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**Radiation biophysical modeling of
the FLASH effect: from
mechanistic understanding
towards clinical endpoint
prediction**

FINAL THESIS

Supervisor:
Dr. Emanuele Scifoni

Candidate:
Marco Battestini

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Abstract

Background

Ultra-High Dose Rate (UHDR) or, commonly, FLASH irradiation is a novel dose-delivery technique based on fast beam release, with typically a total irradiation time < 100 ms and a total mean dose rate > 40 Gy/s for a single dose, usually higher than 10 Gy. Experimentally, compared to conventional dose rate (Conv) irradiation (typically, 0.03–0.1 Gy/s), the UHDR irradiation allows to obtain reduced side effects for normal tissue, for example, with reported evidence of a decrease in memory loss or less intestine necrosis, and, at the same time, the same effectiveness on tumor control, e.g., an indistinguishable tumor response for HBCx-12A xenografts. This FLASH sparing effect has also been confirmed in animals, e.g., no severe fibrotic lesions on mini pig skin. UHDR electron irradiation was successfully tested in a first patient with cutaneous lymphoma. Furthermore, the first clinical trial with proton-FLASH irradiation is ongoing. In addition to that, the FLASH effect has been observed across different radiation qualities, namely, particle types, energies, and linear energy transfer (LET). In particular, it has been demonstrated using electron LINACs, showed for UHDR irradiation a significant sparing of animals from cognitive deficits in learning and memory. The FLASH effect has been seen using proton beams, where the loss of proliferating cells in intestinal crypts or the acute skin damage and radiation-induced fibrosis was reduced at FLASH regime with respect to Conv, for the same tumor control. Lastly, this effect has been confirmed with ions, in particular with helium and carbon beams, both *in-vitro* and *in-vivo*.

Despite a plethora of experiments supporting the FLASH effect, the biological mechanism underpinning it is still unclear and highly debated after 10 years from its discovery. Different possible explanations have been proposed, such as (i) transient hypoxia due to O_2 depletion, (ii) organic radical recombination, (iii) inter-track effects, or (iv) the immune system-driven effect. However, some of these have been discredited by further experiments. Based on these mechanisms, several mathematical models have been proposed. However, most of these have been based on a single driving mechanism and have failed to fully explain the FLASH effect or to reproduce a significant part of the experimental data.

For these reasons, this study is devoted to the development of a novel mechanistic approach to investigate the biological mechanism behind the FLASH effect. At the core of the mathematical model development is the hypothesis that the FLASH effect is due

to a complex interplay of various spatio-temporal scales of the radiation damage action.

Development of a multiscale radiation biophysical model for ultra-high dose rate (UHDR) investigations

The first part of this study focused on the conception, realization, implementation, and validation of a multiscale stochastic radiobiological model for the study of the FLASH effect, based on beam irradiation at UHDR. From the experimental effort, an established consensus is arising in the community that the concurrent involvement of multiple scales of radiation damage is involved. In particular, the crucial role of the chemical environment and the redox system is underlined. For these reasons, we developed the MultiScale Generalized Stochastic Microdosimetric Model (MS-GSM²) [1, 2], a multi-stage extension of the Generalized Stochastic Microdosimetric Model (GSM²) [3, 4, 5, 6]. The GSM² is a probabilistic model that describes the time evolution of DNA lesions in a cell nucleus according to microdosimetric principles, without considering the Poissonian assumption to describe the number of radiation-induced DNA damage.

The MS-GSM² can investigate the combined effects of several chemical species and the formation and time evolution of DNA damage at the UHDR regime, incorporating the complex interplay between different levels of spatio-temporal stochasticity in physics, chemistry, and biology. In particular, our model includes the physical stage, allowing the description of the energy deposition by each single particle of a beam in a microscopic volume, which mimics the cell nucleus. The absorbed dose of every energy deposition event is distributed on the transverse plane of the cell nucleus using an Amorphous Track (AT) model, a parametrization of the radial dose distribution for a particle track hitting the target. The MS-GSM² is capable of describing any dose-delivery time structures and radiation qualities, for particle, electron, and X-ray beams.

Our model considers the homogeneous chemical stage, which consists of an optimized chemical reaction network of a pre-existing reaction kinetics model [7], described by five ODEs. The system involves reactions between chemical species derived from the water radiolysis and organic molecules, for example, radicals of nucleotides, proteins, and lipids, scavengers, such as vitamin E, thiols, and their derivatives, and those between organic species alone. The early pre-chemical and heterogeneous chemical stages are assumed to be instantaneous, and their final effect is incorporated in the model input. In particular, these two stages are described by the G-values, i.e., the number of chemical species produced per 100 eV of energy absorbed by the medium, of the production terms of chemical species in the ODEs' system. The G-values were tabulated using a Monte Carlo particle track structure code.

The MS-GSM² takes into account the bio-chemical stage, which describes the connection between the energy deposited by ionizing radiation, the chemical environment of the cell, and the yield of biological damage. Since the oxygen is among the considered species in the chemical network, we include the impact of the oxygenation levels on the formation of DNA damage. We explicitly incorporate the reduction of radiation-induced DNA damage due to lower oxygen concentration. Starting from [7], we assume that UHDR

irradiation modifies the chemical environment of the cell, reducing the accumulation in time of organic peroxy radicals $\text{ROO}^\bullet$, and thus the cell toxicity. In fact, the persistence of $\text{ROO}^\bullet$ in the cellular environment leads to biological damage [7]. For this reason, we link the different time evolution of the organic peroxy radical concentration $[\text{ROO}^\bullet](t)$ after Conv and UHDR irradiation with the number of indirect damage created, which implies a reduction in the indirect DNA damage yield only at UHDR regime. Thus, the indirect DNA damage yield decreases as the oxygenation level decreases and the dose rate increases.

Lastly, the model includes the biological stage, which considers the possible repair of the radiation-induced DNA damages (the sub-lethal lesions), and the plausible presence of irreparable damages (the lethal lesions), at time t . In particular, the sub-lethal damage can go through three possible biological pathways: it can be left unrepaired and lead to cell inactivation, at rate a , it can interact with another sub-lethal lesion becoming a lethal one, at rate b , or it can be repaired, at rate r . The final biological endpoint predicted by the MS-GSM² is the surviving probability of the cell, allowing a direct comparison of our model predictions with the experimental data.

Comparison of the MS-GSM² predictions with UHDR experiments and proposal of a new mechanism behind the FLASH effect

We studied the impact of different physical parameters (radiation quality, dose, dose rate, and beam structure) on the emergence of the FLASH biological effect. The study was performed at the chemical level, analyzing the different time evolution of the organic peroxy radical $\text{ROO}^\bullet$ between conventional and UHDR irradiation; at bio-chemical level, studying the relative reduction in indirect damage per unit Gy between conventional and UHDR irradiation; at biological level, predicting the cell survival probability for both conventional and UHDR irradiation. We investigated the biological effect of different physical irradiation parameters, i.e., dose-delivery time structures and scanning pathways, for UHDR irradiation, calculating a look-up table in dose, dose rate, LET, antioxidant rate, and oxygenation level, through the MS-GSM².

We compared the MS-GSM² predictions with all the main *in-vitro* experimental results at UHDR regime (DU145 cell line at 10 MeV electrons irradiation [8], A549 cell line at 4.5 keV/ μm helium ions irradiation [9], CHO-K1 cell line at 13 keV/ μm carbon ions irradiation [10]), for different radiation quality, and oxygenation level. All the irradiation parameters (oxygenation, time structure, dose rates, doses, radiation quality, etc.) have been set as reported in the original publication. The three biological parameters (a , b , r) of the MS-GSM² have been calibrated on the experimental data points at standard conditions, namely, conventional dose rate and normoxia (i.e., 21% oxygenation), while all the other points at different oxygenation levels and dose rates have been completely predicted. Thus, we showed remarkable accuracy through a wide range of radiation quality and oxygen concentration.

FLASH irradiation, in a clinical perspective, would have a great impact, because it can be selective on healthy tissue and not on the tumor. However, no theory at the moment can fully explain this. In particular, one of the initially most accredited hypotheses (oxygen depletion) has now been largely discredited. For this reason, we proposed a new consistent mechanism for the FLASH differential effect observed in normal tissues and tumors, highlighting the crucial role of the redox environment. In particular, we analyzed the combined action of oxygenation and antioxidant environment on the emergence of the FLASH effect. From this analysis, we observed how, for a given oxygenation, the FLASH effect decreases for increasing antioxidant rate. Since tumors typically have higher antioxidant levels than healthy tissues, our hypothesis would justify the emergence of the FLASH effect only on normal tissues. Therefore, from our *in-silico* analysis, we underlined the fundamental role of combined effect of the environmental oxygenation level and the redox balance on the appearance of the FLASH effect. Thus, correlating tissue specificities to the onset and the severity of the effect. Therefore, the developed MS-GSM² can consistently describe multiple aspects of the FLASH effect, reproducing the main evidence from the *in-vitro* experimental data.

Development of a mechanistic-driven model for predicting healthy tissue complications and tumor control at Conv and UHDR regime

In the second part of this thesis, to target more clinically relevant endpoints, we focused on the conception, realization, implementation, and first validation of a mechanistic-driven Normal Tissue Complication Probability (NTCP) model and Tumor Control Probability (TCP) model for both Conv and UHDR irradiations. This study aims to extend the MS-GSM² to clinically relevant biological endpoints, integrating the radiation biophysical model MS-GSM² into the Relative Seriality Model [11] for NTCP and TCP predictions, allowing a comprehensive investigation of healthy tissue complications for conventional and UHDR irradiations. The MS-GSM²-driven NTCP model [12] considers single-cell resolution, allowing it to describe heterogeneity at the cell and tissue levels, considering different geometric and functional arrangements of cells, and gradients of oxygen concentration, but also effects due to different radiation quality. Moreover, the developed NTCP model can also account for different fractionation schemes of dose delivery.

We investigated the impact of physical parameters (e.g., radiation quality, total dose, fractionation, partial irradiation), the chemical environment (e.g., oxygenation level), and the biological characteristics of the tissue (e.g., cell line, tissue architecture) on the emergence of possible complications in normal tissue for Conv irradiation. We applied our NTCP model to several experimental scenarios across various particles, in particular, protons, helium, and carbon ions, at both Conv, for example [13, 14, 15], and UHDR regimes, such as [16]. The MS-GSM²-driven NTCP model described different dose-delivery fractionation schemes, e.g., for the rat spinal cord during Conv irradiation. Furthermore, it can reproduce the UHDR experiment with protons for mouse skin injury. Therefore, the

developed NTCP model provide a mechanistic approach to calculate clinically relevant endpoints for both Conv and UHDR irradiation.

Towards FLASH biological treatment planning

The final part of this work focused on possible extensions and improvements of the developed models to enable the clinical translation of FLASH radiotherapy from a computational point of view. The developed radiobiological model is fast, but, at the moment, it does not allow to optimize clinical plans because this would still require too much time. Since FLASH radiation therapy is very innovative and different from a conventional dose-delivery approach, predictive models that are able to calculate the biological effect are needed to optimize FLASH treatment plans. Furthermore, compared to a conventional approach, it is not only the dose that counts but also the dose rate, and therefore it is necessary to predict the biological impact of the scanning pattern for the dose-delivery. So we need fast and efficient models that predict the FLASH effect to figure out the best treatment plan for UHDR irradiation.

A fully mechanistic approach, while fundamental for basic understanding, is computationally expensive and, as a result, is currently impractical for biological optimization of treatment plans at FLASH dose rates. Indeed, a traditional optimization approach requires predicting biological outcomes for a huge number of parameters across various doses, dose rates, LET values, and oxygenation levels. Furthermore, even with fixed parameters, calculating the biological effect on a clinical treatment plan is too expensive. Thus, in our opinion, the first step towards enabling UHDR biological treatment planning is enhancing the mechanistic model using artificial intelligence (AI). Therefore, we developed an AI-enhanced mechanistic model, i.e., a surrogate model of the MS-GSM², for a fast study of the FLASH effect in preclinical and clinical scenarios.

We trained a neural network to replicate the biological outcome predicted by MS-GSM². This enables a rapid and comprehensive exploration of physical and physiological parameters related to the emergence of the FLASH effect. We combined the speed and predictive capability of AI with the robustness of a mechanistic-driven approach to overcome the previously calculated look-up table, by MS-GSM², of biological outcomes across a wide range of physical, chemical, and biological parameters. So, this AI-enhanced MS-GSM², coupled with the previously proposed NTCP model, allows fast prediction of possible healthy tissue complications. Moreover, it allows us to study how different scanning patterns of dose delivery affect the dose rate and the resulting biological effect, considering various environmental conditions of the irradiated tissue. Therefore, the AI-enhanced MS-GSM² can unleash significant breakthroughs in the clinical use of UHDR radiation.

Conclusions and future perspectives

The current version of the MS-GSM² accounts for DNA-damage-related death pathways, which probably play the main role in *in-vitro* biological systems. However, due to the great complexity of *in-vivo* biological systems, death pathways not mediated by DNA, such as

ferroptosis, are known to contribute to the FLASH effect. For this reason, future effort is devoted to the inclusion of lipid peroxidation, which could influence the redox balance of a macroscopic biological system, thus contributing to the emergence of the FLASH effect.

Regarding the clinical translation of FLASH radiotherapy, we are working on the development of a biologically-driven optimizer for UHDR treatment planning. We use the AI-enhanced MS-GSM2 to investigate the biological effect of different physical parameters, taking into account possible gradients in oxygen concentration.

In summary, in this work, we developed a multiscale radiation biophysical model, i.e., the MS-GSM², for *in-vitro* UHDR investigations. We integrated the MS-GSM² into NTCP and TCP models for mechanistic-driven predictions of possible healthy tissue complications and tumor control probability at Conv and UHDR regimes. We enhanced the MS-GSM² using AI for a fast and comprehensive investigation of the impact of physical, chemical, and biological parameters on the emergence of the FLASH effect. Thus, we proposed a consistent picture to get insight into the mechanism underpinning the FLASH effect. Further ongoing work focuses on the inclusion in the MS-GSM² of alternative pathways to DNA damage and on the biological optimization of FLASH treatment plans.

Plan of the work

This thesis is organized as follows: Chapter 1 is an introduction dedicated to the analytic description of the spatio-temporal scales of the radiation damage action, with a focus on the main physical, chemical, and biological processes involved, and on the main modifiers of the biological response, which represent the basic background for the development of a multiscale model, like the present one. Chapter 2 focuses on the presentation of key models and tools to investigate different stages of radiation damage, focusing on those that will later be expanded and used in this work, for physics, chemistry, and biology. Since the latest findings in the topic are fundamental to this thesis, Chapter 3 reports a detailed overview of FLASH radiotherapy, presenting the FLASH effect, the main UHDR dose delivery techniques, the possible definitions of the dose rate, a review of UHDR experimental studies, and the main mechanistic hypotheses and developed models for FLASH. In light of the explosive interest in FLASH radiotherapy raised in latest years, and the growing significance of the experimental evidences to the challenge of its mechanistic understanding, a comprehensive review of the state of the art on the FLASH effect has been conducted. Chapter 4 aims to present and describe the multi-scale radiation biophysical model developed in this work, reporting the main results obtained, such an *in-silico* analysis of the cellular response to UHDR radiation, for different physical, chemical, and biological parameters, a comparison of the model results with the main *in-vitro* experiments, and an investigation of a new, consistent, hypothesis that could explain the main features of the FLASH effect. Chapter 5 reports the development of a mechanistic model to provide a comprehensive investigation of clinically relevant endpoints, describing the main numerical results, with an analysis of the impact of different physical and physiological parameters on the emergence of complications in healthy tissues, and showing a comparison of the model results with *in-vivo* data after conventional irradiation, for different experimental

scenarios. Chapter 6 is devoted to the description of the multi-scale extension of the dose-response model for UHDR investigation, reporting a first comparison of the model results with an *in-vivo* FLASH experiment. Chapter 7 presents the mathematical framework of a surrogate model, which combines artificial intelligence with the developed multi-stage radiobiological model, showing the first results. Chapter 8 introduces the future perspectives of this work and the main ongoing work with preliminary results. Chapter 9 draws the conclusions of the work discussed in this thesis.

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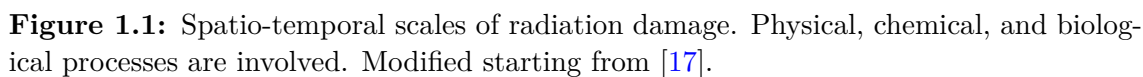
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Introduction

Spatio-temporal scales of radiation damage



3

The main biological target of ionizing radiation is the DNA molecule [18]. The interaction between the radiation and the biological system can be "direct", namely via the direct deposition of energy by the radiation on DNA, or "indirect", i.e. mediated by the harmful chemical species generated after the physical interaction with liquid water, which is the main constituent of the biological systems and organic molecules. Typical distances between ionization points in a track are of the same order as the distances between the base pairs on the DNA molecule (2 – 3 nm).

A major aim of this thesis is to investigate the fundamental basis of a new biophysical effect, namely a modification of the radiation response. Thus, in this Chapter, the spatio-temporal scales of the radiation damage action are presented. In particular, the first three Sections are dedicated to the physics (1.1), chemistry (1.2) and biology (1.3) involved in the radiation-induced biological damage, while the Section 1.4 focuses on the modifiers of the radiation action on biological systems.

1.1 Physics

In this Section, the physical processes involved during the interaction between radiation and biological medium are described.

1.1.1 Physical stage

When ionizing radiation interacts with biological materials, the radiation deposits its energy in the medium through a series of elementary electromagnetic interactions, that is, through excitation and ionization, mainly, of water molecules, depending on the amount of energy transferred by the primary radiation to the electron [20, 21], as we can see in Figure 1.2. These processes are very fast, from attosecond to femtosecond, and involve a broad spatial scale, from Å to μm . The excitation of liquid water H_2O produces the excited water molecule H_2O^* , with an energy threshold in water of ~ 13 eV, while the ionization process generates the ionized water $\text{H}_2\text{O}^{\bullet+}$ and releases an electron e^- , with a threshold of ~ 7 eV. These species are highly unstable, and so are the starting point for a cascade of chemical reactions that will occur during the following chemical stages.

Furthermore, IR can interact directly with bio-molecules, typically represented as RH , producing organic radicals $\text{R}^\bullet$, such as radicals of nucleotides, proteins, and lipids. These species will play a key role during the homogeneous chemical stage, as we will see in Section 1.2.3.

In the case of a particle beam, the energy loss by ions per unit path length, or usually referred to as the Stopping Power, depends mainly on the projectile velocity and medium properties. We can describe it using the so-called Bethe-Bloch formula [23]:

$$-\frac{dE}{dx} = 4\pi\rho N_A \frac{Z_t}{A_t} \left(\frac{e^2}{4\pi\epsilon_0} \right)^2 \frac{Z_p^2}{m_e v^2} \left\{ \ln \left(\frac{2m_e v^2}{I} \right) - \ln(1 - \beta^2) - \beta^2 - \frac{C}{Z} - \frac{\delta}{2} \right\}, \quad (1.1)$$

where Z_t and Z_p are the target and projectile charges, A_t is the target mass number, N_A is the Avogadro number, ρ is the target mass density, e and m_e are the charge and the mass

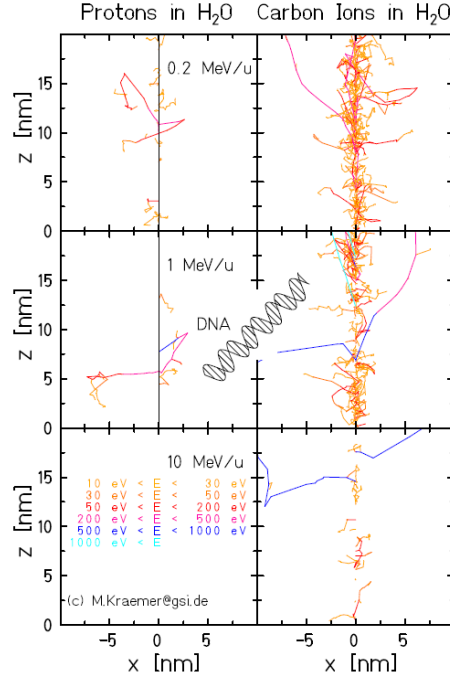


Figure 1.2: Example of energy deposition events in water by different IR. From [22].

of electron, v is the projectile velocity, $\beta = \frac{v}{c}$ is the projectile relative velocity with respect to c , the speed of light, ϵ_0 is the vacuum permittivity, I is the target mean ionization potential. The last two terms in brackets are the shell and density-effect corrections.

Another fundamental quantity is the absorbed dose, namely the energy absorbed by the medium per unit of mass [Gy] = [J/kg], which is linked with the so-called Linear Energy Transfer (LET), as

$$D [\text{Gy}] = 1.6 \cdot 10^{-9} \cdot \frac{dE}{dx} \left[\frac{\text{keV}}{\mu\text{m}} \right] \cdot \frac{1}{\rho} \left[\frac{\text{cm}^3}{\text{g}} \right] \cdot F [\text{cm}^{-2}], \quad (1.2)$$

where $\text{LET} = \frac{dE}{dx}$ and F is the particle fluence. An example of depth-dose curves for different radiation quality is reported in Figure 1.3.

Ions deposit energy through numerous Coulomb collisions, generating secondary electrons primarily in the forward direction. This generates a highly localized microscopic dose distribution around the primary ion track, as we can see in Figure 1.4. Additionally, reducing the LET of the given primary ion we observe a lower single-track dose deposition, resulting in a broader spatial microscopic dose distribution. Therefore, to achieve the same average dose in a given water volume, the number of tracks, i.e., the fluence, increases for decreasing LET.

For completeness, charged particles can also interact with the medium via elastic electromagnetic scattering, resulting in a lateral broadening of the beam due to particle path deviation with a given angular distribution, or via nuclear interaction, which leads to an

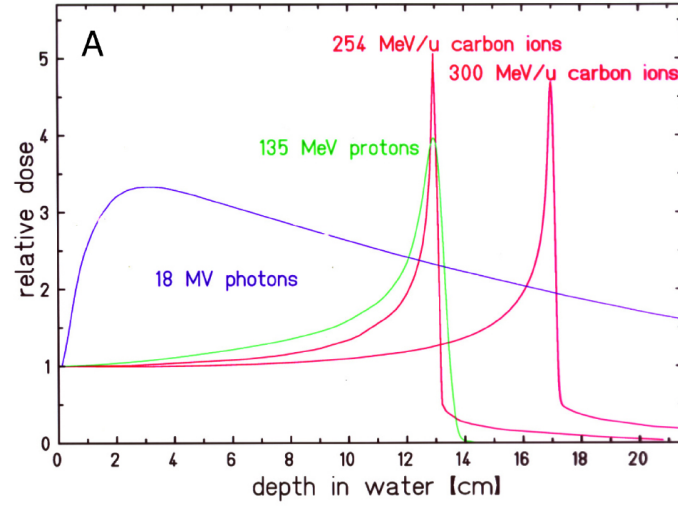


Figure 1.3: Example of depth-dose curve in water for different IR. From [24].

attenuation of primary particles and the production of projectile fragments, generating a mixed radiation field.

Instead, photons interact mainly with the medium via photoelectric and Compton interactions, depositing energy through secondary electrons. Several primary photons are needed to deposit a macroscopic clinical dose. They produce high-energy electrons, which can subsequently undergo further interactions with the surrounding medium, leading to the formation of an electron cascade within the cell nucleus. This generates a flat microscopic dose distribution, as we can see in Figure 1.4, which is completely different from that generated by protons and heavier ions. Thus, the different pattern of energy deposition events and microscopic dose distributions for changing LET values lead to a different biological response at the cellular level. In fact, radiation with higher LET results in a more severe type of DNA lesions compared to photons [25, 26], as we will see in Section 1.4.1.

1.2 Chemistry and physical-chemistry

In this Section, the generation of chemical species and network of chemical reactions that follow the physical stage are shown. Free radicals are generated by the radiolysis of water, that is, by the series of processes that follow the deposition of energy in water, as illustrated in Figure 1.5, or through reactions with environmental molecular oxygen and organic molecules. In particular, the chemical processes can be divided in three distinct spatio-temporal stages, namely the pre-chemical stage, the heterogeneous chemical stage, and the homogeneous chemical stage.

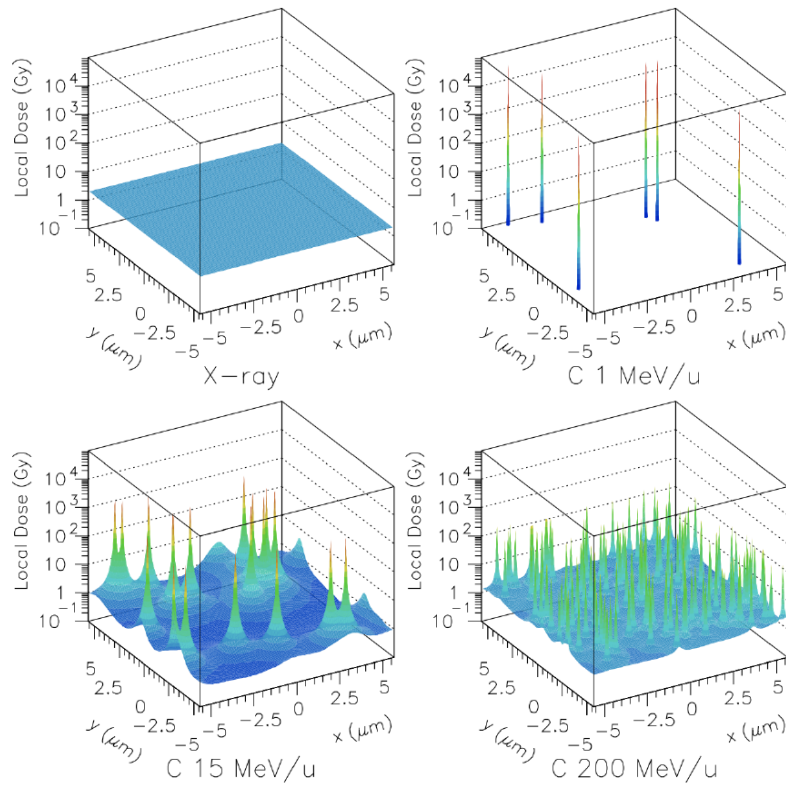


Figure 1.4: Example of spatial microscopic dose distribution for different radiation quality, for an average macroscopic dose of 2 Gy. From [26].

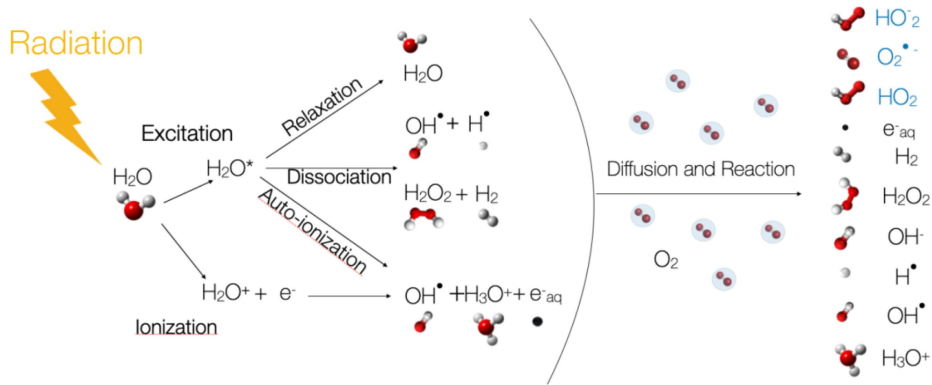


Figure 1.5: Illustration of the water radiolysis processes. From [27].

1.2.1 Pre-chemical stage

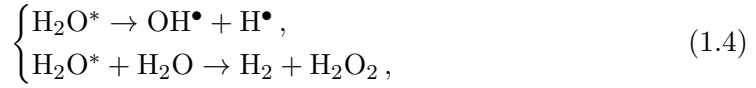
The pre-chemical stage, also-called physico-chemical stage, is a hybrid stage between physics and chemistry. It occurs very short in time, between fs and ps, and involves local processes in space, from Å to nm. The generation of H_2O^* , $\text{H}_2\text{O}^{\bullet+}$, and e^- during the physical stage triggers a series of processes during this stage that lead to the formation of new chemical species, such as ions and radicals. Free radicals are ions, atoms, or molecules with an unpaired electron on an open shell configuration, and they are highly reactive.

In particular, the excited water molecule can go through three possible pathways:

- the relaxation,

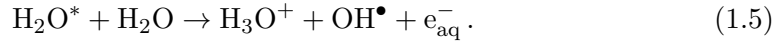


- the dissociation,



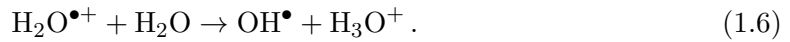
where $\text{H}^\bullet$ is the hydrogen radical, $\text{OH}^\bullet$ is the hydroxyl radical, H_2 is the molecular hydrogen, while H_2O_2 is the hydrogen peroxide, that are some of the main chemical species linked to DNA damage;

- the auto-ionization,



which produces the hydronium ion H_3O^+ , i.e. the hydrated form of the H^+ , and the aqueous or solvated electron e_{aq}^- , which is an electron surrounded by six water molecules;

Instead, the ionized water molecule can react with the water molecule:



In the end, when an electron goes below the excitation energy threshold becomes a sub-excitation electron, it can be captured by water through dipolar interactions,



or it can react with a free hydrogen ion H^+ ,



After the thermalization of these chemical species with the solvent, i.e. the liquid water, all the molecules, atoms, ions, and radicals produced during this stage enter the heterogeneous chemical stage.

1.2.2 Heterogeneous chemical stage

The heterogeneous chemical stage goes, temporally, from ps to around μs , and, spatially, from 10^{-9} m to 10^{-7} m. This stage is characterized by the diffusion of the previously produced chemical species far from the initial primary radiation track, and by the reactions between these chemical species, producing a non-uniform distribution of molecules, ions, and radicals. An example of the time evolution of a track at different time scales is reported in Figure 1.6.

- Diffusion: chemical species can diffuse within the medium via a Brownian motion. Each species ξ can jump in a random direction and is characterized by a specific diffusion coefficient D_ξ in $[\text{m}^2 \cdot \text{s}^{-1}]$, according to the Einstein-Smoluchowski equation, which also depends on the type of environment.
- Reaction: molecules, ions, and radicals can react with each other, producing more stable chemical species. It is possible to represent chemical reactions using chemical kinetics, which describes the change of the concentration of a chemical species in time. For a generic chemical reaction between reagents A, B and products C, D, that is,



we can define the reaction rate as

$$R_1 = -\frac{d[A]}{dt} = k_1[A]^a[B]^b, \quad (1.10)$$

for the direct reaction, while as

$$R_2 = -\frac{d[C]}{dt} = k_2[C]^c[D]^d, \quad (1.11)$$

for the inverse reaction, where $[\xi]$ is the concentration of the species $\xi \in \{A, B, C, D\}$ in $[\text{mol} \cdot \text{l}^{-1}]$, $x \in \{a, b, c, d\}$ is the stoichiometric coefficient, i.e., the amount of each species required to trigger the reaction, while k_i is the rate constant in $[(\text{mol/l})^{-1} \cdot \text{s}^{-1}]$, which describes the speed of the reaction.

At the equilibrium, $R_1 = R_2$, thus

$$K = \frac{[C]^c[D]^d}{[A]^a[B]^b} = \frac{k_1}{k_2}, \quad (1.12)$$

which is the equilibrium constant.

The typical approach to describe the yield of chemical species is by defining the G-value, namely,

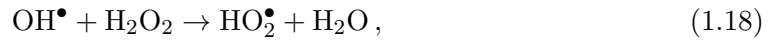
$$G = \frac{\# \text{ molecules}}{100 \text{ eV}}, \quad (1.13)$$

which is the number of chemical species produced per 100 eV of energy absorbed by the medium. It is possible to quantify this parameter experimentally or by using Monte Carlo

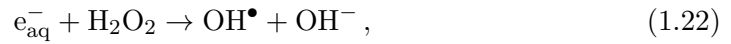
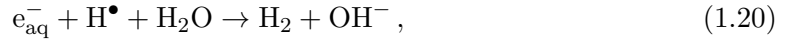
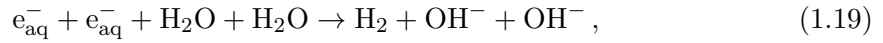
(MC) particle track structure codes, as we will see in Section 2.2.1. An example of the time evolution of G-values for different chemical species is reported in Figure 1.7.

The probability of a reaction to occur is influenced by the quality of the radiation [28], that is, the type, the energy, and LET of the primary radiation. In fact, the denser the primary radiation track is, that is, the higher its LET, the closer the generation of the region with high concentrations of reactive chemical species is, and thus the more frequent the reaction will take place. It is possible to quantify the impact of radiation quality on the generation and annihilation of chemical species using G-values. In fact, as we will see in Section 2.2.1, the radical yields depend on LET.

During this stage, several different reactions can occur [28, 29], starting from the chemical species generated at the pre-chemical stage, described in Section 1.2.1, characterized by rate constants as reported in [28]. In particular, the main reactions that the hydroxyl radical $\text{OH}^\bullet$ can undergo are:



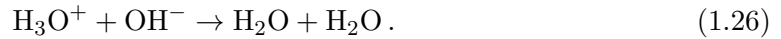
for the solvated electron e_{aq}^- are:



for the hydrogen radical $\text{H}^\bullet$ are:



while for hydronium ion H_3O^+ is:

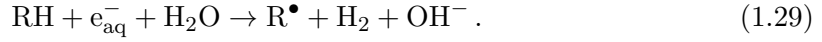


At the end of the heterogeneous chemical stage, around a μs , all chemical species have diffused away from the primary radiation track, producing a situation in which the species are almost uniformly distributed and there is no more memory of the initial track structure, and enter the so-called homogeneous chemical stage.

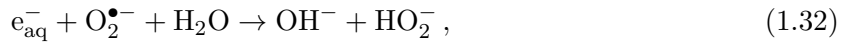
1.2.3 Homogeneous chemical stage

The homogeneous chemical stage starts at about a μs (depending on LET) and goes up to s, while spatially, it takes place between 10^{-8} m and 10^{-6} m, which are dimensions comparable to those of DNA and cell, respectively. This stage is characterized by reactions, in particular, between chemical species derived from water radiolysis, environmental oxygen, and organic molecules RH, for example, radicals $\text{R}^\bullet$ of nucleotides, proteins, and lipids, scavengers, such as vitamin E, thiols, and their derivatives, and those between organic species alone. At this point, there is no more diffusion, and the track structure of the primary radiation is completely lost. It is possible to describe each reaction with a reaction rate and a rate constant, as previously defined in Eq. 1.2.2, as reported in [7].

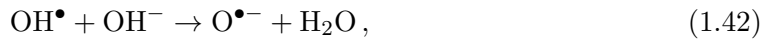
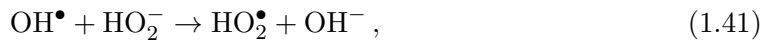
In particular, the organic substrate RH can react with the chemical species produced during the previous stage, such as



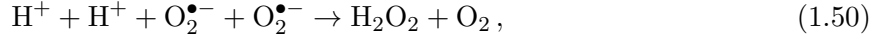
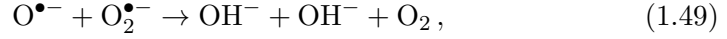
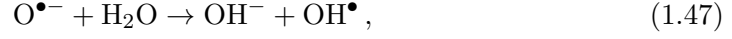
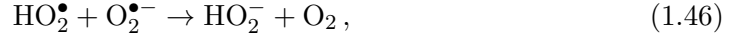
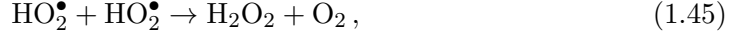
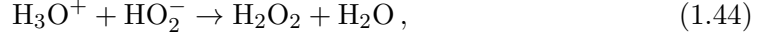
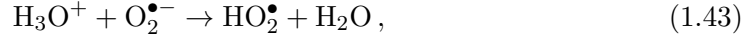
A key species in the homogeneous chemical stage is molecular oxygen O_2 [7]. It can fix biological damage [29]; that is, indirect DNA damage can be permanently "fixed" by O_2 , making the damage irreparable. The capability of oxygen to fix DNA damage increases with increasing oxygen concentrations, leading to increased radiation lethality. Thus, oxygen is one of the main modifiers of radiation efficiency [30]. This aspect will be explored further in Section 1.4. In particular, the oxygen molecule can react with the chemical species generated by water radiolysis, such as with the solvated electron,



the hydrogen radical,



or with other species,



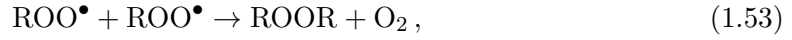
where the last reaction with superoxide $\text{O}_2^{\bullet-}$ occur through the so-called superoxide dismutase (SOD) reaction, or via spontaneous dismutation.

Furthermore, O_2 can also react with organic radicals, such as

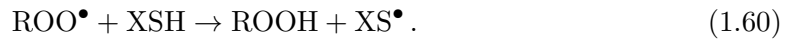


producing the organic peroxy radical $\text{ROO}^\bullet$, which is a harmful reactive species [7], as we will see in detail in the context of a possible FLASH mechanism, in Section 3.5.3.

The organic radical $\text{R}^\bullet$ and the organic peroxy radical $\text{ROO}^\bullet$ can react with each other or with other organic species, such as lipids:

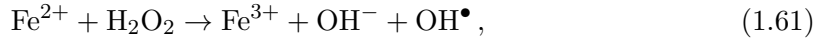


As mentioned above, during the homogeneous chemical stage, the reactions with the biological medium play a key role. In particular, antioxidants XSH, also-called scavengers, are chemical species that can react with reactive species, such as free radicals, e.g., hydrogen radical $\text{H}^\bullet$, hydroxyl radical $\text{OH}^\bullet$, organic radical $\text{R}^\bullet$, etc., or reactive oxygen species (ROS), such as superoxide radical ($\text{O}_2^{\bullet-}$), hydroperoxyl radical ($\text{HO}_2^\bullet$), hydrogen peroxide (H_2O_2), etc., which are all species generated by the reaction between free radicals and molecular oxygen. They are capable of protecting the biological environment from the indirect action of radiation. In fact, the antioxidant protects the biomolecule by scavenging reactive species and preserving it against redox damage. Thus, they maintain the redox balance in the cellular environment. The main reactions are:

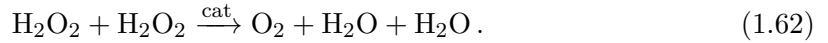


The main example of an antioxidant in the cellular environment is glutathione (GSH), which is a tripeptide consisting of cysteine, glycine and glutamate [7]. It mitigates the oxidative stress of the cell, namely the imbalance between ROS production and antioxidant defenses, and preserves cell redox homeostasis, which is a dynamic process that regulates the balance between reducing and oxidizing reactions. Experimentally, scavengers, such as dimethylsulfoxide (DMSO), are used to selectively study the impact of the direct and indirect action of radiation on DNA damage formation, also for different radiation quality.

Finally, other reactions of the homogeneous chemical stage involved in the consumption of ROS, in particular H_2O_2 , are the Fenton reaction, which involves iron, i.e.,



or other reactions that involve enzymes, such as catalase, i.e.,



1.3 Biology

In this Section, the complex links between physics, chemistry, and biology and the long-term evolution of biological systems after irradiation are presented.

1.3.1 Bio-chemical stage

The bio-chemical stage describes the intermediate zone between chemistry and biology. It goes from seconds to hours, and takes place in a region between 10^{-7} m and 10^{-5} m. After the end of the homogeneous chemical stage, around 10 s, the activity of most of the products generated by ionizing radiation is terminated, resulting in the intracellular accumulation of harmful chemical species such as ROS. Early and rapid biochemical processes, such as DNA damage, membrane lesion, etc., collectively lead to reduced mitosis, disruption of protein signaling networks, membrane rupture, and enzymatic repair processes, which occurs on a time scale of minutes and hours [18].

The main biological target of IR are the DNA molecules in the nucleus of cells. As initially discussed, IR can "directly" induce DNA lesions, depositing its energy in the DNA atoms, or "indirectly", due to the action of organic radicals or free radicals and ROS produced by water radiolysis, leading to the breaking of molecular bonds and the disruption of one or both DNA strands, as illustrated in Figure 1.8a. The percentage of indirect DNA damage with respect to the total depends on the radiation quality of the projectile [31]. In particular, the contribution of the indirect term of radiation-induced DNA lesions decreases with increasing LET, as we can see in Figure 1.8b.

The main types of radiation-induced DNA damage are single-strand break (SSB), when the DNA backbone is broken at a single point, resulting in the breakage of a single DNA strand, and double-strand break (DSB), when there are two close broken points in the DNA backbone [18]. Typically, if two SSBs are close enough, i.e. between 10 base pairs,

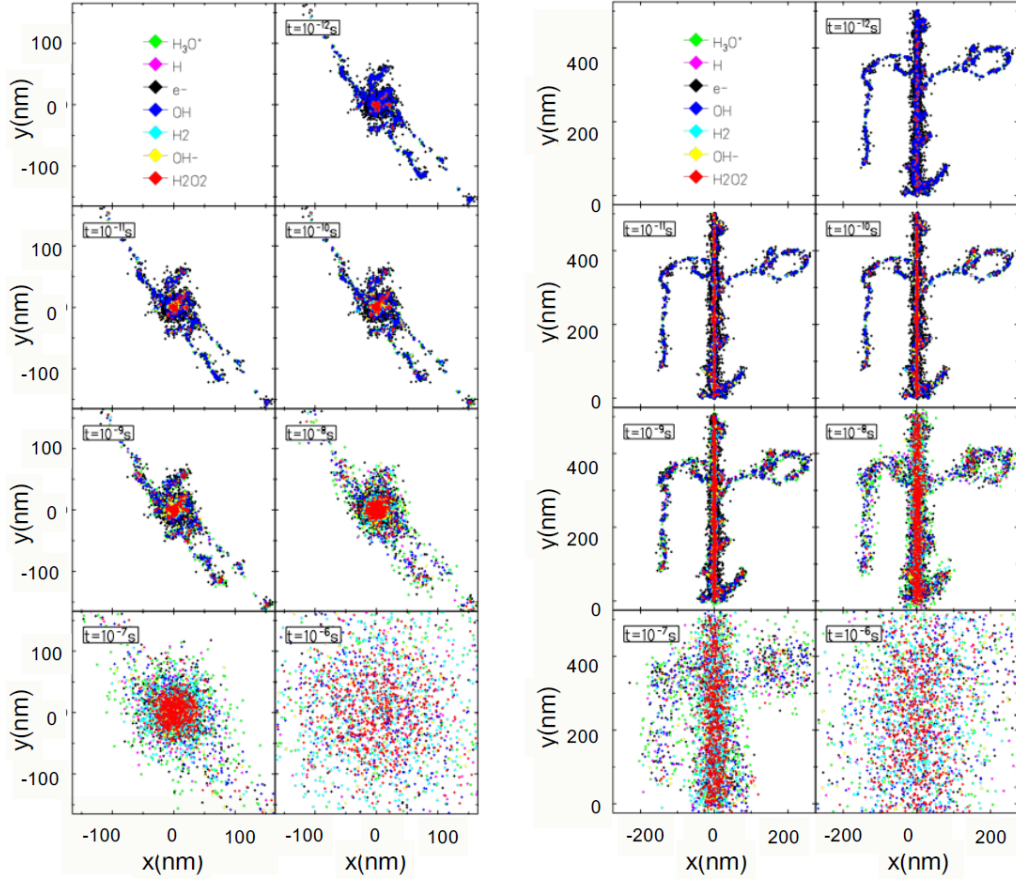


Figure 1.6: Time evolution of chemical species generated by 8 MeV/u carbon ion in water. Simulation from the beginning of the pre-chemical stage (10^{-12} s) to the end of the heterogeneous chemical stage (10^{-6} s), using TRAX-CHEM, a MC track structure code, shown in beam eye view (left panel) and transverse view (right panel). From [28].

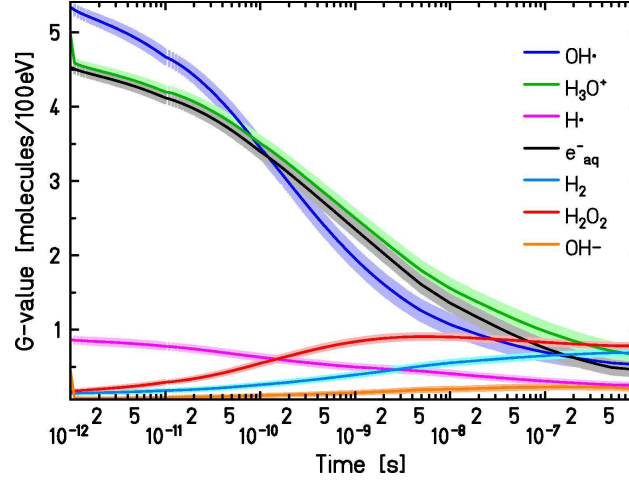


Figure 1.7: Time evolution of G-values for different chemical species generated by 20 MeV/u carbon ion in water. Simulation from the beginning of the pre-chemical stage (10^{-12} s) to the end of the heterogeneous chemical stage (10^{-6} s), using TRAX-CHEM, a MC track structure code. From [28].

these are considered a DSB. Furthermore, DSBs can be classified according to their severity and complexity, considering the distance between the strand breaks and the formation of clusters.

Induction of initial radiation-induced DNA damage is followed by different possible DNA repair pathways, depending on the complexity of DNA lesions. In addition, the probability of repair decreases with increasing severity of the DNA lesion, resulting in gene mutation or cell death.

1.3.2 Biological stage

The biological stage occurs at very long time scales, between hours and days, and involves processes from the cellular level (μm) to the tissue and organ scales (mm - cm). During this stage, we have the biological response from the cellular level, characterized by networking and interaction between cells, to macroscopic systems, like tissue and organs.

A common way to describe the biological effect of radiation on the cellular level is to measure the probability of survival of clonogenic cells as a function of the absorbed dose, where clonogens are cells that have the ability to reproduce, thus producing a colony. The surviving fraction curve is usually described using the Linear-Quadratic (LQ) model, namely

$$S(D) = e^{-\alpha D - \beta D^2}, \quad (1.63)$$

where α [Gy^{-1}] and β [Gy^{-2}] are tissue specific constants, whose ratio, i.e., α/β [Gy] is an indicator of the radiosensitivity of the tissue. An example of a survival curve as a function of the absorbed dose is reported in Figure 1.9.

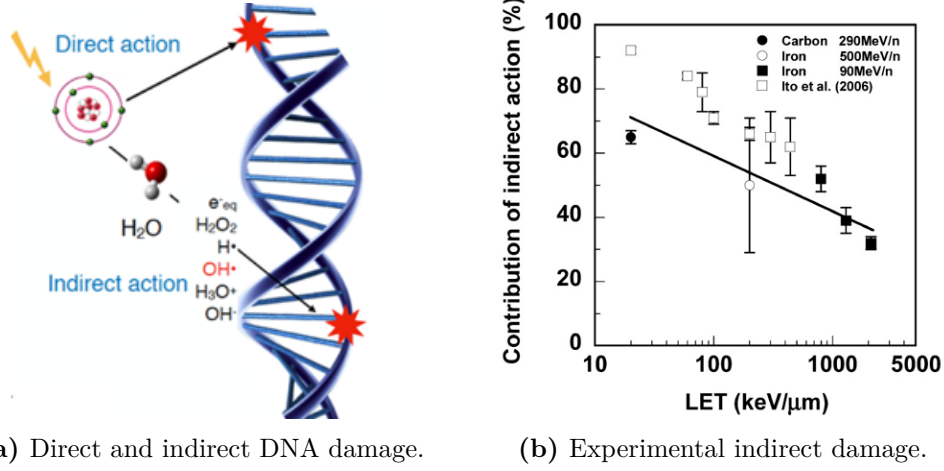


Figure 1.8: **a)** Illustration of direct and indirect contribution of IR on the formation of DNA damage. **b)** Experimental measurements of the portion of indirect DNA damages with respect to the total number of DNA lesions as a function of radiation quality. From [31].

Typically, in RT, the total prescribed dose D is delivered in subsequent n_f fractions of dose $d_f = D/n_f$. For this irradiation scheme, the cell surviving probability proposed in Eq. 1.63 becomes,

$$S(D) = \left(e^{-\alpha d_f - \beta d_f^2} \right)^{n_f}. \quad (1.64)$$

Thus, the biological response to IR is reduced because in the time interval between two consecutive fractions, the cells can repair the radiation-induced damages. It is possible to quantify the effect of fractionation for a given cell radiosensitivity α/β , defining the Biologically Effective Dose (BED) [32, 33] as

$$\text{BED} := \frac{E(D)}{\alpha} = n_f d_f \left(1 + \frac{d_f}{\alpha/\beta} \right). \quad (1.65)$$

where $E(D) = n_f (\alpha d_f - \beta d_f^2)$ is the biological effect according to the LQ model in Eq. 1.64.

1.4 Modifiers and relative effect quantifiers

In this Section the most effective modifiers of the biological response to ionizing radiation are presented. In particular, the effect of the radiation quality, via the Relative Biological Effectiveness (RBE), the environmental oxygen, thanks to the Oxygen Enhancement Ratio (OER), and the dose rate, defining the Dose-Rate Effectiveness Factor (DREF), are described.

1.4.1 Relative Biological Effectiveness (RBE)

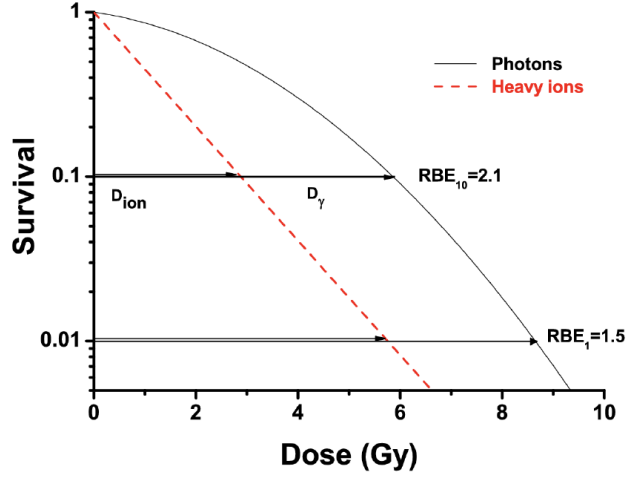


Figure 1.9: Illustration of RBE calculated from survival curves. From [26].

The RBE is defined as the ratio between the dose delivered by the reference radiation, typically photons, $D_{X\text{-ray}}$ and the dose released by ions D_{ion} in order to achieve the same level of biological effect [25]:

$$\text{RBE} := \frac{D_{X\text{-ray}}}{D_{\text{ion}}} \Big|_{\text{isoeffect}}. \quad (1.66)$$

The RBE quantifies the biological response in the case of ion beam irradiation. In particular, we have to increase the X-ray dose to obtain the same radiobiological effect as the ion irradiation. It depends on both physical parameters, such as the absorbed dose and the radiation quality, and biological factors, for example the cell line. In particular, RBE increases for increasing LET, until reaching a maximum value, which corresponds to irreparable DNA damage; then RBE decreases with LET increases due to excess energy deposited in the medium. This decreasing trend is called the *overkill effect*, due to the fact that high-LET IR causes more damage than necessary to kill the cells, as we can see in Figure 1.10. Moreover, for ions with $Z > 1$, the RBE increases with decreasing absorbed dose, up to a maximum value while the dose approaches 0. This point corresponds to the ratio of α values of X-ray and ion.

The RBE is a fundamental quantity for clinical use. In fact, it is used for computing the biological dose D_{bio} , also-called RBE-weighted dose, in treatment planning system (TPS) [34, 35], defined as

$$D_{\text{bio}} := D_{\text{phys}} \cdot \text{RBE}, \quad (1.67)$$

where D_{phys} is the absorbed dose.

Several representations of RBE exist [25]. From the survival fraction curve of Eq. 1.63, it is possible to compute the RBE, using the Eq. 1.66, where the iso-effect in the

definition generally refers to 10% of the cell survival probability, as reported in Figure 1.9. This representation of the biological effectiveness of a radiation is denoted as RBE_{10} , as we can see in Figure 1.10 as a function of different radiation quality.

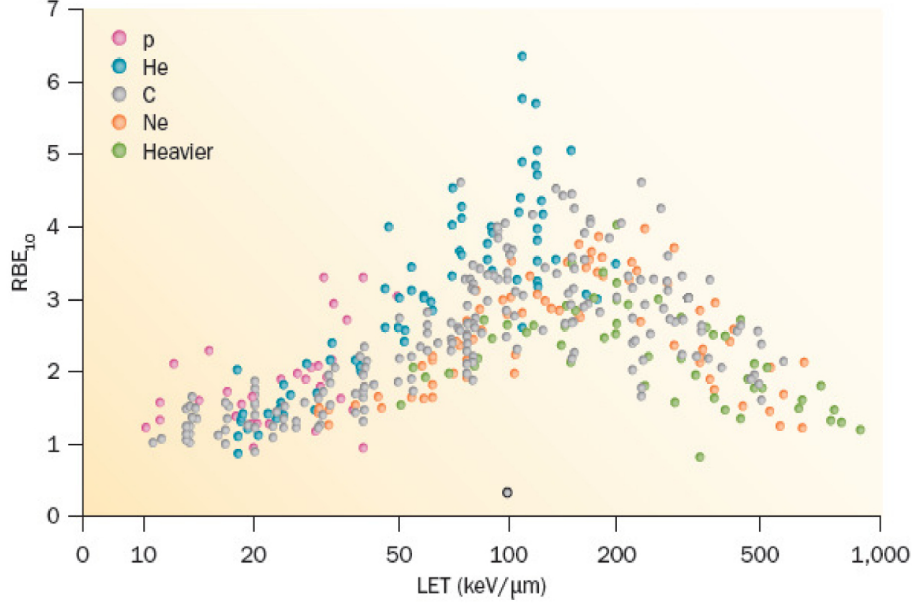


Figure 1.10: Collection of RBE_{10} experimental data as a function of the radiation quality. From [24].

1.4.2 Oxygen Enhancement Ratio (OER)

The OER is defined as the ratio between the absorbed dose at a given oxygenation level $D(p\text{O}_2)$, i.e., $\leq 21\%$ O_2 , and the dose in normoxia condition $D(21\%)$, which is oxygen partial pressure in air, i.e., 21% O_2 , in order to obtain the same given biological effect [30], measured as survival fraction:

$$\text{OER}(p\text{O}_2) := \frac{D(p\text{O}_2)}{D(21\%)} \Big|_{\text{isoeffect}}. \quad (1.68)$$

The OER describes the increasing radioresistance of cells to a decrease in oxygen concentration. In fact, the hypoxia condition in a cell, i.e. reduced oxygen concentration, leads to a decrease of the cell death rate after irradiation. The hypoxia condition is a typical clinical situation, in particular for the cellular environment of the tumor, due to a reduction in blood supply. From a microscopic point of view, this phenomenon occurs because molecular oxygen can fix the biological damage, making it permanent, as previously described in Section 1.2.3. Thus, low concentrations of oxygen reduce the impact of the indirect action of radiation on the generation of DNA lesions.

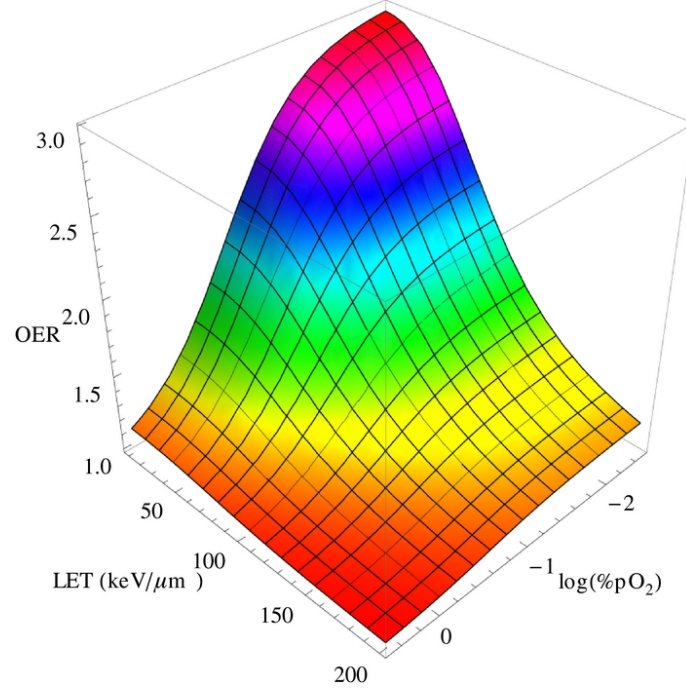


Figure 1.11: Representation of OER as a function of oxygen concentration and radiation quality. From [30].

The first parametrization of the oxygen fixation effect, which has been developed from experimental observations, is the relative radiosensitivity (RR) [30]. RR is the ratio between the sensitivity (RS) at an oxygen tension pO_2 and that in anoxia, i.e. $pO_2 = 0\%$, namely,

$$RR(pO_2) := \frac{RS(pO_2)}{RS(0)} = \frac{MpO_2 + K}{K + pO_2}, \quad (1.69)$$

where M is the maximum value of the curve, and K denotes the oxygen concentration at the midpoint of the curve.

From Eq. 1.68, it is possible to define one of the main parametrizations of oxygen effect [30], which is,

$$OER(pO_2) \approx \frac{M}{RR(pO_2)} = \frac{Mb + pO_2}{b + pO_2}, \quad (1.70)$$

where $b = K/M$.

The OER depends strongly on radiation quality, i.e., on LET. In particular OER decreases with increasing LET [30]. This should be due to the increase in the densities of the track with increasing LET. In fact, for high values of LET, the probability of radical recombination increases, so the importance of the indirect action of radiation decreases, and therefore the dependence on oxygen concentration is attenuated. In [30], they proposed a parametrization of OER in order to also take into account LET dependence, that

is,

$$\text{OER}(p\text{O}_2, \text{LET}) = \frac{b(aM + \text{LET}^\gamma) / (a + \text{LET}^\gamma) + p\text{O}_2}{b + p\text{O}_2}, \quad (1.71)$$

where a and γ are fitting parameters. The dependence of OER on oxygenation and radiation quality is reported in Figure 1.11.

1.4.3 Dose-Rate Effectiveness Factor (DREF)

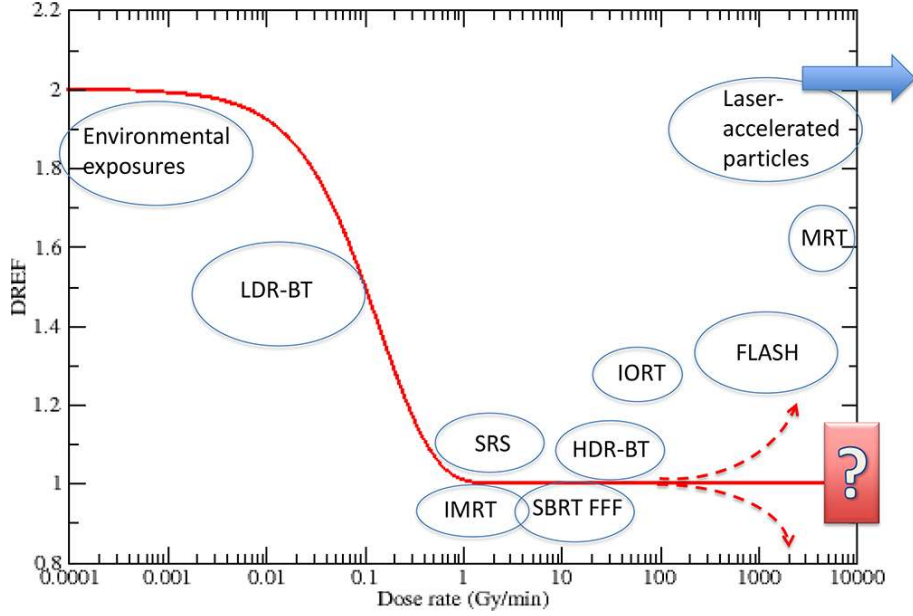


Figure 1.12: Example of DREF as a function of the dose rate. From [36].

The DREF can be defined as the ratio between the absorbed dose at a given dose rate irradiation $D(\dot{D})$, and the dose for the reference dose rate irradiation $D(\dot{D}_{\text{ref}})$ in order to reach the same biological effect [36, 37, 38], i.e.,

$$\text{DREF} := \frac{D(\dot{D})}{D(\dot{D}_{\text{ref}})} \Big|_{\text{isoeffect}}. \quad (1.72)$$

For lower dose rate values with respect to the dose rate of conventional (Conv) radiotherapy (RT), which is ~ 1 Gy/min, DREF begins to increase to achieve a maximum value, around 0.1 Gy/h, where up-regulation of DNA repair mechanisms and activation immune responses start to play a role. This effect is associated with the repair of sub-lethal damage, i.e. lesions that in principle can be repaired. Thus, cells have more time to repair radiation-induced damage, potentially reducing side effects and reaching the maximum sparing effect. This is a well-known phenomenon that is also taken into account in the clinical setting. On the other hand, for an increase in the dose rate, starting from the conventional dose rate, DREF remains constant until it reaches a threshold value, which

is around 40 Gy/s, at which DREF starts to increase, probably achieving a maximum value again. Dose rate values higher than 40 Gy/s are commonly referred to Ultra-High Dose Rate (UHDR) irradiation, and the mechanism behind this biological FLASH effect is still debated and largely unknown, as we will see in Chapter 3.

Another way to quantify the impact of the dose rate in the context of FLASH radiotherapy is to define the FLASH-modifying factor (FMF) [39], as

$$\text{FMF} := \frac{D_{\text{Conv}}}{D_{\text{UHDR}}}\bigg|_{\text{isoeffect}}, \quad (1.73)$$

which is simply the inverse of DREF, where a $\text{FMF} < 1$ describes the FLASH sparing effect. This definition was used in a famous phenomenological study [39], as we will see in Section 3.6.

State of the Art

Chapter 2

Mathematical modeling of radiation damage

The stages of radiation-induced biological damage illustrated in Chapter 1 require the development of mathematical models that can describe and study them. In the literature, a plethora of tools have been proposed using different methodologies, for example, molecular dynamics, Monte Carlo track structure codes, analytical models, and mixed techniques.

Thus, in this Chapter, we show some key examples of models and tools for the investigations of these stages of radiation damage, focusing on those that will later be used in the next Chapters of this thesis. In detail, Section 2.1 reports various methodologies for describing physical processes; Section 2.2 illustrates different approaches to the study of the chemical stage; then, Section 2.3 discusses an example of radiobiological model; finally, Sections 2.4 and 2.4.3 show the most common ways to analyze the dose-response relationship for healthy tissues and tumors, respectively.

2.1 Modeling the physical stage

This Section shows different approaches to describe the physical interactions between ionizing radiation and matter, discussed in Section 1.1. In detail, we present MC track structure codes, with a focus on the TRAX tool [40], we discuss an analytical method, called Amorphous Track (AT) model [41], to describe the radial dose distributions, and we introduce the main concepts of microdosimetry [42].

2.1.1 Monte Carlo 3D track structure codes

The processes that occur during the physical stage can be simulated using different families of MC codes, among which two main groups are:

- (i) **Condensed history codes:** tools that are typically used to compute macroscopic quantities, such as dose distributions or angular distributions of radiation after a series of interactions. These MC codes condense a series of elementary interactions

in a single event. They are used for medical applications, such as in TPSs for dose calculation, imaging analyses, or radiation protection studies. Examples are Geant4 [43, 44], OpenTOPAS [45, 46], FLUKA [47], and FRED [48].

- (ii) **Track structure codes:** tools that describe each interaction that occurs when radiation passes through matter. These MC codes are typically used to compute the positions at each time step of the subexcitation electrons and the ionized and excited species, that are generated by the interaction between ionizing radiation and matter. They also serve as a starting point for studies in chemistry and biology, as we will see in Section 2.2.1. Examples are Geant4-DNA [49], OpenTOPAS-nBio [50], and TRAX [40].

These tools use Monte Carlo approaches in the sense that they employ random numbers to represent the stochastic nature of radiation interactions with matter. For example, quantities such as energies deposition or type of interaction are sampled by specific probability distributions, and the final outcomes are averaged over the number of particle track repetitions.

In particular, TRAX [40] is a track structure MC tool specifically dedicated to the simulation of electrons and ions. It can compute all the physical processes induced by ionizing radiation during the physical stage, given as input projectile parameters, such as type of radiation, initial energy, position of the primary radiation, etc., and target parameters, such as geometrical characteristics, chemical composition, density, etc. The model uses libraries with cross section tables for shell specific excitation and ionization states and elastic scattering event, to describe the physical interactions, as reported in [28]. TRAX can compute different outputs, such as the energy deposition in a given target, the amount and type of physical interactions, the positions of secondary electrons and the corresponding interaction events that generated them, the axial and radial dose distributions, and the electron energy spectra. All of these informations can be used as fundamental input data for further simulations, such as the formation and evolution of chemical species, using the TRAX extension called TRAX-CHEM, as we will see in Section 2.2.1.

2.1.2 The Amorphous Track model

As we saw in the previous Section, MC track structure tools can provide the radial dose distribution for a given projectile as output. Approximations of these distributions can be obtained using analytical methods, namely the so-called AT models, such as the Kiefer–Chatterjee model [51] or the LEM-oriented model [52]. In particular, in this work, we focus on the LEM-oriented model, initially proposed by Scholz and Kraft [52], and used to calculate the local dose in the cell nucleus in the Local Effect Model (LEM) [53, 54], which we will discuss in Section 2.3. The radial dose distribution surrounding the trajectory of charged particles can be described by the inverse of the square of the radial distance r from the track [55], i.e.,

$$D(r) \propto \frac{1}{r^2}. \quad (2.1)$$

In detail, the LEM-oriented model considers the following parametrization to describe the radial dose distribution for a specific charged particle:

$$D(r) = \begin{cases} \lambda \cdot \text{LET}/R_c^2 & \text{if } r \leq R_c, \\ \lambda \cdot \text{LET}/r^2 & \text{if } R_c < r \leq R_p, \\ 0 & \text{if } r \geq R_p, \end{cases} \quad (2.2)$$

where the first formula is the core dose D_c , the second formula describes the penumbra dose D_p , while λ is a normalization constant, so that the integral over the whole track is equal to the LET of the projectile. Considering a medium of density ρ , the normalization constant has the following analytical formula:

$$\lambda = \frac{1}{\pi \rho [1 + 2 \ln(R_p/R_c)]}, \quad (2.3)$$

where R_p [μm] is the penumbra radius, namely the maximum track radius, which is determined empirically by the maximum range of the electrons produced along the primary particle trajectory and it depends on the projectile mass number A and energy E [MeV] [51]. Thus, the penumbra radius is described by the following parametrization:

$$R_p = 0.05 \cdot \left(\frac{E}{A} \right)^{1.7}. \quad (2.4)$$

Typical R_p values are on the order of μm . Instead, $R_c = 0.01 \mu\text{m}$ is the core radius and defines the core region of the track in which the dose is constant.

2.1.3 Brief introduction to microdosimetry

This Section is dedicated to the presentation of the main notions and quantities of microdosimetry. The concept of microdosimetry was originally proposed by Harald H. Rossi, who highlighted the crucial difference between macroscopic absorbed dose due to ionizing radiation and energy deposition at the microscopic level, which is stochastic. These ideas were later formalized in a more advanced manner by Albrecht M. Kellerer [42].

A fundamental quantity in microdosimetry is the *specific energy* z [Gy], which is a stochastic quantity and it is defined as

$$z = \frac{\varepsilon}{m}, \quad (2.5)$$

where ε is the energy imparted by ionizing radiation in the matter of mass m , it's a stochastic quantity, and it can be described as

$$\varepsilon = \sum_i \varepsilon_i, \quad (2.6)$$

where the summation is performed over a finite number of energy depositions events ε_i in a specific volume, which is also a stochastic quantity.

Given a probability density $f(z)$ for the stochastic variable z , the expectation value is

$$\bar{z} = \int_0^\infty z f(z) dz. \quad (2.7)$$

which is called *mean specific energy*. In addition, the specific energy may be caused by one or more energy deposition events. Thus, given the single event distribution $f_1(z)$ of z , the corresponding expectation value is

$$\bar{z}_F = \int_0^\infty z f_1(z) dz, \quad (2.8)$$

which is called *frequency-mean specific energy per event*. Then, given the number of deposition events n , which is typically described by a Poisson distribution, we can define the mean events number as

$$\bar{n} = \frac{\bar{z}}{\bar{z}_F}. \quad (2.9)$$

In addition, given the dose probability density $d_1(z)$, we can define the *dose-mean specific energy per event* as

$$\bar{z}_D = \int_0^\infty z d_1(z) dz, \quad (2.10)$$

where $d_1(z)$ and $\bar{z}_D$ are linked to $f_1(z)$ and $\bar{z}_F$ as

$$d_1(z) = \frac{z}{\bar{z}_F} f_1(z), \quad (2.11)$$

$$\bar{z}_D = \frac{1}{\bar{z}_F} \int_0^\infty z^2 f_1(z) dz. \quad (2.12)$$

The second crucial quantity in microdosimetry is the *lineal energy* y [keV/ μ m], which is a stochastic quantity defined as

$$y = \frac{\varepsilon_1}{\bar{l}}. \quad (2.13)$$

where ε_1 the energy imparted to the matter in a volume by a single energy-deposition event, while $\bar{l}$ is the mean chord length in that volume.

In this case, given the lineal energy distribution $f(y)$ of y , its expectation value is

$$\bar{y}_F = \int_0^\infty y f(y) dy, \quad (2.14)$$

which is called *frequency-mean lineal energy*. Furthermore, given the dose probability density $d(y)$ of y , the corresponding expectation value is

$$\bar{y}_D = \int_0^\infty y d(y) dy, \quad (2.15)$$

which is called *dose-mean lineal energy*, and where $d(y)$ and $\bar{y}_D$ are related to $f(y)$ and $\bar{y}_F$ as

$$d(y) = \frac{y}{\bar{y}_F} f(y), \quad (2.16)$$

$$\bar{y}_D = \frac{1}{\bar{y}_F} \int_0^\infty y^2 f(y) dy. \quad (2.17)$$

More details on theoretical and experimental microdosimetry are reported, for example, in [56].

2.2 Modeling the chemical stage

This Section is dedicated to the presentation of tools for the simulation of chemical processes induced by ionizing radiation, discussed in Section 1.2. In particular, we present MC track structure codes for the investigation of the heterogeneous chemical stage, focusing on the TRAX-CHEM tool [28], and a reaction kinetic model [7] for the description of the homogeneous chemical stage.

2.2.1 Monte Carlo codes for chemistry simulation

The pre-chemical and heterogeneous chemical stages, namely all reactions and diffusion processes that occur after the physical interaction of ionizing radiation with matter, typically represented by water, can be simulated using the family of Monte Carlo tools called “track structure” codes. As discussed in Section 2.1.1, the peculiarity of these tools is that they can follow the evolution of all interactions occurring in a volume of interest, describing each single event. Thus, while this “event-by-event” approach allows for a detailed description of the interaction between radiation and matter, making them ideal candidates for simulation of chemical processes, it also comes with enormous computational costs, which limit its use to small target volumes.

The most common approach for the simulation of reactions and diffusion of chemical species is the so-called *step-by-step* algorithm [57], which can describe, with high precision, the evolution in space and time of each chemical species in the target volume. In recent decades, several MC tools have been developed, such as PARTRAC [58, 59], Geant4-DNA [49], OpenTOPAS-nBio [60, 50], and TRAX-CHEM [28, 29]. In particular, TRAX-CHEM can simulate water radiolysis [28], also considering the impact of environmental oxygenation level [29]. The model receives as input the positions of the subexcitation electrons and the ionized and excited species, generated by ionizing radiation during the physical stage. Then, using a table that contains all the necessary information for the chemistry simulation, such as the dissociation channel probabilities, the thermalization scheme, the diffusion coefficients, and the reaction rate constants, the TRAX-CHEM model can compute reactions and diffusion processes for each molecule, ion, and free radical. In detail, the diffusion of chemical species is a jump in a random direction, and it is described by 3D Gaussian distributions, where the displacement is calculated according to the Einstein-Smoluchowski equation, that is,

$$\lambda = \sqrt{6D\Delta t} \quad (2.18)$$

where D is the diffusion coefficient and Δt the time step. At the same time, two chemical species can react with each other, according to their concentrations and the specific reaction rate constant. The reaction process is described by the definition of the *reaction*

radius R_r , which represents the minimum distance between two chemical species to react, that is,

$$R_r = \frac{k_{12}}{4\pi(D_1 + D_2)} \quad (2.19)$$

where k_{12} is the reaction rate constant, while D_1 and D_2 are the diffusion coefficients. The output of the model are the track position in space and time, the radial distribution, and the time evolution of the G-value, according to Eq. 1.13, for each chemical species. An example of the time evolution of chemical species is reported in Figure 1.6, while an example of G-values is shown in Figure 1.7. Both examples were computed using TRAX-CHEM.

The second main approach to simulate the heterogeneous chemical stage is the so-called *independent reaction time* (IRT) algorithm [61], which is implemented in various tools, such as RITRACKS [62], Geant4-DNA [63], and OpenTOPAS-nBio [64], allowing faster calculations compared to the standard *step-by-step* method, but losing the exact positions of chemical species. The main idea of this approach is that each chemical process is computed as the minimum reaction time from specific time-dependent survival probabilities, which are calculated considering all possible pairs of reactive species in the target volume, starting from the initial positions and distributions of that species. The simulation ends when no other reactions can occur, and produces as output probability density distributions of relative distances between species.

2.2.2 A physicochemical model of reaction kinetics

The homogeneous chemical stage, namely all reactions that occur on a time scale from $\sim \mu\text{s}$ to s, can be described using a system of ordinary differential equations (ODEs) according to Eq. 3.47, such as the reaction kinetic system proposed in [7]. The details of this approach will be presented in Section 3.6, in the context of mathematical modeling of the FLASH effect.

2.3 The radiation biophysical models

In this Section, we discuss the main radiobiological models in RT, in particular, focusing on the main features of the Generalized Stochastic Microdosimetric Model (GSM²), which is the radiation biophysical model that we extensively improve in this thesis. These models are used to describe the biological response induced by ionizing radiation, as discussed in Section 1.3.

The most comprehensive radiation biophysical models in the literature are:

- (i) **Local Effect Model (LEM):** The main assumption of this model is that at the microscopic level, radiation damage is radiation-type independent and is due to the local dose [53, 54]. The LEM model can determine the biological effect of any type of radiation knowing: (1) the average number of lethal events for X-ray irradiation, according to an improved formulation of the LQ model in Eq. 1.63, which is the so-called Linear-Quadratic-Linear (LQL) model; (2) the radial dose distribution for

the specific ion, according to the discussed AT model in Eq. 2.2; (3) the radius of the cell nucleus, considering DNA as the critical target of IR. Therefore, integrating the average number of lethal events over the volume of the cell nucleus and considering the local dose, which determines the biological effect in a small volume element, the survival probability for heavy ions is determined.

- (ii) **Microdosimetric Kinetic Model (MKM):** The MKM model is the most used model in clinics and can predict the survival probability of cell nuclei after exposure to IR, where the radiation quality is described by microdosimetry [65]. The main assumptions of the model are the following: (1) the cell nucleus is divided into a finite number of independent domains; (2) IR can induce two different types of DNA damage, i.e., type *I* and *II*; (3) type *II* lesions cannot be repaired, so they are called lethal lesions. Instead, type *I* lesions can be either repaired or evolve into a lethal lesions either by spontaneous death or by interaction with another sublethal lesion, so they are called sub-lethal lesions; (4) the lesions are confined to the domain in which they are generated; (5) the number of type *I* and *II* lesions in a single domain is proportional to the specific energy z delivered in that volume, according to Eq. 2.5; (6) lethal lesions follow a Poissonian distribution; (7) cell death occurs if at least one domain suffers at least one lethal lesion.
- (iii) **Generalized Stochastic Microdosimetric Model (GSM²):** The GSM² model is a fully probabilistic model that describes the stochastic nature of energy deposition in volumes of interest for cellular systems [3, 4]. The final goal is to overcome existing models, which most assume a Poissonian distribution of the number of radiation-induced DNA damage, to provide a better prediction of biological endpoints relevant to radiotherapy applications. More details are reported in Section 2.3.1.

2.3.1 The Generalized Stochastic Microdosimetric Model (GSM²)

The GSM² has been first introduced in [3], where a detailed description of its structure can be found. The GSM² is a stochastic Markov model that describes the formation and repair of radiation-induced DNA damages in a cell nucleus. The model assumes that the cell nucleus is divided into N_d independent domains d . Within each domain, it is assumed that two types of DNA lesions can be formed, called lethal and sub-lethal lesions. The former represents damage that cannot be repaired leading to cell inactivation, while the latter can be either repaired or become a lethal lesion leading to cell inactivation.

For modeling the time-evolution of the number of lethal and sub-lethal lesions, we will denote by $(Y(t), X(t))$ the state of the system at time t , where X and Y are two $\mathbb{N}$ -valued random variables representing the number of lethal and sub-lethal lesions, respectively.

A sub-lethal lesion can evolve in three different ways: i) it can become a lethal lesion at a rate a , ii) it can be repaired at a rate r , or it can combine with another sub-lethal lesion to become a lethal lesion at a rate b . This scheme can be represented by the following

equations:

$$\begin{aligned} X &\xrightarrow{a} Y, \\ X &\xrightarrow{r} \emptyset, \\ X + X &\xrightarrow{b} Y. \end{aligned} \tag{2.20}$$

where $\emptyset$ represents the ensemble of healthy cells.

On the bases of (2.20), the *Microdosimetric Master Equation* (MME) [3] has been defined as

$$\begin{aligned} \frac{\partial}{\partial t} p(t, y, x) &= (E^{-1,2} - 1) [x(x-1)bp(t, y, x)] + (E^{-1,1} - 1) [xap(t, y, x)] + \\ &+ (E^{0,1} - 1) [xrp(t, y, x)] = \\ &+ \mathcal{E}^{-1,2} [x(x-1)bp(t, y, x)] + \mathcal{E}^{-1,1} [xap(t, y, x)] + \\ &+ \mathcal{E}^{0,1} [xrp(t, y, x)], \end{aligned} \tag{2.21}$$

where the creation operator is defined as

$$\mathcal{E}^{i,j} [f(y, x)] := (E^{i,j} - 1) [f(y, x)] := f(y+i, x+j) - f(y, x).$$

which means a transition in the state defining the number of sub-lethal and lethal damage, namely denotes the increase or decrease in the number of sub-lethal and lethal lesions based on the biological rates a , b , and r . In detail, Eq. 2.21 describes the time evolution of the joint probability density function $p(t, y, x)$, which represents the probability of observing exactly x sub-lethal lesions and y lethal lesions within the cell nucleus at time t .

The MME is coupled with an initial damage distribution derived from microdosimetric spectra, which describe in full detail the radiation field quality, [66, 67, 68]. The single-event distribution of energy deposition on a domain d , referred to as $f_{1;d}(z)$ [42], can be either computed numerically with a MC code or measured experimentally. Given a cell nucleus domain d , the probability that ν events deposit energy z follows a Poissonian distribution of mean $\lambda_n := \frac{z_n}{z_F}$, being z_n the mean energy deposition on the nucleus domain, that is,

$$z_n = \int_0^\infty z f(z|z_n) dz, \tag{2.22}$$

and z_F the first moment of the single event distribution $f_{1;d}$, namely,

$$z_F = \int_0^\infty z f_{1;d}(z) dz. \tag{2.23}$$

Then, assuming a Poissonian probability that a domain registers ν events, the energy deposition distribution is given by

$$f_d(z|z_n) := \sum_{\nu=0}^{\infty} \frac{e^{-\frac{z_n}{z_F}}}{\nu!} \left(\frac{z_n}{z_F} \right)^\nu f_{\nu;d}(z), \tag{2.24}$$

where $f_{\nu;d}(z)$ is the energy deposition distribution resulting from ν depositions and we assumed by convention that $f_{0;d}(z) = \delta_0(z)$, meaning that if no deposition events occur within a given domain, the deposited energy is, by definition, zero.

Denoting by κ and by λ the average yields of sub-lethal and lethal lesions, respectively, for a given energy deposition z , the induced number of lesions are random variables, where the distribution of X given z is a Poisson random variable of mean value κz , while the distribution of Y given z is another Poisson random variable of mean λz . In particular, we assume that the formation of lesions by different deposition events is statistically independent because the events are independent. In addition, the formation of lesions by a single energy deposition event is approximately statistically independent.

Thus, the initial condition for the MME in Eq 2.21 is

$$p(0, y, x) = p_0^X(x) p_0^Y(y), \quad (2.25)$$

where the initial distribution is obtained as

$$\begin{aligned} p_0^X(x) &= \int_0^\infty p_z^X(x|\kappa z) f(z|z_n) dz, \\ p_0^Y(y) &= \int_0^\infty p_z^Y(y|\lambda z) f(z|z_n) dz. \end{aligned} \quad (2.26)$$

in which $p_z^X(x|\kappa z)$ and $p_z^Y(y|\lambda z)$ denote the initial random distribution for the number of sub-lethal and lethal lesions, respectively, given an energy deposition z . In particular, $p_z^X(x|\kappa z)$ and $p_z^Y(y|\lambda z)$ can be any probability distribution. In addition, the initial condition (2.26) is a generalization of the standard assumptions, because both p_0^X and p_0^Y may not be Poisson distributed even if $p_z^X(x|\kappa z)$ and $p_z^Y(y|\lambda z)$ are Poisson random variables. This is the key difference between GSM² and MKM, which typically assumes a Poisson distribution for radiation-induced lethal lesions. In fact, this assumption shows limitations, particularly at high doses and high LET, as shown in [4, 69].

An example of the time evolution of sub-lethal and lethal lesions is reported in Figure 2.1, where we can observe that the mean value may not accurately represent the entire distribution of path realizations.

The final biological endpoint predicted by GSM² is the cell survival probability. For a single domain d , it is defined as

$$S_d(z_n) := \mathbb{P} \left(\lim_{t \rightarrow \infty} Y(t) = 0 \right); \quad (2.27)$$

which can be derived by analyzing the limiting long-term distribution of the MME in Eq 2.21. Thus, no lethal lesions should remain in the cell nucleus after a sufficiently long time after irradiation.

