 8-oxoGua repair (D'Augustin et al. 2020). However, such a drastic effect was also demonstrated in cells deficient with other NER factors, as XPA and CSB. This could be due to the recognition of 8-

oxoGua by XPC, XPA, CSB, DDB1 etc (D'Augustin et al. 2020). Also, several oxidative DNA lesions were reported, leading to bulky lesions that NER could recognize and repair. Hence, ***could our observed results be a direct impact of XPC dysregulation or due to a wonky crosslink between NER and BER?***

Melis et al. compared the effect of oxidative stress on XP-C and XP-A mice and fibroblasts. They detected a higher mutational load, more sensitivity towards oxidants, and higher internal tumor spectrum in the absence of XPC compared to XPA deficiency or normal control (Melis et al. 2008). Furthermore, *xpc*<sup>+/-</sup> mice showed a higher predisposition to UV-induced skin cancer than *xpa*<sup>+/-</sup> mice (Melis et al. 2011). This may be due to the role of XPC as a rate-limiting protein in NER or due to other possible roles. Even though the accumulation of bulky lesions majorly triggers skin cancer, oxidative stress has been suggested to contribute to photocarcinogenesis. For instance, Melanoma and nonmelanoma skin cancer exhibit high oxidative stress and inadequacy in the antioxidant defense system (Sander et al. 2003).

Additionally, some non-bulky lesions, including 8-oxoGua, were reported to be repaired primarily by TC-NER due to a possible partial stalling of RNA polymerase II (Melis et al. 2011). This may indicate a competition between BER and NER. As XPA and XPC are involved in NER, the difference in response towards oxidants suggests a role of XPC in oxidative DNA damage removal by stimulating BER's functionality and/or redox homeostasis, thereby halting internal tumor development. For example, XP-C fibroblasts showed a deficient BER repair of oxidative DNA damage post-visible light excited methylene blue, a ROS stimulator (Berra et al. 2013).

XPC was postulated as a co-factor stimulating and interacting with several BER factors to induce the repair of 8-oxoGua. For instance, XPC interacts with different BER repair factors, including glycosylases [thymine DNA glycosylase (TDG) and SMUG1] and APE1. For example, it interacts with OGG1, a rate-limiting glycosylase in BER, to regulate its turnover and expression (Melis et al. 2011; de Melo et al. 2016).

Similar to XPC, CSB, TC-NER factor is known to interact with different BER proteins as PARP1 and APE1 and regulate the repair of 8-oxoGua and 8-oxoA post-oxidative stress (Melis et al. 2011).

In conclusion, XPC has a direct but not essential role in BER's expression and activity. For example, we showed that oxidized purines were repaired in XP-C cells but slower than the normal control. Therefore, XPC's role in BER affects the efficacy but not the efficiency of repair. A study showed XP-C patients developing only basal and squamous cell carcinomas but not internal cancer, implying an effective BER activity. It also showed two siblings with

the same type of XPC mutation (c.1643-1644delTG), but one developed B-cell lymphoma while the other did not develop internal cancers (Oetjen et al. 2020). This could indicate the need for XPC impairment and an additional stimulant to induce internal cancers. In our case, such a stimulus could be UVB, where we showed a higher and more persistent ROS level in XP-C cells after stress than normal control.

Therefore, *could the halted BER's expression and efficacy be due to a higher redox imbalance and disturbed stimulation of BER in the absence of XPC? Is it possible to reverse such an impact to enhance the background DNA repair?*

Part two of the project allowed us to answer such a question.

- **Part Two “Ameliorating the DNA repair of XP-C cells by modulating their redox state via pharmacological treatments”**

To focus on the redox state in XP-C versus normal cells, we pretreated the cells with different treatments that act differently but work towards the same goal.

**Nicotinamide (NIC)**'s central role is to increase the nicotinamide adenine dinucleotide (NAD<sup>+</sup>) level in cells. A diminution in NAD<sup>+</sup> leads to its precursor's depletion, ATP. This induces cellular energy depletion and dysregulated DNA repair triggering genomic instability, skin aging, and carcinogenesis (Fania et al. 2019). As BER is known to be ATP-dependent, NIC might enhance BER's background status shown in part one via preventing its depletion. NER is also ATP-dependent. For instance, XPB and XPD have ATPase activities (Schärer 2013). Therefore, monitoring NER could also be interesting.

After selecting the most suitable NIC dose (50 µM), we checked the effect of such a treatment on UVB-photosensitivity, BER's gene expression and activity, and NER's activity. No impact of NIC on the photosensitivity of XP-C and normal cells was detected. This could be considered a good indicator. If NIC enhanced an exaggerated cellular viability of XP-C cells carrying mutations, carcinogenesis might be ultimately triggered. Similarly, at low NIC doses (<2 mg/mL), no difference in cellular viability was detected in cervical cancer-associated fibroblasts. Still, a reduction was observed at higher doses (Hassan, Luo, and Jiang 2020). Also, NIC (50µM) showed no effect on HaCaT keratinocytes and primary melanocytes viability in the presence and absence of UV (Thompson, Halliday, and Damian 2015; Thompson et al. 2014). Therefore, at low levels, NIC plays a role other than affecting cellular viability, which may indirectly show no effect of NIC on apoptosis and cell cycle. For instance, treatment with NIC led to similar melanocytes entering S-phase compared to control (Thompson et al. 2014).



To check the other possible protective roles of NIC, we studied the mRNA and protein expression of different BER factors in normal and XP-C cells.

NIC increased the mRNA expression of several BER factors at basal and UVB levels in normal and XP-C cells. It also increased the basal expression of MYH protein in normal cells. This shows that NIC triggered the BER's expression. NIC also induced BER's excision activity post-UVB. Both cell lines had lower initiated an enhanced repair of the oxidized purines. This may show that NIC may play a role in ameliorating BER's background in DNA repair-deficient cells such as XP-C cells. Similar results were observed by Thompson et al., who showed that NIC reduced UV-induced 8-oxoGua in HaCaT keratinocytes and in *ex vivo* human skin. They proposed that NIC does not prevent DNA lesions' formation instead enhances their repair by increasing cellular ATP level (Thompson, Halliday, and Damian 2015).

On the contrary, NIC significantly downregulated oxidized purines instantaneously post-UVB in normal and XP-C cells. This could be due to the different parameters of our experiments. We used a comet±FPG assay that detects 8-oxoGua and other oxidized purines in the cells while they used immunofluorescence that detects 8-oxoGua solely. Also, we used UVB lamp post-incubating cell lines with NIC (50 µM) for 24 hours while they treated cells with NIC (50 µM) before and after solar simulation. Thompson et al. showed that solar simulation-induced 8-oxoGua is repaired better in NIC presence in keratinocytes and human skin. Their comet assay revealed lower 8-oxoGua in the presence of NIC compared to its absence in keratinocytes instantly post-solar simulation (the starting time point of lesion detection, 15 minutes) (Thompson, Halliday, and Damian 2015).

Moving forward, several studies showed that NIC reduces precancerous skin lesions (actinic keratosis) and skin cancers (MSCs and NMSCs) (Snaidr, Damian, and Halliday 2019; Malesu et al. 2020). These cancer incidences are usually triggered by the accumulation of unrepaired bulky lesions [CPDs and (6-4) PPs], and NIC could enhance their repair or prevent their induction. It was demonstrated that NIC does not reduce or prevent the initial UV-induced CPD level in keratinocytes but rather enhances their repair by NER (Thompson, Halliday, and Damian 2015; Snaidr, Damian, and Halliday 2019). We showed that NIC pretreatment enhanced the repair of TT CPDs and TT (6-4) PPs in XP-C cells but not their initiation. On the other hand, NIC did not induce a difference in effect in normal cells. This could indicate that NIC triggers multiple pathways to protect the cells against stress. As XP-C cells lack efficient GG-NER, NIC might be stimulating other alternative excision pathways as a compensation. Interestingly, Topoisomerase I (TOP1), an essential enzyme in resolving torsional stress in the genome, dependent-BER was suggested to substitute a deficient-NER pathway in repairing (6-

4) PPs *in vivo* and *in vitro*. During DNA replication and transcription, TOP1 forms a transient complex with the single-strand DNA (3'-TOP1 DNA adduct). Such complex becomes trapped next to unrepaired (6-4) PPs leading to single-strand breaks due to TDP1/TDP2 enzymes that activate BER. Higher UV-induced bulky lesions were detected in XP-A cells when BER is deficient compared to being proficient (L. K. Saha et al. 2020). NIC was also shown to inhibit protein and lipid oxidation (L. K. Saha et al. 2020).

To check whether other factors participated in the effect of NIC on cells, we monitored the redox status by checking different antioxidants' expression levels and the GSH level. This will allow us to check whether NIC downregulates the initiation of DNA damage in cells and/or enhance their repair. Our results showed that *SOD1* and *SOD2* were significantly higher in NIC-treated normal cells post-UVB, while only *SOD1* was upregulated in the presence of NIC post-UVB in XP-C cells. The difference between both superoxide dismutases is that *SOD1* is usually located in the cytoplasm while *SOD2* is in the mitochondria. Perhaps, no significant change in *SOD2* in XP-C cells was due to this enzyme's known high expression and activity in the absence of XPC (S.-Y. Liu et al. 2010). Phosphorylated Nrf2 plays a role in the transcriptional activation of antioxidants, including the SODs, catalases, etc (Jing Chen, Zhang, and Cai 2014). Studying the gene expression of Nrf2 could provide a more general conclusion about the effect of NIC on the antioxidant defense pathway in the presence and absence of XPC. NIC significantly only downregulated the Nrf2 protein basal level in XP-C cells. This could indicate that NIC enhanced the XP-C cell's resting redox status, which negatively affects Nrf2. This prevents the overactivation of Nrf2 that could lead to abnormal proliferation due to the overexpression of downstream target genes (Schäfer et al. 2014). Furthermore, NIC may act downstream Nrf2 pathway or on Nrf2's posttranslational activity. For example, NAD<sup>+</sup> induces ERK activation to translocate Nrf2 into the nucleus to bind to antioxidants' promoters. Therefore, NAD<sup>+</sup> pretreatment enhances the resistance of cells to UV-induced oxidative stress in an XPC-independent manner by increasing the capacity of antioxidants (J.-K. Kim and Jang 2014). This is well-illustrated where NIC was able to reduce ROS level by time in XP-C cells. Previously, NIC had been shown to alleviate ROS levels in primary human fibroblasts and reduce oxidative cellular damage *in vivo* (Kwak et al. 2015). In addition to neutralizing UV-induced ROS, NIC reduces cells' mitochondrial activity, contributing to lower respiration rate and hydrogen peroxide and superoxide anion levels (Kang, Lee, and Hwang 2006). This could be interesting in XP-C cells that are sensitive to mitochondrially-induced ROS under stressed conditions due to altered H<sub>2</sub>O<sub>2</sub> production/clearance upon impaired mitochondrial complex I and upregulated complex II (Mori et al. 2017).

Additionally, ROS-induced DNA damage triggers PARP1 involved in several cellular functions, including DNA repair, transcription, and cell death. Overactivation of PARP1 in DNA repair-deficient cells leads to ATP and NAD<sup>+</sup> depletion, which can be compensated by NIC treatment (Kwak et al. 2015). A cleavage of PARP1 by caspase-3 also indicates the presence of cell death and genotoxic stress (Touat et al. 2019). Therefore, exploiting the status of PARP1 in our cells could unravel the interaction between NIC and PARP1 in the presence and absence of XPC at basal and UVB levels and could provide an insight into the general effect of NIC on cellular health. We showed that NIC significantly inhibited the cleavage of PARP1 in XP-C cells but not in normal cells. This indicates that NIC could neutralize the severe genotoxicity in XP-C cells which could decrease cell death.

On the other hand, we detected an upregulated PARP1, though not significant, which could be due to the increase in NAD<sup>+</sup> availability and the role of PARP1 in localizing to the DNA lesions to recruit factors as CSB to induce repair. In addition to CSB's role in transcription and TC-NER, it regulates UV-induced oxidative DNA damage and interacts with OGG1 and APE1 (Boetefuer et al. 2018). PARP1 also recruits other DNA repair proteins as XRCC1. This could participate in the enhancement of DNA repair presented in the cells post-NIC treatment.

Therefore, we propose that NIC enhances DNA repair by increasing ATP level and stimulating BER's gene expression. In parallel, it increases the antioxidant defense system (gene expression and GSH) to inhibit ROS and prevent further dramatic initiated DNA damages, particularly in XP-C cells.

**N-acetylcysteine (NAC)** is a potent ROS scavenger, while **BSO/DMF** depletes cellular GSH, triggering higher ROS levels. Thus, pretreatment of cells with these paradoxical drugs could provide an insight into the importance of GSH in normal and XP-C cells at DNA repair level and antioxidants defense level.

NAC pretreatment increased the mRNA expression of some BER factors while BSO/DMF inhibited the mRNA expression of some BER factors in normal and XP-C cells at basal and UVB levels. Surprisingly, NAC and BSO/DMF inhibited some BER protein factors in the studied cells. Nevertheless, the reason for downregulation is different between the two conditions. We suggest that NAC inhibited the oxidative stress and DNA damage which could act as a negative feedback on BER's expression. In contrast, BSO/DMF inhibits BER's expression and activity, worsening the scenario. To prove this hypothesis, we checked the DNA repair activity in cells. NAC enhanced CPD repair and oxidized purines repair in both cell lines, suggesting that NAC protects cells via an XPC-independent mechanism of action while

BSO/DMF had an opposite function. GSH and DNA have a negative charge, so no direct interaction occurs amongst them; however, the former is suggested to either prevent DNA damage or participate in their repair. GSH's concentration had previously been inversely correlated with higher DNA damage due to its role in redox homeostasis directly or indirectly through enzymatic interactions (Chatterjee 2013). As discussed earlier, XPC deficiency could induce internal cancers such as leukemia, and XP-C cells have lower GSH level compared to control. In parallel, defective GSH had been linked to leukemia and the high sensitivity of cells to radiation. This shows that GSH deficiency plays a major role in XP-C patients' phenotype. The observed enhancement of CPD and 8-oxoGua repair in cell lines could be due to lower ROS levels in the presence of GSH. NAC also triggered the upregulation of some antioxidants' mRNA expression post-UVB in normal and XP-C cells; however, depletion of GSH by BSO/DMF triggered an upregulation of their expression in normal cells only. XP-C cells had a downregulated level of some genes. This could indicate a high ROS level that is oxidizing proteins responsible for activating such genes or due to mitochondrial disturbance. SOD2 is localized in the mitochondria, and its impairment post-stress may indicate high oxidative damage and mitochondrial dysfunction. In 2019, Düzenli et al. suggested a combination of NAC and acetyl-L-carnitine, an antioxidant and anti-apoptotic agent, to stimulate DNA repair genes as synergic protection against UV damage (Düzenli et al. 2019). Such treatment seems promising to be tested in XP-C cells.