Assuming independent domains, the survival probability of a single cell can be computed in the following way

$$S_n(z_n) := (S_d(z_n))^{N_d} = \left(\mathbb{P} \left(\lim_{t \rightarrow \infty} Y(t) = 0 \right) \right)^{N_d}. \quad (2.28)$$

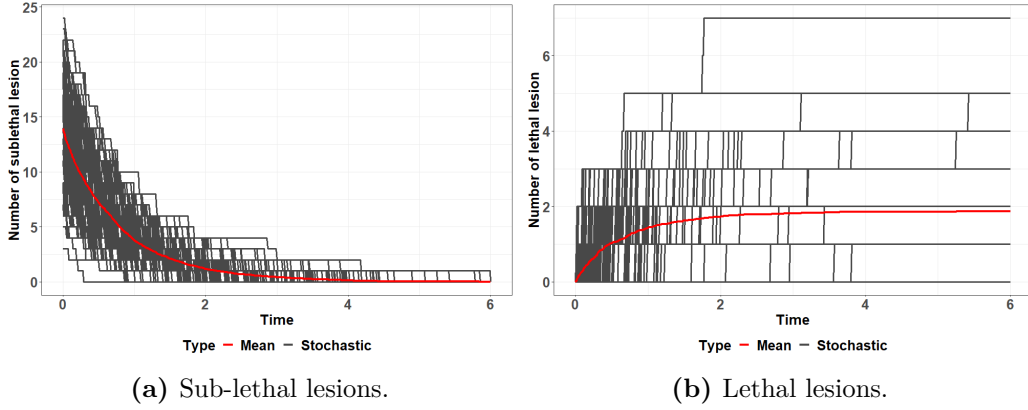


Figure 2.1: **a)** Sub-lethal lesions and **b)** lethal lesions evolution after 0.3 Gy irradiation with 80 MeV protons. GSM² parameters were set to $r = 1$, $a = 0.1$ and $b = 0.01$. The red line represents the average value. From [3].

Defining the multi-event distribution on the whole cell population as

$$f_n(z_n|D) := \sum_{\nu=0}^{\infty} p_n(\nu|D) f_{\nu;c}(z_n), \quad (2.29)$$

where $f_{\nu;c}(z_n)$ is the energy deposition distribution resulting from ν depositions in a single nucleus, which can be obtained convolving the single event distribution $f_{1;c}(z_n)$, and $p_n(\nu|D)$ is the probability that ν energy depositions occur in a nucleus.

Considering a macroscopic absorbed dose D , which can be computed as

$$D = \int_0^{\infty} z_n f_n(z_n|D) dz_n, \quad (2.30)$$

the survival probability of the cell population can be determined as

$$S(D) = \int_0^{\infty} S_n(z_n) f_n(z_n|D) dz_n. \quad (2.31)$$

The GSM² can model *in-vitro* cell survival data for conventional irradiation for different particles [69, 71]. Our recent studies [6] have shown that GSM² can describe the biological effects of radiation types and energies, including protons, helium, and carbon ions, as we can see in Figure 2.2. GSM² integrates microdosimetric information into cell survival predictions, thereby generalizing the MKM that rely on average values. Furthermore, it can describe the trend of the overkilling effect without needing ad hoc corrections [6], such as saturated average values. The GSM² was considerably extended in the present work, as we will see in Chapter 4.

2.4 Dose-effect relation for normal tissues and tumors

This Section is dedicated to the presentation of common approaches used to evaluate the radiation-induced response of healthy tissues and tumors. In Section 2.4.1 we define the

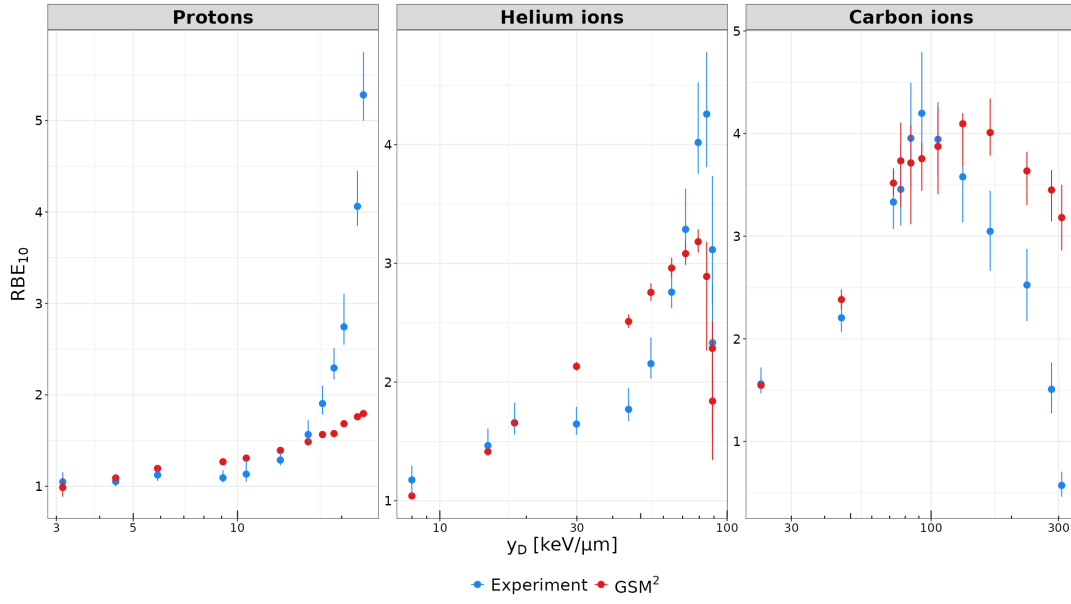


Figure 2.2: RBE_{10} , which is the ratio of the doses for the two different irradiation types necessary to reach a 10% survival probability, against y_D , namely the microdosimetric dose average lineal energy which describes the radiation quality, for H460 cell line divided into three panels corresponding to each type of particle irradiation, namely protons, helium, and carbon ions (from left to right respectively). Blue circles show RBE_{10} data from the experimental work [70], while in red are the predictions from GSM^2 . From [6].

concept of volume effect for an organ; in Section 2.4.2 we report some example of Normal Tissue Complication Probability (NTCP) models; while, in Section 2.4.3 we present Tumor Control Probability (TCP) models.

2.4.1 Tissue architecture and volume effect

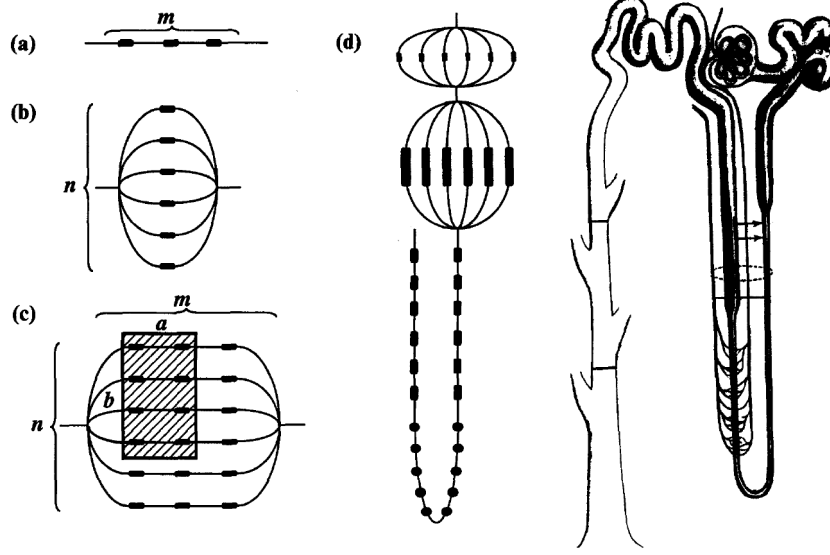


Figure 2.3: Schematic representation of (a) serial, (b) parallel, and (c) complex organ structure. (d) Example of the structure of a nephron, which is the functional sub-unit (FSU) of the kidney. The black points represent the FSUs. From [72].

The study of the radiation response of macroscopic biological structures, such as tissues or organs, is complicated because different types of cells may be present, characterized by different radiosensitivities. Furthermore, a group of cells can be organized by forming a functional sub-unit (FSU) [73], namely a biological structure that has a specific organization and function (e.g. lung alveoli or nephron). The FSUs can be organized in different structural arrangements, forming serial and parallel tissues, as schematized in Figure 2.3. In a serial arrangement, the functional sub-units are connected as the links of a chain, while in a parallel organization, the FSUs are independent of each other. For this reason, in the case of a serial organ, if a single FSU is damaged by IR, this event can trigger the loss of organ functionality, leading to a complication, such as for the spinal cord or the esophagus. Instead, for a parallel organ, the inactivation of a single FSU does not disturb the correct functionality of the organ, such as for the liver, the lung, or the parotid glands; in this case, the inactivation of FSUs above a specific threshold [74], quantifiable by a minimum number of preserved FSUs, can cause a complication.

Therefore, the tissue architectures, i.e. the different FSUs arrangements, can explain the different dose-volume dependencies of radiation response. In this context, we can define the volume effect as the way in which an organ or a tissue responds to a damage

after irradiation, depending on its structural organization. In particular, serial organs are characterized by a small volume effect, due to the fact that the inactivation of a single FSU can lead to organ dysfunction; thus, in this case, the maximum dose D_{max} received by the organ correlates with a possible complication. Instead, for parallel organs, the volume effect is large, that is, the organ can tolerate a certain number of damaged FSUs; for this reason, the possible toxicity of the organ is related to the mean dose D_{mean} . In practice, real complex organs are organized as a combination of serial and parallel architectures, as for the nephron structure, which is illustrated in Figure 2.3d. Furthermore, the same organ can develop several different complications, responding in some cases as a serial organization, while in others as a parallel architecture. The typical example is rectal bleeding compared to diarrhea.

2.4.2 Normal Tissue Complication Probability (NTCP)

The notable improvement of radiotherapy techniques and TPSs in recent years has led to the technical possibility of generating a wide variety of treatment plans and dose distributions, which must be compared in a simple and fast way. Several studies on radiation-induced normal tissue toxicity have been performed. The probability of encountering a side effect has been quantified by proposing the concept of NTCP, a key quantity to classify the goodness of treatment plans. In doing this, however, it is necessary to take into account different practical problems, such as different radiation complications for the same type of organ, different clinical methods for the evaluation of the endpoint (patient's symptoms interview, imaging, laboratory analysis, etc.), and additional clinical factors (surgery, chemotherapy, co-morbidities etc.). All of this led to an intensive modeling study of the NTCP, using different approaches, namely

- (i) **Statistical models:** Based on the correlation between dosimetric or clinical variables and a specific toxicity endpoint, using logistic regression analysis. Few or no biological considerations. E.g. the uni- and multi-variate (MVA) logistic analyses [75].
- (ii) **Mechanistic models:** Based on tissue organization and cellular radiosensitivity. Complex formulation and poor clinical use. E.g. the relative seriality model [76].
- (iii) **Phenomenological models:** Based on a sigmoidal dose-response curve. The most widely used approach. E.g. the Lyman–Kutcher–Burman (LKB) model [76].

The Lyman–Kutcher–Burman (LKB) model

The Lyman–Kutcher–Burman (LKB) model [76] is one of the most used phenomenological models for the description of NTCP curves. The idea of this model is to obtain a curve of the probability of reaching a given clinical endpoint, for a given healthy organ, as a function of a dosimetric index, starting from a 3D dose distribution. In detail, it describes

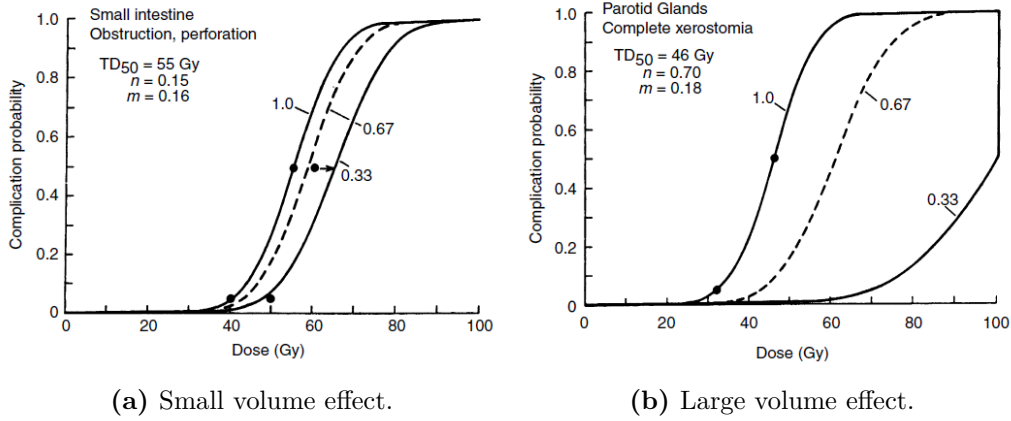


Figure 2.4: Example of Normal Tissue Complication Probability (NTCP) curves as a function of absorbed dose for two organs, one with **a)** a small volume effect, and another with **b)** a large volume effect, for three different irradiation of the organ volume, namely whole organ (1.0), two-thirds (0.67), and one-third (0.33). From [77].

the probability for a certain toxicity as a function of the dose using a sigmoid shaped curve, namely

$$\text{NTCP}(u) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^u e^{-\frac{t^2}{2}} dt \quad (2.32)$$

where the variable

$$u(D) = \frac{D - TD_{50}}{m \cdot TD_{50}} \quad (2.33)$$

contains the parameters which describe the dose-response relationship. In particular, TD_{50} is the whole organ dose corresponding to NTCP of 50%, while m is the slope of the dose-response curve at TD_{50} .

The present LKB model is an evolution of the simpler Lyman model [78], whose main assumption is the uniform irradiation of the whole organ to a certain dose level D . So, in the case of heterogeneous dose distributions, Eq. 2.33 becomes

$$u = \frac{\text{gEUD} - TD_{50}}{m \cdot TD_{50}} \quad (2.34)$$

where gEUD is the Generalized Equivalent Uniform Dose (gEUD), firstly proposed by Niemierko in [79] and used in our previous work [80], which is defined as

$$\text{gEUD} = \left(\frac{1}{N} \sum_{i=1}^N D_i^a \right)^{\frac{1}{a}} \rightarrow \begin{cases} D_{\min} & \text{if } a \rightarrow -\infty \\ D_{\text{mean}} & \text{if } a \rightarrow 1 \\ D_{\max} & \text{if } a \rightarrow +\infty \end{cases} \quad (2.35)$$

where N is the total number of voxels of a given organ, while a is a parameter which quantifies the volume effect of the organ. In particular, $a \simeq 1$ is used for organs with

a large volume effect, i.e. parallel organs, while $a \gg 1$ for organs with a small volume effect, namely serial organs. Instead, $a < 0$ are applied to tumors. The key point of gEUD is the conversion of a heterogeneous dose distribution into a uniform dose associated with a same biological effect.

Figure 2.4 reports an example of NTCP curves for two different volume effects, while Figure 2.5 shows the impact of different partial irradiations of the whole organ volume, calculated according to Eq. 2.32.

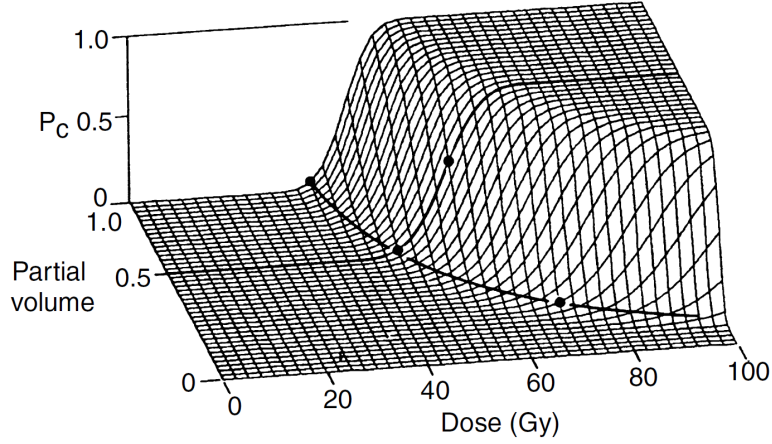


Figure 2.5: Example of 3D surface of Normal Tissue Complication Probability (NTCP) as a function of absorbed dose and partial irradiated volume, for a given organ. From [78].

The relative seriality model

The relative seriality model is a common NTCP model, which was originally proposed by Kallman et al. in [72]. This is a more radiobiologically based NTCP model, compared to phenomenological models such as the LKB model [76]. In fact, it takes into account the architecture of the organ through the definition of a relative seriality parameter. In detail, the model considers a target volume composed of a total number of FSUs $M = n \cdot m$, where n is the number of parallel FSUs, and m is the number of serial FSUs, as illustrated in Figure 2.3c. Thus, for a given organ architecture, the relative seriality parameter s is defined as the number of serial FSUs, m , with respect to the total number of FSUs, M , that is

$$s := \frac{m}{n \cdot m} = \frac{1}{n} \rightarrow \begin{cases} 1 & \text{for fully serial organs} \\ 0 & \text{for fully parallel organs} \end{cases} \quad (2.36)$$

Furthermore, the model describes cell inactivation through Poisson statistics. Thus, considering a fully serial organ, which is made up of m serial FSUs, e.g. that in Figure 2.3a, the dose-response relation, after a uniform irradiation with a dose D , is the following:

$$P_o = 1 - \prod_{i=1}^m (1 - P_i), \quad (2.37)$$

where P_i is the local response of the i -th FSU, which is described by a Poisson statistical model of cell kill, namely

$$P_i = 2^{-\exp\left(e\gamma\left(1-\frac{D}{D_{50}}\right)\right)}, \quad (2.38)$$

where D_{50} is the uniform dose that causes 50% probability of injury and γ is the slope of the response curve at D_{50} . Thus, inactivation of any FSU leads to complications.

Instead, in the case of a fully parallel organ, which contains n parallel FSUs, e.g. that in Figure 2.3b, the dose-response relation becomes

$$P_o = \prod_{j=1}^n P_j. \quad (2.39)$$

So, any FSU remaining preserves organ function.

However, most organs have a complex architecture. Thus, for a generic tissue organization, such as that in Figure 2.3c, the dose-effect relationship can be estimated as

$$\text{NTCP} := P_o = \prod_{j=1}^n \left[1 - \prod_{i=1}^m (1 - P_{ij}) \right], \quad (2.40)$$

In addition, since the true arrangement of FSUs is often unknown, the NTCP formula in Eq. 2.40 can be reformulated in terms of the relative seriality parameter s in Eq. 2.36, that is

$$\text{NTCP} = \left[1 - \prod_{i=1}^M (1 - P_i)^s \right]^{\frac{1}{s}}. \quad (2.41)$$

This model has also been generalized to describe heterogeneous dose distributions. More details are reported in the original work [72].

2.4.3 Tumor Control Probability (TCP)

The primary objective of radiotherapy is to counteract tumor progression, maintaining control over tumor volume growth. As presented in Section 2.4.2 for NTCP models, various mathematical approaches are available to characterize the dose-effect relationship of tumors.

In particular, as discussed in Section 1, the response of a single cell to IR can be described through the LQ model, according to Eq. 1.63, where the DNA molecule is the critical target. In this way, the TCP for a given tumor volume with N_{cell} cells, which are characterized by a specific radio sensitivity α/β , can be defined in the following way

$$\text{TCP} = \exp\left(-N_{cell} \cdot \exp\left(-\alpha D - \beta D^2\right)\right), \quad (2.42)$$

as a function of the delivered dose D .

Instead, in the context of the relative seriality model [72], the tumor is parameterized as a purely parallel organ. Thus, the TCP is defined as the probability of killing the tumor, namely

$$\text{TCP} = \prod_{i=1}^n P_i. \quad (2.43)$$

Chapter 3

The FLASH effect

The goal of technological and biological advances in RT has always been to develop new treatment approaches that are as effective as possible on the tumor and that spare as much healthy tissue as possible. That is, the goal of oncologists is to improve as much as possible the so-called therapeutic window between sufficient efficacy in tumors and damage healthy tissue at an acceptable level.

In this context, in the last decade, a new rapid dose release radiotherapeutic technique has been proposed, which is currently one of the frontier approaches under study and development in the fight against tumors. In particular, this novel RT technique is based on Ultra-High Dose Rate (UHDR) irradiation [81, 82]. Experiments have extensively demonstrated that UHDR RT is capable of reducing side effects in normal tissue [83, 84, 85, 86], preserving the same effectiveness in tumors [87], compared to traditional approaches. This biological effect is commonly known as the FLASH effect, hence the name FLASH RT. Today, the FLASH effect is one of the most active research fields in radiotherapy, as in principle it could be a great step in the fight against tumors [88]. However, the origin and mechanism of this peculiar biological effect remain elusive and largely unknown [82].

In this Chapter, FLASH RT is presented and described in detail, given the crucial role of the present evidences and open questions in this thesis. In particular, the first Section (3.1) is dedicated to the illustration of the FLASH effect, the second Section (3.2) to the description of UHDR dose delivery techniques, and the third Section (3.3) to the possible definitions of dose rate. Then, Section 3.4 focuses on a review of the experimental studies on the biological response to UHDR irradiation. In the end, the main hypotheses of the mechanism behind the FLASH effect and the mathematical models proposed to describe it are discussed in Sections 3.5 and 3.6, respectively.

3.1 Definition and brief historical background

The UHDR or FLASH RT is a novel irradiation approach which delivers the dose on the patient in a very fast way, with typically a total irradiation time < 100 ms and a total mean dose rate > 40 Gy/s for a single irradiation with dose usually higher than 10 Gy

[81, 82], where the dose rate is dose delivered per unit of time, defined as

$$\dot{D} [\text{Gy/s}] = \frac{D [\text{Gy}]}{t [\text{s}]} \quad (3.1)$$

However, the dose and dose rate thresholds are indicative and are still being studied today. Usually, the conventional (Conv) technique releases the dose using a dose rate between 0.03 Gy/s and 0.1 Gy/s. As will be described in detail in the following sections, this innovative technique is based on the concept of FLASH effect, i.e. a greater sparing of healthy tissues for the same tumor efficacy, compared to Conv RT.

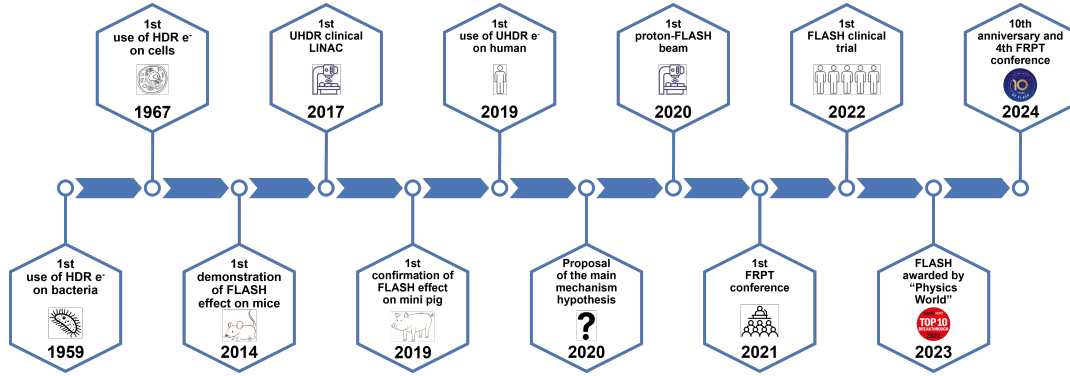


Figure 3.1: Timeline showing the main events regarding the FLASH effect.

FLASH radiotherapy as we know it today was born just over 10 years ago, with the seminal experiment conducted by the Vincent Favaudon team at the Institute Curie in 2014 on mice, using a linear electron accelerator (LINAC) at a high beam current [87]. They demonstrated for the first time the differential response between healthy tissue and tumor in the UHDR regime, inventing the term "FLASH". The radiobiological benefits of the FLASH effect were confirmed by the second fundamental experiment carried out by the Marie-Catherine Vozenin team in CHUV (Lausanne) 2019 on a mini-pig, using electron LINAC [83]. However, the first use of high dose rate irradiation dates back to 1959, by the Research Unit in Radiobiology of Mount Vernon Hospital (Northwood, United Kingdom). They used an electron accelerator, which could release a dose of 10 – 20 krad in 2 μ s, on bacteria [89]. Instead, the first use of radiation in a high dose rate regime in cells occurred in 1967 at the Department of Radiobiology of the Medical College of St. Bartholomew's Hospital (London, United Kingdom), in which they studied the difference in surviving probability of mammalian cells irradiated with 1 or 2 pulses [90]. They used 15 MeV electron LINAC which could produce an electron beam characterized by pulses of duration $\sim 1 \mu$ s. However, we have to go back a few years, specifically in 2017, to see that the production of the first UHDR clinical LINAC took place thanks to the P. G. Maxim and B. W. Loo teams [91].

Another fundamental step occurred in 2019: for the first time, a human patient with a skin tumor was treated with FLASH RT at CHUV, Switzerland [92]. Moreover, between

2019 and 2020, most of the the main hypotheses on the mechanism underlying the biological FLASH effect were proposed: these are the transient hypoxia [93], the inter-track effects [94], the organic radical-radical recombination [7], and the immune system-driven response [95], which will be explored in more detail in the next sections. In 2020, the first FLASH proton beam was developed at U-Penn by the K. Cengel, C. Koumenis, and J. Metz teams [96].

The explosion of research in the field of UHDR radiation culminated in 2021, with the first international conference, i.e., the FRPT (FLASH Radiotherapy & Particle Therapy) conference in Vienna (and online), entirely dedicated to FLASH RT that brought together the entire FLASH community.

2022 marked another fundamental milestone in the progress of research in FLASH RT, namely the start of the first clinical trial involving 10 patients treated with the proton-FLASH beam [97]. This human trial was performed by the J. Breneman and J. Perentesis teams at the University of Cincinnati and Cincinnati Children's Hospital Proton Therapy Center. All these crucial steps in the development of FLASH RT are schematized in Figure 3.1.

3.2 Dose delivery techniques

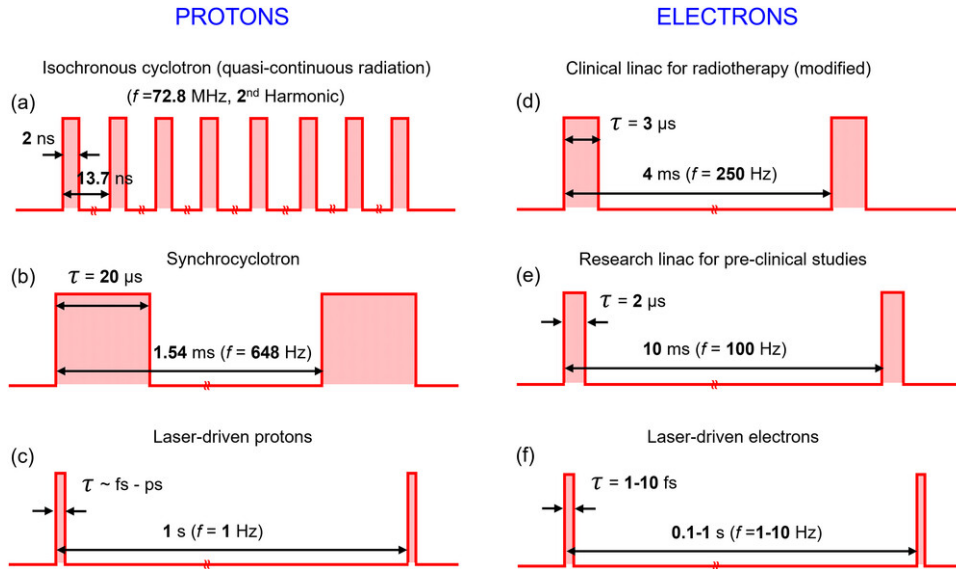


Figure 3.2: Temporal structures of various UHDR beams. From [98].

The development of novel accelerators that are capable of delivering dose at both Conv and UHDR irradiation regime is a fundamental step for the FLASH investigations: on the one hand, for experimental studies to explore and confirm the mechanism behind the FLASH effect, while on the other hand, to produce technologies that are compatible with a typical hospital RT facility. Today, one of the main practical problems for the

clinical translation of FLASH accelerators is that UHDR radiation is incompatible with the shielding setup of most hospital rooms. Moreover, many accelerators have sizes greater than the dimensions of a typical hospital RT facility [99].

Most of the machines already developed for FLASH irradiation have been obtained by upgrading some components of the present accelerators for the Conv radiation regime. The main UHDR beam time structures of several accelerators are reported in Figure 3.2. Moreover, the currently available accelerators range across a wide range of radiation qualities, including electron, photon, and hadron beams [99, 100, 101, 102], as we see in the following paragraphs. A major issue in the development of new UHDR machines is that, nowadays, the physical parameters that trigger the biological FLASH effect are not well known and debated [99, 102, 103]. Thus, producing technologies able to induce an optimal FLASH effect is challenging. The dose delivery time structures that allowed the first experimental observation of the FLASH effect are summarized in Figure 3.3.

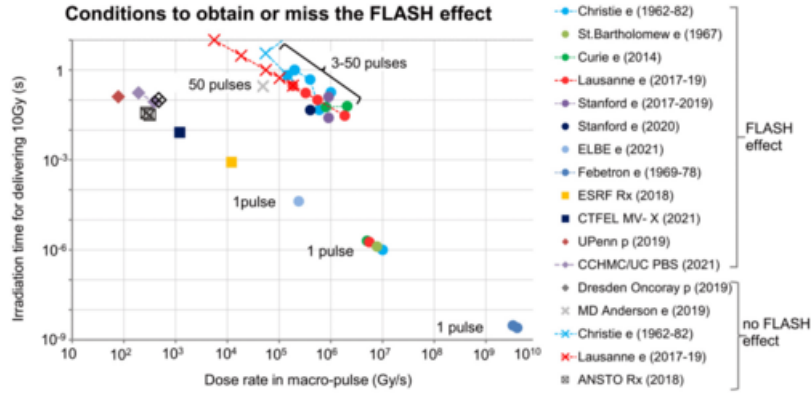


Figure 3.3: Temporal conditions of UHDR beams for positive and negative FLASH effect experimental observations. From [99, 104].

Electron UHDR beams

Electron LINAC is a common technology applied in experimental scenarios for small animals, for example mice, while in clinical practice is used to treat cancer situated superficially in the human body, such as skin tumors, at the Conv radiation regime. The update of these machines for UHDR irradiation is often very simple, just increasing the electron beam current. However, the main limitations of UHDR electron LINAC are low-range dose depositions and scattered dose distributions in biological systems [99].

The main physical parameters that characterized pulsed beam irradiation, as shown in Figure 3.4, are the pulse width or duration t_p [s], the pulse repetition frequencies (PRFs) [Hz] or the corresponding rate f_p , the dose-per-pulse (DPP) D_p [Gy], the total irradiation time t_i [s], the number of pulses n_p , and the total delivered dose $D = n_p D_p$ [Gy] [102, 98].

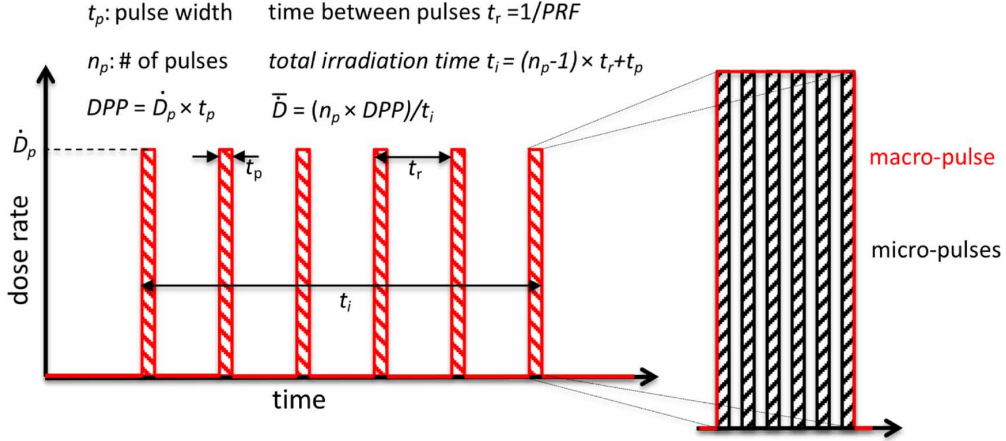


Figure 3.4: Temporal structures of UHDR pulsed beams and parameters. From [102].

From these quantities we can define the average dose rate [98] as

$$\dot{D} = \frac{D f_p}{n_p}, \quad (3.2)$$

and the instantaneous dose rate [98] as

$$\dot{D}_p = \frac{D}{n_p t_p} = \frac{\dot{D}}{f_p t_p}. \quad (3.3)$$

The first modern electron accelerator used at the UHDR regime was the Kinetron (CGR-MeV, Paris, and then PMB-Alcen, Peynier, France). This is a pulsed 4 – 5 MeV electron beam that can operate in both the Conv and the UHDR irradiation regime. It was used for the fundamental experiment with which the FLASH effect has been proven [87], but also for multiple other important experiments, such as [84]. It is characterized by an adjustable triode electron gun that can provide beam currents of 10 – 250 mA, pulse widths of 0.1 – 2.2 μ s, PRFs of 10 – 250 Hz, and average dose rates between 10^{-1} and 10^6 Gy/s in a single pulse.

The Kinetron has been replaced by the Oriatron eRT6 (PMB-Alcen, Peynier, France). It is a flexible machine, with a nominal energy of 6 MeV, whose beam parameters can be easily adjusted: in particular, this device can achieve a peak beam current of 300 mA, pulse widths of 0.5 – 4 μ s, PRFs of 5 – 200 Hz, and average dose rates between 10^{-2} and 10^3 Gy/s in a single pulse. It was used for several physical and biological FLASH experiments, for example, [84, 105, 106, 107, 108, 109, 37].

Other important electron accelerators used for UHDR experiments are those reported in [110, 111, 112, 113, 114, 115, 116, 117, 91, 118, 119, 120, 121, 122, 123, 124].

Very-high energy electron (VHEE) UHDR beams

The development of very-high energy electron (VHEE) beams, namely electron beams with energies > 100 MeV, for clinical purposes is a cutting-edge topic in RT research. This is

due to the physical advantages of VHEE with respect to photon beams [125, 126, 127]. In fact, electrons with energies between 100 and 250 MeV are characterized by a shallower depth-dose falloff and a reduced lateral scattering. Moreover, the electromagnetic scanning of VHEE beams is faster than that with proton beams [99]. However, the main issue associated with the clinical translation of VHEE beams is that the machine size and the power required for it are currently incompatible with typical clinical facilities. Instead, VHEE research accelerators can allow the modification of several physical parameters, beyond the simple dose rate and DPP values, enabling exploration of the full set of beam variables [102].

Several works are ongoing to improve the performance, using less RF power, and the dimensions, producing more compact technology, of VHEE devices, such as those in [128, 129, 130, 131, 132, 102].

Photon UHDR beams

X-ray RT is the most widely used technique for Conv irradiation. Typically, the photon beams are produced via bremsstrahlung production, by electrons striking a high atomic number target. The electrons are produced using the previously discussed LINAC. However, because of the low efficiency of converting electron beam energy to X-rays, LINACs with extremely high beam currents ($\gtrsim 300$ times a typical medical LINAC) are needed to achieve the UHDR regime. Thus, the development of UHDR photon beams is very difficult [99].

One of the most important UHDR X-ray beams is that at the European Synchrotron Research Facility (ESRF, Grenoble, France) [133, 134], which has been used to investigate, for the first time, the FLASH effect for photon irradiation, observing reduced cognitive defects in mice [135]. Several ongoing projects are actually focusing on the development of novel UHDR X-ray beams, such as those reported in [136, 137, 138, 139].

Proton and ion UHDR beams

For Conv dose rate irradiation, hadron therapy, i.e. RT with protons or ions, combines physical and biological advantages with respect to photons and electrons RT.

Typically, in clinics, proton beams are accelerated to a maximum energy of 230 – 250 MeV [141], with a pulsed or quasi-continuous structure, as illustrated in Figure 3.2. Moreover, in principle, any proton therapy machine should reach a mean dose rate of at least 40 Gy/s, just by increasing the beam extraction current [96, 142]. The main issue related to proton-FLASH RT is the energy switching time of the beam, which is in the range of 0.5 – 2 s. In fact, for Conv RT, the tumor is irradiated using the so-called spread-out Bragg peak (SOBP), namely combining several proton beams at different energies and intensities, reaching a uniform dose distribution throughout the tumor volume along the beam direction. However, it is impossible to maintain UHDR for the entire irradiated volume. In order to solve this issue, two possible irradiation approaches have been proposed. The first is the so-called shoot-through, or transmission modality, namely, accumulating several beam directions using the plateau region of the depth-dose distribution of the

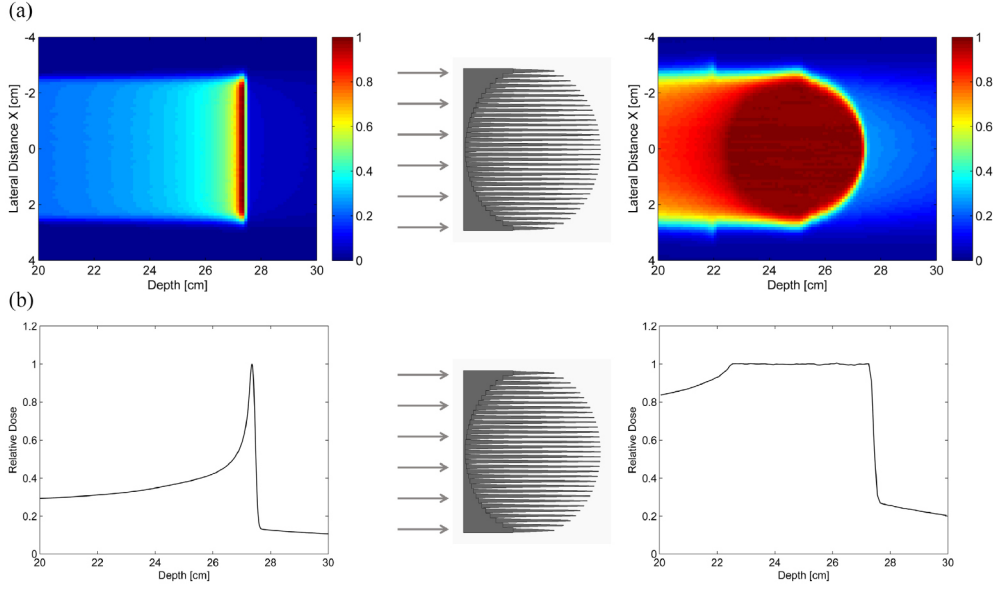


Figure 3.5: *In-silico* application of a spherical 3DRM using a Monte Carlo (MC) simulation code, (a) at the X-Z midplane profile, and (b) at the center line of the depth-dose distribution, for a 400 MeV/u carbon ion beam. From [140].

proton beam at the maximum energy, for example, as in [143]. The second approach consists of the use of a so-called 3D range modulator (3DRM) [140], a passive device used to decrease the energy of the proton beam, which is produced at the maximum energy, continuing to exploit the SOBP features, as described in [17] and shown in Figure 3.5. In both approaches, protons are accelerated at high energy because the beam transport is more efficient, thus producing the beam at its maximum dose rate.

The second important issue with proton-FLASH RT is the irradiation of the transverse plane of the tumor, which usually requires dose distribution with sizes greater than the typical beam spread. The most widely used irradiation technique to cover the transverse plane in Conv RT is the pencil-beam scanning (PBS) approach, in which two dipole magnets are used to deflect the beam in the transverse plane [141], as shown in Figure 3.6. However, the dead time between the irradiation points, commonly called spots, in the same energy layer is on the order of ms. The second technique used in clinics to spread the beam in the lateral direction is the passive scattering approach, in which the proton beam is enlarged by passive scattering material [141]. However, the issue related to both approaches is that the dose rate is drastically reduced, due to scanning irradiation time for the first approach, while beam intensity degradation for the second technique. Several computational and experimental studies are already published about proton-FLASH with PBS, such as [144, 145, 146, 147, 148, 149, 16, 150, 151, 152].

The main clinical proton-FLASH machines are (i) the IBA Proteus Plus system (Ion Beam Applications S.A, Louvain-La-Neuve, Belgium), which has been used for several FLASH experiments with mice, such as [96, 148, 153], or with zebrafish [154]; (ii) the

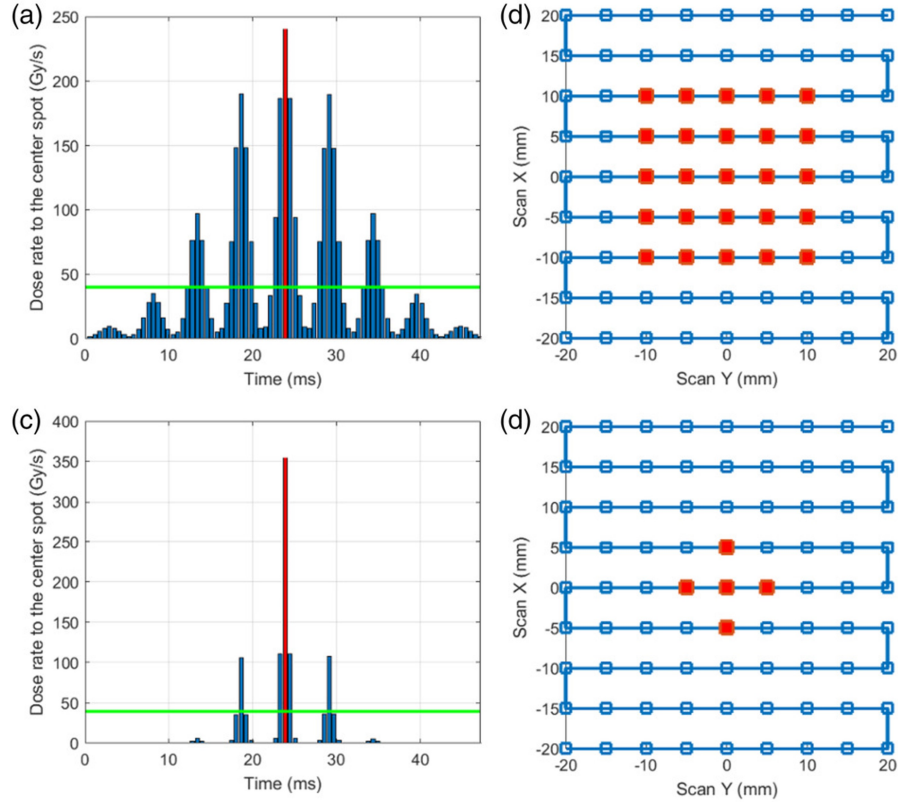


Figure 3.6: Dose delivery time structure for pencil beam scanning (PBS) with proton-FLASH devices, in the entrance plateau (a) and at the Bragg peak (c). Corresponding spot positions and scanning pathway, in the entrance plateau (b) and at the Bragg peak (d). The red points correspond to dose rate values > 40 Gy/s. From [142].

Varian ProBeam (Palo Alto, CA, USA), which has been applied in various FLASH experiments with mice [147, 149, 16, 150, 155], and has been used for the first clinical trial (FAST-01) at the Cincinnati Children's/UC Health Proton Therapy Center [88, 97], and now it is used for a second trial (FAST-02) [156].

Regarding research facilities, various proton-FLASH beams have been developed or are currently under design, such as those reported in [157, 158, 159, 160].

Beyond protons, the possibility of heavy-ions-FLASH devices has also been exploited. In particular, using the carbon ion beam at GSI (Helmholtz Centre for Heavy Ion Research; Darmstadt, DE) [161], and at HIT (Heidelberg Ion-Beam Therapy Center, Heidelberg, DE) [10]. The FLASH effect has also been demonstrated using the helium ion-FLASH beam, at HIT using a PBS irradiation technique [9], and in [162]. Recently, the feasibility of using oxygen ion-FLASH beam at HIT has also been demonstrated [163].

3.3 Dose rate definitions

As we have seen previously, FLASH RT is based on the fundamental concept of dose rate. However, even today, there is no univocal definition that allows one to correctly describe all the possible experimental scenarios, in particular in connection to a specific biological effect which is still far to be associated to an exact delivery metric (see below). In recent years, several mathematical metrics have been proposed. The simplest is certainly the so called average or field dose rate (ADR), which is defined as

$$\text{ADR}_i := \dot{D}_i^{\text{field}} = \frac{D_i}{t_f}, \quad (3.4)$$

where D_i is the total absorbed dose at i -th voxel, while t_f is the total delivery time. This allows us to obtain a 3D dose rate map for the whole irradiated volume, as shown in Figure 3.7a. Moreover, averaging over all voxels gives the mean dose rate $\bar{D} := \bar{D}^{\text{mean}}$, used for the definition of UHDR irradiation. This definition of dose rate allows us to give an indication of the type of irradiation used. However, it cannot discriminate between different temporal structures of dose delivery.

A step towards more informative definitions is certainly the proposal of the instantaneous dose rate (IDR). This metric focuses on the time structure of dose delivery at the voxel level. In particular, it is defined as

$$\text{IDR}_i := \dot{D}_{ij} = \frac{D_{ij}}{t_{ij}}, \quad (3.5)$$

where D_{ij} is the dose per j -th pulse, and t_{ij} is the j -th pulse duration, for the i -th voxel. By plotting the cumulative dose or the IDR versus time, as in Figure 3.7b, it is possible to observe at the voxel level the impact of the type of dose delivery over time.

Another option is the so called dose-averaged dose rate (DADR) [143], which is defined

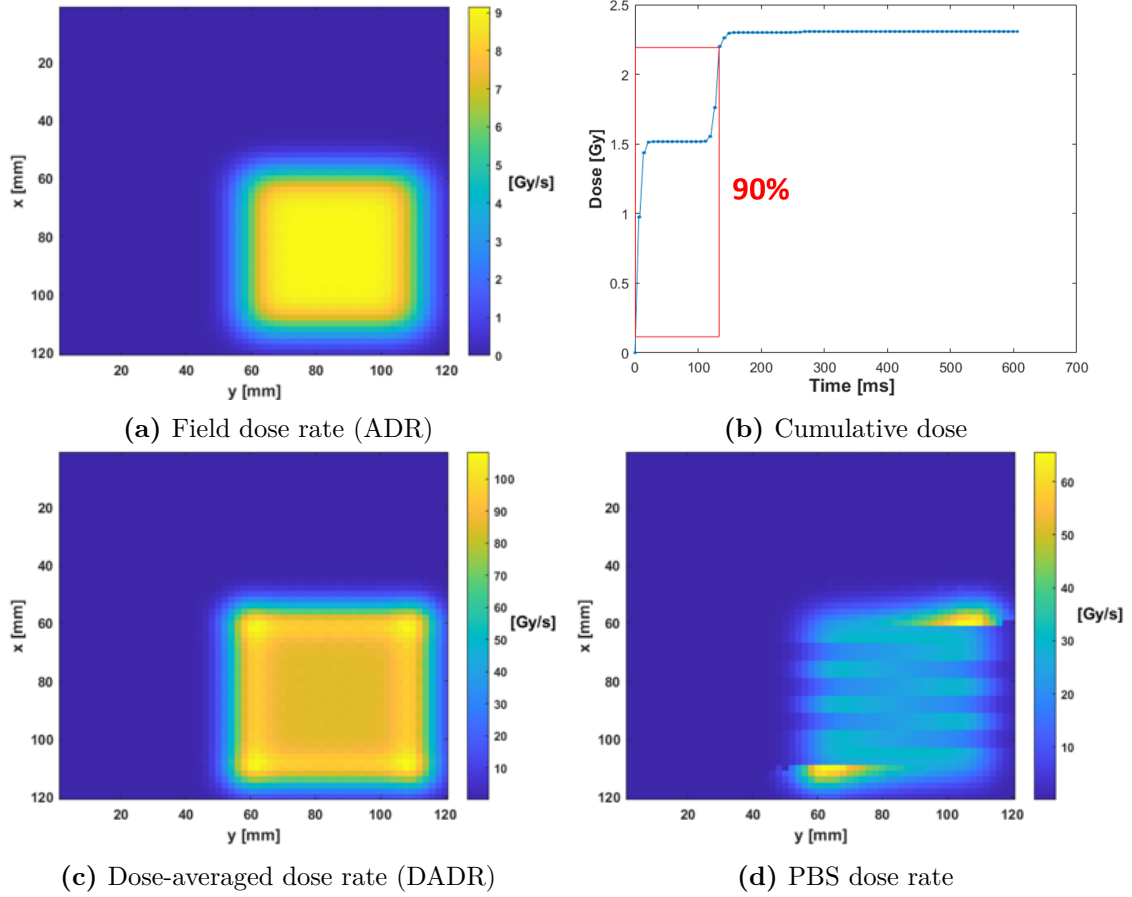


Figure 3.7: (a) Slice of a 3D average or field dose rate (ADR) map, calculated. (b) Cumulative dose as a function of time for the voxel at the center of the 3D dose map. The red label describes the time window which corresponds to a dose accumulation of 90% of the total absorbed dose. (c) Slice of a 3D dose-averaged dose rate (DADR) map. (d) Slice of a 3D pencil beam scanning (PBS) dose rate map, considering a time window corresponding to a cumulative dose of 90% of the total dose. All the dose rate maps are calculated starting from a 3D dose map for each pencil-beam obtained with TOPAS MC simulation of 230 MeV proton beams in water.

as

$$\text{DADR}_i = \frac{\sum_{j=1}^n (d_{ij} w_j) (d_{ij} B I_j)}{\sum_{j=1}^n (D_{ij} w_j)}, \quad (3.6)$$

where d_{ij} is the ij -th term of dose-influence matrix in [Gy/protons], w_j is the j -th spot weight in [protons], while $B I_j$ is the j -th beam intensity in [protons/s]. That is, DADR is a 3D map of the instantaneous dose rate averaged by spots and weighted by the dose delivered by each beam in the voxel, as proposed in Figure 3.7c. The limitation of this definition is that it completely neglects the spatial and temporal pattern of dose delivery in the target volume, and most importantly doesn't give any weight to the time segments delivering no dose.

Perhaps the most accurate definition of dose rate is probably the PBS dose rate [144]. It is computed as

$$\dot{D}_i^{\text{PBS}} = \frac{D_i - 2D_t}{T_i}, \quad (3.7)$$

Here D_i is delivered dose in the i -th voxel at the end of the irradiation, D_t is a dose threshold, $T_i = t_1 - t_0$ is an effective irradiation time for the i -th voxel, where t_0 and t_1 are defined from the accumulated dose $D_i(t)$ in the i -th voxel at time t , i.e. $D_i(t_0) = D_t$ and $D_i(t_1) = D_i - D_t$, respectively, as illustrated in Figure 3.7d. This metric allows us to describe different situations of temporal structures and scanning pathways of dose delivery, as shown in Figure 3.7d.

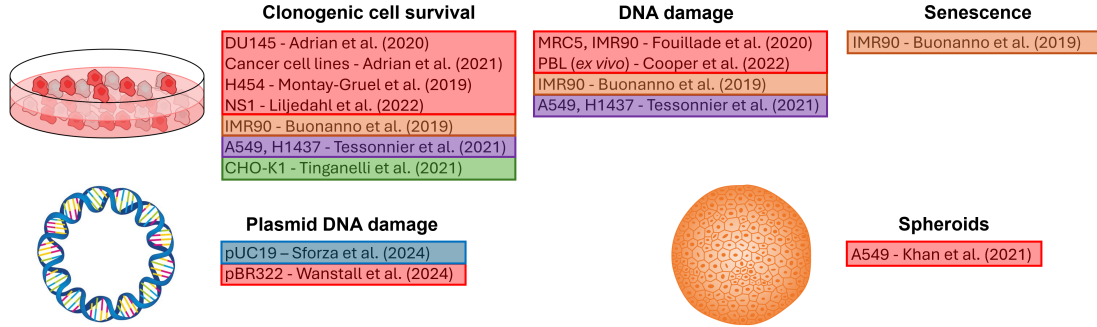
For completeness, in recent years additional dose rate metrics have been proposed; however, they are much more complicated and less used by the FLASH community, such as the dose-threshold dose rate (DTDR) [164], the FLASH trigger window [146], or the sliding window [152]. For a complete review, see [165].

As it can also be seen in the numerical values examples reported in Figures 3.7a, 3.7c, and 3.7d, ADR is the most pessimistic metric and tends to underestimate the dose rate values; instead DADR overestimates the dose rate values and is probably the most unrealistic definition, while PBS DR is placed in the middle of the range of dose rate values and has the ability to take into account the dose delivery pattern.

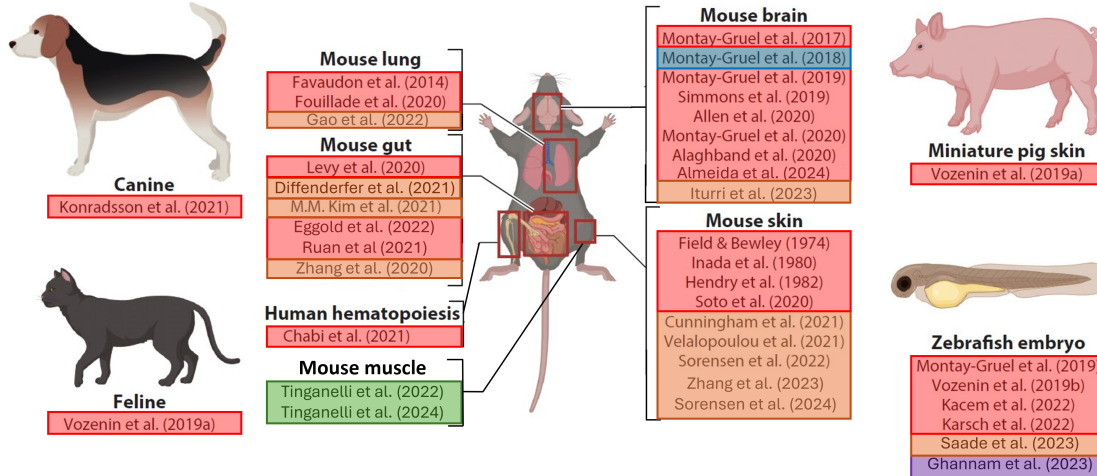
The real issue lies in the lack of a clear and unified definition of dose rate, in that even today, the essential physical parameters responsible for triggering the FLASH effect remain unknown. Therefore, at the moment, the most used approach for experiments is to indicate the mean dose rate of the irradiation and the physical parameters of the beam, such as type of accelerator, type of particle, energy, LET, irradiation with or without scanning, pulsed irradiation or not, specifying also in this case the dose per pulse, the pulse repetition frequency, the time of each pulse, the total irradiation time, etc. Therefore, even in this context, the development of mathematical models that allow us to analyze the aforementioned physical parameters that play a role in the manifestation of the FLASH effect is essential.

3.4 Experimental evidences

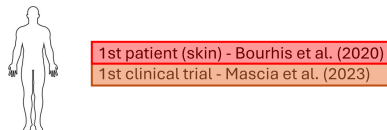
In vitro experiments



In vivo experiments



Human patients



UHDR beams

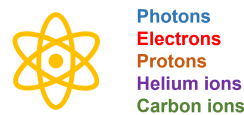


Figure 3.8: Summary of the main *in-vitro* and *in-vivo* evidences of the FLASH effect, and the feasibility of UHDR irradiation in human patients. Modified starting from [82].

The FLASH effect is an *in-vivo* biological phenomenon that occurs for given UHDR irradiation, as defined in Section 3.1. The FLASH sparing effect, namely the reduction of specific side effects occurring in healthy tissues under the UHDR regime, emerged in several animal studies, in particular for the brain, lung, gut, and skin, using photon, electron, proton, and carbon ion beams, as reported in [99, 166, 102], and illustrated in Figure 3.8. In addition, the same effectiveness of Conv and UHDR irradiation on tumors

was experimentally demonstrated.

However, experimental investigations of UHDR irradiation in *in-vitro* scenarios are fundamental to obtain information on the elusive mechanism behind the FLASH effect, as has historically always happened for other biological effects. Several results have shown that the FLASH sparing effect also occurred at the cellular level [167]. The main findings are reported in Figure 3.8.

Lastly, the feasibility of UHDR irradiation has been shown in human patients [92], especially during clinical trials [88, 97]. This is a crucial step for the clinical translation of FLASH RT.

The main *in-vitro* and *in-vivo* findings, and the clinical trials under the UHDR irradiation regime are presented in detail below.

3.4.1 *In-vitro* studies

The role of *in-vitro* experiments in the context of FLASH RT is highly debated [99, 167]. Historically, *in-vitro* assays have always played a crucial role in radiobiology, allowing, also thanks to the high throughput, the investigation of many biological mechanisms triggered by IR. In particular, they allowed the study of cellular response to IR, for example, cell cycle progression, DNA damage formation and repair, lipid peroxidation and ferroptosis, and the role of modifiers described in Section 1.4, such as radiation quality, oxygenation, low dose rate, and radio-sensitizers. Moreover, what emerges from a large portion of classical radiobiological experiments at Conv irradiation is the key role of DNA damage [168, 169], based on our current understanding of DNA damage kinetics, in particular its formation and evolution in time. For these reasons, if *in-vitro* experiments were not useful in the case of UHDR irradiation, it would be the signal of a completely new radiobiology, which is highly unlikely. Therefore, although the FLASH effect is *in-vivo* effect, a relevant part of the FLASH community believes in the importance of *in-vitro* experiments. For example, from a biological point of view, simple *in-vitro* models can allow us to exclude possible pathways or get some insights about the FLASH mechanism. In addition, *in-vitro* assays also allow the exploration of possible modification of the chemical environment of the cell under FLASH irradiation, such as the oxygen depletion [93], i.e., a possible change of the time evolution of the oxygen concentration due to UHDR radiation, or the role of scavengers, as described later in Section 3.5. Although clearly useful for specific insights, the clinical implications and proven utility of UHDR *in-vitro* experiments remain uncertain and are actively debated within the scientific community.

Clonogenic cell survival

The main biological endpoint at cellular level is the survival fraction, namely the probability for clonogenic cells to survive after IR, as described in Section 1.3.2. Experimentally speaking, the survival fraction is defined from the concept that a single clonogen survives if, proliferating, has formed a colony of at least 50 cells. However, the main issue related to the clonogenic assay irradiated in the UHDR regime is the statistical uncertainty. This challenge is related to the fact that UHDR irradiation are performed at high doses to

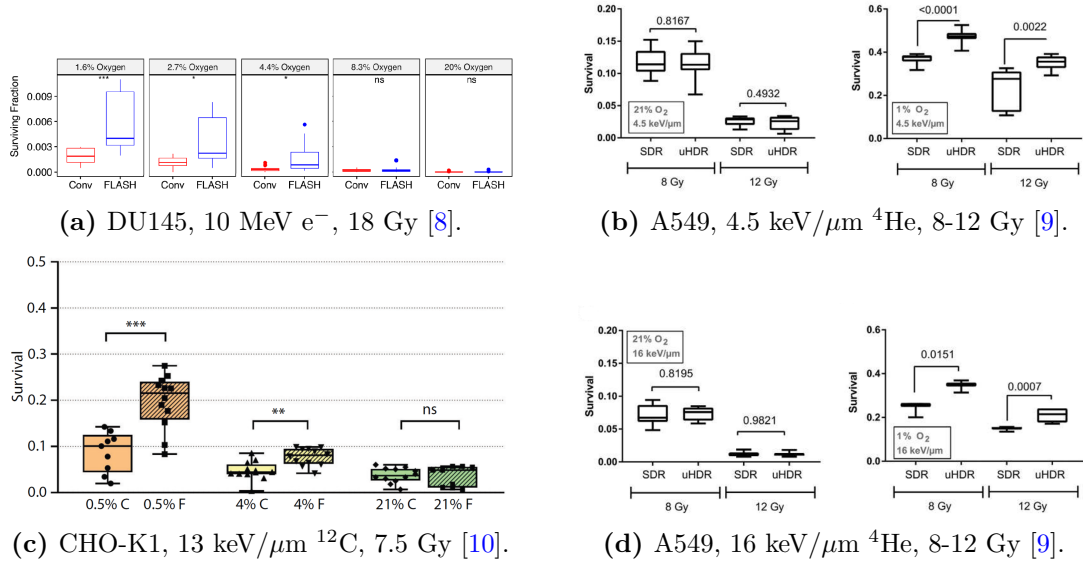


Figure 3.9: Main hypoxic and physioxenic FLASH *in-vitro* evidence in terms of clonogenic cell survival, for different radiation quality and oxygenation. Images from [8, 9, 10].

trigger the FLASH sparing effect. Thus, reducing the number of surviving colonies to just a few.

Several experiments have been carried out under the UHDR irradiation regime, with a focus on the surviving probability of both normal and cancer cell lines, using different radiation qualities (i.e., electrons, protons, helium and carbon ions beams), and at different oxygen concentrations (from severe hypoxia (0.5% O₂) to normoxia (21% O₂)), as summarized in [167].

The first irradiation of cells at an ultra-high dose rate happened in 1967, delivering the dose with the 15 MeV Mullard LINAC [90]. This machine was able to deliver an electron beam in pulses of duration 1.3 μs , with a variable pulse repetition frequency (PRF). They irradiated HeLa S3 cells in a dose range of up to 25 Gy, with an instantaneous dose rate of $\sim 3.5 \cdot 10^7$ Gy/s, in a single or two pulses to study the impact of the dose rate using a clonogenic assay. With this approach, they observed a protective effect in normoxia condition between the two irradiation modalities [90].

Other experiments were performed between 1969 and 1978 using UHDR electron beams, with different cell lines (i.e., HeLa, HeLa S3, CHO), and also under hypoxia conditions. However, they delivered the dose only on a high dose rate regime [170, 171, 172, 173]. Thus, it is impossible to quantify the probable sparing effect as a result of the lack of data for Conv irradiation. In fact, the goal of these experiments was to study a peculiar phenomenon observed in cell survival for UHDR irradiation and high doses, and not to compare UHDR and Conv irradiation. In particular, Town in 1967 [90] observed a discontinuity in the cell survival curve, namely a decreased radio-sensitivity, only if delivering the dose with a single pulse. He hypothesized that this effect was due to oxygen deple-

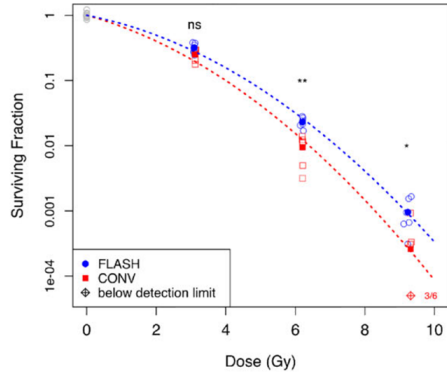
tion [90]. In 1972, the Epp team managed to demonstrate the role of oxygen depletion in the appearance of this discontinuity only for severe hypoxia, with an initial oxygen concentration between 0.26% and 0.91% [172].

In 1968, the Berry team irradiated Chinese hamster and HeLa S3 cells using a 2.0 MV photon beam, delivering a dose of up to 15 Gy with a single pulse. They observed a protective effect in both cell lines for a UHDR of $\sim 10^9$ Gy/s [174].

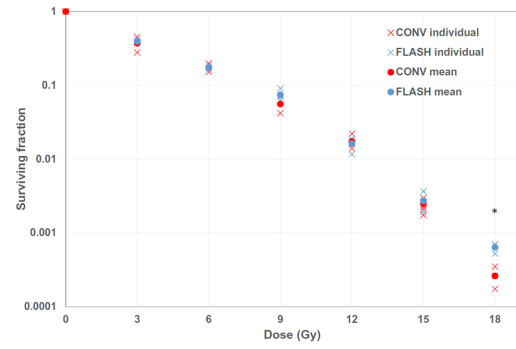
The first modern UHDR experiment that shows the FLASH sparing effect in cells was carried out in 2019 by Montay-Gruel and published in his important PNAS work [85]. They irradiated the H454 murine glioblastoma assay under two different oxygen concentrations, namely 4% (physioxia) and 21% (normoxia) O_2 , using a 6MeV LINAC. This device is capable of producing pulsed electron beams with a mean dose rate ranging from 0.1 Gy/s (Conv) up to 1000 Gy/s (UHDR), corresponding to a dose-per-pulse between 0.01 and 10 Gy. They observed an increased cell survival probability for UHDR irradiation under both oxygen conditions, delivering 20 Gy [85], as can be observed in Figure 3.10c.

In the same year, another important publication demonstrated the protective effect of UHDR on a cell assay using electron beams. In particular, in Adrian et al. work [8], they irradiated the DU145 prostate cancer cell line under several oxygenation levels, from hypoxia (1.6%) up to normoxia (20%), using a hypoxia chamber (InVivo $_2$ Hypoxia Work Station 400, Baker Ruskinn Technology Ltd, Bridgend, UK), as we can see in Figure 3.9a. They used the modified 10 MeV Elekta Precise LINAC, which delivers pulsed electron beams in both the Conv regime, with a mean dose rate of 14 Gy/min, a pulse width of $3.5\mu s$, and a pulse repetition frequency of 200 Hz, and the UHDR regime (600 Gy/s), with a dose-per-pulse of 3 Gy, and a PRF of 200 Hz. After a 18 Gy irradiation, they observed a significant difference in the surviving fraction between the Conv and UHDR irradiation under hypoxia conditions (1.6% and 2.7% O_2), and at a physiological oxygen level (4.4% O_2). They also concluded that there was no sparing effect at 8.3% and 20% O_2 due to the lack of significant statistical difference derived from the fit using the linear-quadratic model (see Eq. 1.63) [8]. However, they imposed that the parameter β was the same for FLASH and Conv under normoxic (20%) and anoxic (0%) conditions, keeping the parameter α fixed during the fitting procedure of the LQ model on the experimental data.

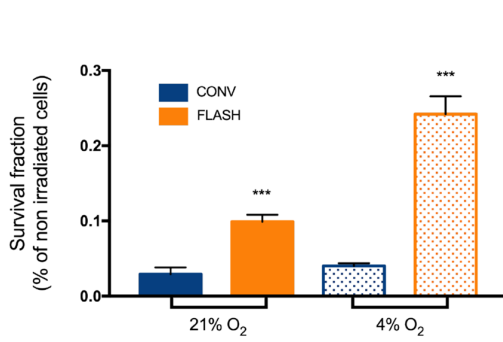
The same research group repeated the experiments for several other cancer cell lines (that is, MCF7, MRC-5, Lu-HNSCC4, MDA-MB-231, HeLa $_{earlypassage}$, HeLa $_{subclone}$, WiDr), but only under normoxia conditions, delivering a dose up to 12 Gy [175]. They observed the FLASH sparing effect for all cell lines, with significant statistical differences for four of the seven cell lines, also at low doses (between 6 Gy and 9 Gy), as can be observed in the example shown in Figure 3.10a. They also calculated a Dose Modifying Factor (DMF), as defined in Eq. 1.72, at 10% of the cell survival probability between Conv and UHDR irradiation. They obtained a DMF $_{10\%}$ that ranges from 1.1 to 1.3, depending on the cell line [175]. They concluded that the magnitude of the FLASH sparing effect depends on the cell line. We already anticipate here that this conclusion aligns with our hypothesis regarding the mechanism underlying the FLASH effect, namely that it relies on the unique antioxidant capacity of each cell line to remove potentially harmful chemical species from its cell environment. Similarly to [8, 175], another finding that suggest the existence of a



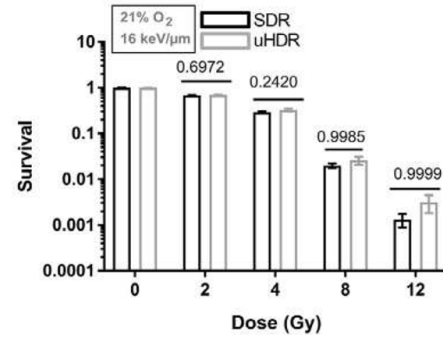
(a) MDA-MB-231, 10 MeV e^- , 0-10 Gy [175].



(b) NS1, 10 MeV e^- , 0-18 Gy [176].



(c) H454, 6 MeV e^- , 20 Gy [85].



(d) H1437, 16 keV/ μm ^4He , 0-12 Gy [9].

Figure 3.10: Main normoxic FLASH *in-vitro* evidence in terms of clonogenic cell survival, for different radiation quality. Images from [175, 85, 176, 9].

dose-threshold for the emergence of the FLASH sparing effect, also in normoxic condition, is that carried out by Liljedahl et al. [176]. They irradiated NS1 Glioblastoma cells at 21% O_2 using the 10 MeV electron LINAC (Elekta) with mean dose rates of 8 Gy/min (Conv) and ~ 90 Gy/s, delivering doses in the range of 0 – 21 Gy. They observed the FLASH sparing effect, namely a statistically significant difference between Conv and UHDR, at 18 Gy, as shown in Figure 3.10b.

Moving on to particle irradiation, another fundamental demonstration of the occurrence of the FLASH sparing effect at the cellular level is due to the experiment carried out by the Tinganelli team from GSI [10]. They irradiated Chinese hamster ovary CHO-K1 cells at different oxygen concentrations (0.5%, 4%, and 21% O_2) using the GSI hypoxic chambers, as we can see in Figure 3.9c. They delivered 7.5 Gy under both Conv (with a mean dose rate of 0.6 Gy/s) and UHDR (i.e. 70 Gy/s) regime with a carbon ion beam of 13 keV/ μ m using the HIT accelerator. They observed a sparing effect for all oxygenation levels, with a high significant statistical difference under hypoxia conditions and no significant difference in normoxia. However, as discussed with the authors of [10], who supplied us with the experimental raw data, excluding certain low-statistics points at normoxia for both Conv and UHDR irradiation, the FLASH sparing effect also emerges for this situation, even if it is attenuated in magnitude.

An experiment with a helium ion beam has been performed at HIT by Tessonnier et al. [9] using human lung epithelial cells A549 and lung carcinoma cells H1437. They irradiated the cells under hypoxia (1% O_2) and normoxia conditions, obtained through a hypoxia chamber (C-chamber, Biospherix), using 146 MeV helium ion beams in the entrance region (4.5 keV/ μ m) and also at the peak of a SOBP (16 keV/ μ m), which was produced using a 2D range modulator. In particular, for the entrance channel experiment, they delivered 8 Gy for both Conv, with a mean dose rate of 0.12 Gy/s and the UHDR regime, with a mean dose rate of 205 Gy/s and 12 Gy at 0.18 Gy/s (Conv) and 139 Gy/s (UHDR), as we can see in Figure 3.9b. Instead, for the SOBP experiment, they delivered 2, 4, 8, and 12 Gy, with dose rates between 0.04 – 0.21 Gy/s for Conv irradiation, and UHDR values from 141 Gy/s up to 216 Gy/s, for increased dose values delivered, as reported in Figure 3.9d. In the case of the A549 cell line, they observed a FLASH sparing effect for both dose values (8 and 12 Gy) and both LET values (4.5 and 16 keV/ μ m) under 1% O_2 , while no significant differences emerged under normoxia conditions for the same irradiation scenario. Instead, with the H1437 cell line, they carried out the experiment only in SOBP. They observed an increased survival probability with UHDR with respect to Conv irradiation at doses > 4 Gy for both oxygenation levels, as can be observed in the example shown in Figure 3.10d. However, they concluded that there was a significant statistical difference only for the hypoxia condition.

A more controversial experiment was performed by Buonanno et al. [158] with 4.5 MeV proton beams at RARAF. They irradiated normal human diploid lung fibroblasts IMR90 under normoxia conditions, delivering doses in the range of 0.5 – 10 Gy at three different mean dose rates, i.e. 0.05 (Conv), 100, and 1000 Gy/s (UHDR). They observed a slight difference in survival probability between Conv and both UHDR irradiations only for the highest dose. However, they concluded that this difference is not statistically

significant. The controversial aspect of this experiment is that it showed a significant difference between Conv and UHDR irradiation in terms of DNA damage, as we will see in the next section.

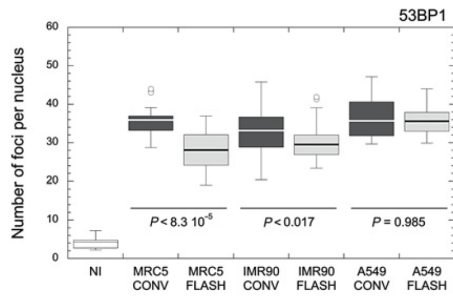
In conclusion, the main *in-vitro* FLASH experiments showed an increase in cell survival under hypoxia conditions for UHDR irradiation relative to the Conv regime [85, 8, 10, 9], while, for normoxia condition, most of them reported a reduced [90, 174, 85, 8, 175, 176], a slight but not statistically significant [10, 9, 158], or no FLASH sparing effect [9]. All of these experimental data clearly indicate that the FLASH sparing effect also occurs at the cellular level. Furthermore, interestingly, the protective effect is manifested both in healthy cell lines and tumor cell lines. However, because of the challenges of comparing experiments conducted under varying irradiation conditions, it is impossible to draw definitive conclusions about the underlying mechanism of this effect. In addition, there are also some negative results, both in hypoxic [177] and in normoxic [178, 179, 177] conditions. Nevertheless, it is evident that a trigger at the cell level plays a role in the manifestation of the protective effect in the UHDR regime. Furthermore, it can reasonably be assumed that the magnitude of this effect depends on the cellular environment, including factors such as oxygenation and cell type. Therefore, we strongly emphasize the significance of *in-vitro* experiments and recommend that, in the future, they will be carried out systematically to assess the role of physical parameters of irradiation, as well as chemical and biological environmental conditions, in the emergence of the sparing effect for the UHDR irradiation. We point out how some data, especially the experiments carried out before the birth of FLASH (2014), are affected by uncertain dosimetry. For this reason, in this work, as we will see in Chapter 4, only modern experiments have been considered, given that the observed effects are highly dependent on precise dose ratios.

DNA damage and other cellular endpoints

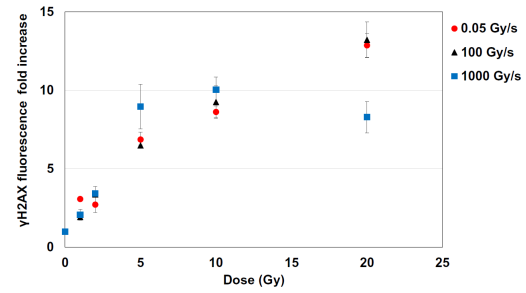
The effects of UHDR radiation in terms of DNA damage are one of the most debated subjects in the FLASH community. In fact, the *in-vitro* experiments carried out up to now have reported conflicting results, and in some cases even contradictory to what was seen in terms of surviving probability.

The work by Buonanno et al. [158] discussed previously is a clear example of this. In fact, they observed a statistically significant reduction in DNA DSB damage delivering 20 Gy at the highest dose rate of 1000 Gy/s, as we can see in Figure 3.11b. They carried out this experiment measuring the formation of γ H2AX foci 30 min after a proton beam irradiation, which is a typical method to investigate DNA damage in standard radiobiology. γ H2AX is a molecular marker of DNA DSB induction [181]. From these results, they speculated that the UHDR radiation modulates the complexity of the induced DNA damage. However, as previously discussed, they did not observe a statistically significant increase in the survival fraction for the same irradiation scenario.

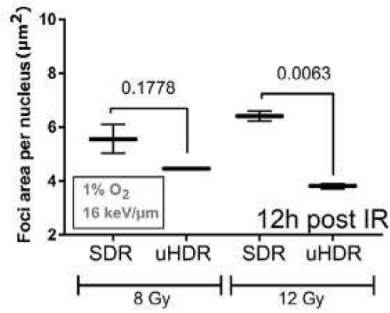
Fouillade et al. [180] irradiated two non-transformed human fibroblast cell lines MRC5 and IMR-90 and a non-small cell human lung cancer cell line A549 using the 4.5 MeV electron LINAC (Kinetron). They measured the induction of DNA DSB after 30 min post irradiation from the foci of both γ H2AX and TRP53BP1 (53BP1) using immunofluores-



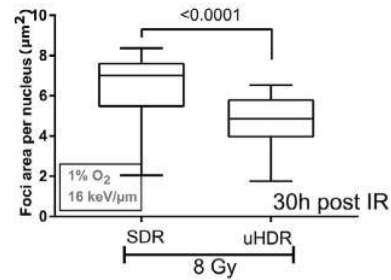
(a) 53BP1, 30 min, MRC5, IMR90, and A549, 4.5 MeV e^- [180].



(b) γ -H2AX, 30 min, IMR90, 8.6 keV/ μ m p [158].



(c) γ -H2AX, 12 h, A549, 16 keV/ μ m ⁴He [9].



(d) γ -H2AX, 30 h, A549, 16 keV/ μ m ⁴He [9].

Figure 3.11: Main FLASH *in-vitro* evidence in terms of cell DNA damage for different radiation quality. Images from [180, 158, 9].

cence. TRP53BP1 (53BP1) is another biological marker for DNA DSB. They delivered 5.2 Gy at 0.03 Gy/s (Conv) and 40 Gy/s (UHDR). They observed a statistically significant reduction only in 53BP-1 foci formation using UHDR radiation for the two fibroblast cell lines MRC5 and IMR-90, while they did not detect a significant difference between UHDR and Conv for the A549 cell line, as reported in Figure 3.11a. Furthermore, for the radiation-induced foci of the γ H2AX protein, there is no significant difference between the two irradiation modalities.

Adrian et al. [175] also measured the formation of radiation-induced foci with the 53BP1 marker at 2 h and 24 h after 3 Gy irradiation with Conv and UHDR electron beams of Lu-HNSCC4, MDA-MB-231, and HeLa_{subclone} cell lines. They observed similar foci levels between Conv and UHDR irradiation at both time points. However, the dose delivered is very low, so it is challenging to draw a definitive conclusion about the impact of UHDR irradiation on DNA damage formation.

In the paper by Tessonnier et al. [9], previously mentioned for the survival fraction experiment, they also measured the formation of foci of the protein γ H2AX at the DNA damage site for the A549 and H1437 cell lines, after helium ion beam irradiation in both the Conv and UHDR regime. They observed the same level of radiation-induced foci in the case of 21% O₂, while a statistically significant reduction in the formation of foci for UHDR irradiation compared to Conv under 1% O₂. In particular, they detected this significant difference for both cell lines and both LET values, 12 h and 30 h after 8 Gy irradiation, as shown in Figures 3.11c and 3.11d.

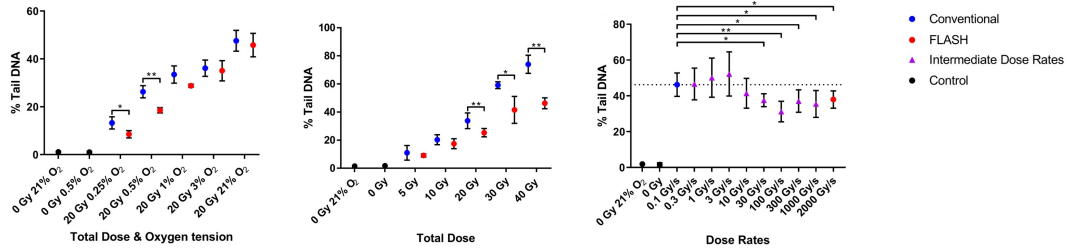


Figure 3.12: Main FLASH *ex-vivo* evidence in terms of formation of DNA damage in human PBL cells after 6 MeV e⁻ irradiation. Images from [182].

In addition, Cooper et al. [182] clearly showed a protective effect on the formation of DNA damage induced by UHDR radiation. In particular, they irradiated *ex-vivo* human peripheral blood lymphocytes (PBL), under different oxygen concentrations (0.25, 0.5, 0.75, 1, 3, 21% O₂), using a 6 MeV electron LINAC, also delivering doses in the range of 0 – 40 Gy with mean dose rates between 0.1 Gy/s (Conv) and 2000 Gy/s (UHDR). They observed interesting features, as reported in Figure 3.12. First of all, they showed a general trend of reduced DNA damage for UHDR irradiation compared to Conv RT, with significant differences at 0.25% and 0.5% O₂. Furthermore, they reported an increased magnitude of sparing effect in terms of DNA damage with increasing dose and dose rate values, where significant differences emerged for doses ≥ 20 Gy and dose rates ≥ 30 Gy/s for irradiation under 0.5% O₂.

Recently, two interesting experiments were carried out to analyze the impact of UHDR irradiation on DNA damage formation using plasmids, which are small circular DNA molecules in solution. Thus, an assay able to detect the DNA damage without including any role for the repair mechanism, being deprived by the relevant protein system. The first experiment was performed using the VHEE beam at CLEAR [129]. They irradiated the plasmid DNA (pBR322) within a scavenging environment to mimic the cellular environment, at three dose rate values: Conv (0.08 Gy/s), HDR (96 Gy/s) and UHDR ($2 \cdot 10^9$ Gy/s). They used two different concentrations of scavengers in order to modulate the impact of the indirect action of the radiation on the formation of DNA damage. They observed a significantly reduced level of DNA damage for UHDR irradiation with respect to the Conv regime, as we can see in Figure 3.13a. In particular, in the paper they show that the magnitude of the reduction in DNA damage depends on the scavenging capacity of the irradiated environment. Therefore, they underlined the impact of the scavenging capacity of the environment and the indirect action of radiation for UHDR irradiation.

The second experiment [183] was performed using a kV X-ray beam at Conv (0.1 Gy/s), HDR (25 Gy/s) and UHDR (55 and 125 Gy/s), with a supercoiled DNA plasmid (pUC19) subjected to various environmental conditions. They observed that both UHDR values induced lower amounts of isolated strand breaks and clustered DNA damage for dose values > 40 Gy in an oxygenated environment with respect to Conv and HDR irradiation, as shown in Figure 3.13b. Furthermore, they did not detect a difference in terms of the amount of DNA damage between Conv and UHDR irradiation in the case of a fully scavenged environment or in anoxia conditions. Thus, they concluded that the environmental condition of the irradiated medium is crucial for UHDR irradiation. Moreover, they speculated on the importance of radical-radical reactions for the emergence of the FLASH sparing effect. This mechanistic hypothesis will be discussed in detail in Section 3.5.

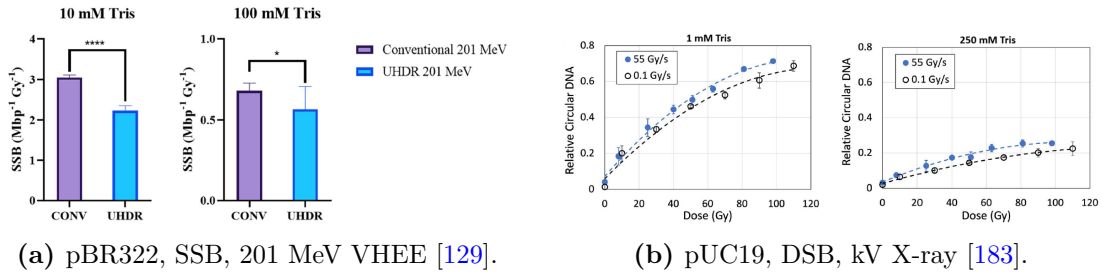


Figure 3.13: Main FLASH *in-vitro* evidence in terms of plasmid DNA damage for different radiation quality and scavenger concentration. Images from [129, 183].