GSH also plays a role in suppressing apoptosis (Chatterjee 2013). In view of this, we checked the effect of NAC and BSO/DMF on PARP1 due to its previously described essential roles in cell cycle and DNA repair. NAC dramatically decreased cleaved PARP1 in XP-C cells while BSO/DMF increased it only in normal cells. This may be due to the high level of cleaved PARP1 in XP-C cells before treatment with BSO/DMF. Therefore, GSH could enhance the cell status via buffering ROS levels leading to more minor macromolecular damages.

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## *Conclusion and Perspectives*

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As a conclusion, XP-C cells have a weakened BER gene expression and activity due to the high ROS imbalance that could play a significant role in XP-C patients' phenotypes.

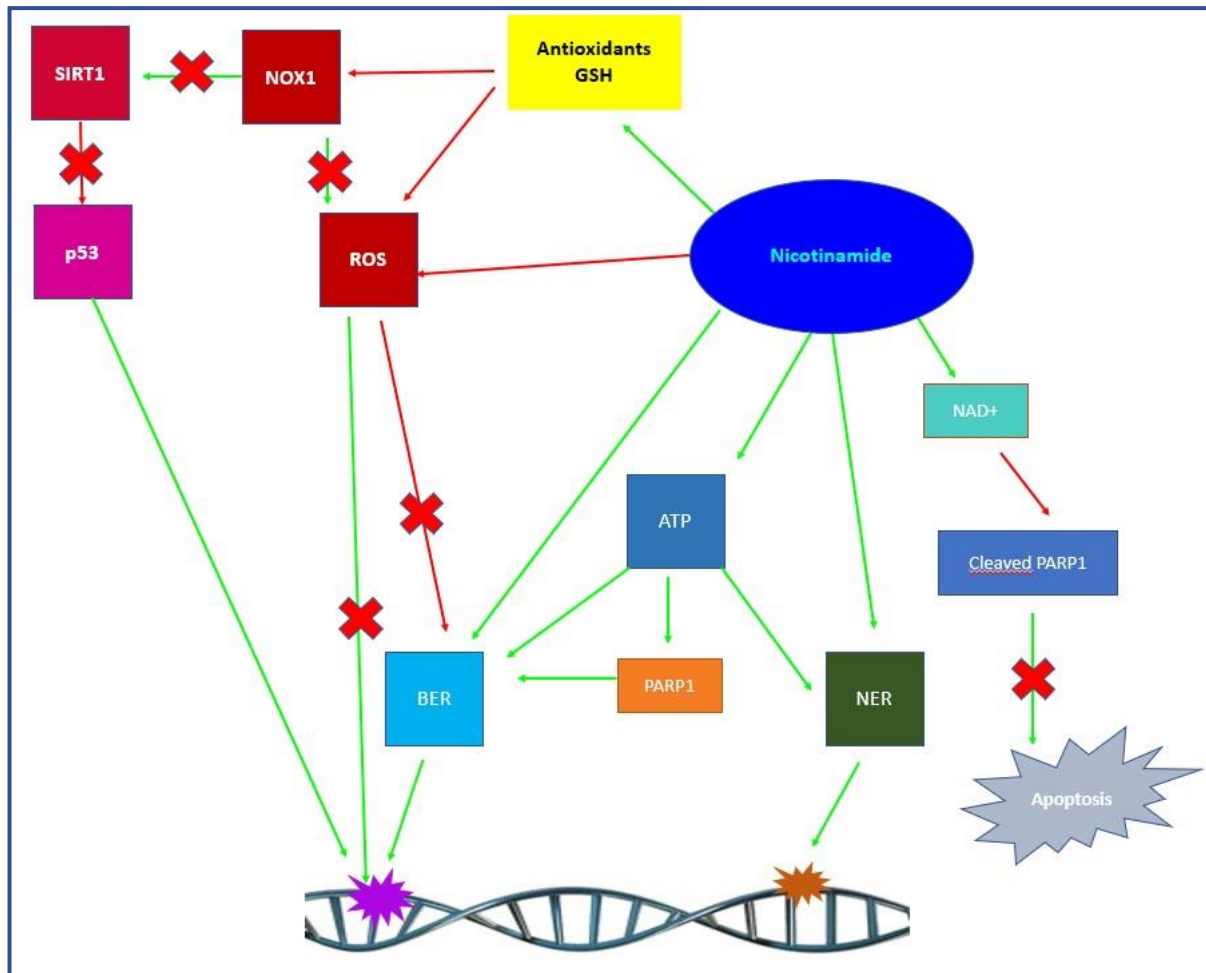
NIC and NAC represent potential preventive methods to protect and treat XP-C patients per se and other DNA-repair deficient patients. This is mainly by boosting the antioxidant defense mechanism and buffering ROS level via GSH, which could participate in BER's higher capacity in accommodating daily life threats and oxidative stressors. Based on the observed results (gene expression, activity..) NAC could be proposed to act more as a preventive pretreatment rather than therapeutic, while NIC could be proposed as preventive and therapeutic pretreatment.

To further validate our hypothesis, these results should be confirmed in primary cells (fibroblasts and keratinocytes), in vivo models (mice), and 3D-reconstructed skin models. This will provide further realistic and robust conclusions about the efficacy of these treatments in the absence of DNA repair. Additionally, checking the treatments' different interactions more detailed via proteomic analysis could enhance our understanding of their mechanisms and provide insights for different targeted therapies. Another interesting study could be to investigate the efficiency of a combination treatment and whether it could have a synergetic protective effect. Perhaps the combination of NIC and NAC could provide synergic protection and damage prevention on cells that could help XP patients have a better/healthier life.

*Proposed schematic summary*

Based on our results and bibliography, we propose the following mechanism of action of NIC in cells (figure 107).

Since NAC has a similar goal and mechanism of action (enhancing GSH..), we propose a similar action mechanism to this treatment. BSO/DMF acts the opposite to both drugs by depleting GSH, increasing DNA damage and apoptosis, and inhibiting DNA repair.



**Figure 108. Suggested mechanism of action of NIC on XP-C cells.** It increases NAD<sup>+</sup>, ATP, and antioxidants (SOD, GSH..) levels. This leads to lower ROS and cleaved PARP1 (apoptosis hallmark) levels, higher PARP1, and upregulated DNA repair's (NER and BER) expression and activity to remove DNA oxidized and bulky lesions. The enhancement in antioxidants inhibits NOX1, which is highly present in XP-C cells. This will reduce ROS level, thereby preventing BER's inhibition, oxidative DNA damage, and an increase of SIRT1, which usually halts P53. This will allow P53 to increase and function normally in initiating DNA repair (NER and BER). The red cross= inhibition of path, green arrow=activates, red arrow=inhibits.

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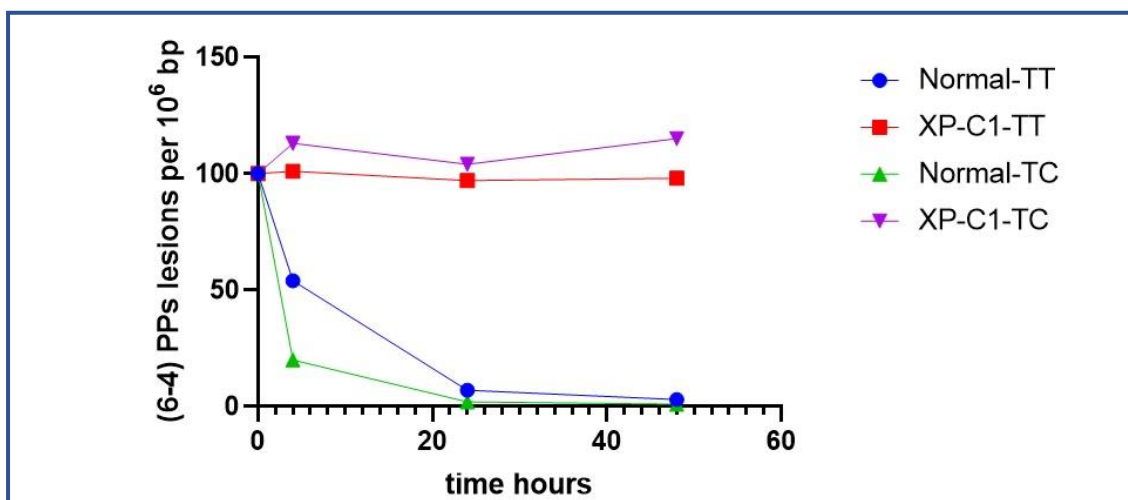
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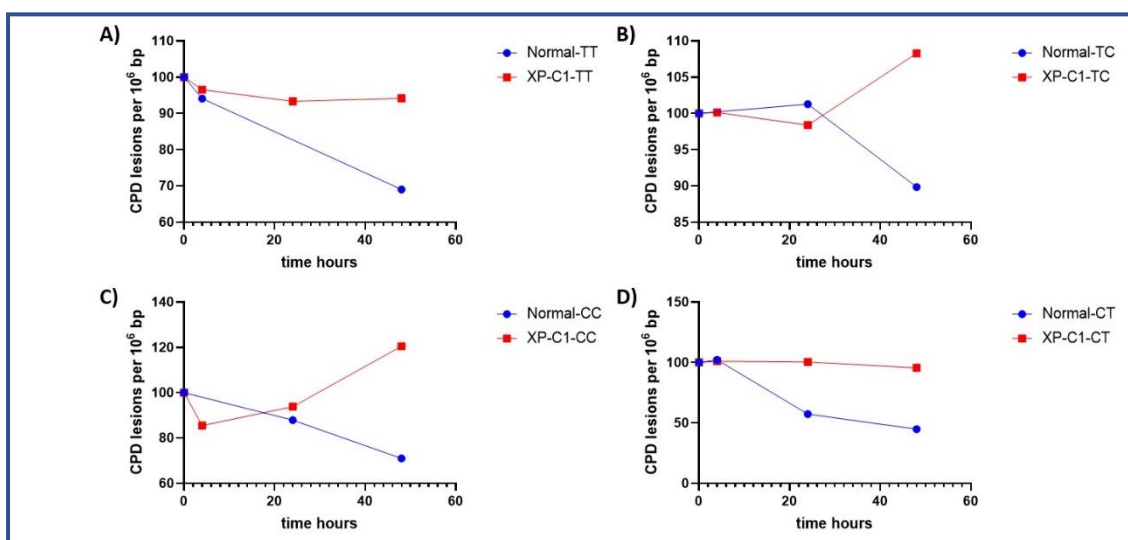
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## *Annex 1- Preliminary Results*

- **Part One “Deciphering the Role of XPC in BER and Oxidative Stress”**
  - **Bulky lesions repair in normal and XP-C1 primary fibroblasts (HPLC-MS/MS)**



Supplementary figure 1. TT and TC (6-4) PPs kinetic repair in normal vs XP-C1 fibroblasts.



Supplementary figure 2. TT, TC, CC, CT CPDs kinetic repair in normal vs XP-C1 fibroblasts.

*Note: We tested another drug (Acetohexamide) but we did not add it to have a coherence in our manuscript where we focused on drugs that have similar mechanism of action.*



# Xeroderma Pigmentosum C (XPC) Mutations in Primary Fibroblasts Impair Base Excision Repair Pathway and Increase Oxidative DNA Damage

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Xeroderma Pigmentosum C (XPC) is a multi-functional protein that is involved not only in the repair of bulky lesions, post-irradiation, via nucleotide excision repair (NER) *per se* but also in oxidative DNA damage mending. Since base excision repair (BER) is the primary regulator of oxidative DNA damage, we characterized, post-Ultraviolet B-rays (UVB)-irradiation, the detailed effect of three different XPC mutations in primary fibroblasts derived from XP-C patients on mRNA, protein expression and activity of different BER factors. We found that XP-C fibroblasts are characterized by downregulated expression of different BER factors including *OGG1*, *MYH*, *APE1*, *LIG3*, *XRCC1*, and *Poβ*. Such a downregulation was also observed at *OGG1*, *MYH*, and *APE1* protein levels. This was accompanied with an increase in DNA oxidative lesions, as evidenced by 8-oxoguanine levels, immediately post-UVB-irradiation. Unlike in normal control cells, these oxidative lesions persisted over time in XP-C cells having lower excision repair capacities. Taken together, our results indicated that an impaired BER pathway in XP-C fibroblasts leads to longer persistence and delayed repair of oxidative DNA damage. This might explain the diverse clinical phenotypes in XP-C patients suffering from cancer in both photo-protected and photo-exposed areas. Therapeutic strategies based on reinforcement of BER pathway might therefore represent an innovative path for limiting the drawbacks of NER-based diseases, as in XP-C case.