Another interesting biological endpoint is senescence, which is a complex stress-induced proliferation arrest of cells. This phenomenon is usually triggered by an inflammatory response after radiation-induced DNA damage, due to a high production of ROS and harmful chemical species [184]. In the work by Buonanno et al. [158], they show a reduced amount of senescence in normal human lung fibroblasts IMR90 after 1 month from 20 Gy irradiation at both UHDR dose rates compared to the Conv regime, suggesting reduced

long-term effects. In particular, they measured it using β -galactosidase staining, which is a typical biomarker of senescence. In addition, they observed a reduction in the cytokine TGF- β expression 24 h and 1 month after UHDR 20 Gy irradiation compared to Conv, as shown in Figure 3.14. This is interesting because the cytokine TGF- β is the main pro-fibrotic factor that regulates cell division, proliferation, and differentiation. Thus, in principle, this could correlate with reduced radiation-induced inflammatory responses under FLASH regime, mitigating the insurgence of fibrosis, which is a chronic inflammatory diseases. Therefore, because both senescence and fibrosis are strictly related to ROS production, oxidative stress, antioxidant capacity, and redox environment, these results could suggest the crucial involvement of chemistry-mediated processes for the UHDR irradiation regime. The impact of UHDR radiation on TGF- β has also been observed *in-vivo*, as we will see in Section 3.4.2.

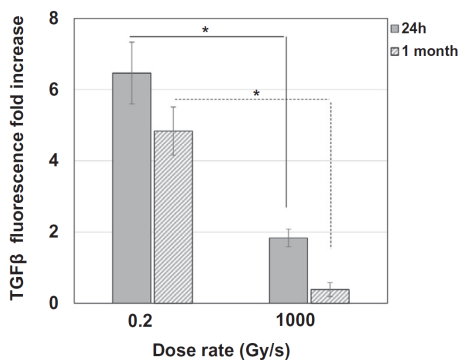


Figure 3.14: Main FLASH *in-vitro* evidence in terms of expression of pro-fibrotic marker TGF- β . IMR90, 8.6 keV/ μ m p. Image from [158].

In conclusion, *in-vitro* experiments have shown a reduction in the formation of DNA damage after UHDR irradiation compared to the Conv approach in terms of formation of radiation-induced foci [158, 180, 9, 182]. However, these results appear to show contradictions both with respect to the corresponding survival measures, as in [158, 175], and in terms of the method of acquiring DNA damage (γ H2AX VS TRP53BP1 (53BP1)), such as for [180]. Furthermore, recent experiments reported a reduction in the amount of DNA damage for UHDR irradiation using plasmid DNA models, for which the magnitude of the reduction depends on the environments, namely the scavenging capacity and oxygenation, of the irradiated medium [129, 183], indicating the importance of the indirect action of radiation in the UHDR regime. In addition to that, reduced senescence cells and pro-fibrotic factors suggest the involvement of the redox environment in the emergence of the sparing effect for UHDR irradiation [158]. However, all of these results do not allow us to completely exclude or confirm possible mechanisms underlying the FLASH sparing effect. Although, they suggest the involvement of chemically mediated DNA damage.

Spheroids

Tumor spheroids is a novel *in-vitro* assay which consists in the aggregation of cancer cells, producing 3D cultures that mimic better the physiological conditions rather than the 2D cell cultures. Typically, the inner core of the spheroid is quiescent or necrotic, while the cells in the outer layer can proliferate. For these reasons, this assay is ideal for investigating possible modulators of the FLASH effect, such as oxygenation and immune response.

The main FLASH experiment on spheroids is that carried out by Khan et al. [179]. They developed spheroids that aggregate lung adenocarcinoma A549 cells with a gradient of oxygen concentrations, where the core was hypoxic. They uniformly irradiated them using 16 MeV electron beams at both Conv (with a reported mean dose rate of 0.0747 Gy/s) and UHDR (90 Gy/s) regime, delivering doses in the range of 5 – 20 Gy. After the irradiation, they dissociated the spheroids for clonogenic assay. They observed a large sparing effect on cell survival for UHDR irradiation compared to the Conv regime, as reported in Figure 3.15. They calculated a DMF as in Eq. 1.72, obtaining a maximum value of 1.3 for doses > 10 Gy, which is coherent with the *in-vivo* results, as we will see in the following Section 3.4.2.

These results are very promising and, given the versatility of the spheroids, further analyses may suggest important insights into the mechanism behind the FLASH effect.

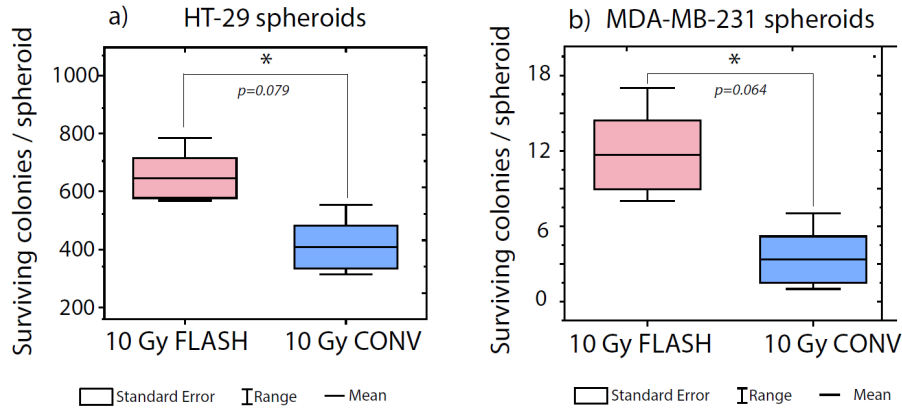


Figure 3.15: Main FLASH *in-vitro* evidence in terms of 3D spheroid with oxygenation gradient. A549, 16 MeV e^- . Images from [179].

3.4.2 *In-vivo* studies

The FLASH effect, as previously defined, is an *in-vivo* radiobiological effect. Thus, *in-vivo* experiments play a crucial role for a desirable clinical translation of FLASH RT [103]. In fact, UHDR experiments on animals allow for the investigation of the elusive mechanism behind the FLASH effect. Furthermore, they permit the study of the optimal physical parameters in order to trigger both the differential-sparing effect (healthy tissue vs tumor) for UHDR irradiation. Finally, they enable the exploration of the optimal biological targets

for the emergence of the FLASH effect.

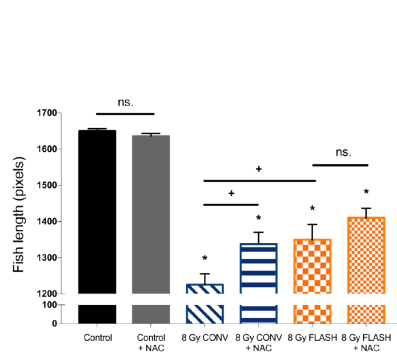
In this Section, we will present key experimental studies in animals that demonstrate the benefits of FLASH RT [38, 99, 102], as shown in Figure 3.8, categorizing the findings based on the specific tissues and organs irradiated. In particular, the most investigated biological structures are the lung, the gut, and primarily, the brain, and the skin. The majority of UHDR experiments have been carried out on mice and rat, using electron-FLASH beams, due to the easier experimental set-up from both physical and biological point of views. Other experiments have been performed on mini-pigs, cats, and dogs, using also photon, proton, and carbon ion beams at the UHDR regime. Furthermore, several FLASH experiments have been carried out on zebrafish embryos, because of their simple biological structure, high throughput, and their genetic similarities to humans.

Zebrafish embryo

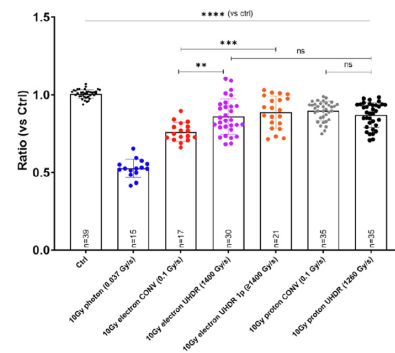
The zebrafish is a small freshwater fish that is usually used in radiobiological experiments [185] because of its several advantages. In fact, zebrafish have metabolic and genetic similarities to humans, allow whole-body investigation, their radiobiological response is fast (5 days), their embryos are transparent, allowing direct and real-time observations, and they are cheaper compared to large animals. Thus, we can consider zebrafish an intermediate biological model between cell cultures and large animals.

Various FLASH experiments have been carried out in zebrafish using electron [85, 109, 187], proton [186], and helium ion [162] UHDR beams. In particular, Montay-Gruel et al. [85] studied the morphological alterations of zebrafish embryos induced by 8 Gy electron beam irradiation (see Section 3.4.1 for irradiation details). They observed systematically significantly fewer alterations in zebrafish length in the case of FLASH irradiation, as we can see in Figure 3.16a. In addition, they repeated Conv and UHDR irradiation of embryos that had been pre-incubated in the presence of antioxidant. They observed fewer alterations in body length with antioxidant for Conv irradiation, while there was no difference for UHDR irradiation with or without antioxidant. The interesting point here is that the reduction in body length in the presence of a potent scavenger for Conv irradiation is significantly greater than FLASH irradiation without antioxidant. Thus, from this result, they concluded that FLASH irradiation reduces ROS production, such as H_2O_2 . However, confirming this hypothesis requires further specific studies.

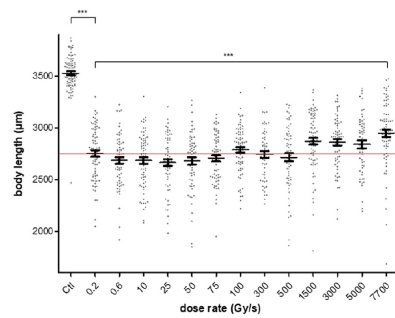
Another interesting work that confirmed the previous findings is that by Kacem et al. [109]. They irradiated zebrafish embryos at physioxia conditions using photons (Xrad 225CX/225 keV, Pxi Precision X-ray, with mean dose rate of 0.037 Gy/s), electrons (eRT6/Oriatron 5.5 MeV electron beam, at 0.1 Gy/s and 1400 Gy/s), and protons (PSI Comet Cyclotron 235 MeV, in transmission, at 0.1 Gy/s and 1260 Gy/s) FLASH RT, studying also the impact of the delivered dose on the emergence of the FLASH effect. In particular, in the case of 10 Gy irradiation, they observed a sparing effect, i.e. small or no body length alterations, in the case of UHDR electron beams irradiation compared to the Conv approach, as reported in Figure 3.16b, while they obtained the same body deformation levels in the case of Conv and UHDR protons irradiation, which are also comparable with the results obtained for UHDR electrons irradiation. They concluded



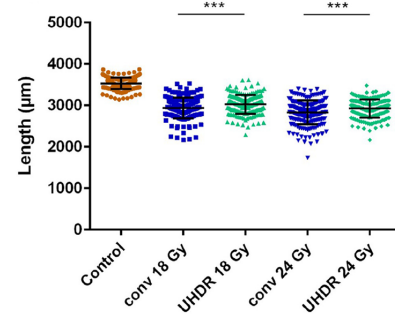
(a) 6 MeV e^- , 8 Gy [85].



(b) 5.5 MeV e^- , 10 Gy [109].



(c) 68 MeV p, 40 Gy [186].



(d) 67.4 MeV ^4He , 18-24 Gy [162].

Figure 3.16: Main FLASH *in-vivo* evidence in terms body length of zebrafish embryo for different radiation quality. Images from [85, 109, 186, 162].

that proton beams could intrinsically induce less side effects for this particular model study, for example because of this specific dose-delivery modality or beam characteristic, but this remains to be demonstrated. Furthermore, they observed that the ratio between apoptotic cells and proliferated cells levels for UHDR electrons and both Conv and UHDR protons returned to the level of non-irradiated embryos at 72 h after irradiation, namely, apoptosis was compensated by proliferation. Finally, they tried to correlate the production of H_2O_2 , a typical ROS as described in Section 1.2.3, with alterations in the body length of zebrafish. In particular, they obtained a decreasing production of H_2O_2 for an increase in radiation quality and an increasing dose rate. However, these measurements are performed in pure water, which is a strong approximation.

The electron results in [109, 85] have been confirmed by Karsh et al. [187], who irradiated zebrafish embryos using the 30 MeV electron LINAC (ELBE), observing a preserving effect that depends on the mean dose rate. They also confirmed the feasibility of electron-FLASH in reducing side effects in zebrafish, as shown in [132]. In contrast to the results in [109], they observed a protective effect in terms of body length for irradiation with UHDR proton beams, delivering the dose using the experimental 224 MeV proton beam of UPTD with a mean dose rate of 300 Gy/s.

This sparing effect for UHDR protons irradiation was also confirmed by Saade et al. [186]. They irradiated zebrafish embryos with 68 MeV protons beams produced by an IBA isochronous cyclotron. They observed a significantly longer body length of zebrafish for UHDR irradiation compared to the Conv regime for a delivered dose of 40 Gy, as shown in Figure 3.16c. Furthermore, they showed a reduction in pericardial edema formation in the case of 30 Gy UHDR irradiation compared to Conv. In addition to that, they did not observe differences between UHDR and Conv for spine curvature. In their first experiment with zebrafish [154], they did not reach the FLASH effect. However, in that work, they probably used a too low mean dose rate (100 Gy/s) for the proton-FLASH beam and a too high oxygenation, as discussed in [99].

The protective effect of FLASH irradiation in terms of body length of zebrafish embryos has also been confirmed using a 67.4 MeV helium ion beam [162], in the plateau region, produced by the isochronous ARRONAX cyclotron (IBA Cyclone 70XP). Ghannam et al. delivered 18 Gy and 24 Gy with mean dose rates of 0.25 Gy/s (Conv) and $9.7 \cdot 10^3$ Gy/s (UHDR). They observed significantly longer body lengths for zebrafish irradiated at both doses under the UHDR regime compared to Conv, as we can see in Figure 3.16d. Moreover, as in the protons experiment [186], they obtained a statistically significant reduction in spine curvature for 24 Gy UHDR helium ion irradiation with respect to the Conv regime. However, in contrast to [186], they observed reduced edema for 24 Gy UHDR irradiation. These protective effects did not appear for 18 Gy UHDR irradiation.

In conclusion, the protective effect of UHDR radiation in reducing the body length of zebrafish embryos has been confirmed by various experiments using different radiation quality (electrons, protons, and helium ions). However, contradictory results emerged in terms of edema formation and spine curvature. Furthermore, there is no proof of the mechanism behind the emergence of these sparing effects in the UHDR regime. Therefore, these promising results demonstrate the feasibility of achieving the FLASH effect

in-vivo, for this specific simple animal model, for various irradiation conditions, but more, systematically organized research is needed in zebrafish with UHDR radiation.

Skin

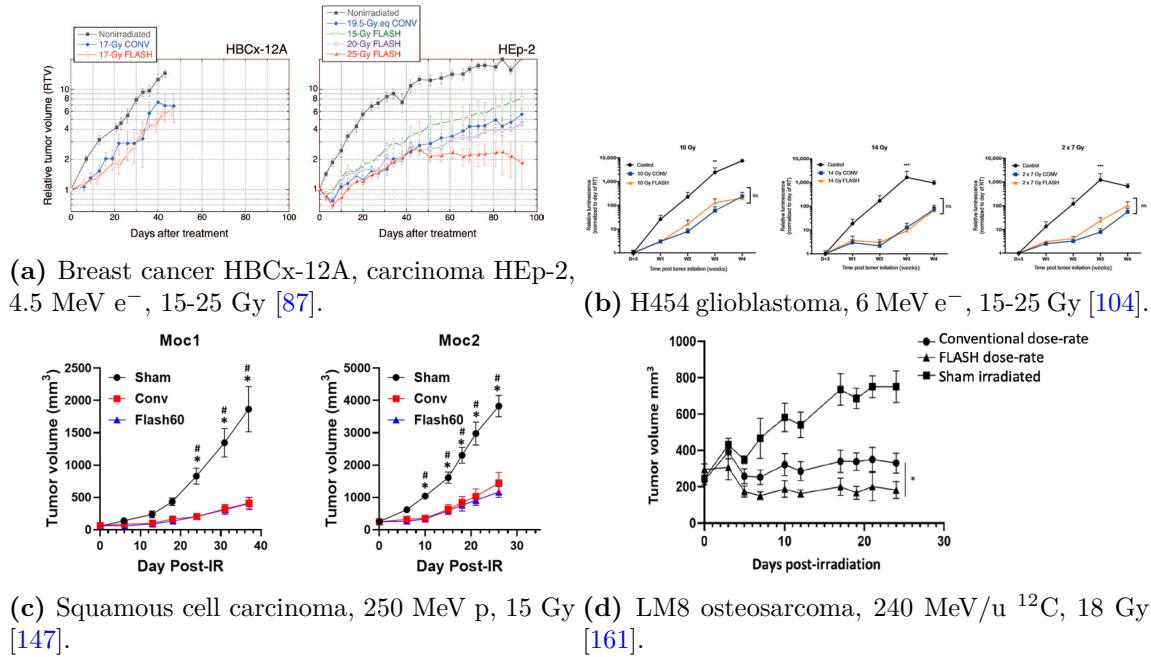
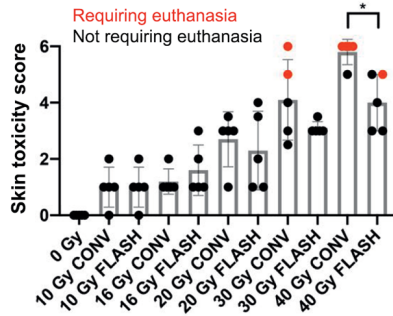


Figure 3.17: Main FLASH *in-vivo* evidence in terms of tumor control for different cancer cell lines and radiation quality. Images from [87, 104, 147, 161].

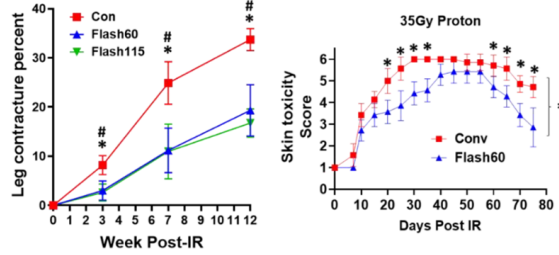
Radiation-induced skin injury is the most common toxicity due to RT, in particular in patients with head and neck, breast, or skin cancer. In humans, the skin is the largest organ, with a total area of $\sim 1.8 \text{ m}^2$, and is structured in three main layers: the epidermis, the dermis, and the hypodermis. Each of these layers has specific biological characteristics and functions. IR can induce two main types of toxicity: acute and late effects. The acute effects occur between hours and weeks after irradiation. The dose threshold to trigger different lesion severity depends on radiation quality and skin area sensitivity. The most common acute lesions are erythema, desquamation, and ulceration, as a consequence of radiation-induced DNA damage. The biological effect is modulated by the balance between oxidative stress and antioxidant. ROS and other harmful free radicals are initially produced by IR. However, they can increase in concentration following an increase in pro-inflammatory cytokines, which induce the release of them, for example, from mitochondria. The inflammatory process can be attenuated by the action of the antioxidant enzyme system, which can reduce the impact of ROS [184]. The main biological pathways for acute effects are necrosis, i.e., an uncontrolled cell death, apoptosis, i.e. a programmed cell death, and senescence, i.e. a stress-induced cell growth arrest. All these processes

are regulated by the $p53$ protein, which controls cell cycle check-points. Instead, late effects appear between weeks and years after irradiation. The most common toxicity is the fibrosis, which is a complex chronic inflammatory disease based on an excessive production of collagen and other extracellular matrix components in the dermis. The typical biomarker of fibrosis is the cytokine TGF- β , which is a signaling protein that regulates cell division, proliferation, and differentiation, and it is secreted by senescent cells.

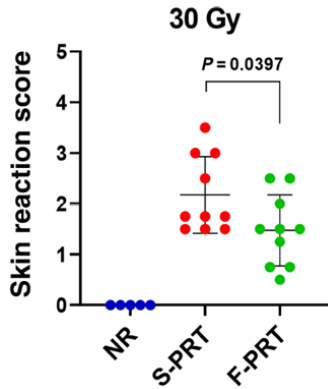
The first investigation regarding the occurrence of skin toxicities after delivering IR at UHDR regime dates back to the 1970s-1980s [188, 189, 190]. In particular, they irradiated rats with electron beams at high dose rates ($> 10^5$ Gy/s) and observed a reduction of acute (e.g., dermatitis) and late (e.g. necrosis) effects in the skin. However, the aim of these works was to study the possible oxygen depletion effect by UHDR irradiation, as we will discuss in Section 3.5.1, and not to investigate the beneficial effects that we know today of the FLASH RT.



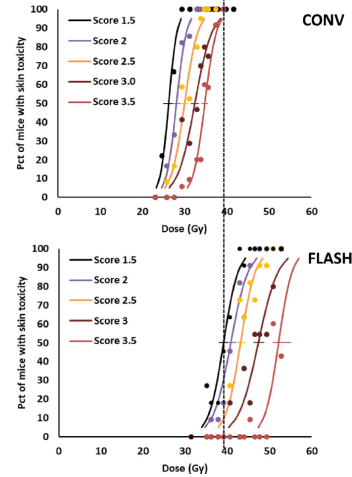
(a) 16 MeV e^- , 0-40 Gy [191].



(b) 244-250 MeV p, 35 Gy [147].



(c) 230 MeV p, 30 Gy [153].



(d) 244-250 MeV p, 0-60 Gy [150].

Figure 3.18: Main FLASH *in-vivo* evidence in terms of skin acute toxicity for different radiation quality. Images from [191, 147, 153, 150].

In 2019, the Vozenin team demonstrated the protective effect of FLASH RT in reducing skin toxicities in a mini-pig and cats in their seminal work [83]. They irradiated a mini-pig using an electron LINAC, delivering doses in the range of 22 – 34 Gy for the Conv (~ 5 Gy/min) and UHDR (~ 300 Gy/s) regime. They followed-up the skin for 48 weeks after irradiation and observed just a transient depilation after 3 weeks and preservation of the hair follicles in the area of the skin irradiated with UHDR RT for doses ≤ 31 Gy. Instead, for Conv irradiation they obtained a permanent depilation and destruction of follicles, and also the emergence of severe late skin fibro-necrosis. Furthermore, they also irradiated the nose of 6 cats with Squamous Cell Carcinoma (SCC) of the nasal planum, with doses ranging between 25 Gy for the first cat and 34 Gy for the last animal. They observed permanent depilation for all cats in the irradiated area, which started 3 weeks after irradiation. Furthermore, in their follow-up study [192], they reported severe acute side effects in cats, in particular maxillary bone necrosis, and severe late skin necrosis in pigs.

The first modern experiment entirely dedicated to the study of skin reactions after UHDR irradiation is that of Soto et al. [191]. They irradiated mice using a 16 MeV electron beam, delivering doses in the range of 10 – 40 Gy with a mean dose rate of 180 Gy/s for UHDR and 0.0747 Gy/s for the Conv regime. They observed the manifestation of ulcers 8 weeks after irradiation of the mice group that received 30 – 40 Gy Conv and also 40 Gy UHDR, but with reduced severity compared to Conv, as reported in Figure 3.18a. The interesting point is that the group of mice irradiated with UHDR RT showed a reduced mortality 184 days after irradiation. Thus, the experiment showed the protective effect of FLASH in terms of the severity of skin toxicity and late effects.

These findings have also been confirmed by proton beams in the UHDR regime. Cunningham et al. [147] demonstrated the feasibility of PBS proton-FLASH RT in reducing acute and late effects in mice skin. In particular, they delivered 35 Gy to the leg of mice with mean dose rates of 1 Gy/s (Conv), 57 Gy/s and 115 Gy/s (UHDR), using the 250 MeV proton beam in the initial plateau region at the Varian ProBeam clinical gantry (CCHMC/UC proton center). They measured TGF- β 1 levels, namely a biomarker of radiation-induced fibrosis, on day 1 and 4 after irradiation. In the case of Conv RT, the TGF- β 1 level increases in both days, while for FLASH RT, on day 1 the value of this marker was higher than the standard level but lower than that of Conv irradiation, as we can see in Figure 3.19a. Moreover, on day 4 this level returned to the normal one. Thus, this suggests the development of reduced radiation-induced late side effects in the case of UHDR irradiation. They also monitored for 12 weeks possible leg contracture after RT, as reported in Figure 3.18b. Both Conv and UHDR RT induced contracture, however, the mice irradiated with FLASH RT showed a significant decrease contractures from 3 weeks post-irradiation, with no difference between the two FLASH dose rates. Furthermore, they observed a reduced induction of skin lesions for UHDR irradiation compared to Conv, and a delayed development of moist desquamation and, at the same time, faster healing. In the end, they also studied the response of both irradiation modalities in terms of tumor response. They delivered 15 Gy at 1 Gy/s and 57 Gy/s in mice, obtaining the same control of tumor growth (squamous cell carcinomas) between Conv and UHDR RT,

as shown in Figure 3.17c. Therefore, they demonstrated the emergence of the FLASH effect with protons both in terms of reduction of skin toxicity and in terms of equivalent effectiveness in tumor control compared to Conv RT.

The results of the previous proton-FLASH experiment are in agreement with those obtained by Velalopoulou et al. [153]. They irradiated mice using the entrance region of the 230 MeV IBA Proteus Plus beam with dose rates of 69 – 124 Gy/s (UHDR) and 0.39 – 0.65 Gy/s (Conv). They obtained reduced skin toxicities and equivalent tumor control for UHDR RT compared to Conv RT. In particular, mice showed fewer severe skin lesions for 30 Gy UHDR irradiation, as we can see in Figure 3.18c. In addition to that, they observed that specific biological pathways were up-regulated only by Conv RT, e.g., those related to apoptosis or keratinocyte differentiation. Interesting, they confirmed previous results about TGF- β 1 signal, which is higher in Conv RT than in UHDR RT in both murine and canine skin, as reported in Figure 3.19b. In particular, they delivered 8 – 12 Gy in the leg skin of the dog. These findings could be correlated with reduced inflammation and late effects in the case of FLASH RT.

Singers-Sørensen et al. [149, 16, 150] have also observed reduced acute and late skin reactions with proton-FLASH RT. In particular, in [149], they irradiated the hind limb of mice using the 250 MeV ProBeam (Varian Medical Systems, Palo Alto, CA, USA), delivering protons in the plateau region with the PBS approach in the Conv and UHDR regimes, with mean dose rates in the range of 0.35 – 0.73 Gy/s and 65 – 92 Gy/s, respectively. They observed reduced moist desquamation, that is, reduced epidermal thickness, in the skin for UHDR RT compared to Conv RT, with a DMF of 1.44 – 1.58 at all scoring levels of severity of the lesions, which corresponds to an increase of 44 – 58% in dose for FLASH irradiation to obtain the same toxicity level of Conv RT, as shown in Figure 3.18d. Furthermore, in [16], they obtained a reduced occurrence of severe fibrosis in mice, with a DMF of 1.14 and the same tumor control for C3H mouse mammary carcinoma between Conv and UHDR RT. In addition to that, in [150], they studied the impact of repainting and split-dose irradiation on the emergence of the FLASH sparing effect. The repainting experiment consisted of delivering a total dose of 39.3 Gy scanning the pencil beam multiple times over the target volume, comparing these results with those obtained by delivering the dose with a single scanning. They observed higher toxicity levels for the same mean dose rate with the repainting approach compared to that without repainting, which corresponds to higher dose rate values requested in the case of repainting to obtain the same toxicity level. Instead, the split-dose experiment is based on delivering a total dose of 39.3 Gy splitting them into different delivers with pauses of 2 min. They observed a slight reduction in the FLASH sparing effect releasing the dose in 2 delivers, while for a higher number of delivers the FLASH sparing effect was largely diminished until vanishing for lower toxicity levels. Thus, in principle, these findings could compromise the clinical application of FLASH RT because in this work they showed how a decrease in the mean dose rate or the splitting of dose delivery. However, more studies are needed to investigate the existence of a minimum dose-rate threshold to maintain the FLASH effect in clinical settings.

Finally, Zhang et al. [193] studied the role of oxygenation in the manifestation of the

FLASH sparing effect, decreasing and increasing the oxygen concentration in the skin. They irradiated the legs of mice using a 230 MeV proton beam, delivering doses between 25 Gy and 45 Gy at both Conv (0.4 Gy/s) and UHDR (130 Gy/s) dose rates. Interestingly, they observed a disappearance of the protective effect induced by UHDR radiation in mice whose normal oxygen levels have been disrupted.

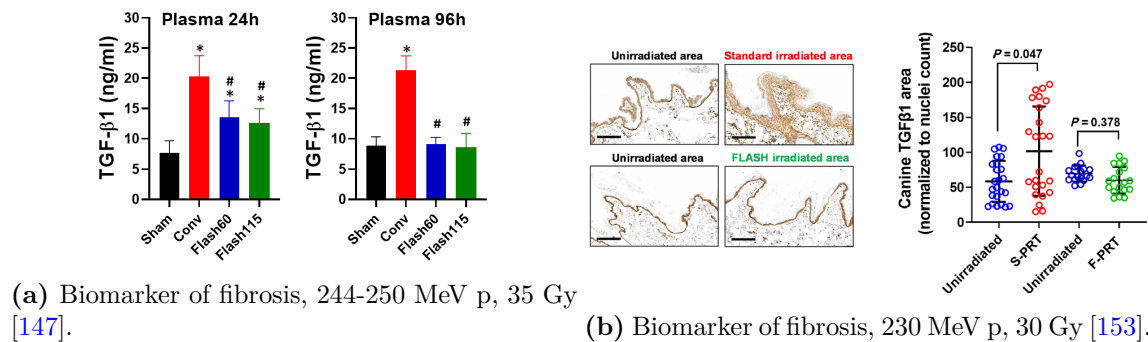


Figure 3.19: Main FLASH *in-vivo* evidence in terms of skin late toxicity for different radiation quality. Images from [147, 153].

In summary, these studies reported reduced acute side effects in terms of depilation, leg contracture, moist desquamation, and ulceration [83, 191, 147, 153, 149, 16, 150, 193], and late effects, such as fibrosis [83, 191, 16]. Furthermore, other studies reported on down- or up-regulation of specific biological pathways could be associated with a reduction of severe late effects [147, 153]. In addition to that, there appears to be a minimum dose-rate threshold [150] and an oxygenation dependence [193] for the emergence of the FLASH effect. However, [192] reported severe acute and late effects after UHDR irradiation. In the end, the same tumor efficacy was demonstrated between Conv and UHDR RT [147, 153, 16].

Brain

Brain injury is the main radiation-induced toxicity in patients with head and neck cancers. In general, brain toxicities consist of functional and cognitive alterations due to neuronal, glial, and vasculature lesions. Cognitive impairments typically begin to manifest about one month after irradiation with 10 Gy Conv. Given the significant impact on quality of life, there is great interest in finding radiotherapeutic techniques that can reduce these cognitive toxicities. For this reason, the application of FLASH therapy for brain cancer is one of the main areas of study.

The first study on cognitive impairments after FLASH RT is that of Montay-Gruel et al. [194] with the protocol developed in the Limoli team. They delivered 10 Gy in mice with complete brain irradiation using electron LINAC accelerators (Oriatron 6e and Kinetron) in both the Conv and UHDR regime, with mean dose rates in the range of 0.1 – 500 Gy/s. They evaluated cognitive alterations using the Novel Object Recognition (NOR) test, which is a behavioral experiment that consists of distinguishing between

an object known to the animal and a new one. They observed an increasing cognitive deficiency for a decrease in dose rate 2 months after irradiation, as shown in Figure 3.20c. In particular, they did not observe memory alterations for dose rates ≥ 100 Gy/s, while they detected a significant decrease for dose rates < 30 Gy/s. They also investigated the proliferation of cells in the brain hippocampal using BrdU immunostaining, which is a nucleoside that is used to label newly generated neurons to evaluate neurogenesis. They observed a relative preservation of neurogenesis in the case of FLASH RT compared to Conv irradiation, as we can see in Figure 3.20d.

These findings were also confirmed using photon-FLASH RT. In [135], Montay-Gruel et al. carried out a whole brain irradiation experiment in mice, delivering 10 Gy in the Conv (0.05 Gy/s) and UHDR (37 Gy/s) regime. They did not notice memory alterations in FLASH-irradiated mice using the NOR test 2 and 6 months after irradiation, as reported in Figure 3.20a, while they detected a drop in cognitive deficit in Conv-irradiated mice. They also performed BrdU immunostaining of the irradiated hippocampus and observed a preservation of mitotic activity, i.e., cell proliferation, for FLASH RT, which could lead to a relative preservation of neurogenesis. In addition, they measured the number of astrocytes after irradiation, monitoring GFAP expression, which is the typical biomarker of astrocytes. Astrocytes are glial cells that play a crucial role in brain homeostasis. A significant increase in astrocytes is typically observed after RT due to radiation-induced stress. In this experiment, they observed an attenuated increase in the number of astrocytes for UHDR irradiation compared to Conv. Thus, this could correlate with the preservation of cognitive activity in mice in the case of FLASH RT. These results on GFAP expression and astrocytes were also confirmed using electron irradiation in [195]. In this work, they showed how FLASH RT did not induce astrogliosis, namely a hypertrophic morphology of astrocytes, compared to Conv RT. In particular, they demonstrated that biomarkers linked to astrogliosis and immune signaling are expressed at lower levels following FLASH RT, in contrast to Conv RT. These findings corroborate the hypothesis of the involvement of different oxidative stress and inflammatory processes between Conv and FLASH RT.

Additional results by Montay-Gruel et al. [85] confirmed the protective beneficial effect of electron FLASH irradiation on the mouse brain in terms of alterations in neurocognitive and behavioral characteristics, highlighting the critical role of tissue oxygen concentration. Furthermore, in this study, they found that FLASH RT did not trigger neuroinflammation, a process typically driven by oxidative stress. This specific finding on neuroinflammation was confirmed by Simmons et al. [118], using a modified LINAC (Varian). In particular, they noticed a considerable growth in 5 of 10 pro-inflammatory cytokines in the hippocampus for electron beam irradiation in the Conv regime, delivering 30 Gy with a mean dose rate of 0.13 Gy/s. In contrast, FLASH RT (200-300 Gy/s) was linked to smaller increases in just three cases. They also confirmed the preservation of the cognitive functionality of mice for FLASH RT using the NOR test. Furthermore, they detected a marked reduction in the density of dendritic spin in the hippocampus for Conv RT compared to FLASH RT. This may be related to memory deficits in the case of Conv irradiation.

All these results on the neuroprotective effects of FLASH RT were also confirmed by

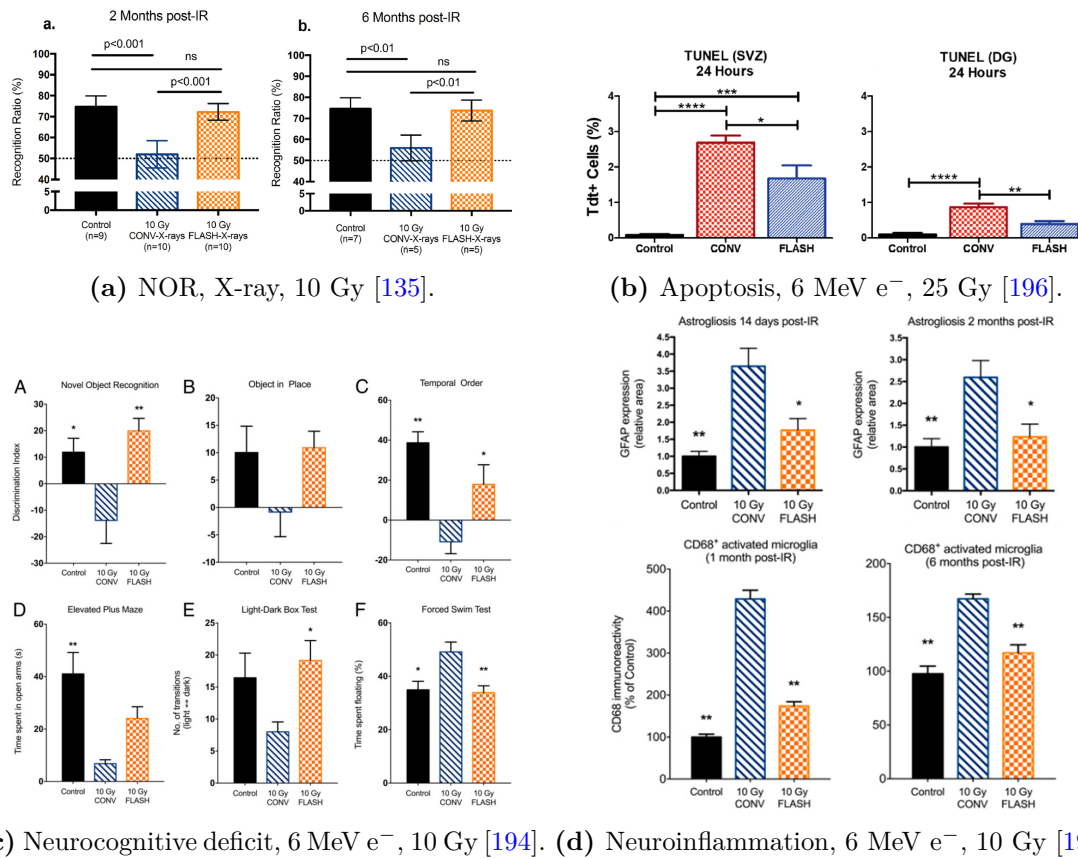


Figure 3.20: Main FLASH *in-vivo* evidence in terms of brain toxicity for different biological endpoints and radiation quality. Images from [135, 196, 194].

Alaghband et al. [197]. They carried out a whole brain irradiation of mice using an electron LINAC (Oriatron 6e), delivering 8 Gy in the Conv (mean dose rate of 0.077 Gy/s) and UHDR ($4.4 \cdot 10^6$ Gy/s) regime. They observed protective effects of UHDR irradiation in terms of several behavioral indices, such as novel object recognition, objects in updated location, fear extinction, light-dark box, and social interaction. In addition, they confirmed reduced neuroinflammations in the case of FLASH RT compared to Conv RT.

Allen et al. [196] also showed other interesting results about the sparing effect of whole brain irradiation with FLASH RT. They delivered 25 Gy to mice using Oriatron 6e, with mean dose rates of 0.1 Gy/s (Conv) and 2500 Gy/s (UHDR), observing an increase in the volume of the brain vessel and immunoreactivity of eNOS, which is a marker of vessel dilation and edema formation, only for Conv RT 1 week after irradiation. They also measured altered protein expression, particularly for OCLN and CLDN5, which are key proteins to maintain tight junctions between cells and markers of lesions in the blood-brain barrier, only for Conv RT. Furthermore, they showed reduced induction of apoptosis in the neurogenesis-active areas of the brain for FLASH RT, as reported in Figure 3.20b. Finally, they also investigated the involvement of vascular alterations in the occurrence of long-term cognitive side effects, delivering 10 Gy at 0.09 Gy/s (Conv) and $5.6 \cdot 10^6$ Gy/s (UHDR), a known dose that can trigger late effects. They observed reduced tight junction lesions and no vascular dilation in the case of UHDR RT compared to Conv 1 month after irradiation. These findings suggested the involvement of the brain vascular system in preserving cognitive activities. In this context, FLASH RT can cause less damage to blood vessels, resulting in reduced acute and long-term cognitive effects.

In addition to their previous results, Montay-Gruel et al. [104] also studied the impact of hypofractionation in the context of FLASH RT. Using Oriatron 6e, they performed different irradiation schemes with mean dose rates of 0.1 Gy/s for Conv and in the range of $1.9 - 5.6 \cdot 10^6$ Gy/s for FLASH. In particular, they delivered single doses of 10 Gy and 14 Gy, 2×7 Gy and 4×3.5 Gy with intervals of 24 h, and 3×10 Gy with intervals of 48 h. Interestingly, they noticed a beneficial effect for FLASH RT in terms of acute cognition diseases, quantified using the NOR assay, only for some irradiation schemes, in particular for 10 Gy, 2×7 Gy, and 3×10 Gy. Furthermore, these two fractionated schemes have shown the ability to slow down tumor growth (H454 orthotopic glioblastoma tumors), with similar results between Conv and UHDR RT, as reported in Figure 3.17b. This experiment was the first indication of the existence of possible dose-threshold for triggering the FLASH effect. At the same time, these findings enable the possibility of using fractionated approaches also with FLASH RT.

The feasibility of fractionated electron-FLASH RT was confirmed by Liljedahl et al. [176]. They irradiated immunocompetent rats with 2×8 Gy for subcutaneous tumors (NS1 glioblastoma) and with 2×12.5 Gy for intracranial tumors at both Conv and UHDR regime, using the 10 MeV Elekta beam. For the rats with subcutaneous tumors, which were irradiated with mean dose rates of 8 Gy/min and 66 Gy/s, they obtained the same tumor control for Conv and UHDR irradiation, and, at the same time, a huge reduction of tumor TIMP-1 in serum, which is a key protein for enhancing tumor growth and tumor invasion,

and also for suppressing apoptosis, for FLASH RT compared to the control. Instead, the difference in TIMP-1 between Conv RT and control was not statistically significant. This result may indicate prolonged survival. In the case of rats with intracranial tumors (dose rates of 8 Gy/min and 74 Gy/s), they observed the same increase in survival for Conv and FLASH RT with respect to the control, but without reaching the cure. There is no clear explanation of the different capacity of reaching the cure between subcutaneous and intracranial tumors, but the authors suggested the involvement of different level of integrity of the blood–brain barrier. However, answering to this open question goes beyond the focus on the differential effect between Conv and FLASH RT, where in this experiment, the two approaches have proven to be equally effective against tumor growth.

A more recent work by Almeida et al. [198] confirmed reduced brain toxicities and equal tumor control for GL261 glioblastoma mouse model with electron-FLASH delivered with Oriatron 6e. In addition to that, interestingly for the first time, they achieved the FLASH effect for brain irradiation of the same animal model using a 170 MeV proton-FLASH beam at PSI. In particular, they delivered 10 Gy in transmission mode with mean dose rates of 0.1 Gy/s (Conv) and 110 Gy/s (UHDR) on healthy mice. They performed the NOR test to investigate the cognitive activity after irradiation, observing the same response between control and FLASH, while reduced capacity in animals irradiated with Conv RT. Furthermore, they obtained the same tumor control between Conv and UHDR, delivering 20 Gy on the primary tumor volume with the previous dose rates.

In addition, Iturri et al. [199] demonstrated the emergence of FLASH effect with 226 MeV UHDR-proton RT (ICPO). In particular, they used the entrance channel of a proton beam for irradiating the brain of rats at both Conv and UHDR regime, with mean dose rates of 4 Gy/s and 257 Gy/s, respectively, and delivering doses of 15 – 25 Gy. The rats were anesthetized using two different methods, namely with or without adding oxygen, to study the impact of oxygenation. They observed reduced brain lesions and preservation of cognitive activity in the case of UHDR RT without oxygenation, while the addition of oxygen during anesthesia led to the highest level of brain toxicities. Interestingly, they noticed a significant reduction of immune cell infiltration into the tumor volume (RG2 glioma cell line) for Conv RT when the anesthesia is performed with oxygen addition. Instead, adding oxygen has less impact with UHDR RT. Therefore, this finding suggests a varying impact of oxygenation on the induction of the immune response, depending on the dose rate regimes. Furthermore, these results were one of the main proofs for oxygen depletion as a triggering mechanism of the FLASH effect, as we will see in Section 3.5.1.

In summary, all of these studies showed reduced side effects for irradiation of the entire brain of mice in the case of UHDR RT compared to Conv irradiation. In particular, FLASH RT preserves neurogenesis and behavioral performance [194, 135, 85, 118, 197, 196, 198, 199] and reduces inflammatory processes [135, 85, 118, 195, 197], which is strictly related to oxidative stress. Furthermore, various experiments [104, 176, 196, 199] demonstrated the feasibility of FLASH-RT in controlling the tumor growth.

Lung

The lung is a complex hierarchical biological structure that plays a vital role in the process of respiration. The development of radiation-induced side effects in the lung is very common in the case of treatment of lung cancer, breast cancer, and esophageal cancer. The most common acute IR-induced toxicity in the lung weeks after irradiation is radiation pneumonitis, while the typical late effect is lung fibrosis, which can manifest months after irradiation.

The seminal work of Favaudon et al. [87] studied the impact of UHDR irradiation in the development of side effects in the lung of mice, in particular, in terms of lung fibrogenesis. They mice was irradiated using the 4.5 MeV electron LINAC (Kinetron), delivering 17 Gy at the Conv and UHDR regimes. In terms of acute responses, they observed the induction of apoptosis in vascular and bronchial smooth muscle cells 1 h after just 7.5 Gy dose deposition for Conv irradiation, as shown in Figure 3.21a, and also inflammatory processes 24 h after irradiation. Instead, FLASH RT induced rare inflammation for a dose of 17 Gy, while they observed the same level of side effects of 7.5 Gy Conv RT only for 30 Gy UHDR irradiation. This finding about apoptosis in the lung could be the trigger of late responses, such as fibrosis. This suggestion has been confirmed by further studies in terms of late complications. In fact, 8 weeks after Conv RT, they observed the formation of fibrogenic patterns in the lung, which gradually deteriorated to dense fibrosis devolvment 24 weeks after irradiation, in which the lung manifested large collagen formation and activation of the biomarker TGF- β . Instead, for UHDR RT, they did not observe pulmonary fibrosis from histological images and TGF- β levels. They also performed a dose escalation experiment, delivering doses in the range of 16 – 30 Gy in the UHDR regime. They observed mice with cachexia, which is severe weight loss and muscle wasting due to metabolic changes, within 32 weeks after ≥ 23 Gy UHDR irradiation and the formation of lung edema, fibrotic patches, and inflammatory processes after 24 weeks of 30 Gy UHDR irradiation. In this work, they also demonstrated for the first time the same efficacy of Conv and UHDR RT to control tumor growth, using human breast cancer HBCx-12A tumor xenografts and human head and neck carcinoma HEp-2 xenografts, as we can see in Figure 3.17a.

The greater lung protection with FLASH RT compared to Conv RT was confirmed by Fouillade et al. [180]. They performed a bilateral thorax irradiation of mice using Kinetron. They noticed a reduced induction in the expression of pro-inflammatory genes and proliferation of progenitor cells in the case of UHDR irradiation. Furthermore, they noticed a decrease in the expression of three senescence markers for UHDR irradiation compared to Conv RT, as shown in Figure 3.21b, leading to a smaller number of senescent cells. This could suggest reduced late effects for FLASH RT, due to the key role of senescent cells in the induction of fibrosis. In addition to that, they observed a significantly higher number of damaged cells in terms of the number of cells with 53BP1 foci and the number of foci per cell in the case of 1 week after Conv irradiation than FLASH RT, as reported in Figure 3.21c, where 53BP1 is a protein that binds to the DNA region with chromosomal lesions. The number of cells with DNA damage foci did not change 3 months after FLASH irradiation, but there was a significant increase in the case of Conv RT. At the same time,

the number of foci per cell decreased 3 months after FLASH RT, while in the case of Conv this number increased. This suggests that Conv RT caused spontaneous DNA damage after irradiation, whereas FLASH RT resulted in lower persistence of DNA damage.

The FLASH effect also emerged for the photon beam, which is produced by a 6 – 8 MeV superconducting LINAC (CTFEL) [139]. Gao et al. irradiated tumor volume (EMT6 mouse breast cancer cells) with mean dose rates of 0.01 Gy/s (Conv) and ~ 1000 Gy/s (UHDR). Regarding tumor control, they obtained a statistically significant improvement for 18 Gy UHDR RT compared to 15 Gy Conv irradiation in terms of tumor growth, with no difference in survival, leading to DMF = 1.2. In addition, they carried out whole-thorax irradiation for the lung study, delivering 30 Gy (UHDR) and 24 Gy (Conv). They observed reduced severity of alveolar fibrosis, as reported in Figure 3.21d, higher integrity of alveolar structures, and better survival for UHDR irradiation compared to Conv RT, obtaining DMF = 1.25.

Therefore, these findings [87, 180, 139] suggest reduced acute and late effects in the lung in the case of FLASH RT compared to Conv irradiation. In particular, in [87, 180], they noticed less expression of biomarkers related to fibrosis induction, in terms of apoptosis and senescence, inflammatory processes, collagen accumulation, TGF- β activation, and DNA damage 53bp1 foci, for FLASH irradiation.

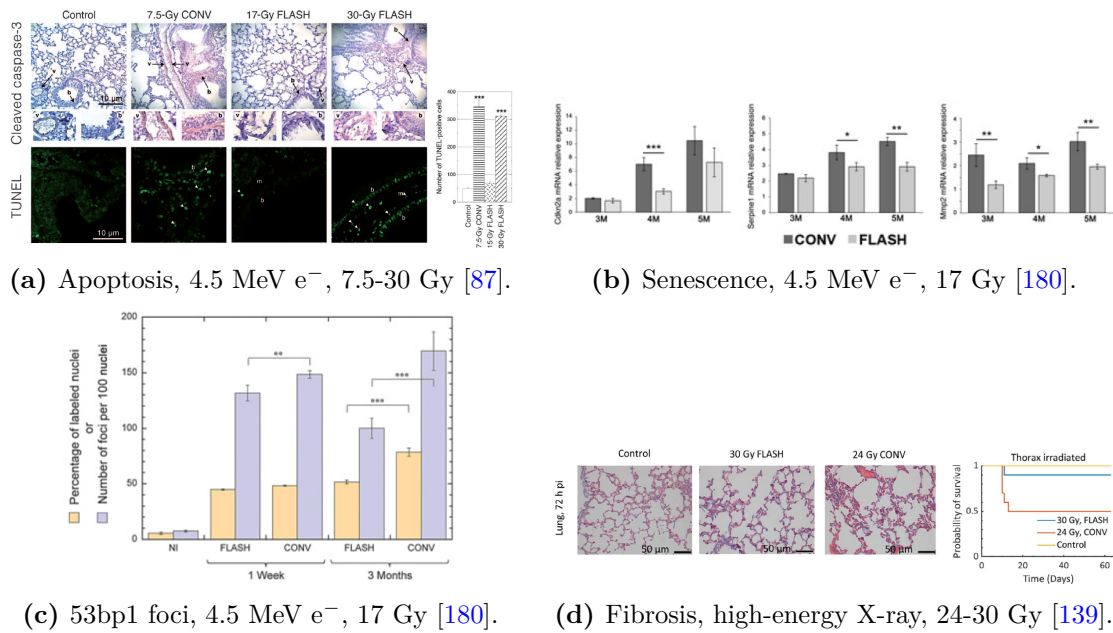


Figure 3.21: Main FLASH *in-vivo* evidence in terms of lung toxicity for different biological endpoints. Images from [87, 180, 139].

Abdominal and pelvic organs

Abdominal organs are those located in the abdominal cavity, such as the gut, stomach, liver, pancreas, kidneys, etc., and are fundamental for metabolism and digestive activities. Intestinal injury is the most common radiation-induced toxicity in the case of radiation treatment of abdominal and pelvic cancers, such as stomach, pancreas, colon, and intestinal tumors, or metastatic ovarian cancer for women and prostate cancer for men. The typical acute side effect for these cancer patients is gastrointestinal toxicity, which manifests itself in nausea, abdominal pain, diarrhea, and fatigue. Instead, the most common late effect is delayed radiation enteropathy, which is a chronic inflammation and IR-induced dysfunction of the intestine. In particular, radiation-induced intestinal lesions occurred as a result of the damage of the intestinal epithelium.

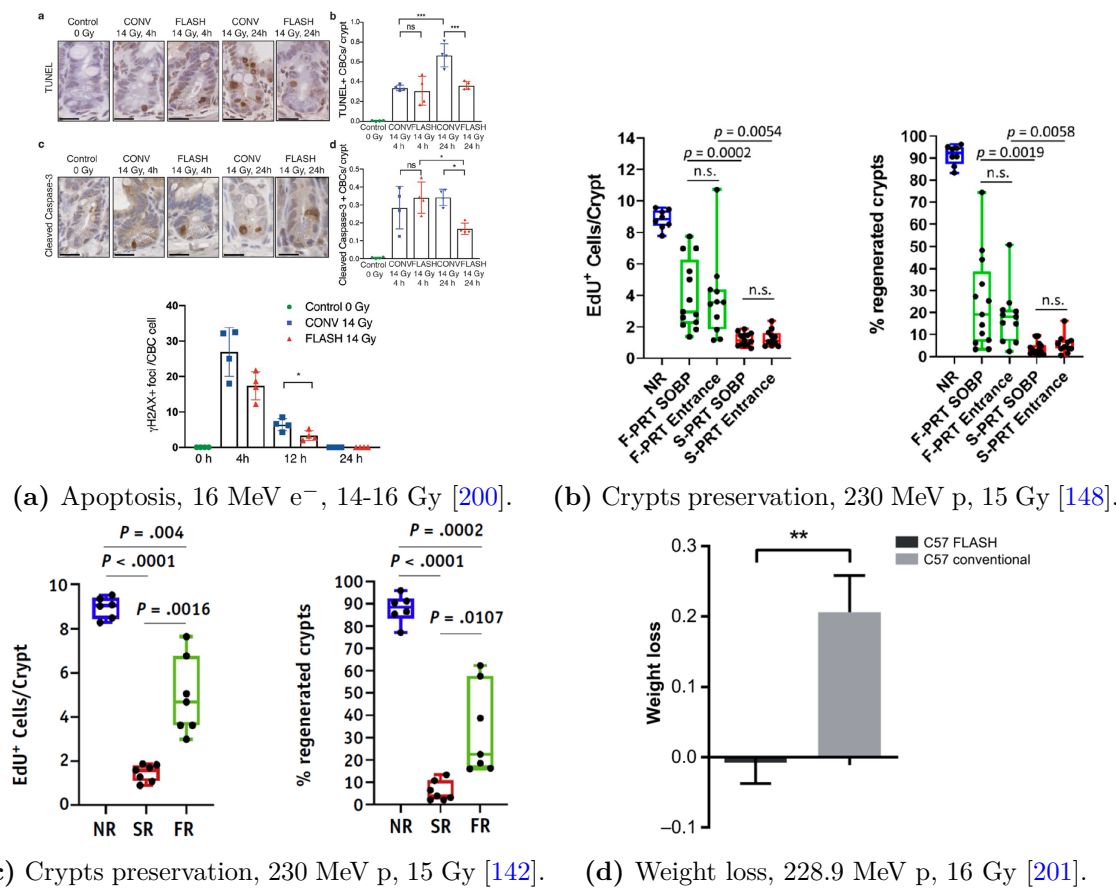


Figure 3.22: Main FLASH *in-vivo* evidence in terms of abdominal and pelvic toxicity for different biological endpoints and radiation quality. Images from [200, 148, 142, 201].

The main experiment entirely dedicated to the study of the occurrence of side effects in the case of FLASH RT is that carried out by Levy et al. [200], where they performed a total abdominal irradiation of mice, delivering 14–16 Gy using a 16 MeV electron LINAC

(Varian) in both the Conv and UHDR regime, with mean dose rates of 0.079 Gy/s and 216 Gy/s, respectively. They observed a higher survival for mice irradiated with FLASH RT, which, after an initial decrease in their body weight, recovered 90 days after irradiation, compared to mice that had received Conv RT. Furthermore, mice irradiated with Conv RT showed mucosal lesions, while those treated with FLASH exhibited the same histological characteristics as control mice 12 weeks after irradiation. Furthermore, they monitored the proliferation process of crypt cells, namely cells of the intestinal glands in the intestine, labeling mice with BrdU, which is a marker of DNA replication, and those of crypt base columnar (CBC) cells, the intestinal stem cells responsible for the regeneration of the gut. They noticed a more efficient recovery process of the crypts and a greater protection of stem cells against radiation-induced depletion, in the case of UHDR RT compared to Conv RT. In addition, they studied the apoptotic process for intestinal cells and they observed same apoptotic levels for the total population of crypt cells, but a reduction in the occurrence of apoptosis for CBC cells after FLASH irradiation, as we can see in Figure 3.22a. Thus, FLASH RT leads to a higher protection against apoptosis for intestinal stem cells, which could be mediated by a reduction in radiation-induced DNA damage compared to Conv irradiation. To support this hypothesis, they examined the formation of DSBs in CBC cells using the γ -H2AX assay, which serves as a biomarker for this process. They observed a statistically significant decrease in the formation of γ -H2AX foci at 4 h and 8 h after FLASH irradiation, while no γ -H2AX foci after 24 h, probably because the γ -H2AX method reached saturation, becoming inefficient in distinguishing between the two modes of irradiation. For this reason, the γ -H2AX assay should be combined with the 53bp1 method, which allows the monitoring of damage over longer periods, as used in [180]. Thus, this result is a sign of a possible reduction in initial DNA damage in the case of UHDR irradiation compared to Conv. In the end, they obtained the same tumor control in the case of the ID8 ovarian peritoneal tumor, which can also metastasize in the abdomen. Therefore, they demonstrate the emergence of the FLASH effect for the whole abdominal UHDR irradiation in a mouse model.

Another electron-FLASH experiment in the abdomen region from the previous group is that carried out by Eggold et al. [202], which focuses on the immunomodulatory properties of FLASH RT. Using the previously described electron beam (Varian) with dose rates of 0.126 Gy/s (Conv) and 210 Gy/s (UHDR), they delivered 14 Gy to mice with ID8 ovarian cancer cells. They confirmed the results obtained in [200], in terms of tumor control and intestinal crypt protection with electron-FLASH RT. Furthermore, they noticed an earlier increase for intratumoral T cell infiltration for FLASH compared to Conv RT. This could suggest that FLASH RT may stimulate an early immune response.

For the first time, Ruan et al. [110] interestingly investigated the impact of the dose-delivery time structure on the emergence of FLASH effect for whole abdomen irradiation of mice under 95%O₂. They changed the number of electron pulses in the range of 1 – 1250 or the time interval between pulses in the range of $3.3 \cdot 10^{-3} - 50$ s, using a 6 MeV electron LINAC (Oxford). These intervals correspond to FLASH mean dose rates up to $\sim 3 \cdot 10^6$ Gy/s and a Conv mean dose rate of 0.25 Gy/s. In general, they confirmed the higher sparing of intestinal crypts for FLASH irradiation compared to Conv RT, for dose

in the range of 7.5 – 12.5 Gy. The most interesting aspect is that this protective effect was reduced or lost by increasing the number of pulses or the time between pulses. For example, for a total dose of 11.2 Gy, a drop in the percentage of surviving crypts was already observed between 1 and 2 pulses. In more details, they observed that the FLASH effect is maximized for the highest dose per pulse, the minimum number of pulses, and the shortest pulse width, while it was maintained for a combination of parameters that correspond to a mean dose rate > 280 Gy/s. Furthermore, they obtained a $DMF = 1.1$, which is slightly smaller compared to the previous discussed results, probably due to the 95%O₂ condition (i.e., hyperoxia). In addition, they noticed a reduced impact on the microbiota using FLASH RT.

Kim et al. [148] confirmed these interesting findings by performing proton-FLASH irradiation in both SOBP ($\sim 2\text{--}3$ keV/ μm) and shoot-through (~ 0.4 keV/ μm) modalities. In particular, using the 230 MeV IBA Proteus Plus device, they carried out a whole abdomen irradiation of mice in both Conv, with mean dose rates of ~ 0.82 Gy/s and ~ 0.83 Gy/s for SOBP and entrance irradiation, respectively, and the UHDR regime, with mean dose rates of ~ 108 Gy/s (SOBP) and ~ 107 Gy/s (entrance). They studied the occurrence of acute gastrointestinal side effects by delivering 15 Gy for each irradiation method, in particular focusing on the proliferation process of crypt cells using the EdU assay, which is the typical technique for measuring cell proliferation. They observed a sparing effect in terms of survived cells in the crypts, as shown in Figure 3.22b, and a more efficient recovery process for UHDR irradiation than Conv, as for the Levy et al. [200] experiment. Furthermore, interestingly, they did not notice significant differences between the shoot-through and SOBP irradiation modalities. In addition, they studied the efficacy of FLASH RT against MH641905 mouse pancreatic tumor, delivering 18 Gy for every irradiation approach. They obtained the same efficacy between Conv and UHDR RT, with a slight increase in tumor control, but without a significant difference, in the case of SOBP compared to the entrance of both dose rate modalities due to an increase in the proton RBE, from ~ 1.0 (entrance) to ~ 1.1 (SOBP).

The emergence of the FLASH effect in the gut with proton-FLASH RT was confirmed by Diffenderfer et al. [96]. They delivered 15 Gy throughout the abdomen of mice using the 230 MeV IBA Proteus Plus machine with mean dose rates of ~ 0.9 Gy/s (Conv) and ~ 78 Gy/s (UHDR). They measured cell proliferation in crypts using the EdU assay, observing a substantially higher number of proliferating cells per crypt, as reported in Figure 3.22c, and a more effective crypt regeneration in the case of UHDR irradiation compared to Conv. Thus, FLASH RT reduced acute intestinal side effects. In contrast to [200], they also analyzed the devolvement of late effects, particularly in terms of intestinal fibrosis. They performed a focal irradiation, that is, delivering the dose (18 Gy) only in the tumor region and in the upper intestine, with both Conv and UHDR techniques. They observed reduced fibrosis induction 8 weeks after FLASH RT compared to Conv RT, applying Masson's trichrome staining, which is a common histological technique to distinguish between different tissues. In the context of fibrosis, this approach allows us to differentiate between collagen and muscle. In this experiment, they observed a significant increase in the thickness of the average muscle layer, which is a marker of the occurrence

of fibrosis. Finally, the same tumor growth for FLASH and Conv irradiation was obtained in the case of a pancreatic flank tumor model, for two different prescribed doses (12 Gy and 18 Gy).

Zhang et al. [201] performed partial abdomen irradiation in mice using an experimental proton-FLASH beam (Harvard facility). They delivered 13 – 22 Gy with mean dose rates of 1.9 – 4.5 Gy/min (Conv) and 122 – 128 Gy/s (UHDR). They observed a statistically significant FLASH sparing effect only for 16 Gy in terms of weight loss 9 days after irradiation, as shown in Figure 3.22d, and survival with DMF = 1.23, while for higher doses, all mice died within 15 days for both Conv and UHDR irradiation. For the occurrence of late effects, they noticed a trend of higher regeneration processes for the submucosa and muscle layer of the intestinal rolls for FLASH RT compared to Conv RT, which is an indication of reduced long-term intestinal toxicities. Moreover, 1 mouse developed a necrotic lesion, and only 2 mouse survived among those irradiated with Conv RT. For this reason, these findings for late effects are not statistically significant. However, with a later and larger study from the same group [203], they showed no sparing effects in healthy intestinal tissue, and no difference in lymphocyte depletion, for 228 MeV protons irradiation at ~ 120 Gy/s. They also highlighted the challenge of evaluating toxicity exclusively in the intestine, given the nature of abdominal irradiation, which affects multiple types of tissue and organ at the same time. They also recommend exploring alternative irradiation approaches, such as total gut or total abdominal irradiation, to more effectively evaluate the possible occurrence of the FLASH effect in the intestine.

For abdominal organs, there are also at least other two negative results. The first experiment is that performed by Venkatesulu [204], using an updated 20 MeV Varian clinical electron LINAC, which delivers 16 Gy in mice. They showed no protection for lymphocytes after cardiac and splenic irradiation, no protection for crypts, and induction of gastrointestinal diseases after whole-abdominal irradiation, in the case of FLASH RT. However, these negative findings are probably due to the too low mean dose rate used (35 Gy/s), which is lower than that used in [200]. Thus, it may be insufficient to trigger the protective effect of FLASH RT. The second negative result is that obtained by Smyth et al. [205], using a photon-FLASH device with the same mean dose rate (37 Gy/s) as that used by Montay-Gruel et al., with which they showed the FLASH effect for brain irradiation [135]. However, as discussed in [99], this negative finding is attributed to the use of the same physical irradiation parameters for acute and late responding organs and/or small and large irradiated volumes. Therefore, higher dose rates may be required to trigger the FLASH effect due to the different biological characteristics of the irradiated target, suggesting the presence of a tissue-dependent dose-rate threshold for the protective effect.

In contrast to [205], Gao et al. [139] showed the emergence of the FLASH effect also with 6 – 8 MeV photon beams, previously described for the lung experiment. They carried out whole-abdominal irradiation to study possible side effects in the intestine, delivering 15 Gy (UHDR) and 12 Gy (Conv). They observed higher inflammatory processes and more diffused collagen fibers, which is an indication of higher fibrosis induction, in the case of Conv compared to FLASH RT, with DMF = 1.25. However, both Conv and FLASH irradiated animals died after 4~5 days due to severe radiation-induced enteritis.

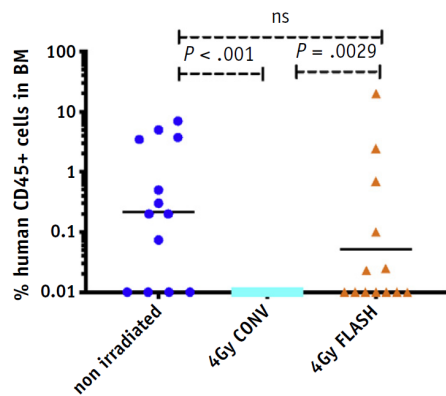
In summary, these results proved the manifestation of the FLASH effect in the case of whole abdominal irradiation of mice with electro- and proton-UHDR beams. In particular, they showed a reduction in the appearance of acute gastrointestinal effects, in terms of reduced stem cell apoptosis, enhanced crypt cell survival and proliferation, and increased gut regeneration [180, 148, 96, 202, 110, 201]. These findings are an indication of a reduction in radiation-induced DNA damage. Furthermore, it has been observed that UHDR irradiation also induced less late effects, which were quantified in relation to fibrosis development [96, 139, 201]. In addition, in all of these experiments, the same efficacy has been verified between UHDR and Conv RT in controlling tumor growth [180, 148, 96, 202, 139]. However, some studies have reported negative results, such as [204, 203, 205], making the manifestation of the FLASH effect for abdominal irradiation one of the most debated topics, highlighting the need for further investigation.

Other tissues and organs

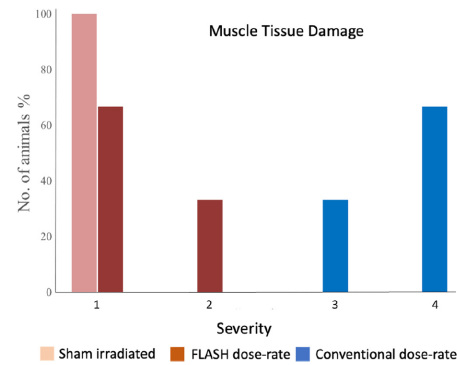
Experiments have also been carried out to investigate the manifestation of the FLASH sparing effect in various other tissues and organs, such as muscles, bones, and blood cells, also using heavy-ion therapy.

In this context, the first verification of the emergence of FLASH with carbon ion beams was obtained by Tinganelli et al. [161]. They irradiated the legs of mice after the injection of osteosarcoma LM8 cells, which typically produced several metastases in the lung. They delivered 18 Gy with a 240 MeV/u carbon ion beam at the entrance region, which is produced by a SIS18 synchrotron (GSI), with mean dose rates of ~ 0.3 Gy/s (Conv) and ~ 100 Gy/s (UHDR). They observed a higher preservation of muscle tissue organization for carbon-FLASH irradiation compared to Conv, as reported in Figure 3.23b. In particular, via histological analysis, they noticed abnormal alterations in the morphology of myofibril, which is a long fiber that allows muscle contraction, for Conv irradiation compared to FLASH RT. Interesting, in terms of tumor control, they noticed a reduced growth of the primary tumor, that is, a higher control, in the case of FLASH RT with respect to Conv irradiation, as shown in Figure 3.17d. Furthermore, UHDR irradiation leads to a significant reduction in the number of lung metastases compared to Conv RT, as we can see in Figure 3.23c. Therefore, carbon-FLASH RT can efficiently reduce side effects in muscle tissues. In addition, surprising, carbon-FLASH beams appear to be more effective both in controlling the primary tumor and in reducing lung metastases.

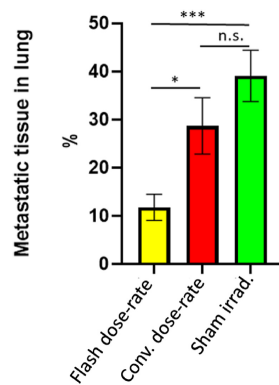
They carried out another experiment on mouse models with 240 MeV/u carbon-ion beams, but using the Bragg peak to investigate the feasibility of a more realistic clinical scenario [207]. In particular, the LET values were in the range of $65 - 85$ keV/ μ m. They delivered 20 Gy on mice' limbs (one healthy and one with osteosarcoma LM8 cells), with mean dose rates of ~ 20 Gy/min (Conv) and > 100 Gy/s (UHDR). They observed the preservation of the FLASH effect also for high-LET radiation, in terms of the same efficacy in controlling tumor growth and higher protective effects in healthy muscles. In particular, they found the same spatial distribution of collagen between UHDR and control, while there was a significant increase in collagen formation in the case of Conv irradiation, which is a marker of radiation-induced fibrosis, as shown in Figure 3.23d. Furthermore,



(a) Bone marrow, 6 MeV e^- , 4 Gy [206].



(b) Muscles, 240 MeV/u ^{12}C , 18 Gy [161].



(c) Lung metastases, 240 MeV/u ^{12}C , 18 Gy (d) Muscles, 240 MeV/u ^{12}C (65–85 keV/ μm on target), 20 Gy [207].

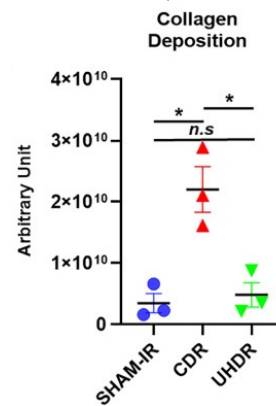


Figure 3.23: Main FLASH *in-vivo* evidence in terms of bones, muscles, blood toxicity, and lung metastases for different biological endpoints and radiation quality. Images from [206, 161, 207].

they confirmed the reduction of lung metastases in the case of FLASH RT.

Another interesting FLASH work is that carried out in blood cells by Chabi et al. [206]. They carried out a whole body irradiation, using a 6 MeV/u LINAC (Oriatron e6), in immunodeficient mice, with low radiosensitivity, after injection of human thymocytes from acute lymphoblastic leukemia (M106 T-ALL cells). They delivered 4 Gy in both the Conv and the UHDR regime, with mean dose rates of ~ 0.072 Gy/s and 200 Gy/s, respectively, observing the same tumor control 20 days after irradiation. They performed the same irradiation in another immunodeficient mouse model with high radiosensitivity. They observed a reduction in the growth of M106 T-ALL cells for FLASH RT compared to Conv RT, as shown in Figure 3.23a. They repeated the experiment with other human leukemic cells (M114 and M108), obtained a better tumor control for M114 using FLASH RT, while for M108 using Conv RT. Therefore, these findings suggest that the appearance of the FLASH effect depends on the intrinsic biological characteristics of the irradiated target. Interestingly, they observed greater protection on hematopoietic stem and progenitor cells (HSPC), which are capable of differentiate into blood cells, i.e., red blood cells, white blood cells, and platelets.

Therefore, the emergence of the FLASH effect has been demonstrated in other organs and tissue, different from the common skin, brain, lung, and intestine. In particular, FLASH RT also induces greater sparing of muscle fibers and HSPC of the blood, and can control the progression of osteosarcoma and leukemia [161, 207, 206].

3.4.3 Clinical data

In general, clinical trials are a fundamental step towards the clinical translation of novel techniques. In particular, for FLASH RT, clinical studies of UHDR radiation in human patients show to be a crucial aspect due to the higher doses delivered to trigger the FLASH effect with respect to the Conv approach. Furthermore, in principle, they can allow a systematic investigation of the optimal physical parameters for clinical UHDR beams. However, as discussed in [37, 208, 209], additional biological experiments and technological developments are indispensable for the clinical translation of FLASH RT.

In this scenario, the first 75-year-old human was treated in 2019 at CHUV (Switzerland) [92]. This patient had a CD30+ T-cell skin lymphoma diagnosed in 1999. It was treated several times using different techniques (chemotherapy, traditional RT, etc.). However, no treatments were able to control this disease, which spread throughout the skin. Localized RT was previously used more than 110 times for various ulcerative and painful skin lesions (with a size in the range of 1 – 5 cm) that progressed despite systemic treatments. For these reasons, in 2018 the patient agreed to the experimental use of FLASH RT. This patient resulted in being the perfect case for applying FLASH RT, according to preclinical results, because FLASH RT could guarantee the same probability of tumor control while reducing the appearance of possible side effects on the skin. Thus, the most resistant and progressive skin tumor (3.5 cm) was treated with Oriatron eRT6 5.6 MeV LINAC (Lausanne University Hospital, Switzerland), specifically designed for FLASH RT (PMB/Alcen, Peynier, France). They delivered on the tumor a dose of 15 Gy in 90 ms. This specific dose value was chosen because it was believed to be high enough to trigger a



Figure 3.24: First FLASH evidence in human patient. CD30+ T-cell cutaneous lymphoma, 5.6 MeV e^- , 15 Gy. Image from [92].

FLASH effect, with the hope of reducing the toxicity in healthy skin and reaching a tumor control. After 3 weeks from irradiation, which is typically the peak of the reactions, the patient showed signs of mild asymptomatic grade 1 epithelitis along with a transient grade 1 edema under the skin surrounding the tumor region, as reported in Figure 3.24. In addition to that, the tumor response was rapid (shrinking at 10 days), complete (at 36 days), and durable after 5 months. While promising, these results represent only a preliminary step toward the clinical translation of FLASH RT, requiring further studies, including direct comparisons with conventional irradiation protocols, to quantify a potential benefit of UHDR irradiation for this lesion.

The first clinical non-randomized trial (FAST-01) involving 10 patients (5 men and 5 women, with ages between 27 and 81 years, and a median age of 63 years) started in 2022 at the Cincinnati Children's/UC Health Proton Therapy Center (USA), treating them using 250 MeV Varian ProBeam proton-FLASH device [88, 97]. All patients presented up to 3 painful metastases in the extremities, with different histological diagnoses (e.g. lung cancer, breast cancer, multiple myeloma, etc.). They irradiated 12 different metastatic sites with a mean dose rate of 51 – 61 Gy/s. All patients were followed in the range of 2.3 – 13.0 months, with a median of 4.8 months. They showed mild adverse effects, which were comparable to those after Conv RT. In addition, 33% of the patients reported transient pain flares, 67% pain relief, and 50% a complete response without pain. Therefore, this study showed the efficacy in the tumor and the safety in healthy tissue of FLASH RT, supporting further clinical trials. Nonetheless, without a direct comparison to Conv irradiation, the actual benefit cannot be properly evaluated. Thus, these promising findings allowed the start of a second clinical trial (FAST-02) for the treatment of painful bone metastases in the thorax with proton-FLASH RT, which is currently ongoing [156], while several others are planned or in the recruitment stage.

3.5 Main FLASH mechanistic hypotheses

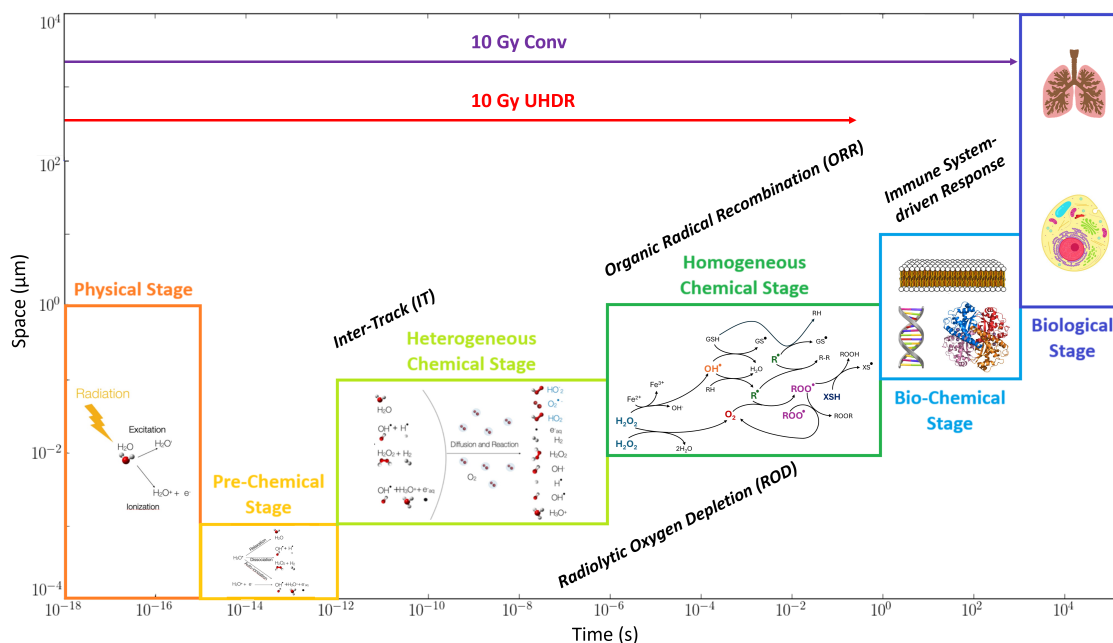


Figure 3.25: Spatio-temporal scales of radiation damage, typical Conv and UHDR irradiation times, and the main FLASH mechanistic hypotheses. Modified starting from [17].

The primary unresolved issue related to UHDR irradiation is the underlying mechanism of the FLASH effect. This significantly hinders the clinical translation of FLASH RT, as the optimal parameters required to trigger the protective effect and their dependence are still far to be fully understood. In recent years, the FLASH community has made significant efforts to unravel this enigma [99, 166, 210], yet the increase in experiments has only led to more contradictions and uncertainties. Numerous hypotheses have been proposed, but all remain highly contested, with some of them already disproved by further experiments [211, 95, 82, 99, 212]. The greatest challenge is that, to date, no hypothesis has fully explained this mysterious biological effect; at best, they have only managed to account for parts of it.

A key aspect of understanding the mechanism behind the FLASH effect, as mentioned previously, is investigating the optimal irradiation parameters to trigger it. The challenge in determining these physical parameters is evident in the numerous proposed metrics intended to best represent the concept of dose rate, as presented in Section 3.3. As highlighted by the experiments discussed earlier in Section 3.4.2, some studies suggested that the dose per pulse and the instantaneous dose rate are the crucial parameters, while others contend that the best metric is the average dose rate. This uncertainty significantly hinders both technological development, such as the development of new accelerators or dose delivery systems, and the design of new biological experiments aimed at investigating

the mechanism of the FLASH effect.

After 10 years from its discovery, the only certainty at present is that the FLASH effect is an *in-vivo* biological phenomenon [99], triggered by a different physical (dose-delivery structure) parameter. However, several complications arise immediately. As shown in Section 3.4.1, the protective effect induced by UHDR irradiation can also manifest itself *in-vitro*. The second complication is that, while for the *in-vivo* scenario there is a differential effect between healthy and tumor tissue, that is, the sparing effect occurs only in normal tissues, for *in-vitro* settings, the protective effect can emerge in both healthy and tumor cell lines, as previously shown. Lastly, another significant complication is that it is generally assumed that the FLASH sparing effect does not occur in normoxia, yet numerous *in-vitro* experiments have shown the opposite, as already discussed. For all these reasons, in this thesis opinion, the first step should be to fully understand the basis of the UHDR dependent sparing effect that occurs at the cellular level, in order to better investigate the highly complex biological mechanisms at the macroscopic level, for organs and tissues.

One of the aspects of the FLASH effect that was immediately evident is the different involvement of spatio-temporal scales of radiation-induced damage, as presented in Section 1, between UHDR irradiation and Conv RT [17]. As extensively discussed, FLASH irradiation is typically defined by a sufficiently high single dose delivered with a mean dose rate > 40 Gy/s and a total irradiation time < 100 ms. As shown for simplicity in Figure 3.25, typical UHDR irradiation can significantly impact the chemical stages, due to the overlap of the time scales of these stages with FLASH irradiation. For this reason, many leading hypotheses about the FLASH mechanism are based on various aspects of the chemical stages. Thus, the involvement of multiple scales of radiation-induced damage is one of the most widely accepted speculations so far [17], although the exact details of how this occurs remain unclear.

In recent years, several hypotheses have been proposed. The main possible explanations proposed are (i) transient hypoxia due to oxygen depletion [93, 213], (ii) organic radical-radical recombination [214, 7], (iii) inter-track effects [58, 64], or (iv) immune system-driven effect [215, 216]. As can be seen, three of these major hypotheses involve directly chemical species generated by the IR, given the plausible importance of the chemical stages for UHDR radiation [17]. Additional hypotheses refer to the role of lipid peroxidation and ferroptosis [214, 217], oxidative metabolism [218], mitochondrial activity [82, 219, 220], etc. In the following paragraphs, all these possible mechanisms will be presented and discussed.

3.5.1 Transient hypoxia and radiolytic oxygen depletion

Transient hypoxia, also known as radiolytic oxygen depletion (ROD), is one of the first proposed mechanisms to explain the FLASH effect [93, 211]. At the same time, this is the most contradictory and debated hypothesis. The basic idea is that UHDR radiation can theoretically trigger an increase in radioresistance through a huge depletion of oxygen, through reactions with radicals generated in water and in the medium, which leads to an acute transient hypoxia condition. In fact, hypoxic cells are well known to be more radioresistant than those under normoxic conditions, due to the role of oxygen in fixing

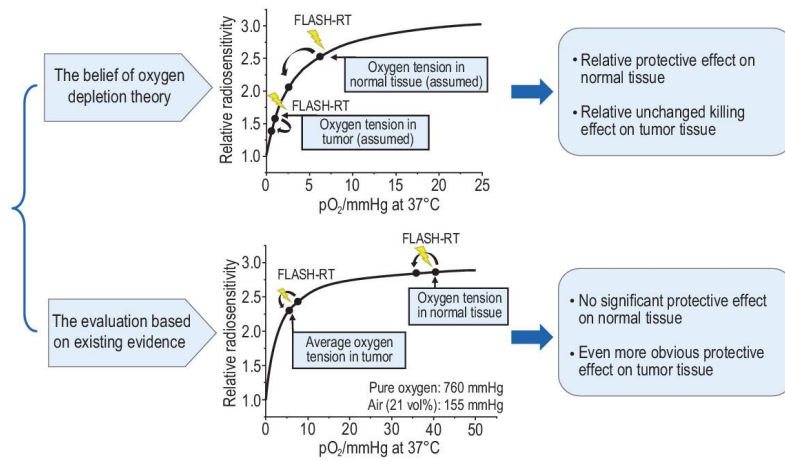


Figure 3.26: Schematic representation of the radiolytic oxygen depletion (ROD) hypothesis. From [212].

radiation-induced DNA damage [30]. This effect for Conv RT is parametrized by OER, as discussed in Section 1.4.2.