**Keywords:** Xeroderma Pigmentosum C, nucleotide excision repair, base excision repair, ultra violet (UV) light, oxidative DNA damage, oxidative stress, skin cancer

## INTRODUCTION

Skin is considered a first line of defense protecting the human body against several chemical and physical stressors (such as microbial infections, irradiation, toxic substances, pollutants) that can generate molecular DNA lesions at a rate of 1,000 to 1,000,000 lesions per cell per day (Kelley, 2012). Such lesions could be repaired via different repair systems [such as base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR)] that are specialized to remove DNA

damage and maintain genome integrity. Ultraviolet B rays (UVB) (280–315 nm), an environmental stress, could act as a carcinogen that triggers tumor-initiation, -promotion, and progression. UVB, by inducing both direct and indirect DNA damage, is capable of causing genomic instability, thus leading to acute- or delayed-skin lesions (D'Orazio et al., 2013; Melis et al., 2013a). Direct lesions, including pyrimidine (6–4) pyrimidone photoproducts [(6–4) PPs] and cyclobutane pyrimidine dimers (CPDs), can cause UV-signature mutations (C > T and CC > TT transition mutations). Such mutations usually contribute to a dominant phenotype as in the case of *p53* gene mutations that are dominant in skin cancer (Ravanat et al., 2001; Kemp et al., 2017). Indirect damages, such as 8-oxoguanine (8-oxoGua), are oxidative DNA damage occurring at a rate of  $10^4$  hits per cell per day in humans and are usually triggered by UV-induced reactive oxygen species (ROS) that will also damage protein and lipid cellular molecules. However, unlike these molecules, DNA lesions are not replaced with new molecules rather repaired (Ravanat et al., 2001; Rezvani et al., 2006; Melis et al., 2013b). If left unrepaired, 8-oxoGua may give rise to the oxidative stress hallmark, GC→TA transversion mutation, subsequently, sporadic and hereditary cancerogenesis (Hegde et al., 2008). Therefore, these oxidized bases are repaired via BER pathway for maintaining genome integrity and survival, consequently preventing cancer and aging (David et al., 2007; Hegde et al., 2008; Krokan and Bjørås, 2013). In general, BER corrects small base lesions from oxidation, deamination and alkylation in the nucleus and mitochondria. First, depending on the type of lesions and cell's physiological state, a selective DNA glycosylase will recognize and remove the base lesion, leaving an abasic site that is further processed by short-patch or long-patch repair. The subsequent steps are incision, end-processing, repair synthesis, and ligation (Krokan and Bjørås, 2013). Meanwhile, direct bulky photoproducts (CPDs and (6–4) PPs) are removed by NER pathway to prevent UV-mediated mutagenesis and maintain cell and tissue viability post-stress. NER is regulated by DNA damage-induced signaling pathway (DDR pathway) and subdivided into global genome NER (GG-NER) and RNA-polymerase dependent transcriptional coupled NER (TC-NER) (Park and Kang, 2016; Kemp et al., 2017). Both sub-pathways differ in their recognition step, speed, and efficiency. Hereditary alterations in NER-genes may result in severe diseases, such as Cockayne syndrome, Trichothiodystrophy, and Xeroderma Pigmentosum (XP) (Park and Kang, 2016).

Xeroderma Pigmentosum is a rare, recessive, cancer-prone, autosomal genodermatosis with an incidence rate of 1 in 250,000 in North America, and 1 in 1,000,000 in Europe (Lehmann et al., 2011; Pázmándi et al., 2019). Its prevalent symptoms include photosensitivity, cutaneous atrophy, dry pigmented-freckled skin, and a 2,000 and 10,000-fold incidence increase of melanoma and non-melanoma skin cancers, respectively. It is characterized by the accumulation of mutations either in proto-oncogenes (such as *BRAF* and *MYC*) or tumor suppressor genes (such as *P53* and *PTCH1*) that persist due to NER defect whereby neither DNA repair nor apoptosis occurs (Daya-Grosjean and Sarasin, 2004; Murray et al., 2016; Zebian et al., 2019). Amongst XP patients, XPC patients have a lost or mutated XPC protein, the main initiator of GG-NER. Not only

do they suffer from cancer in photo-exposed areas, but also XP-C patients are characterized by a 10 to 20-fold increased risk of developing internal malignancies in photo-protected sites (Zebian et al., 2019). Analysis of these internal tumors indicated that mutations are most likely caused by unrepaired oxidative DNA damages (Melis et al., 2013b). Remarkably, primary internal tumors (such as lung, uterus, thyroid, breast, and thyroid malignancies) have also been reported in XP-C patients (Hosseini et al., 2015). This predicts that XPC is involved in pathways other than NER. In this context, it has been demonstrated that NER is engaged in processing oxidative DNA lesions that are usually repaired by the BER pathway (Hutsell and Sancar, 2005). It is suggested that this role could be done by XPC (Murray et al., 2016). Researchers had started to suggest a direct link between XPC, BER, and oxidative DNA damage. For instance, it has been reported that XPC mutation leads to 8-oxoguanine (8-oxoGua) persistence, where this effect can be inverted by XPC-overexpression (D'Errico et al., 2006). Moreover, it has been demonstrated that XPC knockdown in normal keratinocytes leads to metabolism alterations through NADPH-oxidase-1 (NOX1) and ROS upregulation (Rezvani et al., 2011). Furthermore, a previous study proposed that XPC recognizes oxidative DNA damage directly, and thus it will be recruited solely without other GG-NER factors (Hosseini et al., 2015). Others demonstrated that XPC stimulates the activities of distinct glycosylases (such as OGG1 and MPG) (Zebian et al., 2019). This may explain the different cancer etiology in patients where increased intracellular oxidative DNA damage may function synergistically with altered DNA repair response to promote tumorigenesis and/or premature aging (one of the major XP-C disorder's clinical features) (Hosseini et al., 2014, 2015).

In this study, we deciphered, post-UVB-irradiation, the effect of three different XPC mutations on the expression status and activity levels of different components of BER pathway. This unraveled the adaptation of BER components to XPC mutations and could enable: (1) better understanding of skin and internal cancers' etiology; (2) identification of risk factors in XP-C patients; and (3) provide better insights toward designing novel therapeutic or preventive strategies.

## MATERIALS AND METHODS

### Primary Fibroblasts Isolation and Culture

Three unrelated patients clinically classified as classical XP-C with no associated neurologic or extracutaneous findings were included in this study (Supplementary Table S1). After the concerned Ethical Committee agreed to perform the analysis and the patients' parents gave their informed consent, XP-C fibroblasts were isolated from punch biopsies obtained from non-exposed patients' body sites followed by their sequencing as previously described (Soufir et al., 2010). These cells are compatible with our aim in explaining the reason for cancer development in photo-protected areas of XP-C patients. They were compared to normal primary fibroblasts ( $n = 3$ ) extracted by our laboratory (SyMMES, CIBEST, CEA).

Fibroblasts were cultured in DMEM medium (DMEM, high glucose, GlutaMAX<sup>TM</sup> Supplement, +Pyruvate, Thermo

Fisher Scientific) with 10% SVF and 1% penicillin/streptomycin in falcon flasks (75 cm<sup>2</sup>) at 37°C in 5% CO<sub>2</sub> incubator. 3000 cells/cm<sup>2</sup> were seeded 7 days to reach 80% confluency.

### Short-Term Cytotoxicity Assay, MTT

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (SIGMA) was used to evaluate cell viability 24 h post-UVB-irradiation. 200 µL MTT (5 mg/mL)/well were added to six well-plates followed by a 2 h-incubation at 37°C and discarding the supernatant. Next, 2 mL DMSO/well was added along with 20 min shaking. Solutions were then transferred to 96 well microplates and the absorbance of formazan crystals at 560 nm was measured by a spectrophotometer (Spectramax M2; from Molecular Devices) allowing us to quantify the number of living cells. All data were normalized by comparison with the yield of MTT conversion in non-irradiated control samples set at 100% viability.

### Immunofluorescence and Associated Microscopy

Cells were seeded in 96 well microplates, after that exposed to UVB-irradiation at a dose of 0.03 J/cm<sup>2</sup>. Following that, cells were fixed at different time points (0 and 24 h) with 4% paraformaldehyde, and then permeabilized with 0.2% Triton X-100. After washing with PBS, DNA was denatured with 2 M HCL, followed by blocking with 3% FBS in PBS. The primary anti-pyrimidine (6–4) pyrimidone photoproducts (64M-2, Cosmo Bio) and secondary antibodies (Alexa Fluor 488 goat anti-mouse, Invitrogen) were diluted in 1% FBS and incubated with three washing steps between them. Finally, nuclear DNA was counter-stained with Hoechst (Sigma-Aldrich). Cell images were acquired by the Cell-insight NXT high content screening platform at 10× magnification. Data were normalized against non-irradiated samples.

### Treatment and Cell-Pellets Preparation

Sub-confluent cells (80%) were either exposed or not to 0.05 J/cm<sup>2</sup> UVB-irradiation. Fibroblasts were harvested 4 h post-irradiation [real-time quantitative PCR (qRT-PCR) assay, western blot], centrifuged, and rinsed with PBS (Invitrogen, Carlsbad, CA, United States). Pellets were rapidly frozen at –80°C until further use.

For comet assay, cells were either exposed or not to 0.05 J/cm<sup>2</sup> UVB-irradiation then harvested after 0, 2, and 24 h. Cells were then centrifuged, rinsed with PBS, and dissolved in freezing buffer (pH = 7.6) to be stored at –80°C.

### Reverse Transcription and Real-Time Quantitative PCR (qRT-PCR) Analysis

Total RNA was isolated using GenElute<sup>TM</sup> Mammalian Total RNA Miniprep kit (Sigma-Aldrich) and then quantified using Nanodrop 1000 from Thermo scientific to check its integrity. Another method for assessing RNA integrity was to add 5 µL of sample/well (each sample tube consisted of 1 µL RNA, 9 µL water, and 2 µL DNA gel loading dye) in agarose gel using LT4

DNA ladder. Total RNA was considered intact when two acute 28S and 18S bands were visualized.

RNA (2 µg) was reversely transcribed to cDNA (Superscript<sup>®</sup> III Reverse Transcriptase, Invitrogen, Carlsbad, CA, United States) in the presence of random primers (100 ng/µL, Promega, Charbonnières, France), dNTP mix (10 mM, Sigma-Aldrich, Saint-Quentin-Fallavier, France), 5×-First-strand buffer (Invitrogen, Carlsbad, CA, United States), DTT (0.1 M, Invitrogen, Carlsbad, CA, United States), ribonuclease inhibitor (45 U/µL, Sigma-Aldrich, Saint-Quentin-Fallavier, France) and SuperScript III enzyme (200 units, Invitrogen, Carlsbad, CA, United States).

Next, 5 µL of each cDNA (25 ng/µL) was used in qPCR reactions with gene-specific primers (**Supplementary Table S2**), and qPCR was performed by MESA Blue qPCR MasterMix Plus for SYBR<sup>®</sup> Assay with low ROX (Eurogenetic, Angers, France). Samples were run in triplicates through Bio-Rad CFX96<sup>TM</sup> Real-time Sys (C1000 Touch<sup>TM</sup> Thermal Cycler). At the end of each run, the integrity of amplification was verified by a single melt-curve peak per product. Expression levels of target genes were normalized to those of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Calculations for determining the relative level of gene expression were made using the  $\Delta\Delta CT$  method for quantification as reported by Livak and Schmittgen (2001).

### Western Blot

Total proteins were extracted from fibroblasts upon adding 100 µL of lysis buffer to the cell pellet followed by vortexing and incubation on ice for 30 min (vortexed every 10 min). The mixture was then transferred to 1.5 mL eppendorf and centrifuged at 16000 rpm for 15 min at 4°C. Total proteins were dosed by microBC assay protein quantification kit according to the manufacturers' instructions. Western blotting was performed as previously described (D'Errico et al., 2006). Briefly, equal amounts of proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane (*Trans-Blot<sup>®</sup> Turbo<sup>TM</sup>* Transpack, Bio-Rad), followed by blocking the membrane with 5% lyophilized milk and the addition of 1/1000 diluted primary anti-XPC (mouse monoclonal antibody; Thermo Fisher Scientific), 1/50000 diluted primary anti-OGG1 (rabbit monoclonal antibody; Abcam), 1/250 diluted primary anti-MYH (rabbit monoclonal antibody; Novus Biologicals), and 1/1000 diluted primary anti-APE1 (rabbit monoclonal antibody; Sigma Aldrich). Incubation at 4°C for overnight was done, followed by incubation with mouse or rabbit anti-HRP (1/10000 diluted secondary antibody) and the addition of clarity<sup>TM</sup> western ECL substrate (Bio-Rad). The membrane was visualized through Bio-Rad Molecular Imager<sup>®</sup> ChemiDoc<sup>TM</sup> XRS + using Image Lab<sup>TM</sup> software. After a stain-free total protein detection, target proteins' expression was normalized to the total protein extract.

### Comet Assay ± FPG

Comet assay is a single-cell gel electrophoresis assay that is used to measure the DNA lesions of cell extracts, and consequently, can monitor the excision repair capacity when it is employed at various time points post-treatment of cells. More specifically,

upon adding FormamidoPyrimidine [fapy]-DNA Glycosylase (FPG) enzyme, we were able to detect 8-oxoguanine excision activity. Hydrogen peroxide ( $H_2O_2$ , 400  $\mu M$ ) was used as an internal positive control. Cells were plated in 100 mm dishes as triplicates and irradiated with 0.05  $J/cm^2$  UVB-irradiation then collected after 0, 2, and 24 h. Immediately following treatment, cells were harvested, counted, and suspended at a concentration of 200,000 cells in 100  $\mu L$  freezing buffer. Samples were stored at  $-80^\circ C$  until use.

Briefly, slides were prepared with normal agarose coating 1 day in advance. On the day of the experiment, cells were deposited on the slides with 0.6% solution of low-melting agarose followed by adding a coverslip, immersing in lysis buffer, and incubation for 1 h after which a three times wash with Tris-HCL 0.4 M (neutralizing buffer) was done. FPG 0.05  $u/\mu L$  (1.25  $\mu L/slide$ ) was then prepared in which 100  $\mu L$  FPG solution with or without FPG enzyme was deposited on the slides and covered with coverslips. The slides were set on a humidified bed and added in a  $37^\circ C$  incubator for 40 min. The reaction was stopped by incubation for a few minutes on ice. After digestion, the slides were transferred to an electrophoresis tank filled with electrophoresis buffer pre-chilled at  $4^\circ C$ . The slides were left at room temperature for 30 min, and electrophoresis was subsequently done for 30 min at 25 V and 300 mA. The slides were then rinsed 3 times with Tris-HCL 0.4 M. 50  $\mu L$  of Gel Red was added per slide, and a coverslip was added for reading the next day. We read the slides using a  $10\times$  objective microscope and Comet Assay IV software (Perceptive Instruments, Suffolk, United Kingdom). 50 randomly selected nuclei were scored

in each slide and triplicate slides were processed for each experimental point. The extent of damage was evaluated by the Tail DNA value defined as the percentage of DNA in the tail of the comet. The normalization was done by doing a ratio of irradiated/non-irradiated at each condition.