In the case of healthy tissues, which are characterized by higher oxygenation levels, i.e. physoxic conditions $\sim 4 - 7\%$ O_2 , compared to tumor regions, which are typically affected by a given level of hypoxia $< 1\%$ O_2 , UHDR radiation should induce a huge and rapid depletion of environmental oxygen, leading to a temporary hypoxic condition and an increase of radiation resistance. Instead, in the case of tumors, since they are already in hypoxic conditions, the impact of UHDR would be minimal in terms of oxygen depletion, thus not inducing a modification of the radioresistance of the cancer tissues. This hypothesis would in principle explain the differential protective effect between healthy and tumor tissues induced by FLASH RT.

Due to its simplicity, this possible mechanism, which is based on a well-known oxygen-mediated damage-fixing effect in Conv irradiation, has attracted significant attention within the FLASH community, which, beside some authors still supporting some potential or partial role for it [211, 213, 8, 221, 222, 223, 224, 225, 226], has been almost completely dismissed following the substantial evidence against its feasibility [27, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238].

The study of oxygen as an efficient modifier of the biological response to IR dates back to the 60s and 70s of the last century, with famous experiments carried out in bacteria and cells under hypoxic conditions with UHDR radiation [89, 90, 170], as discussed in Section 3.4.1. Their findings suggested a correlation between oxygen concentration and radioprotection for UHDR irradiation. However, already in the 1970s, Epp speculated about the significant role of oxygen depletion as the trigger for increased radioresistance after UHDR irradiation only in the case of severe hypoxia, when the initial environmental oxygenation was between 0.26% and 0.91% [172]. These findings were followed by several other works, such as [239, 173, 240], who reported a ROD in the range of 0.21-0.42 mmHg/Gy. It

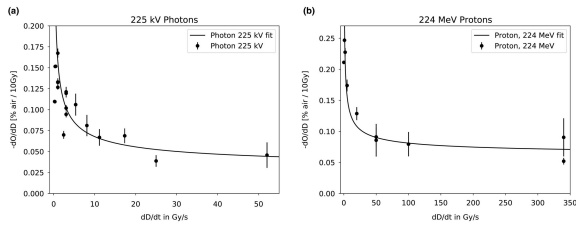
has been estimated that a dose of 100 Gy is required to cause a 3% decrease in oxygen concentration in cells, which is approximately an order of magnitude greater than that required to trigger the FLASH effect.

In recent years, Pratz et al. [93] took up this concept, proposing a computational model of ROD for UHDR irradiation. Subsequently, other analytical models have been developed for the investigation of ROD, such as those proposed in [213, 221, 222]. More details will be presented in Section 3.6. We point out that these studies rely on certain unrealistic assumptions, such as an initial oxygen concentration of the environment that is excessively low and a depletion rate which is too high and unrealistic compared to what emerged from the experiments.

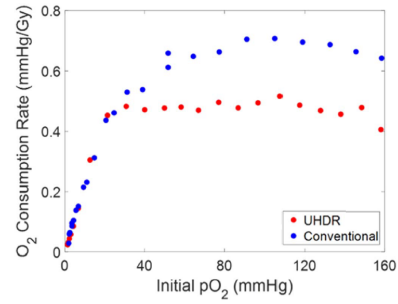
Instead, from a biological experiment point of view, Montay-Gruel et al. [85] were the first to emphasize the importance of oxygen concentration in tissues in triggering the FLASH effect. In particular, as discussed in Section 3.4.2, they showed the loss of protective effect induced by FLASH irradiation against brain toxicity under oxygenated conditions. Thus, they speculated that adequate levels of radioprotective hypoxia were not reached for 10 Gy UHDR irradiation. To this day, this remains the most compelling *in-vivo* experimental evidence that supports an impact of the oxygenation in the FLASH effect. Other *in-vivo* data, such that in Zhang et al. [193], underlined the fact that the FLASH effect is modulated by oxygenation. Furthermore, the importance of initial oxygen concentration and the possibility of ROD as a reason for the manifestation of the FLASH effect are also intensively investigated *in-vitro*, such as in [8], as described in Section 3.4.1.

In addition, in these years, several chemical experiments have been carried out to investigate the impact of UHDR radiation to deplete oxygen both *in-vitro* and *in-vivo* scenario. In particular, Jansen et al. [232] delivered 10 Gy in pure water using different radiation qualities, with mean dose rates up to 340 Gy/s, obtaining an oxygen depletion of 0.04% atm - 0.18% atm for 225 kV photons, 0.04% atm - 0.25% atm for 224 MeV protons, and 0.09% atm - 0.17% atm for carbon ions, depending on dose rate values. They showed that the magnitude of oxygen depletion depends on dose, dose rate, and radiation quality (see Figure 3.27a), concluding that the consumed oxygen was not sufficient to trigger radioprotection for clinically relevant doses and dose rates. In [234], they also investigated the impact of different dose-delivery time structure of a 30 MeV electron beam in modulating the oxygen depletion, observing a reduction in the consumed oxygen with increasing mean dose rates.

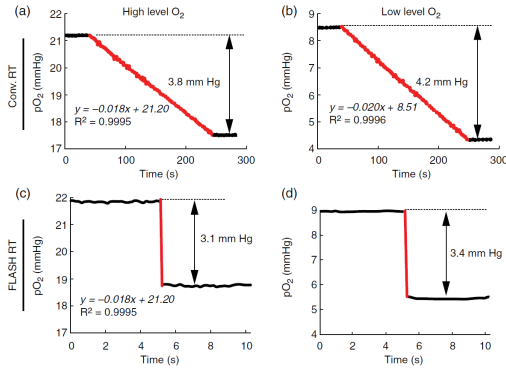
The conclusion that the oxygen consumed is not sufficient to induce a different biological effect has also been confirmed by several other *in-vitro* experiments, such as by Cao et al. [233], who obtained an oxygen depletion of 0.16-0.17 mmHg/Gy for UHDR irradiation (200 Gy/s) and 0.19-0.21 mmHg/Gy for Conv irradiation (~ 0.17 Gy/s) depending on the initial oxygenation level, irradiating with electrons a medium containing 5% Bovine Serum Albumin (BSA) to mimic the cellular medium, as we can see in Figure 3.27c. However, their method may have had insufficient temporal resolution, potentially leading to an underestimation of the depletion. El Khatib et al. obtained a ROD of $\sim 0.5 \mu M/Gy$ in a 5% BSA solution using a 230 MeV proton beam at a dose rate of ~ 110 Gy/s. In addition, Sunnerberg et al. showed [236] a ROD of ~ 0.49 mmHg/Gy in a saturated



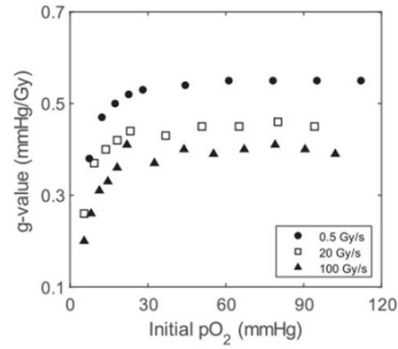
(a) 255 kV photons and 224 MeV p, water medium [232].



(b) 10 MeV e^- , water medium [236].



(c) 10 MeV e^- , BSA medium [233].



(d) 230 MeV p, Koch medium [235].

Figure 3.27: Main *in-vitro* experimental evidence against the feasibility of the oxygen depletion hypothesis, for different medium, initial oxygen level and radiation quality. Images from [232, 233, 235, 236].

solution of BSA, under initial normoxic condition, delivering 21.5 Gy at 250 Gy/s with a 9 MeV electron beam (see Figure 3.27b). Furthermore, Van Slyke et al. [235] irradiated an aqueous solution containing several nutrients and scavengers, which is the so-called Koch medium. Using a 230 MeV proton beam, they delivered 30 Gy with dose rates of 0.5 Gy/s (Conv) and 100 Gy/s (UHDR). They obtained an oxygen depletion of 0.55 mmHg/Gy for Conv and 0.37 mmHg/Gy for UHDR, as we can see in Figure 3.27d.

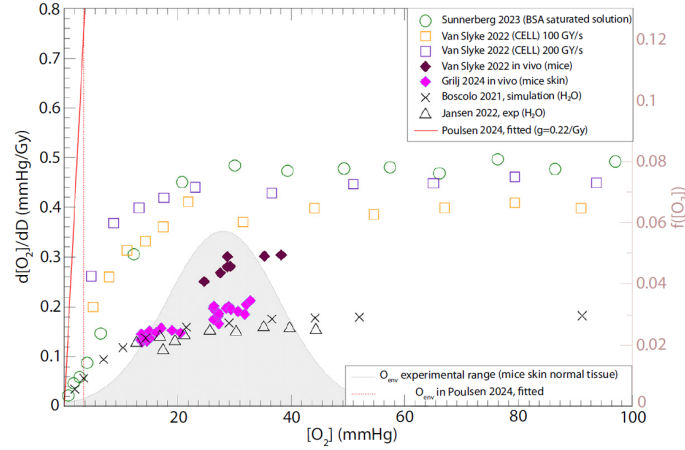


Figure 3.28: Summary of experimental and *in-silico* evidence against the feasibility of the oxygen depletion hypothesis. From [238].

Insufficient oxygen consumption for a biological effect has also been demonstrated *in-vivo* by Cao et al. [233]. In particular, for 10 MeV electron irradiation, they observed a depletion of oxygen of 2.3 mmHg in normal tissue and 1 mmHg in the tumor, delivering 20 Gy with a mean dose rate of 270 Gy/s. Instead, El Khatib [241] showed a larger depletion, which is ~ 8 mmHg, delivering 30 Gy at ~ 100 Gy/s with protons, probably due to a different oxygen heterogeneity in the tissue, and a worse measurement temporal resolution, perhaps not sufficient, in [233]. Furthermore, Van Slyke et al. [235] observed a ROD of 0.28 mmHg/Gy, delivering 30 Gy under the UHDR regime with a 230 MeV proton beam in normal tissue of mice. Recently, Grilj et al. [242] showed a depletion of 0.1-0.2 mmHg/Gy in healthy skin of mice using a 6 MeV UHDR-electron beam.

The unrealistic nature of this hypothesis has also been demonstrated computationally. The main work is that of Boscolo et al. [27] using the TRAX-CHEM MC code. They showed a ROD of $0.33 \mu\text{M}/\text{Gy} = 0.19 \text{ mmHg}/\text{Gy}$ in pure water for 1 MeV electron irradiation (see Figure 3.28), in agreement with the experimental data reported in [232]. This consumption rate means that to obtain a significant oxygen depletion of 2.4%, 100 Gy would be needed, a dose that is absolutely unrealistic compared to those released experimentally to observe the FLASH effect.

To date, *in-vivo*, *in-vitro*, and *in-silico* studies consistently indicate that the oxygen depletion hypothesis is highly unlikely to be the underlying mechanism of the FLASH effect, as summarized in Figure 3.28. However, what clearly emerges is that the initial

oxygen concentration plays a significant role in modulating the protective effect within the UHDR regime. These findings against the ROD hypothesis are schematized in Figure 3.26: based on the discussed results, it is evident that, contrary to the initial hypothesis, transient hypoxia induced by UHDR radiation is not sufficient to cause a significant change in radiosensitivity and thus to explain the FLASH effect.

3.5.2 Inter-track effects

The inter-track effects hypothesis is based on the potential impact of spatial and temporal proximity of primary particle tracks on the chemical environment. This hypothesis is based on the key concept that the interaction between the UHDR particle tracks can induce substantial changes in the chemical environment. In particular, UHDR irradiation could modify the yields of chemical species generated by IR, such as free radicals, inducing different radiobiological effects, for example, reducing DNA damage compared to Conv irradiation, thus explaining the FLASH sparing effect.

The importance of ion tracks that overlap in space and in time in terms of modification of chemical yields and DNA damage was initially investigated in [58]. This concept was taken up by Ramos-Méndez et al. [64] in the context of FLASH RT, to try to explain the mechanism underlying the biological effect induced by UHDR radiation. This work was followed by a series of *in-silico* studies [224, 229, 243, 244, 245, 246, 247, 248, 249, 250], most of them using MC track-structure approaches, which showed contradictory results on the biological impact of inter-track effects for FLASH irradiation, as we will see in Section 3.6.

In Conv RT, where the typical mean dose rate is in the range of 0.03 – 0.1 Gy/s, the probability of interaction in a cell between two consecutive particles is negligible because the physical process ended in a few femtoseconds, as we saw in Section 1.1. The same conjecture applied for the alteration of the chemical environment during the heterogeneous chemical stage, which ends around microseconds, as shown in Section 1.2.2. In fact, it is unrealistic for two reactive chemical species produced through water radiolysis from two consecutive primary ions to interact with each other, for Conv DR.

Inter-track effects cannot be ruled out in advance in the case of FLASH RT. In fact, as discussed previously in Section 3.5, the typical arrival time between two consecutive tracks for UHDR irradiation is on the time scale of chemical processes. Therefore, in principle, this could induce changes in the chemical environment of the cell. For example, the arrival of the second primary particle can induce a local increase in the yield of chemical species that are directly generated by IR, such as $\text{OH}^\bullet$, e_{aq}^- , H_3O^+ , or can trigger a temporary decrease in the yield of chemical species that are produced by further chemical reactions, such as H_2O_2 , H_2 , OH^- , $\text{H}^\bullet$, as showed for example in Figure 3.29.

The depletion or enhancement of a specific chemical species depends on various factors, such as, obviously, the spatial and temporal proximity of arriving for two consecutive primary particles, which can be in competition, temporally, with the specific time scale of each reaction or, spatially, with the characteristic diffusion coefficient of each chemical species. Moreover, it also depends on the radiation quality, i.e. particle type or LET, of the projectiles which can be characterized by different energy deposition events and different

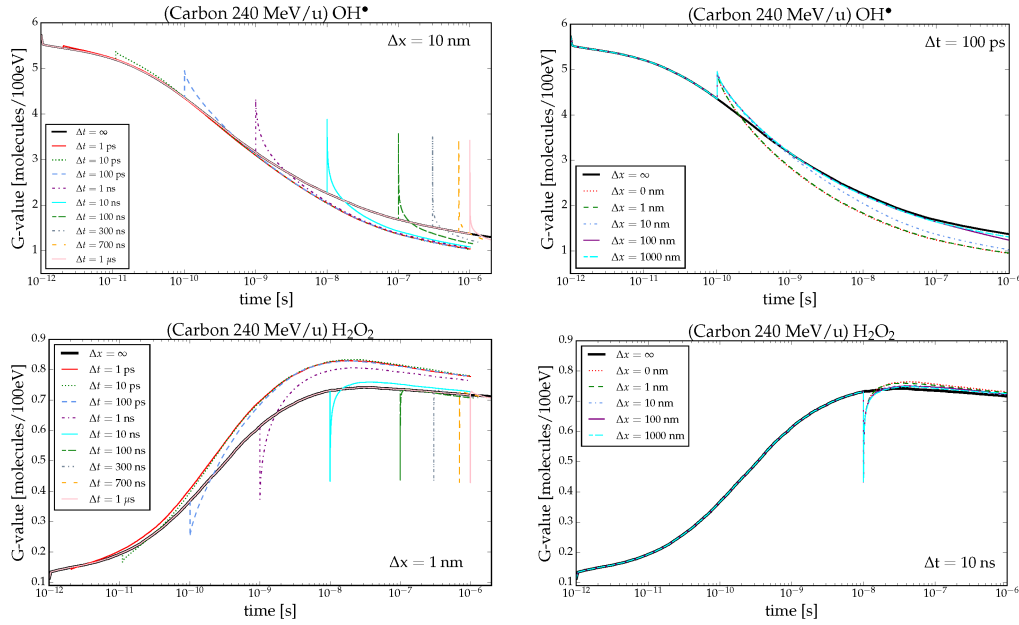


Figure 3.29: Time evolution of G-values of $\text{OH}^\bullet$ and H_2O_2 for different spatial and temporal distances between tracks, using TRAX-CHEM. From [250].

shape and density of track. In fact, the reactions between radicals generated by different tracks is in competition with the reactions between chemical species produced by the same primary particle, depending on the density of the core of the track. Therefore, in principle, these alterations of the chemical environment could influence the biological response of cells to UHDR radiation, for example, through increased radical-radical interactions during the heterogeneous chemical stage.

For example, Kreipl et al. [58], using the PARTRAC MC code, showed that the biological impact of inter-track effects would be negligible for realistic clinical doses in terms of modification of induced DNA lesions, even in the very high FLASH regime. Ramos et al. [64], using the TOPAS-nBio MC code, suggested that the inter-track effects could have a biological impact for UHDR-protons with low LETS ($< 2 \text{ keV}/\mu\text{m}$), allowing them to support the FLASH protective effect and suggesting a decrease or loss of this effect for increasing LET values. In more recent works, D-Kondo et al. [247, 249], using TOPAS-nBio, studied the impact of the biological medium on the evolution of chemical species, underlining its importance for *in-silico* investigations of the FLASH effect. Derksen et al. [246] confirmed the possible role of inter-track effects in the induction of the FLASH effect using the TOPAS-nBio MC code. In particular, they observed modifications in the yields of chemical species, increasing the number of primary particles simulated simultaneously, as a surrogate of increasing dose rate values, for different radiation qualities. Instead, Thompson et al. [245] concluded that the spatial distance between consecutive primary protons is too large to induce variations in the chemical environment. Baikarov et al. [251] speculated on the importance of inter-track interactions in triggering the FLASH effect,

using MC simulations. They observed critical dose and dose rate thresholds, depending on radiation quality, to obtain significant inter-track effects to trigger a FLASH effect. However, they showed that these critical thresholds are not comparable with the typical irradiation parameters for pulsed-electrons irradiation in the UHDR regime. Castelli et al. [250], using the TRAX-CHEM MC code in water medium, showed that, in general, inter-track effects are negligible in the context of FLASH RT. In particular, they did not observe significant chemical perturbations for distance $> 1 \mu\text{m}$ at the microsecond scale. However, they observed substantial changes in the chemical environment occurring in areas with tight spatial and temporal proximity. Abolfath et al. [243] suggested higher inter-track effects for UHDR irradiation compared to Conv radiation, concluding that the negative results reported in other studies using different MC codes can be attributed to an underestimate of the diffusion constants of chemical species. Finally, Lai et al. studied the impact of oxygenation as a modulator of inter-track effects [229].

As detailed in Section 3.6, the wide variation in results is mainly due to differences in how inter-track effects are accounted for, but also the presence or absence of environmental chemical species such as oxygen or scavengers, and the significant uncertainty in the reaction and diffusion constants of the considered chemical species.

It should be noted that all the results mentioned for the inter-track interaction were obtained with computational methods. The major challenge in UHDR radiation chemistry is the limited availability of experimental data at time scales $< 1 \mu\text{s}$. In fact, the main experimental techniques are not feasible in the UHDR regime, due to low temporal resolution [250]. However, only experimental measurements will be able to clarify the inconsistencies between the various *in-silico* results and help to understand the actual impact of the inter-track in triggering the biological effect.

In this context, one of the main open questions is the so-called mystery of the H_2O_2 yield. In detail, there is an inconsistency in the behavior of the H_2O_2 time evolution between the experimental findings (see Figure 3.30) and the *in-silico* results. In particular, MC simulations showed a reduction in the chemical yield of $\text{OH}^\bullet$ and an increase in the G-value of H_2O_2 with increasing dose rates, due to the higher recombination of $\text{OH}^\bullet$ for tracks with increasing spatial and temporal proximity. In this connection, very advanced *in-silico* studies are those carried out by Thompson et al. [245], Derksen et al. [246], Baikalov et al. [244], D-Kondo et al. [249], and Shin et al. [248] using the TOPAS-nBio MC code, and by Castelli et al. [250] using TRAX-CHEM. However, the experimental data reported the opposite trend, namely a decrease in H_2O_2 yield moving from Conv to UHDR irradiation [252, 109, 253, 254, 255]. This strange behavior could be due to chemical reactions competing with $\text{OH}^\bullet$ recombination that are neglected or not fully described by the cited Monte Carlo approaches. In this context, in [248], they were able to describe some H_2O_2 experiments, using an extended TOPAS-nBio chemistry framework. In [250], using the TRAX-CHEM MC tool, they observed non-monotonic effects for some chemical yields as a function of the temporal distance between consecutive relatively high-LET tracks, in particular for H_2O_2 , in agreement with experimental trends [252, 253].

Further experimental and computational efforts are needed to clarify this ambiguous behavior of the H_2O_2 yield.

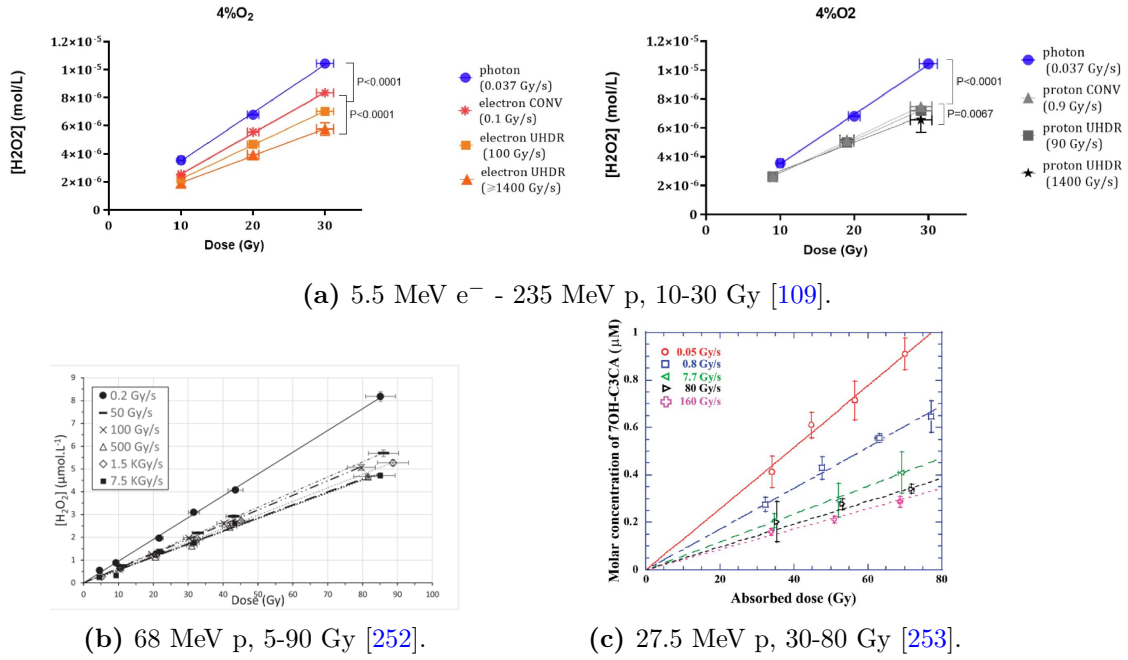


Figure 3.30: Main FLASH experimental evidence in terms of H_2O_2 yield modification for different radiation quality. Images from [109, 252, 253].

3.5.3 Organic radical-radical recombination

The organic radical-radical recombination hypothesis was initially proposed by Favaudon et al. [214] and then further developed by Labarbe et al. [7]. In particular, the key chemical species involved in this process is the organic peroxy radical $\text{ROO}^\bullet$, which is a very harmful chemical species that plays a crucial role during the homogeneous chemical stage and the bio-chemical stage, thus focused on a later stage than the precedent, as discussed in Section 1.2.3. In fact, it is well known that this radical is responsible for the damage to DNA and lipids [214, 7]. In particular, $\text{ROO}^\bullet$ can induce two adjacent DNA damages by extracting hydrogen from the deoxyribose residue, which is the sugar of the DNA molecule. Thus, it leads to an accumulation of irreparable double-strand-breaks and a reduction in the efficiency of the repair pathway driven by DNA glycosylase, which is a fundamental enzyme involved in the repair process of base lesions. In addition, $\text{ROO}^\bullet$ can induce chromosomal breaks and mutations, leading to cell death. Furthermore, $\text{ROO}^\bullet$ can induce lipid peroxidation of unsaturated phospholipids in membranes, producing organic hydroperoxides ROOH and leading to cell damage. In particular, alterations in the membrane structure or modifications in lipid metabolism can induce inflammatory processes that can trigger, for example, cell apoptosis.

The organic peroxy radical $\text{ROO}^\bullet$ is the product of the rapid reaction between the organic radical $\text{R}^\bullet$ and molecular oxygen O_2 ,



This production reaction in Eq. 3.8 is competition mainly with the self-rearrangement of alkyl radicals, which is also called truncation, dimerization, or disproportionation, i.e.,



and with the protective action of antioxidants, such as glutathione, which are hydrogen donors, that is,



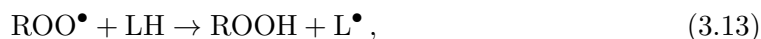
Alkyl $R^\bullet$ is a short-lived radical, which is derived from the direct ionization of the generic carbon-centered substrate RH, such as nucleotides, proteins, and lipids, as discussed in Section 1, that is,



and also by the action of the hydroxyl radical $OH^\bullet$,



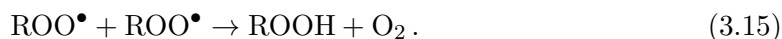
The alkylperoxyl radical $ROO^\bullet$ is a relatively long-lived reactive chemical species ($t_{1/2} \simeq 17$ s) that can undergo different possible chemical reactions. In particular, mainly, $ROO^\bullet$ can react with the lipid substrate LH, which is the so called chain propagation,



or it can react with antioxidants, namely the so called chain termination,



or, finally, it can auto-annihilate, which is the so called recombination reaction, releasing molecular oxygen, that is,



These three reactions, i.e. Eqs. 3.13, 3.14, and 3.15, are all decay processes for $ROO^\bullet$. In addition to competing with each other, they also compete with the production reaction itself, i.e. Eq 3.8. Furthermore, this self-annihilation process, involving $ROO^\bullet$ and releasing O_2 , limits the transient oxygen depletion process, which begins earlier after UHDR irradiation through different possible reactions, as discussed in Section 3.5.1. Thus, in this hypothesis, oxygen plays a fundamental role in the balance between the generation and annihilation of organic peroxyl radicals. However, it is not the primary factor that is affected by a different type of irradiation modality. Therefore, it acts as a modulator of the magnitude of the FLASH effect.

The basic idea of the organic radical-radical recombination hypothesis is that UHDR irradiation could induce more $R^\bullet$ recombination and less production of $ROO^\bullet$, leading to a decrease in harmful chemical species for biological tissues. Therefore, according to this hypothesis, the FLASH effect is due to the competition between reactions that cause cellular damage and reactions that lead to environmental detoxification, for example,

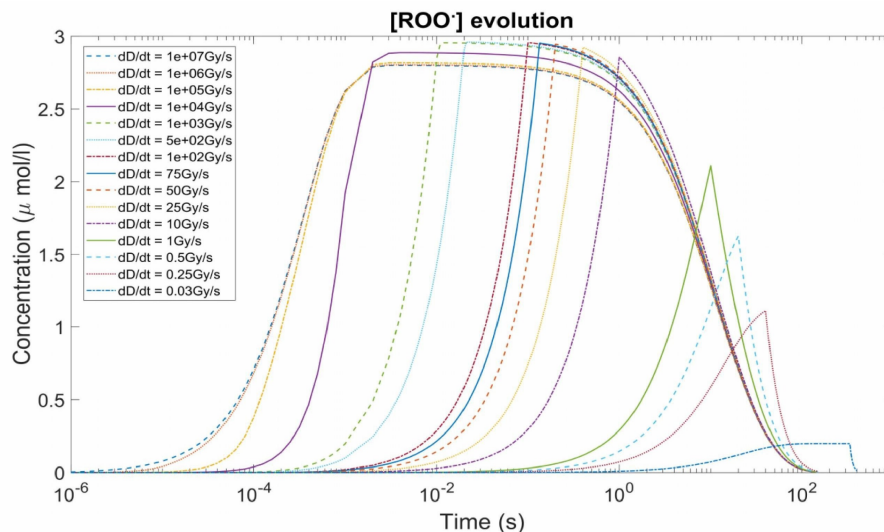


Figure 3.31: Time evolution of the organic peroxy radical concentration for different dose rates for a 10 Gy electrons irradiation. From [7].

through the recombination of $\text{ROO}^\bullet$. Labarbe et al. [7] proposed a method to parameterize this phenomenon: they assumed that the adverse action of $\text{ROO}^\bullet$ on cell homeostasis was the result of persistence in time of a sufficiently high concentration of organic peroxy radical, represented by the integral in time of this concentration. This approach is similar to the method used in pharmacokinetics to investigate the effect of drug in the human body over time. Thus, UHDR irradiation causes a more rapid increase in the concentration of $\text{ROO}^\bullet$, but, at the same time, induces a more rapid decrease due to recombination reactions, as we can see in Figure 3.31. So, cell exposure to $\text{ROO}^\bullet$ results to be shorter for UHDR compared to Conv RT, leading to reduced cell damage for FLASH RT.

In this way, in principle, they can explain the FLASH sparing effect as a reduction in cell exposure to $\text{ROO}^\bullet$. However, the major limitation of this method is that they lack in the explanation of the FLASH differential effect between normal tissues and tumors. They simply speculated that the differential action of UHDR radiation is due to interconnected mechanisms that involve different biological pathways related to the formation and repair of DNA lesions. But they have neither explained the cause of this nor developed a model for it. However, the hypothesis of organic radical-radical recombination remains one of the most widely considered mechanisms by the FLASH community regarding the manifestation of the protective effect induced by UHDR radiation. In addition to being the starting point for the development of the work presented in this thesis.

More details of the mathematical models used to describe the chemical reaction kinetics of the homogeneous chemical stage and the correlation between the action of $\text{ROO}^\bullet$ and the induction of cellular damage will be presented and discussed in Section 3.6.

3.5.4 Immune system-driven response

The immune system-driven response is the last main hypothesis proposed in the literature [215, 216, 95] and is the only one that does not directly involve chemistry in some way. In fact, it is entirely based on the potential greater protection of the immune system, which could trigger a systematic immune response and a reduction of inflammatory processes, as illustrated in Figure 3.32. A possible speculation about this is that proposed in [216, 215] about the greater sparing of circulating immune cells in blood induced by FLASH RT. In particular, Jin et al. [216] showed a higher number of surviving lymphocytes after UHDR irradiation compared to Conv, leading, in principle, to a higher repair efficiency of damaged tissues. From a computational point of view, Cucinotta et al. [256, 257, 258] developed a mathematical model to investigate the influence of dose rate in surviving lymphocytes, as we will see in Section 3.6. They showed the induction of greater protection of them in the case of UHDR radiation. However, this idea seems to be denied by various negative results, such as [204, 203, 259].

Other experimental studies suggested the involvement of the immune system in the biological response of FLASH RT, but without definitively proving it. For example, in healthy tissues, various works [87, 147, 153, 158] showed suppression of the TGF- β 1 cytokine, which is the main biomarker of radiation-induced fibrosis, as discussed in Section 3.4.2 and a potent immunosuppressive agent, in the case of UHDR irradiation. In addition, other studies reported a modification in the production of other cytokines, as in [118, 147]. Furthermore, some experimental findings suggested the local influence of UHDR irradiation on tumor microenvironment. For example, some experiments [155, 260, 261, 202] showed higher T lymphocytes recruitment and their higher infiltration in tumor volume. However, negative findings about T lymphocytes recruitment also emerged, such as [259, 262, 263]. It is worth mentioning, in addition, that in [104], the FLASH effect also emerged in nude mice.

To date, experimental evidence on the immune system-driven hypothesis has yielded conflicting results, making it impossible to confirm or rule out the immune system's involvement. However, it is clear that the immune effect alone is unlikely to fully account for the entire FLASH mechanism.

3.5.5 Other mechanisms and biological targets

In recent years, several alternative mechanisms, beyond the more commonly discussed ones, have been proposed, such as lipid peroxidation and ferroptosis, oxidative metabolism, mitochondrial activity, and protein targets, as collected in [82]. The main ones are outlined below.

Lipid peroxidation, apoptosis and ferroptosis

Favaudon et al. [214] speculated about the involvement of lipids as the primary critical target for UHDR irradiation, rather than DNA as in standard radiobiology for Conv irradiation. As previously mentioned in Section 3.5.3 in the context of the organic radical-radical recombination hypothesis, they suggested that UHDR radiation induced less lipid perox-

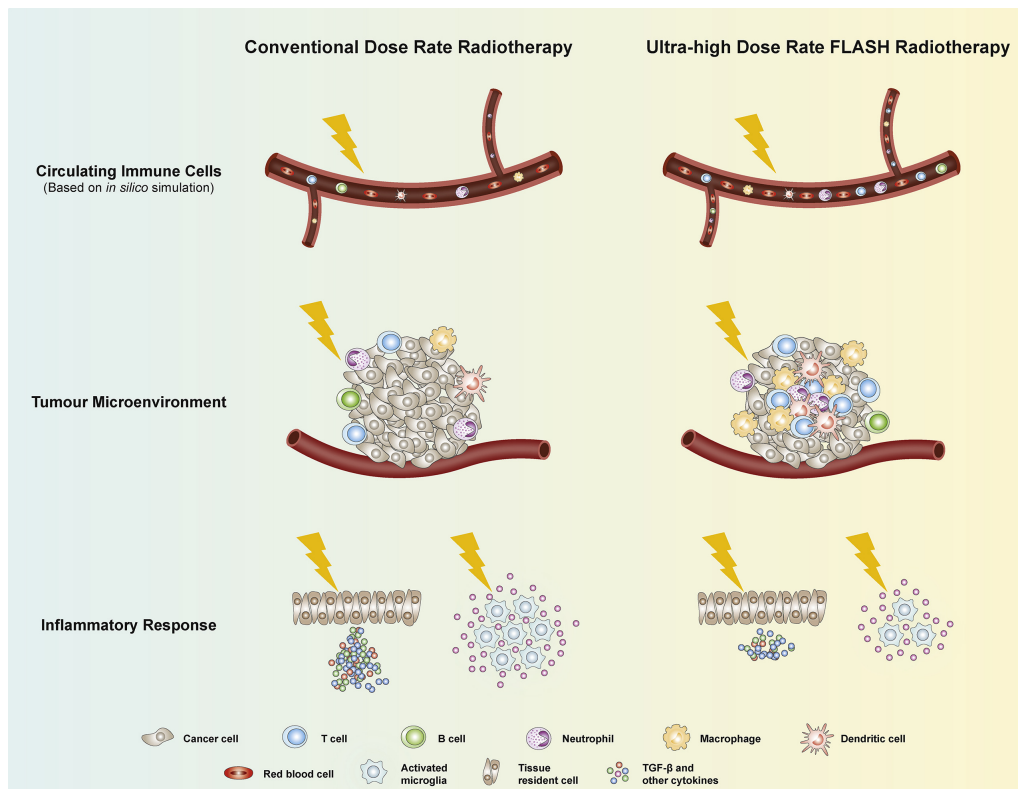


Figure 3.32: Illustration of the immune system driven hypothesis in the induction of FLASH effect, in terms of preservation of circulating and tumor-infiltrating immune cells, and reduction of inflammatory responses. From [215].

idation in membranes compared to Conv radiation. Lipid peroxidation is the chemical process that consists of the degradation of unsaturated phospholipids in cell membranes, mediated by free radicals, in particular ROS. The reactions between ROS and lipid induce the generation of a lipid radical $L^\bullet$, which, consuming oxygen O_2 , produces the harmful lipid peroxy radical $LOO^\bullet$, analogously to the reactions described in Section 3.5.3. This can induce the formation of additional lipid radicals, continuing the reaction chain, or can react with antioxidants or other substrates, producing lipid hydroperoxide $LOOH$ and terminating the chain. All of these can lead to a reduction in cell death for UHDR radiation, because alterations in membrane integrity and functionality can trigger inflammatory processes and thus the apoptotic pathway.

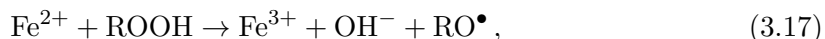
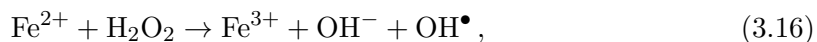
This hypothesis was corroborated by the experimental work of Froidevaux et al. [217]. They irradiated linoleic acid micelles and phosphatidylcholine liposomes, which mimic the cell membrane, to investigate the impact of lipid peroxidation for UHDR-electron beams compared to Conv RT, using 5.5 MeV Oriatron eRT6. The measured concentrations of $LOOH$ and malondialdehyde (MDA), which are markers of lipid peroxidation and oxidative stress. They did not observe the occurrence of lipid peroxidation in the case of FLASH RT, whereas the Conv radiation induced lipid peroxidation, whose magnitude increases linearly with the dose under normoxia conditions. Instead, for 4% O_2 , they observed a decrease in lipid peroxidation for Conv RT.

However, this experiment presents some limitations. The main one is that they considered an *in-vitro* situation where only ROSs could trigger lipid peroxidation, while *in-vivo* there are various enzymes that favor alternative pathways for lipid peroxidation. In particular, lipid peroxidation can be induced by perturbations of the GPX4 system, which is an enzyme involved in the protection of cells by oxidative stress, reducing lipid hydroperoxides in the cell membrane. This severe lipid peroxidation can trigger ferroptosis, which is an alternative cell death pathway to apoptosis, which is mediated by labile iron Fe^{2+} . The second main issue related to this hypothesis is that even if these results were applicable *in-vivo*, they would only explain the FLASH sparing effect induced by UHDR radiation and not the FLASH differential effect between healthy tissue and tumor. They speculated that due to the different iron metabolism between healthy tissue and tumor, where in particular tumors have higher iron concentrations compared to normal cells, cancer tissue could still be very affected even by low levels of lipid peroxidation induced by UHDR irradiation, contrary to normal tissue, as discussed in [217, 99]. This hypothesis for the differential effect could also be seen in the context of a reduced detoxifying capacity that characterizes tumors, as proposed by Spitz et al. [218], whose idea will be presented in the next paragraph. However, to date this proposal about lipids as the main target for UHDR irradiation has not been proven and requires further experimental evidence to be confirmed.

Oxidative metabolism

Spitz et al. [218] tried to explain the FLASH differential effect due to the different oxidative metabolism between healthy and cancer tissues, leading to a different accumulation of $ROOH$. In particular, they speculated that FLASH irradiation induces an initial higher

production of ROOH due to the complete consumption of environmental oxygen, according to the hypothesis of oxygen depletion. However, after reaching the maximum concentration, ROOH decreases in different ways between normal tissues and tumors, due to the different ability to detoxify the cellular environment. According to them, this process is due to the different concentrations of reactive iron Fe^{2+} available in cancer cells by a factor 2 – 4 compared to normal cells [264]. This higher level of labile iron triggers the Fenton reactions, i.e.



that produce especially hydroxyl radicals $\text{OH}^\bullet$, leading to higher levels of oxidative cellular damage in the case of tumors compared to normal tissues. However, this mechanism proposed by Spitz et al. [218] presents several critical issues, as discussed in [265, 266]. The first main issue with this approach is that UHDR irradiation appears to induce more cellular damage to the tumor compared to Conv RT, which is never observed in experimental studies. The second main issue is that they assumed a complete depletion of oxygen in order to produce the difference ROOH accumulation between healthy and tumor cells, which is highly unlikely, as discussed previously in Section 3.5.1.

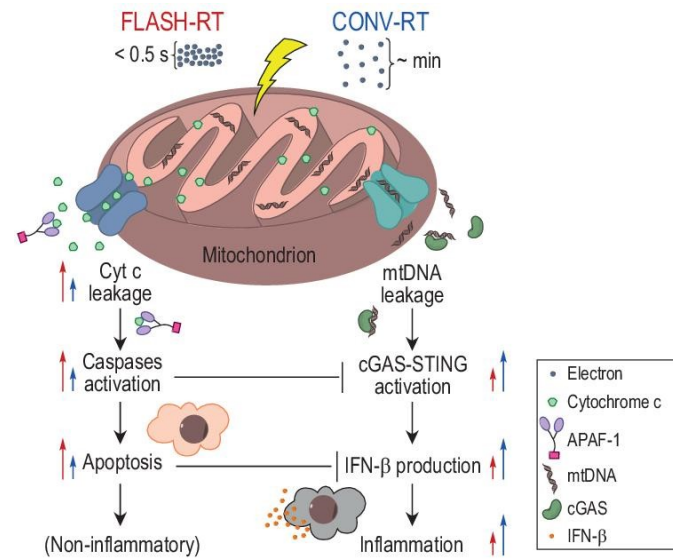
Despite the challenges and inconsistencies associated with this hypothesis, the work proposed in [218] remains significant in understanding the mechanism underlying the FLASH effect, as it was among the first to suggest the involvement of radiation chemistry.

Mitochondrial activity

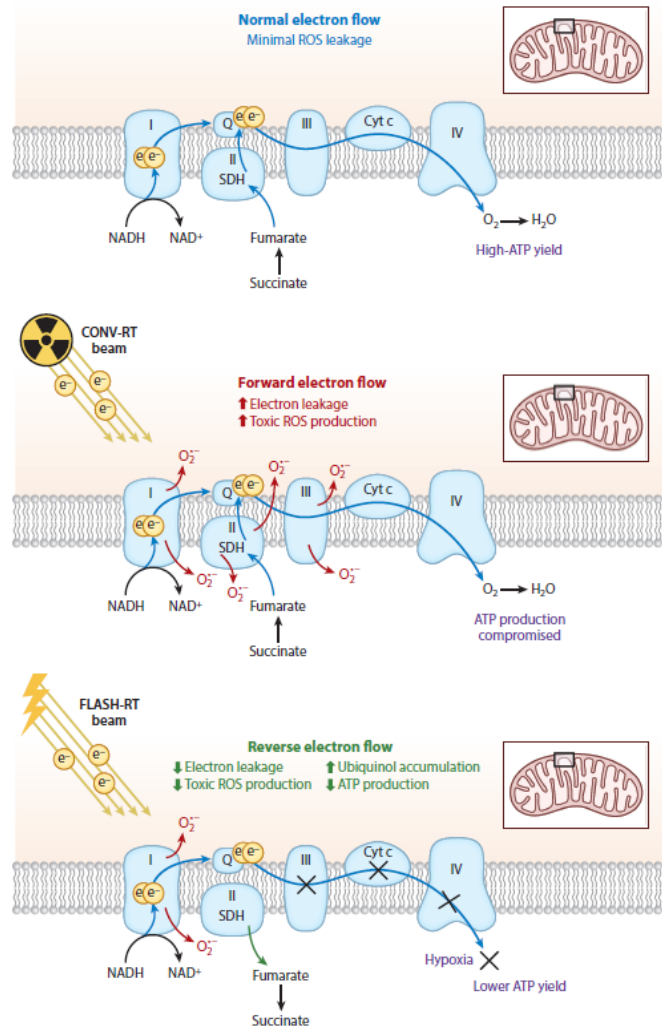
Mitochondria play several important roles in the correct functioning of cells. For example, they are involved in different death pathways, such as apoptosis mediated by cyt c protein, in several pro-inflammatory response pathways, in energy metabolism acting as an ATP reserve, and in ROS production mediated by the electron transport chain (ETC), which is extremely sensitive to IR. For these reasons, the involvement of mitochondrial activity in the mechanism of the FLASH effect cannot be ruled out in advance, as proven by the FLASH experiments carried out in [219, 220].

In particular, Han et al. [219] showed a modulation of apoptotic and necrotic pathways in the case of cyt c-deficient cells after UHDR irradiation, while Guo et al. [220] suggested that the main path of cell death in FLASH RT is apoptosis, while in the case of Conv RT it is necrosis. Thus, they concluded that FLASH RT could better preserve the mitochondrial functionality of healthy cells compared to Conv RT, as schematized in Figure 3.33a.

In [82], they speculated about the role of the ETC in the context of FLASH RT. They suggested that Conv RT could induce higher ROS production by mitochondria, compromising ATP production, because of lack of electron transfer via the ETC pathway. Instead, UHDR irradiation could reduce ROS production, as illustrated in Figure 3.33b. However, this hypothesis cannot explain the differential effect between healthy tissue and tumor.



(a) Cyt c protein and apoptosis.



(b) Electron transport chain (ETC) and reactive oxygen species (ROS).

Figure 3.33: Illustration of possible pathways in mitochondria involved in the activation of FLASH effect. Images from [212, 82].

Therefore, the involvement of mitochondria in the activation of the FLASH effect is highly debated and requires a larger amount of dedicated specific experiments to be elucidated.

3.6 Proposed FLASH models and tools in literature

Mechanism	Endpoint	Particle	Methodology	Supported	Reference
ROD	S, δ_{ROD}	e^-	A	-	[93]
ROD	S, $\overline{RR}$	e^-	A	yes	[213]
ROD	S, dO_2/dD	e^-	A	yes	[221, 222]
ROD	S, dO_2/dD	e^- , p, 4He , ^{12}C	MC, A	no	[29, 27]
ROD	S, dO_2/dD	e^- , p	MC, A	no	[231]
ROD	dO_2/dD	e^-	MC, A	no	[230]
ROD	dO_2/dD	e^- , p	MC	no	[228]
ROD	dO_2/dD	γ , e^-	MC	no	[227]
ROD	dO_2/dD	^{12}C	MC	yes	[225]
ROD	S	e^-	A	yes	[226]
ROD, IT	dO_2/dD , ΔG	p	MC	yes, yes	[224]
ROD, IT	dO_2/dD , ΔG	e^-	MC	no, yes	[229]
IT	DNA, ΔG	p, 4He , ^{12}C	MC	no	[58]
IT	ΔG	p	MC	yes	[64]
-	dO_2/dD , ΔG	e^- , p	MC	-	[247, 249, 248]
IT	ΔG	e^- , p	MC	yes	[246]
IT	$\#_{overlaps}$	p	MC, A	no	[245]
IT	$\Phi(t)$	e^-	A, MC	no	[244]
IT	ROS, NROS	p	MD, MC, A	yes	[243, 267]
IT	ΔG	p, 4He , ^{12}C	MC	no	[250]
ORR	NTCP	e^-	A	yes	[7]
ORR	NTCP	e^-	MC, A	yes	[268]
ORR	ΔAUC	e^- , p	A	yes	[269]

Table 3.1: *In-silico* FLASH studies in literature. Mechanisms: ROD = Radiolytic Oxygen Depletion, IT = Inter-Track, ORR = Organic Radical Recombination. Methodologies: A = Analytical model, MC = Monte Carlo code, MD = Molecular Dynamics. Endpoints: S = surviving probability, δ_{ROD} = relative reduction in radiosensitivity induced by ROD (Eq. 3.21), $\overline{RR}$ = averaged relative radiosensitivity (Eq. 3.28), dO_2/dD = oxygen consumption yield, ΔG = normalized difference between G-values (Eq. 3.46), $\#_{overlaps}$ = expected number of overlaps (Eq. 3.39), $\Phi(t)$ = relative change of chemical environment induced by inter-track (Eq. 3.44), ROS = reactive oxygen specie, NROS = non-reactive oxygen specie (Eq. 3.45), NTCP = normal tissue complication probability (Eq. 3.52), ΔAUC = normalized difference of area-under-the-curve (Eq. 3.54).

This Section will present, in association to the previously introduced hypothetical pathways, the main mathematical models and computational tools that have been devel-

oped in recent years for the study of the mechanism behind the FLASH effect. These models and tools are based on different methodologies, for example, molecular dynamics [270], Monte Carlo track structure codes [271, 27, 58, 64, 248, 247, 249, 224, 229, 246, 250, 231, 230, 228, 227, 225], analytical models [7, 269, 93, 213, 221, 222, 226], and mixed techniques [243, 267, 268, 272, 244, 245, 273, 1, 2]. Furthermore, each of these models or tools aims to investigate one of the main hypotheses regarding the mechanism underlying the FLASH effect, such as oxygen depletion and transient hypoxia [221, 222, 213, 270, 27, 272, 226, 231, 230, 228, 227, 225], organic radical-radical recombination [7, 268, 269, 1, 2], and inter-track effects [58, 224, 243, 267, 64, 248, 247, 249, 229, 245, 246, 244, 250]. These mathematical models and computational tools provided important insights, while were still unable to provide a unified explanation. Phenomenological models have also been developed in an attempt to parameterize the FLASH effect, such as [39, 272]. All the *in-silico* FLASH studies in literature are reported in Table 3.1. In the following paragraphs, the main mechanistic models among those previously mentioned will be presented.

Analytical models and Monte Carlo track structure codes for the investigation of the transient hypoxia hypothesis

As mentioned in Section 3.5, Pratz et al. [93] developed a computational model to study oxygen depletion under the UHDR regime. First, they separate two dose rate regimes: the ultra-high dose rate (> 100 Gy/s) and the very-high dose rate (> 10 Gy/s).

In the case of the UHDR regime, they assumed that the rate of oxygen depletion is higher than the possible alteration in oxygen concentration of the tissue induced by oxygen diffusion and cellular metabolism. The starting point is the quantification of OER. They opted for an OER parameterization [274] that differs from the standard model described in Section 1.4.2, that is

$$\text{OER}(p) = 1 + \frac{\Phi_0}{\Phi_D} (1 - e^{-\varphi p}) , \quad (3.18)$$

where p is the oxygen tension, $\Phi_O/\Phi_D = 1.63$, which is the ratio between the fraction of death cells via radical fixation Φ_O and the fraction of cells lacking oxygen fixation Φ_D , and $\varphi = 0.26$.

They calculated the oxygen tension p after the delivering of a dose D as

$$p = p_0 - L_{ROD}D , \quad (3.19)$$

where p_0 is the initial oxygen concentration, and $L_{ROD} \approx 0.42 \approx \text{mmHg/Gy}$ is the depletion rate according to the experimental data.

Then, they calculated the cell survival fraction after UHDR irradiation. After some mathematical steps, they obtained the following expression:

$$\log \left(\frac{N_p}{N_0} \right) = (1 - \delta_{ROD}) \log \left(\frac{N_p}{N_0} \right) \Big|_{L_{ROD}=0} , \quad (3.20)$$

where N_0 is the initial number of cells, N_p is the number of cells after a dose D_p , while δ_{ROD} represents the relative reduction in radiosensitivity induced by ROD, which is computed

as

$$\delta_{\text{ROD}} = \frac{\text{OER}(p_0)D_p - \int_0^{D_p} \text{OER}(p_0 - L_{\text{ROD}}D)dD}{\text{OER}(p_0)D_p} \quad (3.21)$$

where $\text{OER}(p) = \alpha(p)/\alpha(0)$, i.e. the ratio of radiosensitivities. Moreover,

$$\log \left(\frac{N_p}{N_0} \right) \Big|_{L_{\text{ROD}}=0} = -\alpha(p_0)D_p, \quad (3.22)$$

is the the survival fraction for Conv irradiation, where they assumed no ROD.

Instead, in the case of very-high dose rate regime, they also included the impact of oxygen diffusion and cellular metabolism. They defined the time evolution of the oxygen tension p as

$$\frac{\partial p}{\partial t} = D_{\text{O}_2} \frac{1}{r} \frac{\partial}{\partial r} \left(r \frac{\partial p}{\partial r} \right) - m - \frac{L_{\text{ROD}}D_p}{T}, \quad (3.23)$$

where $D_{\text{O}_2} = 2 \cdot 10^{-5} \text{ cm}^2/\text{s}$ is the diffusion coefficient, $m = 3 \text{ mmHg/s}$ is the metabolic consumption rate, and T is the pulse width. Then, they computed the survival fraction in a similar way as in the previous case. More details are reported in [93].

Using these models, they speculated that the magnitude of the protective effect induced by UHDR radiation should decrease with increasing pulse duration, up to being lost for a time width of 10 s. Moreover, they suggested that a pulsed dose-delivery should be more effective than a continuous pulse with the same total irradiation time. However, they have not been able to provide compelling evidence in favor of oxygen depletion as the mechanism underlying the FLASH biological effect.

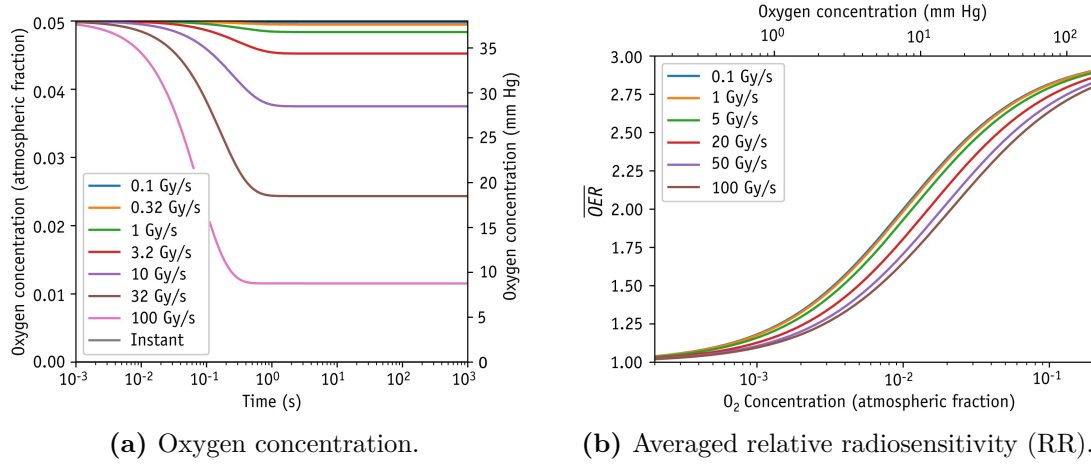


Figure 3.34: Main predictions of oxygen depletion using an analytical model. Images from [213].

Another analytical model for the investigation of ROD is that proposed by Petersson et al. [213]. They parametrized the time evolution of the oxygen concentration taking into account the depletion process, which depends on the dose rate, and the reoxygenation

pathway due to diffusion from the surrounding environment. Thus, they described these processes using an ordinary differential equation for the oxygen tension $[O_2](t)$ at time t , namely

$$\frac{d[O_2](t)}{dt} = -g\dot{D}[O_2](t) + \lambda([O_2]_{env} - [O_2](t)) \quad (3.24)$$

$$= -\gamma[O_2](t) + \lambda[O_2]_{env}, \quad (3.25)$$

where $\dot{D} = \frac{dD}{dt}$ is the dose rate, g is the depletion rate constant, λ is the recovery rate, $[O_2]_{env}$ is the initial environmental oxygenation, and $\gamma = g\dot{D} + \lambda$ represents the rate of change in oxygenation after irradiation.

Solving the Eq. 3.24, they obtained an analytical expression for the time evolution of oxygen tension, that is,

$$[O_2](t) = [O_2]_{env} \left(\frac{\lambda}{\gamma} + \left(1 - \frac{\lambda}{\gamma} \right) e^{-\gamma t} \right) = \begin{cases} [O_2]_{env} & \text{if } \dot{D} \rightarrow 0 \\ 0 & \text{if } \dot{D} \rightarrow \infty \end{cases}. \quad (3.26)$$

In Figure 3.34a we can see the time evolution of oxygen tension $[O_2](t)$ for different dose rates $\dot{D}$.

Then, they considered a standard formulation of the relative radiosensitivity (RR) (see Section 1.4.2), namely,

$$RR([O_2]_{env}) = \frac{K + m[O_2]_{env}}{K + [O_2]_{env}}, \quad (3.27)$$

where $m \approx 3$ is the maximum effect, and K is the concentration that corresponds to a half maximum effect.

However, because they wanted to consider the possible alteration of oxygenation after UHDR irradiation, starting from Eqs. 3.27 and 3.26, they defined the averaged RR for a constant mean dose rate $\dot{D} = D/T$ as

$$\overline{RR} = \frac{1}{T} \int_0^T \frac{K + m[O_2](t)}{K + [O_2](t)} dt = \quad (3.28)$$

$$= 3 - \frac{2K}{T(\gamma K + \lambda[O_2]_{env})} \log \left(\frac{(\gamma - \lambda)[O_2]_{env} + (\gamma K + \lambda[O_2]_{env})e^{\gamma t}}{\gamma(K + [O_2]_{env})} \right), \quad (3.29)$$

where D is the dose delivered, and T is the irradiation time. In Figure 3.34b we can see the impact of different dose rates $\dot{D}$ in the averaged relative radiosensitivity $\overline{RR}$.

In addition, in Adrian et al. [8], they proposed a way to account for oxygen depletion in the survival fraction. In particular, they fixed the α parameter of the LQ model of survival probability to a constant value, assuming that oxygenation and dose rate influence only the β term with the following parametrization:

$$\beta_i(pO_2) = \beta_0(1 - f_i(pO_2)) + \beta_{20}f_i(pO_2), \quad (3.30)$$

where

$$f_i(pO_2) = \left(\frac{pO_2}{20} \right)^{\gamma_i}, \quad (3.31)$$

with i equal to Conv or UHDR, where γ_i is a fitted parameter. Using this approach, they tried to replicate their *in-vitro* experimental data [8], concluding that there was no FLASH sparing effect in normoxic condition. However, they decided *a priori* that survivals would be identical in anoxia and normoxia, imposing the same values of the β parameter between Conv and UHDR at 0% and 20% pO_2 .

Petersson et al. [213] applied Eq. 3.28 to reproduce the survival fractions obtained in their previous study [8]. Fixing $\lambda = 1 \text{ s}^{-1}$ and m parameters, they fitted the LQ parameters, i.e., α and β , and also two of their Eq. 3.28 parameters, i.e., k and g . In particular, they obtained an oxygen depletion rate of $g \simeq 0.053 \text{ Gy}^{-1}$, which is extremely high and unfeasible compared to the experimental findings, as discussed in [238]. Furthermore, they correlate their $\overline{RR}$ calculation using Eq. 3.28, with the above mentioned NOR experimental results [194] as a function of the dose rate. In this case, they fitted the oxygen recovery rate $\lambda \simeq 7.9 \text{ s}^{-1}$, and estimated a higher oxygen depletion rate compared to the previous one, without being able to quantify it due to the unavailability of the reference dose-response curves. Finally, this model has been used in [223], but several criticalities have been highlighted [238], such as excessively low initial environmental oxygenation and a huge and unrealistic oxygen depletion rate.

The oxygen depletion model just discussed was subsequently included in the radiobiological model called UNIVERSE by Liew et al. [221, 222] to further investigate the mechanistic basis of the FLASH effect. They considered the function of the time evolution of the oxygen concentration $[O_2](t)$ obtained in Eq. 3.26, including it in another formulation of the oxygen fixation effect as a function of time t , which is the hypoxia reduction factor (HRF), or also known as one of the definitions of OER (see Section 1.4.2), i.e.,

$$\text{HRF}([O_2](t)) = \frac{mK + [O_2](t)}{K + [O_2](t)}, \quad (3.32)$$

This parametrization was used to take into account the effects of oxygenation $[O_2]$ at time t in the formation of DSBs, through the definition of the reduced DSB yield, that is,

$$\alpha_{DSB}([O_2](t)) = \frac{\alpha_{DSB}}{\text{HRF}}, \quad (3.33)$$

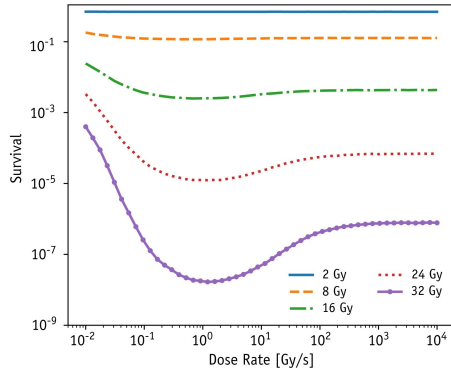
where $\alpha_{DSB} = \alpha_{DSB}(21\%)$ is the average yield of DSB per cell and unit dose in normoxia.

Then, using Eq. 3.33, they could compute the expected number of DSBs after delivery of a dose D with a mean dose rate $\bar{D}$, which is,

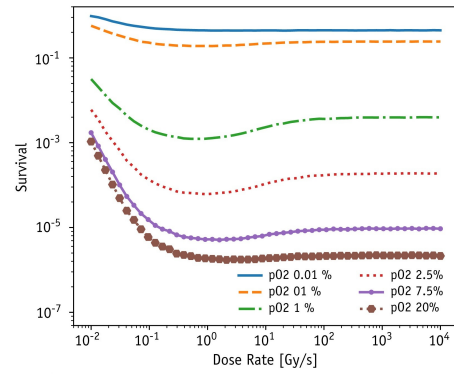
$$\langle N_{DSB} \rangle = \alpha_{DSB}([O_2](t)) \cdot D. \quad (3.34)$$

Finally, from Eq. 3.34, they could estimate the survival probability, after some mathematical considerations, as reported in detail in [221].

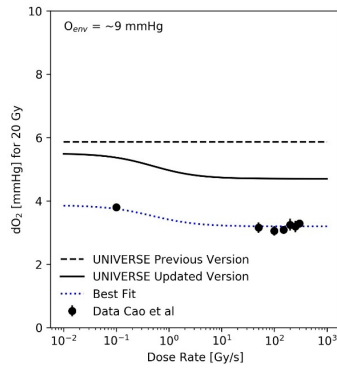
In [221], they showed their predictions of survival probability as a function of oxygenation, dose, and dose rate (see Figure 3.35a). Hypoxic survival curves, after $\sim 1 \text{ Gy/s}$, grow as a function of dose rate, reaching a maximum value at $\sim 100 \text{ Gy/s}$. However, these curves appear to have strange behavior at low dose rates: in fact, the survival probability begins to decrease rapidly from $\leq 1 \text{ Gy/s}$ with decreasing dose rate values, so too early to



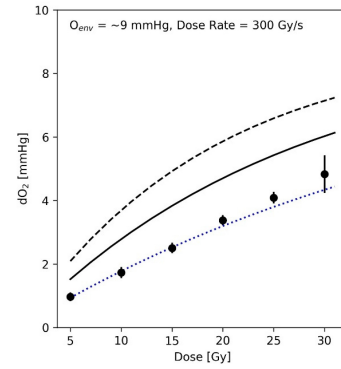
(a) Surviving fraction, different doses.



(b) Surviving fraction, different oxygenations.



(c) Depletion rate constant.



(d) Oxygen depletion.

Figure 3.35: Predictions of cell survival using the UNIVERSE model for different dose, dose rate, and oxygenation. Different parametrization of oxygen depletion rate constant and corresponding oxygen depletion. Images from [221, 222].

see the up-regulation of the DNA repair mechanism, as presented in Section 1.4.3. In the same study, they also showed the comparison of their model prediction with some *in-vitro* and *in-vivo* experimental data, using, however, the questionable values of the depletion rate constant and the reoxygenation constant obtained in [213], i.e., $g = 0.053 \text{ Gy}^{-1}$ and $\lambda = 1.0 \text{ s}^{-1}$.

In [222], they extended the UNIVERSE model, for example, including the impact of pulsed beams in the description of oxygen depletion. Thus, after a dose delivery with a dose per pulse D_p , the oxygen depletion expression becomes

$$[\text{O}_2](D_p) = [\text{O}_2]_{pre} \cdot e^{-gD_p}, \quad (3.35)$$

where $[\text{O}_2]_{pre}$ is the oxygen tension before the pulse, while the reoxygenation function between pulses changes into

$$[\text{O}_2](t_{post}) = [\text{O}_2]_{post} \cdot e^{-\lambda t_{post}} + [\text{O}_2]_{env} \cdot (1 - e^{-\lambda t_{post}}), \quad (3.36)$$

where $[\text{O}_2]_{post}$ and t_{post} are the oxygen concentration and the elapsed time after the last pulse, respectively.

However, the main update is the introduction of an empirical parametrization of the depletion rate constant g as a function of the dose rate $\dot{D}$, according to the experimental observations of Cao et al. [233]. They obtained the following expression with a sigmoidal shape:

$$g(\dot{D}) = \Gamma \cdot \frac{\mu\kappa + \dot{D}}{\kappa + \dot{D}} = \begin{cases} \Gamma \cdot \mu & \text{if } \dot{D} \rightarrow 0 \\ \Gamma & \text{if } \dot{D} \rightarrow \infty \end{cases}, \quad (3.37)$$

where Γ , μ , and κ are three phenomenological parameters. Initially, they chose $g = 0.037 \text{ Gy}^{-1}$ at UHDR regime, on the basis of their previous findings with *in-vitro* data of Epp et al. [172], as we can see in Figure Figure 3.35b. However, with this value, they were not able to reproduce the oxygen depletion data of Cao et al. [233], obtaining a higher oxygen depletion value of $\sim 1 \text{ mmHg}$ (i.e. $0.13\% \text{ O}_2$) for 10 Gy irradiation and $[\text{O}_2]_{env} \sim 9 \text{ mmHg}$. In addition, using the parameter $g = 0.058 \text{ Gy}^{-1}$ from [213], they obtained a depletion value even above of $\sim 2 \text{ mmHg}$ to the experimental data of [233]. For this reason, they decided to fit the free parameters on these experimental data, obtaining $\Gamma = 0.022$, $\mu = 1.28$, and $\kappa = 0.45$. Thus, in the UHDR regime, the fitted $g = 0.022 \text{ Gy}^{-1}$ is lower than that used in [213] by a factor 2.4. These results are reported in Figures 3.35c and 3.35d.

A more recent analytical model, proposed by Zhu et al. [226], studied the role of the pO_2 profile in the biological response after UHDR irradiation in the context of the ROD hypothesis. They described cell survival using the LQL model, modifying the α and β parameters to take into account the impact of oxygenation, which is modeled via OER. They reproduced *in-vitro* experiments [240, 173] and showed the impact of the full pO_2 profile on normal human tissues and tumors. They emphasized the importance of analyzing the pO_2 profile instead of relying solely on the median pO_2 . In addition, they reported a patient and tissue-dependent biological response after FLASH RT, considering oxygen depletion as the main mechanism.

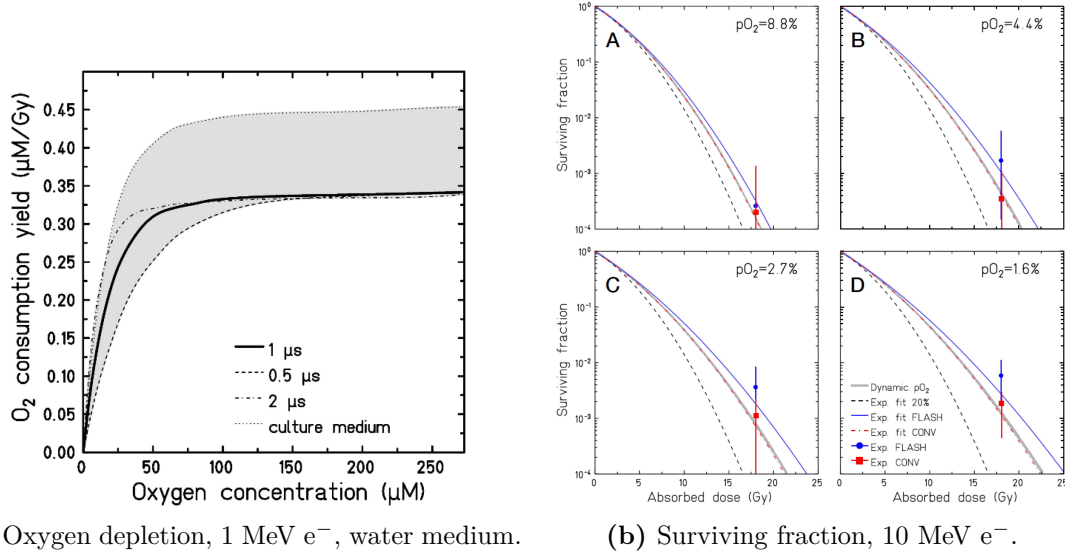
(a) Oxygen depletion, 1 MeV e^- , water medium.(b) Surviving fraction, 10 MeV e^- .

Figure 3.36: Main *in-silico* evidence against the feasibility of the oxygen depletion hypothesis. Images from [27].

The oxygen depletion hypothesis has also been investigated using MC track structure tools, as discussed in Section 3.5.1. In this context, the main work is that performed by Boscolo et al. [27]. They used TRAX-CHEM [28, 29] to compute oxygen depletion in pure water for low-LET radiation, simulating the heterogeneous chemical stage with the step-by-step approach up to the microsecond. They found a depletion of 0.19 mmHg/Gy, as we can see in Figure 3.36a, which is coherent with experimental findings [232]. Thus, a huge dose of 100 Gy is needed to obtain a significant depletion of 2.4%. Furthermore, they estimated the impact of oxygen depletion using the parametrization of the β parameter (see Eq. 3.30) proposed in [8]. They showed that, using the TRAX-CHEM predictions of oxygen depletion, it is impossible to reproduce the FLASH sparing effect observed in the [8] experiment, because the oxygen consumption is insufficient to trigger a significant macroscopic biological effect (see Figure 3.36b). Taking all this significant evidence, they concluded that the ROD hypothesis is highly unrealistic to explain the FLASH effect [27]. These findings have been corroborated by other studies using different MC tools, such as Geant4-DNA [227, 228, 229], NASIC [230, 231], and gMicroMC [229]. However, other MC studies have reported results supporting ROD, especially those that employed the IONLYS-IRT code [224, 225].

Monte Carlo tools and analytical approaches for the investigation of the inter-track effects hypothesis

The first MC track structure codes (see Chapter 2 for general information on MC codes for radiation simulations) used to investigate the impact of inter-track effects on the radiation-induced biological response was PARTRAC [59]. In the work [58], Kreipl et al., before the

FLASH challenge started, but dedicated to the laser-driven beams at ultra-high intensities, extended the MC codes, enabling the simulation of multiple parallel tracks, also taking into account the temporal feature of the track evolution in the physical stage. The effects of the inter-track were investigated by calculating the G-values for the chemical species produced by IR, as defined in Section 1.2.2, and their impact in inducing DNA damage. In fact, the code can estimate DNA lesions dividing them into base damage (BS), SBS, DSB, and clustered lesion (CL). They simulated in pure water primary particles with different radiation qualities, from electrons to helium ions. They assumed negligible inter-track effects during the physical and pre-chemical stage, due to the independence of these events from each other. As for the other MC codes, PARTRAC can trace the particle evolution using a step-by-step algorithm, considering Brownian diffusion and chemical reactions for the heterogeneous chemical stage. Regarding DNA damage yield, PARTRAC can consider the impact of both direct and indirect action of IR, where the biological target is a spherically shaped lymphocyte cell nucleus, considering also the presence of scavengers. The direct strand-break DNA damage is scored for the direct hit of the primary particle with a probability that depends on its energy deposition at the phosphate deoxyribose backbone. Instead, PARTRAC can consider an indirect strand-break for reactions between $\text{OH}^\bullet$ and the sugar moiety, with a fixed probability. The severity of DNA lesions between SBS, DSB, and CL can be determined according to the geometric characteristic positioning of the SSBs.

They investigated inter-track effects for primary particle track pairs with different radiation qualities, comparing these results with those obtained without inter-track considerations, namely two independent evolution of particle tracks. They considered spatial separations of 0, 1, 10, 10^2 , 10^3 nm and temporal separations of 0, 1, 10, 10^2 , 10^3 , 10^4 ps between track pairs, repeating the simulations several times to improve statistical fluctuations. They simulated 20 MeV protons, 0.25, 5 MeV/u helium ions, 5 MeV/u carbon ions as projectiles for G-values calculations, while 20 MeV protons and 20 MeV/u helium ions as projectiles for DNA damage assessment.

They observed increased inter-track effects in terms of chemical yields for primary particle with track event density due to the enhancement of the probability for reactions between chemical species generated by different incident tracks. Furthermore, they observed negligible impact of time separation between consecutive incident particles for a large spatial distance. They found that the G-values and DNA damage patterns were affected by inter-track interactions only for sufficiently small spatial and temporal separation between incident particle tracks. However, the threshold that was found for spatial and temporal distances was not compatible with the typical spatio-temporal characteristics of a clinical irradiation. For example, they estimated that for 20 MeV protons in water with homogeneous spatial separations of 100 nm, the minimum dose value in order to observe inter-track effects is 50 Gy, which is extremely larger than a typical clinical prescribed dose of 2 – 3 Gy. They also concluded that even for ion pulses with picoseconds time-width, inter-track effects did not affect the yield of DNA damage.

Ramos-Mendéz et al. [64] adopted the concept of inter-track effects and applied it within the framework of FLASH RT. They used another MC tool, TOPAS-nBio [50], but

differently from PARTRAC, they simulated reaction kinetics using the IRT method, which can allow faster investigation of chemical processes induced by IR compared to the step-by-step method (see additional details in Section 2.2). In principle, TOPAS-nBio does not allow by default the possibility of simulation of inter-track effects, because the chemical reactions can occur only between chemical species generated by the same primary particle. In fact for each history, which in this case corresponds to a single primary particle, each event, such as reactions, is simulated independently from the others. To get around this problem they used the IRT approach as we will see below.

They performed simulations of chemical yields in a micrometer cube of pure water delivering protons with energies in the range of 1 – 100 MeV uniformly on the surface of the cube of water, with their initial direction randomly oriented toward the inside of the box. The inter-track effects started to be considered at the end of the pre-chemical stage, as in PARTRAC, using the chemical species currently available in the environment as the input for the IRT method. The time separation between the primary tracks was randomly sampled from a uniform distribution characterized by a time width of 1 ns, 1 μ s, or 10 μ s. These correspond to dose rates in the range of $5 \cdot 10^4 - 5 \cdot 10^8$ Gy/s. They repeated the simulations of the physical and pre-chemical stages up to the accumulation of 50 cGy in the target. Then, they computed the chemistry simulation using IRT for the set of particles corresponding to that absorbed dose. They observed an impact of inter-track effects in terms of the alteration in the G-values of the chemical species generated by IR for clinical-relevant pulse widths, with the maximum modification occurring in the case of particles delivered in a zero-width pulse, i.e., instantaneous dose-delivery, similarly to what was observed in [58]. Furthermore, they showed that the dependence of G-values on inter-track interactions decreases with increasing LET because the chemical species generated by a single high-LET primary particle interact more with each other than with radicals produced by other tracks, due to high density of chemical species generated in the proximity of the track core. Thus, these modifications of chemical yields result in significant effects for particles that arrive extremely close in space and in time. An example of their results is showed in Figure 3.37. Therefore, the actual biological impact remains to be evaluated and, above all, under realistic irradiation conditions from a clinical point of view.

D-Kondo et al. [247] extended the TOPAS-nBio code, including the impact of the biological medium. In particular, they computed the yields of several chemical species, after Conv (0.3 Gy/s) and UHDR (500 Gy/s) pulsed irradiation with 100 MeV protons, delivering doses between 5 and 20 Gy. They considered both pure water and biological medium as the target. They showed significant modifications of chemical yields, in particular for H_2O_2 , and depletion of O_2 , induced by UHDR radiation compared to Conv, for pure water. The interesting aspect is the impact of the biological medium, which significantly reduces these effects. In addition, a recent work by Shin et al. [248] showed the comparison of computational results (i.e. H_2O_2 chemical yield and O_2 depletion) with experimental data for different irradiation scenarios.

These modifications in the yields of chemical species, induced by irradiation with particles hitting the target with spatial and temporal proximity, were confirmed by Derksen et

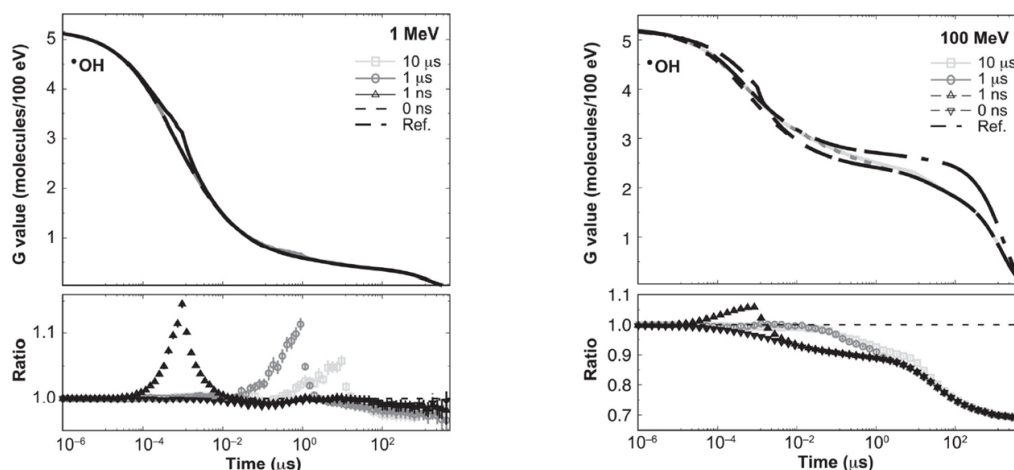


Figure 3.37: Example of impact of inter-track effects on time evolution of G-values of $\text{OH}^\bullet$ for different radiation quality, using TOPAS-nBIO MC tool. Images from [64].

al. [246] using the TOPAS-nBio MC code. In particular, they modeled the inter-track effects differently from [64]. In fact, they applied the step-by-step method to simultaneously simulate N different histories in a single event, enabling the reactions between chemical species produced by different primary particles, where N is proportional to fluence and dose rate. Changing N , they modified the number of possible tracks between which inter-track effects can happen. They investigated possible inter-track effects for 10 eV electrons, and 10 MeV and 100 MeV protons, in water, with $N = 2, \dots, 60$, with different spatial displacements between the primary particles, in the range of 1 – 100 nm. They observed significant modifications in the G-values, in particular for $\text{OH}^\bullet$. Furthermore, these alterations depend on the distance between tracks, the number of simultaneous tracks N , and the radiation quality of the primary particle. However, the main limitation of this study is that there is no direct relation between N and the dose rate, because the number N of histories have been simulated simultaneously in a single event.

The impact of inter-track interactions was also studied through other MC codes. For example, Alanazi et al. [224], using the IONLYS-IRT MC code for the irradiation of 300 MeV protons with dose rates up to 10^{10} Gy/s, observed significant modifications in product yields, while Lai et al. [229], using gMicroMC, i.e. a GPU-based MC tool, and including the impact of environmental oxygen, observed changes in G-values for all chemical species. Both works were carried out as preliminary studies for the investigation of the oxygen depletion hypothesis. Even in these cases, the biological impact of FLASH irradiation remains to be evaluated.

Nevertheless, Thompson et al. [245] showed opposite results using the TOPAS-nBio MC tool. They did not observe the impact of inter-track interactions for realistic spatial and temporal distances between primary tracks. However, they applied a different approach to investigate the modifications of the chemical environment induced by spatio-temporal overlaps of tracks. In particular, they simulated protons irradiation with energies

in the range 0.5 – 100 MeV, focusing on the time evolution of the radial track dimensions during the heterogeneous chemical stage. They estimate the geometric probability of tracks overlap, developing an analytical model whose input is the track areas at different time points, repeating the procedure for different delivered doses, as a surrogate of inter-track interaction. Thus, they wrote the probability of track overlap for a dose corresponding to N tracks, as

$$P_{\text{overlap}} = 1 - \left(1 - \frac{A_{\text{track}}}{A_{\text{target}}}\right)^N, \quad (3.38)$$

where the target area is a circle of radius $5 \mu\text{m}$, to mimic the cross-section of a generic cell nucleus. From Eq. 3.38 they could obtain the expected number of overlaps as

$$\#_{\text{overlap}} = \frac{A_{\text{track}}}{A_{\text{target}}} \cdot \frac{N - 1}{2}. \quad (3.39)$$

From Eq. 3.39 they could estimate the dose threshold at which inter-track interaction became significant for each irradiation condition. However, the main limitation is that with this approach they considered instantaneous hitting of all particles, allowing only the investigation of spatial separation effects. Despite this, they showed negligible inter-track effects at each time scale and for every radiation quality of the projectile, for clinical-relevant 2 Gy irradiation. Furthermore, they observed an increase in the probability of track overlap with increasing time point, projectile energy, and imparted dose. In particular, they obtained a dose threshold of 23 Gy to trigger significant track overlaps. Thus, they concluded that the spatial distances between protons are too large to induce significant inter-track interactions that lead to modifications of chemical environment and biological response in the case of instantaneous irradiation of all primary particles.

Baikalov et al. [244] developed an analytical model to investigate the impact of inter-track interaction on the chemical environment. They described the spatial and temporal evolution of a track using a reaction-diffusion equation, that is,

$$\frac{\partial c_i(\vec{r}, t)}{\partial t} = \alpha \nabla^2 c_i(\vec{r}, t) - k_e c_i(\vec{r}, t) - k_r \sum_{i,j}^N c_i(\vec{r}, t) c_j(\vec{r}, t), \quad (3.40)$$

where $c_i(\vec{r}, t)$ is the probability density function (PDF) of chemical species at point $\vec{r}$ and time t , α is the diffusion coefficient, k_e is the rate constant of the scavenging environment, k_r is rate constant of track-track interaction. The track-track term describes intra-track interaction for $i = j$ and inter-track interaction term for $i \neq j$. Then, they calibrated the model parameters using TOPAS-nBio.

They described the effect of inter-track interaction defining the total inter-track conversion, namely

$$I_{\text{inter}}(t) = \frac{N(t)}{2} \cdot \int_0^t \lim_{R \rightarrow \infty} N_R(t') \cdot \langle \omega_{1,2}(t') \rangle dt', \quad (3.41)$$

where $N(t)$ is the total number of tracks on the target at time t , $N_R(t)$ is the expected number of tracks within any circular area of radius R , which represents the interaction

surface, and $\omega_{1,2}(t)$ is the interaction rate, which describes the modification of chemical species at time t induced by interaction of two tracks, and it is defined as

$$\omega_{1,2}(t) = \int k_r \cdot c_1(\vec{r}, t) \cdot c_2(\vec{r}, t) dV. \quad (3.42)$$

They also quantified the intra-track interaction, namely self-interaction of tracks, defining the total intra-track conversion as

$$I_{intra}(t) = \sum_i^{N(t)} \int_0^t \omega_{i,i}(t') dt'. \quad (3.43)$$

Finally, they evaluated the impact of inter-track interaction with respect to intra-track interaction, defining the relative change that inter-track interactions induce to the chemical environment at time t , that is,

$$\Phi(t) = \frac{I_{inter}(t) + I_{intra}(t)}{I_{intra}(t)} - 1 = \frac{I_{inter}(t)}{I_{intra}(t)}. \quad (3.44)$$

In particular, they considered significant modifications of the chemical environment caused by inter-track interactions if $\Phi(t)$ becomes larger than a critical value Φ_c , which was obtained fitting their results with experimental data, as discussed in detail in [244].

They simulated electron tracks in oxygenated water and in cell scavenging conditions, observing negligible modification of the chemical environment for 12 Gy irradiation. Furthermore, they estimated significant chemical changes due to the effects of inter-tracks only if they delivered a dose > 13 Gy in a pulse of $0.5 \mu\text{s}$ for oxygenated water (4%), and even a huge dose > 90 Gy in a pulse of $0.5 \mu\text{s}$ in cellular scavenging conditions. Therefore, they concluded that inter-track effects could not trigger the FLASH effect.