## Statistical Analysis

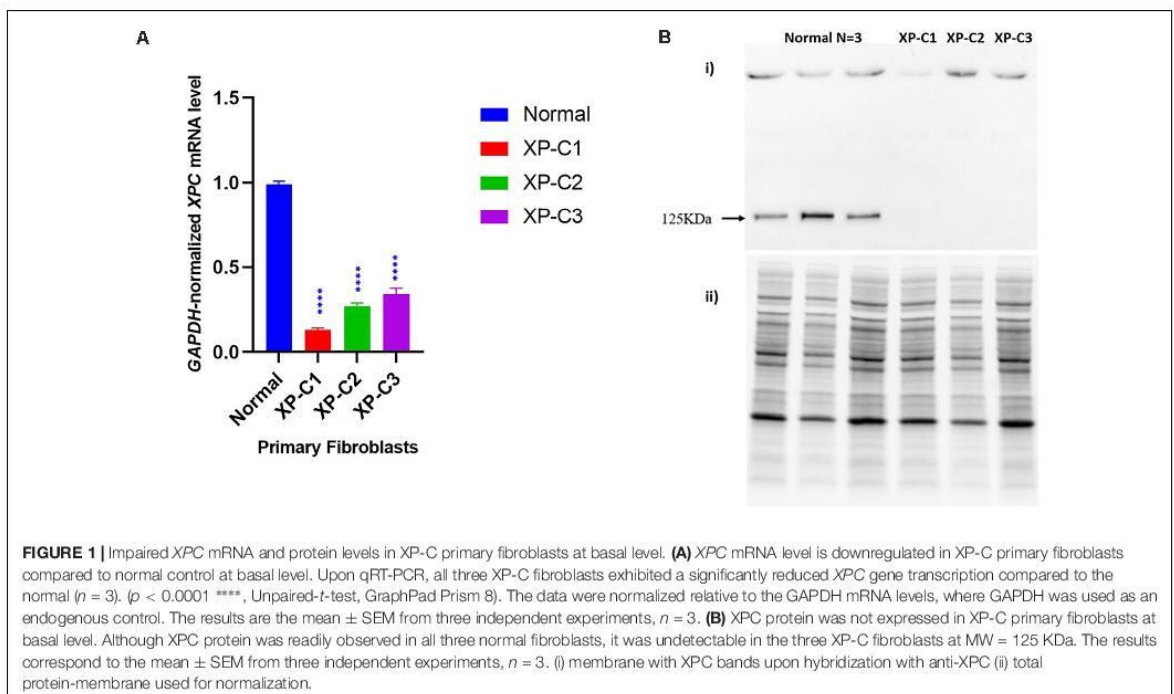
The data were expressed as mean  $\pm$  SEM for three independent experiments. Statistical significance of data was assessed using the student's *t*-test (GraphPad Prism 8) after checking variance homogeneity with the Levene's test and normality by normality test. Student paired-*t*-test allows us to compare each sample between two different conditions while student unpaired-*t*-test allows us to compare different samples at each condition. Results were considered significant for  $p$ -value  $\leq 0.05$ .

## RESULTS

### Characterization of Normal and XP-C Primary Fibroblasts

#### Dysregulated XPC mRNA and Protein Expression Levels in XP-C Fibroblasts Compared to Normal

We first examined using qRT-PCR the mRNA levels of *XPC* in the normal versus XP-C fibroblasts at basal state. As shown in **Figure 1A**, XP-C1, XP-C2, and XP-C3 exhibited a drastically significant ( $p < 0.0001$ ) lower *XPC* mRNA levels than normal fibroblasts ( $n = 3$ ) (~ 8, 4, and 3-fold downregulation,





respectively). In a next step, western blot was performed to validate this expression profile at protein level. As seen in **Figure 1B**, XPC protein (band size = 125 KDa) was detected in normal cells but was totally absent in the three XP-C fibroblasts. This indicates that all the three different XPC mutations led to an impairment in XPC gene expression and absence at the protein level.

#### Similar Photosensitivity Between Normal and XP-C Primary Fibroblasts

We checked whether XP-C and normal fibroblasts differ in their photosensitivity as the former are suspected to be hyper-photosensitive. Hence, we did a cytotoxicity test 24 h post-UVB-irradiation. The viability of the different cells was gradually decreasing in a manner dependent on the increasing UVB doses. Generally, there was no significant difference in photosensitivity between the control and XP-C fibroblasts (**Figure 2**). At the highest dose (1.5 J/cm<sup>2</sup>), less than 20% of cells survived.

The LD<sub>50</sub> was determined for all primary fibroblasts (**Supplementary Table S3**) using regression analysis.

Based on this cytotoxicity test, we decided to perform our experiments at 0.05 J/cm<sup>2</sup> UVB dose. It is a moderate cytotoxic dose which kills <50% of cells and is thus suitable for the investigation of DNA oxidative lesions and their repair.

#### Dysregulated Photoproducts' Repair in XP-C Primary Fibroblasts Compared to Normal

Xeroderma Pigmentosum C protein does not recognize the lesion itself but rather binds to the associated helix distortion. Hence, XPC binds with a high affinity to pyrimidine (6–4) pyrimidone photoproducts [(6–4) PPs], inducing high helical alterations (Nemzow et al., 2015). For that, we were interested in following the kinetics of repair of (6–4) PPs by immunocytochemistry, where an anti-(6–4) PP was used to detect (6–4) PPs at 0 and 24 h. 24 h post-UVB-irradiation around 70% of lesions were repaired in normal fibroblasts. However, this was not the case in the three XP-C fibroblasts where elevated levels persisted. Almost 20% were repaired in XP-C1, XP-C2, and XP-C3 as shown in **Figure 3** and **Supplementary Figure S1**. This lesion persistence was significant in XP-C1, XP-C2, and XP-C3 compared to normal fibroblasts ( $p < 0.001$ ,  $p < 0.001$ ,  $p < 0.05$ , respectively).

#### Dysregulated BER-Associated Gene Expression in XP-C Fibroblasts Compared to Normal, Post-UVB-Irradiation

We examined the mRNA levels of a series of genes involved in BER between normal and XP-C primary fibroblasts 4 h post-UVB-irradiation (**Figure 4**). We scanned the whole BER pathway starting from the initiation and base removal steps (*OGG1*, *MYH*), passing by abasic sites removal (*APE1*), and to newly synthesized nucleotide (*PoIB*) and ligation (*LIG3*, *XRCC1*).

We observed that 8-oxoguanine glycosylase (*OGG1*), MutY Adenine DNA Glycosylase (*MYH*), apurinic endonuclease 1 (*APE1*), ligase 3 (*LIG3*), and X-ray repair cross-complementing 1, *LIG3*'s cofactor, (*XRCC1*) were characterized by a significantly

lower mRNA levels in all three XP-C fibroblasts compared to normal cells ( $p < 0.01$ ). On the other hand, *PoIB* transcription levels were significantly downregulated in XP-C1 and XP-C3 ( $p < 0.01$ ) but not XP-C2.

#### Dysregulated BER-Associated Protein Expression in XP-C Fibroblasts Compared to Normal, Post-UVB-Irradiation

To better understand the effect of XPC mutation on BER's regulation, we studied the difference in *OGG1*, *MYH*, and *APE1* protein levels between normal and XP-C primary fibroblasts 4 h post-UVB-irradiation (**Figure 5** and **Supplementary Figure S2**). Such proteins were selected due to their main role in initiating BER of oxidative damage. *OGG1* and *MYH* were characterized by a significantly ( $p < 0.05$ ) lower protein levels in all three XP-C fibroblasts compared to normal cells. Meanwhile, *APE1* protein expression was significantly downregulated in XP-C2 ( $p < 0.0001$ ) but not XP-C1 and XP-C3.

#### Lower Intrinsic Base Excision-Repair Capacities in XP-C Primary Fibroblasts Compared to Normal

Standard alkaline comet (–FPG) is a genotoxic assay that measures DNA single-strand breaks (SSBs) and alkali-labile sites (ALS). Once FPG glycosylase is added, oxidized purines (including 8-oxoguanine) can be evaluated. This is done by the excision of FPG-sensitive sites (oxidized purines) converting abasic sites into DNA SSBs. The net cleavage sites (oxidized purines) generated by FPG activity are calculated by subtracting the value of DNA damage at alkaline conditions from that with FPG treatment (as presented in **Figure 6C**). This FPG enzyme is functionally similar to *OGG1* where both recognize oxidized purines, majorly Fapy and 8-oxoGua (Hu et al., 2005).

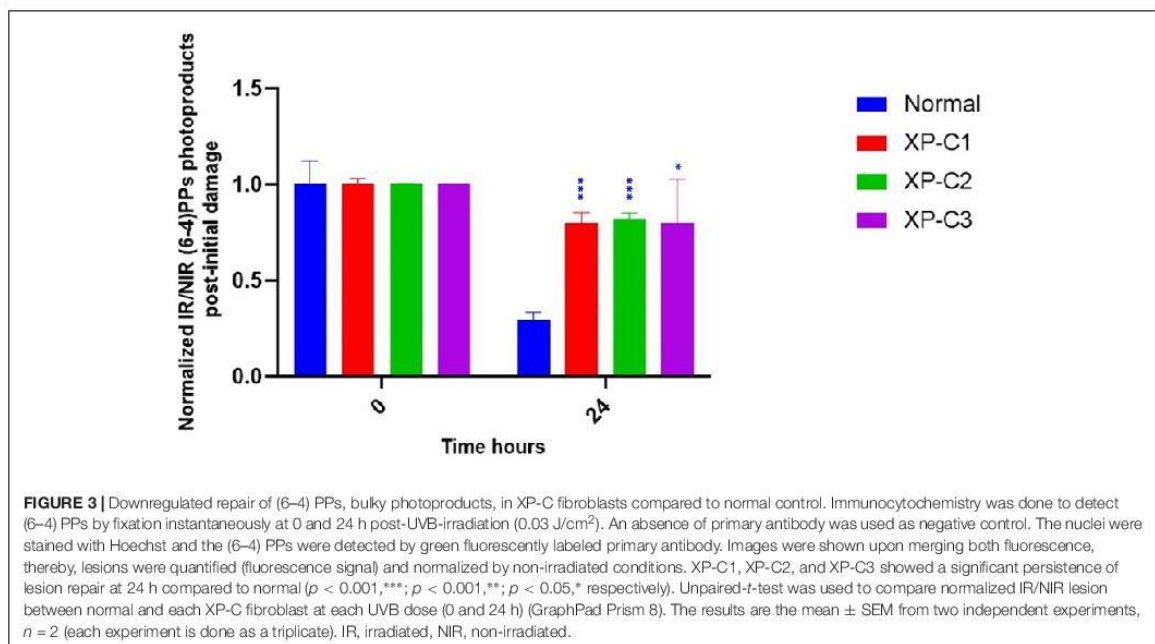
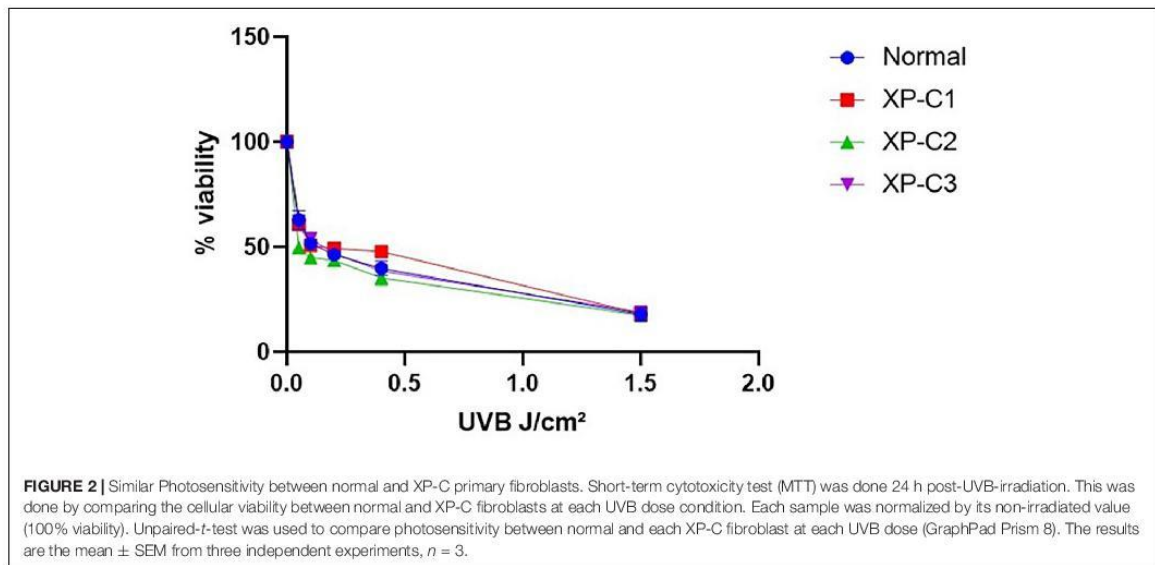
**Figure 6A** shows an example of comets done  $\pm$  FPG in normal and XP-C1 fibroblasts, and the positive control, H<sub>2</sub>O<sub>2</sub>. The head of comet represents intact DNA, meanwhile, the tail represents damaged DNA.

#### In Absence of FPG

Ultraviolet B rays-irradiation increased SSBs to the maximum at time = 0 h in all primary fibroblasts. However, this was significantly higher in XP-C1 and XP-C2 compared to the control ( $p < 0.01$ , \*\*). On the contrary, the increase of DNA damages in XP-C3 was not significant compared to the control. During the course of the experiment, lesions were repaired faster in the normal fibroblasts. Similarly, at times = 2 and 24 h, XP-C1 and XP-C2 had significantly higher DNA lesions compared to normal ( $p < 0.01$  and  $p < 0.05$ , respectively) (**Figure 6B**).

#### In Presence of FPG

Upon adding FPG, % mean tail intensity increased due to more breaks in DNA where FPG will specifically excise oxidized purines (oxidative DNA lesions, as 8-oxoguanine and Fapy). This was clearly demonstrated upon a significant increase in intensity when comparing the samples with/without FPG at times = 0 and

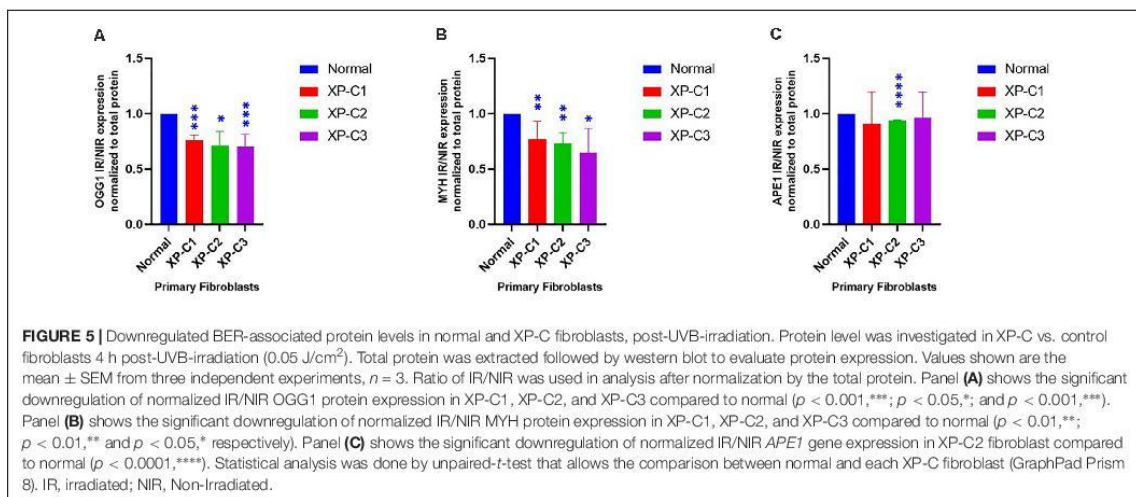
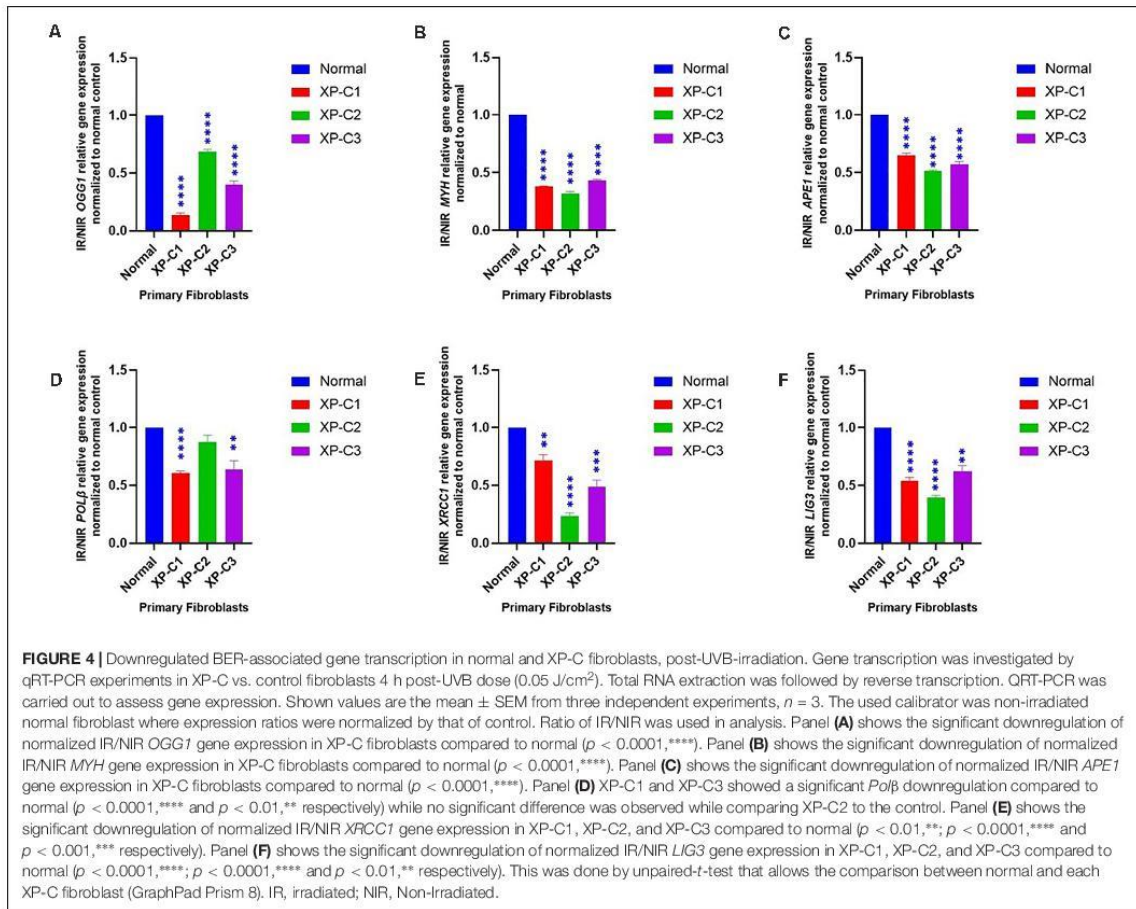


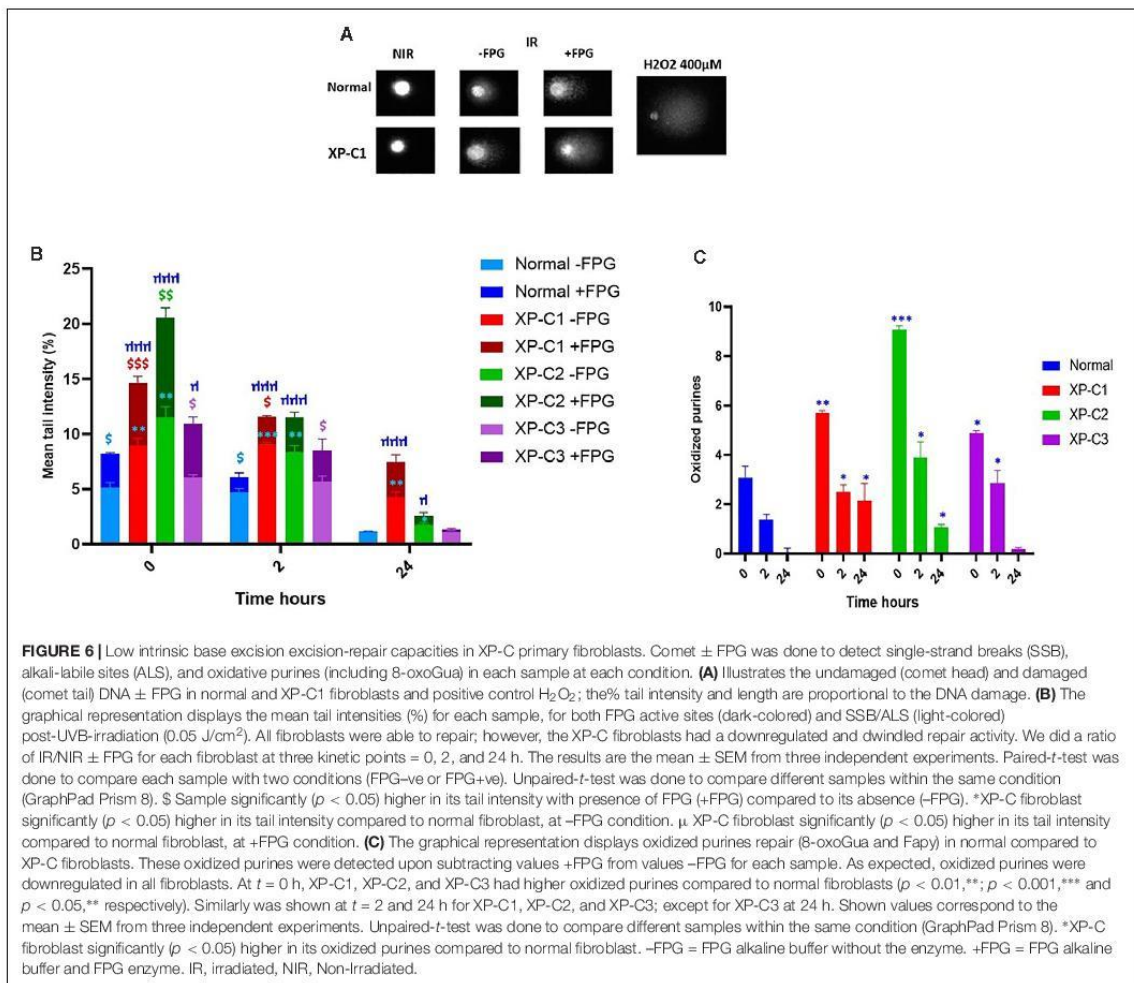
2 h ( $p < 0.05$ , \$). When comparing control cells to each XP-C fibroblast: significantly higher DNA lesions were observed in XP-C1 and XP-C2 at  $t = 0$ , 2 and 24 h. Meanwhile, XP-C3's higher DNA lesions were only significant at  $t = 0$  h ( $p < 0.05$ ,  $\mu$ ) (Figure 6B).

Figure 6C is a zoom in to Figure 6B. It represents oxidized purines present in each sample and its kinetic repair follow up. At  $t = 0$  h, XP-C1, XP-C2, and XP-C3 had higher oxidized purines

compared to normal control ( $p < 0.01$ ,  $p < 0.001$ , and  $p < 0.05$ , respectively). Similarly, at  $t = 2$  and 24 h, XP-C fibroblasts showed significantly higher oxidized purines compared to control ( $p < 0.05$ ); except for XP-C3 at 24 h.

On the contrary to Berra et al. (2013), the repair was not similar between XP-C deficient and XP-C-proficient fibroblasts (normal control). Induction of single strand breaks and oxidized purines was more prominent and persistent (slower rate of





repair) in XP-C fibroblasts compared to control at 0, 2, and 24 h where both lesion types are usually repaired by BER.

## DISCUSSION

It is well described that several cancer-prone diseases result from defective nucleotide excision DNA repair. This is well known for XPC mutations that are associated with high rate of basal cell carcinomas (BCCs), squamous cell carcinomas (SCCs), and melanoma in photo-exposed skin (De Boer and Hoeijmakers, 2000).

In a previous report, Agar et al. was able to detect 8-oxoGua and its correlated G:C  $\rightarrow$  T:A transversion, thus indicating the contribution of such oxidative DNA damage to skin cancer (D'Errico et al., 2006). Hence, they speculated that the increased UV-induced skin cancer could be attributed not only to reduced

NER but also to impaired BER, the foremost oxidative DNA damage repair system (D'Errico et al., 2006). Besides, XP patients also suffer from internal cancers that could be contributed to ROS accumulation and oxidative stress. Indeed, exploiting the long term follow-up of XP patients at NIH (National Institute of Health) from 1971 to 2009, Bradford et al. (2010) have reported that internal cancers (17%,  $n = 5$ ) could be considered as the third factor [besides skin cancer (34%,  $n = 10$ ) and neurologic degeneration (31%,  $n = 9$ )] leading to XP patients' death. Of note, among XP patients in their cohort, only patients belonging to XP-C group (6 out of 12) died due to internal cancers, including central nervous system cancers ( $n = 3$ ), peripheral nerve cancer ( $n = 1$ ), lung cancer ( $n = 1$ ) and endocervical adenocarcinoma of the uterus ( $n = 1$ ). Furthermore, Live imaging data has shown that XPC is rapidly recruited to the oxidized bases, independent on the recruitment of downstream NER factors (Menoni et al., 2012).

Thus, our study aimed to assess the effect of different XPC-mutations on BER in order to decipher how its phenotype is linked to a defect in BER's DNA repair capacity.

### Characterization of Normal and XP-C Primary Fibroblasts

In this study, we used fibroblasts isolated from patients with different clinical manifestations. XP-C1 had frame-shift mutation (c.1643\_1644del). Meanwhile, XP-C2 and XP-C3 patients were compound heterozygotes for *XPC* mutations (Supplementary Table S1). As shown in Figure 1, while the full-length XPC protein was undetectable in fibroblasts from the three XP-C patients, XPC mRNA was expressed in these cells although at a significantly lower level than that of control fibroblasts. This suggests that XPC-mutated mRNAs may contain premature termination codons (PTCs) that induce non-sense mediated mRNA decay (NMD pathway) as a protective method to prevent deleterious-truncated proteins' expression. Our observations are in agreement with what was described by Chavanne et al. (2000); Khan et al. (2005), and Senhaji et al. (2012) who reported that mutations in the *XPC* gene are expected to cause protein truncations as a result of non-sense, frameshift, and deletion events. Thus, XPC mRNA levels may be considered as a predictive-diagnostic biological marker protecting from skin cancer since its low expression level is linked to an increased susceptibility to cancer in XPC-mutation carriers (Khan et al., 2005).