Instead, Abolfath et al. [243] tried to combine step-by-step MC simulations using Geant4-DNA with a Molecular Dynamics (MD) approach to study inter-track effects in the context of FLASH RT. They predicted much higher diffusion constants using a MD approach compared to those usually used in the main MC codes, trying to explain why no significant impacts due to inter-track interactions have been seen up to that point using other MC codes. Upon application of these new diffusion coefficients, they observed an increase in inter-track chemical reaction rates, and at the same time a decrease in intra-track reaction rates for the UHDR irradiation regime.

In addition, in another work, Abolfath et al. [267] extended the work reported in [244], developing a couple of partial differential equations (PDE) that describe reaction–diffusion processes between two chemical species, i.e., a reactive oxygen species (ROS) and a non-reactive oxygen species (NROS), that are

$$\begin{cases} \frac{\partial u}{\partial t} = G(\vec{r}, t) + \vec{\nabla} \cdot (D_f(\vec{r}) \vec{\nabla} u) - k_1 u + k_2 v - 2k_3 u^2 - k_{12} uv \\ \frac{\partial v}{\partial t} = k_1 u - k_2 v + k_3 u^2 \end{cases}, \quad (3.45)$$

where $u = u(\vec{r}, t)$ is the ROS densities, e.g. $[\text{OH}^\bullet]$, and $v = v(\vec{r}, t)$ is the NROS densities, e.g. $[\text{H}_2\text{O}_2]$, $G(\vec{r}, t)$ is the ROS production yield, is the diffusion constant $D_f(\vec{r})$ of ROS, k_1

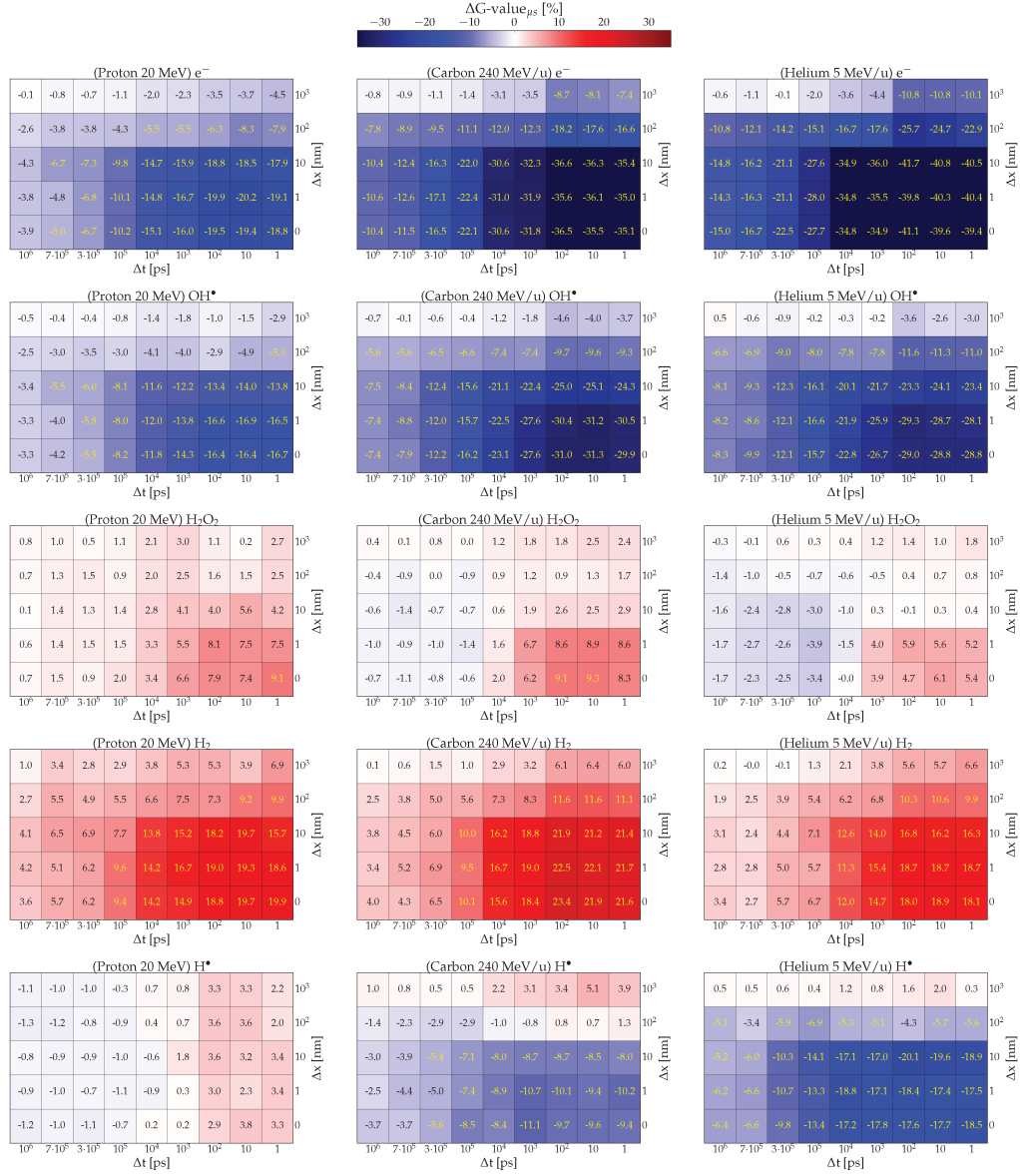


Figure 3.38: ΔG – value $_{\mu s}$ for different chemical species, spatial and temporal distances, and radiation qualities. From [250].

and k_2 are the reaction rate constants for scavenging effect on ROS and NROS, respectively, k_3 is the reaction rate constant for the recombination of ROS, and k_{12} is the rate constant for the reaction between ROS and NROS. They applied this model to study the role in inter-track interactions in ordered and disordered mediums. The ordered medium is a homogeneous and uniform medium, such as water, to represent healthy tissue, while the disordered medium is characterized by inhomogeneity and fractal-type porosity, to mimic the tumor environment. They showed decreasing inter-track effects with increasing complexity of the medium. Thus, they speculated on the role of different geometrical structures between normal tissues and tumors in the emergence of the FLASH effect. However, there is no experimental evidence to prove this.

Finally, Castelli et al. [250] extended the TRAX-CHEM MC code to simultaneous consideration of several particle tracks that are characterized by user-defined spatial and temporal separations. In particular, they focused their attention to the impact of intra-track interactions compared to the non-interacting (NI) tracks simulation, predicting the yields of chemical species for various combinations of spatial and temporal distances for couple of ion tracks and for different radiation qualities of the initial projectile. In detail, they simulated 20 MeV protons, 240 MeV/u carbon ions and 5 MeV/u helium ions in pure water with relative spatial distance Δs of 0, 1, 10, 10^2 , and 10^3 nm and temporal shift Δt of 1, 10, 10^2 ps, 10, 10^2 , $3 \cdot 10^2$, $7 \cdot 10^2$, 10^3 ns.

To quantify the alteration of the chemical environment induced by inter-tracks effects they defined the normalized difference between G-values, namely

$$\Delta G - \text{value}_{\mu s} = \frac{G - \text{value}(1\mu s + \Delta t) - G - \text{value}_{NI}}{G - \text{value}_{NI}}, \quad (3.46)$$

where $G - \text{value}(1\mu s + \Delta t)$ is the G-value at time $1\mu s + \Delta t$ considering inter-track interactions, while $G - \text{value}_{NI} = (G - \text{value}_{NI}(1\mu s + \Delta t) + G - \text{value}_{NI}(1\mu s))/2$ is the G-value at time $1\mu s + \Delta t$ considering NI condition.

They obtained negligible influence of inter-track interactions for distances between tracks $> 1 \mu m$, while they found a significant impact on chemical yields for temporal shifts $\leq 1 \mu s$, enhanced at higher LET values, as we can see in Figure 3.38. In contrast to the approaches proposed in [244, 245], they emphasized the fact that inter-track effects encompass not only geometric considerations but also intricate spatio-temporal dynamics.

In addition, the TRAX-CHEM MC tool has been further extended with an analytical approach, i.e. TRAX-CHEMxt [273], to speed up the study of the possible impact of FLASH irradiation in the homogeneous chemical stage, i.e. for times longer than the microsecond. The investigation is still ongoing.

A physicochemical model of reaction kinetics for the investigation of the organic radical-radical hypothesis

As discussed in Section 3.5.3, Labarbe et al. [7] developed a model to describe the hypothesis of organic radical-radical recombination proposed in [214, 7]. They developed a pure analytical physicochemical model to describe the kinetics of the complex network of reactions that occur during the homogeneous chemical stage, as schematized in Figure

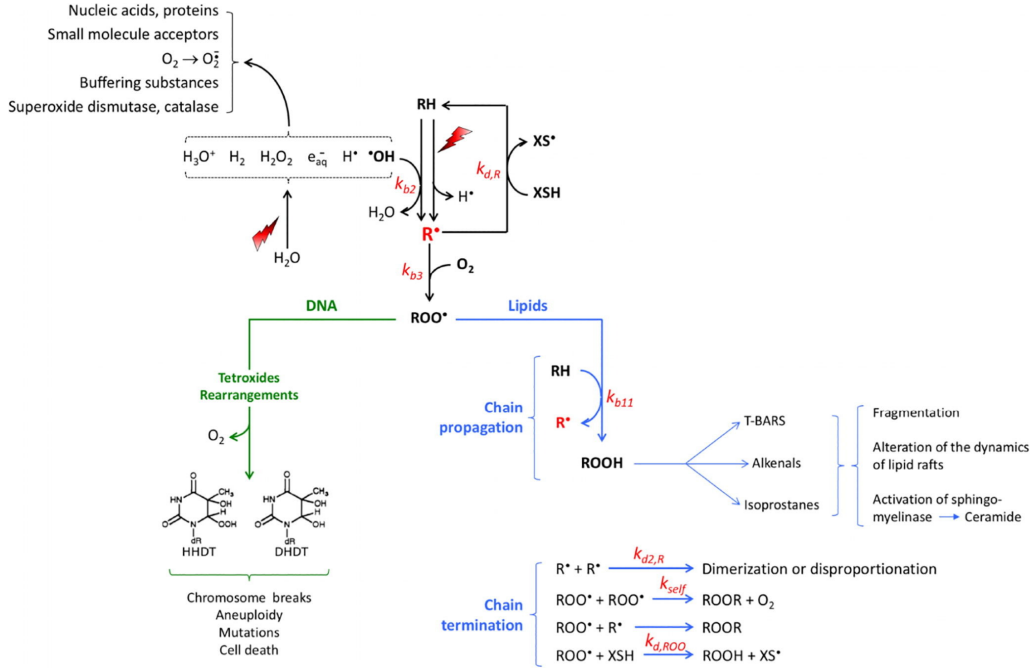


Figure 3.39: Chemical reactions network considered to investigate the hypothesis of organic radical-radical recombination. From [7].

3.39. They described the reaction rates of nine chemical species using a system of ODEs such as,

$$\frac{d\boldsymbol{\xi}(t)}{dt} = \mathbf{f}(\xi_1(t), \dots, \xi_9(t)) + \mathbf{G}\rho \frac{dD(t)}{dt}, \quad (3.47)$$

where $\boldsymbol{\xi}(t) = ([e_{aq}^-], [O_2], [C_{H_2O_2}], [C_{OH^\bullet}], [H^\bullet], [H_2], [C_{O_2^{\bullet-}}], [R^\bullet], [ROO^\bullet]) (t)$ is the vector of concentration at time t for each chemical species; ρ is the water density; $\mathbf{G}$ is the vector of G-values for each chemical species at microsecond, as defined in Section 1.2.2, which describe the chemical system at the end of the heterogeneous chemical stage; $\mathbf{f}(\xi_1(t), \dots, \xi_9(t))$ is the vector of the reaction rates for each chemical species, as defined in Eq. 1.2.2, which describe the complex network of chemical reactions and are a function of the concentration of the nine chemical species $\boldsymbol{\xi}(t)$ and depend on concentrations of other chemical species $\bar{\boldsymbol{\xi}}$ that are considered constant during irradiation, and also on rate constants $\mathbf{K}$, as defined in Section 1.2.2. Furthermore, the instantaneous dose rate is defined as

$$\dot{D}(t) := \frac{dD(t)}{dt} = \begin{cases} \bar{D} & \text{if } t \leq T_{irr} \\ 0 & \text{if } T_{irr} < t \leq T_{sim} \end{cases}, \quad (3.48)$$

namely is considered constant and equal to the mean dose rate $\bar{D}$, where T_{irr} is the total irradiation time and T_{sim} is the total simulation time, which is equal to the longest time between 150 s and $120\% \cdot T_{irr}$. With this approach, all the physical and chemical events that occur before the start of the homogeneous chemical stage, namely, for times

shorter than a microsecond, are considered instantaneous and parametrized in this model using, for example, the G-values. This system was implemented in *MATLAB* and solved numerically using a default function. They used standard values from the literature for the rate constants, equilibrium constants, concentrations, and G-values. All these values are reported in the supplementary material file of [7].

To account for the cells exposure to $\text{ROO}^\bullet$, which is consider the key quantity for injuries in lipids and DNA, that triggers the manifestation of the FLASH effect, as discussed in Section 3.5.3, they defined the area-under-the-curve (AUC) of $\text{ROO}^\bullet$ as

$$\text{AUC}_{\text{ROO}^\bullet} = \int_0^{T_{\text{sim}}} [\text{ROO}^\bullet](t) dt, \quad (3.49)$$

which is the integral over time of the concentration of the organic peroxy radical at time t , between 0 s and T_{sim} , in order to also consider the relaxation of the radical concentration after the end of the irradiation.

They computed $\text{AUC}_{\text{ROO}^\bullet}$ for several dose rates in the range $[0.03 - 10^7]$ Gy/s, doses in $[0 - 30]$ Gy, and initial oxygen conditions in the interval $[0\% - 21\%]$, setting the initial conditions for the other eight species equal to 0. Then, they normalized $\text{AUC}_{\text{ROO}^\bullet}$ between 0 and 1 for all dose rate and dose values in the case of physioxia initial conditions (4% O_2 at 0 s), namely,

$$\text{AUC}_{\text{norm}}(D, \bar{D}) = \frac{\text{AUC}_{\text{ROO}^\bullet} - \text{AUC}_{\text{ROO}^\bullet}^{\min}}{\text{AUC}_{\text{ROO}^\bullet}^{\max} - \text{AUC}_{\text{ROO}^\bullet}^{\min}}, \quad (3.50)$$

where $\text{AUC}_{\text{ROO}^\bullet} = \text{AUC}_{\text{ROO}^\bullet}(D, \bar{D})$, $\text{AUC}_{\text{ROO}^\bullet}^{\min} = \text{AUC}_{\text{ROO}^\bullet}(D^{\min}, \bar{D}^{\max})$, and $\text{AUC}_{\text{ROO}^\bullet}^{\max} = \text{AUC}_{\text{ROO}^\bullet}(D^{\max}, \bar{D}^{\min})$.

Finally, they fitted the results for AUC_{norm} , obtaining the following analytical function,

$$\text{AUC}_{\text{norm}}(D, \bar{D}) = a(D) + \frac{b(D)}{\sqrt{\bar{D}}}, \quad (3.51)$$

where $a(D)$ and $b(D)$ are polynomial coefficients that are fitted to the simulated values for AUC_{norm} . The shape of AUC_{norm} is consistent with FLASH observations, namely AUC_{norm} started from a maximum value, then decreased with increasing dose rates. Moreover, its dependence on dose rates is more marked with increasing dose values. The dependence of AUC_{norm} on dose and dose rate is shown in Figure 3.40 (a).

In order to compare their *in-silico* results with the experimental findings, they assumed that the NTCP curve, which is the typical method to quantify the biological response of normal tissue to IR (see Section 2.4 for more details), is a sigmoid function of AUC_{norm} , that is,

$$\text{NTCP}(\text{AUC}_{\text{norm}}(D, \bar{D})) = \frac{1}{1 + \exp(-\gamma \cdot (\text{AUC}_{\text{norm}}(D, \bar{D}) - \text{AUC}_{50}))}, \quad (3.52)$$

where γ and AUC_{50} are the fitted parameters on the experimental data points. In particular, they calibrated their model on the experimental results of the famous work on brain

toxicity with UHDR-electrons by Montay-Gruel et al. [194]. These experimental data can be described using the following expression,

$$\text{NTCP}_{\text{exp}}(\text{RR}(\bar{D})) = 1 - \frac{\text{RR}(\bar{D}) - \text{RR}^{\min}}{\text{RR}^{\max} - \text{RR}^{\min}}, \quad (3.53)$$

where RR is the recognition ratio, i.e., the measured biological endpoint in the experiment for 10 Gy irradiation, $\text{RR}^{\min} = \min(\text{RR})$, and $\text{RR}^{\max} = \max(\text{RR})$. In conclusion, using their devolved NTCP model, they can reproduce the trend of the experimental results as a function of the mean dose rate $\bar{D}$ in [194], as shown in Figure 3.40 (b).

After this calibration, they investigated the impact of different mean dose rates, doses, and initial oxygen concentrations on the emergence of the FLASH effect, in terms of NTCP predictions. In physioxia and for a fixed dose starting from ~ 7 Gy, they observed that TD_{50} , which is the dose that corresponds to $\text{NTCP} = 0.5$, is constant up to a dose rate value of ~ 1 Gy/s. Then, in the range of $\sim 1 - 10^2$ Gy/s, TD_{50} gradually shifts towards higher doses, which corresponds to the appearance of the FLASH sparing effect, with DMF ~ 1.4 , which is consistent with that obtained in experiments, such as in [87, 180, 194]. After a dose rate on the order of $\sim 10^2$ Gy/s, TD_{50} remains constant for increasing dose rates, namely, the FLASH sparing effect is maintained. These results are reported in Figure 3.40 (c). Furthermore, fixing $D = 10$ Gy, they observed a shift of TD_{50} towards higher oxygenation levels and a modification in the steepness of the NTCP curve with increasing dose rate values, starting from ~ 1 Gy/s, in agreement with the trend observed in various experiments, such as in [85, 83], as shown in Figure 3.40 (d).

The model also has some limitations, such as the numerical uncertainties in the rate constants and G-values, the uncertainty of having considered all the main chemical reactions, the exact end of the heterogeneous chemical stage at the microsecond, the lack of a specific characterization of bio-molecules, and the use of only continuous irradiation. However, given the lack of experimental evidence and the significant uncertainty, especially in chemistry experiments, these limitations are currently secondary to the overall impact of the model, which is nowadays one of the few models that has successfully described some of the typical trends observed in FLASH experiments. The main limitation of the present model is undoubtedly the inability to quantitatively explain the FLASH differential effect between healthy tissue and tumor under UHDR irradiation. In this context, Espinosa et al. [268] extended the model, for example by studying the impact of pulsed irradiation, and confirming Labarbe's findings.

Tan et al. [269] investigated the feasibility of this model in predicting oxygen depletion findings. They used the same equations, reactions and constants discussed by Labarbe et al. [7], with the exception of the rate constant for the $\text{ROO}^\bullet$ decay, following the suggestion proposed by Wardman [275]. In particular, they compared their *in-silico* results with the main *in-vitro* data of oxygen consumption after UHDR irradiation. They showed that this physicochemical model [7] can reproduce the experimental results of oxygen depletion, in particular [233, 241], as we can see in Figure 3.41.

Then, in agreement with Labarbe et al., they speculated that different cell exposure to $\text{ROO}^\bullet$ could explain the FLASH effect. Moreover, they also took into account the impact

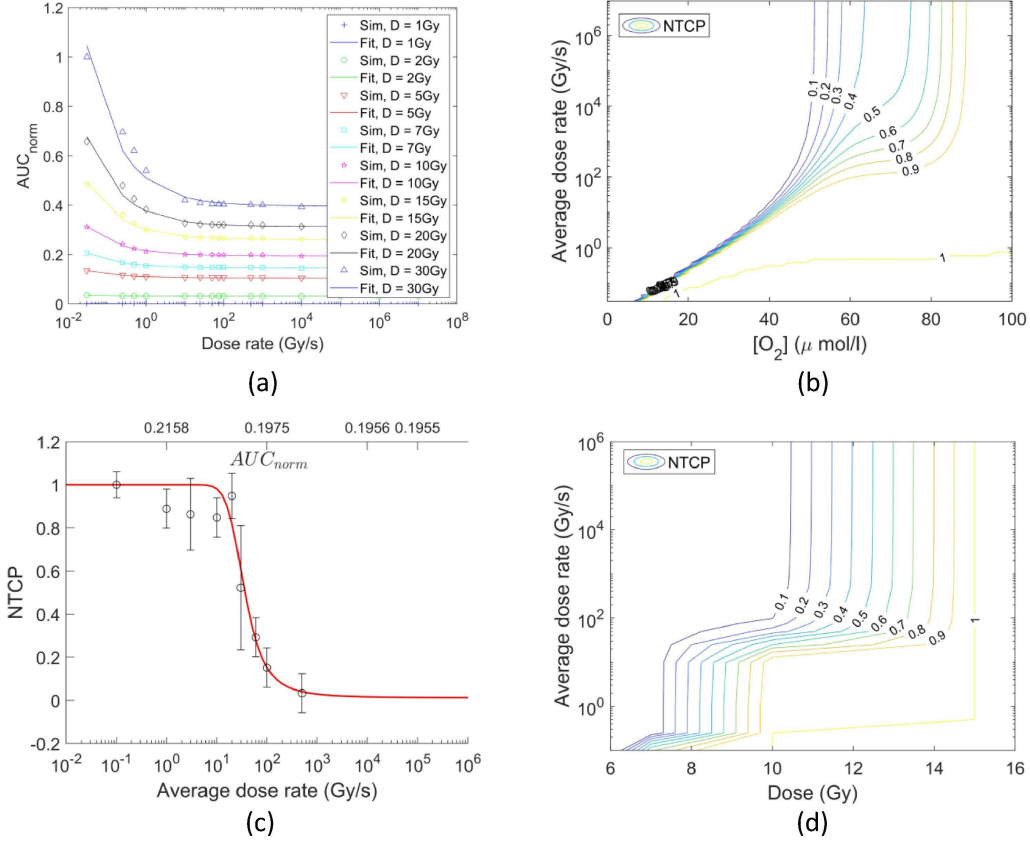


Figure 3.40: Main results obtained investigating the organic radical-radical recombination hypothesis. (a) Normalized area-under-the-curve AUC_{norm} , according to Eq. 3.50 (points) and Eq. 3.51 (lines), as a function of dose rate for different doses, at 4% O_2 . (b) Simulated Normal Tissue Complication Probability (NTCP), according to Eq. 3.52, as a function of dose rate and initial oxygenation level, for 10 Gy irradiation with electrons. (c) Experimental NTCP (points), according to Eq. 3.53, and simulated NTCP (line) as a function of dose rate and AUC_{norm} . (d) Simulated NTCP as a function of dose and dose rate. From [7].

of O_2^- on the emergence of the sparing effect. In order to compare their results with the experimental data, they defined the normalized difference of AUC between Conv and UHDR irradiation, that is,

$$\Delta AUC_\xi = \frac{AUC_\xi^{Conv} - AUC_\xi^{UHDR}}{AUC_\xi^{Conv}}, \quad (3.54)$$

where $\xi = ROO^\bullet$, O_2^- , which can be seen as a parametrization of the FLASH sparing effect. In particular, they observed (i) a correlation between AUC_ξ of both chemical species and memory preservation data in [84]; (ii) a correlation of ΔAUC_ξ for both radicals with TGF- $\beta 1$, leg contracture, and cytokine IL-6 measured by [147]; (iii) a similar trend between ΔAUC_ξ for both radicals and the findings about the preservation of intestinal crypts after beam entrance or mid-SOBP irradiation observed by [148]. However, it is not entirely clear how they correlated their *in-silico* results with the experimental data, particularly in terms of the biological endpoints considered.

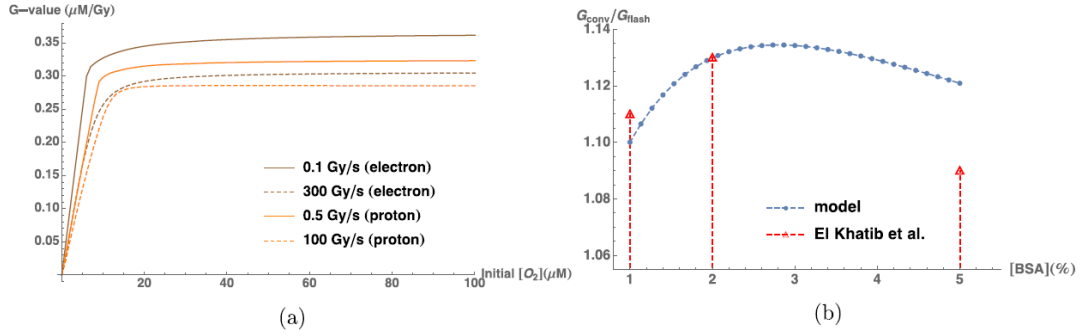


Figure 3.41: Comparison of the computational results obtained for oxygen depletion using the kinetic model of [7] with the main experimental data [233, 241]. From [269].

The physicochemical model developed by Labarbe et al. [7] was also the starting point for the development of the multiscale radiation biophysical model of this thesis [1, 2], as we will see in Section 4.

An analytical model for the investigation of the hypothesis of an immune system-driven response

In Section 3.5.4, we have discussed the possible involvement of the immune system in the activation of the FLASH effect, in particular, focusing on the key role of blood lymphocytes. In this context, Cucinotta et al. [256, 258, 257] developed an analytical model to study the impact of FLASH RT in the preservation of lymphocytes in the blood. In particular, they described the surviving fraction of lymphocytes using algebraic equations, taking into account several physiological, biophysical, and physical parameters, such as the properties of the blood flow (i.e. the total blood volume, blood volume coming from the heart per unit of time, and the blood circulation time), the fraction of blood circulatory

system irradiated, the concentration of lymphocytes in blood, the specific radiosensitivity of lymphocytes and the characteristics of the irradiation beam (i.e. dose, dose rate, irradiation time). All the mathematical formulas and calculations are reported in [256, 258, 257].

The main point of this work is that they were able to speculate the influence of the dose rate in the survival of blood lymphocytes. In particular, in [256], they showed that the surviving fraction of blood lymphocytes increases with increasing dose rate values, suggesting the involvement of lymphocytes protection in trigger the FLASH effect. Furthermore, they predicted that surviving fraction increases by reducing the fraction of the blood volume in the irradiated region of the blood circulatory system. In addition, they showed that to optimize the protective effect of human blood lymphocytes, the dose rate should be > 40 Gy/s for doses delivered in the range of $10 - 40$ Gy, considering a blood circulation time of ~ 60 s, which is extremely higher than the typical irradiation time. Finally, they speculated that for mice the dose rate threshold to maximize the lymphocytes protection is higher than in humans due to the shorter blood circulation time.

However, at present, there is no experimental evidence clearly supporting the protection of blood lymphocytes by FLASH RT, as discussed in Section 3.5.4. Therefore, further experimental studies are needed to validate the proposed mathematical model.

Phenomenological models for the investigation of the FLASH effect

In the previous paragraphs we have discussed about the various mathematical models and MC tools to study the feasibility of the main hypotheses proposed to describe the FLASH effect, attempting to understand what is the mechanistic basis of this elusive biological effect. Instead, in this paragraph we focus on the main phenomenological models proposed in the literature whose aim is the parametrization of the FLASH effect, such as [39, 272].

Zou et al. [272], starting from the equations proposed by [93, 213] for the time evolution of oxygen concentration, they updated the model, obtaining,

$$\frac{\partial [\text{O}_2](\vec{x}, t)}{\partial t} = -K_1 \dot{D}(\vec{x}, t) \frac{[\text{O}_2](\vec{x}, t)}{[\text{O}_2](\vec{x}, t) + l} + -K_2 \frac{[\text{O}_2](\vec{x}, t)}{[\text{O}_2](\vec{x}, t) + \lambda} + K_3 \nabla^2 [\text{O}_2](\vec{x}, t), \quad (3.55)$$

where K_1 is the oxygen depletion rate, K_2 is the metabolic oxygen consumption rate, K_3 is the oxygen diffusion constant from tissues, while l and λ are additional parameters to modulate the depletion and consumption terms. Additionally, the dose rate is defined as

$$\dot{D}(\vec{x}, t) = \begin{cases} 0 & \text{if } t \leq 0 \\ \bar{D} & \text{if } 0 < t \leq T \\ 0 & \text{if } t > T \end{cases}, \quad (3.56)$$

where T is the irradiation time.

Then, considering the OER formulation proposed by [30] and reported in Eq. 1.70, and the Eq. 3.55 for the evolution in time of oxygenation, they defined the cumulative normoxic-equivalent dose (CNED), which is the cumulative dose at a given oxygen level equivalent to obtain the same biological effect in normoxia, namely,

$$\text{CNED}(\vec{x}) = \int_0^T \frac{\dot{D}(\vec{x}, t)}{\text{HRF}(\vec{x}, t)} dt, \quad (3.57)$$

where HRF is the hypoxia reduction factor, which is another name for OER.

Finally, they defined the FLASH effective-dose-modifying factor (FEDMF), which is a way to quantify the FLASH effect taking into account the dynamic oxygen effect, that is,

$$\text{FEDMF} = \frac{\text{CNED}_{\text{Conv}}(\vec{x})}{\text{CNED}_{\text{UHDR}}(\vec{x})}, \quad (3.58)$$

namely, the ratio of CNED between Conv and UHDR irradiation, where they considered the manifestation of the FLASH effect if $\text{FEDMF} \geq 1.2$.

Using this approach, they studied the impact of dose rate, oxygenation, and vasculature spacing as the main modulators of the magnitude of the FLASH effect [272].

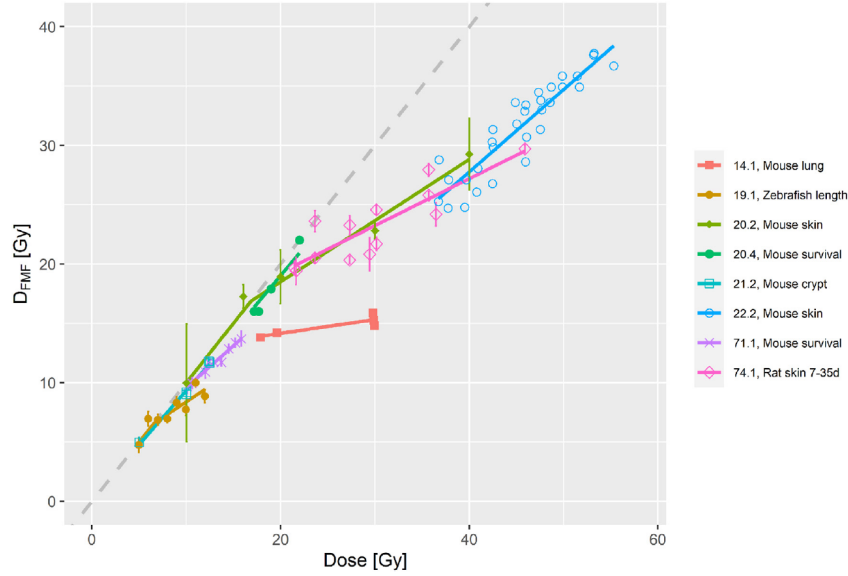


Figure 3.42: *In-vivo* experimental data as a function of the single-fraction UHDR dose D and the estimated isoeffective FMF-weighted dose D_{FMF} . From [39].

Böhlen et al. [39] proposed a pure phenomenological model to analyze the experimental findings after FLASH irradiation. In particular, starting from their definition of FMF, as illustrated in Eq. 1.73, they defined the isoeffective FMF-weighted dose as,

$$D_{\text{FMF}} = D \cdot \text{FMF}, \quad (3.59)$$

which is the dose delivered in Conv regime to achieve the same biological effect of a dose D delivered in UHDR regime.

Then, they applied the FMF definition to all *in-vivo* experiments in the literature up to October 2021 to derive FMF values. In addition, they proposed a phenomenological parametrization for these FMF values, starting from the definition of NTCP curve, which is,

$$\text{FMF}_{\text{FMF}^{\min}, D_T}(D) = \begin{cases} 1 & \text{if } D \leq D_T \\ \left(1 - \text{FMF}^{\min}\right) \frac{D_T}{D} + \text{FMF}^{\min} & \text{if } D > D_T \end{cases}, \quad (3.60)$$

where

$$\text{FMF}^{\min} = \frac{\gamma_{50}^{\text{UHDR}}}{\gamma_{50}^{\text{Conv}}} , \quad (3.61)$$

is the minimum FLASH effect, and

$$D_T = \frac{D_{50}^{\text{Conv}} - D_{50}^{\text{UHDR}} \cdot \frac{\gamma_{50}^{\text{UHDR}}}{\gamma_{50}^{\text{Conv}}}}{1 - \frac{\gamma_{50}^{\text{UHDR}}}{\gamma_{50}^{\text{Conv}}}} , \quad (3.62)$$

is the minimum threshold dose needed to trigger the FLASH effect, in which γ_{50}^i is the slope of a NTCP curve at 50% complication probability with i equal to Conv or UHDR. They defined the NTCP curve using two typical parametrizations, that are the logistic function, namely,

$$\text{NTCP}_{D_{50}^i, \gamma_{50}^i}(D_i) = \frac{1}{1 + e^{-\gamma_{50}^i(D_i - D_{50}^i)}} , \quad (3.63)$$

or the probit function, that is,

$$\text{NTCP}_{D_{50}^i, \gamma_{50}^i}(D_i) = \frac{1}{2} \left(1 + \text{erf} \left(\frac{\gamma_{50}^i(D_i - D_{50}^i)}{\sqrt{2}} \right) \right) , \quad (3.64)$$

where D_{50}^i is the dose at 50% complication probability.

Applying Eq. 3.60, they found mean FMF values in the range 0.71 – 0.96, depending on the specific organ or biological endpoint considered, and on the dose delivered, thus showing that the magnitude of the FLASH effect depends on tissue type and dose, as we can see in Figure 3.42.

Model Development and Results

Chapter 4

The MultiScale-Generalized Stochastic Microdosimetric Model (MS-GSM²) for FLASH investigations

The mechanism behind the FLASH effect is extremely complex, elusive, and highly debated, as we have seen in Chapter 3. Both *in-silico* and experimental studies suggest that spatial and temporal effects related to radiation chemistry are highly significant in activating this biological mechanism under UHDR radiation. Thus, this Chapter is entirely dedicated to the conception, realization, implementation, and validation of a multiscale radiation biophysical model for the investigation of the FLASH effect. This work is the main core of the present thesis and is the foundation for the studies that will be proposed in the following chapters. The MultiScale-Generalized Stochastic Microdosimetric Model (MS-GSM²) [1, 2] is a multi-stage stochastic radiobiological model that describes the formation and time evolution of DNA lesions in a cell nucleus according to microdosimetric principles, taking into account the indirect action of radiation in the generation of DNA damage. The MS-GSM² can describe spatio-temporal effects on DNA damage, considering possible modifications of the chemical environment induced by UHDR radiation. The peculiarity of this model is that MS-GSM² can consider different levels of spatial and temporal stochasticity, in particular, in physics, chemistry, and biology, as we will see in detail in the following Sections.

The MS-GSM² can handle different physical, chemical, and biological scenarios. In particular, from a physical point of view, the MS-GSM² can manage different radiation quality of the beam, that is, type, energy, and LET of radiation, and any dose-delivery time-structure of the beam, considering, in principle, different mean dose rate, instantaneous dose rate, dose per pulse, pulse width, and pulse repetition frequency. Furthermore, talking about chemistry, the MS-GSM² model includes a complex network of chemical reactions that involve chemical species that come from water radiolysis, environmental

oxygen, biomolecules, and organic radicals. In this way, the model can consider the influence of several chemical agents on DNA damage, such as the action of harmful chemical species, the fixation effect of the molecular oxygen, and the scavenging effect of the antioxidant environment. Finally, from the point of view of biology, the MS-GSM² model outlines three potential biological pathways for irradiated cells, which may either recover or die after sustaining radiation-induced injuries [3, 4]. Furthermore, the final biological outcome of MS-GSM² is the cell survival fraction, enabling easy comparison with *in-vitro* experimental data.

The main contributions that will be discussed in the present Chapter are the following:

- (i) to introduce a general stochastic model able to provide a unified framework encompassing physics, chemistry, and biology;
- (ii) to propose a mechanism underpinning the manifestation of the FLASH effect, which includes three potential mechanistic pathways, i.e. organic radical - radical recombination, oxygenation and scavengers activity on DNA damage formation, and spatial and temporal dose deposition effects;
- (iii) to show how the interplay of the above different mechanisms can in turn produce a cell survival sparing at a sufficiently high dose and dose rate, for different radiation quality, and environmental chemical conditions;
- (iv) to reproduce the main *in-vitro* UHDR experiments, for different radiation quality and oxygenation;
- (v) to try to explain the different *in-vivo* response between healthy tissues and tumors on the basis of different redox environments;
- (vi) to suggest a consistent picture explaining the most relevant features related to the FLASH observations.

In particular, this Chapter is structured as follows: Section 4.1 presents the development of the mathematical framework of the radiobiological model; Section 4.2 reports the numerical implementation of the multiscale tool; Section 4.3 shows the main numerical results for the physical, chemical, and biological stages; Section 4.4 demonstrates the feasibility of the model in reproducing the main *in-vitro* experimental data reported in literature; Section 4.5 reveals a new possible hypothesis that could explain the main features of the FLASH effect, which involves the redox environment, bridging the next chapters; finally, Section 4.6 discusses the main results of the MS-GSM².

4.1 Theory and calculations

This Section is completely dedicated to the description of the mathematical framework of the MS-GSM². This tool is a multi-stage probabilistic radiation biophysical model, resulting from the inclusion of dose rate and chemical effects in the GSM², which is the radiobiological model discussed in Section 2.3.1. The GSM² [3, 4] is a Markov model

that generalizes the standard Poisson statistics for the number of radiation-induced DNA damages [5, 276]. Specifically, the GSM² encompasses the randomness of radiation energy deposition, DNA damage formation, and the temporal repair of these damages within a singular framework.

Typically, standard radiobiological models discussed in Section 2.3, such as LEM, MKM, and the more recent GSM², do not take into account fast temporal processes, such as the production of chemical species, e.g., radicals, or reactions between that species or with the cellular environment. Furthermore, they are not capable of managing the temporal structure of dose delivery, but only spatial features of the beam. The core idea of MS-GSM² is exactly to fill this gap between physical and biological processes, by bridging different spatial and temporal scales. In particular, spatially, it can describe scales in the order of cell and nucleus dimensions, between $10^{-6} - 10^{-5}$ m. Instead, temporally, it can account for fast events in the order of microseconds, i.e., from the beginning of the homogeneous chemical stage, and slow biological events up to hours and days. Thus, MS-GSM² can explicitly incorporate both temporal and spatial effects, coupling energy deposition in the cell nucleus by ionizing particle tracks with a chemical characterization of the cellular environment.

From a mathematical point of view, the main core of the MS-GSM² model is the description of the formation and evolution in time of direct and indirect DNA damage in a cell nucleus, which are described by an extended version of the MME discussed in Section 2.3.1, as we will see in detail later. In order to apply this approach, the first step is the description of the cell nucleus in terms of mathematical domains, following that proposed in [3], and analogously to the MKM model.

The cell nucleus, which is the target of the model, is represented by a cylinder of radius R_N in the xy -plane and depth $2R_N$ in the z -direction, which coincides with the projectile direction. The cylinder is divided into N_z circular layers, and each circle is subdivided into N_{xy} square domains, with a typical side in the order of micrometer. Thus, the cylinder is composed in total of $N_d = N_z \cdot N_{xy}$ independent cubic domains d . The subdivision into domains allows for consideration of physical, chemical, and biological spatial information. In fact, in this way, it is possible to have a discretized spatial distribution of energy at the micrometer scale, acquire information regarding possible spatial inhomogeneities in the chemical environment dependent on the quality of the radiation, and take into account these energy and chemical species distributions in the local onset of DNA damage.

We can think of the MS-GSM² developed model as a sequence of four stages of radiation-induced damage. The processes that lead to cell death through the formation of DNA lesions start with (i) the physical stage, which is characterized by energy deposition by the primary ionizing projectile hitting the surface of the cell nucleus. The deposition event is extremely fast and is considered instantaneous for this model. Then, the energy deposited is spatially distributed in the horizontal plane of the cell nucleus. This dose deposition process is followed by (ii) the chemical stage, which is typically divided into pre-chemistry, heterogeneous chemistry, and homogeneous chemistry, as we have already seen in Chapter 1. In particular, the fastest chemical processes occurring during the pre-chemical and heterogeneous chemical stages, such as water radiolysis, are considered

instantaneous and are parameterized as input information for the model. This stage is characterized by the generation of harmful chemical species, such as ROS and organic radicals, induced by IR, and the occurrence of chemical reactions between these species. Then, (iii) the bio-chemical stage occurs. This stage connects the physical processes, that is, the energy deposition events by IR, and the chemical stage, namely, the reactions between environmental chemical species and radicals produced by IR, with the slowest biological processes, such as the time evolution of DNA lesions. During this stage, DNA damages are caused by the indirect action of IR, mediated by free radicals. Instead, direct DNA damages are instantaneously generated by IR. The last step is (iv) the biological stage, where the DNA lesions can undergo three different possible biological pathways, such as the repair process or two different death events. Thus, the final biological endpoint of the model is the probability that cells survive after irradiation. The mathematical framework of MS-GSM² is schematically illustrated in Figure 4.1, where it is possible to appreciate the connection between the different components of the MS-GSM² model, each of which describes a specific stage. In the following paragraphs, we will analyze in detail the mathematical construction of each of these stages of radiation-induced biological damage.

Stage	Temporal scale	Spatial scale	Stochasticity
Physics	instantaneous	$10^{-6} - 10^{-5} \text{ m}$	track hitting and energy deposition
Homogeneous chemistry	$10^{-6} - 10^0 \text{ s}$	10^{-6} m	time τ for next deposition event $\vec{d}$
Direct damage formation	instantaneous	10^{-6} m	$Z_{Y;\text{dir}}^{(c,d)}$ and $Z_{X;\text{dir}}^{(c,d)}$ according to (4.25)
Indirect damage formation	$10^{-6} - 10^0 \text{ s}$	10^{-6} m	$Z_{Y;\text{indir}}^{(c,d)}$ and $Z_{X;\text{indir}}^{(c,d)}$ according to (4.25)
Damage evolution	$10^0 - 10^5 \text{ s}$	10^{-6} m	processes a, b, r according to (4.36)
Cellular endpoint	$> 10^5 \text{ s}$	10^{-5} m	—

Table 4.1: Stages and corresponding spatio-temporal scales of radiation damage, as considered in this work, and the related levels of stochasticity taken into account by the MS-GSM². Table from [1].

(i) The physical stage

The first scale of radiation-induced biological damage that is taken into account by the MS-GSM² model is the physical stage. This stage is characterized by the hitting of the projectile track on the circular surface of the cell nucleus, and the subsequent deposition of energy by the ionizing particle. The primary particle is characterized by an atomic number A , an initial energy E , and a linear energy transfer LET. Temporally, the MS-GSM² model considered physical events instantaneous, while spatially, it describes the dose distribution in the order of $10^{-6} - 10^{-5} \text{ m}$, which is the typical size of the cell nucleus, as summarized in Table 4.1.

The paths of the primary particles are assumed to be parallel to each other and to the cylinder axis, i.e. the particles perpendicularly hit the circular surface of the cell nucleus. Similarly to what is done in [277], energy depositions are described via a Monte Carlo sampling of particle track hitting the target. The energy can be deposited directly by the

core of the projectile track or by the secondary particles that characterized the so-called penumbra of the track. In addition, the impact point can be within the surface area of the cell nucleus of radius R_N or outside the cylinder, i.e., in the outer circular ring around the nucleus. The maximum impact dimension $R_{max} > R_N$ from the center of the nucleus is chosen so that the projectile deposits at least the minimum energy in the cell nucleus. For each hitting track, the primary particle deposits an energy z , which is the energy imparted by a single event. The dose imparted by each primary particle can be spatially distributed in the N_{xy} domains of the horizontal plane according to an AT model [41], discussed in Section 2.1.2. Assuming a cylindrical symmetry, the analytical description of the radial dose imparted by a track is the following,

$$D(r) = \begin{cases} \frac{1}{\pi R_c^2} \left(\frac{\text{LET}}{\rho(1+2 \ln(R_p/R_c))} \right) & r \leq R_c \\ \frac{1}{\pi r^2} \left(\frac{\text{LET}}{\rho(1+2 \ln(R_p/R_c))} \right) & R_c < r \leq R_p \\ 0 & r > R_p \end{cases} , \quad (4.1)$$

where r is the radial distance from the center of the track, R_c is the radius of the track core centered on the impact point, $R_p = 0.05 \cdot (E/A)^{1.7}$ is the maximum radius of the track penumbra, which depends on the energy per nucleon E/A of the primary radiation, according to [52], LET is the linear energy transfer of the primary particle, which characterizes its radiation quality, and ρ is the water density. Thus, the AT model considers a uniform dose distribution in the core of the particle track, while the radial dose decreases proportionally to r^{-2} in the penumbra region.

Typically, for low-LET radiation, the variations in trajectory and speed of the primary particle are neglected along the z -direction, i.e. the LET is constant within the cylinder of length $2R_N$. This is the so-called "track-segment conditions", in analogy to that used in [41]. Thus, the N_z layers observed the same energy deposition event z and the identical radial dose distribution $D(r)$. Instead, for the high-LET regime, this approximation is not applicable. Thus, we must take into account the loss of energy along the z -direction by the primary particle. For ions, this can be computed analytically, according to the Bethe-Bloch formula of Eq. 1.1, or using MC tools. Knowing the energy loss by ions per unit of path length in each layer in the z -direction of the cylinder, it is possible to compute the corresponding radial dose distribution in the xy -plane for the layer d_z and for each deposition event ν .

An usual practical limitation of this analytical approach for the radial dose is that the AT model also considers the propagation of secondary electrons until they completely lose their energy and are stopped by interactions with the water medium. This maximum distance traveled by these electrons corresponds to the radius R_p of the track, at which the radial dose is several orders of magnitude lower than the dose in the core region. Thus, this significantly increases the number of primary particles, i.e., the number of energy deposition events ν , required to deliver a given macroscopic dose D in the cylinder. A possible feasible approximation is that proposed in [17], where the maximum radius of a track is described by a reduced value corresponding to the extent where 80 % of the dose

is deposited by the track, that is,

$$R_{80} = R_c \cdot \exp\left(\frac{0.8(1 + 2 \ln(R_p/R_c) - 1)}{2}\right). \quad (4.2)$$

This approximation neglects the residual extension of the dose until the maximum distance allowed to the secondary electrons propagation, which is spread along a broader circular crown.

A total macroscopic dose deposition D is characterized by a series of ν energy deposition events $z_1, \dots, z_\nu$ on the whole cell nucleus. Given a cell nucleus domain d , the probability that ν events deposit energy z follows a Poissonian distribution of mean $\lambda_n := \frac{z_n}{z_F}$, where z_n is the mean energy deposition in the nucleus domain d and z_F is the first moment of the single-event distribution of energy deposition $f_{1;d}$, which can be either computed numerically with MC tools or measured experimentally, according to microdosimetry [42], as discussed in Section 2.1.3.

Then, assuming a Poissonian probability that a domain registers ν events, the energy deposition distribution is given by

$$f_d(z|z_n) := \sum_{\nu=0}^{\infty} \frac{e^{-\frac{z_n}{z_F}}}{\nu!} \left(\frac{z_n}{z_F}\right)^{\nu} f_{\nu;d}(z), \quad (4.3)$$

where $f_{\nu;d}(z)$ is the energy deposition distribution resulting from ν depositions. We assumed by convention that $f_{0;d}(z) = \delta_0(z)$, meaning that in the absence of deposition events within a given domain, the deposited energy is necessarily zero.

Furthermore, assuming that these energy deposition events are statistically independent, the distribution resulting from ν events can be computed convolving ν times the single event distribution [42]. Therefore, the distribution $f_{\nu;d}$ of the imparted energy z is computed iteratively as

$$\begin{aligned} f_{2;d}(z) &:= \int_0^{\infty} f_{1;d}(\bar{z}) f_{1;d}(z - \bar{z}) d\bar{z}, \\ &\dots, \\ f_{\nu;d}(z) &:= \int_0^{\infty} f_{1;d}(\bar{z}) f_{\nu-1;d}(z - \bar{z}) d\bar{z}. \end{aligned} \quad (4.4)$$

Thus, using this mathematical formulation for the energy deposition, we provide a general multi-domain cell nucleus description, along the lines of [3].

The average number of primary particles N_p imparted to deliver a macroscopic dose D to the cell nucleus is defined by the dose-LET relation and can be obtained by inverting the Eq. 1.2, i.e.

$$D = 1.6 \cdot 10^{-9} \cdot \frac{\text{LET} \cdot N_p}{\pi(R_N + R_{80})^2 \cdot \rho}, \quad (4.5)$$

considering a uniform distribution for the impact position of the projectile on a circle of radius $R_N + R_{80}$ on the xy -plane, in order to account for dose depositions due to particles passing outside the cell nucleus but delivering a significant contribution inside it.

The macroscopic dose D is delivered in a total irradiation time T_{irr} , with a mean dose rate $\dot{D} = D/T_{irr}$, according to Eq. 3.4. The elapsed time τ between two consecutive deposition events is sampled from an exponential distribution, which depends on the dose rate and the mean energy deposited in a single event, as we will see in Section 4.2. Thus, the actual total number of primary particles is obtained by summing the number of deposition events ν up to the end of the irradiation, at time T_{irr} .

The MS-GSM² model can handle any dose-delivery time structure, including those described in Section 3.2. In particular, in the case of irradiation with ions, the dose is delivered in a continuous way, as shown in Section 3.2, considering a unique pulse described by a macroscopic dose prescription D and an overall mean dose rate $\dot{D}$, according to Eq. 3.4. The number of discrete energy deposition events of this pulse are calculated according to the previous description. On the contrary, for electron beams, according to the experimental dose-delivery time structure shown in Section 3.2, the irradiation is characterized by a sequence of an integer number of pulses n_p , identified by a dose-per-pulse D_p and a pulse time t_p , interspersed with pauses, defined by a pulse repetition frequency f_p . Combining these physical parameters of the beam is possible to compute the mean and the instantaneous dose rates, according to Eqs. 3.2 and 3.3, respectively. The actual number of energy deposition events is sampled as before for each pulse.

Therefore, the final outcome of the MS-GSM² model at the end of the physical stage is the discretized radial and depth dose distributions for each energy deposition event administered by the respective primary particle in the cell nucleus, with a voxel-based resolution of the order of few μm^3 , and the elapsed times for each pair of consecutive particles. All of these results are obtained given the physical characteristics of the irradiation, such as the radiation quality of the primary particle, the prescribed macroscopic dose, the mean dose rate, the time structure of the beam, and the geometrical properties of the irradiated target, and serve as input for the next step of the model, which is the chemical stage.

(ii) The chemical stage

The second stage included in the MS-GSM² model is the chemical stage, which encompasses the pre-chemistry and the heterogeneous chemistry in an implicit way, while the homogeneous chemistry with an explicit approach, as we will see in detail in this Section. In fact, this stage describes the chemical events that occur from microseconds up to the scale of seconds, which corresponds to the duration of the homogeneous chemical stage as presented in Section 1.2.3, and on a spatial scale of the order of micrometers, as summarized in Table 4.1. As discussed previously, the homogeneous chemical stage is characterized, in particular, by the reactions with environmental oxygen and bio-molecules. In addition, it is possible to assume that there is no more diffusion of chemical species generated by IR.

We include chemistry in the development of the MS-GSM² model because we hypothesize that the homogeneous chemical stage is the crucial scale of UHDR radiation-induced biological damage to try to explain the mechanism underlying the FLASH effect. We start from the hypothesis of organic radical recombination proposed in [7], which is based on the concept that a specific radical could accumulate differently between normal tissue and

tumor under the UHDR regime. In particular, in [7] it was suggested that the different temporal evolution of the peroxy radical concentration $[\text{ROO}^\bullet]$ at a high dose rate could explain the sparing effect. The peroxy radical $\text{ROO}^\bullet$ is generated from the reaction between the alkyl radical $\text{R}^\bullet$ and O_2 and it is known to be an important source of adverse effects on lipids and DNA [7, 214, 278].

This chemical stage describes the network of chemical reactions illustrated in Figure 4.1. Following the approach proposed in [7], we described the chemical reaction network using a physicochemical analytical model of reaction kinetics. In particular, we included in the MS-GSM² model the time-evolution and reaction of radicals and other molecular species, describing them by a system of ODEs with the form of

$$\frac{d\boldsymbol{\xi}^d(t)}{dt} = \mathbf{f}(\boldsymbol{\xi}^d(t)) + \mathbf{G}\rho\dot{z}^d, \quad (4.6)$$

where $\boldsymbol{\xi}(t) = ([\text{O}_2], C_{\text{H}_2\text{O}_2}, C_{\text{OH}^\bullet}, [\text{R}^\bullet], [\text{ROO}^\bullet]) (t)$ is the vector of concentration at time t in the domain d for each chemical species; ρ is the water density; $\mathbf{G}$ is the vector of G-values for each chemical species at microsecond, as defined in Section 1.2.2, which describe the chemical system at the end of the heterogeneous chemical stage; $\mathbf{f}(\boldsymbol{\xi}^d(t))$ is the vector of the reaction rates for each chemical species, as defined in Eq. 1.2.2, which describe the complex network of chemical reactions illustrated in Figure 4.1 and are a function of the concentration of the five chemical species $\boldsymbol{\xi}^d(t)$ and depend on concentrations of other chemical species $\bar{\boldsymbol{\xi}}$ that are considered constant during irradiation, and also on rate constants $\mathbf{K}$, as defined in Section 1.2.2. In addition, this system is coupled with a set of initial conditions $\boldsymbol{\xi}_0$ at the impact time τ of each primary particle, as we will see in detail in Section 4.2.

As we can see from Eq. 4.6, the time evolution of a chemical species ξ in the domain d depends on two main terms: the first one is the reaction term, which describes the reactions between ions, molecules, free radicals, and environmental species; the second one is the generation term, which describes the formation of free radicals and other chemical species induced by IR.

In addition, Eq. 4.6 is a generalization of that proposed in Eq. 3.47. In fact, differently to [7], we do not assume a continuous dose-rate effect, which is represented by $\frac{dD(t)}{dt}$ in the original equations, but rather the effect of energy deposition is modulated by the tracks arrival time. In particular, $\dot{z}^d$ is a purely discontinuous jump process that describes the local energy deposition within the domain d , depending on the radiation quality of the primary radiation and the mean dose rate of the beam. This allows extreme flexibility in the modelization of the effect of dose rate so that any dose-rate structure can be implemented into the MS-GSM². Specifically, the terms $\dot{z}$ are characterized by random impulses in both time and magnitude, which depict the stochastic nature of the time delivery structure.

Furthermore, also for the chemistry, we provide a general multi-domain cell nucleus description, to take into account possible spatial heterogeneity in the generation of chemical species. In this way, the MS-GSM² model can take into account both spatial effects, due to different radiation quality, and temporal effect, due to different dose-delivery time structure, in the formation and evolution of chemical species in a cellular environment.

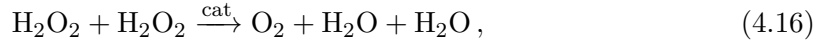
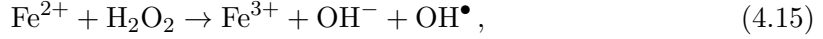
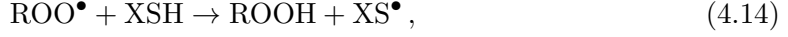
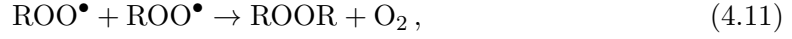
Thus, the complete system of ODEs that describes the chemical environment of the cell, considered by MS-GSM² for each domain d , is the following

$$\left\{ \begin{array}{l} \frac{d}{dt} [\text{O}_2]^d(t) = k_1 \cdot [\text{R}^\bullet]^d(t) \cdot [\text{O}_2]^d(t) + \\ \quad + k_2 \cdot [\text{ROO}^\bullet]^d(t) \cdot [\text{ROO}^\bullet]^d(t) + \\ \quad + k_3 \cdot \frac{[\text{cat}]}{k_m + [\text{H}_2\text{O}_2]^d(t)} \cdot [\text{H}_2\text{O}_2]^d(t), \\ \frac{d}{dt} C_{\text{H}_2\text{O}_2}^d(t) = k_4 \cdot [Fe^{2+}] \cdot [\text{H}_2\text{O}_2]^d(t) + \\ \quad - 2k_3 \cdot \frac{[\text{cat}]}{k_m + [\text{H}_2\text{O}_2]^d(t)} \cdot [\text{H}_2\text{O}_2]^d(t) + \\ \quad + G_{\text{H}_2\text{O}_2} \rho \dot{z}^d, \\ \frac{d}{dt} C_{\text{OH}^\bullet}^d(t) = -k_5 \cdot [\text{RH}] \cdot [\text{OH}^\bullet]^d(t) + \\ \quad + k_4 \cdot [Fe^{2+}] \cdot [\text{H}_2\text{O}_2]^d(t) + \\ \quad - k_6 \cdot [\text{XSH}] \cdot [\text{OH}^\bullet]^d(t) + \\ \quad + G_{\text{OH}^\bullet} \rho \dot{z}^d, \\ \frac{d}{dt} [\text{R}^\bullet]^d(t) = k_5 \cdot [\text{RH}] \cdot [\text{OH}^\bullet]^d(t) + \\ \quad - k_1 \cdot [\text{R}^\bullet]^d(t) \cdot [\text{O}_2]^d(t) + \\ \quad - k_7 \cdot [\text{XSH}] \cdot [\text{R}^\bullet]^d(t) + \\ \quad - 2k_8 \cdot [\text{R}^\bullet]^d(t) \cdot [\text{R}^\bullet]^d(t) + \\ \quad + G_{\text{R}^\bullet} \rho \dot{z}^d, \\ \frac{d}{dt} [\text{ROO}^\bullet]^d(t) = k_1 \cdot [\text{R}^\bullet]^d(t) \cdot [\text{O}_2]^d(t) + \\ \quad - k_9 \cdot [\text{XSH}] \cdot [\text{ROO}^\bullet]^d(t) + \\ \quad - 2k_2 \cdot [\text{ROO}^\bullet]^d(t) \cdot [\text{ROO}^\bullet]^d(t), \end{array} \right. \quad (4.7)$$

where $[\text{H}_2\text{O}_2]^d(t) = \frac{C_{\text{H}_2\text{O}_2}^d(t)}{1 + \frac{K_{\text{H}_2\text{O}_2}}{[\text{H}^+]}}$ and $[\text{OH}^\bullet]^d(t) = \frac{C_{\text{OH}^\bullet}^d(t)}{1 + \frac{K_{\text{OH}^\bullet}}{[\text{H}^+]}}$, that describe the acid-base equilibria, in analogy to [7]; $\mathbf{K} = (k_1, \dots, k_9)$ is the vector of rate constants; while G_ξ are the G-values for each chemical species ξ , according to TRAX-CHEM simulations [28, 29], as discussed in Section 2.2.1, computed at 1 μs , under the assumption of independent tracks throughout the heterogeneous chemical stage. In this way, as announced at the beginning of this Section, the model can take into account implicitly the faster scales of the chemistry, namely the pre-chemical stage and the early heterogeneous stage of water radiolysis, which we assume to be instantaneous, and are incorporated through the production terms as described by G-values of chemical species in the system of equations. The evolution of the chemical species is followed up to a time $T = T_{\text{irr}} + T_{\text{off}} > 500$ s, where T_{off} is the time for the relaxation of the chemical system, once the irradiation is finished, i.e. $\dot{d} = 0$.

In addition, the set of chemical reactions that occur during the homogeneous chemical stage and that is considered by the model in an explicit way is the following





where Eq. 4.8 describes the generation of the organic radical $R^\bullet$ from the organic substrate RH; Eq. 4.9 refers to the production of the organic peroxy radical $ROO^\bullet$; Eqs. 4.10 and 4.11 are the self-annihilation of organic radicals and organic peroxy radicals, respectively; Eqs. 4.12, 4.13, and 4.14 are the scavenging action of the antioxidants XSH, which had been partially neglected in [7]; Eq. 4.15 is the Fenton reaction mediated by labile iron; Eq. 4.16 is the scavenging action of catalase on hydrogen peroxide H_2O_2 .

In summary, the output of the MS-GSM² model for this chemical stage is the time evolution of the concentrations of five chemical species for a given cell, which is influenced by the evolution of the previous physical stage, depending on the characteristic of the irradiation beam. In fact, as we can see from Eq. 4.6, the physical and chemical stages are strictly connected by the production term. In particular, the time evolution of the chemical environment depends on the imparted dose, the dose rate, and the radiation quality of the primary particle via the term $\dot{z}^d$ of the ODEs' system. The chemical environment will influence the induction of indirect DNA damage during the next stage, which is the bio-chemical stage.

Optimization of the chemical reaction network

The derived ODEs system shown in Eq. 4.7 is a severe optimization of the one proposed in [7], on the one hand by simplifying some equations, cutting away some negligible reactions, on the other hand by adjusting some reaction constants, among all of those listed in Sections S.1 and S.2 of [7], as we will see later in Section 4.2. In particular, in [7], the reactions involving only chemical species derived from the water radiolysis, such as e_{aq}^- , $OH^\bullet$, $H^\bullet$, H_2 , $O_2^{\bullet-}$, have much lower rates than both the reactions between these species with organic molecules, and those between organic species alone. Indeed, it is possible to neglect these types of reactions, thus separating the two timescales: a faster one which we assume to be instantaneous, i.e., the early heterogeneous stage of water radiolysis, and that of the reactions involving the various organic chemical species, such as RH, $R^\bullet$, $ROO^\bullet$, vitamin E, thiols and their derivatives, described explicitly by the reaction rates. It is worth stressing that the $ROO^\bullet$ species is the one which we directly link to the FLASH sparing effect, while the antioxidants modulate the intensity of the biological response at UHDR regime, as we will see later. Furthermore, from a computational point of view, this optimization of the chemical network has a huge impact for the numerical resolution of

the system of ODEs. In fact, even if after this optimization, for example considering the number of domains in the xy -plane $N_{xy} = 52$, we have a system of five ODEs describing chemical kinetics, coupled with biological processes (see Eq. 4.36), for each domain.

Starting from the definition of the AUC of the time evolution of the organic peroxy radical $\text{ROO}^\bullet$, which is shown in Eq. 3.49 and initially proposed by Labarbe et al. in [7], we consider the AUC relative variation of a given reaction kinetic model with respect to the original Labarbe model, that is

$$\delta\text{AUC}_i = \frac{\text{AUC}_i - \text{AUC}_{\text{LM}}}{\text{AUC}_{\text{LM}}}$$

where $i \in \{\text{MS} - \text{GSM}^2, \text{WM}\}$. In particular, with AUC_{LM} we refer to the AUC calculated using the original system proposed by Labarbe et al. in [7]; with $\text{AUC}_{\text{MS} - \text{GSM}^2}$ we refer to the results obtained through the optimized system of ODEs reported in Eq. 4.7; instead, with AUC_{WM} we refer to the findings using the approximated reaction network discussed by Wardman in [275]. In detail, Wardman speculated on the possibility of using only the reactions in Eqs. 4.8, 4.9, 4.11, and 4.14 to describe in a complete way the chemical system proposed by Labarbe.

In Figure 4.2, we report the relative variation of AUC of the time evolution of the organic peroxy radical $\text{ROO}^\bullet$, i.e. δAUC_i , as a function of the mean dose rate, for continuous electron irradiation at different macroscopic doses (1, 10, and 30 Gy) and initial oxygenation levels (0.5, 4, and 21 % O_2). In particular, we show a comparison between the numerical results obtained through our model, comparing them with the results obtained with the approximated model of Wardman in [275]. From these plots, we can appreciate how our model is able to reproduce the results obtained with the Labarbe's model better than the Wardman's model.

In addition, our model also consistently describes the temporal evolution of other chemical species, such as O_2 and H_2O_2 , in agreement with Labarbe's model, as shown in Figure 4.3.

(iii) The bio-chemical stage

The bio-chemical stage takes place on a time scale up to a few seconds with spatial resolution of the cell domain, as summarized in Table 4.1. This is a crucial stage because it describes the interconnections between the deposition of radiation energy and the chemical environment through the yield of biological damage. In particular, the MS-GSM² is capable to handle both the direct and the indirect action of radiation in the formation of DNA damage, taking into account the impact of the radiation quality, the dose-delivery time structure, and the environmental conditions of the cell.

From a mathematical point of view, as we discussed in Section 2.3.1, the peculiarity of the GSM² is that it overcomes the usual assumptions of a Poissonian distribution of the number of radiation-induced DNA damage, which limits the possible predictions of biological endpoints, as demonstrated in [69]. The GSM² assumes the possibility of generation of two types of DNA damage induced by IR within each domain d : the lethal lesion Y , i.e., irreparable damage, leading to cell inactivation, and sub-lethal lesion X ,

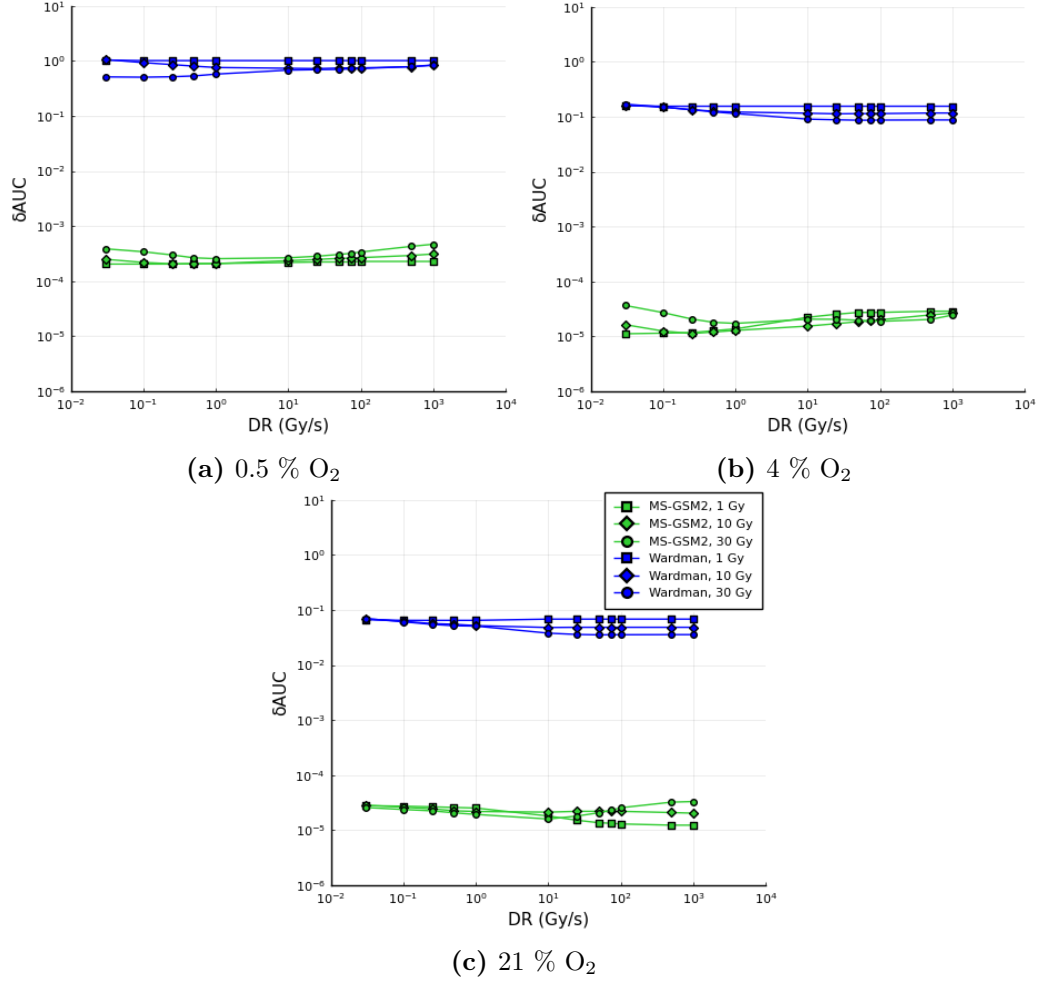


Figure 4.2: Relative variation of the area under the curve (AUC) of the time evolution of the organic peroxy radical $\text{ROO}^\bullet$, which is defined in Eq. 4.1, as a function of the mean dose rate. Comparison between our optimized model (green) in Eq. 4.7, and the approximated model by Wardman (blue) in [275] with respect to the original model by Labarbe in [7], in the case of a continuous electron irradiation for different macroscopic doses (1, 10, and 30 Gy) and initial oxygenation levels (0.5, 4, and 21 % O₂).

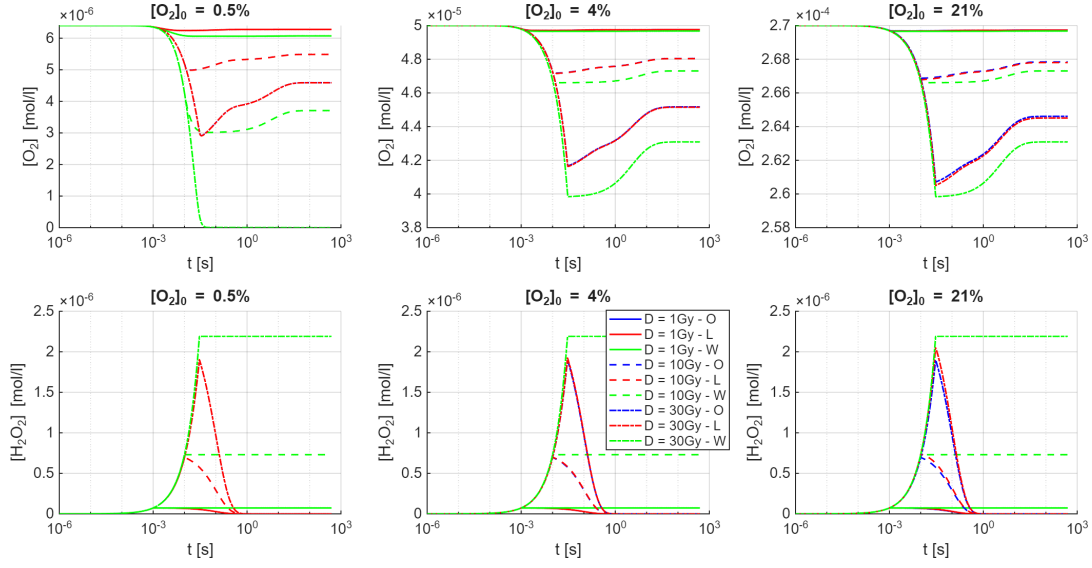


Figure 4.3: Comparison of the time evolution of **a)** oxygen O_2 and **b)** hydrogen peroxide H_2O_2 concentration between our optimized model (blue) in Eq. 4.7, the approximated model by Wardman (green) in [275], and the original model by Labarbe (red) in [7], in the case of a continuous electron irradiation with a mean dose rate of 1000 Gy/s, for different macroscopic doses (1, 10, and 30 Gy) and initial oxygenation levels (0.5, 4, and 21 % O_2).

i.e., repairable damage, which can be either repaired or become a lethal lesion, where Y and X are two $\mathbb{N}$ -valued random variables.

For simplicity, as a first step in developing the multiscale extension of the GSM² model, we neglect the impact of chemistry, focusing only on the formation of direct DNA damage by IR. Starting from the energy deposition distribution $f_d(z|z_n)$ defined in Eq. 4.3, and denoting by $p_z(x, y|z)$ the initial joint distributions for the number of sub-lethal and lethal lesions for a given energy deposition z , we obtain

$$p_0(x, y) = \sum_{\nu=0}^{\infty} \int_0^{\infty} \cdots \int_0^{\infty} p_z(x, y|z_1, \dots, z_{\nu}) \frac{e^{-\frac{z_n}{z_F}}}{\nu!} \left(\frac{z_n}{z_F}\right)^{\nu} f_{\nu;d}(z_1, \dots, z_{\nu}) dz_1 \dots dz_{\nu}, \quad (4.17)$$

which is the initial distribution of damages.

Consider thus the quantity $p_z(x, y|z_1, \dots, z_{\nu})$ which gives the probability of observing x sub-lethal lesions and y lethal lesions resulting from the energy depositions $z_1, \dots, z_{\nu}$. Denote by $\kappa(z_1, \dots, z_{\nu})$ and by $\lambda(z_1, \dots, z_{\nu})$ the average yields of sub-lethal and lethal lesions respectively, for the energy depositions $z_1, \dots, z_{\nu}$. The formation of lesions by different deposition events is statistically independent because the events are independent. The formation of lesions by a single energy deposition event is approximately statistically independent. It follows that the distribution of the number of lesions that result from the

i -th deposition event is approximately Poissonian, that is

$$p_z(x_\nu, y_\nu | z_1, \dots, z_\nu) := e^{-\kappa(z_1, \dots, z_\nu)} \frac{(\kappa(z_1, \dots, z_\nu))^{x_\nu}}{x_\nu!} e^{-\lambda(z_1, \dots, z_\nu)} \frac{(\lambda(z_1, \dots, z_\nu))^{y_\nu}}{y_\nu!}. \quad (4.18)$$

The probability that x and y total lesions will be produced if energy depositions $z_1, \dots, z_\nu$ occur is

$$p_z(x, y | z_1, \dots, z_\nu) := \sum_{\substack{x_1, \dots, x_\nu \\ y_1, \dots, y_\nu}} p_z^X(x_1, y_1 | z_1) p_z^X(x_2, y_2 | z_1, z_2) \dots p_z^X(x_\nu, y_\nu | z_1, \dots, z_\nu), \quad (4.19)$$

where above summation runs over $x_1, \dots, x_\nu$ and $y_1, \dots, y_\nu$ such that $x_1 + \dots + x_\nu = x$ and $y_1 + \dots + y_\nu = y$. Let's denote for short

$$\bar{\kappa}(z_1, \dots, z_\nu) := \sum_{i=1}^{\nu} \kappa(z_1, \dots, z_i), \quad \bar{\lambda}(z_1, \dots, z_\nu) := \sum_{i=1}^{\nu} \lambda(z_1, \dots, z_i), \quad (4.20)$$

so that the probability of x and y lesions is given by

$$p_z(x, y | z_1, \dots, z_\nu) = e^{-\bar{\kappa}(z_1, \dots, z_\nu)} \frac{(\bar{\kappa}(z_1, \dots, z_\nu))^x}{x!} e^{-\bar{\lambda}(z_1, \dots, z_\nu)} \frac{(\bar{\lambda}(z_1, \dots, z_\nu))^y}{y!}. \quad (4.21)$$

The standard assumption for conventional irradiation is that the average yield of lesions is proportional to the dose, that is

$$\bar{\kappa}(z_1, \dots, z_\nu) = \kappa(z_1 + \dots + z_\nu) = \kappa z, \quad \bar{\lambda}(z_1, \dots, z_\nu) = \lambda(z_1 + \dots + z_\nu) = \lambda z \quad (4.22)$$

for positive constants $\kappa > 0$ and $\lambda > 0$. In [3, 4] the same choice has been made, implying that the average yield of the lesion for a given dose D is given by

$$\sum_{x \geq 0} x p_0(x, y) = \kappa D. \quad (4.23)$$

The quantity $\kappa(z_1, \dots, z_\nu)$ is generally taken to be either a linear or quadratic function that summarizes several physical, chemical, and biological effects. It is a function of the type of ionizing particle, the LET, details of the track structure, radical diffusion, and reaction rates, the phase in the cell cycle, and the chemical environment of the cell. Additionally, as it is widely acknowledged that the temporal and spatial distribution of various particle tracks and energy deposition significantly influence the emergence of the FLASH effect, our forthcoming approach is geared towards explicitly incorporating both temporal and spatial stochasticity, as reported in Table 4.1. This was achieved by closely coupling the energy deposition with a rapid chemical characterization of the surrounding environment, as we discussed in the previous Section.

The second fundamental step in extending the GSM² model is the introduction of a protracted dose-rate term $\dot{d}$, which modulates the energy deposition events and subsequent lesion creation, according to that proposed in [3]. This term explicitly models the series of energy deposition in the whole cell nucleus $z_1, \dots, z_\nu$, where ν , in this case, is the number

of events on the nucleus. The damage creation yield can be described by the following pathways:



where Z_X and Z_Y are two Poisson random variables, with average, respectively, κz and λz , for the sub-lethal and lethal damage, respectively, that are described via unitary Poisson jump processes $P_d^{(c)}$ for the cell c . These Poisson processes account for energy deposition and are, therefore, inter-domain processes that can affect more than a single domain at once. Thus, each single energy deposition can induce a random number $Z_X^{(c,d)}$ and $Z_Y^{(c,d)}$, of sub-lethal or lethal lesions, respectively, on each domain $d = 1, \dots, N_d$. In detail, according to the above description, given an energy deposition z subsequent to a track deposition, a certain number of DNA damages are created within the cell nucleus, $Z_X^{(c,d)}$ and $Z_Y^{(c,d)}$. These random variables follow a distribution

$$\begin{aligned} p(x|z) &= e^{-\kappa(z)} \frac{(\kappa(z))^x}{x!} \\ p(y|z) &= e^{-\lambda(z)} \frac{(\lambda(z))^y}{y!}. \end{aligned} \quad (4.25)$$

for the sub-lethal and lethal lesions, respectively.

The next crucial step in the construction of the MS-GSM² model is the inclusion of the indirect action of radiation, which is mediated by free radicals produced during the chemical stages. Thus, we performed several generalizations regarding the creation of DNA damage, to include the influence of dose rate, oxygenation, and scavengers. In the following paragraphs, we will discuss in detail these extensions.

First of all, we include the impact of environmental oxygenation in the induction of DNA damage, to consider the so-called "oxygen fixation" effect, discussed in Section 1.4.2. Thus, the average damage yield consequent to an energy deposition z is calculated as

$$\kappa(\text{LET}, [\text{O}_2]) z = \frac{\text{DSB}(\text{LET})}{\text{OER}(\text{LET}, [\text{O}_2])} z. \quad (4.26)$$

Above, $\text{DSB}(\text{LET})$ is the analytical formula representing the average yield of damage obtained via track-structure simulations in [279], whereas $\text{OER}(\text{LET}, [\text{O}_2])$ represents the oxygen enhancement ratio, i.e., the ratio of doses giving the same biological effect in the presence of a given concentration of oxygen $[\text{O}_2]$ as compared to the full oxygenation (normoxia), characterizes a well-documented experimental phenomenon in radiation biophysics where damage fixation increases at higher oxygen concentrations, leading to enhanced radiation lethality, as discussed in detail in Section 1.4.2. The OER is described in [30, 172] as

$$\text{OER}(\text{LET}, [\text{O}_2]) = \frac{K_{\text{O}_2} \cdot \frac{K_{\text{LET}} \cdot M + \text{LET}^\gamma}{K_{\text{LET}} + \text{LET}^\gamma} + [\text{O}_2]}{K_{\text{O}_2} + [\text{O}_2]}, \quad (4.27)$$

where M represents the maximum effect, K_{O_2} is the flex point for $[O_2]$, as outlined in [172], K_{LET} denotes the flex point for LET, that is, the average energy deposited per unit path length, which characterizes the radiation quality of the ionizing particle, and γ is an additional fitted parameter, as emphasized in [30].

Then, we explicitly take into account the impact of the chemical environment of the cell, and thus the influence of the dose rate in the formation of DNA lesions. We formally introduce a fast component that describes the evolution of the chemical environment and, further modeling explicitly the dose rate effect, we will assume that the function κ depends on the chemical environment as modified by radiation. To consider the impact of the chemical environment, the damage yield is divided into a *direct contribution*, which describes the lesions formed immediately from energy deposition regardless of the chemical environment, and an *indirect contribution* resulting from the action of radicals and other molecular species. Thus, we explicitly include in the functions $\kappa(z)$ and $\lambda(z)$ the effects that the chemical environment and LET have on the number of damages created. In particular, we assume that the average yield of sub-lethal lesion per unit Gy under the conventional dose rate is $\kappa > 0$. Then, we assume that a fraction $q \in [0, 1]$ of κ is due to indirect damage, i.e., radical-mediated. In contrast, the remaining $1 - q$ is due to direct damages, and this fraction depends on LET, according to several experimental data such as [280]; we assume that only the number of indirect damages created depends on the chemical environment.

Formally, the formation of indirect DNA damage is described by an additional unitary Poisson jump process $P_{indir}^{(c,d)}$, in analogy to that previously introduced for the direct DNA damage $P_{dir}^{(c,d)}$. This process depends explicitly on the chemical environment and on energy depositions. We assume that following an energy deposition event z , on average, a proportion $(1 - q)\kappa(LET, [O_2])$ yield a direct damage. In contrast, the remaining $q\kappa(LET, [O_2])$ will yield an indirect damage. These proportions are again calculated using the formulas given in [279].

Thus, being the direct damage independent of the chemical environment, we describe the number of induced lesions per unit Gy with a Poisson random variable $Z_{X;dir}^{(c,d)}$ of average

$$\kappa_{dir}(LET, [O_2]) = (1 - q)\kappa(LET, [O_2]) , \quad (4.28)$$

which is described by the following unitary Poisson jump

$$P_{X;dir}^{(c)}(\dot{d}t) . \quad (4.29)$$

Regarding indirect damages, we assume that the number of such damages depends on the chemical environment and the irradiation pattern. We assume that the concentration of peroxy radical $[ROO^\bullet]$ is directly linked to the number of indirect damage created, so we describe this process with a Poisson random variable $Z_{X;indir}^{(c,d)}$ of average per unit Gy

$$\kappa_{indir}(LET, [O_2]) = q\kappa(LET, [O_2]) , \quad (4.30)$$

which is modulated by the dose rate effect, via the following unitary Poisson jump

$$P_{X;indir}^{(c,d)}\left(\varrho \int_0^t [ROO^\bullet](s) ds\right) . \quad (4.31)$$

for a suitable constant $\varrho > 0$. This formulation enables us to quantify the contribution of organic peroxy radicals to DNA damage formation, as a function of dose, dose rate, radiation quality, and oxygenation level. In fact, following [7], we hypothesize that the different temporal evolution of the peroxy radical concentration $[\text{ROO}^\bullet]$ at a high dose rate is at the origin of the FLASH effect. In particular, differently from [7], we conjecture that the number of lethal and sub-lethal damages depends on the local peroxy radical concentration $[\text{ROO}^\bullet]$, since the contribution of $[\text{ROO}^\bullet]$ to the damage of biological structures, including DNA, is well known [7]. Thus, our idea of the mechanism behind the FLASH effect is that UHDR regimes modify the $[\text{ROO}^\bullet]$ evolution which can, in turn, change the indirect damage yield, according to Eq. 4.31, that eventually leads to a cell survival reduction. This effect is incorporated via the time integral of $[\text{ROO}^\bullet]$ in Eq. 4.31. This formulation captures not only the instantaneous absolute concentration of $\text{ROO}^\bullet$, but also its time-dependent behavior, thereby capturing the persistence of the cellular environment's toxicity over time. This mathematical treatment is analogous to the standard pharmacokinetic description of drug delivery, as outlined in [7].