Even though photosensitivity is a XP-linked symptom, it was not the case when we did the short-term cytotoxicity test on XP-C fibroblasts. Similarly to De Waard et al. (2008), all fibroblasts shared similar moderate UVB-photosensitivity. It was clearly demonstrated that *XPC* mutations and deletions do not shorten lifespan in mice rather perhaps could be early events that induce late onset, slow growth/progression of tumor (Hollander et al., 2005). This suggests that cells harboring accumulated UVB-induced DNA damage are not eliminated with apoptosis. In agreement, Hollander et al. (2005) had shown that *Xpc*<sup>-/-</sup> mice develop spontaneous lung tumors at old age, due to an overtime accumulating effect passing the threshold of cancer risks. Also, Rezvani et al. (2006) showed that XP-C cells underwent spontaneous tumoral transformation owing to their susceptibility to accumulate DNA damage. XPC protein is not essential for cellular viability, proliferation or development as its dysfunction does not result in stalling RNA replication fork; hence, the difference between normal and mutated fibroblasts is the persistence of mutations in the latter arising to genomic instability and abnormal survival. In accordance, XP-C patients have been reported to suffer less from acute burning on minimal sun exposure than other XP complementary groups (Chavanne et al., 2000; Sethi et al., 2013). Indeed, it has been suggested that XP-C patients are diagnosed later in their life owing to their normal sunburn reactivity compared to the other XP groups. Consequently, XP-C patients are less likely to adhere to ultraviolet radiation protection and precaution early in their lives. As the inevitable consequence, they later develop a more aggressive phenotype compared to other XP-groups (Fassihi et al., 2016).

This implies that sensitivity to sunburn may not always be an adequate clinical marker of an individual's skin cancer risk rather BER capacity, GG-NER in particular, may be a better predictor (Berg et al., 1998).

Hence, we monitored the repair of bulky lesions in normal and XP-C fibroblasts to investigate XPC mutations' effect on GG-NER repair activity. XPC protein's binding affinity to DNA correlates with the extent of helical distortions. Although it recognizes (6–4) PPs and CPDs, it binds with more specificity to the former that are bulkier than CPDs (Melis et al., 2008). Thus, we decided to study the effect of XPC mutations on the repair of (6–4) PPs at 0 and 24 h post-UVB-irradiation. Repair synthesis in XPC-mutated cells ranges between 10 and 20% compared to normal (Chavanne et al., 2000). In agreement with that, we showed that only ~20% of pyrimidine (6–4) pyrimidone photoproducts [(6–4) PPs] were repaired in XP-C fibroblasts after 24 h. This is consistent with what Courdavault et al. (2004) had published. (6–4) PPs were repaired efficiently within less than 24 h in cultured dermal human primary fibroblasts.

### Downregulation of Different BER-Associated Gene and Protein Levels in XP-C Fibroblasts Compared to Normal

Besides skin cancers, XP patients have a 10 to 20-fold increased risk of developing internal malignancies, such as lung, tongue, brain, and liver cancer (Bowden, 2004; DiGiovanna and Kraemer, 2012; Zhang et al., 2015; Murray et al., 2016; Zebian et al., 2019). These incidences cannot be explained unless XPC acts as a multi-functional protein involved in roles beyond GG-NER initiation. It recognizes both NER targeted lesions and base lesions which may provide it the power of determining the eventual repair type. Once cells are irradiated by UVB, stress-mediated alterations in mitochondrial and nuclear functions and oxidative unbalance will arise since almost 50% of UVB-induced lesions attribute to the formation of ROS, consequently oxidative DNA damage and cancer. These events are highly pronounced once XPC protein is dysregulated (Rezvani et al., 2011; Wölflle et al., 2011; Melis et al., 2013a; Hosseini et al., 2014; Zhang et al., 2015). For instance, there is an association between the increased lung tumor incidence and oxidative stress in *Xpc*-knock out mice (Liu et al., 2010). D'Errico et al. (2006) showed that primary keratinocytes and fibroblasts derived from XP-C patients are hypersensitive to DNA-oxidizing agents and that could be inverted by the re-expression of XPC. In good agreement, the activity of catalase, an enzyme protecting the cell from oxidative DNA damage through the conversion of H<sub>2</sub>O<sub>2</sub> into oxygen and water, was found to be decreased in XP patients (Rezvani et al., 2006). XPC has been reported to affect oxidative and energy metabolism. For instance, *in vitro* studies displayed an elevated sensitivity in *Xpc*<sup>-/-</sup> mouse embryonic fibroblasts (MEFs) to oxidative DNA-damaging agents compared to control (Melis et al., 2008). It is estimated that up to 100,000 8-oxoGua lesions can be formed daily in DNA per cell (Ba and Boldogh, 2018). They are recognized by OGG1 and MYH. The former excise 8-oxoGua directly, meanwhile, the latter removes misincorporated adenines

in front of 8-oxoguanine (8-oxoGua) during DNA replication. These excision activities will result in an AP site that will be cleaved by APE1 for the synthesis and ligation to be carried out by POL $\beta$  and LIG3, respectively (Campalans et al., 2015). It has been shown that MYH-OGG1 deficient cells are sensitive to oxidants and ROS (Xie et al., 2008). As ROS accumulation negatively regulates the activity of several important DNA repair proteins, including OGG1, its production may lead to the increase of DNA damage which supports the important role of ROS in carcinogenesis. Not only it induces oxidative DNA damage but also prevents its repair (Srinivas et al., 2018). Mutations in the *OGG1* gene can lead to lung and kidney tumors and the S326C polymorphism appears to be associated with an increased risk of esophageal, lung and prostate cancers (Thibodeau et al., 2019). In parallel, mutations in the *MYH* gene are associated with lung, colorectal and breast cancers (Hollander et al., 2005; Viel et al., 2017; Thibodeau et al., 2019). In light of these studies, several questions arise. Would this occur through lack of XPC-BER interaction? If yes, how profound is XPC's influence on BER effectiveness and expression?

Therefore, we were interested in highlighting the role of XPC as an interplay between NER and BER. For that, real-time-qPCR was done where primers anneal to each of the following BER components: *OGG1*, *MYH*, *APE1*, *POLB*, *XRCC1*, and *LIG3*. Afterward, we did western blot analysis on OGG1, MYH, and APE1. These proteins were selected due to their essential role in initiating oxidative DNA lesions' repair.

OGG1, MYH gene and protein expressions were significantly inhibited upon UVB-irradiation ( $p < 0.05$ ). Although APE1 showed a significantly inhibited gene expression in all XP-C fibroblasts ( $p < 0.0001$ ), such an inhibition was not significant at protein level except in XP-C2. These results indicate a downregulation in stimulation. In agreement with our results, it has been shown that XPC is recruited to 8-oxoguanine lesions to induce a partial removal of the oxidative DNA damage and regulate cellular stress-response (Zhou et al., 2001; Miccoli et al., 2007). This is done by stimulating OGG1's protein expression and catalytic activity and physically interacting with APE1 (D'Errico et al., 2006; De Melo et al., 2016). Hence, XPC mutation affects them directly. For example, there is an evidence that XPC P334H substitution can prevent stimulation of BER factor OGG1 (Melis et al., 2013b). However, little is mentioned in the literature about the effect of XPC on *MYH*, *LIG3*, *POLB*, and *XRCC1*. Perhaps *MYH* was barely studied because it is an indirect secondary actor in the repair of 8-oxoGua, functioning downstream of OGG1 and removing adenine bases misincorporated opposite 8-oxoGua (Forestier et al., 2012). However, due to its role, there might be a direct link between MYH and XPC. This was seen at both gene and protein levels. Meanwhile, other BER factors' mRNA downregulation could be explained by the fact that an interaction and cross-talks amongst BER factors is crucial for the recruitment to the site of repair and optimum repair efficiency (Campalans et al., 2015). Hence, since, as shown in our results, OGG1, MYH, and APE1 are affected, a stimulation to trigger the expression of downstream factors could be inhibited or slowed down where the coordination amongst the protein complexes is similar to passing of the

baton, where the repair product is handed over from an enzyme to the next one.

### Downregulation in Excision Activity of BER-Associated Enzymes in XP-C Fibroblasts Compared to Normal

Some studies have demonstrated that ROS-induced 8-oxoguanine formation, primarily in guanine-rich gene regulatory regions, inactivates OGG1's enzymatic activity (Hao et al., 2018), resulting in GC to TA transversion mutations (De Rosa et al., 2012). Hence, 8-oxoGua accumulation might be considered as a diagnostic marker for BER malfunction (Tinaburri et al., 2018). For example, OGG1-Cys enzymatic activity decreases under oxidative stress due to redox-sensitive residues in accordance with our results where there is a reverse correlation between OGG1 activity and oxidative stress (Bravard et al., 2009; De Rosa et al., 2012; Tinaburri et al., 2018). Moreover, D'Errico et al. (2006) had shown that XPC plays a role as a cofactor for the efficient 8-oxoGua excision by OGG1. XPC/P334H mutation weakens the interaction between OGG1 and XPC, resulting in a decreased glycosylase activity and turn-over (Melis et al., 2013b). Additionally, studies had demonstrated that APE1 and XRCC1 are involved in the repair of SSBs containing 3'-8-oxoGua and SSBs, respectively in human cell extracts (Okano et al., 2000; Parsons et al., 2005). Both were shown, in our data, to be downregulated at mRNA level in absence of XPC. Decreased expression of several BER factors in XP-C cells could explain why at time = 0 h, more single strand breaks and oxidative DNA damage were found in these cells compared to control. Our results also showed that repair of the oxidative damage was much lower and slower in XP-C cells than normal cells. In agreement, it had been shown that XPC deficiency impairs the repair of oxidative DNA damage induced by visible light and methylene blue where XPC had been proven to bind much better oxidative base damage than direct SSBs (Menoni et al., 2012; Melis et al., 2013b). Similarly, the level of 8-oxoGua in cells treated with KBrO<sub>3</sub> (40 mM) at different time points after exposure was much higher in XP-C cells compared to their control counterparts (D'Errico et al., 2006). Despite the dysregulation in BER's efficiency in XP-C fibroblasts, a bashful repair occurred. This could be explained by two complement scenarios: Although OGG1 is the main preferred actor in BER, other multiple of backup-glycosylases will step up once it is function becomes incompetent (Hegde et al., 2008). On the other hand, as mentioned before, XPC enhances OGG1's turnover i.e., efficiency of activity (De Melo et al., 2016). In the absence of XPC, OGG1 is stable and able to remove oxidized lesions with a less competency and slower rate.

These results suggest that increased susceptibility to internal tumors in XP-C patients and spontaneous tumors in *Xpc* mice may be due to incompetent oxidative DNA lesions repair.

It is evident now that repair of both endogenous and induced oxidative DNA damage are essential for maintaining genomic integrity and homeostasis. This involves complex interactions among BER proteins and between them and other proteins, mainly XPC (Hegde et al., 2008).

## CONCLUSION

The difference in XPC mutations among our samples allowed us to have a general and more confirmed conclusion about the effect of such protein on the expression and activity status of distinct BER system components to repair oxidized DNA damage.

Characterization of the interplay between BER factors and XPC may provide new insights about the occurrence of non-skin cancer upon XPC-deficiency. Furthermore, the synergic effects of amassed oxidative DNA damage and impaired BER could explain heterogeneity in the clinical spectrum of XP-C patients.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**. For further inquiries contact the corresponding author.

## AUTHOR CONTRIBUTIONS

NF performed all the experiments (cell culture, short-term cytotoxicity assay, qRT-PCR, western blot, Immunocytochemistry, and comet  $\pm$  FPG assay) and wrote the manuscript. WR conceived the project, grant funding, supervised the project, and revised the manuscript. HRR contributed to formal analysis and manuscript editing and revision. MF-K and BB aided in the manuscript revision. HF-K participated in

the statistical analysis and manuscript revision. FK assisted in the immunocytochemistry experiment. DB assisted in certain experiments. WM prepared the XP-C fibroblasts. They were sequenced and had their mutation identified by CG and FM-P. All authors read and approved the final version of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2020.561687/full#supplementary-material>

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Immunology and Cancer Biology

### **Book Chapter**

## **Link between Base Excision Repair (BER), Reactive Oxygen Species (ROS), and Cancer**

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### **ROS: Sources, Targets, Cancer, and Defense Mechanisms**

Different cellular organelles contribute to the production of free electron-radical and stable non-radical ROS (Reactive Oxygen Species). This includes lysosomes, cytoplasm, peroxisomes, and

endoplasmic reticulum [1]. Most importantly, eukaryotic mitochondrial membrane (complex I, complex II, complex III, inner membrane components) produces ROS through the electron transport chain. As shown in Reaction 1, it reduces oxygen into superoxide ( $O_2^{\cdot-}$ ) that can further be converted into hydrogen peroxide ( $H_2O_2$ ) via dismutation [2].



**Reaction 1:** The Conversion of Oxygen ( $O_2$ ) into different ROS due to mitochondrial enzymes and processes.

At physiological levels, ROS have a vital role in fertilization, intra/intercellular signaling pathways, and inflammatory immune responses against infections [3,4]. For instance, upon pathogen invasion, T-lymphocytes and phagocytes (eosinophils, neutrophils, and macrophages) release hydrogen peroxide, NADPH oxidase-dependent superoxide anion, and its derivatives [3]. Other cell types (fibroblasts, keratinocytes, endothelial, and muscle cells) produce ROS as a regulator of cellular signaling pathways, including growth, differentiation, progression, and cell death [3,5]. ROS can stimulate NF $\kappa$ B-pathway, a cell-cycle and pro-inflammatory pathway, directly by inhibiting its cysteine residues' phosphorylation and oxidation or indirectly by inactivating and degrading I $\kappa$ B $\alpha$  through inducing (1) phosphorylation of its tyrosine residues, (2) Cysteine-179 residue's S-glutathionylation, (3) and ubiquitination. NF $\kappa$ B-pathway can also regulate ROS by inducing the expression of antioxidants as superoxide dismutase (SOD) and glutathione peroxidase (GPx) [6]. Similarly, mitogen-activated protein kinase (MAPK) pathway is regulated by ROS via (1) kinases [extracellular signal-regulated kinases (ERK), and c-JUN N-terminal kinases (JNK)], and/or (2) p38 activation. Keap1-Nrf2-ARE signaling pathway is regulated through inhibiting Keap1 (Nrf2 inhibitor), increasing  $Ca^{2+}$  influx, or activating kinases (protein kinase C and MAPK...) that will activate Nrf2. Such a process detoxifies ROS by upregulating antioxidants. It is worth mentioning that high level of ROS can induce Nrf2 phosphorylation and degradation through GSK3 $\beta$ . Other regulated pathways include ubiquitination/proteasome,

mitochondrial permeability transition pore (mPTP), and protein kinase pathways [6,7].