A completely analogous argument has been used to form lethal lesions Y , with the corresponding function λ . In this case, the random variables used to describe the formation of direct lethal and indirect lethal lesions are labeled as $Z_{Y;\text{dir}}^{(c,d)}$ and $Z_{Y;\text{indir}}^{(c,d)}$, respectively.

Therefore, based on the formulation discussed above, the MS-GSM², through the sub-lethal and lethal damage yields, i.e. κ and λ , respectively, can capture various effects induced by IR, such as the impact of radiation quality, the oxygen-dependent fixation of damage, and the modulation of cellular toxicity triggered by UHDR.

Overall, the damage creation yield proposed in Eq. 4.24 is now updated and it can be summarized by the following network

$$X \xrightarrow{d} \begin{cases} X + Z_{X;\text{dir}} & \text{with probability } 1 - q \in [0, 1], \\ X + Z_{X;\text{indir}} & \text{with probability } q \in [0, 1], \end{cases} \quad (4.32)$$

for sub-lethal lesions, while by

$$Y \xrightarrow{d} \begin{cases} Y + Z_{Y;\text{dir}} & \text{with probability } 1 - q \in [0, 1], \\ Y + Z_{Y;\text{indir}} & \text{with probability } q \in [0, 1], \end{cases} \quad (4.33)$$

for lethal lesions.

Therefore, the final result of the bio-chemical stage of MS-GSM² is the initial distributions of sub-lethal and lethal lesions in a cell nucleus, taking into account the effect of physical parameters, such as radiation quality, dose, and dose rate, and the chemical characteristics of the cellular environment, in particular, the oxygenation and scavenging properties. In fact, the formulation described above offers a comprehensive overview of the process of radiation-induced damage formation, distinguishing between direct and indirect lesions. It introduces an explicit dependency on the chemical environment through the OER formulation and explicitly incorporates the impact of radicals on the damage yield. Consequently, as the pattern of radiation energy deposition significantly influences this factor, the formulation enables us to depict the dual dependency of damage yield on

oxygenation and the presence of organic radicals. Thus, this novel formulation of damage formation included in MS-GSM² allows us to investigate the impact of progressively higher dose rates on the biological stage, as we will see in the following Section, with a particular focus on UHDR irradiation in the context of FLASH RT.

(iv) The biological stage

The final scale of radiation-induced DNA damage included in the MS-GSM² model is the so-called biological stage. The model describes the slow biological events that occur on a time scale from seconds to days, at the cellular level of a few micrometers, as summarized in Table 4.1. In particular, it describes the time evolution of sub-lethal and lethal lesions that are generated during the previous stage by the direct action of IR or by the indirection action, which is mediated by radicals. Formally, the MS-GSM² model describes the state of the system at time t considering two random variables $(Y(t), X(t))$, i.e., the number of lethal and sub-lethal lesions at time t , respectively. In particular, as discussed in Section 2.3.1, a sub-lethal lesion can undergo three different pathways,

$$\begin{cases} X \xrightarrow{r} \emptyset, \\ X \xrightarrow{a} Y, \\ X + X \xrightarrow{b} Y, \end{cases} \quad (4.34)$$

where a is the rate of the evolution of a sub-lethal lesion to a lethal one, b describes the rate of pairwise interaction of two sub-lethal lesions, while r represents the repair rate, with $\emptyset$ the ensemble of healthy cells. These processes are described by $P_h^{(c,d)}$, with $h \in \{a, b, r\}$, that are independent unitary Poisson jump-processes acting on a single domain d of the cell c , [281].

First of all, in order to generalize the GSM² model to include the impact of dose rate and chemistry in the biological stage, we reformulate the original model, giving a pathwise description for the MME, described in Section 2.3.1 in Eq. 2.21, in terms of jump-type *Stochastic Differential Equations* (SDE). In particular, it can be seen, [281, Chapter 13], that Eq. 2.21 describes the time evolution for the probability density function associated with the following SDE for each domain $d = 1, \dots, N_d$, of the cell c ,

$$\Sigma^c : \begin{cases} Y^{(c,d)}(t) = Y_0^{(c,d)} + P_a^{(c,d)} \left(\int_0^t a X^{(c,d)}(s) ds \right) + \\ \quad + P_b^{(c,d)} \left(\int_0^t b X^{(c,d)}(s) (X^{(c,d)}(s) - 1) ds \right), \\ X^{(c,d)}(t) = X_0^{(c,d)} - P_a^{(c,d)} \left(\int_0^t a X^{(c,d)}(s) ds \right) + \\ \quad - P_r^{(c,d)} \left(\int_0^t r X^{(c,d)}(s) ds \right) + \\ \quad - 2P_b^{(c,d)} \left(\int_0^t b X^{(c,d)}(s) (X^{(c,d)}(s) - 1) ds \right), \end{cases} \quad (4.35)$$

where $X_0^{(c,d)}$ and $Y_0^{(c,d)}$ are the initial distributions of sub-lethal and lethal lesions induced by IR, respectively, while the terms $P_h^{(c,d)}$, with $h \in \{a, b, r\}$, are independent unitary Poisson jump-processes, [281], that describe the evolution in time of DNA lesions, according to the biological pathways in Eq. 4.34.

Thus, including all the extensions proposed in the previous discussed stages, we can generalize the SDE description of GSM² in the case of the MS-GSM² model for UHDR investigations. In summary,

- (i) For the physical stage, we include a protracted dose-rate term that accounts for the energy deposition events and subsequent lesion creation, according to Eq. 4.24; such a term explicitly models the series of energy deposition in the whole cell nucleus $z_1, \dots, z_\nu$, where ν , in this case, is the number of events on the nucleus.
- (ii) Then, for the bio-chemical stage, we split the creation of direct and indirect DNA damage according to Eqs. 4.32 and 4.33, whereas the former happens instantaneously at the energy deposition, the latter depends on the chemical environment and how organic radicals recombine in time. This latter effect is influenced by the time structure of the dose delivery.
- (iii) Subsequently, to describe the formation of indirect DNA damages, we include the chemical stage, which is described by a set of differential equations, i.e., Eqs. 4.7, that models the time-evolution and reaction of radicals and other molecular species, for different oxygenation and scavenger conditions.
- (iv) At last, for the bio-chemical stage, we take into account the so-called "oxygen fixation" effect in the formation of DNA damages, according to Eq. 4.26.

Thus, the resulting MS-GSM² model is an extremely general biophysical model, able to provide a mechanistically grounded description of several effects of random processes in space and time.

In the end, we obtained the following system of equations Σ_{MS}^c for the MS-GSM² for each domain $d = 1, \dots, N_d$ of the cell c ,

$$\Sigma_{\text{MS}}^c : \begin{cases} Y^{(c,d)}(t) &= P_a^{(c,d)} \left(\int_0^t a X^{(c,d)}(s) ds \right) + \\ &+ P_b^{(c,d)} \left(\int_0^t b X^{(c,d)}(s) (X^{(c,d)}(s) - 1) ds \right) + \\ &+ Z_{Y;\text{dir}}^{(c,d)} P_{Y;\text{dir}}^{(c)} \left(\dot{d} t \right) + \\ &+ Z_{Y;\text{indir}}^{(c,d)} P_{Y;\text{indir}}^{(c,d)} \left(\varrho \int_0^t [\text{R}\bar{\text{O}}\text{O}\bullet](s) ds \right), \\ X^{(c,d)}(t) &= -P_a^{(c,d)} \left(\int_0^t a X^{(c,d)}(s) ds \right) + \\ &- P_r^{(c,d)} \left(\int_0^t r X^{(c,d)}(s) ds \right) + \\ &- 2P_b^{(c,d)} \left(\int_0^t b X^{(c,d)}(s) (X^{(c,d)}(s) - 1) ds \right) + \\ &+ Z_{X;\text{dir}}^{(c,d)} P_{X;\text{dir}}^{(c)} \left(\dot{d} t \right) + \\ &+ Z_{X;\text{indir}}^{(c,d)} P_{X;\text{indir}}^{(c,d)} \left(\varrho \int_0^t [\text{R}\bar{\text{O}}\text{O}\bullet](s) ds \right), \\ \frac{d}{dt} \xi^d(t) &= f_{\xi^d} \left(\xi^d(t) \right) + G_{\xi^d} \rho \dot{z}^d, \end{cases} \quad (4.36)$$

where the first two equations in 4.36 is a pathwise description in terms of jump-type SDE of the processes in Eqs. 4.32, 4.33, and 4.34, while the last equation represents the random ODE system of Eq. 4.7 describing the chemical environment. The multiscale nature of the

MS-GSM² model clearly emerges from these driving equations, where the slow biological processes are described by discrete stochastic differential equations, whereas the chemical kinetics are continuous ordinary differential equations.

The final biological endpoint predicted is the cell survival probability [4], defined as

$$S := \mathbb{P} \left(\lim_{t \rightarrow \infty} \left(Y^{(c,1)}(t), \dots, Y^{(c,N_d)}(t) \right) = \mathbf{0} \right), \quad (4.37)$$

allowing a strict validation of the MS-GSM² model on experimental data, as we will see in Section 4.4.

We emphasize that all the enhancements discussed in this Section render the MS-GSM² highly flexible and comprehensive, enabling the examination of various scenarios: physically, it accommodates any radiation dose-delivery complex time structure, type, and energy without simplification on the dose rate structures as done in several existing mechanistic models where an average macroscopic dose rate is mostly assumed; chemically, it considers all chemical and environmental conditions, such as oxygenation and scavenger concentrations; biologically, it describes multiple effects, from well-established ones like the oxygen effect on DNA damage fixation during conventional irradiation, to widely debated ones like the sparing effect seen at UHDR regimes across various cell lines. The main characteristics of the MS-GSM² are summarized in Figure 4.4.

However, the current version of the model, fully described by Eq. 4.36, also has certain limitations regarding the radiation-induced chemical and biological effects that it can represent. For example, it currently cannot describe cell death pathways that are not mediated by DNA damage, such as ferroptosis, which is triggered by lipid membrane damage, or apoptosis, which is regulated by key proteins such as p53 and p21. Moreover, the modeled cellular environment does not fully capture the complexity of the actual biochemical environment, which remains only partially understood to this day. Finally, the model does not currently account for possible UHDR-induced effects occurring during the heterogeneous chemical phase, such as inter-track effects. These and other limitations, along with potential directions for extending the model, will be discussed in more detail in Section 4.6.

4.2 Numerical implementations

Stepwise construction of the MS-GSM²

The aim of this Section is to calculate a numerical solution to the Σ_{MS}^c system, which is reported in Eq. 4.36 and illustrated in Figure 4.5B. In order to do this, we performed the following steps, which are also schematized in Figure 4.5A:

1. We consider a cylinder of radius R_N and length $2R_N$ that mimics the cell nucleus. We divided it into N_d total domains, i.e., N_{xy} horizontal domains and N_z longitudinal layers, of side l_d ;

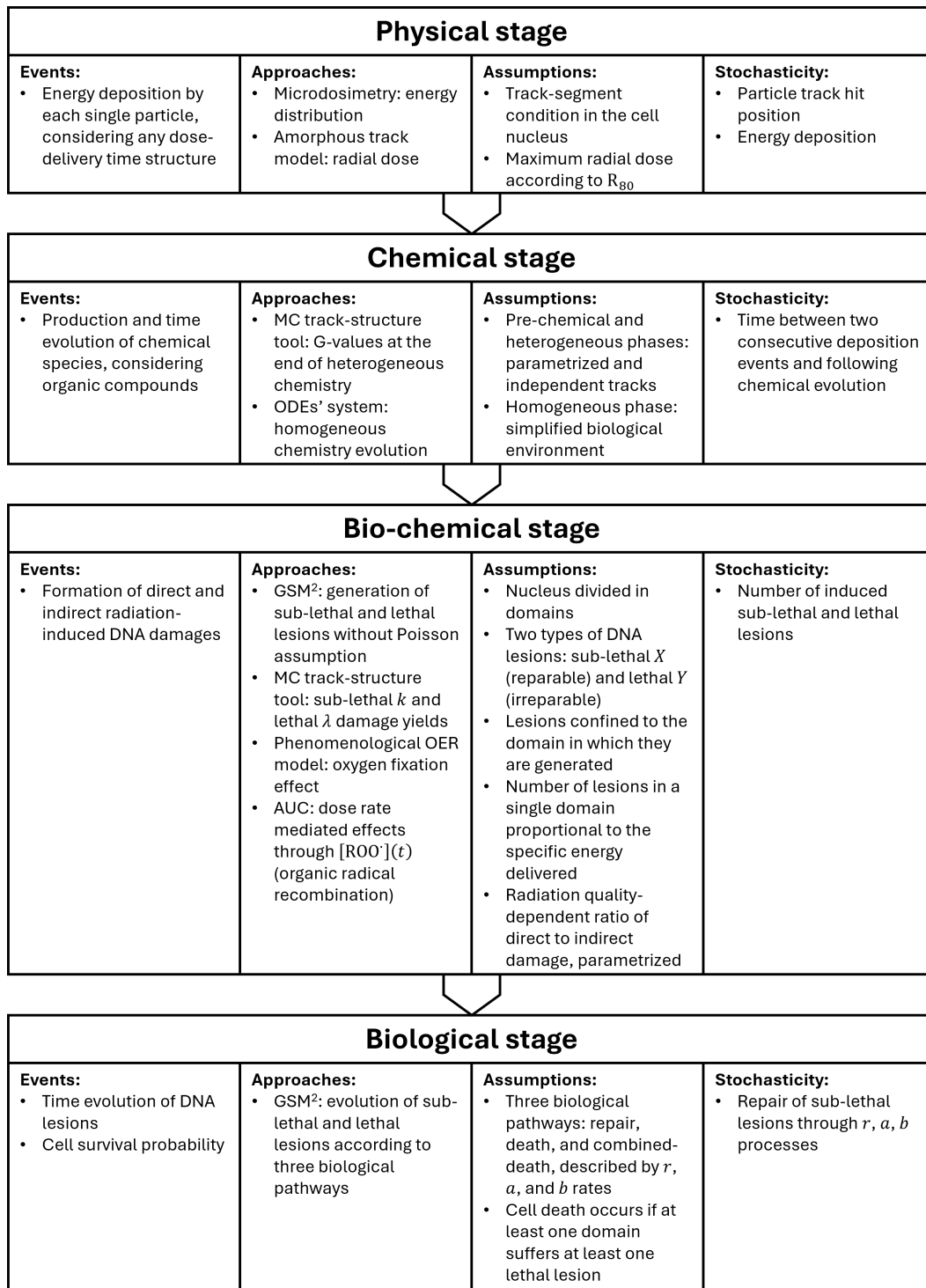


Figure 4.4: Summary of the main characteristics of the here implemented MS-GSM². The table reports the radiation-induced events, the modeling approaches, the main assumptions, and the stochasticity levels considered by the model, for each spatio-temporal stage. MS-GSM² = MultiScale-Generalized Stochastic Microdosimetric Model, MC = Monte Carlo, ODE = Ordinary Differential Equation, AUC = Area-Under-the-Curve, OER = Oxygen Enhancement Ratio.

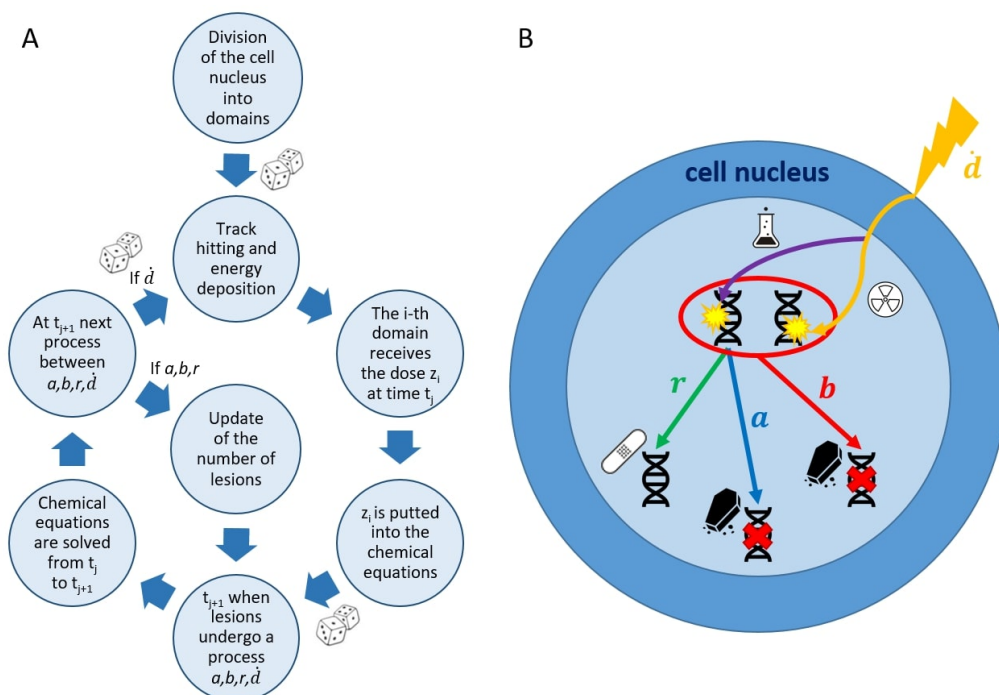


Figure 4.5: Schematic representation of the MS-GSM² workflow. **(A)** Simulation steps of the MS-GSM². Energy deposition according to microdosimetry, dose z distributed with the amorphous track; joint evolution of slow biological processes and fast chemical processes; **(B)** Possible evolution of DNA lesions in a cell nucleus. a , b , r , $\dot{d}$ are the death rate, the pairwise death rate, the repair rate, and the dose rate, respectively. Both direct and indirect damage actions of ionizing radiation are taken into account. Image from [2].

2. Given the radiation quality of the primary radiation, a macroscopic prescribed dose D , and a mean dose rate $\dot{D}$, we calculate the impact point of the track hit and the corresponding single-event energy deposition z ;
3. We calculate the radial dose distribution on the cell nucleus according to the AT model in Eq. 4.1, obtaining the absorbed dose z^d for each particle ν in each domain d at time τ_j ;
4. We put the energy deposition into the chemical system of Eqs. 4.7 for each domain d .
5. We sample the time τ_{j+1} at which the following event occurs, which could be another energy deposition event, according to the pathways 4.32 and 4.33, or could be a biological event, according to the pathways 4.34. The elapsed time τ_{j+1} between two consecutive events is sampled from an exponential distribution, which depends on the dose rate and the mean energy deposited in a single event;
6. We solve the chemistry up to the following event at time τ_{j+1} ;
7. We sample the next process according to the physical and biological pathways in Eqs. 4.32, 4.33, and 4.34.;
8. If the sampled event is a physical process, we repeat the cycle from point (2), updating the number of sub-lethal and lethal lesions according to $Z_{Y;\text{dir}}^{(c,d)}$, $Z_{X;\text{dir}}^{(c,d)}$, $Z_{Y;\text{indir}}^{(c,d)}$, and $Z_{X;\text{indir}}^{(c,d)}$, while if it is a biological process, we simulate the evolution pathways via a *stochastic simulation algorithm* (SSA), given the initial number of lesions; then, in this case, we repeat the cycle from point (5);
9. We repeat all steps from (2) to (3) until the end of the irradiation at time T_{irr} . We assume irradiation in the time interval $[0, T_{irr}]$ so that we have $\dot{d} = 0$ for $t > T_{irr}$, and a terminal time $T > T_{irr} + T_{off}$ sufficiently large so that the system Σ_{MS}^c reaches convergence. Thus, after that time T_{irr} , only biological processes can occur.
10. We calculate the survival probability of the cell from the lesions distributions obtained at the end of step (9), according to Eq. 4.37.

The previous steps cannot be computed independently for each domain because physical processes involved more domains at the same time. A possible stepwise construction of the MS-GSM² model and the corresponding pseudocode (1) are reported in the Appendix 9 of this thesis.

Computational information and model parameters

The MS-GSM² model was implemented both using *Julia* [282], which is the final version, and *MATLAB* [283], which is the preliminary version. In the case of Julia, we use a *Rosenbrock method*, in particular the *Rodas4* solver of the `DifferentialEquations.jl`

package, for the numerical integration of the chemical system of ODEs, that is coupled with the parameters reported in Table 4.3, and with all initial conditions set to zero, except for the oxygen, as shown in Table 4.3. This assumption is because while oxygen is an environmental chemical species, all the others are directly produced by radiation or the reactions between chemical species. Instead, in the case of *MATLAB* we apply the function `ode15s.m` for the numerical integration of the ODEs.

Common values and ranges for (i) geometrical and physical parameters, such as the nucleus radius, the side and the number of domains, the particle type and energy, the macroscopic doses, and mean dose rates, are reported in Table 4.2; (ii) chemical parameters, such as rate constants and chemical concentrations, are reported in Table 4.3; (iii) biological parameters, such as the sub-lethal and lethal damages, fraction of indirect damages, and pathways rates of damage evolution, are reported in Table 4.4.

In the case of numerical results shown in Section 4.3, we considered the physical and physiological parameters reported in Tables 4.2, 4.3, and 4.4. For the comparison with *in-vitro* experiments reported in Section 4.4, we considered the geometrical parameters in Table 4.2 and the chemical parameters in Table 4.3. Instead, the physical parameters are those reported in the original experimental publications, while the biological parameters are selected from the literature based on the experiment under consideration ($\text{yield}_{\text{DSB}}$ and q) or fitted to the experimental data (a , b , and r).

Parameter	Description	Value/Range
R_N	Nucleus radius	$8 \mu m$
l_d	Domain side	$2 \mu m$
N_d	Number of cubic domains	$52 (xy) \times 8 (z)$
X	Particle type	$e^-, {}^1\text{H}, {}^4\text{He}, {}^{12}\text{C}$
D	Dose	$\sim 0 - 40 \text{ Gy}$
$\dot{D}$	Mean dose rate	$\sim 0.03 - 200 \text{ Gy/s}$

Table 4.2: Common geometrical and physical stage parameters considered by the MS-GSM² model. Table from [1].

4.3 Numerical results

This Section is entirely dedicated to the presentation of the numerical results obtained through the MS-GSM² model. In particular, we divide the numerical results into physical, chemical and biological findings, showing them in Sections 4.3.1, 4.3.2, and 4.3.3, respectively.

To verify the feasibility of the MS-GSM² model to potentially investigate the main features of the FLASH effect at the cell level, we performed simulations for different radiation qualities of the primary radiation, characterized by type, energy, and LET, macroscopic doses, and mean dose rates. In particular, we repeated the simulation over a wide range of macroscopic doses D , from 1 Gy to 30 Gy, and the mean dose rate $\dot{D}$, from

Parameter	Description	Value/Range	Reference
k_1	Rate constant of (4.9)	$5 \cdot 10^7 \text{ (M} \cdot \text{s)}^{-1}$	[7, 218, 284, 285, 286]
k_2	Rate constant of (4.11)	$10^4 \text{ (M} \cdot \text{s)}^{-1}$	[7, 287]
k_3	Rate constant of (4.16)	$6.62 \cdot 10^7 \text{ (M} \cdot \text{s)}^{-1}$	[7]
k_4	Rate constant of (4.15)	$10^3 \text{ (M} \cdot \text{s)}^{-1}$	[7]
k_5	Rate constant of (4.8)	$10^9 \text{ (M} \cdot \text{s)}^{-1}$	[7]
k_6	Rate constant of (4.12)	$10^{10} \text{ (M} \cdot \text{s)}^{-1}$	[7]
k_7	Rate constant of (4.13)	$4.62 \cdot 10^4 \text{ (M} \cdot \text{s)}^{-1}$	[7]
k_8	Rate constant of (4.10)	$5 \cdot 10^7 \text{ (M} \cdot \text{s)}^{-1}$	[7]
k_9	Rate constant of (4.14)	$10^2 \text{ (M} \cdot \text{s)}^{-1}$	[218, 275]
$[\text{O}_2]_0$	Initial oxygenation	0 – 21 %	[8, 9, 10]
[RH]	Organic substrate	1 M	[7]
[cat]	Catalase	$0.08 \cdot 10^{-6} \text{ M}$	[7]
$[\text{Fe}^{2+}]$	Labile iron	$8.9 \cdot 10^{-7} \text{ M}$	[7]
[XSH]	Antioxidant	$6.5 \cdot 10^{-3} \text{ M}$	[7]

Table 4.3: Common chemical stage parameters considered by the MS-GSM² model. Table from [2].

Parameter	Description	Value/Range	Reference
$\text{yield}_{\text{DSB}}$	Double-strand break yield	$\geq 6.8 \text{ Gy}^{-1} \text{ GBp}^{-1}$	[279]
q	Fraction of indirect damage	≤ 0.8	[279]
a	Death rate	$10^{-3} - 10^{-1} \text{ 1/h}$	[3, 4]
b	Double-death rate	$10^{-3} - 10^{-1} \text{ 1/h}$	[3, 4]
r	Repair rate	$2.0 - 5.0 \text{ 1/h}$	[3, 4]

Table 4.4: Common biological stage parameters considered by the MS-GSM² model.

0.03 Gy/s (Conv) to 100 Gy/s (UHDR). To illustrate the main characteristics of the MS-GSM² model and to highlight the impact of radiation quality on the manifestation of the sparing effect between Conv and UHDR irradiation, we report the physical, chemical, and biological results for two different scenarios in terms of radiation quality. In particular, we considered protons at 18.6 MeV (low LET radiation, i.e. $\sim 2.8 \text{ keV}/\mu\text{m}$) and carbon ions at 149 MeV/u (higher LET value, i.e. $\sim 19.6 \text{ keV}/\mu\text{m}$). These two specific energy values were chosen because they have already been used in other applications of the standard GSM² model.

4.3.1 Physics

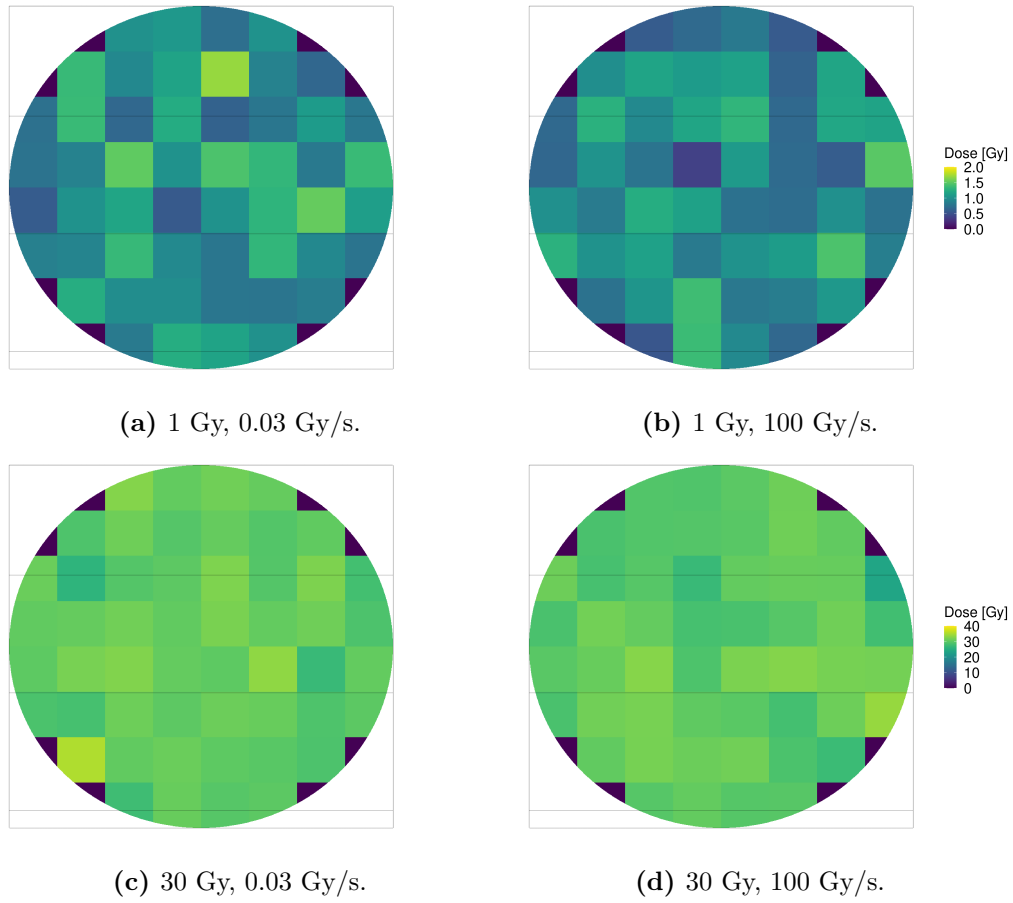


Figure 4.6: Domain distribution of absorbed dose by the cell nucleus for protons at 18.6 MeV at different macroscopic doses and mean dose rates. Images from [1].

Figure 4.6 shows the microscopic absorbed dose for protons at 18.6 MeV over the cell nucleus in different domains for different (macroscopic) average doses: the top row reports 1 Gy, the bottom row reports 30 Gy, the left column reports 0.03 Gy/s (Conv) whereas

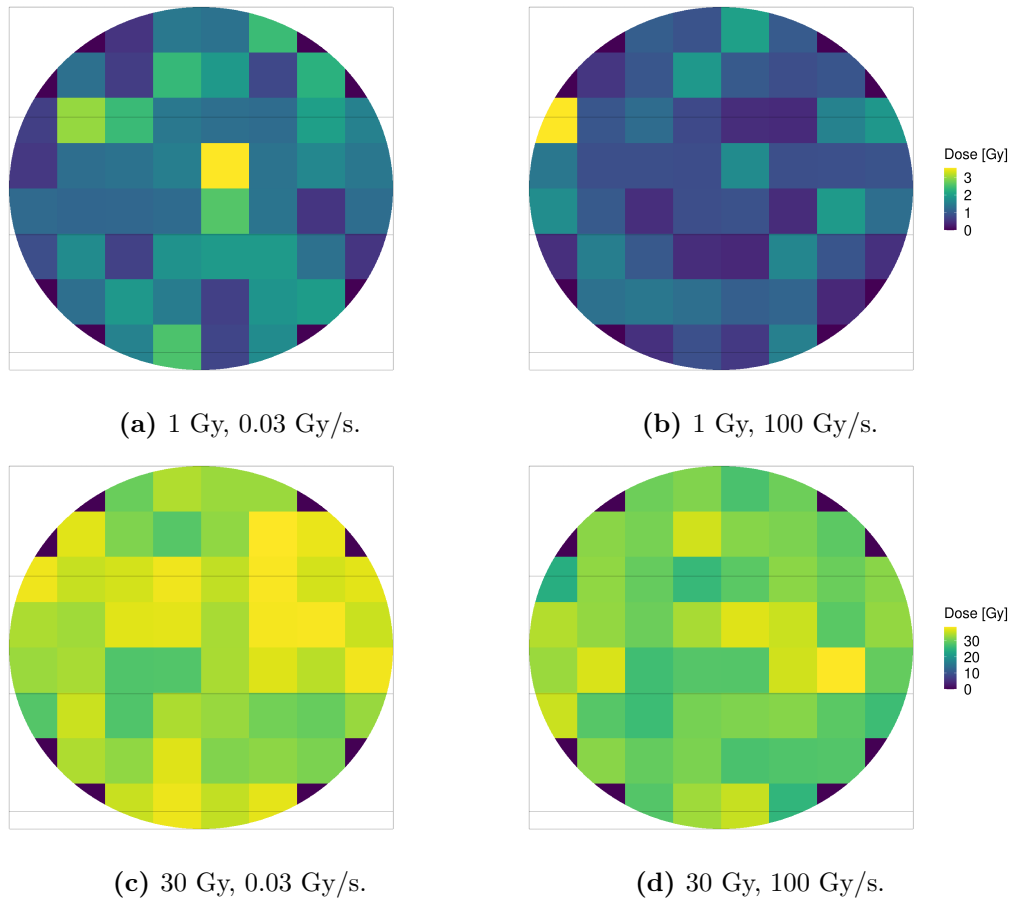


Figure 4.7: Domain distribution of absorbed dose by the cell nucleus carbon ions at 149 MeV/u at different macroscopic doses (1 and 30 Gy) and mean dose rates (0.03 and 100 Gy/s). Images from [1].

the right column reports 100 Gy/s (UHDR). Figure 4.7 shows the same results for the case of carbon ions at 149 MeV/u. Both figures 4.6 and 4.7 highlight how the case of 1 Gy is characterized by a more spatially heterogeneous dose distribution over the cell nucleus, whereas, on the contrary, at 30 Gy the dose distribution is more homogeneously distributed. Further, differences in the absorbed dose over cell domains are more significant in the case of carbon ions, characterized by high local energy deposition in a single domain even at 30 Gy, with a non-uniform dose distribution. In particular, in the case of 1 Gy is evident the impact of the energy deposited by a single track depositing almost 3 Gy in a single domain, as opposed to an overall dose deposition of 1 Gy in the cell nucleus. Furthermore, it is clear from both figures that the dose rate has no impact on the total dose distribution, as expected.

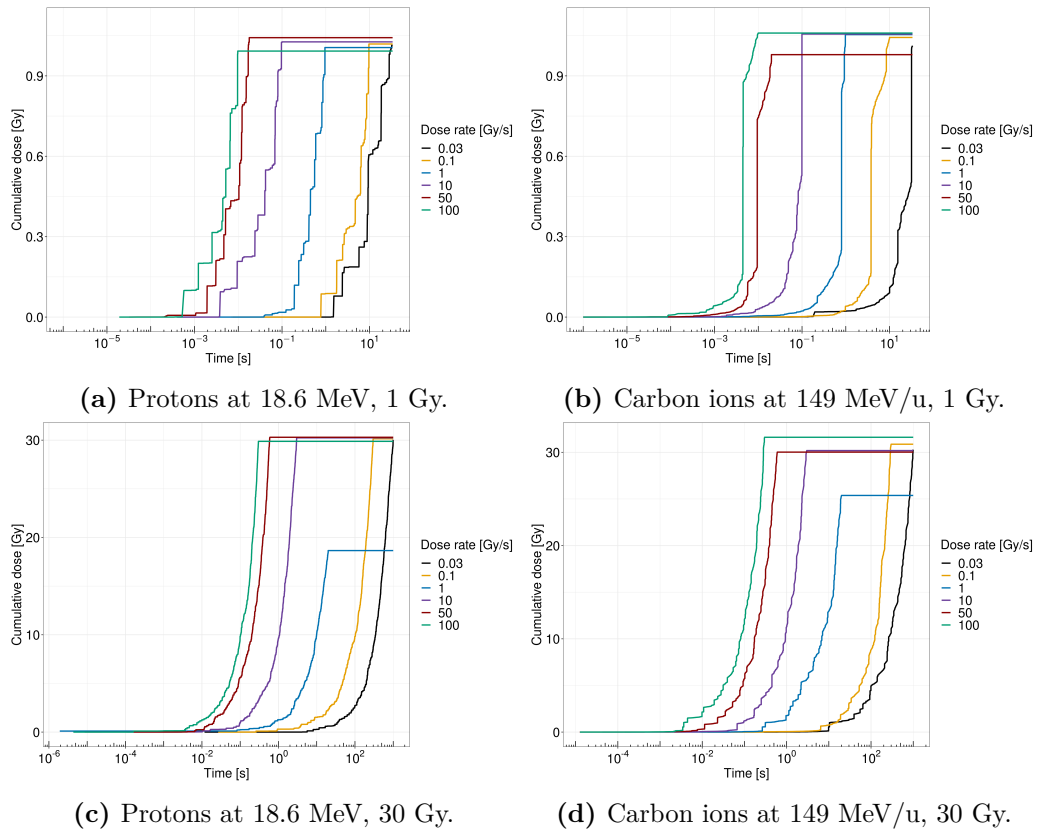


Figure 4.8: Cumulative absorbed dose by the cell nucleus for different radiation qualities (protons at 18.6 MeV and carbon ions at 149 MeV/u) over time for a single domain at 1 or 30 Gy at different mean dose rates (0.03, 0.1, 1, 10, 50, and 100 Gy/s). From [1].

Figure 4.8 shows the cumulative absorbed dose in a single domain over time for different dose rates. Both top panels report the case of a total dose of 1 Gy (4.8a and 4.8b) in the domain, while the bottom panels report the case of 30 Gy (4.8c and 4.8d). The left panels show the case of protons at 18.6 MeV (4.8a and 4.8c) whereas the right panels consider

the case of carbon ions at 149 MeV/u (4.8b and 4.8d). The figure shows how the pattern of local energy deposition is different for the two ions considered, which is emphasized for the case of 1 Gy irradiation (4.8a and 4.8b).

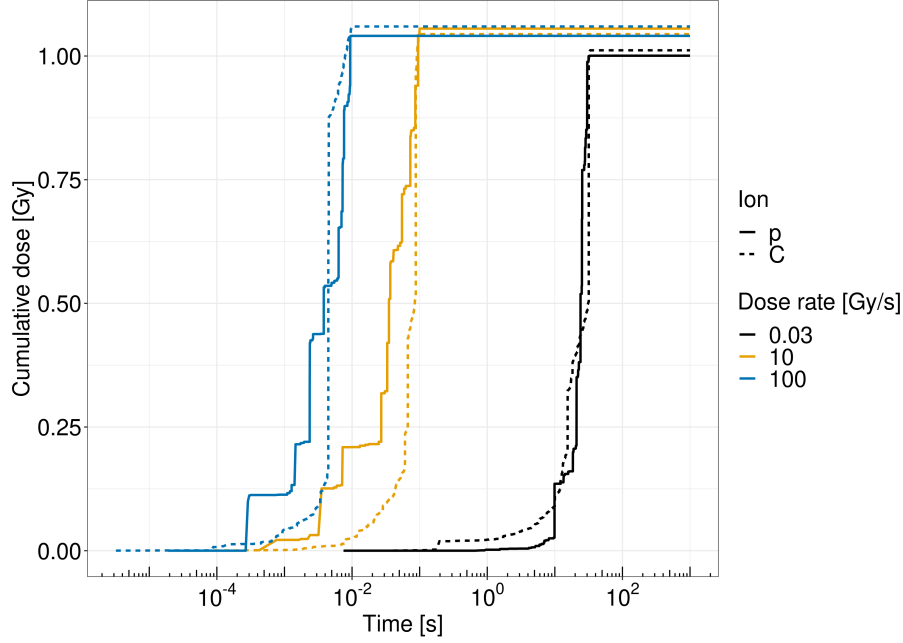
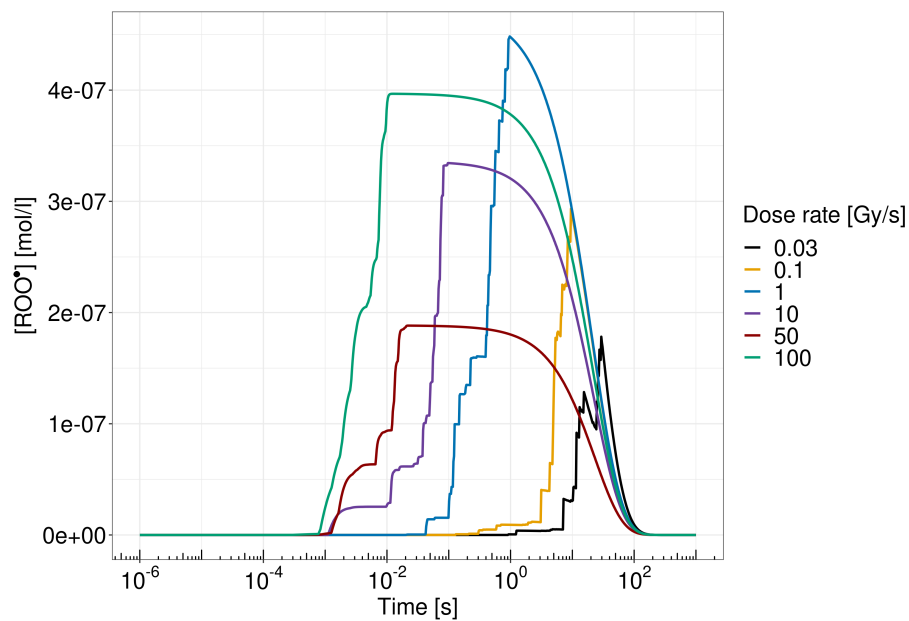


Figure 4.9: Comparison of cumulative absorbed dose by the cell nucleus for different radiation qualities (protons at 18.6 MeV and carbon ions at 149 MeV/u) over time for a single domain at 1 Gy at different mean dose rates (0.03, 10, 100 Gy/s).

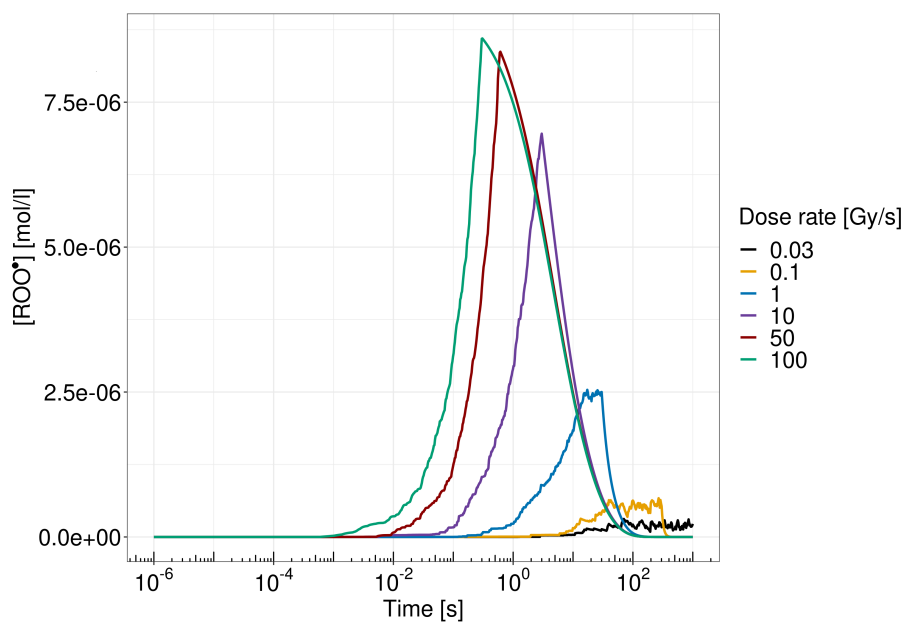
Figure 4.9 shows the comparison of cumulative absorbed dose between the two different radiation qualities, i.e., protons at 18.6 MeV and carbon ions at 149 MeV/u, in the case 1 Gy irradiation, for three different mean dose rates, i.e., 0.03, 10, 100 Gy/s. Protons are characterized by a higher amount of energy depositions, each of which releases a small amount of energy. On the contrary, carbon ions exhibit fewer depositions with a clear impact of single tracks hitting the nucleus. In fact, jumps in the cumulative absorbed dose due to high energy depositions are clearly visible.

4.3.2 Chemistry

Figure 4.10 shows the $\text{ROO}^\bullet$ concentration over time for a single domain for proton at 18.6 MeV at 1 Gy (top panel) and 30 Gy (bottom panel) for different dose rates. From figure 4.10 is visible how both dose and dose rate play a crucial role in the peroxy radical concentration evolution. The higher dose case is characterized by a higher concentration of $\text{ROO}^\bullet$. Also, high dose rates show an almost continuous pattern of energy deposition whereas on the contrary low dose rates are characterized by more jagged behavior due to the longer time elapsing between consecutive energy depositions. Moreover, $\text{ROO}^\bullet$



(a) 1 Gy.



(b) 30 Gy.

Figure 4.10: Organic peroxy radical $\text{ROO}^\bullet$ concentration over time for a single domain for proton at 18.6 MeV for two macroscopic doses (1 and 30 Gy) at different mean dose rates (0.03, 0.1, 1, 10, 50, and 100 Gy/s). Images from [1].

concentrations at 1 Gy are more similar for the different dose rates while in the 30 Gy case there is a clear different time evolution of $[\text{ROO}^\bullet]$ for the dose rates considered, both in terms of maximum concentration and in terms of growth and relaxation rate of the chemical species.

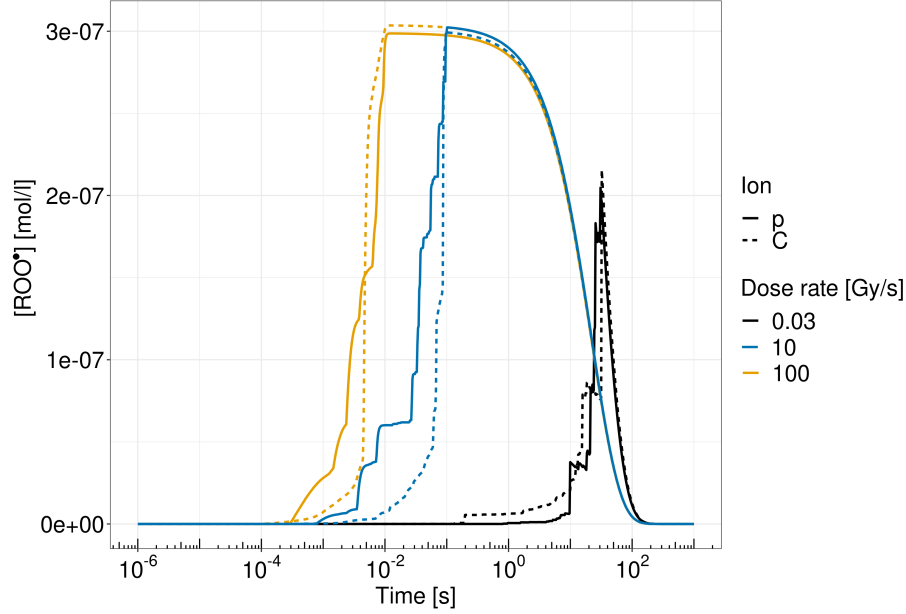
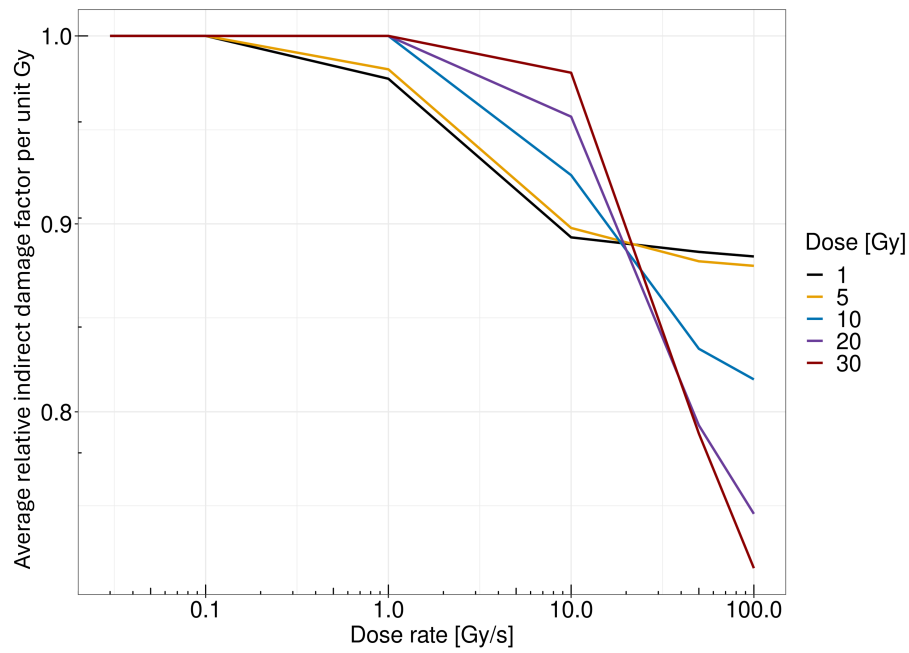


Figure 4.11: Comparison of organic peroxy radical $\text{ROO}^\bullet$ concentration over time for different radiation qualities (protons at 18.6 MeV and carbon ions at 149 MeV/u) for a single domain at 1 Gy at different mean dose rates (0.03, 10, 100 Gy/s).

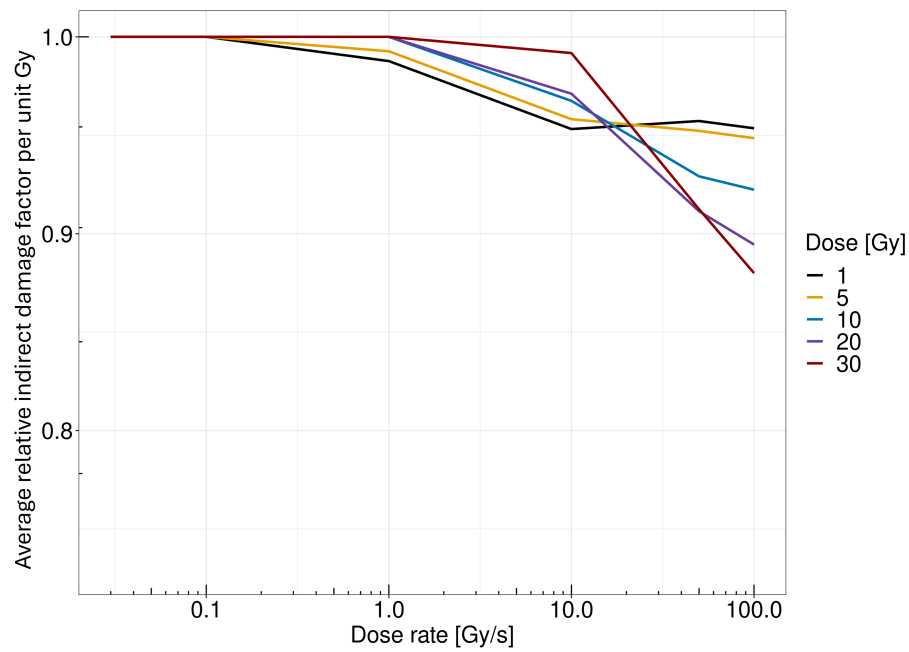
Figure 4.11 shows the comparison of $\text{ROO}^\bullet$ concentrations over time between the two different radiation qualities, i.e., protons at 18.6 MeV and carbon ions at 149 MeV/u, a single domain where for both irradiation the same absolute maximum concentration is reached, for a macroscopic prescribed dose on the whole cell nucleus of 1 Gy. The figure shows how the pattern of local energy deposition for the two different ions, discussed in Figure 4.9, influences the increase in concentration of $\text{ROO}^\bullet$.

4.3.3 Biology

Figure 4.12 reports the average relative factor for the indirect damage yield per unit Gy κ_{indir} in Eq. 4.30 versus dose rate for different doses. Specifically, it quantifies the relative change in the indirect damage yield per unit Gy between a given dose rate and the minimum dose rate, averaged across the cell domains. The top panel shows the case of protons at 18.6 MeV whereas the bottom panel shows the case of carbon ions at 149 MeV/u. A more severe reduction is evident in the case of protons, where at 30 Gy and 100 Gy/s the indirect damage yield is reduced to 30% compared to the conventional dose

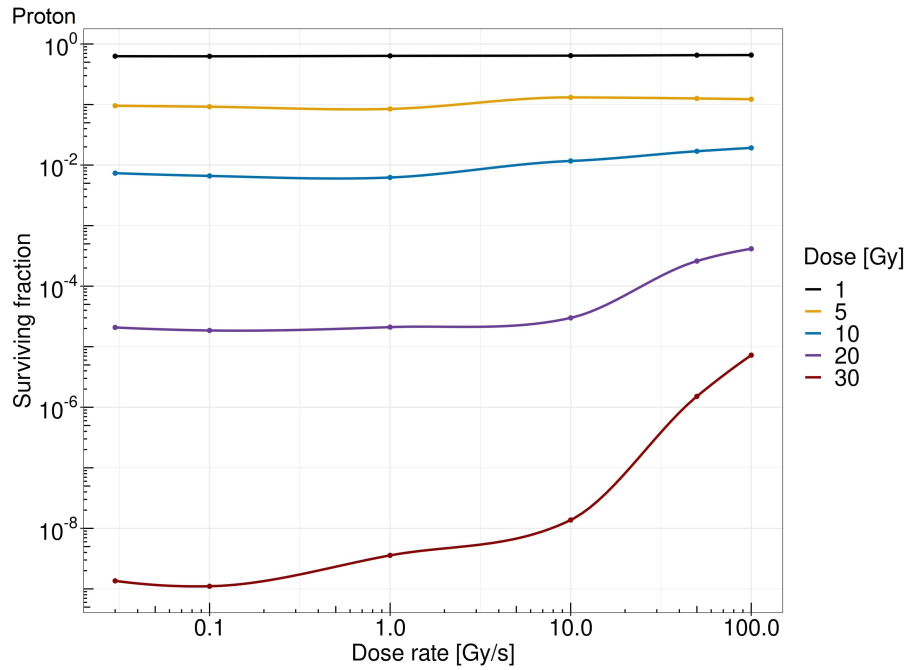


(a) Protons at 18.6 MeV.

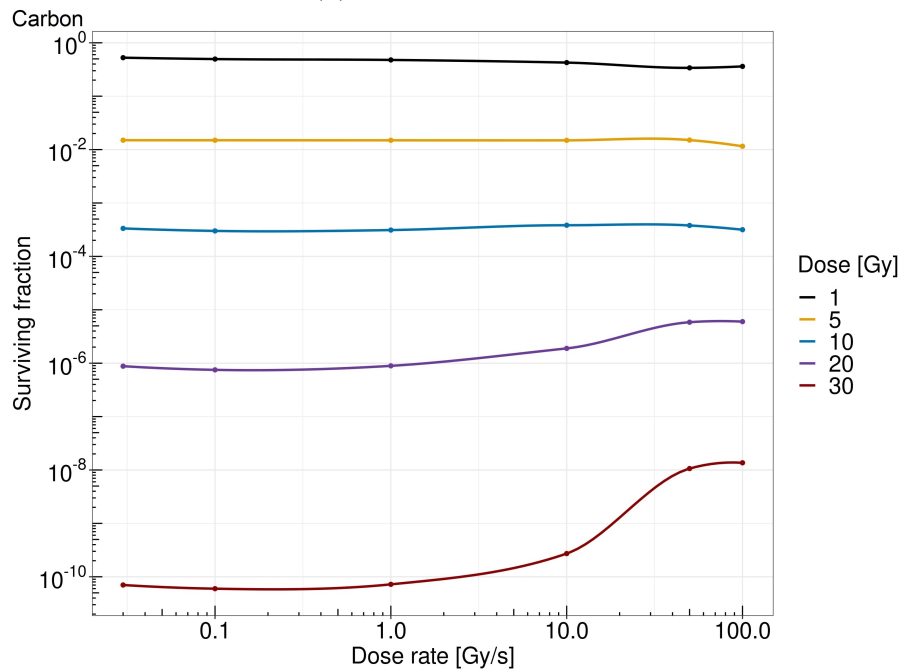


(b) Carbon ion at 149 MeV/u.

Figure 4.12: Dose rate versus average relative indirect damage factor per unit Gy for different radiation qualities (protons at 18.6 MeV and carbon ion at 149 MeV/u) at different macroscopic doses (1, 5, 10, 20, and 30 Gy). Images from [1].



(a) Protons at 18.6 MeV.



(b) Carbon ion at 149 MeV/u.

Figure 4.13: Dose rate versus survival fraction for different radiation qualities (protons at 18.6 MeV and carbon ion at 149 MeV/u) at different macroscopic doses (1, 5, 10, 20, and 30 Gy). Images from [1].

rate of 0.03 Gy/s. In the case of carbon ions, the relative reduction reaches at most 12% in the same situation. This is consistent with what was predicted in [288], pointing to a lower entity of the FLASH effect for higher LET beams. However, this aspect is still open and needs to be further investigated. Also, in both ions considered, there is a clear pattern of reversed behavior where up to 10 Gy/s increasing the imparted dose yields a higher amount of indirect damage whereas after this dose rate, the pattern is the opposite. In fact, above 50 Gy/s higher doses are associated with less damage induced per unit Gy, due to the reduction of DNA damage yield at UHDR regime. In general, low doses are characterized by a steady low reduction of damage yields per unit Gy whereas high doses show almost no reduction up to 10 Gy/s followed by a sharp fall-off with high indirect damage yield reduction per unit Gy.

Figure 4.13a shows the survival fraction of Human Salivary Gland (HSG) cells computed with the MS-GSM² versus dose rate at different doses in the case of protons at 18.6 MeV. Figure 4.13b reports the same result in the case of carbon ions at 149 MeV/u. Both figures are characterized by a steady flat surviving fraction from the conventional dose rates up to 10 Gy/s after which an increase in the surviving fraction is visible. The sparing effect emerges only at high doses: for protons, this effect is slightly visible at 10 Gy and severe at 30 Gy, whereas for carbon ions the effect clearly emerges after 20 Gy and it is more attenuated.

4.4 Comparison with *in-vitro* experiments

This Section is dedicated to the presentation of the comparison between experimental *in-vitro* data and MS-GSM² predictions. To stress MS-GSM² validity and predictive power, the model predictions have been compared with all the main available experimental data at UHDR for different oxygenation. Such experimental data are of broadly different types, using all the parameters (oxygenation, time structure, doses, radiation quality, etc.), as reported in the original publications. The selection of experiments was made as follows: (i) we only chose modern experimental data, i.e. after the birth of FLASH definition (2014), to avoid potential issues with dosimetry as reported in the original publication; (ii) we only considered experiments that present additional oxygenations besides normoxia to be able to perform a benchmark of model predictions at lower oxygenations which are relevant for realistic *in-vivo* systems; (iii) in the case of irradiation with ions, we only selected experiments using the beam in the entrance channel to avoid possible modeling issues with the SOBP irradiation, which is characterized by a complex mixed radiation field.

Figures 4.14, 4.15, and 4.16 highlight how the MS-GSM² can describe the trend of the experimental data at different oxygenation levels in terms of oxygenation and sparing at different dose rates and for different radiation quality. For each of these experiments, we calibrated the biological parameters of our model, i.e., a , b , and r , on the experimental points at standard conditions, i.e., for conventional irradiation and normoxia, that is 21% oxygenation, highlighted by a red square in Figures 4.14, 4.15, and 4.16. The MS-GSM² has completely predicted all the other results, i.e. for different oxygen levels and dose

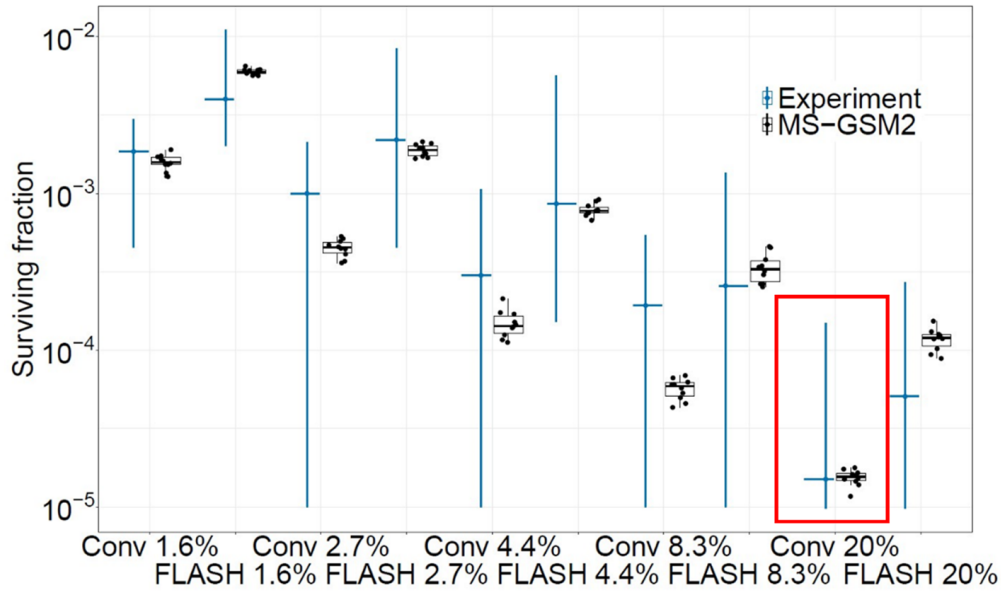


Figure 4.14: Comparison of the MS-GSM² results with experimental data [8]. The MS-GSM² parameters a , b and r are calibrated on conventional dose rate at normoxic condition, highlighted by a red square in the picture. DU145 cell survival in the case of 10 MeV electrons irradiation, at 18 Gy for both Conv (14 Gy/min) and FLASH (600 Gy/s) dose rates, for different oxygenations (1.6%, 2.7%, 4.4%, 8.3%, 20% O₂). Image from [2].

rates. The values obtained for the a , b , and r parameters are reported in Table 4.5. The statistical variations of model predictions reported in Figures 4.14, 4.15, and 4.16 were obtained by repeating the biological prediction, due to the stochasticity that underlies the MS-GSM², in terms of formation and evolution of DNA lesions, as discussed in Section 2.3.1. The box plots display the minimum, the 25th, 50th, and 75th percentiles, and the maximum of the data points.

Figure 4.14 shows the comparison between the experimental cell survival fractions of prostate cancer cell line DU145 [8] (in blue) and MS-GSM² prediction (in black). The horizontal axis lists different conditions, not to scale, reporting the different levels of oxygenation (1.6%, 2.7%, 4.4%, 8.3%, 20.0% O₂) as indicated in the original publication, for electron at 10 MeV in Conv (mean dose rate of 14 Gy/min) and UHDR (mean dose rate of 600 Gy/s) irradiations of 18 Gy. In particular, the dose was delivered with an integer number of pulses, a pulse time of 3.5 μ s, and a pulse repetition frequency of 200 Hz in the case of Conv irradiation, while with an integer number of pulses, a dose-per-pulse of 3 Gy, and a pulse repetition frequency of 200 Hz at UHDR regime. The experimental irradiation structure has been incorporated within the MS-GSM² framework. As illustrated in Figure 4.14, the MS-GSM² encompasses the OER effect, evidenced by the elevated cell survival fraction at reduced oxygen concentrations for both conventional and FLASH irradiation, as well as the FLASH sparing effect distinguishing the conventional and UHDR regimes.

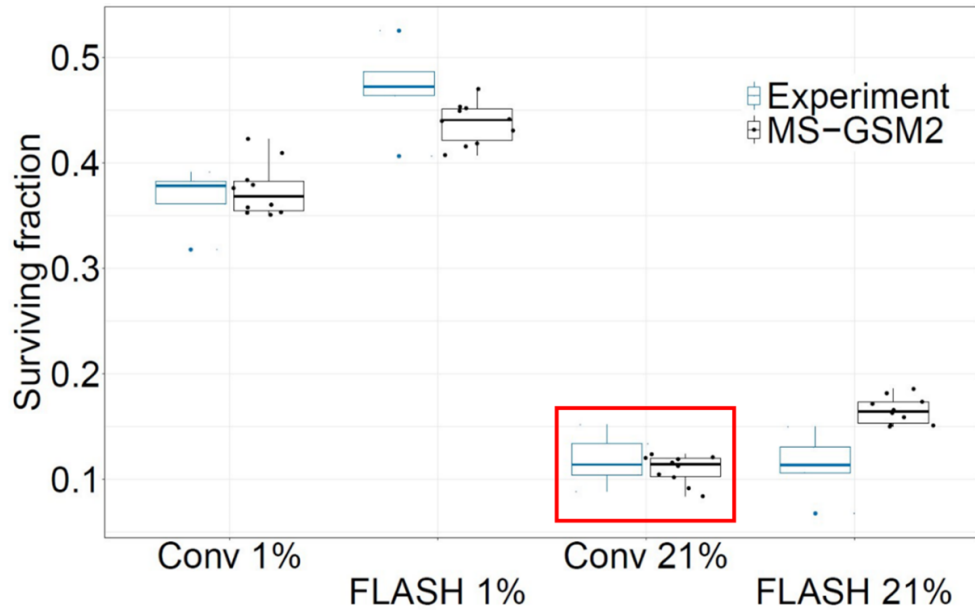


Figure 4.15: Comparison of the MS-GSM² results with experimental data [9]. The MS-GSM² parameters a , b and r are calibrated on conventional dose rate at normoxic condition, highlighted by a red square in the picture. A549 cell survival in the case of 4.5 keV/ μ m helium ions irradiation, at 8 Gy for both Conv (0.12 Gy/s) and FLASH (205.13 Gy/s) dose rates, for different oxygenations (1%, 21% O₂). Image from [2].

Figure 4.15 reports the survival fractions of human lung epithelial cells A549 (experimental [9] results (in blue) plotted against MS-GSM² prediction (in black)), at hypoxia and normoxia conditions (1.0% and 21.0% O₂), for Conv and UHDR irradiations of 8 Gy, in the case of helium ions impinging the cells at a LET of 4.5 keV/ μ m. In particular, the dose was delivered with a mean dose rate of 0.12 Gy/s for Conv, with a mean dose rate of 205.13 Gy/s.

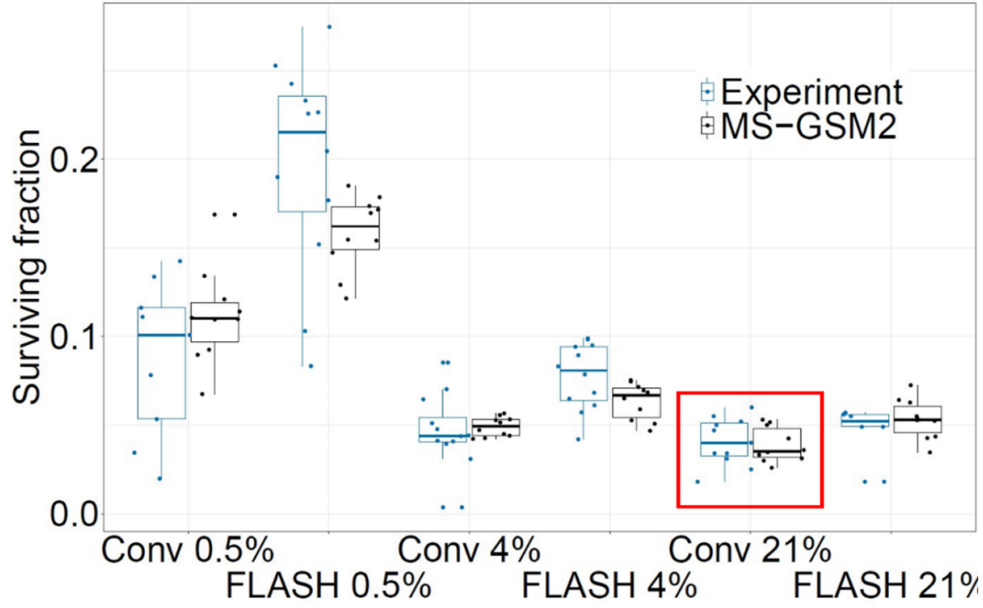


Figure 4.16: Comparison of the MS-GSM² results with experimental data [10]. The MS-GSM² parameters a , b and r are calibrated on conventional dose rate at normoxic condition, highlighted by a red square in the picture. CHO-K1 cell survival in the case of 13 keV/ μ m carbon ions irradiation, at 7.5 Gy for both Conv (0.6 Gy/s) and FLASH (70 Gy/s) dose rates, for different oxygenations (0.5%, 4%, 21% O₂). Image from [2].

Figure 4.16 shows the comparison of survival fractions of Chinese hamster ovary cells CHO-K1, between the MS-GSM² prediction (in black) and the experiment [10] (in blue), at different oxygen concentrations (0.5%, 4.0%, 21.0% O₂). Carbon ion irradiation has been performed for both Conv (mean dose rate of 0.6 Gy/s) and UHDR (mean dose rate of 70 Gy/s) irradiations of 7.5 Gy, in the case of carbon ions of 13 keV/ μ m.

Data analyses

For the comparison of the MS-GSM² results with the *in vitro* experiments, shown in Figures 4.14, 4.15, and 4.16, we optimize the biological rates, reported in Table 4.5, on experimental data at standard conditions, i.e., conventional irradiation and normoxia (21 % O₂). In particular, we use a *cross-entropy* method to obtain the best combination of a , b , and r rates, allowing us to describe all the experimental points. We start from

Experiment	Reference	a [1/h]	b [1/h]	r [1/h]
DU145, e ⁻	[8]	$7.82 \cdot 10^{-3}$	$1.83 \cdot 10^{-2}$	3.23
A549, ⁴ He	[9]	$4.70 \cdot 10^{-3}$	$1.34 \cdot 10^{-2}$	4.51
CHO-K1, ¹² C	[10]	$4.21 \cdot 10^{-3}$	$2.43 \cdot 10^{-2}$	3.68

Table 4.5: Fitted biological rates for the survival curves calculated via MS-GSM² for the experimental data points at standard conditions, i.e., conventional irradiation and 21 % O₂. Table from [2].

an interval $[l_j, u_j]$ of possible realistic values for these biological rates $j \in a, b, r$, i.e. $a \in [10^{-3}, 10^{-1}]$ 1/h, $b \in [10^{-3}, 10^{-1}]$ 1/h, and $r \in [2.0, 5.0]$ 1/h. We randomly sample 10^3 values of $\log a$, $\log b$, and $\log r$ from uniform distributions $[\log l_j, \log u_j]$. We compute the *normalized mean square error* (NMSE), i.e.

$$\text{NMSE} := \text{mean} \left(\frac{(S_{\text{exp}} - S_{\text{MS-GSM}^2})^2}{S_{\text{exp}}} \right), \quad (4.38)$$

as proposed in [289], between S_{exp} and $S_{\text{MS-GSM}^2}$, which are the experimental and the predicted survivals, respectively, and we consider the best 50 values, that we call NMSE₅₀. We calculate the biological rates

$$\begin{cases} l_{j,\min} &= l(\min(\text{NMSE}_{50})) \\ u_{j,\max} &= j(\max(\text{NMSE}_{50})) \end{cases}, \quad (4.39)$$

with $j \in a, b, r$. We consider the new intervals, i.e. $[\log l_{j,\min}, \log u_{j,\max}]$, and we repeat N -times the previous procedure, selecting, in the end, the best combination of a , b , and r .

As discussed in Section 4.1, the creation process of indirect DNA damages is described by a Poisson random variable of average $\kappa_I(\text{LET}, [\text{O}_2], [\text{ROO}^\bullet]_t)$ as defined in Eq. 4.30. The modulation term $\varrho \int_0^t [\text{ROO}^\bullet](s) ds$ of DNA lesions is normalized to 1, on average over the nucleus, to be consistent with the DNA damage yield at standard conditions [279], i.e., conventional irradiation and 21 % O₂. At the same time, it naturally decreases as the oxygenation level decreases and the dose rate increases, in such a way that the average damage yield $\kappa(\text{LET}, [\text{O}_2], [\text{ROO}^\bullet]_t)$ is in agreement with the OER formulation [30, 172] for conventional irradiation.

To quantify the accuracy of the model's predictions, we compute the *normalized mean square error* between MS-GSM² results and experimental data reported in Figures 4.14, 4.15, and 4.16, to quantify the accuracy of the model's predictions. We applied two different metrics, i.e.,

$$\text{NMSE}_m := \frac{\left(S_{\text{exp}}^{\text{mean}} - S_{\text{MS-GSM}^2}^{\text{mean}} \right)^2}{S_{\text{exp}}^{\text{mean}}}, \quad (4.40)$$

and

$$\text{NMSE}_\sigma := \frac{\left(S_{\text{exp}}^{\text{mean}} - S_{\text{MS-GSM}^2}^{\text{mean}} \right)^2}{\sigma_{\text{exp}}}. \quad (4.41)$$

Oxygen level	Irradiation type	NMSE _m	NMSE _σ
1.6 %	Conv	$3.8 \cdot 10^{-5}$	$1.1 \cdot 10^{-4}$
1.6 %	UHDR	$4.7 \cdot 10^{-6}$	$1.2 \cdot 10^{-5}$
2.7 %	Conv	$4.0 \cdot 10^{-4}$	$8.6 \cdot 10^{-4}$
2.7 %	UHDR	$8.3 \cdot 10^{-4}$	$1.5 \cdot 10^{-3}$
4.4 %	Conv	$1.2 \cdot 10^{-4}$	$1.7 \cdot 10^{-4}$
4.4 %	UHDR	$4.9 \cdot 10^{-4}$	$6.1 \cdot 10^{-4}$
8.3 %	Conv	$9.4 \cdot 10^{-5}$	$1.4 \cdot 10^{-4}$
8.3 %	UHDR	$2.3 \cdot 10^{-5}$	$1.8 \cdot 10^{-5}$
20.0 %	Conv	$5.7 \cdot 10^{-9}$	$2.5 \cdot 10^{-9}$
20.0 %	UHDR	$8.7 \cdot 10^{-5}$	$7.0 \cdot 10^{-5}$

Table 4.6: Normalized mean square error NMSE_m and NMSE_σ between MS-GSM² results and experimental data for the electron experiment [8] reported in Figure 4.14. Table from [2].

Oxygen level	Irradiation type	NMSE _m	NMSE _σ
1.0 %	Conv	$3.8 \cdot 10^{-5}$	$6.4 \cdot 10^{-4}$
1.0 %	UHDR	$2.5 \cdot 10^{-3}$	$2.6 \cdot 10^{-2}$
21.0 %	Conv	$2.1 \cdot 10^{-3}$	$6.7 \cdot 10^{-3}$
21.0 %	UHDR	$4.5 \cdot 10^{-2}$	$1.6 \cdot 10^{-1}$

Table 4.7: Normalized mean square error NMSE_m and NMSE_σ between MS-GSM² results and experimental data for the electron experiment [9] reported in Figure 4.15. Table from [2].

Oxygen level	Irradiation type	NMSE _m	NMSE _σ
0.5 %	Conv	$2.5 \cdot 10^{-3}$	$5.3 \cdot 10^{-3}$
0.5 %	UHDR	$7.6 \cdot 10^{-3}$	$3.1 \cdot 10^{-2}$
1.0 %	Conv	$6.1 \cdot 10^{-5}$	$3.0 \cdot 10^{-4}$
1.0 %	UHDR	$3.7 \cdot 10^{-3}$	$1.3 \cdot 10^{-2}$
21.0 %	Conv	$7.6 \cdot 10^{-5}$	$2.2 \cdot 10^{-4}$
21.0 %	UHDR	$7.6 \cdot 10^{-4}$	$6.9 \cdot 10^{-3}$

Table 4.8: Normalized mean square error NMSE_m and NMSE_σ between MS-GSM² results and experimental data for the electron experiment [10] reported in Figure 4.16. Table from [2].

The computed values of NMSE_m and NMSE_σ for the three experiments [8, 9, 10] are reported in Tables 4.6, 4.7, and 4.8, respectively, highlighting in bold those below a threshold of 10^{-2} , which is an arbitrary constant chosen to reflect the observed qualitative trend. Smaller values indicate greater agreement between model predictions and experimental data.

4.5 Translation to *in-vivo*

In-silico evidence of a new mechanism for the FLASH differential effect

Figure 4.17 reports the combined action of oxygenation and antioxidant environment on the emergence of the FLASH effect for electron irradiation. In particular, the color map distribution describes the relative variation of the surviving probability for UHDR irradiation with respect to the conventional one, i.e. the *relative sparing effect*

$$\text{RSE} := \frac{\ln S_{\text{Conv}} - \ln S_{\text{FLASH}}}{\ln S_{\text{Conv}}}, \quad (4.42)$$

as a function of the environmental oxygen (in the horizontal axis) and the rate of antioxidants (in the vertical axis), i.e., the product between the concentration of an antioxidant species [XSH], such as glutathione, and the reaction constant between the antioxidant XSH and the organic peroxy radical $\text{ROO}^\bullet$. Thus, the rate of antioxidants measures the cell's scavenging ability to eliminate harmful chemical species. We obtained this 2D plot via the MS-GSM², by repeating the simulation of the irradiation with 10 MeV electrons for several levels of antioxidant concentrations and at different oxygen conditions and considering the same physical (mean dose rate of 14 Gy/min, Conv, and 600 Gy/s, UHDR) and biological (prostate cancer cell line DU145) parameters that we used for [8], for two different prescribed doses, i.e. 3 and 18 Gy. In particular, light colors correspond to a high differential effect between conventional and UHDR irradiation, while dark colors correspond to a low differential effect.

4.6 Discussion

In this Section, we discuss the main results that we obtained using the MS-GSM² model, presented in Sections 4.3 and 4.4. In addition, we comment on the main limitations of the developed model, and we speculate on possible future developments.

Analysis of the results

Starting from the numerical results obtained through the MS-GSM² model in Section 4.3, the aim of this first part of the work is to present the main components of this novel tool that allows, among the many radiation biological effects, to capture possible effects of DNA damage during UHDR irradiation. In particular, with these calculations, we want to show how this multi-stage radiation biophysical model is able to describe the sparing

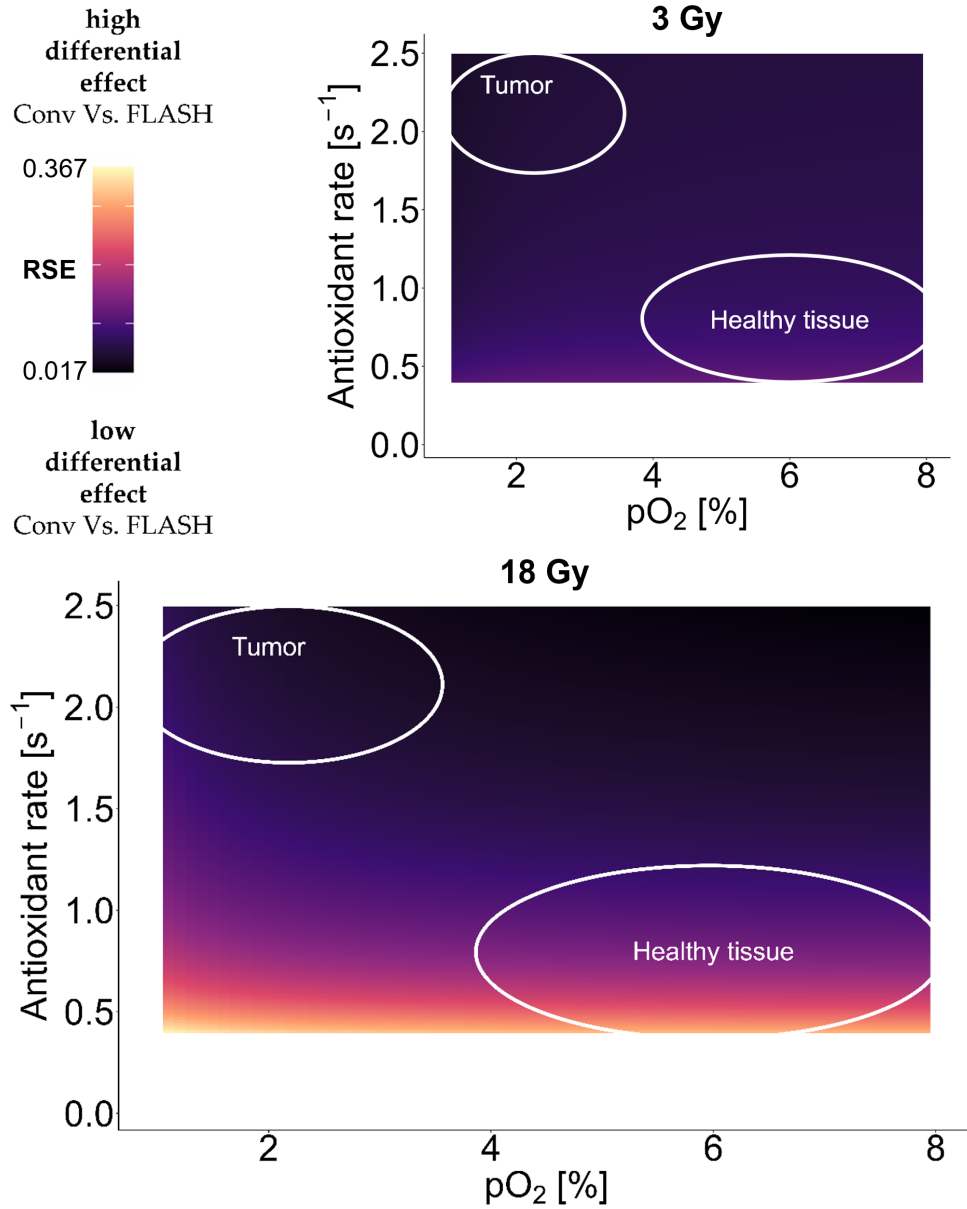


Figure 4.17: Perspective analysis of the impact of the chemical environment on the emergence of the FLASH effect for electron irradiation, using the MS-GSM². Relative change in cell survival between Conv and UHDR irradiation, i.e., $RSE := (\ln S_{\text{Conv}} - \ln S_{\text{FLASH}}) / \ln S_{\text{Conv}}$, as a function of the environmental oxygen (in the horizontal axis) and the rate of antioxidants (in the vertical axis). DU145 cell line, 10 MeV electrons irradiation, at 3 and 18 Gy for both Conv (14 Gy/min) and FLASH (600 Gy/s) dose rates. Light colors correspond to a high differential effect, while dark colors correspond to a low differential effect. *In-vivo* - healthy tissue, and *in-vivo* - tumor regions are highlighted in white. Image from [2].

effect of healthy tissues at high doses and high dose rates - an effect contradicting conventional radiobiological modeling outcomes, which typically may explain larger damage in occurrence on a more temporally dense dose delivery - and not to explain the FLASH effect. In fact, at this initial stage of the study, we want to underline how the trend of the survival curves that we obtained is, in any case, in agreement with the few experimental *in-vitro* data currently available, such as [10].

In addition, it is worth stressing how different stochastic effects and time scales are in fact included in the here derived MS-GSM², as summarized in Table 4.1. The MS-GSM² construction highlights the several stochasticities that are taken into account in the MS-GSM². First, the time between consecutive energy deposition is stochastic, so both the evolution of the chemical environment and the yield of DNA damage depend on the time elapsing between two consecutive energy deposition events. Further, the spatial position of the track hitting the biological target, which we recall, in complete generality, is larger than the cell nucleus, is again stochastic so that in turn, each domain of the cell nucleus experiences a different energy imparted at any deposition event. It is worth noticing that, mostly in the case of high LET radiation, some domains could also have null energy deposited. Then again, both the chemical environment and the damage yield are affected by the stochastic energy deposition, so that, not only the time elapsed between consecutive tracks is stochastic but also the actual energy imparted is a random variable. Even further, the indirect damage yield depends on the chemical environment which thus accounts for a further stochasticity. At last, after the chemical environment reaches stability after the end of the irradiation, only the biological pathways of repair and clustering, described by MS-GSM² via r , a , and b , remain, so that the predicted cell survival probability can be computed. This represents the final level of stochasticity, influencing the time evolution of DNA lesions based on the previously discussed biological rates.