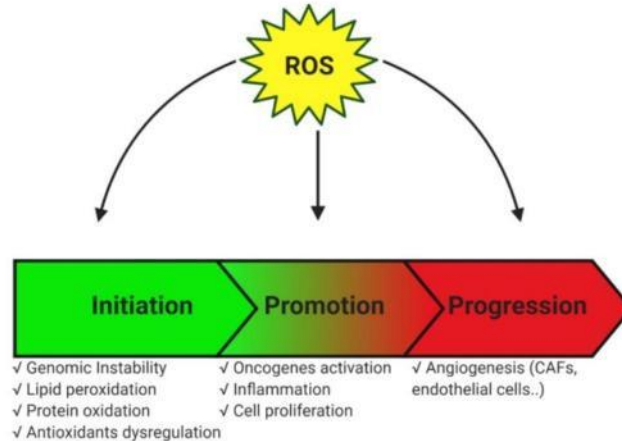
Elevated ROS level induces oxidative stress, a misbalance in the redox homeostasis that causes uncontrolled proliferation and damages to all cellular components, including proteins, lipids, and DNA. This could lead to aging and various pathologies (neurological, cardiovascular diseases, obesity, Fanconi anemia, cancer...). Zablocka-Słowińska, K. et al. found that lipid peroxidation is higher in lung cancer patients compared to control subjects. This may be due to the direct/indirect antioxidant role of some components involved in lipid metabolism that are altered by the misbalanced redox state. Peroxidized products [4-hydroxynonenal (4-HNE), Malondialdehyde (MDA)...] downregulate antioxidants' serum level (vitamin E,  $\alpha$ -tocopherols) and generate point mutations in tumor suppressor genes that will trigger the necessary inflammatory response for cancer [8]. Similarly, protein and DNA high oxidation levels have been proven to contribute to gastric and colorectal carcinoma mechanism [9,10].

Furthermore, oxidative stress is involved in most cancerogenesis steps. As presented in Figure 1., it acts as the hallmark of cancer initiation, promotion, progression, and metastasis via inducing a prime tumor-microenvironment [11,12]. These cancers could be internal (breast, lung, liver, colon, prostate, ovary, and brain) or skin cancers [non-melanoma (NMSC) and melanoma skin cancers (MSC)] [13].

In addition to genomic instability, ROS induce the upregulation and stabilization of hypoxia-inducible transcription factors (HIFs) that will orchestrate expression of oncogenes, glycolytic enzymes, and proteins (GLUT1, GLUT3, hexokinases), and trigger angiogenesis [12,14]. HIF upregulates expression of vascular endothelial growth factor (VEGF) that will induce endothelial cell proliferation via the stimulation of several signaling cascades, including extracellularly regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway [12]. Endothelial cell proliferation could also be triggered by CAFs (cell-associated fibroblasts). Chan et al. proved that  $H_2O_2$

triggers the transformation of normal fibroblasts into CAFs, *invivo*, and *invitro*, through the NF $\kappa$ B signaling pathway. Then, CAFs will produce more ROS and promote the onset, propagation, and metastasis of tumors [15].

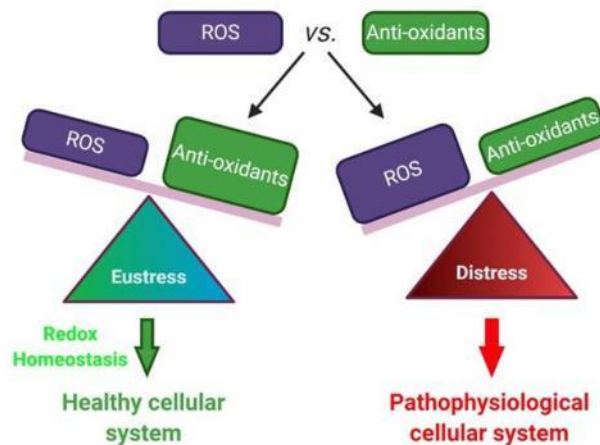
Such a ROS-dependent microenvironment also induces immunosuppression. Despite that, as mentioned before, a low level of ROS does not impede immune cells; a high level prevents them to act. Both T-cell and natural killer cell proliferation and function are impaired. For example, ROS induced undifferentiated myeloid-derived suppressor cells and regulatory T cells (Tregs) inhibiting T-lymphocytes and inducing their apoptosis [16]. ROS can also inhibit T-lymphocytes activation and metabolism by inhibiting the mTOR signaling pathway and inducing Granzyme B-dependent cell death [16]. It is noteworthy to point out that this ROS-induced apoptotic mechanism varies in sensitivity amongst the different T-lymphocytes subsets as follows: effector T cells > regulatory T cells > naive T cells > memory T cells. This shows that effector T cells are the least sensitive to ROS-induced apoptosis, while memory T cells are the most sensitive [16].



**Figure 1:** ROS induce cancer's initiation, promotion, and progression. (1) Initiation of cancer occurs upon genomic instability, accumulation of mutations, and protein and lipids damage. (2) Then ROS trigger promotion upon inflammation (activation of immunological cells), oncogenes activations, and uncontrolled cellular proliferation. (3) Finally, metastasis/progression

occurs due to angiogenesis, tumor-environment, and activation of several signaling cascades.

As shown in Figure 2., the antioxidants defense system is present to neutralize such an upregulation. This could be done via antioxidant proteins like superoxide dismutases (SOD1, SOD2...), glutathione peroxidase (GPx) that induce protective intracellular mechanisms, or through synthetic/natural consumable products (strawberry, pomegranate, carotenoids..). Such products include ROS scavengers that could strengthen the defense against oxidative stress [6,17].



**Figure 2:** The balance between ROS and antioxidants. At the physiological state, the antioxidant defense mechanism can induce redox homeostasis by preventing ROS's upregulation. However, once an imbalance between ROS and antioxidants occurs in favor of the former, disruption of signaling mechanisms and molecular damages induce a pathophysiological state (pathologies and cancer).

## Base Excision Repair (BER) and ROS

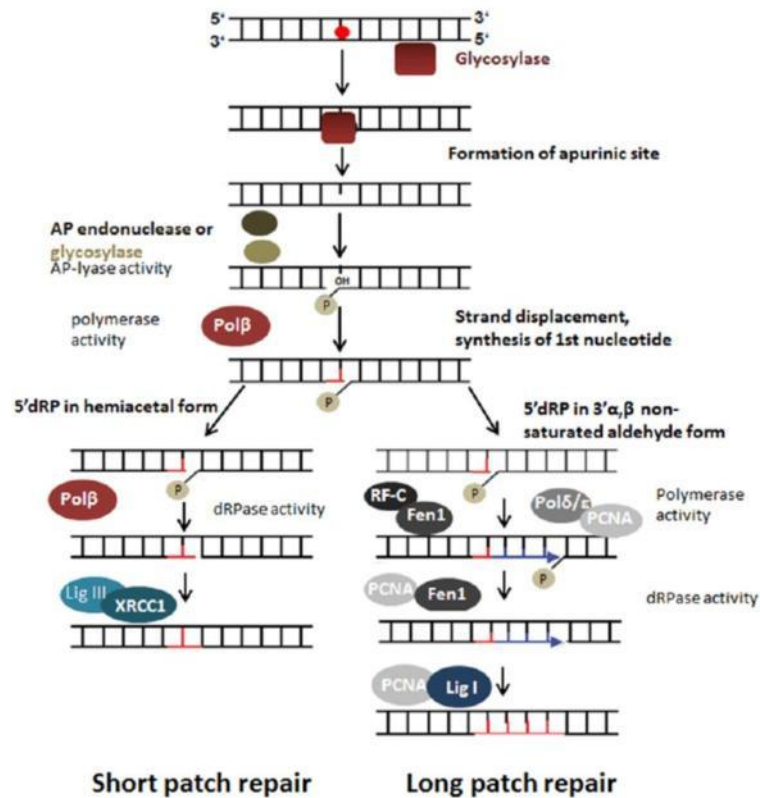
### Overview of BER

One of the primary DNA repair pathways involved in genome integrity is base excision repair (BER). It is responsible for repairing single-strand breaks or small DNA base damages, including oxidized, deaminated, and alkylated DNA damage

[18]. Such damages occur numerously each day, and their repair failure leads to highly mutagenic risk, pathologies, aging, and cancer [19]. As shown in Figure 3., it is divided into 2 sub-pathways: short-patch BER (SP-BER) that repairs a single nucleotide and long-patch BER (LP-BER) that repairs 2 or more nucleotides. At least 11 different substrate specific-DNA glycosylases are known to be involved in the lesion splicing [18]. OGG1(8-oxoGuanine glycosylase) and MYH (MutY DNA glycosylase) are the most studied nuclear glycosylases due to their role in recognizing and removing ROS-induced oxidative DNA lesions, including 8-oxoGuanine.

Contrary to OGG1, MYH does not directly identify the lesion but rather recognizes the adenine nucleotide opposing 8-oxoGuanine [18]. Such an excision process will form an apurinic/apyrimidinic site (AP) site where APE1 (apurinic/apyrimidinic endonuclease 1) will cleave the 5'phosphodiester bond generating a single-strand break with 3' hydroxyl and 5' dRP termini. This will prepare the intermediate for the DNA polymerase that will insert the correct nucleotides followed by nick ligation via DNA ligase and its scaffold protein, X-ray repair cross-complementing protein 1 (XRCC1) [18].





**Figure 3:** Schematic representation of base excision repair (BER) mechanism [20]. BER involves two sub-pathways (short patch repair and long patch repair). Both involve lesion recognition and incision through glycosylases and/or AP endonuclease, polymerization through polymerase activity, and ligation. Polβ (Polymerase-Beta), Polδ (Polymerase-Delta), Polε (Polymerase-Epsilon), Lig III (Ligase III), XRCC1 (X-ray repair cross-complementing protein 1), RF-C (Replication factor-C), PCNA (Proliferating cell nuclear antigen), Fen1 (Flap-endonuclease 1), Lig I (ligase I).

### BER and Human Disorders

Since BER repairs oxidative DNA damage, its dysregulation is the main driver of ROS-dependent pathologies due to an accumulation of point mutations. Such a dysfunction is cytotoxic and could be the cause of aging and its consequences: stress, genomic instability, and appearance of variants [21]. This could be seen in various neurodegenerative diseases such as in

Alzheimer-patients were ligases, polymerases, endonuclease (APE1), and glycosylases (OGG1, MYH, NEIL1) were shown to be downregulated [22]. Meanwhile, APE1 was shown to be dysregulated in amyotrophic lateral sclerosis (ALS) patients [21,23]. Similarly, OGG1's glycosylase activity is downregulated in Parkinson disease, another neurodegenerative disease [24]. Another type of disorders linking BER, oxidative stress, and oxidative DNA damage is currently observed in diabetes, where studies showed a decreased OGG1 protein expression [25]. In addition, recently, BER deficiency at expression and activity level was linked to Gorlin and Xeroderma Pigmentosum C diseases [26,27].

Unfortunately, BER's dysregulation could generate dramatic consequences such as cell transformation. 8-oxoGuanine is the most common oxidative DNA damage that is induced upon oxidative stress [28]. If left unrepaired, it can pair with adenine forming G: C to T: A transversion and induce double-strand breaks during replication [28]. Such a damage is found in numerous internal cancers (prostate, lung, liver, esophageal, and kidney cancers) and skin cancer [29-33].

#### **BER Variants/Mutations and Cancer (Skin and Internal)**

As mentioned previously, studies have shown a link between BER alterations and cancerogenesis [34].

Loss of OGG1 protein has been studied by many researchers who shared a similar conclusion, linking OGG1's absence to tumorigenesis. Upon OGG1 knockout, lung adenocarcinoma, and skin tumors (squamous cell carcinoma and sarcoma) developed in mice [35,36]. A double knockout in OGG1 and MYH induces a high frequency (65.7%) of lung and ovarian tumors and lymphomas [37-40]. Missense mutations in OGG1 at codons 85, 131, and 232 were linked to lung and kidney cancers [41]. Also, missense and nonsense mutations in OGG1 (Cys326Ser, Val159Gly; Gly221Arg; and Trp375STOP) were related to breast cancer [42]. Moreover, single nucleotide variations in OGG1 have been shown to be linked to an increase in cancer risk. This may be due to a lower activity level

compared to the wild type. Benitez-Buelga et al. showed that the rs2304277 variant downregulates its transcriptional expression, thereby contributing to ovarian cancer risk in BRCA1 mutation carriers [43]. Similarly, Tayyaba et al. showed that such a variant contributes to urothelial bladder carcinoma [44]. In parallel, rs1052133 *OGG1* variant was linked to various cancers as breast, colon, stomach, kidney, orolaryngeal, bladder, colorectal cancers, and leukemia [45-50]. Rs113561019 *OGG1* was suggested as a low penetrance contributor to colorectal cancer [51].

Surprisingly, an upregulation in OGG1 protein is seen in various cancers as esophageal squamous carcinoma and ulcerative colitis-associated cancer [52]. This may indicate a persistence of oxidative stress and the inefficiency in OGG1's activity despite its high expression.

Studies have also shown a dramatic increase in cancer in the absence of MYH. A study involving MYH knockout in mice revealed significantly higher spontaneous tumorigenesis compared to control. Upon treatment with KBrO<sub>3</sub>, an oxidizing agent, small intestinal tumor incidences were substantially higher in MYH's absence compared to its presence [53]. P.Tyr179Cys *MYH* variant showed a breast cancer risk while *MYH* Gln324His polymorphism showed a direct link with lung cancer risk in the Japanese population [54,55]. *MYH* monoallelic mutations, including Y179C and G396D, have been shown to have a higher risk in colorectal, endometrial, gastric, and liver cancers, while biallelic *MYH* mutations increase urinary bladder and ovarian cancer [56,57].