The obtained results, summarized in Figure 4.13, show a cell survival increase with a consequent tissue sparing at sufficiently high doses and dose rates; in particular, it is worth highlighting how both the dose and the dose rate play a crucial role in the sparing effect at the UHDR regime, consistently with most preclinical literature, interestingly collected as a general trend in [39]. In this sense, in the proposed model, several results point toward the conclusion that the dose and the dose rate play a different role in inducing the FLASH effect:

- (i) A sufficiently high dose rate allows for close enough energy depositions over time so that the chemical environment is modified appropriately;
- (ii) A sufficiently high dose permits spatial interaction of subsequent energy depositions;
- (iii) Relevant enough, as expected, the ion and its energy play a role in both dose- and dose-rate-related effects.

In fact, as shown in the results Figure 4.13, the dose and dose rate at which the FLASH effect emerges, and the magnitude of the sparing shown, depends strongly on the quality of the radiation considered. For both ions considered and more clear in the case of carbon ions, 4.6–4.7, it is evident that high local dose deposition is not sufficient to trigger the

FLASH effect, whereas, on the contrary, a uniform dose must be reached over the cell nucleus in order to give rise to the FLASH effect. In other words, enough domains must be above a certain dose threshold to have a significant response, since the FLASH effect is not a linear one. Energy depositions happen not only due to direct hit of tracks of the cell nucleus but also due to tracks passing nearby. This translates into more localized energy deposition for high LET and in a more spatially homogeneous dose pattern in lower LET. This is evident in Figures 4.6-4.7, where carbon ions are characterized by high local energy deposition. The same effect emerges in Figure 4.8, with sudden jumps in the cumulative deposited dose due to tracks hitting the cell nucleus.

Regarding the chemical environment, again it emerges from Figure 4.10 how both dose and dose rate concur to trigger the FLASH effect. Heuristically speaking, the main idea of the proposed model is, following the main mechanism already proposed in [7], to relate a cell survival sparing to the peroxy radical concentration $\text{ROO}^\bullet$ evolution in time. Therefore, the dose rate concurs to rise $[\text{ROO}^\bullet]$ in a short time, so that, if the absorbed dose is sufficiently high, causing $[\text{ROO}^\bullet]$ to be high enough, organic radical-radical recombination, during the chain termination in the homogeneous chemical stage, is more likely to happen with a consequent lower probability of interaction with the DNA. This reduced radical interaction probability with the DNA has a consequent DNA damage yield reduction, as emerges in fact in Figure 4.12.

This combined effect of dose and dose rate is also coherent with the inversion of the indirect damage yield pattern that emerges between 10 and 50 Gy/s. In fact, at a chemical level, a higher dose corresponds to a higher production of $\text{ROO}^\bullet$, which is clearly more marked at high dose rates, where the radiation-induced formation of $\text{ROO}^\bullet$ outpaces the chemical reactions responsible for its consumption. Indeed, only the combined effect of high dose and high dose rate leads to a different temporal evolution of $[\text{ROO}^\bullet]$: in particular, the high dose rate allows a faster growth of $[\text{ROO}^\bullet]$ but at the same time also a more rapid decrease due to the recombination of these chemical species. In fact, at high dose rates, the dose deposition events are much closer to each other in time and therefore this leads to competition with radical recombination effects. On the contrary, if the dose rate is not high enough, increasing the dose would only increase the rate of damage induction per unit Gy, i.e. the standard trend of damage dependence on the dose, which due to the well-known linear quadratic behavior, will result simply in an increasing derivative at larger dose. This is due to the fact that, at a low dose rate, the cell exposure to a high concentration of this harmful organic radical takes place for a prolonged time; this effect is similar to what occurs with drugs [290], as described in [7]. If, on the contrary, a sufficiently high dose is imparted over a short time period, organic radical-radical recombination is favored over interaction with DNA, leading to cell tissue sparing, which means that a high dose rate will trigger an additional quenching effect on the dose, on top of the standard one above recalled, while with conventional radiation, this latter one will simply not occur. Again, the sparing effect is more pronounced in the low LET case, whereas as the LET increases, the highly localized dose deposition pattern, implies that a local disappearance of some harmful chemical species occurs, however, this process does not happen on the whole cell, i.e. a higher dose is required to reach a sufficiently

spatially uniform dose needed to trigger the FLASH effect.

The above points also highlight some key differences between the originally proposed model in [7], discussed in Section 3.6, and its generalization proposed in the current research. The main novelty, compared to [7], is to provide a more direct link from the chemical environment to its biological effect. Radical concentration is thus related to indirect damage induction. Therefore, the general linear or quadratic relation between energy deposition and damage yield, already proposed decades ago in [291], is generalized in the present work so that a more direct link between the concentration of the radical and the consequent damage yield is considered. Also, the multiscale nature of the proposed model clearly emerges in several plots, such as in Figures 4.8-4.10. In fact, a purely discontinuous time pattern in energy deposition is evident. This translates, differently to what is considered in [7], into highly jagged radical concentration at low dose rates, which naturally turn continuous as the dose rate increases.

Therefore, with the proposed approach, we are able to directly link the radical concentration, which is directly modified by the energy deposition pattern depending on both dose and dose rate, to the DNA damage formation. Notably enough, conventional dose rates do not provide any changes in the indirect damage yield, whereas, as the dose rates increase, a reduction in the damage emerges. The magnitude of the damage reduction depends on the absorbed dose. Also, the overall pattern in the indirect damage yield reduction strongly depends on the radiation quality. In this sense, it is worth stressing that, Figure 4.12, reports the relative reduction in the indirect damage yield per unit Gy. Therefore, since the proportion of indirect damage compared to the direct damage in the high LET radiation is lower than in the low LET case, the overall reduction in the total damage yields would be even higher.

All the above discussion eventually emerges in the different sparing at the cell survival level. Figure 4.13 shows that a sufficiently high dose and dose rate results in cell survival sparing. Further, the highly localized energy deposition of high LET results in a lower sparing at 30 Gy compared to protons. In fact, the magnitude of the sparing of carbon ions at FLASH dose rates at 30 Gy is in the order of the sparing at FLASH dose rates for protons at 20 Gy. This is in agreement with the discussion that emerged above considering how the local pattern of energy deposition affects the chemical environment. It is worth stressing that the surviving fraction remains almost constant up to 10 Gy/s, after which there is an uprise of the surviving fraction. For protons, a slight increase in the surviving fraction emerges at 10 Gy and it is clear at higher doses, such as 30 Gy; this is in agreement with what was experimentally observed, [39]. On the contrary, carbon ion requires a higher dose to observe the FLASH effect, which is in agreement with [288]. Differently from the results derived in [221] and discussed in Section 3.6, where the predicted surviving fraction steadily decreases in the conventional dose rate regimes, reaching a minimum around 1 Gy/s, the predicted surviving fraction of the MS-GSM² remains almost unchanged in the range below 10 Gy/s, after which the FLASH effect is triggered.

Moving on to the comparison between the predictions obtained through the MS-GSM² model and the experimental data, presented in Section 4.4, a very interesting aspect arises from Figure 4.14: the survival level at 1.6 % O₂ for conventional irradiation is comparable

with those at 2.7 % O₂ for UHDR irradiation. This means that the rise of the surviving fraction in the case of FLASH irradiation, due to the reduction of indirect damage caused by the higher recombination of organic radicals, can have the same biological effect of a reduction of oxygenation. Environmental oxygen plays a crucial role in the emergence of the FLASH effect. We can see from the reaction network illustrated in Figure 4.1 and from the reactions system in Eq. 4.7 that oxygen is essential for producing organic radicals. However, it is important to note that this effect is not directly related to oxygen depletion. In the initial years of FLASH mechanistic investigations, the prevailing hypothesis was proposing a transient hypoxia induced by oxygen depletion [93, 213]. Still, this hypothesis has been widely discredited [238] by both theoretical calculations [27] and experimental evidence [232, 233], as we discussed in Section 3.5.1.

Another crucial point from Figures 4.14, 4.15, and 4.16 is that the sparing effect, i.e., the increase in cell survival at UHDR, is visible for every oxygenation level. In particular, the FLASH effect is also visible at 21 % O₂ condition, e.g., for electrons irradiation, as in Figure 4.14 or, in general, in [8, 175] for several cell lines, but also in the case of carbon ions, although at much reduced intensity compared to lower oxygenations, as in Figure 4.16. In contrast, in the case of *in-vivo* experiments, the FLASH effect was not observed at the highest oxygen levels [85]. In addition, unlike *in-vivo*, in the case of *in-vitro* measurements, the sparing effect occurs in both healthy and tumor cells. We speculate about a new possible mechanism for the FLASH differential effect. In particular, we hypothesize that these two aspects highlight how, most likely, the FLASH effect *in-vivo* is described by the action of at least two combined effects:

- (i) A protective effect depending on the dose rate;
- (ii) Another effect modulating its magnitude, depending on the redox environment.

Indeed, as already mentioned previously, we hypothesize that the sparing effect for UHDR irradiation, which is also visible *in-vitro*, is due to the different recombination of organic radicals, which decreases the toxicity of the cellular environment. Therefore, our idea is that this leads to a reduction of the indirect action of radiation on the formation of DNA damages, as recently suggested by two experiments on plasmids [129, 183], because the time evolution of organic peroxy radicals is strictly related to cellular damage [7]. At the same time, we speculate that the action of the redox environment [214], particularly the presence of antioxidants such as thiols, is the basis for the differential effect between healthy and tumor tissue observed for *in-vivo* experiments. This second aspect of the overall FLASH mechanism, which involves the antioxidant ability of the cell and may result in leveling out the magnitude of the DNA damage reduction, is much more complex than the mechanism underlying the sparing effect, which occurs on an intra-cellular level and results in a variation of the indirect DNA damage yield. This scavenging action may involve a more macroscopic regulation of the redox environment *in-vivo* compared to *in-vitro*.

We performed an *in-silico* investigation of our hypothesis about the mechanism behind the emergence of the FLASH effect, as shown in Section 4.5, trying to highlight the main experimentally observed differences between *in-vitro* and *in-vivo* scenarios discussed above.

It is important to emphasize that we do not claim our analysis replicates an *in-vivo* system. Instead, we are generating an *in-silico* hypothesis on the origin of the differential effect between normal and tumor tissue, quantifying them via the definition of RSE in Eq. 4.42. In particular, in Figure 4.17 we can identify the two regions for healthy and cancer tissues as jointly characterized by the environmental oxygenation level but also by a different redox balance, which is represented by the rate of antioxidants. It is possible to appreciate how in the normal tissue region there is a gradient of the effect, in the case of the tumor region there is no effect. Indeed, we can distinguish these two areas, e.g., due to the different concentrations of glutathione (GSH) between normal tissue and tumor [292, 293, 294, 295, 296], and also considering that $[O_2]_{\text{tumor}} < [O_2]_{\text{healthy}}$. From this Figure 4.17, in the case of 18 Gy, it is also possible to appreciate how, for a given level of antioxidants, the sparing effect decreases as the oxygen concentration increases, in agreement with the experiment predictions, e.g., [10]. Therefore, we hypothesize that the varying responses of normal and tumor tissues are attributable to distinct redox environments. Given that various tissues and tumors may possess differing concentrations of antioxidants, which are seldom quantified, our hypothesis could account for the inconsistent experimental observations regarding the FLASH effect. Moreover, our analysis suggests a more intricate role than solely oxygenation effects. Indeed, our findings indicate that many parameters contribute to the emergence of the sparing effect. For instance, a lower antioxidant level, typically observed in *in-vitro* cells, thus placing them in the lower region of the plot in Figure 4.17, requires a reduced dose to trigger a sparing effect, as we can appreciate by comparing the two plots corresponding to two different macroscopic doses. Thus, we also hypothesize that the gradient of the sparing effect of the *in-vitro* region explains both the fact that the FLASH effect is observed with different magnitude for the various cell lines and that it emerges for both healthy and cancer cells, e.g., as in [175]. Conversely, as antioxidant levels increase, as seen in healthy tissue or even more so in tumors, a significantly higher dose is necessary to induce the effect. This aligns with experimental observations, where *in-vitro* studies demonstrate a pronounced effect at lower doses (around 8 Gy), in contrast to *in-vivo* experiments that require doses exceeding 10 Gy. Furthermore, from the two plots in Figure 4.17, it can be observed that there is a dose threshold required to trigger the FLASH effect, given a fixed oxygenation level and antioxidant rate. Specifically, in the case of 3 Gy irradiation, the sparing effect is largely negligible compared to 18 Gy.

Therefore, we hypothesize that the emergence of the FLASH effect results from a complex interplay of competing reactions during the homogeneous chemical stage, where factors such as radiation quality, dose, dose rate, environmental oxygenation, and antioxidant activity influence the relative significance of different chemical processes. Experimental evidence indicates that three components modulate the FLASH effect:

- (i) A high dose rate is needed to activate the FLASH effect;
- (ii) A high dose is required;
- (iii) Tumor and healthy tissues exhibit differential responses.

The previous *in-silico* analysis, reported in Section 4.3, suggests that a sufficiently high dose and high dose rate are crucial to ensure particles' spatial and temporal proximity. This

proximity favors the recombination of organic chemical species during the homogeneous chemical stage rather than their interaction with DNA, resulting in an overall reduction in DNA damage. The differential effect is the main aspect that makes the FLASH effect so powerful and is currently the main open problem in the community, following the disproof of the oxygen depletion hypothesis [238]. Our analysis suggests a different redox balance between tumor and healthy tissue can modulate the FLASH effect. In particular, a higher scavenger capacity with higher antioxidant levels, typical of tumor tissue, can prevent the radical-radical recombination that we hypothesize to be at the origin of the FLASH effect, yielding an overall unvaried biological effect independently of the dose rate. In this work, we demonstrated how this hypothesis allows us to predict experimental results at different oxygen levels and dose rates by calibrating the biological parameters of the MS-GSM² model on the experimental data at standard conditions and highlighted the key role of the cellular chemical environment. Specifically, the oxygenation level and scavenging activity, through chemical reactions occurring during the homogeneous chemical stage, compete with recombination reactions, resulting in a modulation of the intensity of the FLASH effect, as shown in Figure 4.17.

We thus provided a novel mechanistic mathematical framework spanning all relevant scales, able from one side to be consistent with the current *in-vitro* data at UHDR and to suggest a consistent picture explaining the most relevant features related to the FLASH observations. Among the novel possibilities enabled by this work is applying the MS-GSM² to investigate specific collections of *in-vivo* data, as we will see in Chapters 5 and 6 for Conv and UHDR experiments, respectively.

Current limitations of MS-GSM² and future perspectives

Although the presently developed MS-GSM² model is general enough in the sense that it includes several effects of random processes in space and time inherent to three different domains such as physics, chemistry, and biology, it also presents some limitations. In particular, next, we discuss some possible future lines of research to strengthen the developed model.

It is worth stressing that $\sim 10^2$ Gy/s represents a limit for the proposed model. In fact, above $\sim 10^2$ Gy/s, the time elapsing two consecutive energy depositions significantly overlaps with the heterogeneous chemical stage. Therefore, in order to accurately considering high dose rates would require a more sophisticated chemical treatment that goes beyond the homogeneous chemical stage as considered in the current approach. Thus, a logical next step in extending the model would be to incorporate the heterogeneous chemical stage, considering the explicit impact of spatial diffusion of chemical species and faster reactions. However, such an extension would require a more sophisticated spatial description of the physical, chemical, and biological processes involved, moving from a domain-like formulation to a complete spatial model [297]. This would allow us to assess the role of radiation quality and LET in considering higher LET values. As LET increases, the importance of the late heterogeneous chemical scale becomes more relevant, necessitating an extension beyond the homogeneous chemical stage to perform a realistic model-based analysis, such as the one conducted in this study. The current study gen-

erally relates the FLASH effect to a sparing of indirect damage arising from spatial and temporal effects on organic radicals. Since higher LET is characterized by a more localized pattern of spatial energy deposition and a decreased importance of indirect effects, it is unclear how high LET would influence the FLASH effect. This is especially true given the limited experimental studies investigating such a scenario [99]. However, we are currently extending the MS-GSM² model to include higher LET cases to study the potential role of LET on the FLASH effect, as we will see in Chapter 7.

Building on the previous discussion on heterogeneous chemistry, another limitation of our model is that we do not consider possible inter-track effects that may arise during the heterogeneous chemical stage. In principle, such effects could be addressed either by explicitly incorporating this stage into MS-GSM², though this would involve the challenges outlined above, or by parameterizing them through the modulation of G-values for various chemical species up to the microsecond timescale, as suggested in [250]. However, the latter approach would involve substantial computational demands, as it would necessitate accounting for the influence of each individual particle depositing energy within the cell nucleus on the surrounding heterogeneous chemical environment. For example, in [250], inter-track effects were analyzed for only two particles due to such complexity. Furthermore, it would be essential to evaluate the actual biological relevance of any resulting changes in the chemical environment. Indeed, the same study demonstrated that, in general, at least for aqueous environments, inter-track effects are likely negligible in the context of FLASH RT. Nevertheless, we do not exclude the possibility of exploring inter-track effects in future work, particularly in relation to biologically relevant environments.

It is also important to note that our model, by design, does not account for the full complexity of the cellular environment, which remains only partially understood. In addition, the repair process is represented by the pathway in Eq. 4.34, governed by the rate r , which is a simplification of the actual mechanism. Nonetheless, we believe that the assumptions made offer a reasonable compromise between a realistic bio-chemical description and computational feasibility. However, as we will discuss in Section 8, ongoing work aims to expand the chemical reaction network, for example by explicitly incorporating the effects of lipid membrane damage and additional antioxidant-mediated chemical pathways.

As well known, the FLASH biological effect can be described by two different and presently not explained aspects, i.e. the sparing effect of the healthy tissues and the differential effect between normal tissues and tumors. In the first part of this work, whose results are reported in Section 4.3, we focus on the first aspect of the UHDR irradiation, i.e. the higher sparing of the healthy tissues, at high doses and high dose rates, with respect to the conventional irradiation; nevertheless, our model is not in contradiction with some of the most accredited hypotheses that try to explain the differential effect between normal tissues and tumors. The first hypothesis is based on a combination of two mechanisms linked to DNA damage, i.e. a different damage formation, due to the reduction of a specific oxygen-dependent DNA damage, which is consistent with a decrease of the organic peroxy radical concentration, and a defective repair process, such as a pathologic chromosome rearrangements due to an aberrant DNA double-strand breaks repair, as described by [7]. The second conjecture postulates that a different accumulation of a specific chemical

species between normal tissue and tumor is the basis for the differential effect, as proposed by [218]. In particular, this hypothesis states that normal tissues have a greater ability to detoxify from reactive oxygen species, reducing oxidative injury, with respect to tumors, as described in [214]. In fact, normal cells have a more rapid elimination, mediated by enzymes, of peroxidized compounds compared to tumor cells, [218]. We may test these hypotheses in future work. However, as seen in Section 4.5 and discussed previously, our idea is that the redox environment is crucial in determining the differential effect between healthy and tumor tissues.

Currently, the MS-GSM² model only includes DNA-damage-related death pathways, which are believed to play a major role in *in-vitro* biological systems. However, *in-vivo* biological systems are more complex, and other lethal pathways, not necessarily related to cell death, may also be involved. Lipid peroxidation and ferroptosis [218, 7, 214, 99] are extensively investigated and are believed to also contribute to the emergence of the FLASH effect. Since the current version of the model accounts only for DNA damage, our main investigation has been based on *in-vitro* data, whose results are reported in Section 4.4. Extensions to include other lethal pathways are underway, as we will see in Section 8.1. Nevertheless, we believe DNA damage still plays a fundamental role in *in-vivo* studies. Given that lipids and ferroptosis are highly influenced by the chemical environment and redox balance, these pathways could be modified at UHDR in a parallel way by the same biological mechanism proposed in our research in Section 4.5, as also suggested in [7]. We hypothesize that *in-vivo*, the same mechanism could simultaneously reduce different death pathways, supporting the idea that the FLASH effect impacts multiple biological pathways at once. We believe that a chemical mechanism is at the origin of the FLASH effect, as it would uniquely affect multiple pathways simultaneously.

Another aspect we are currently investigating with the MS-GSM² model is the role of the temporal dose rate structure in the emergence of the FLASH effect. The scientific community is actively working to understand which notion of dose rate most closely correlates with the FLASH effect. This has immediate implications for translating FLASH into clinical practice, as it is known that different delivery patterns ensure dose uniformity but profoundly different dose rate structures. As described in Section 4.1, this model allows us to consider single deposition events. Thus, our model does not require any parameterization of the dose rate time structure, neither as an average dose rate nor as an intra-pulse dose rate. Instead, the stochastic simulation algorithm that was developed considers the entire dose rate time structure. This, in principle, would allow us to consider any temporal structure of dose delivery. Consequently, we are investigating which aspects of dose rate time structure are most relevant, performing an *in-silico* analysis on simplified treatment plans to understand how different dose delivery patterns, as we will see in Section 8.3, and, therefore, different dose rate time structures affect the overall FLASH effect.

The last natural generalization of the model is the extension to the investigation of the FLASH effect of macroscopic biological systems, thus considering slower and more complex biological processes and spatial scales of the order of tissues and organs. In this context, we are integrating the radiobiological model developed here into a model for TCP and NTCP predictions, as reported in Chapters 5 and 6. This can enable a more mechanistic

analysis of the phenomena underlying the FLASH effects, studying the possible impact of oxygenation gradients, different fractionation schemes, dose-delivery time structures, partial irradiations, and volume effects. A major step forward is exploiting the present tool to facilitate the clinical translation of the FLASH radiation therapy. Indeed, the MS-GSM², in principle, can straightforwardly be used to enable FLASH treatment planning through a specific biologically-driven optimization of the plan, including organ volume effects, such as in [80], as we will discuss in Section 8.3.

Chapter 5

The GSM²-driven NTCP and TCP models for conventional irradiation

In conventional radiotherapy, the probability of controlling tumor growth is quantified using TCP models, as presented in Section 2.4.3. Instead, the probability of experiencing a side effect after the irradiation of healthy tissues and organs is typically assessed using the concept of NTCP, a crucial metric for evaluating and comparing treatment plans, as discussed in Section 2.4. As we have seen previously, there are two types of approach: the first is the phenomenological approach, which aims to describe the probability of a given toxicity occurring based on clinical tolerance doses for different irradiated volumes. The second is the mechanistic approach, which seeks to model the response to ionizing radiation by considering tissues and organs' functional and anatomical organization. However, these latter types of models are often based on the assumption of Poisson statistics for cell kill [72].

This Chapter is entirely dedicated to the conception, realization, implementation, and validation of a general mechanistic model to describe the effects of radiation on different tissue organizations without Poissonian assumptions, extending the GSM², which is the stochastic radiobiological model reported in Section 2.3.1, to the study of macroscopic biological systems. This work falls within the scope of extending a traditional radiobiological model including additional stages of radiation-induced biological damage. While in Chapter 4 intermediate spatio-temporal scales between the physical and biological ones, namely, the homogeneous chemical stage, were included, in this Chapter, we extend the biological stage of the GSM² model to larger spatial and temporal scales, involving cell populations with a specific geometric and functional architecture.

In particular, the GSM²-driven NTCP and TCP models are mechanistic-driven dose-response models [12] that integrate the GSM² into the RSM model [72], allowing a comprehensive investigation of clinically relevant endpoints, such as healthy tissue complications and tumor growth predictions for conventional irradiation. The model's single-cell resolution allows it to account for energy deposition and tissue heterogeneity, considering different organ volume effects and oxygen gradients for different radiation qualities of the beam, that is, type, energy, and LET of radiation. Additionally, the model can compute

damage repair for each cell over time, enabling the implementation of various fractionation schemes. Thus, thanks to their extreme generality, the GSM²-driven NTCP and TCP models can exploit several clinically relevant parameters for Conv RT.

The main contributions that will be derived in the present Chapter are the following:

- (i) to introduce general mechanistic-driven models able to provide a comprehensive investigation of clinically relevant endpoints;
- (ii) to show the interplay between physical and environmental parameters on the induction of side effects on healthy tissues;
- (iii) to reproduce *in-vivo* Conv experiments in healthy tissues, for different radiation qualities and fractionation schemes;
- (iv) to propose a flexible mathematical framework to be potentially extended also to the study of radiation-induced toxicity in UHDR regime.

In particular, this Chapter is structured as follows: Section 5.1 presents the development of the mathematical framework of the mechanistic-driven NTCP and TCP models; Section 5.2 reports the numerical implementation of the dose-response tools; Section 5.3 shows the main numerical results obtained in terms of prediction of complications; Section 5.4 demonstrates the feasibility of the dose-effect model in reproducing *in-vivo* findings reported in the literature for different experimental scenarios; finally, Section 5.5 discusses the main results of the GSM²-driven NTCP model. Thus, the main focus of this Chapter will be on the dose-effect relations for healthy tissues because the NTCP tool developed here will be extended to FLASH irradiation in Chapter 6.

5.1 Theory and calculations

This Section is dedicated to describing the mathematical framework of the GSM²-driven dose-response models, as illustrated in Figure 5.1.

As we can see in Figure 5.2, we consider a cell population of N_c cells, which are considered spheres of radius R_{cell} . The cells are arranged on a 3D grid of the target volume, with the centers of the cells spaced $2R_{cell}$ apart from each other. Each cell has a cylindrical nucleus of radius R_N and length $2R_N$, and it is divided into N_d domains, in analogy to the approach presented in Section 4.1 for the multi-scale model, but in this case using the standard GSM² model [3, 4] for the cell response to IR. Since this approach is the multi-cell generalization of the model discussed in Chapter 4.1 for the computation of the energy deposition, DNA damage formation, and evolution, but without considering chemistry, in the following paragraphs, we particularly focus on the description of the mathematical framework of the model for describing the organ's response.

(i) The energy deposition model

Depending on the considered application, a region of the cell population, which can also be the entire cell population, is irradiated with a circular beam of radius R_{beam} with a

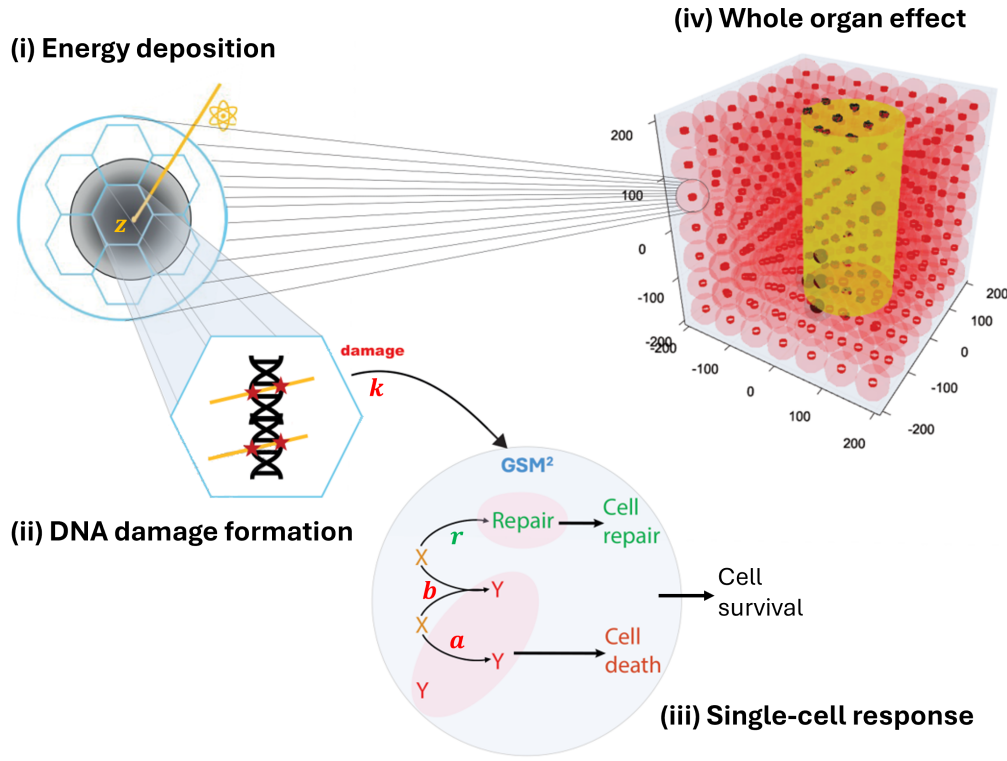


Figure 5.1: Schematic representation of the GSM²-driven NTCP and TCP workflow. The model takes into account different spatial and temporal scales of radiation-induced biological damage, that are (i) the physical stage (energy deposition), (ii) the bio-chemical stage (DNA damage formation), and (iii-iv) the biological stage (single-cell response and whole organ effect). The physical stage describes the energy deposition on a cell nucleus according to microdosimetry. The bio-chemical stage encompasses radiation-induced sub-lethal and lethal DNA damages, incorporating modulation effects driven by radiation quality and oxygen availability. The biological stage models the temporal evolution of DNA lesions through three distinct biological pathways, i.e. repair, death, and combined-death cellular mechanisms, and the macroscopic response of the organ, considering its specific biological architecture, which is described by the seriality parameter s .

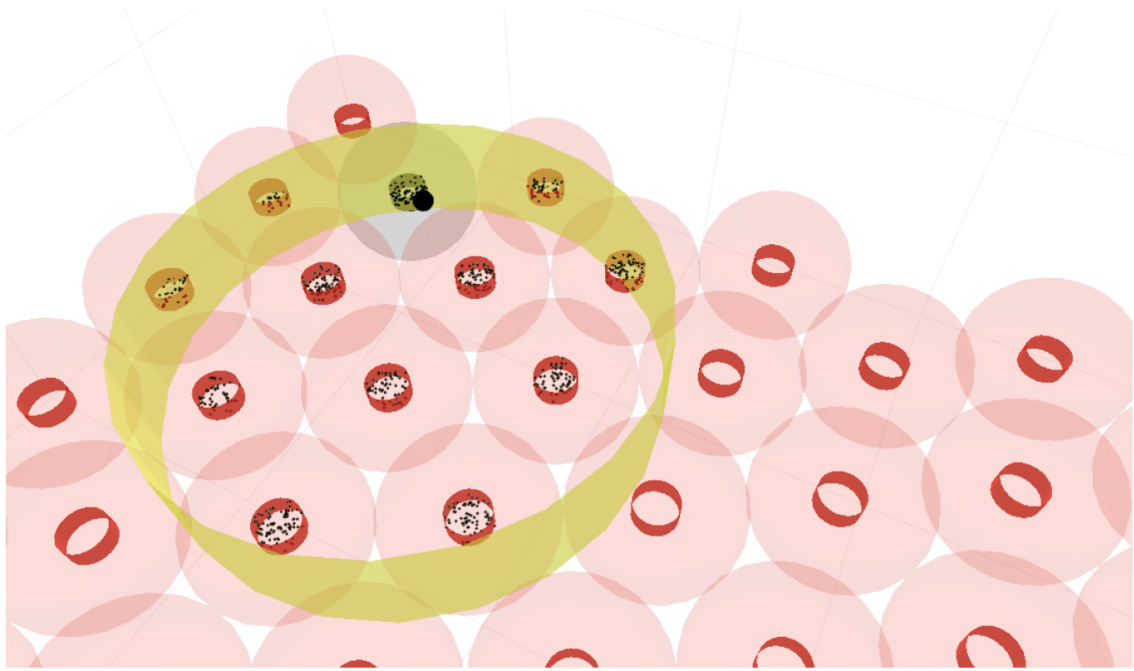


Figure 5.2: Illustration of a typical application of the GSM² to multi-cellular systems. Irradiation of the target volume with 10^6 particles uniformly sampled in the cylindrical beam in yellow, and corresponding damages distribution in the cell nuclei. The pink spheres are the cells, the red cylinders are the nuclei, the small black dots are the damage in the cell nuclei. The cells in black are considered dead.

certain LET spectrum, which characterizes the radiation quality, and dose D . The beam penetrates the tissue along the z -direction.

The number of incident particles is randomly sampled from a Poisson distribution of average fluence $(\pi R_{beam}^2)^{-1}$. Each particle is randomly distributed along the planar xy -plane inside the beam. Also, the corresponding LET is sampled from the LET spectrum for each particle.

The absorbed energy of every energy deposition event z is distributed on each cell nucleus according to an AT model [52], as defined in Section 2.1.2. The AT model postulates energy deposition by a particle with cylindrical symmetry, with a uniform energy distribution across a circle of radius R_c centered at the impact point. This distribution diminishes proportionately to r^{-2} , where r represents the distance from the impact point. The model assumes no energy is deposited beyond a specific distance, denoted as R_p . The radial energy distribution is thus computed according to Eq. 4.1. Thus, in this way, we can know the absorbed dose at each domain in the xy -plane inside each cell nucleus, as discussed in Section 4.1.

Sampling the impact position of the primary particles and the LET gives an energy stochastic deposition described by microdosimetry principles, presented in Section 2.1.3. Therefore, each cell will experience a different energy deposition.

For low-LET radiation, we assume the so-called "track-segment condition" within each nucleus, meaning that the energy of the incoming particle is high enough that energy loss inside the cell nucleus can be disregarded. Thus, the LET is constant within the cylinder of length $2R_N$, in analogy to [41]. On the contrary, energy loss is accounted for by different layers of cells along the penetration direction z of the beam. This can be computed analytically for ions, according to the Bethe-Bloch formula of Eq. 1.1. Knowing the energy loss by ions per unit of path length in the z -direction, it is possible to compute the corresponding radial dose distribution in the xy -plane for each layer. The total energy deposited in each cell is thus calculated, summing up all the energy deposition by the single particle energy deposition.

(ii) The DNA damage formation model

The model assumes the creation of two possible types of radiation-induced DNA damage, X and Y , which are the sub-lethal and the lethal lesions, respectively, according to the GSM² model, as presented in Section 2.3.1. The mathematical description of the formation of DNA lesions, including the impact of the environmental oxygenation level, but without considering dose rate mediated effects, is reported in Section 4.1.

(iii) The single-cell response model

Given the lethal and sub-lethal lesions X and Y , respectively, distributions within each domain d of each cell, the repair and death pathways can be determined as described by the GSM² model. Specifically, we consider the three usual biological pathways, which are a repair pathway stating that each sub-lethal lesion is repaired at rate r , a death pathway of sub-lethal lesions X to become a lethal one Y at rate a , and another death pathway for

the pairwise interaction of two sub-lethal lesions X at rate b , according to Eq. 4.34, and as described in Section 4.1. Finally, the model predicts the survival probability S_j of each cell, according to Eq. 4.37, which is the input of the organ response model.

(iv) The whole organ effect model

We extend the single-cell GSM² model, discussed in Section 2.3.1, to include the general serial/parallel organ model as in [72]. In this way, the model can consider volume effects, namely, how an organ responds to damage depending on its tissue architecture. We assume that an organ comprises FSUs constituting the organ's basic functioning units, as defined in Section 2.4.1. Thus, a single FSU is the minimum ensemble of clonogenic cells that preserves organ functionality. Different organs have different arrangements and sizes of FSUs: [298] reports the typical size considered for different organs relevant to radiotherapy. In this study, for simplicity, we model the FSUs as cubic boxes with a given side length l_{FSU} . However, it is possible to describe FSUs of any geometric shape and size.

As standard, [299], we assume that a single surviving clonogen in an FSU is enough for the i -th FSU to repopulate and survive. We define $P_i^{\text{FSU}}(D_i, \text{LET}_i, [\text{O}_2]_i)$ as the probability that all clonogens in the FSU die given an average dose D and LET, also considering an oxygenation level $[\text{O}_2]$. Modeling the FSU as outlined, we can calculate its biological response to IR as

$$P_i^{\text{FSU}}(D_i, \text{LET}_i, [\text{O}_2]_i) := \prod_{j=1}^{N_{\text{cell}}} \left(1 - S_j(z_n^j, \text{LET}^j, X^j, Y^j | D_i, \text{LET}_i, [\text{O}_2]_i) \right), \quad (5.1)$$

where the index j spans all clonogens N_{cell} inside the FSU.

Also, $S_j(z_n^j, \text{LET}^j, X^j, Y^j | D_i, \text{LET}_i, [\text{O}_2]_i)$ represents the survival probability of the j -th clonogen after receiving a local dose z_n^j and radiation quality LET^j , considering an average macroscopic dose D and LET, along with a distribution of sub-lethal and lethal lesions X^j and Y^j , respectively. A notable distinction from most existing models is that each clonogen possesses a unique survival probability due to the stochastic nature of energy deposition and resulting damage induction. Moreover, all clonogens are correlated through the average macroscopic dose D and LET.

The organ response to radiation is thus determined by the organ structure, described by how these FSUs are geometrically and/or functionally interconnected. In particular, it is defined as a parallel organ that requires all of its FSUs to die to be inactivated. On the contrary, a serial organ is inactivated as soon as a single FSU is inactivated, as discussed in Section 2.4.1. Figure 5.3 reports different organ structures: a serial, a parallel, and a complex organ structure.

The response of a completely serial organ composed by m FSUs, e.g. Figure 5.3 (a), each of which received an average dose D_i and LET_i , is computed as

$$P^{\text{serial}} = 1 - \prod_{i=1}^m \left(1 - P_i^{\text{FSU}}(D_i, \text{LET}_i, [\text{O}_2]_i) \right). \quad (5.2)$$

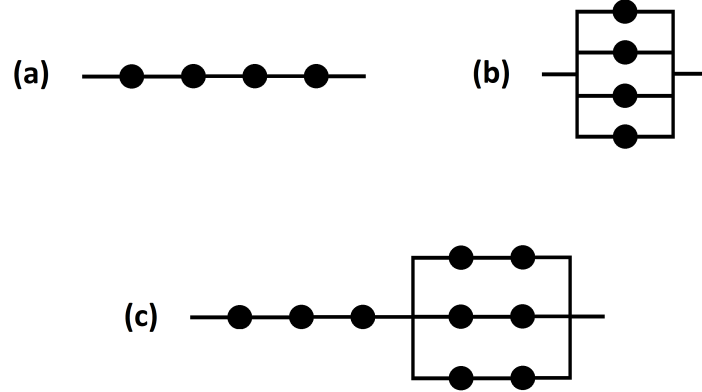


Figure 5.3: Schematic representation of (a) serial, (b) parallel, and (c) complex organ structure. The points represent the functional sub-units (FSUs), while the solid lines represent the connections between FSUs.

Thus, inactivating any FSU leads to complications. In this case, we typically refer to it as a *small volume effect*.

On the contrary, the response to radiation of a parallel organ with n FSUs, as reported in Figure 5.3 (b), is calculated as

$$P^{\text{parallel}} = \prod_{i=1}^n P_i^{\text{FSU}}(D_i, \text{LET}_i, [\text{O}_2]_i) . \quad (5.3)$$

So, any FSU remaining active preserves organ function. For this reason, we usually talk about a *large volume effect*.

In addition, the intermediate situation can be considered as the response of an organ composed of m serial FSUs and n parallel FSUs, e.g. Figure 5.3 (c). So we define the NTCP as

$$\text{NTCP} = \prod_{k=1}^n \left[1 - \prod_{i=1}^m (1 - P_{i,k}(D_{ik}, \text{LET}_{ik}, [\text{O}_2]_{ik})) \right] . \quad (5.4)$$

which is a generalization of the RSM model [72].

The tumor is considered a purely parallel organ, so the TCP is defined as the probability of killing the tumor. Given the tumor parallel nature we obtain

$$\text{TCP} = \prod_{i=1}^n P_i(D_i, \text{LET}_i, [\text{O}_2]_i) . \quad (5.5)$$

Regarding normal tissue, since complete knowledge of an organ structure is not always possible to be known, the above organ response can be parameterized in terms of a seriality

parameter s , which is defined as the number of serial FSUs, m , with respect to the total number of FSUs, which is $M = n \cdot m$, i.e.

$$s := \frac{m}{n \cdot m} = \frac{1}{n} \quad (5.6)$$

according to [72]. In particular, $s = 1$ corresponds to a fully serial organ, while $s \rightarrow 0$ describes a fully parallel organ. Thus, the NTCP can also be computed as

$$\text{NTCP} = \left[1 - \prod_{i=1}^M (1 - P_i(D_i, \text{LET}_i, [\text{O}_2]_i))^s \right]^{\frac{1}{s}}. \quad (5.7)$$

Equations 5.2, 5.3, 5.4, 5.5, and 5.7 mark a substantial difference with existing models, where $P_i(D_i, \text{LET}_i)$ is often calculated assuming Poisson statistics, averaging over the entire cell population, as in [72, 300].

We further defined the gEUD received by an organ or a tumor volume as

$$\text{gEUD} = \left(\frac{1}{M} \sum_{i=1}^M D_i^a \right)^{\frac{1}{a}}, \quad (5.8)$$

where D_i is the dose received by the i -th FSU. For $a \rightarrow -\infty$, $\text{gEUD} \rightarrow D_{\min}$, allowing the description of tumors; for $a \rightarrow \infty$, $\text{gEUD} \rightarrow D_{\max}$, which is the case for serial organs; for $a \rightarrow 1$, $\text{gEUD} \rightarrow D_{\text{mean}}$, which is used for parallel organs. This quantity allows us to describe a heterogeneous dose distribution in terms of a single dose value [80, 301], as discussed in Section 2.4.2.

In summary, the developed approach for the GSM²-driven NTCP and TCP models discussed in this Section allows the investigation of several experimental scenarios. In detail, it can take into account several parameters and effects, such as

1. radiation quality (particle type, energy and LET);
2. dose prescription;
3. fractionation scheme;
4. environmental oxygenation level;
5. cell line;
6. tissue architecture and volume effect.

Furthermore, physically, the model can take into account energy deposition heterogeneity and stochasticity, while biologically, it describes multiple effects at different scales, from well-known ones at the cellular level, such as "oxygen fixation effect" on DNA damage formation, to complex whole organ response.

5.2 Numerical implementations

This Section aims to show the stepwise construction and computational information of the GSM²-driven NTCP and TCP models, reported in Eqs. 5.4, 5.7, and 5.5. In order to do this, we performed the following steps:

1. We consider a parallelepiped of water, which mimics the organ volume, divided by m by n cubic boxes of side length l_{FSU} , i.e. $M = m \cdot n$ FSUs, that represent the structural arrangement of the tissue. The organ is characterized by a volume effect parameter s , according to Eq. 5.6, and an oxygenation level $[O_2]$. Each of these FSUs contains N_{cell} cells, i.e. a cell population, as previously described;
2. Given the radiation quality of the primary radiation, the radius of the beam R_{beam} , and a macroscopic prescribed dose D , we calculate the impact point of the track hit and the corresponding single-event energy deposition z ;
3. We calculate the radial dose distribution on the nucleus of each cell, and according to the AT model in Eq. 4.1, we obtain the absorbed dose z_d for each particle ν in each domain d ;
4. We simulate the evolution pathways via a SSA, given the initial number of lesions, according to the biological pathways in Eq. 4.34;
5. We calculate the survival probability of each cell from the lesion's distributions.
6. We compute the probability that all clonogens in the FSU die according to Eq. 5.1, repeating the procedure for each FSU.
7. We predict the NTCP or the TCP according to Eqs. 5.4-5.7 or 5.5, respectively, for the irradiated organ or tumor, respectively.

The previous physical processes cannot be computed independently for each cell or FSU because they simultaneously involve more cells or FSUs. Instead, the computation of each FSU response can be performed independently.

From a computational point of view, the GSM²-driven dose-response models were implemented using *Julia* [282] following the above stepwise construction.

5.3 Numerical results

This Section presents the numerical results obtained through the GSM²-driven NTCP model. We focus on studying the impact of several physical and biological parameters in modulating the dose-effect relationship. In particular, we investigate the feasibility of the GSM²-driven NTCP model to describe various experimental scenarios, performing simulations for different radiation qualities of the projectile, characterized by particle type, energy, and LET. We analyze the impact of different macroscopic doses, fractionation schemes, and partial irradiations (PIs) on the emergence of side effects. In addition, we

consider different levels of environmental oxygen and various organs with specific volume effects.

In particular, to illustrate the main features of the GSM²-driven NTCP model, we consider a parallelepiped of water, which mimics an organ of volume 0.9 mm^3 , for simplicity, divided by m by n cubic boxes, i.e. $M = m \cdot n$ FSUs, that represent the structural arrangement of the tissue. Each FSU contains N_{cell}^{FSU} cells of radius $10 \text{ }\mu\text{m}$, i.e., a cell population with a nucleus radius of $8 \text{ }\mu\text{m}$, as previously described. For a fixed value of the organ and FSU volumes, we study the impact of different arrangement of the M FSUs, i.e. the different tissue seriality according to Eq. 5.6, on the biological outcome, i.e. the NTCP, for the whole organ, in the case of uniform irradiation of the organ for different doses per fraction, considering 30 total fractions. We want to underline how, in the case of a completely parallel organ, i.e. for $s \rightarrow 0$, the dose-response curve corresponds to the TCP. For this *in-silico* study, we consider realistic values for the three biological rates of the GSM², namely $a = 0.01$, $b = 0.3$, and $r = 4.3$.

We also study the role of the partial irradiation of the organ, delivering an uniform dose only on a fraction of the whole organ, repeating the procedure for different dose levels, tissue seriality parameters, radiation qualities (particle type, energy and LET), and environmental oxygen concentrations, on the emergence of a complication for that organ.

In the following, we present the main results obtained from the simulation with the GSM²-driven NTCP model. In detail, Figure 5.4 illustrates the impact of radiation quality on the dose-response relationship. In particular, the NTCP curves against the dose per fraction were obtained simulating different primary radiation, i.e., from 0.86 to $52.0 \text{ keV}/\mu\text{m}$, irradiating the whole target volume, that is $\text{PI} = 100\%$, and fixing the volume effect parameter at $s=1$ for a fully serial organ, and the environmental oxygenation level at $21\% \text{ O}_2$.

Next, we will focus on the results obtained for two representative cases of radiation quality: 80 MeV protons and 40 MeV/u carbon ions. Figure 5.5 displays the influence of the environmental oxygen concentration on the NTCP as a function of the dose per fraction. We consider 30 fractions and doses per fraction in the $0\text{-}3 \text{ Gy}$ range. The curves in Figures 5.5a and 5.5b were obtained repeating the simulation for different oxygenation levels, i.e., from 0.5% to $21\% \text{ O}_2$, for 80 MeV proton beam and 40 MeV/u carbon ion beam irradiation of the entire target, respectively, and fixing the volume effect parameter to $s=0.03$.

Figures 5.6 and 5.7 report the NTCP as a function of dose per fraction considering 80 MeV proton beam and 40 MeV/u carbon ion beam irradiation, respectively, for different PI, i.e., 3% and 100% , and volume effects, i.e., $s = 0.03, 0.07, 0.1, 0.17, 0.33, 0.5$, and 1.0 , considering an environmental oxygenation level at $21\% \text{ O}_2$. In particular, Figures 5.6a and 5.7a show the dose-response relationship for irradiation of the whole target volume, i.e. $\text{PI} = 100\%$, while Figures 5.6b and 5.7b reveal the impact of different volume effects of the irradiated organ in the case of $\text{PI} = 3\%$ of total target volume, for 80 MeV proton beam and 40 MeV/u carbon ion beam irradiation, respectively.

Figure 5.8 shows the dose-effect relationship simulating 80 MeV proton beam irradi-

ation, respectively, for different PI, i.e., from 3% to 100%, fixing the volume effect. In particular, Figures 5.8a and 5.8b show the NTCP curves as a function of dose for two volume effect parameters s , i.e., 0.03 and 1.0, respectively, considering an oxygen concentration of 21% O_2 . The impact of partial irradiation is also emphasized in the 2D plots in Figure 5.9, using the same parameters previously described.

Figure 5.10 reports the computation times for 1 Gy irradiation of a box of 1 mm^3 that contains 8217 cells with a beam of radius 707 mm for different radiation qualities, in particular proton, helium ion, and carbon ion beams with various LET values. We use an Intel(R) Core(TM) i9-14900, 2.00 GHz, RAM 64,0 GB, 31 cores.

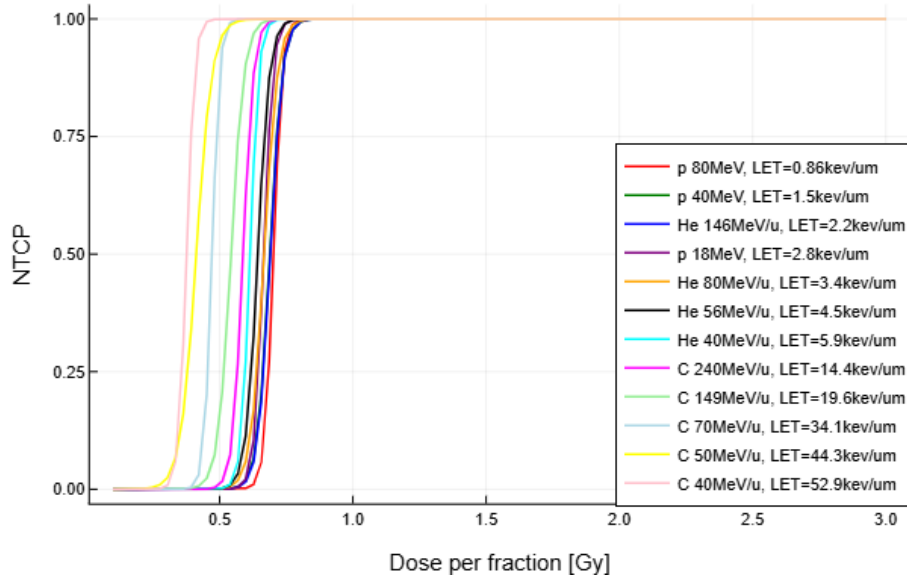
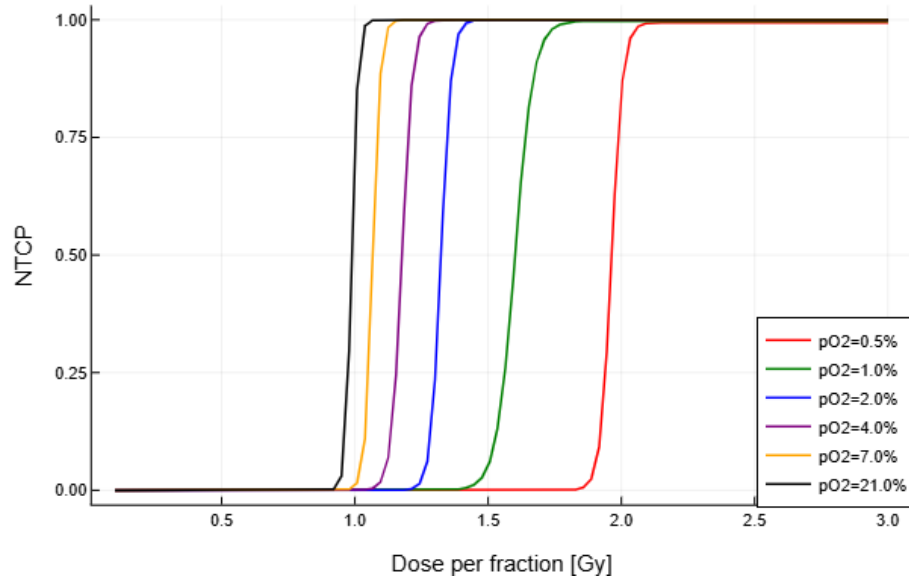


Figure 5.4: Normal Tissue Complication Probability (NTCP) as a function of dose per fraction in the case of an irradiation of the whole target volume in 30 fractions, an environmental oxygenation level at 21% O_2 , and a volume effect parameter $s=1$, for different radiation qualities, i.e., from 0.86 to 52.0 $\text{keV}/\mu\text{m}$.

5.4 Comparison with *in-vivo* experiments

This Section compares experimental *in-vivo* data of rat spinal cord tolerance [13, 14, 15], which is a fully serial organ, and GSM²-driven NTCP predictions. To underline the predictive power of the GSM²-driven NTCP model, the model predictions have been compared with some experimental data available in the literature, i.e. [13, 14, 15], in the case of Conv RT, for different radiation qualities (protons, helium, and carbon ions) and fractionation schemes, and for fixed total dose. For such experiments, we considered all the parameters (irradiation volume, number of fractions, dose per fraction, radiation quality, etc.), as reported in the original publications. In particular, we considered an irradiated



(a) p 80 MeV.

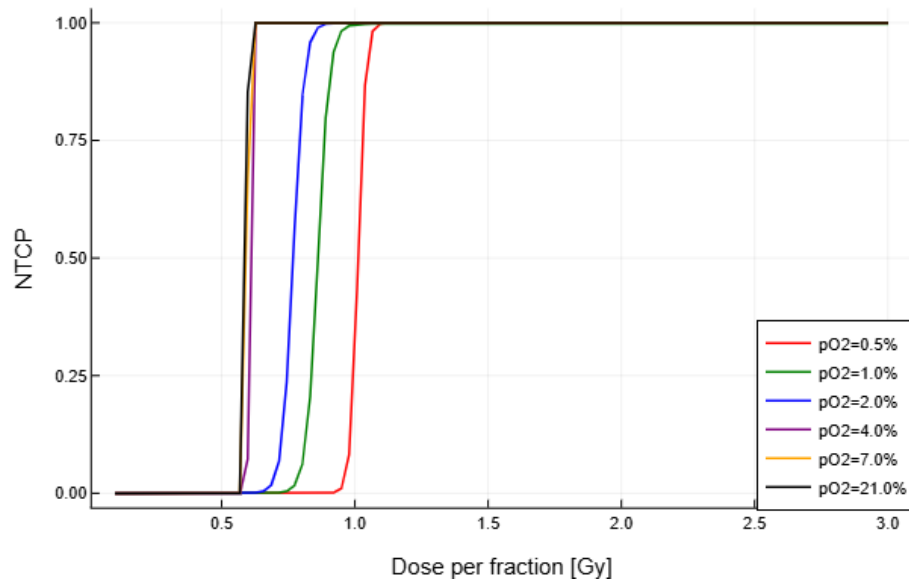
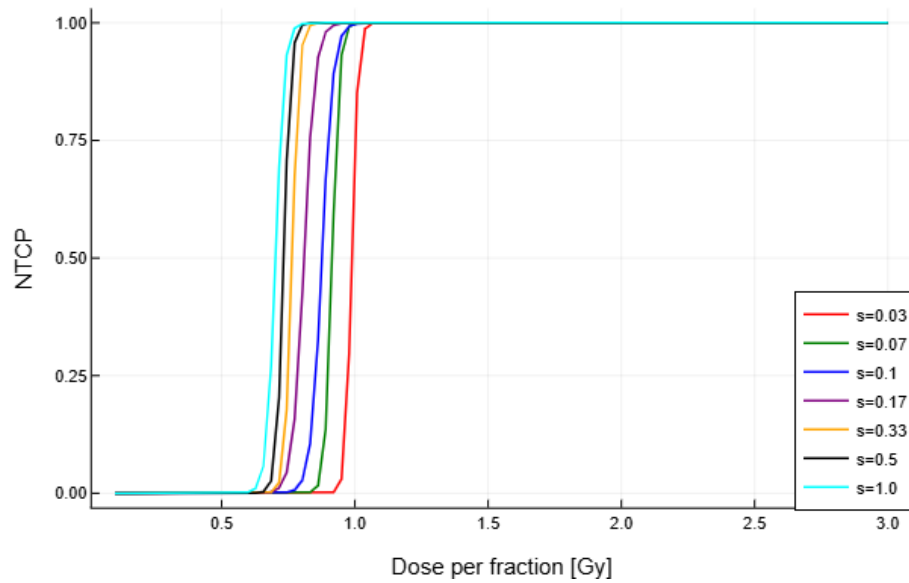
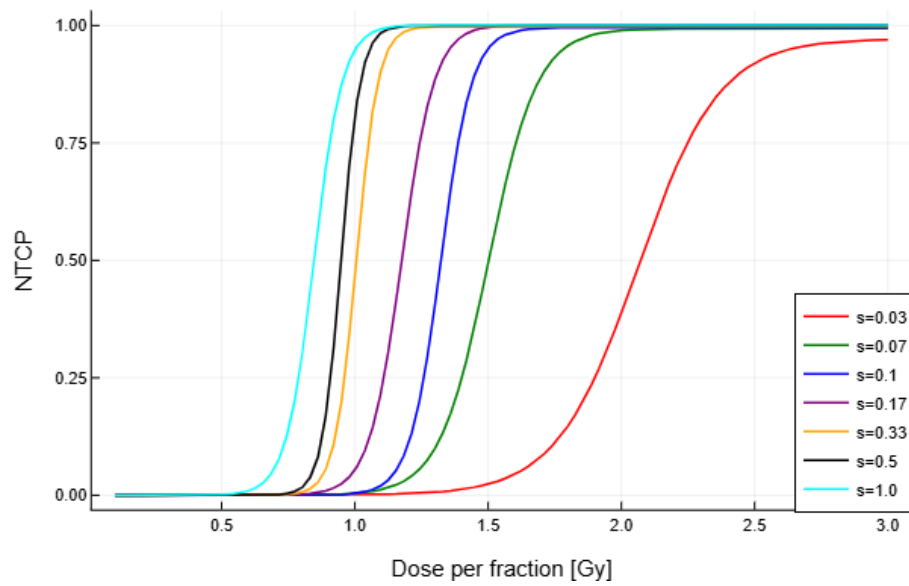
(b) ^{12}C 40 MeV/u.

Figure 5.5: Normal Tissue Complication Probability (NTCP) as a function of dose per fraction for (a) 80 MeV proton beam irradiation, and (b) 40 MeV/u carbon ion beam irradiation, for different environmental oxygenation levels, ranging from 0.5% to 21% O_2 , in the case of an irradiation of the whole target volume in 30 fractions, for volume effect parameter $s=0.03$.

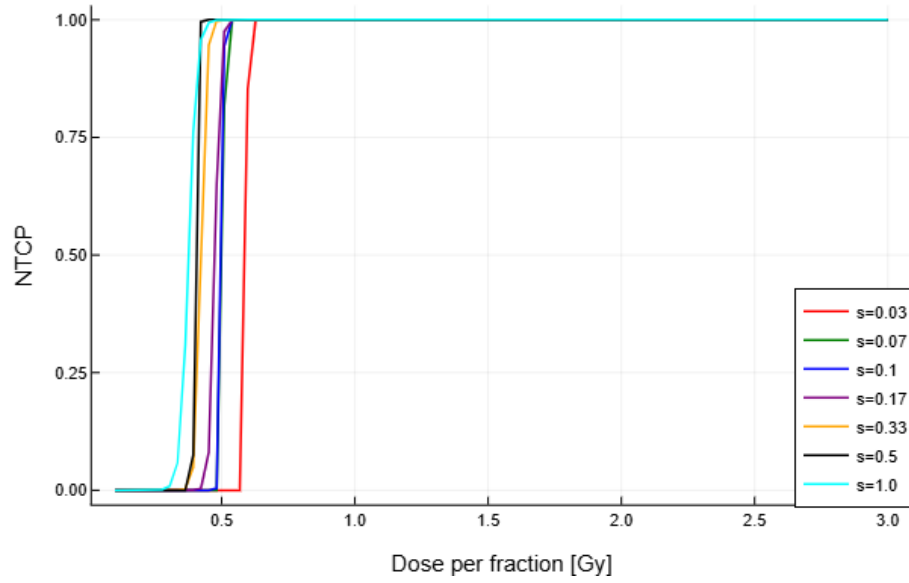


(a) p 80 MeV, PI = 100%.

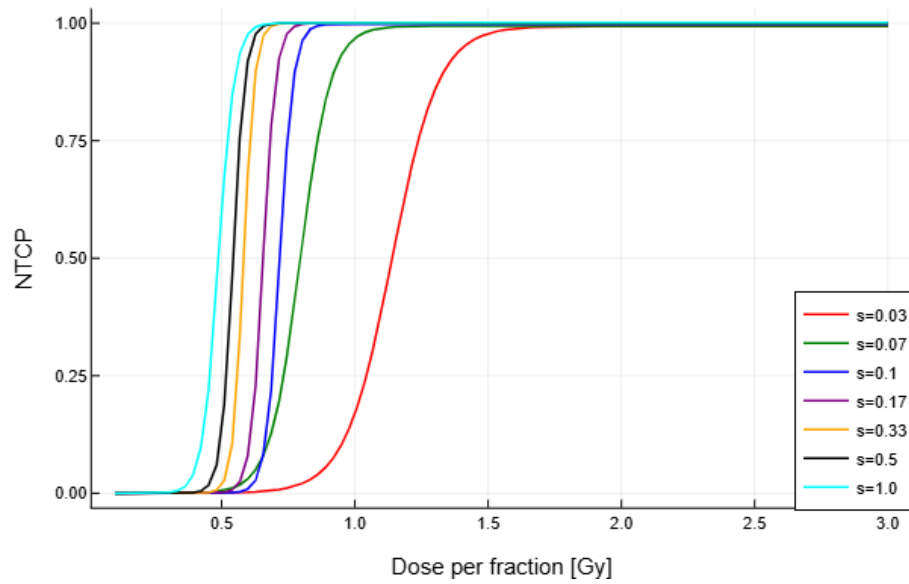


(b) p 80 MeV, PI = 3%.

Figure 5.6: Normal Tissue Complication Probability (NTCP) as a function of dose per fraction for 80 MeV proton beam irradiation in 30 fractions, for an environmental oxygenation level at 21% O_2 , in the case of (a) irradiation of the whole target volume, and (b) partial irradiation (PI) of 3% of the target volume, for different volume effect parameters s , i.e., from 0.03 to 1.0.



(a) ^{12}C 40 MeV/u, PI = 100%.



(b) ^{12}C 40 MeV/u, PI = 3%.

Figure 5.7: Normal Tissue Complication Probability (NTCP) as a function of dose per fraction for 40 MeV/u carbon ion beam irradiation in 30 fractions, for an environmental oxygenation level at 21% O_2 , in the case of (a) irradiation of the whole target volume, and (b) partial irradiation (PI) of 3% of the target volume, for different volume effect parameters s , i.e., from 0.03 to 1.0.

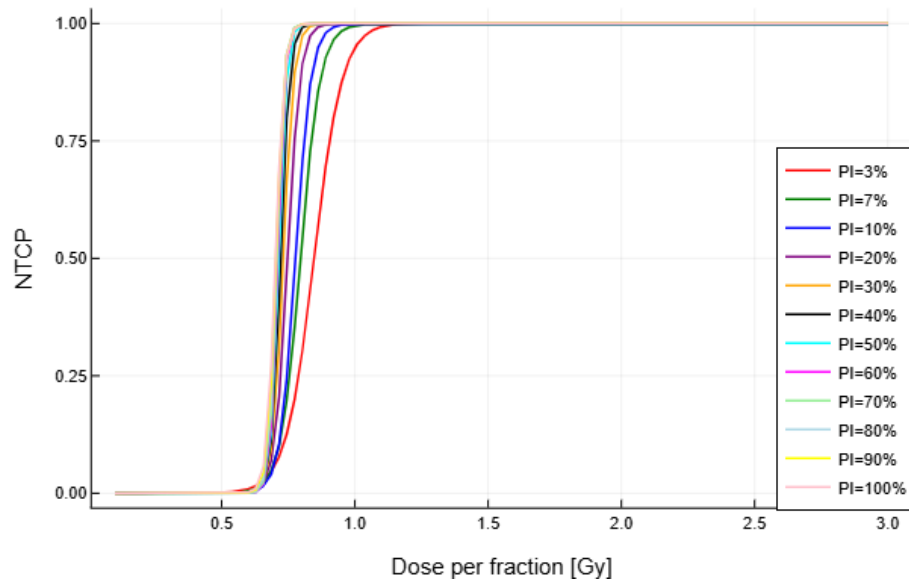
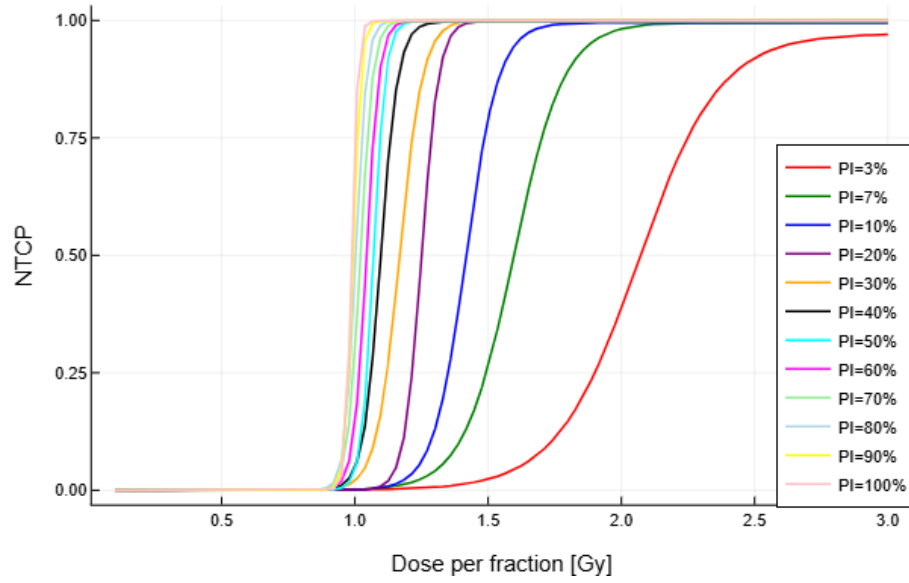
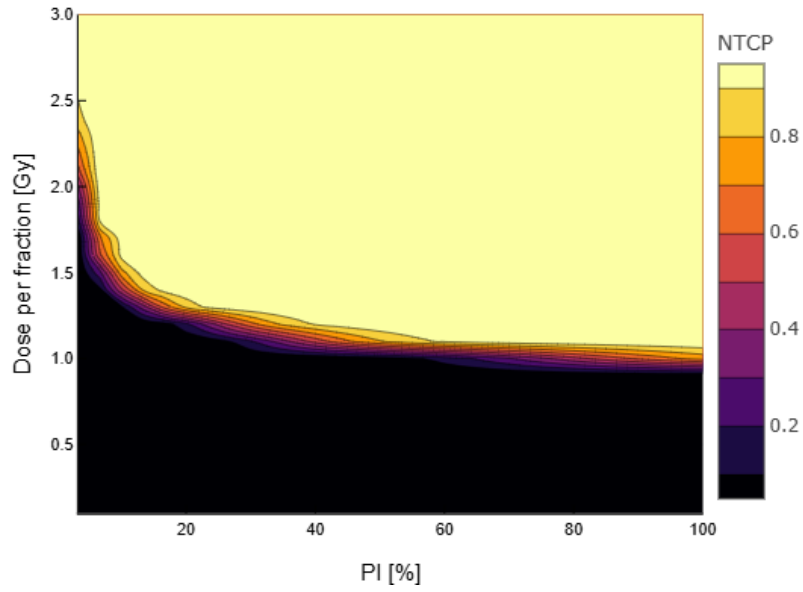
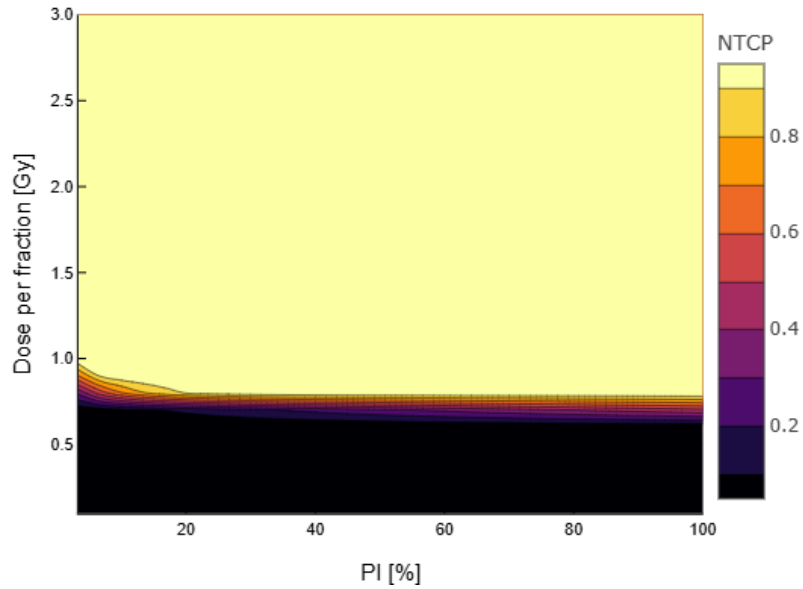


Figure 5.8: Normal Tissue Complication Probability (NTCP) as a function of dose per fraction for 80 MeV proton beam irradiation in 30 fractions, for an environmental oxygenation level at 21% O_2 , in the case of (a) a large volume effect, i.e. $s = 0.03$, and (b) a small volume effect, i.e. $s = 1$, for different partial irradiation (PI) of the target volume, i.e., from of 3% to 100%.



(a) p 80 MeV, $s = 0.03$.



(b) p 80 MeV, $s = 1.0$.

Figure 5.9: Normal Tissue Complication Probability (NTCP) as a function of partial irradiation (PI) (vertical-axis) and dose per fraction (horizontal-axis) for 80 MeV proton beam irradiation in 30 fractions, for an environmental oxygenation level at 21% O_2 , in the case of (a) a large volume effect, i.e. $s = 0.03$, and (b) a small volume effect, i.e. $s = 1$.

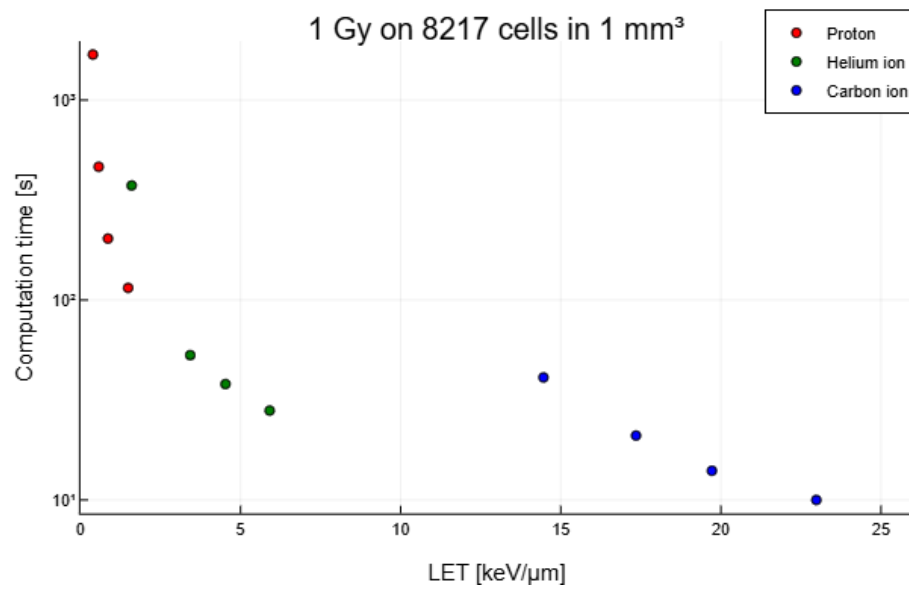


Figure 5.10: Computational times for 1 Gy delivered on a box of 1 mm³ that contains 8217 cells. Irradiation beam of radius 707 mm for different radiation qualities in particular proton (red points), helium ion (green points), and carbon ion (blue points) beams with various LET values. Calculation with an Intel(R) Core(TM) i9-14900, 2.00 GHz, RAM 64,0 GB, 31 cores.

volume $V_{irr} = 450 \text{ mm}^3$, which corresponds to a total number of irradiated clonogens $N_{cell} \sim 5.6 \cdot 10^7$ at physiological oxygenation level, in agreement with the typical cell density of $10^4 - 10^5 \text{ cells/mm}^3$ [72].

Figures 5.11, 5.12, and 5.13 highlight how the GSM²-driven NTCP model can describe the trend of the experimental data [13, 14, 15] at different fractionation schemes in terms of radiation quality and dose per fraction, according to Eq. 5.7. We calibrated the biological parameters of the GSM² model, i.e., a , b , and r in Eq. 4.34, on the experimental points for carbon ions irradiation with different fractionation schemes, considering the relative seriality parameter $s = 1$ in Eq. 5.6, i.e. a completely serial organ, according to [302]. Instead, the GSM²-driven NTCP model has completely predicted all the other results, i.e., for different radiation qualities at different fractionations, using the same a , b , and r parameters.

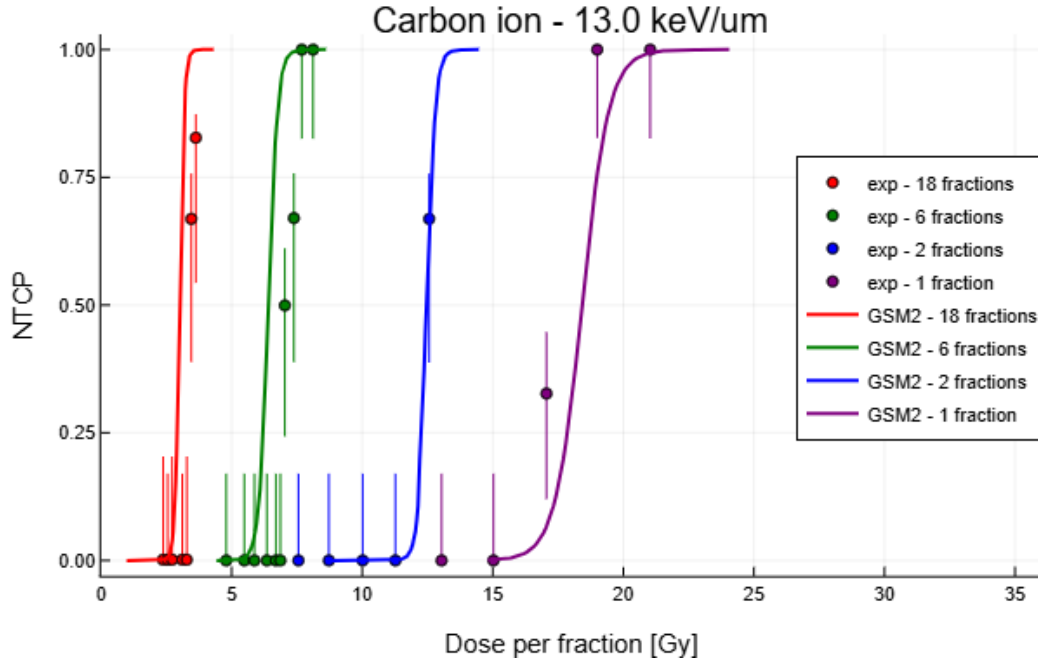


Figure 5.11: Comparison of the GSM²-driven NTCP results (solid lines) with experimental data (points) with error bars. Normal Tissue Complication Probability (NTCP) of rat spinal cord tolerance as a function of dose per fraction for fixed total dose, in the case of $13.0 \text{ keV}/\mu\text{m}$ carbon ions irradiation, for different fractionation schemes, i.e., 1 (purple), 2 (blue), 6 (green), and 18 (red) fractions.

Figure 5.11 reports the comparison between the experimental NTCP of rat spinal cord tolerance [13, 14, 15] (with points and error bars) and GSM²-driven NTCP predictions (with solid lines), as a function of the dose per fraction. In particular, the spinal cord was irradiated with $13.0 \text{ keV}/\mu\text{m}$ carbon ions beam, using different fractionation schemes, i.e., 1 (purple), 2 (blue), 6 (green), and 18 (red) fractions. As illustrated in Figure 5.11,

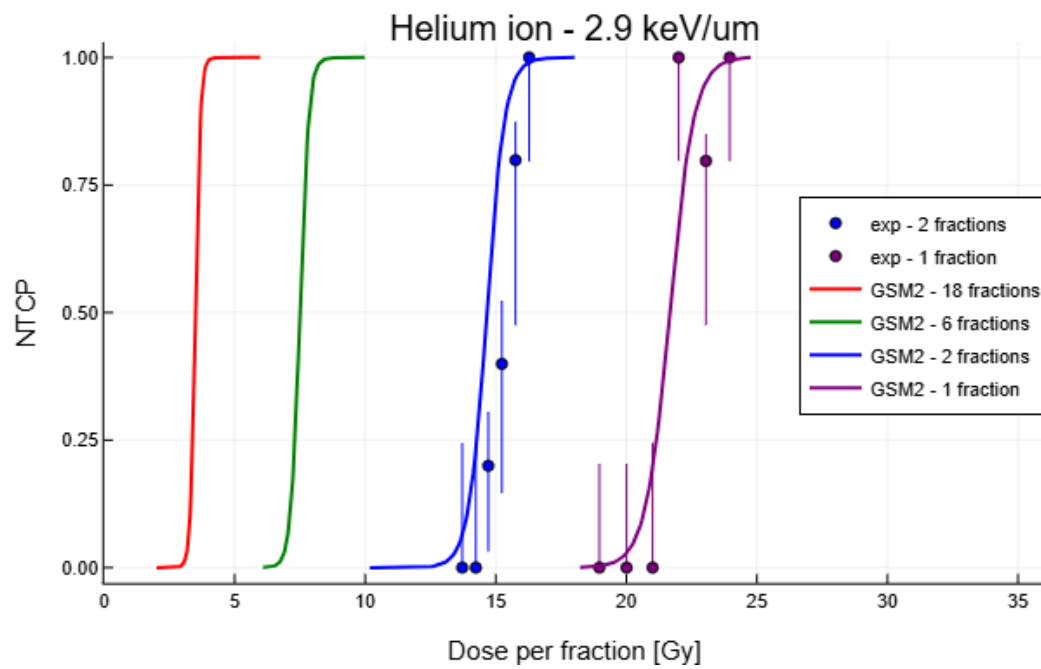


Figure 5.12: Comparison of the GSM²-driven NTCP results (solid lines) with experimental data (points) with error bars. Normal Tissue Complication Probability (NTCP) of rat spinal cord tolerance as a function of dose per fraction for fixed total dose, in the case of 2.9 keV/ μ m helium ions irradiation, for different fractionation schemes, i.e., 1 (purple), 2 (blue), 6 (green), and 18 (red) fractions.

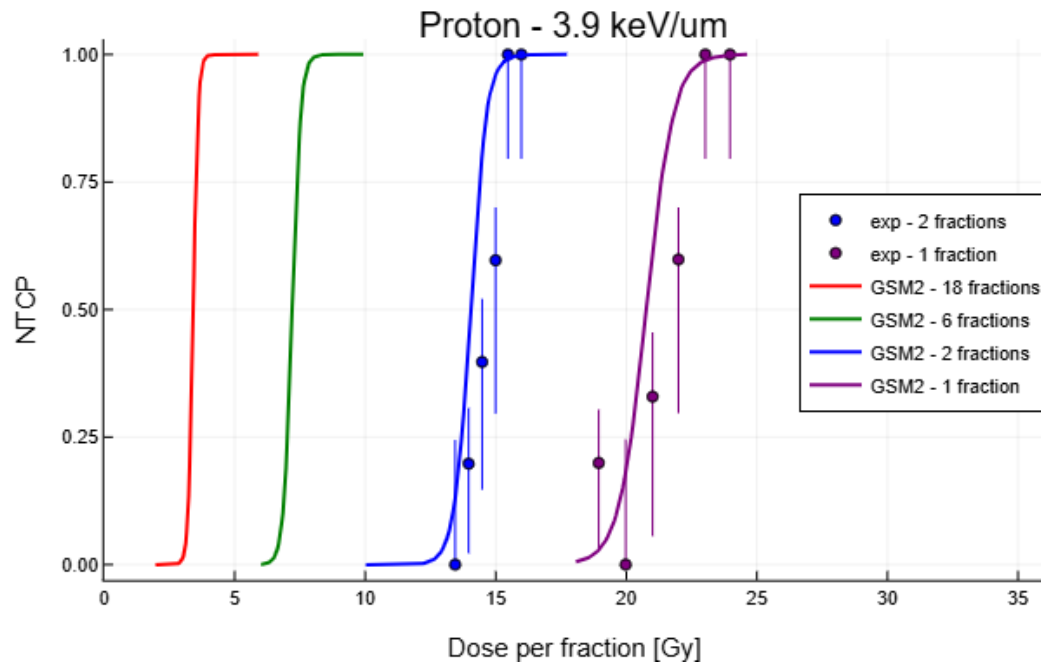


Figure 5.13: Comparison of the GSM²-driven NTCP results (solid lines) with experimental data (points) with error bars. Normal Tissue Complication Probability (NTCP) of rat spinal cord tolerance as a function of dose per fraction for fixed total dose, in the case of 3.9 keV/ μ m protons irradiation, for different fractionation schemes, i.e., 1 (purple), 2 (blue), 6 (green), and 18 (red) fractions.

the GSM²-driven NTCP model can describe macroscopic fractionation effects from cell survival predictions through GSM², calibrating only the parameters of the three biological pathways of cell response.

Figure 5.12 shows the experimental NTCP curves in the case of the rat spinal cord after 3.9 keV/ μm helium ions irradiation at Conv regime (with points and error bars), compared to the GSM²-driven NTCP predictions (with solid lines), as a function of dose per fraction. In detail, two different fractionation schemes, 1 (purple) and 2 (blue) fractions, were applied during the experiment. In addition, dose-response predictions via GSM²-driven NTCP for two other fractionations were reported, i.e., 6 (green) and 18 (red) fractions. Furthermore, we underline that all the model results reported in Figure 5.12, are completely predicted by the GSM²-driven NTCP model, using the same biological pathways parameters calibrated on the carbon ion data.

Figure 5.13 shows the comparison of experimental NTCP curves in for the rat spinal cord (with points and error bars) with the GSM²-driven NTCP predictions (with solid lines), as a function of dose per fraction. In particular, the experiment has been performed using 3.9 keV/ μm protons beam in the Conv regime, considering two different fractionation schemes, which are 1 (purple) and 2 (blue) fractions. As shown in Figure 5.13, we include two additional dose-effect curves predicted via GSM²-driven NTCP for two additional fractionation schemes, i.e., 6 (green) and 18 (red) fractions. As previously mentioned, all the model predictions are completely predicted by the GSM²-driven NTCP model, considering the same a , b , and r parameters calibrated on the carbon ion data.

Data analyses

Experiment	Reference	a [1/h]	b [1/h]	r [1/h]
Rat spinal cord	[13, 14, 15]	$1.21 \cdot 10^{-2}$	$2.50 \cdot 10^{-2}$	3.23

Table 5.1: Fitted biological rates for the dose-effect response curves calculated via the GSM²-driven NTCP model for the experimental data points in the case of carbon ions irradiation.