Other BER factors' variants/mutations were also linked to increased cancer occurrence. For example, APE1 Asp148Glu (rs3136820) is associated with breast and lung cancer [48,58]. XRCC1 Arg194Trp, Arg280His, and Arg399Gln were linked to various cancers like lung, stomach, bladder, and breast cancers [48]. Additionally, PARP-1 Ala762Ala and P53 Arg72Pro are significantly associated with cervical cancer [34]. While T889C Polβ point mutation increases membrane progesterone receptors, consequently, gastric cancer [59]. Polymerase mutations could

inhibit its expression/activity, thereby triggering the aggressiveness of cancer as breast cancer [60].

On the other hand, BER's overexpression (as APE1 and/or XRCC1) has also been detected in solid tumors and poor survival [61]. APE1's overexpression promotes ovarian cancer, and polymerase's overexpression (particularly Pol $\beta$ ) induces ovary, stomach, and prostate cancers [63,64].

### **Base Excision Repair Targeted Treatment**

Cancer therapy is a modified chemotherapy that involves ionizing radiations or other types of DNA damaging cytotoxic process in addition to molecules that could augment the tumor-killing and reduce resistance to therapy. Since base excision repair is an essential pathway in repairing DNA, targeting its factors could be interesting to kill tumor cells selectively.

**Methoxyamine** is a small molecule that targets apurinic sites (AP) sites to block their repair by APE1. It is used as a potential targeting molecule in parallel to other chemotherapeutic agents as temozolomide (TMZ) or 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) that induce such sites in the DNA. This leads to the accumulation of DNA cytotoxic damages, thereby an induction of apoptosis. An important discovery about methoxyamine was its p53-independence, where usually loss of p53 is a common cancer resistance trait. Another strength point is its ability to target APE1 that plays an essential role in BER and regulating redox signaling. A phase 1 clinical trial has been conducted studying the combination of methoxyamine and TMZ in targeting solid tumors [65,66]. Other phase 1 and phase 2 studies are ongoing [63]. In 2019, Khoei et al. showed that methoxyamine enhances colon cancer cell lines' sensitization to the modified combined chemo- and radiation therapy. Such therapy included gamma radiation and 5-fluorouracil (5-FU), a cytotoxic molecule targeting DNA and RNA. Methoxyamine was shown to improve the efficacy of such a combined treatment by increasing the cytotoxicity and genotoxicity without increasing the gamma radiation dose [65]: **CRT0044876** was conducted as another APE1-inhibitor that reduced the survival of

fibrosarcoma cells in combination with TMZ. Unfortunately, this was not the case in combination with ionizing radiation. It had a poor potentiation [63]. Two interesting agents, **E3330** and **Gossypol/AT101**, NF $\kappa$ B and BCL2 inhibitors, respectively, were shown to bind to APE1 and inhibit its redox activity. Such agents could induce cytotoxicity solely or in combination with other cancer therapies in lung, lymphoma, prostate, adrenocortical, and glioblastoma cancers. More than 20 clinical trials are currently ongoing [63].

Loss of OGG1's activity has also been registered as a potential process towards sensitizing cells to chemo- and radiotherapy [67]. After screening almost 25975 potential compounds, Tahara et al. found a promising new compound, **SU0268**, that could bind specifically to OGG1 in HEK293T and HeLa cells and inhibit its activity [67]. Similarly, **SU0383** was developed to target MTH1, another 8-oxoguanine glycosylase. By inhibiting both glycosylases, oxidized bases will accumulate, forcing cancer cells to apoptosis. The effect of such inhibitors on animal cancer models is in progress [63]. **TH5487**, a recent OGG1 inhibitor that prevents its binding to oxidized purines and prevents inflammatory responses, seems promising but needs further experimental studies within cancer cell models [63].

Researchers have also targeted PARP1 due to its role in activating OGG1's expression and regulating oncogenes and tumor suppressors expression. Some PARP1 inhibitors are already on the market as **talazoparib** and **niraparib**, targeting BRCA-deficient cancer cells (breast, ovarian, and peritoneal cancers) [63,68]. However, drug resistance through increasing PARP1 levels and activation of repair pathways as homologs recombination has emerged. Hence, researchers are trying to develop other inhibitors that could have better efficacy [63]. Other dual anti-cancer molecules, **PD0332991** and **LEE011**, had shown a promising efficiency in targeting lung cancer cells by reducing PARP1 transcriptional expression and impairing OGG1-dependent BER, consequently inducing oxidative-cell death. Such treatment has its drawbacks. It depends on a link between RB1 (retinoblastoma protein), PARP1, and OGG1. However, RB1 is mutated in some cancer types as

retinoblastomas and small cell lung cancers. This may interfere with its efficacy. Therefore, further genomic and transcriptomic screenings are required before administration of this dual treatment as a potential PARP1 inhibitor can be foreseen [68].

## Conclusion

Base excision repair plays a significant role in protecting cells against oxidative DNA damage, single-strand breaks, and ROS-cancerogenesis. Any variation/mutation or expression dysregulation in BER could alter its function in favor of cancerogenesis. However, targeting such a repair system to induce massive oxidative DNA damage in cancer cells could be a potential therapeutic process forcing cancers to cell death. Nevertheless, such an advanced targeted therapy seems challenging and needs further studies to avoid targeting normal cells and compensating their inhibition by activating other repair systems [63]. Immunotherapy and BER inhibitors combination could be one of the solutions to target cancer cells specifically by targeting specific cancer cell receptors and inhibiting repair to induce cancer apoptosis. Such combinatorial therapy has already resulted in intriguing and exciting findings.

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## *Annex 4 - Other Activities*

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- **Other published articles:**

**Isoconazole and Clemizole Hydrochloride Partially Reverse the Xeroderma Pigmentosum C Phenotype.** *International Journal of Molecular Sciences*/ 2021

Farah Kobaisi, Eric Sulpice, Caroline Barette, Nour FAYYAD, Marie-Odile Fauvarque, Bassam Badran, Mohammad Fayyad-Kazan, Hussein Fayyad-kazan, Xavier Gidrol, Walid Rachidi

**Impairment of Base Excision Repair in Dermal Fibroblasts Isolated from Nevoid Basal Cell Carcinoma Patients.** *Frontiers in Oncology*/ 2020

Aurélie Charazac, Nour Fayyad, David Beal, Sandrine Bourgoïn-Voillard, Michel Sève, Sylvie Sauvaigo, Jérôme Lamartine, Pascal Soularue, Sandra Moratille, Michèle T. Martin, Jean-Luc Ravanat, Thierry Douki, Walid Rachidi

**Xeroderma Pigmentosum C a valuable tool to decipher the signaling pathways in skin cancers.** *Oxidative Medicine and Cellular Longevity*/ 2021

Ali Nasrallah, Nour Fayyad, Farah Kobaisi, Bassam Badran, Hussein Fayyad-Kazan, Mohammad Fayyad-Kazan, Michel Sève, Walid Rachidi

**High-throughput synthetic rescue for exhaustive characterization of suppressor mutations in human genes.** *Cellular and Molecular Life sciences*/ 2020

Farah Kobaisi, Nour Fayyad, Eric Sulpice, Bassam Badran, Hussein Fayyad-Kazan, Walid Rachidi, Xavier Gidrol

**Signaling Pathways, Chemical and Biological Modulators of Nucleotide Excision Repair: The Faithful Shield against UV Genotoxicity.** *Oxidative Medicine and Cellular Longevity*/ 2019

Farah Kobaisi, Nour Fayyad, Hamid.R. Rezvani, Mohammad Fayyad-Kazan, Eric Sulpice, Bassam Badran, Hussein Fayyad-Kazan, Xavier Gidrol, Walid Rachidi

- **Oral and poster communications:**

**European Society for Dermatological Research (ESDR)**| September 18<sup>th</sup>-21<sup>st</sup>, 2019 - Bordeaux, France: Oral presentation and poster

**International Workshop on Radiation Damage to DNA (IWRDD)**| May27<sup>th</sup>-June 1<sup>st</sup>, 2018 -Aussois, France: Oral presentation and poster

- **Trainings and Workshops:**

**Elected member of the “Chemistry, Biology, Health” research council**| 2018-2020 Grenoble Alpes University-Grenoble

**Member of the “Doctorants Engagés” doctoral students’ group**| 2018-2020 Grenoble Alpes University-Grenoble

**Biological risk formation**| 2017 CEA-Grenoble

**Chemical risk formation** | 2017 CEA-Grenoble

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## Abstract

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**X**eroderma pigmentosum C (XPC) protein initiates global genome-nucleotide excision repair (GG-NER) pathway to remove UV-induced DNA lesions such as pyrimidine (6-4) pyrimidone photoproducts [(6-4) PPs] and cyclobutane pyrimidine dimers (CPDs). XPC deficient (XP-C) patients show a persistence of such lesions triggering high skin cancer incidences. They also suffer from internal cancers that could be due to the accumulation of oxidative DNA damage. Such base lesions, including 8-oxoguanine (8-oxoGua), are usually repaired by the base excision repair (BER) pathway. Despite growing evidence about how XPC enhances the activity of several BER DNA glycosylases, the effect of XPC mutations on other BER factors and their activities is still elusive. Herein, we seek to answer this open question by characterizing normal and XP-C fibroblasts derived from patients, optimizing the conditions, and dividing our project into two parts.

In part one, we showed a global downregulation of BER's genes in XP-C cells post-UVB compared to normal controls. Furthermore, the major proteins linked to oxidative DNA damage repair (OGG1, MYH, and APE1) were downregulated. This led to an ineffectiveness of BER in excising UVB-induced oxidative DNA damage. In part two, we investigated whether balancing the cellular redox state by treating XP-C cells with different drugs could boost their BER's activity post-UVB. We showed that nicotinamide (NIC) and N-acetyl cysteine (NAC) pretreatments increase glutathione level, decrease ROS level, and enhance BER's gene expression and activity. Meanwhile, buthionine sulfoximine/dimethylfumuate (BSO/DMF) pretreatment depletes glutathione level, increases ROS level, and impairs BER's gene expression and activity.

Based on these results, we propose that pretreatment with drugs that could enhance glutathione's level may protect XP-C cells from an imbalanced redox state that affects the DNA repair. This could pave the way for therapeutic strategies for XP-patients and other DNA repair-deficient patients.

Future work is required to check the efficiency of such treatments on 3D reconstructed skin and *in vivo* models. Additionally, studying the interactome linking XPC and glutathione signaling could be interesting.

**Keywords:** *Ultraviolet irradiation-B (UVB), xeroderma pigmentosum C (XPC), nucleotide excision repair (NER), bulky lesions [CPDs and (6-4) PPs], base excision repair (BER), oxidative DNA lesions (8-oxoguanine), reactive oxygen species (ROS), oxidative stress, glutathione (GSH), nicotinamide (NIC), N-acetylcysteine (NAC), buthionine sulfoximine/dimethyl fumarate (BSO/DMF)*

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## Résumé

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La protéine Xeroderma pigmentosum C (XPC) initie la réparation globale du génome par excision de nucléotides (GG-NER) pour éliminer les lésions de l'ADN induites par les rayonnements UV, telles que les photoproduits de pyrimidine (6-4) [(6-4) PPs] et les dimères de cyclobutane de pyrimidine (CPDs). Les patients déficients en XPC (XP-C) présentent une persistance de ces lésions, déclenchant ainsi une forte incidence de cancers cutanés. Ces patients souffrent également de cancers internes qui pourraient être dus à l'accumulation de lésions d'oxydation de l'ADN. Ces dernières, dont la 8-oxoguanine (8-oxoGua), sont généralement réparées par excision de bases (BER). Malgré les preuves, de plus en plus tangibles, concernant l'implication de la protéine XPC dans l'activité de plusieurs glycosylases clés de la voie BER, l'effet des mutations de *XPC* sur les autres facteurs de cette voie reste encore peu connu. Le but de ce travail de thèse est de répondre à cette question ouverte en caractérisant la modulation de la voie BER dans les cellules normales et les cellules XP-C issues de patients.

Dans un premier temps, nous avons montré un effondrement global de l'expression de plusieurs gènes importants de la voie BER dans les cellules XP-C par rapport aux cellules témoins après irradiation aux UVB. En outre, les principales protéines liées à la réparation des dommages d'oxydation de l'ADN (OGG1, MYH, et APE1) ont été dérégulées. Cela a conduit à une inefficacité du BER dans l'excision des purines oxydées induites par les UVB. Dans un deuxième temps, nous avons cherché à savoir si la modulation de l'état redox en traitant les cellules avec différents médicaments pharmacologiques pouvait restaurer l'activité de BER après irradiation aux UVB. Nous avons montré que les prétraitements par le nicotinamide (NIC) et le N-acétyl cystéine (NAC) augmentent le niveau de glutathion, diminuent la génération des espèces réactives de l'oxygène (ROS), et augmentent l'activité du BER après irradiation aux UVB. Cependant, le prétraitement à la buthionine sulfoximine/diméthylfumate (BSO/DMF) inhibe le glutathion, augmente la production des ROS, et diminue l'activité du BER.

Sur la base de ces résultats, nous pourrions proposer que le prétraitement avec des médicaments qui pourraient augmenter le niveau de glutathion puisse protéger les cellules XP-C d'un état redox déséquilibré qui affecte la réparation de l'ADN. Cela pourrait ouvrir la voie à des stratégies thérapeutiques pour les patients XP et d'autres patients souffrant des maladies génétiques de réparation de l'ADN.

Des travaux futurs sont nécessaires pour vérifier l'efficacité de ces traitements au niveau de la peau reconstruite en 3D et sur des modèles pré-cliniques *in vivo*. En outre, l'étude de l'interactome reliant XPC et la signalisation du glutathion pourrait être intéressante.

*Mots-clés : Rayonnement ultraviolet B (UVB), xeroderma pigmentosum C (XPC), réparation par excision de nucléotides (NER), lésions de l'ADN [CPD et (6-4) PP], réparation par excision de bases (BER), lésions d'oxydation de l'ADN (8-oxoguanine), espèces réactives de l'oxygène (ROS), stress oxydatif, glutathion (GSH), nicotinamide (NIC), N-acétylcystéine (NAC), buthionine sulfoximine/fumarate de diméthyle (BSO/DMF)*