To compare the GSM²-driven NTCP results with the *in-vivo* experiments on the rat spinal cord, reported in Figures 5.11, 5.12, and 5.13, we optimize the biological rates, on experimental *in-vivo* data in the case of carbon ions irradiation. In detail, we apply a *cross-entropy* method, in analogy to that used in Section 4.4, to obtain the best combination of a , b , and r rates, enabling the correct prediction of all the other experimental points. We start from an interval $[l_j, u_j]$ of possible realistic values for these biological rates $j \in a, b, r$, i.e. $a \in [10^{-3}, 10^{-1}]$ 1/h, $b \in [10^{-3}, 10^{-1}]$ 1/h, and $r \in [2.0, 5.0]$ 1/h. We randomly sample 10^3 values of $\log a$, $\log b$, and $\log r$ from uniform distributions $[\log l_j, \log u_j]$. We compute

the *normalized mean square error*, i.e.

$$\text{NMSE} := \text{mean} \left(\frac{\left(TD_{50}^{\text{exp}} - TD_{50}^{\text{GSM}^2} \right)^2}{TD_{50}^{\text{exp}}} \right), \quad (5.9)$$

where TD_{50}^{exp} and $TD_{50}^{\text{GSM}^2}$ are the doses that correspond to $\text{NTCP} = 0.5$ for the experiment and our model, respectively, as proposed in [289] for cell survival predictions and as applied in Section 4.4, and we consider the best 50 values, that we call NMSE_{50} . We calculate the biological rates

$$\begin{cases} l_{j,\min} &= l(\min(\text{NMSE}_{50})) \\ u_{j,\max} &= j(\max(\text{NMSE}_{50})) \end{cases}, \quad (5.10)$$

with $j \in a, b, r$. We consider the new intervals, i.e. $[\log l_{j,\min}, \log u_{j,\max}]$, and we repeat N -times the previous procedure, selecting, in the end, the best combination of a , b , and r , which is reported in Table 5.1.

5.5 Discussion

This Section presents the key results obtained using the GSM^2 -driven NTCP model, as detailed in Sections 5.3 and 5.4. Additionally, we discuss the main limitations of the developed model and explore potential future advancements.

Analysis of the results

The first key aspect that we want to highlight is that incorporating the GSM^2 model into a mechanistic NTCP model enables the resulting tool to retain the core features of GSM^2 . Specifically, it preserves the ability to account for different stochasticity levels in physics and biology, particularly in energy deposition and the formation and progression of DNA damage, as discussed in Section 2.3.1. Additionally, the present tool goes beyond the Poisson assumption on the formation of initial DNA damage and the subsequent induction of complications, as discussed in Section 5.1, distinguishing it from typical dose-response models, such as [72].

Regarding the numerical application of the developed mechanistic NTCP model, in Section 5.3, we presented all the main computational results obtained through the GSM^2 -driven NTCP model. The initial *in-silico* analysis aims to show the main features of the developed mechanistic-driven dose-response model. In particular, we want to underline how this tool is capable of considering, from a physical point of view, various irradiation schemes, considering different radiation quality of the beam, partial irradiation experiments, and fractionation schemes; while, from a biological point of view, the tool can investigate different cell lines, oxygenation effects, and macroscopic biological systems with characteristic volume effects. Instead, with the second part of this work, presented in Section 5.4, the goal is to show a first comparison of the model results with experimental

data, which gives a first confirmation regarding the feasibility of the model to include different physical and biological effects simultaneously in realistic situations.

The first feature of the model that we want to highlight is that it can handle any radiation quality, i.e., type, energy, and LET, of the irradiation beam. This aspect emerges in Figures 5.4, where we can observe the impact of radiation quality on the emergence of a side effect for normal tissues. In detail, for a fixed organ type, characterized by cell line, volume effect, and oxygenation level, and for a given irradiation approach, the probability curve of the occurrence of a specific complication for that organ shifts towards higher doses with decreasing LET values, as expected. This is a well-known effect, documented in several works in the literature selected for our investigation, namely [13, 14, 15]. This is due to increased biological effectiveness as a function of radiation quality, which is typically parameterized in terms of RBE, as presented in Section 1.4.1. As recently demonstrated in [6], the GSM² model can describe the different biological effects induced by different qualities of radiation at the cellular scale. In this work, we provide the first demonstration of the GSM² model's predictive capability in describing biological effects induced by various radiation qualities, even in complex biological systems on macroscopic scales, as shown in Figures 5.11, 5.12, and 5.13. We emphasize once again that the biological rates a , b , and r of GSM², were calibrated using experimental data from carbon ion irradiation, as shown in Figure 5.11, while the dose-response curves for helium ions and protons, presented in Figures 5.12 and 5.13, were entirely predicted by the GSM²-driven NTCP model. Thus, the developed model demonstrates remarkable accuracy in replicating various experimental scenarios across a broad spectrum of radiation qualities.

The second key feature of the present model we wish to emphasize is its capability to describe different fractionation schemes. This is particularly evident in Figures 5.11, 5.12, and 5.13, where, using the same set of biological parameters, the model successfully predicts the well-known fractionation effect [32], as presented in Section 1.3.2, for four different numbers of fractions, up to 18. Notably, the agreement is excellent for the curves where the three biological rates were fitted (i.e., carbon ions beam) and for the other experimental datasets corresponding to helium ions and protons irradiation. In particular, from Figures 5.11, 5.12, the probability of radiation-induced complications in normal tissues significantly decreases as the number of fractions increases. This is because delivering the dose in a fractionated scheme allows cells more time to repair damage.

Another interesting aspect is that the GSM²-driven NTCP model can take into account the oxygen fixation of DNA damage [30], which is described via OER, as discussed in Section 1.4.2. From Figure 5.5, we can appreciate the impact of different oxygenation levels on the induction of a side effect. In particular, as expected, the probability of a normal tissue complication decreases due to reducing the environmental oxygen concentration for a fixed given dose. Furthermore, the OER effect decreases with increasing radiation quality, according to Eq. 4.27. This effect emerges by comparing the dose-response curves for the two different radiation qualities, i.e., p 80 MeV and 12C 40 MeV/u, reported in Figures 5.5a and 5.5b, respectively.

In addition, our mechanistic-driven NTCP model can exploit the impact of partial irradiation and volume effect on the manifestation of a side effect in healthy tissues. In

detail, from Figures 5.6, 5.7, and 5.8, we can observe the combined action of partial irradiation and volume effect. For example, fixing the irradiation approach, i.e., whole-organ or partial irradiation, the considered organ responds to the radiation in different ways, depending on its characteristic biological architecture. In particular, Figures 5.6a-5.7a illustrate that the influence of tissue architecture is minimal for whole-organ irradiation. In contrast, Figures 5.6b-5.7b highlight the emergence of volume effects in cases of partial organ irradiation, qualitatively aligning with experimental findings [77], as shown in Section 2.4. This is because irradiating only a portion of the target volume, in the case of a parallel organ, reduces the probability of complications. Furthermore, the emergence of volume effects is emphasized by the dose-response curves in Figure 5.8, which represent slices of the 2D plot in Figure 5.9, with the irradiated volume percentage fixed at specific values. It is worth highlighting that our results qualitatively agree with widely recognized findings in the literature, such as those in [303], as illustrated in Section 2.4.

The developed NTCP model provides a mechanistic approach to compute clinically relevant endpoints for various experimental scenarios across different radiation qualities of the beams, irradiation volumes, fractionation schemes, environmental oxygenation levels, and tissue architectures.

Current limitations of GSM²-driven NTCP model and future perspectives

The previous discussion highlights the generality and flexibility of the developed GSM²-driven NTCP model. However, the present model has some limitations. Furthermore, we are developing extensions describing the biological response of complex systems exposed to UHDR irradiation, as described in Chapter 6.

The first point that we want to discuss is the computational cost of the model. This is not a problem for low doses or relatively small volumes, such as those considered in this study, with a cell density range of $10^4 - 10^5$ per mm^3 . In these cases, generating an NTCP curve in just a few seconds to minutes is possible. This conclusion is confirmed by the computational times reported in Figure 5.10. However, the situation could become more complex when considering very high doses, of several tens of Gy, or significantly larger target volumes, on the order of several cm^3 , arising from the single-cell resolution of the developed model. To address this, we are developing a GPU-based approach for predicting NTCP curves under different experimental conditions, ensuring extremely fast computational times.

The second main limitation of the present model is the biological description of organs with complex structures or intricate radiation-induced complications. In this preliminary study, we consider a single cell type, for simplicity, calibrating the organ response model on that cell line. This approximation may present limitations in the description of more sophisticated biological structures. In such cases, it becomes necessary to consider different cell types with specific biological characteristics, as we will show in detail for the skin in Section 8.2. For this reason, we are focusing on reproducing the dose-response relationship for a relatively simple and well-established biological structure, such as the spinal cord. In addition, the model does not differentiate between the various DNA damage-mediated cell death pathways in this version. In this context, we are working to incorporate additional

biological pathways, such as senescence and apoptosis, by integrating the proteins p21, p53, or TGF- β , which mediate these alternative cell-death processes [184]. We are also considering the impact of ferroptosis, another form of cell death that does not involve DNA damage, by accounting for the role of membrane lipids, as we will discuss in Chapter 8. This extension will enable us to compare our model predictions with additional experimental biological endpoints while also considering more complex volume effects beyond the spinal cord, including radiation-induced complications for the skin or lungs.

Furthermore, this first work focused on predicting and investigating the dose-response relationship for healthy tissues as a starting point for the UHDR extension. However, we are working on a feasibility study of the ability of the GSM²-driven TCP model to describe the tumor response to conventional irradiation. In particular, the developed model can allow us to investigate spheroids, i.e., aggregates of tumor cells, also considering oxygenation gradients.

Finally, developing the present predictive mechanistic model with single-cell resolution for the radiation response of macroscopic biological systems makes it an ideal candidate for the UHDR extension to investigate the FLASH effect. The main idea is to include the chemistry, as already performed with developing the MS-GSM² model and detailed in Chapter 4. This topic will be explored in detail in the next Chapter 6.

Chapter 6

The UHDR extension of the mechanistic-driven NTCP model

As discussed in Chapter 4, *in-vitro* investigation can be crucial for better understanding the intricate mechanism behind the FLASH effect. In particular, developing a multiscale radiation biophysical model is essential for understanding and describing a biological effect as elusive and complex as the FLASH effect. However, the logical next step is the extension of the MS-GSM² to studying the FLASH effect for macroscopic *in-vivo* biological systems. Therefore, this Chapter is dedicated to the conception, realization, implementation, and first validation of a mechanistic-driven NTCP model to investigate radiation-induced *in-vivo* side effects in the case of UHDR irradiation. In this way, we include additional spatio-temporal scales of radiation-induced biological damage: on one hand, the homogeneous chemical stage, which is fundamental for describing UHDR radiation, and on the other hand, complex multicellular biological systems, characterized by specific volume effects.

In particular, the main idea of this work is to merge the MS-GSM² model presented in Chapter 4 with the mechanistic-driven NTCP model shown in Chapter 5, enabling a comprehensive investigation of clinically relevant endpoints for UHDR radiation, bridging the gap from the mechanistic-driven hypothesis to clinically relevant predictions. Thus, our proposed mathematical framework can take into account effects with single-cell resolution, such as energy deposition variability and chemical and biological environmental heterogeneity, and, at the same time, macroscopic effects, such as organ volume effects and fractionation schemes. In addition, the model can handle any radiation quality of the beam, that is, type, energy, and LET of radiation, as well as dose-delivery time-structure. Thus, due to its great flexibility, the MS-GSM²-driven NTCP model can be the ideal candidate for investigating various experimental scenarios in the UHDR regime.

The main contributions that will be discussed in this Chapter are as follows:

- (i) to propose a general multi-stage mechanistic model capable of offering a comprehensive investigation of clinically relevant toxicity outcomes for UHDR irradiation;
- (ii) to show the feasibility of the model to reproduce *in-vivo* UHDR experiments, beyond the previously established agreement with *in-vitro* data.

Specifically, this Chapter is organized as follows: Section 6.1 details the development of the mathematical framework of the multi-stage mechanistic-driven NTCP model; Section 6.2 reports the numerical implementation of the NTCP tool; Section 6.3 shows the model's ability to replicate UHDR *in-vivo* results from the literature, for a representative example; finally, Section 6.4 discusses the main findings of the MS-GSM²-driven NTCP model and future possible investigations.

6.1 Theory and calculations

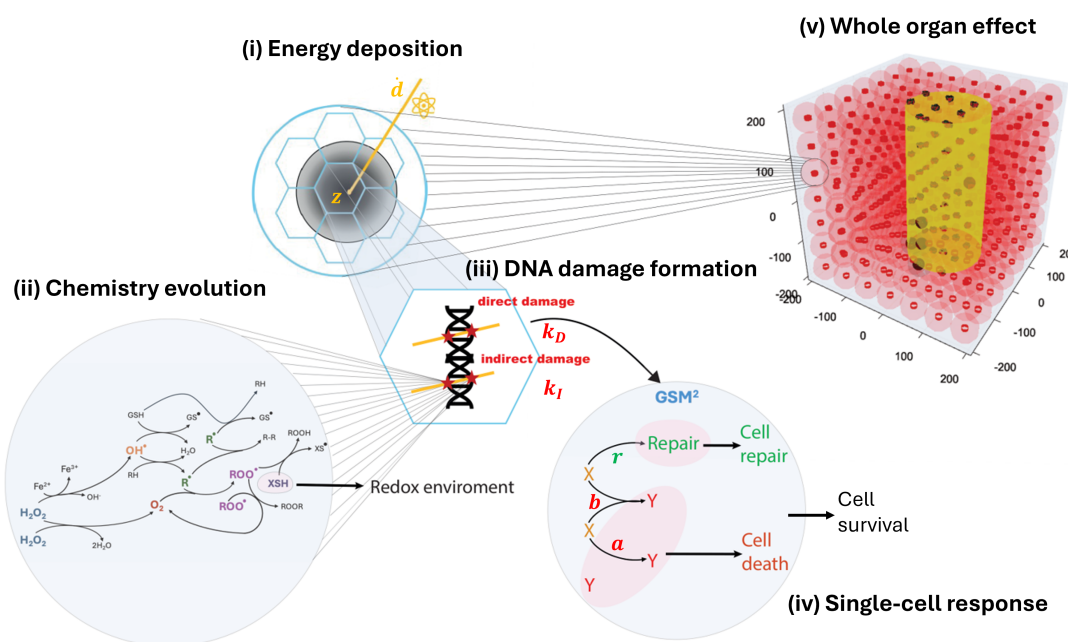


Figure 6.1: Schematic representation of the MS-GSM²-driven NTCP and TCP workflow. The model takes into account different spatial and temporal scales of radiation-induced biological damage, which are (i) the energy deposition, (ii) the chemistry evolution, (iii) the DNA damage formation, (iv) the single-cell response, and (v) the whole organ effect. The proposed workflow combines the approaches presented in Chapters 4 and 5.

This Section is focused on the description of the mathematical framework of the MS-GSM²-driven NTCP model, which is a multi-stage mechanistic-driven model for the investigation of radiation-induced side effects in the UHDR regime on different tissues and organs. In particular, this multi-cell response model is the multi-scale extension of the NTCP model developed in Chapter 5, in a similar manner to what has already been done for the single-cell response in Chapter 4, including the explicit impact of the chemistry for the investigation of the FLASH effect in clinical relevant situations.

From Figure 6.1, it is possible to appreciate the various spatial and temporal scales

of radiation-induced damage this model considers. In detail, on one hand, compared to the MS-GSM² model, we included the multi-cellular stage, while on the other hand, compared to the GSM²-driven NTCP model, we added the chemical network. Thus, all these processes involved, that is, (i) the energy deposition, (ii) the chemistry evolution, (iii) the DNA damage formation, (iv) the single-cell response, and (v) the whole organ effect, are precisely mathematically described as outlined in Sections 4.1 and 5.1. In this way, we can describe physical, chemical, and biological processes that occur on a spatial scale in the range between μm and mm-cm, while temporally on a scale between μs and days-weeks, also taking into account physical, chemical, and biological stochasticities, as reported in Table 4.1.

Thus, formally, the probability that all clonogens N_{cell} in the i -th FSU die given an average dose D , a mean dose rate $\dot{D}$, and LET, and for a given cellular environment, characterized by the oxygenation level $[\text{O}_2]$ and the antioxidant concentration $[\text{XSH}]$, is a generalization of Eq. 5.1 proposed in Section 5.1. In particular, this probability becomes

$$\begin{aligned} P_i^{\text{FSU}}(D_i, \dot{D}_i, \text{LET}_i, [\text{O}_2]_i, [\text{XSH}]_i) &:= \\ &= \prod_{j=1}^{N_{\text{cell}}} \left(1 - S_j \left(z_n^j, \left(\dot{d}^j(t) \right)_{t \in [0, T]}, \text{LET}^j, X_{\text{dir/indir}}^j, Y_{\text{dir/indir}}^j | D_i, \dot{D}_i, \text{LET}_i, [\text{O}_2]_i, [\text{XSH}]_i \right) \right), \end{aligned} \quad (6.1)$$

where S_j represents the survival probability of the j -th clonogen after receiving a local dose z_n^j , and radiation quality LET^j , with a local dose rate $\left(\dot{d}^j(t) \right)_{t \in [0, T]}$, considering an average macroscopic dose D , a mean dose rate $\dot{D}$, a total irradiation time $T = D/\dot{D}$, and LET, along with a distribution of direct sub-lethal and direct lethal lesions X_{dir}^j and Y_{dir}^j , respectively, and indirect sub-lethal and indirect lethal lesions X_{indir}^j and Y_{indir}^j , respectively, as shown in Sections 4.1 and 5.1.

Thus, we extend the radiation response of the whole organ, which is characterized by a generic tissue architecture, composed by m serial FSUs and n parallel FSUs, e.g., that in Figure 5.3 (c), in the case of UHDR irradiation. In particular, the NTCP formula becomes

$$\text{NTCP} = \prod_{k=1}^n \left[1 - \prod_{i=1}^m \left(1 - P_{i,k} \left(D_{ik}, \dot{D}_{ik}, \text{LET}_{ik}, [\text{O}_2]_{ik}, [\text{XSH}]_{ik} \right) \right) \right], \quad (6.2)$$

which is a multi-scale generalization described in Section 5.1.

Alternatively, the NTCP can be calculated in terms of the seriality parameter s , which is defined in Eq. 5.6, obtaining

$$\text{NTCP} = \left[1 - \prod_{i=1}^M \left(1 - P_i \left(D_i, \dot{D}_i, \text{LET}_i, [\text{O}_2]_i, [\text{XSH}]_i \right) \right)^s \right]^{\frac{1}{s}}, \quad (6.3)$$

where $M = n \cdot m$ is the total number of FSUs for a given organ volume.

6.2 Numerical implementations

The stepwise construction of the MS-GSM²-driven NTCP model, reported in Eqs. 6.2 and 6.3, is the same as that discussed in Section 5.2 for Conv irradiation, with the addition of the dose rate pathway and chemistry evolution, which are included as shown in Section 4.2 for the single-cell MS-GSM². However, given the very high computational cost, the chemistry is simulated independently because it is faster than the biological pathways in Eq. 4.34, initially applying the MS-GSM² model to calculate a look-up table (LUT) of the DNA damage yields reported in Eqs. 4.28-4.31, varying several parameters, including radiation quality, dose, mean dose rate, oxygen and antioxidant levels, as will be presented in detail in Chapter 7. In other words, by fixing the physical characteristics of the specific irradiation, such as dose, dose rate, and radiation quality, and selecting a particular cell line, we can build a dataset of cell survival probabilities by exploring the impact of the remaining free parameters, namely oxygenation and antioxidant levels, on the evolution of the chemical environment. These LUTs act as inputs for the NTCP calculation in the UHDR regime, as illustrated above.

From a computational point of view, the MS-GSM²-driven NTCP model was implemented using *Julia* [282].

6.3 Comparison with *in-vivo* experiments

This Section reports the first application of the MS-GSM²-driven NTCP model on a real *in-vivo* experimental scenario to investigate the feasibility of the model for describing the response of macroscopic biological systems to UHDR radiation. In particular, we consider one of the main experiments with a proton-FLASH beam for a murine skin toxicity model [16]. The dose is delivered with the entrance channel in a single fraction with prescribed doses in the range ~ 25.0 -53.5 Gy, in a volume of ~ 60 mm³, considering a uniform mean dose rate of 0.37 Gy/s (Conv) and 80 Gy/s (UHDR), for simplicity. In addition, since the experimental oxygenation is not reported in the original publication, we consider a typical physiological oxygenation level for healthy tissues of 4% [19].

Figure 6.2 shows the results we obtained applying the MS-GSM²-driven NTCP model to [16] for the score 1.5, after performing a fitting process for the following parameters. We calibrated the biological rates of the MS-GSM² model, i.e., a , b , and r in Eq. 4.34, and the relative seriality parameter s on the experimental points for Conv irradiation. At the same time, we estimate the antioxidant concentration [XSH] needed to predict the experimental data for UHDR irradiation. All remaining parameters were taken from the literature and held constant throughout the simulations.

Data analyses

To compare the MS-GSM²-driven NTCP results with the *in vivo* experiment on the mouse skin, reported in Figure 6.2, we optimize the biological rates on experimental data in the case of Conv irradiation. In detail, we apply a *cross-entropy* method, in analogy to that

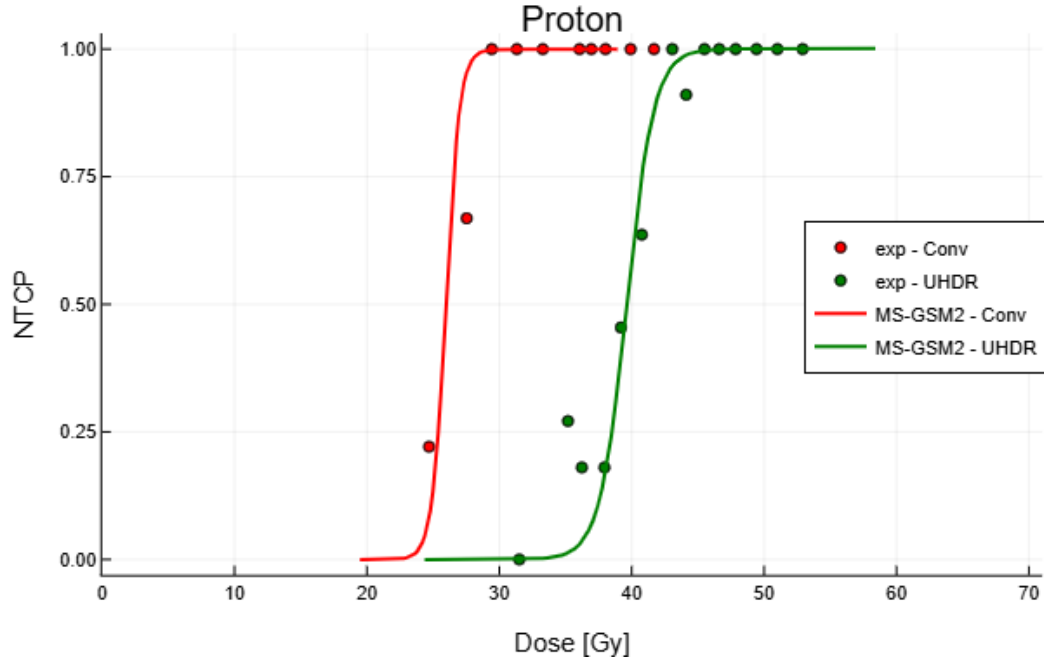


Figure 6.2: Comparison of the MS-GSM²-driven NTCP results (solid lines) with experimental *in-vivo* data (points). Normal Tissue Complication Probability (NTCP) of mouse skin desquamation (score = 1.5) as a function of dose, for 250 MeV proton beam irradiation in the entrance channel, at Conv (red) and UHDR (green) regime.

Experiment	Reference	a [1/h]	b [1/h]	r [1/h]	s
Mouse skin	[16]	$1.50 \cdot 10^{-2}$	$1.08 \cdot 10^{-2}$	4.17	0.7

Table 6.1: Fitted biological rates and volume effect parameter for the dose-effect response curves calculated via the MS-GSM²-driven NTCP model for the experimental data points in the case of Conv irradiation.

used in Section 4.4, to obtain the best combination of a , b , and r rates, enabling the correct prediction of all the other experimental points. We start from an interval $[l_j, u_j]$ of possible realistic values for these biological rates $j \in a, b, r$, i.e. $a \in [10^{-3}, 10^{-1}]$ $1/h$, $b \in [10^{-3}, 10^{-1}]$ $1/h$, and $r \in [2.0, 5.0]$ $1/h$. We randomly sample 10^3 values of $\log a$, $\log b$, and $\log r$ from uniform distributions $[\log l_j, \log u_j]$. We compute the *normalized mean square error*, i.e.

$$\text{NMSE} := \text{mean} \left(\frac{(TD_{50}^{\text{exp}} - TD_{50}^{\text{MS-GSM}^2})^2}{TD_{50}^{\text{exp}}} \right), \quad (6.4)$$

where TD_{50}^{exp} and $TD_{50}^{\text{MS-GSM}^2}$ are the doses that correspond to $\text{NTCP} = 0.5$ for the experiment and our model, respectively, as proposed in [289] for cell survival predictions and as applied in Section 4.4, and we consider the best 50 values, that we call NMSE_{50} . We calculate the biological rates

$$\begin{cases} l_{j,\min} &= l(\min(\text{NMSE}_{50})) \\ u_{j,\max} &= j(\max(\text{NMSE}_{50})) \end{cases}, \quad (6.5)$$

with $j \in a, b, r$. We consider the new intervals, i.e. $[\log l_{j,\min}, \log u_{j,\max}]$, and we repeat N -times the previous procedure, selecting, in the end, the best combination of a , b , and r , which is reported in Table 6.1. At the same time, we calibrate the volume effect parameter s on the experimental data point for Conv irradiation. We consider an interval for $s \in [0.05, 1.0]$, with steps of 0.05. We compute

$$\text{NMSE} := \text{mean} \left(\frac{(m_{50}^{\text{exp}} - m_{50}^{\text{MS-GSM}^2})^2}{m_{50}^{\text{exp}}} \right), \quad (6.6)$$

where m_{50}^{exp} and $m_{50}^{\text{MS-GSM}^2}$ are the slopes of the dose-response curves at TD_{50} for the experiment and our model, respectively, obtaining the best s parameter, which is 0.7. The last step is predicting the UHDR dose-response curve to reproduce the experimental DMF of ~ 1.5 . Using the same a , b , r , and s parameters of Conv irradiation, we consider realistic values for the antioxidant concentration [XSH] in the range $[1.0, 50.0]$ mM, which is a modulator of the FLASH sparing effect, as discussed in Section 4.5. Then, we compute the NTCP curve for each of these values in the case of UHDR irradiation, as described in Section 6.2. Finally, we calculate the corresponding NMSE according to Eq. 5.9 to obtain the best [XSH] value that can reproduce the experimental FLASH sparing effect, that is ~ 2 mM, [XSH], which corresponds to a reduction in the DNA damage yield of 35%, as defined in Section 4.1. As outlined in Section 4.5, antioxidants play a crucial role in regulating the extent of the sparing effect observed under UHDR conditions. By keeping all other physical and environmental parameters fixed, we can reproduce the observed UHDR-induced sparing effect by tuning the concentration of antioxidants, thereby calibrating the corresponding reduction in indirect DNA damage yield. Although this specific value is not directly accessible from the current experiment [16], its typical range is well documented in the literature [7, 292, 293, 294, 295, 296].

6.4 Discussion

This Section discusses the main results obtained using the MS-GSM²-driven NTCP model, shown in Section 6.3. We also address the key limitations of the developed model and examine possible future improvements.

Analysis of the results

First of all, we would like to emphasize the generality of the MS-GSM²-driven NTCP model, which is the culmination of the various multi-scale extensions implemented in the GSM² model and discussed in the previous Chapters 4 and 5. In particular, in this Chapter, we have, on the one hand, integrated chemistry and the dose rate effects through the multi-stage model developed in Chapter 4, i.e., the MS-GSM². On the other hand, we have moved from describing *in-vitro* cellular systems to macroscopic *in-vivo* biological systems by implementing a mechanistic model to study the dose-response relationship, as presented in Chapter 5. Therefore, the model can consider a wide range of irradiation types, e.g., the type, energy, and LET of the radiation, temporal structure of the dose-delivery, fractionation schemes, and the stochasticity of energy deposition. From a chemical point of view, the model can consider the impact of the dose rate on the chemical environment, as well as the different environmental conditions of the cell, such as its antioxidant activity or the specific partial pressure of oxygen. Furthermore, from a biological perspective, the model can account for the stochastic nature of DNA damage formation and its progression over time through three possible biological pathways. Finally, the model can describe various cell lines and organ types while considering potential volume effects.

In Figure 6.2, it emerges that the model successfully describes the biological response of the skin after UHDR irradiation, using realistic physical, chemical, and biological parameters. However, the substantial experimental DMF of ~ 1.5 between the Conv and UHDR curves, in our model, corresponds to a 35% reduction in DNA damage yield, which appears slightly high. This result is intended as a proof of principle for the proposed approach and obviously requires further analysis and improvements, some of which will be discussed in the following Section.

Current limitations of GSM²-driven NTCP model and future perspectives

While in the previous paragraph we discussed the first results obtained with the present model, in the next paragraphs we will focus on the various ongoing developments.

The first point we want to discuss is the current numerical implementation of the model. As described in Section 6.2, the explicit simulation of the chemistry for each cell for a macroscopic system with a cell density of $10^4 - 10^5$ per mm^3 , is highly computationally expensive. Therefore, in this case, we bypassed the problem by calculating a LUT of the damage yields as a function of various physical, chemical, and biological parameters. One possible way to optimize the numerical implementation of the model developed here is to integrate Artificial Intelligence (AI) into the MS-GSM² model, training a neural network (NN) [304, 305] to replicate the MS-GSM² predictions or to explicitly simulate

the chemical network described by the system of ODEs in Eq. 4.7, via physics-informed NN [306, 307, 308, 309]. We can overcome the calculated LUT by combining AI's speed and predictive capability with the robustness of a mechanistic-driven approach. A key strength of this AI-based approach lies in its ability to retain the mechanistic integrity of the original model, i.e. the MS-GSM², since the NNs are trained directly on its predictions. Still under development, this approach will be explored in more detail in Chapter 7.

One of the main advantage of the presented approach is that the model can take into account inter- and intra- cellular effects. In fact, the model can handle different levels of heterogeneity, from a physical, chemical, and biological point of view. In particular, our method allows to consider, for example, specific spatial and temporal dose distributions, oxygenation gradients, antioxidants distributions, ensembles of different cell lines, etc. Thus, this approach enables a more realistic representation of the biological response to IR. However, it also demands a significantly more detailed understanding of both the irradiation process and the biological environment, often only partially characterized, along with additional parameters calibration. For these reasons, the results presented here were obtained assuming uniform initial distributions of oxygen and antioxidants, as well as a single cell type.

The other main limitation in the present version of the model is the description of more complex biological architectures and other biological endpoints, as already discussed in Section 5.5. We propose that the *in-vivo* sparing effect observed with UHDR irradiation, unlike *in-vitro*, is not solely attributable to a reduction in DNA damage. Instead, it likely involves a more complex and coordinated biological response, potentially affecting alternative cell-death pathways beyond DNA damage. In this context, we are working on the inclusion of ferroptosis to also take into account membrane lipid damage, which seems to be able to play a role in the emergence of the FLASH effect, as proposed in [7, 214] and presented in Section 3.5. In this regard, we are already working on a more detailed description of the biological structure of the skin, considering its typical layered architecture, with each layer exhibiting specific chemical and biological characteristics. These extensions of the model will be explored in detail in Chapter 8. In addition, we are working on incorporating alternative pathways of cell death, such as apoptosis and senescence, by including proteins that mediate these processes, such as p21 and p53. This will enable us to describe other skin scores and biological endpoints, including fibrosis.

Chapter 7

AI-enhanced MS-GSM²

Artificial Intelligence (AI) methods have experienced a fast growth in recent years, extending even to everyday tools. Interestingly, medical applications [310], especially in radiotherapy for imaging tools, radiobiological models [311], and treatment planning [312], have also integrated AI into their practices.

A rapidly emerging application of AI is its integration with mechanistic models [305], such as mechanistic learning and scientific machine learning [313, 314]. The AI-enhanced models, which are often referred to as surrogate models, act as a fast approximation of complex mechanistic models [304]. In particular, this approach can be very useful in situations requiring numerous simulations or instant responses. Recently, the main surrogate models have been developed to describe epidemiology [305] and complex biological processes [304], such as metabolic processes, interactions between biomolecules, cellular organelle activities, and macroscopic cellular responses. From a mathematical point of view, the surrogate models have typically been employed to replicate various types of mathematical frameworks, such as ODEs, PDEs, agent-based approaches, SDEs, etc. Training on tabular datasets generated by mechanistic models with various input parameters, these surrogate models captured the relationships between inputs and outputs, allowing faster predictions for complex systems. A key aspect of surrogate models is interpretability. In particular, recent Explainable Artificial Intelligence (XAI) techniques can investigate the impact of input features on output predictions, helping us to interpret key behaviors of complex systems [315], thus providing a tool which from one side goes beyond a black box, but provides some level of interpretability and thus of insights in the data trends, and on another a more safe and robust predictor which can be adopted with a reasonable justification even in a clinical scenario. So, how can they help us in the context of FLASH therapy?

One of the main open questions in the context of FLASH RT is how we can efficiently translate the FLASH effect into clinical practice from a computational point of view. Undoubtedly, a fundamental step is the development of efficient radiobiological models that account for the biological effects induced by UHDR radiation in treatment planning for FLASH RT. A crucial procedure in conventional radiation therapy is optimizing the treatment plan. The main idea is optimizing the beam's physical properties, particularly

in inverse planning, the number of particles for each pencil-beam to generate a 3D dose distribution that meets the required dosimetric constraints. This process must be accurate and fast, requiring efficient dose calculation tools, such as those in [48, 312], and radiobiological models.

While standard radiobiological models are used in the clinic to calculate biological outcomes, particularly for inverse planning, predicting the FLASH effect is highly complex and computationally expensive, as discussed extensively in Chapters 4 and 6. Thus, this Chapter aims to develop an AI-enhanced radiobiological model, which combines AI's speed and predictive capability with the fundamental underpinnings of the mechanistic approach for FLASH investigations.

Thus, the main idea of this work is to train a neural network to replicate the biological outcome predicted by our original mechanistic model, i.e., the MS-GSM² developed in Chapter 4, across a wide range of physical, chemical, and biological parameters. This allows us to continuously navigate a set of physical and physiological parameters, enabling a rapid and comprehensive exploration of the FLASH effect's potential. In principle, thanks to the AI-enhanced model, we can exploit our NTCP model, proposed in Chapter 6, for several experimental scenarios in the UHDR regime in a fraction of the time compared to the traditional approach. Beyond this, the AI-enhanced model enables us to explore the multi-parameter surface, opening the path for developing a fast, efficient, and mechanistically-driven biological optimizer for FLASH treatment plans, as we will see in Chapter 8.

The main contributions that will be discussed in this Chapter are the following:

- (i) to propose a surrogate model of the multi-stage mechanistic model MS-GSM², enabling fast investigation of the FLASH effect in preclinical and clinical scenarios;
- (ii) to show how we train a neural network to replicate the biological outcome predicted by the MS-GSM², given as input a LUT of different physical, chemical and biological parameters;
- (iii) to show the feasibility of the AI-enhanced mechanistic model to efficiently predict cell surviving fraction for different physical and physiological parameters.

In detail, this Chapter is organized with the following structure: Section 7.1 shows the mathematical framework of the AI-enhanced mechanistic model and its numerical implementation; Section 7.2 reports the main numerical results obtained applying the developed surrogate model; finally, Section 7.3 discusses the main findings of the AI-enhanced mechanistic model and future improvements.

7.1 Theory and numerical implementations

This Section is dedicated to presenting the main concept of the AI-enhanced MS-GSM² and describing the mathematical framework behind its development.

The AI-enhanced MS-GSM² model is a surrogate model of the multi-stage mechanistic model developed in Chapter 4. It is designed to replicate the predictions of the original

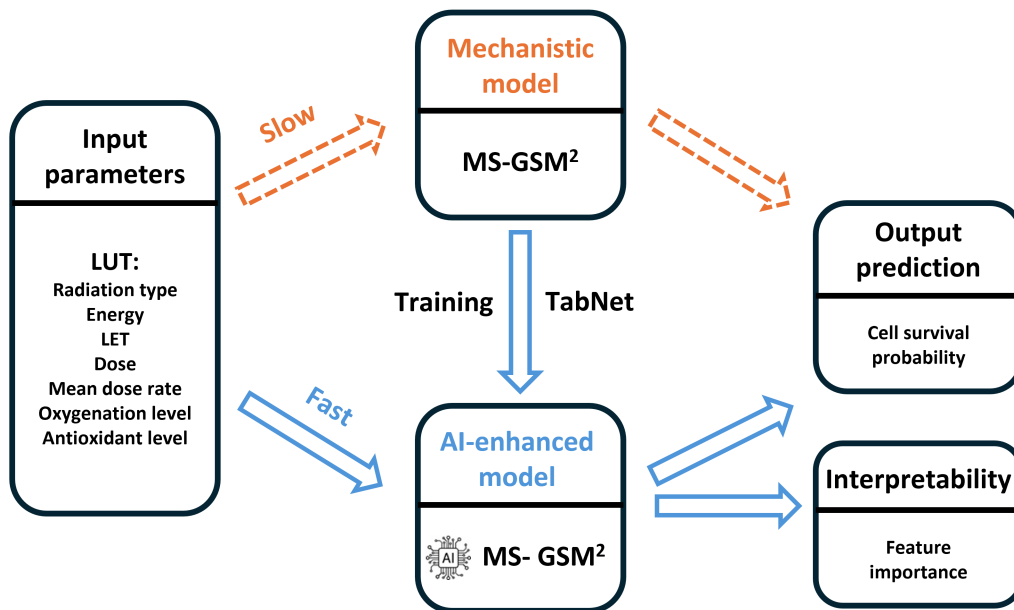


Figure 7.1: Schematization of the AI-enhanced MS-GSM² mathematical framework.

multiscale radiation biophysical model for fast investigations of the FLASH effect, training it on a dataset, which the MS-GSM² model produces. In particular, the input is a LUT of cell survival predictions for different physical, chemical, and biological parameters, including the main characteristics of the irradiation beam, such as radiation quality, dose, and dose rate, or properties of the cell environment, for example, oxygenation and scavenging ability. Given this look-up table, the surrogate model would predict a continuous output, namely, the cell survival probability under different physical and physiological conditions, particularly after UHDR irradiation. The second key point we wish to highlight is that the surrogate model can help us identify which parameters of the mechanistic model play the most significant role in the predicted output. In the context of FLASH, this characteristic can enable the investigation of the main parameters that modulate the sparing effect triggered by UHDR radiation.

Typically, decision tree (DT)-based algorithms [316] are among the most used approaches to tabular data because they are efficient in handling the features of a table by defining the decision boundary, are interpretable by tracing decision steps, and train quickly. However, this may lead to data being overfitted, and large data tables tend to lose efficiency. In addition, they generally struggle to generalize the model output for intermediate values between the discrete points used during the training procedure. On the other hand, deep neural network (DNN) methods are mostly used for unstructured data, such as images and audio, because they encode several data with non-linear dependencies. Furthermore, they require less pre-processing and initial feature manipulation. However, while DNNs are not ideal for tabular datasets, where tree-based models perform

at least as well, if not better, than neural networks [316], notable advancements in this area have recently been made. A representative example is the development of TabNet [317], a DNN-based method that can efficiently handle tabular datasets. The key aspect of this novel architecture is that, using a specific type of DNN algorithm, TabNet can train the model using a tree-based learning approach, combining the benefits of both the DT and DNN methods. We will see in detail how it does this. The second main point of TabNet is interpretability, which enables the algorithm to determine the importance of features and understand the model results. In summary, the main properties of TabNet are as follows:

1. pre-processing of the dataset not needed;
2. training process using a gradient descent-based optimization;
3. pre-selection of the features needed for learning using a sequential attention mechanism [318];
4. learning through feedback from previous results, in analogy to a tree-based model;
5. novel network architecture, called Transformer, based on attention mechanisms, which can capture global dependencies between input features and output results[318];
6. local and global interoperability, namely the acquisition of information about importance and relation between features, and contribution of each feature to the training process, respectively.

Thus, the idea is to apply TabNet to the MS-GSM² model. In the following paragraphs, we will examine in detail the two fundamental steps for building the AI-enhanced model, namely, creating the LUT and using it to train the model.

(i) The input dataset: the MS-GSM² look-up table (LUT)

^AX	E [MeV/u]	LET [keV/ μm]	D [Gy]	$\dot{D}$ [Gy/s]	O_2 [%]	[XSH] [mM]
^1H	19.0, 40.0, 80.0	2.7, 1.5, 0.86	1.0-30.0	0.1-200.0	1.0-7.0	6.5-50.0
^4He	11.5, 56.0	16.2, 4.5	1.0-30.0	0.1-200.0	1.0-7.0	6.5-50.0
^{12}C	149.0, 280.0	19.6, 13.1	1.0-30.0	0.1-200.0	1.0-7.0	6.5-50.0

Table 7.1: Example of physical and physiological input parameters for calculating a look-up table (LUT) of survival probability via the MS-GSM² model, which are used as features input of the TabNet algorithm.

We consider a range of realistic absorbed dose and mean dose rate values, i.e. $D \leq 30$ Gy and $\dot{D} \leq 200$ Gy/s, respectively, for various qualities of the radiation, namely the type $^A\text{X} \in \{^1\text{H}, ^4\text{He}, ^{12}\text{C}\}$, energy $E \leq 280$ MeV/u, and $\text{LET} \leq 19.6$ keV/ μm of the primary particle. Furthermore, we consider a wide range of antioxidant and oxygenation levels, i.e.,

$[XSH] \leq 50$ mM and $O_2 \leq 7\%$, respectively, that characterize the cellular environment. Then, considering several combinations of these parameters, we predict the cell survival probability through the MS-GSM² model, fixing the three rates of biological pathways described in Section 4.1 with realistic values, namely $a = 7.8 \cdot 10^{-3} \text{ 1/h}$, $b = 1.8 \cdot 10^{-2} \text{ 1/h}$, and $r = 3.2 \text{ 1/h}$, in agreement with those previously used.

Thus, the input dataset is a table with different columns, namely features, and several lines, which correspond to the input and output values of the mechanistic model under different conditions. The resulting dataset will serve as input features for the TabNet algorithm, as we will see later. Table 7.1 shows an example of this.

(ii) The AI-based model: the AI-enhanced MS-GSM²

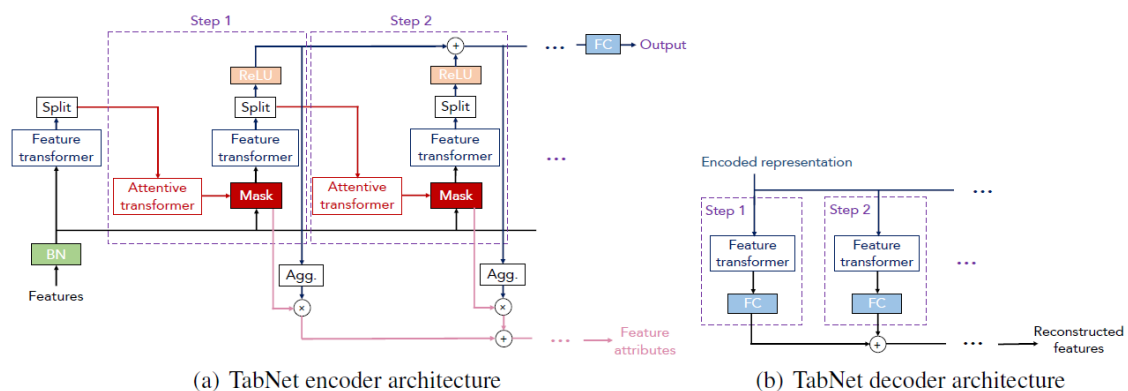


Figure 7.2: TabNet architecture. From [317]

We train the TabNet algorithm [317] in the LUT previously described to develop the AI-enhanced mechanistic model. Now, we illustrate in detail the main steps of TabNet.

The main core of the TabNet architecture is the encoder block to process tabular datasets, as described in Figure 7.2a. It comprises a feature transformer, an attentive transformer, and feature masking. The peculiarity of TabNet is that it combines a sequence of encoder blocks, in analogy to a tree ensemble, leveraging the output of one encoder as feedback for subsequent encoders to optimize feature selection for learning. The first step of the encoder is the batch normalization (BN) process, which normalizes the input features for a more stable learning process. Then, there is a first feature transformer, which converts the input features into an embedding space for learning. This is followed by a split block, which divides the processed features into two channels, particularly as inputs for the attentive transformer and the fully connected layer (FC). The attentive transformer produces mask values, a fundamental step in the feature selection process. The subsequent masking process reduces the dimensionality of the input data, solving the issue of sparsity that typically characterizes tabular datasets, thus increasing the robustness of the model. At this stage, the attention mechanism [318] comes into play. In particular, it computes feature-wise weights to quantify their relative importance using the so-called instance-wise

mechanism. This acts as feedback for the next encoder to guide the selection of the most relevant features and as a component of the model's interpretability mechanism. Instead, the FC layer concatenates all the outputs from the feature transformer of each decision step after a rectified linear unit (ReLU) process, a common activation function in NN for the aggregation process. Thus, after a sequence of decision steps, we obtain the final predictions: the model output and the feature attributes, which describe the importance of the global features, thus enabling interpretability.

The second important block of TabNet is the decoder. This architecture can reconstruct the tabular features starting from the encoded representation, using a feature transformer and an FC layer for each decision step, as shown in the scheme in Figure 7.2b. More details on the workings of TabNet can be found in [317].

Therefore, by providing a dataset like the one shown in Table 7.1 as input to the TabNet architecture, the algorithm produces predictions of cell survival along with the relative importance of each parameter in the prediction process. In this way, we obtain the previously called AI-enhanced MS-GSM². We report some results predicted by this model in the following Section.

7.2 Numerical results

This Section reports the main findings that we obtained using the AI-enhanced MS-GSM² model, in order to highlight its most important features, such as predictive capacity and interpretability. In particular, we predicted the cell survival probability starting from the parameter dataset reported in Table 7.1. In addition, we studied the importance of the various physical and physiological parameters in predicting the survival fraction. We want to emphasize that the main goal of this Section is to show the feasibility of the approach, as we will see in detail below.

Figure 7.3 shows the surviving fraction as a function of the dose absorbed by the cell nucleus, up to 30 Gy, for different radiation qualities, which are 0.86 and 2.7 keV/ μm protons, 16.2 keV/ μm helium ions, and 19.6 keV/ μm carbon ions, for three different mean dose rates, namely a conventional value (0.1 Gy/s), an intermediate value (1.0 Gy/s), and an UHDR value (200.0 Gy/s). Furthermore, we report the impact on the survival probability of two oxygen conditions, i.e., 1.0% and 7.0% O₂, and the modulation of the sparing effect due to two different antioxidant levels, that are 6.5 and 50.0 mM.

Figure 7.4 reports the estimated isoeffective $\overline{\text{DREF}}$ -weighted dose, which is the product between the absorbed dose and the inverse of the dose-rate effectiveness factor ($\overline{\text{DREF}}$), which is defined as the ratio between the dose at a given dose rate and the dose at a reference dose rate (i.e., Conv) in order to obtain the same biological effect, that is

$$\overline{\text{DREF}} := \frac{D(\dot{D}_{\text{Conv}})}{D(\dot{D})} \Big|_{\text{isoeffect}} = \text{DREF}^{-1}. \quad (7.1)$$

So, it is the inverse of the metric defined in Eq. 1.72 and is equal to FMF, according to Eq. 1.73, in the case of UHDR radiation. It is worth mentioning that the present definition we chose is different from previous formulations with the same name DREF [319], mostly used

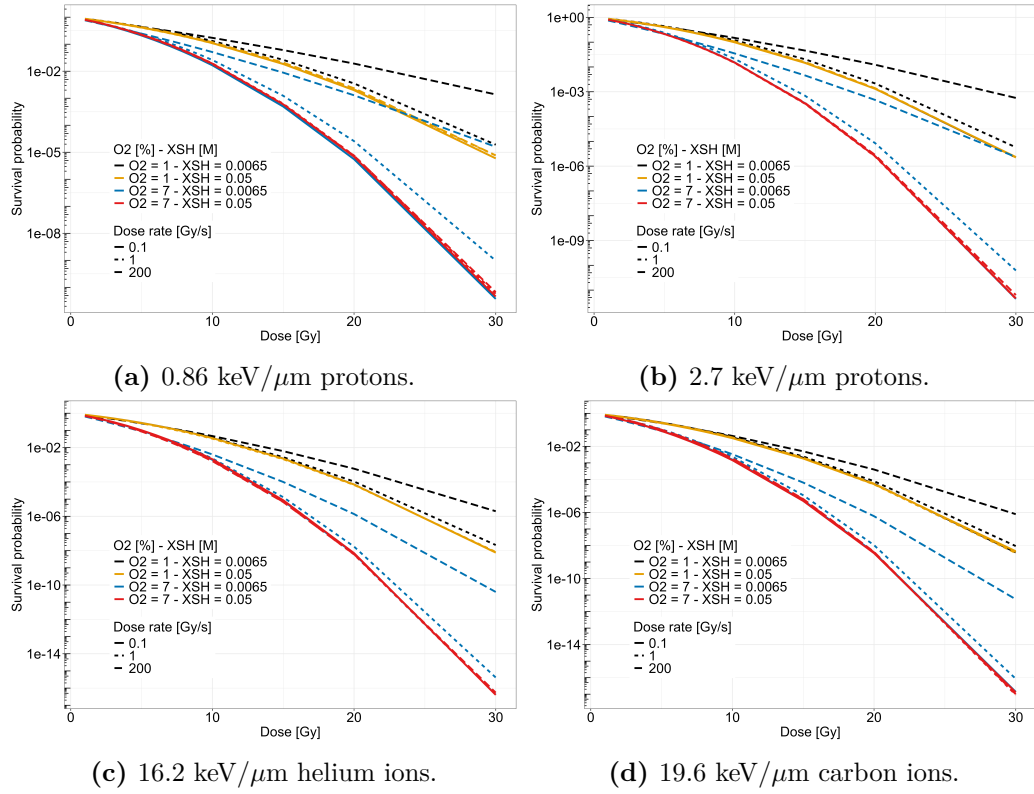


Figure 7.3: Cell survival probability as a function of prescribed dose, computed via the AI-enhanced MS-GSM² model, for different radiation qualities (0.86 and 2.7 keV/μm protons, 16.2 keV/μm helium ions, and 19.6 keV/μm carbon ions), mean dose rates (0.1, 1.0, and 200.0 Gy/s), oxygenation levels (1 and 7%), and antioxidant concentrations (6.5 and 50.0 mM).

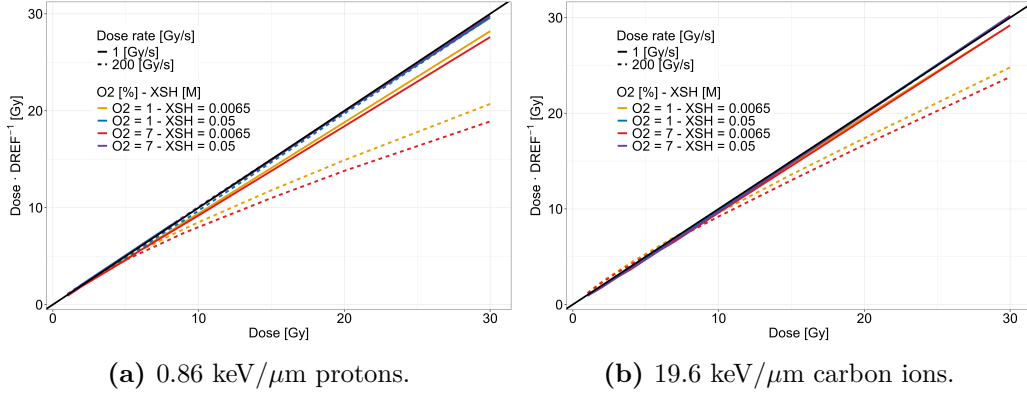


Figure 7.4: 2D representation of the relation between the UHDR dose and the isoeffective $\overline{\text{DREF}}$ -weighted dose, estimated via the AI-enhanced MS-GSM² model, for different radiation qualities (0.86 keV/μm protons and 19.6 keV/μm carbon ions), mean dose rates (1.0 and 200.0 Gy/s compared to 0.1 Gy/s), oxygenation levels (1 and 7%), and antioxidant concentrations (6.5 and 50.0 mM).

in space radiation environment and dedicated to compare ratio of effectiveness rather than doses, at a same dose and for low level of dose rates. In other words, the $\overline{\text{DREF}}$ -weighted dose is the dose delivered in the conventional regime needed to achieve the same biological effect as the dose delivered with a higher dose rate. The values reported here are calculated from the survival fraction and dose values shown in Figure 7.3. In an exemplary manner, Figure 7.4 shows the predictions for two different radiation qualities, namely 0.86 keV/μm protons and 19.6 keV/μm carbon ions, highlighting the impact of two oxygenations, which are 1.0% and 7.0% O₂, and two antioxidant concentrations, i.e., 6.5 and 50.0 mM. In addition, we compare the predictions for two different mean dose rates, which are 1.0 Gy/s and 200.0 Gy/s, where the dose rate value equal to 0.1 Gy/s is considered as the reference for the calculation of $\overline{\text{DREF}}$ for each irradiation and environmental condition reported here.

Figure 7.5 shows the main examples of the predictive capability of the AI-enhanced MS-GSM² model, for different radiation qualities. In particular, given a set of input parameters, the model can predict the cell survival for various LET values within a range bounded by two LET points on which the model was trained. In other words, it represents how the model can generalize the data. In detail, Figures 7.5a and 7.5b report survival probabilities as a function of dose, for LET values in the range of 0.86 and 2.7 keV/μm for protons, and 13.1 and 19.6 keV/μm for carbon ions, respectively, considering a mean dose rate of 200.0 Gy/s, an oxygenation level of 1%, and an antioxidant concentration of 6.5 mM. Instead, Figures 7.5c and 7.5d illustrate the calculation of the corresponding 2D representation of the relationship between the UHDR dose and the isoeffective $\overline{\text{DREF}}$ -weighted dose, for protons and carbon ions, respectively, considering mean dose rates of 1.0 and 200.0 Gy/s compared to 0.1 Gy/s, an oxygenation level of 1%, and an antioxidant concentration of 6.5 mM.

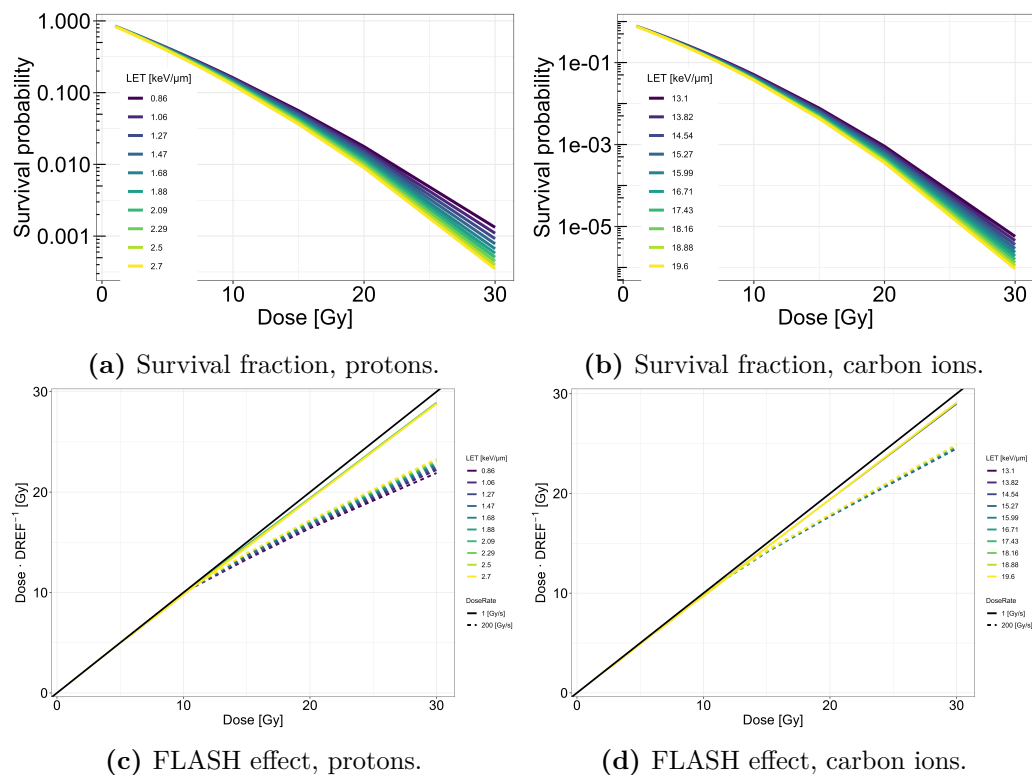


Figure 7.5: Predictions via the AI-enhanced MS-GSM² model for different radiation qualities up to 2.7 keV/μm for protons, and up to 19.6 keV/μm for carbon ions. **(a, b)** Cell survival probability as a function of prescribed dose, considering a mean dose rate of 200.0 Gy/s, an oxygenation level of 1%, and an antioxidant concentration of 6.5 mM. **(c, d)** Corresponding 2D representation of the relation between the UHDR dose and the isoeffective DREF-weighted dose, considering mean dose rates of 1.0 and 200.0 Gy/s compared to 0.1 Gy/s, an oxygenation level of 1%, and an antioxidant concentration of 6.5 mM.

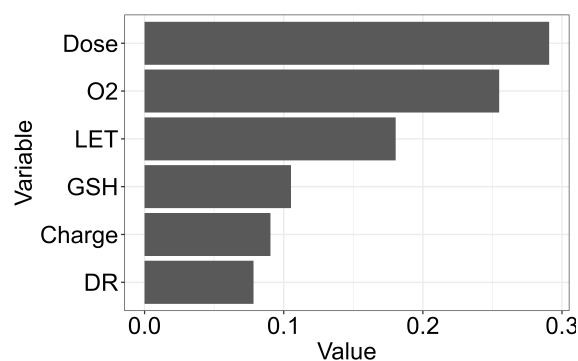


Figure 7.6: Interpretability of the AI-enhanced MS-GSM² model. Average attention weights of each input feature.

Figure 7.6 illustrates the interpretability characteristic of the AI-enhanced MS-GSM² model. In particular, we show the relative importance of input features, between ion type, LET, dose, mean dose rate, oxygen and antioxidant concentrations, on the predicted output, which is the survival probability. The importance is assessed through the average of the attention weights assigned at each step of the TabNet architecture, for each input feature.

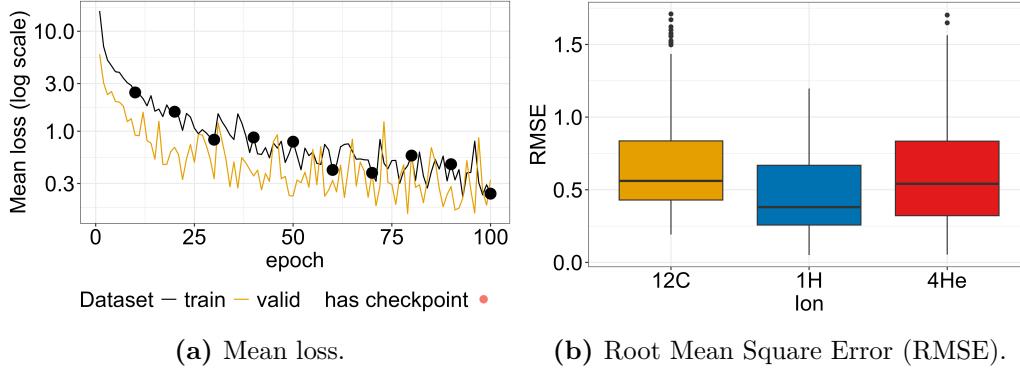


Figure 7.7: Error assessment of the AI-enhanced MS-GSM² model. (a) Mean loss, which is quantified by the Root Mean Square Error (RMSE), as a function of the number of epochs for the training and validation processes. (b) Final RMSE between the model’s predictions and the target values, with the contributions of the three particles considered separately.

Figure 7.7 reports the error assessment of the AI-enhanced MS-GSM² model. In particular, Figure 7.7a shows the mean loss as a function of epochs for the training and validation processes. The loss function compares the predictions of the AI-driven model and the target values, which are the survival values computed via the standard MS-GSM² model, during the training and validation procedures. So, the mean loss measures how well TabNet models the training data. The epoch is a typical parameter of NN that describes the number of times the model parameters are optimized and the weights are updated over the entire dataset using TabNet. In this work, the maximum number of epochs is set to 100. These processes aim to minimize the loss and to avoid overfitting. Instead, 7.7b represents the mean for each particle of the Root Mean Square Error (RMSE), which is defined as

$$\text{RMSE}_i := \sqrt{\frac{1}{N_D} \sum_{j=1}^{N_D} \left(\log \hat{S}_i(D_j) - \log S_i(D_j) \right)^2} \quad (7.2)$$

where N_D is the number of dose points for a given experiment, which is defined by a combination of parameters (^AX , LET, $\dot{D}$, O_2 , [XSH]), $\hat{S}_i$ and S_i are the cell survivals at dose D_j predicted by the AI-enhanced MS-GSM² model and by the MS-GSM² model, respectively, for the i -th set of parameters, in analogy to that proposed in [311]. In particular, the yellow box describes the RMSE of carbon ion data, the blue box refers to

the RMSE of proton data, and the red box represents the RMSE of helium ion data. This metric also quantified the loss during the model's training.

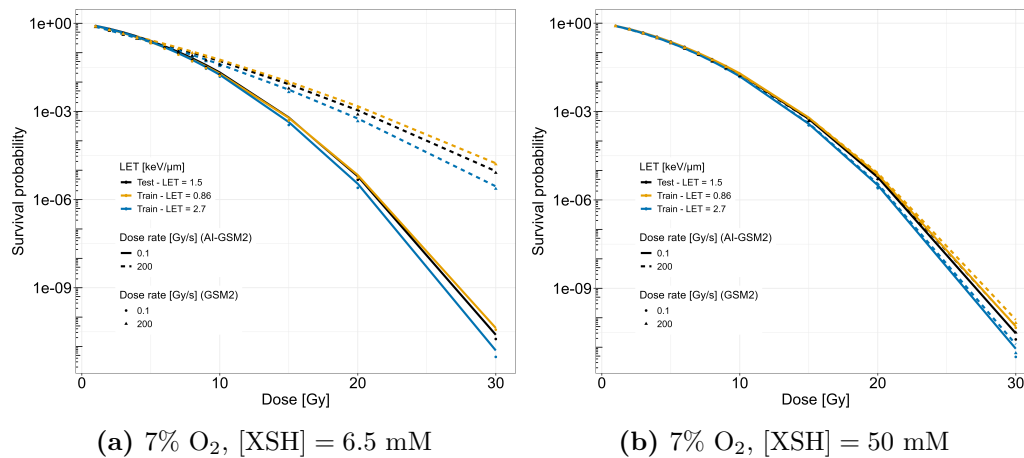


Figure 7.8: Comparison of cell survival probabilities as a function of prescribed dose, between the standard MS-GSM² model (points) and the AI-enhanced MS-GSM² model (lines), for different radiation qualities, i.e. 0.86 and 2.7 keV/μm protons (training set), and 1.5 keV/μm protons (test set), mean dose rates (0.1 and 200.0 Gy/s), and antioxidant concentrations (6.5 and 50.0 mM), for 7% oxygenation level.

Figure 7.8 shows the surviving fraction as a function of dose, up to 30 Gy, for different radiation qualities, which are 0.86, 1.5, and 2.7 keV/μm protons, for two different mean dose rates, i.e. 0.1 Gy/s (Conv) and 200.0 Gy/s (UHDR), and for two different antioxidant levels, that are 6.5 and 50.0 mM, for an oxygen condition of 7.0% O₂. In particular, we compare the original results of the standard MS-GSM² model (points), with the predictions of the AI-enhanced MS-GSM² model (lines). The surrogate model is trained on the dataset for 0.86 and 2.7 keV/μm protons, while it is validated on the dataset for 1.5 keV/μm protons.

7.3 Discussion

This section outlines the main findings predicted by the AI-enhanced MS-GSM² model, reported in Section 7.2. We also discuss the main limitations of the developed model and explore potential future enhancements.

Analysis of the results

As we have already discussed in detail previously in Chapter 6, we can appreciate the impact of the various parameters in determining the trend of the surviving fractions as a function of the absorbed dose from the survival probability curves. In detail, we can notice the combined action of radiation quality, dose, dose rate, oxygenation, and antioxidant concentration in modulation of cell survival curves, as shown in Figure 7.3.

As discussed in Sections 1.4.3 and 3.4, there is no universally accepted univocal method to quantify the FLASH effect. One of the simplest and most commonly used approaches is to characterize it in terms of FMF, as defined by [39]. Our definition of $\overline{\text{DREF}}$ matches the FMF in the case of UHDR irradiation. From this analysis, as illustrated in Figure 7.4a, it is evident that, for UHDR radiation, the maximum achievable DREF is approximately in the range 1.4–1.5, which is in agreement with typical experimental values [82, 39], as discussed in Section 3.4. In addition, this value decreases slightly with increasing LET values of the primary radiation, as shown in Figure 7.4b.

A peculiarity of an AI-based approach is the ability of the developed model to generalize the data. As shown in Figure 7.5, the AI-enhanced MS-GSM² model can predict, in a continuous way, the radiation-induced cellular response for various LET values different from those it was trained on, keeping all other input parameters fixed. Thus, the AI-enhanced approach not only provides significantly faster computation, but, unlike a simple optimization of a standard modeling workflow to improve the computational efficiency, the developed surrogate model enables continuous prediction capabilities once the AI-enhanced MS-GSM² has been trained on a suitably constructed discrete dataset.

In contrast to the classical method previously discussed, the surrogate model's interpretability allows us to identify the contribution of each parameter to the prediction of survival curves. Notably, Figure 7.6 highlights that the most significant parameter for predicting cell survival is the dose, as we probably could have expected, followed by the oxygenation level, a well-known and significant modifier of the biological response to IR. LET and antioxidant level also play an important role, confirming the findings from the mechanistic analyses discussed in the previous Chapters. Interestingly, from these results, it seems that the mean dose rate is the least important parameter in defining the shape of the surviving curve. As a first hypothesis, this could be because, unlike all the other parameters considered, the dose rate has an almost binary effect on modulating the survival curve, as observed as a general trend from experiments [82, 39]. Furthermore, the maximum biological effect triggered by UHDR irradiation is often less intense than other biological effects, such as those caused by hypoxia or those induced by high-LET radiation. These hypotheses still need to be thoroughly tested.

From Figure 7.7, and in particular from the loss trend, we can observe the performance of the developed model. Specifically, the training and validation loss curves decrease as the number of epochs increases. Furthermore, given the small gap between the training and validation curves, we can rule out any overfitting effects. Thus, we can exclude an improper training of the model. This conclusion is supported by Figure 7.8, where we can appreciate the comparison between survival points predicted by the MS-GSM² model and predictions through the AI-enhanced MS-GSM² model, which is trained on the entire protons dataset, excluding all points at the intermediate LET, which is used for its validation.

Current limitations of AI-enhanced MS-GSM² model and future perspectives

This study is an illustrative example to demonstrate the feasibility of the proposed analysis. The ongoing work aims to conduct a more systematic investigation of the beam's physical

parameters. The idea is to incorporate additional parameters, including instantaneous dose rate, pulse frequency, dose per pulse, and other relevant parameters, for more complex dose-delivery time structures. The work also explores different radiation qualities, in particular, the impact of high LET values.

Currently, the AI surrogate model enables us to assess the relative importance of different features to the prediction of radiation-induced biological responses, as illustrated in Figure 7.6. Moving forward, we aim to leverage this capability to investigate how certain physical and physiological parameters influence the magnitude of the FLASH effect, for example, by examining the spatial and temporal patterns of dose delivery. In this context, once the LUT has been sufficiently expanded to ensure proper training of the surrogate model, the AI-enhanced approach could be employed to investigate potential threshold values of the physical, chemical, and biological parameters involved in the emergence of the FLASH effect.

We are trying to improve the predictive capability of the model. In particular, we are working on improving the optimization procedure of the model parameters to reduce the error in predicting the survival curve. An important aspect in this context is the size of the LUT generated using the standard MS-GSM² model, which underpins the AI surrogate model. At present, the LUT includes nearly 7000 cell survival entries, corresponding to different combinations of the physical and physiological parameters listed in Table 7.1. We are currently expanding this dataset by both increasing the number of survival points, i.e., adding more rows to Table 7.1 for new combinations of the existing features, and introducing additional parameter types, effectively extending the columns of Table 7.1.

Furthermore, at this initial stage of the work, we limited ourselves to testing the model's ability to generalize data based on radiation quality, as it is the most controllable parameter. The idea is to test the model for other parameters as well, such as oxygenation or dose rate.

As we will see in detail in Chapter 8, the development of an AI-enhanced mechanistic model will allow faster and more effective studies of complex biological systems under UHDR irradiation conditions. In particular, the idea is to include the AI-enhanced MS-GSM² model in our NTCP model as discussed in Section 6.4, to investigate the radiation-induced response of macroscopic systems, such as for skin injury, as we will see in Section 8.2. Furthermore, the second future step is to apply this model to investigate the role of the main physical parameters in triggering the FLASH effect in realistic clinical scenarios. Thus, this AI-enhanced approach provides a tool to study the optimization of these parameters, enabling the development of an optimizer for the FLASH TPS, as we will discuss in Section 8.3.

Conclusions and Future Perspective

Chapter 8

Outlook

This Section is dedicated to the presentation and discussion of future perspectives and the main ongoing works with preliminary results originated by this Thesis, in particular, regarding additional extensions of the MS-GSM² model. In detail, in Section 8.1, we focus on the inclusion in the MS-GSM² model of alternative radiation-induced cell-death pathways to DNA damage, including also the explicit description of lipids; then, in Section 8.2, we explore a potential characterization of the geometric and functional structure of the skin to expand the study of radiation-induced complications; finally, in Section 8.3, we propose a possible biological optimization scheme of FLASH treatment plans using the MS-GSM² model.

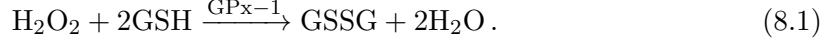
8.1 Alternative cell-death pathways: inclusion of membrane lipids and glutathione peroxidase

Glutathione peroxidase (GPx) is a family of antioxidant enzymes that protect the cell from oxidative stress [320]. In particular, they act against lipid peroxidation, protecting the cell from ferroptosis, which is an alternative cell-death pathway. As seen in Section 3.5, the central role of ferroptosis in the emergence of the FLASH effect has been proposed and discussed in particular by Favaudon and Labarbe in [7, 214].

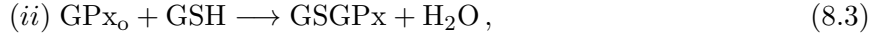
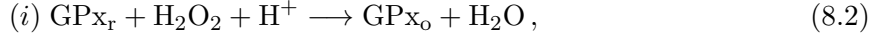
Our idea is that lipid membrane damage may play a role in the complex *in-vivo* scenario of the biological response to UHDR radiation, providing an alternative pathway to the main biological pathways of cell death mediated by DNA damage. As discussed in Chapter 4, the mechanism underlying the FLASH effect could be the result of the combined action of several factors, such as the recombination of organic radicals, and the modulation of radiation-induced damage due to environmental oxygen and the redox balance of the cell. In the case of macroscopic biological systems, additional pathways, such as ferroptosis, could be involved in the complex response to UHDR radiation, as mentioned in Section 6.4.

In this context, we are working on the inclusion of two enzymes belonging to the glutathione peroxidase family. First of all, we are adding an additional channel for the

reduction of hydrogen peroxide H_2O_2 , which is mediated by glutathione peroxidase 1 (GPx-1), using GSH as a cofactor. The kinetics of GPx-1 can be described by the following overall reaction:



Thus, GPx-1 catalyzes this reaction, producing oxidized glutathione GSSG and water H_2O . Eq. 8.1 can be described by a sequence of three reactions, namely



where GPx_o and GPx_r are the oxidized form and reduced form of GPx-1, while GSGPx is an intermediate.

The three reactions reported in Eq. 8.1 can be described by the following system of ODEs [321]:

$$\begin{cases} \frac{d[\text{H}_2\text{O}_2]}{dt} = -k_{\text{GPx}_r} \cdot [\text{GPx}_r] \cdot [\text{H}_2\text{O}_2] \\ \frac{d[\text{GPx}_r]}{dt} = k_{\text{GSGPx}} \cdot [\text{GSH}] \cdot [\text{GSGPx}] - k_{\text{GPx}_r} \cdot [\text{GPx}_r] \cdot [\text{H}_2\text{O}_2] \\ \frac{d[\text{GPx}_o]}{dt} = k_{\text{GPx}_r} \cdot [\text{GPx}_r] \cdot [\text{H}_2\text{O}_2] - k_{\text{GPx}_o} \cdot [\text{GSH}] \cdot [\text{GPx}_o] \\ \frac{d[\text{H}_2\text{O}]}{dt} = k_{\text{GPx}_r} \cdot [\text{GPx}_r] \cdot [\text{H}_2\text{O}_2] + k_{\text{GPx}_o} \cdot [\text{GSH}] \cdot [\text{GPx}_o] \\ \frac{d[\text{GSGPx}]}{dt} = k_{\text{GPx}_o} \cdot [\text{GSH}] \cdot [\text{GPx}_o] - k_{\text{GSGPx}} \cdot [\text{GSH}] \cdot [\text{GSGPx}] \\ \frac{d[\text{GSSG}]}{dt} = k_{\text{GSGPx}} \cdot [\text{GSH}] \cdot [\text{GSGPx}] \end{cases} \quad (8.5)$$

where $[\text{GSH}]$ is considered constant, in analogy to [7]. The same assumption can be applied to $[\text{H}_2\text{O}]$, as the concentration is much larger than all the others.

In Figures 8.1 and 8.2, we show the numerical results that we obtained solving the reaction kinetic system in Eq. 8.5 for the various chemical species. In particular, in Figure 8.1 we show the impact of the initial level of the antioxidant GSH, while in Figure 8.2 we illustrate the influence of the initial concentration of the enzyme GPx_r . As we can see, there is (i) a temporary transition of GPx-1 from the reduced form GPx_r to the oxidized form GPx_o , with a simultaneous consumption of H_2O_2 , according to Eq. 8.2. Then, (ii) the oxidized GPx_o reacts with the cofactor GSH, generating an intermediate species, namely GSGPx, according to Eq. 8.3, necessary for the advancement of the overall reaction in Eq. 8.1. The last step (iii) is the release of the enzyme from the intermediate species, which leads to the formation of GSSG and the return of GPx-1 to its reduced form. From Figures 8.1 and 8.2 we can also see how the initial concentrations of GSH and GPx_r impact the reaction rate of Eq. 8.1, particularly in H_2O_2 depletion and GSSG generation. The slight variation in $[\text{H}_2\text{O}]$ can likely be attributed to numerical error, given that H_2O is typically maintained at a constant level during chemical system resolution.

The second enzyme belonging to the glutathione peroxidase family that we want to include is the glutathione peroxidase 4 (GPx-4). This enzyme, in combination with GSH, catalyzes the lipid hydroperoxides LOOH, producing the stable lipid alcohol LOH, through

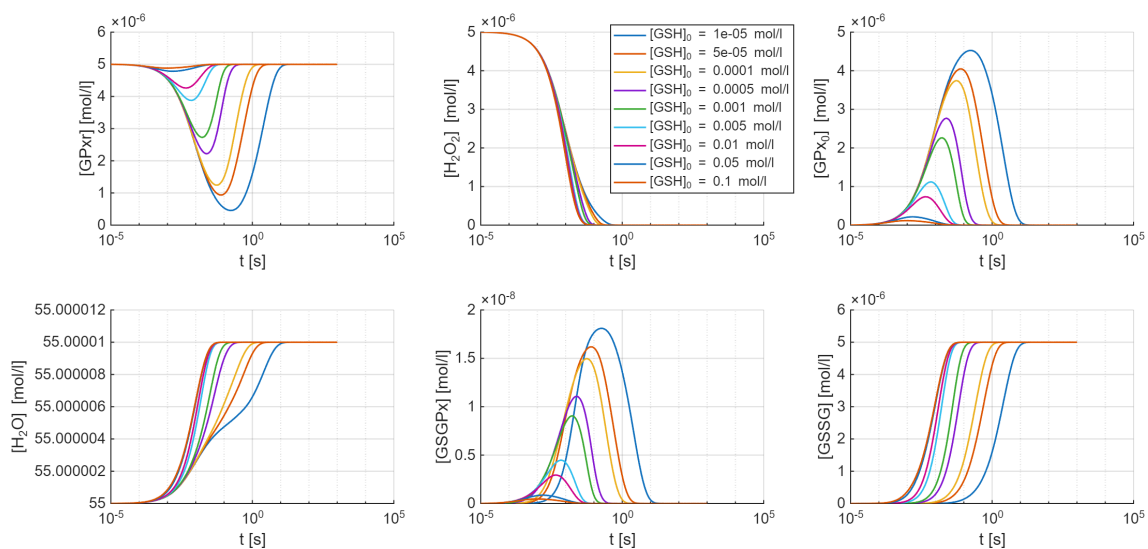


Figure 8.1: Time evolution of the concentration of the six chemical species (H_2O_2 , GPx_r , GPx_o , H_2O , GSGPx , and GSSG), for different concentration of GSH .

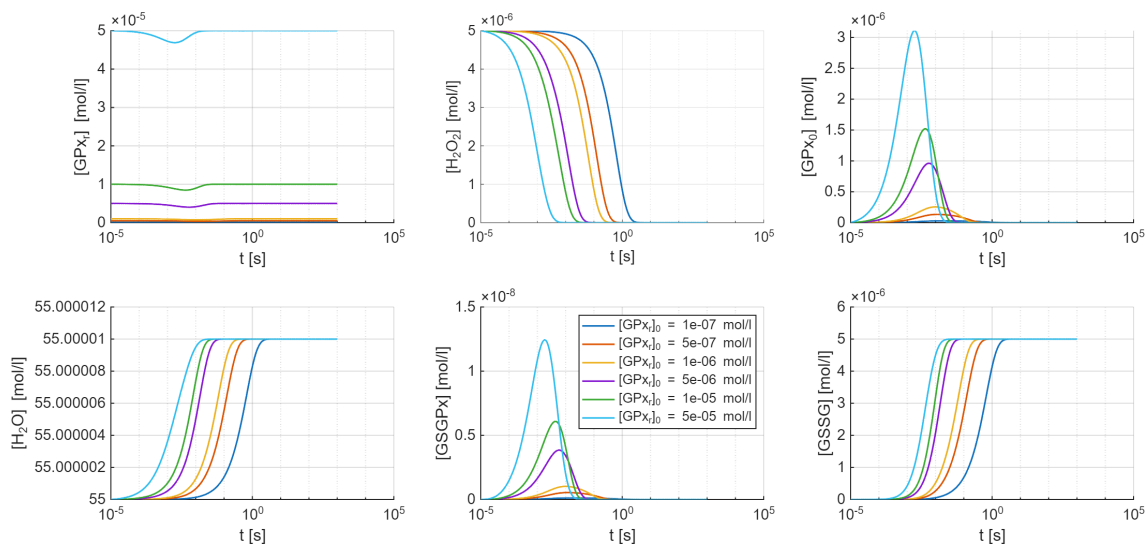
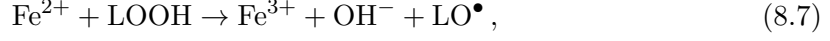


Figure 8.2: Time evolution of the concentration of the six chemical species (H_2O_2 , GPx_r , GPx_o , H_2O , GSGPx , and GSSG), for different concentration of GPx_r .

the following reaction [322]:



In this reaction, glutathione plays a fundamental protective role against ferroptosis, consuming the membrane lipid hydroperoxides. In fact, it is in competition with the Fenton reaction, illustrated in [218] and discussed in Section 3.5:



which initiates membrane lipid peroxidation, leading to ferroptosis.

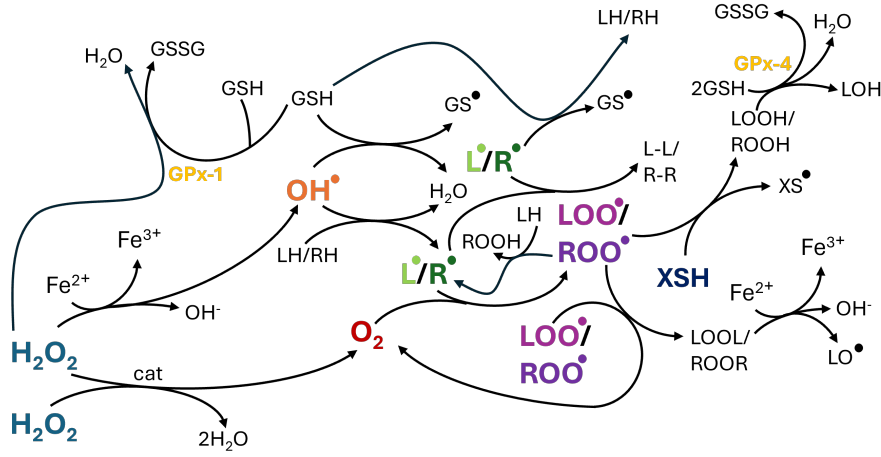


Figure 8.3: Updated chemical reactions network, including reactions that involve membrane lipids, and pathways mediated by glutathione peroxidase.

Thus, we are working on the incorporation of the previous reactions, namely Eq. 8.1 and 8.6, in our system of chemical reactions described in Section 4.1. Before doing this, we must explicitly include membrane lipids LH in our chemical system, starting from what was proposed in [7]. Lipid membranes can be directly damaged by ionizing radiation, producing the lipid radical $\text{L}^\bullet$. This radical can also be generated by the reaction with the organic peroxy radical $\text{ROO}^\bullet$, as proposed in [7], according to Eq. 3.13. Then $\text{L}^\bullet$ can react with molecular oxygen, producing the lipid peroxy radical $\text{LOO}^\bullet$, or it can recombine through various pathways, analogous to the reactions described in Section 3.5 for organic radicals. In addition, the lipid membrane can be influenced by the action of labile iron, through the Fenton reaction [218], according to Eq. 8.7. All these reactions are summarized in our updated scheme of homogeneous chemistry in Figure 8.3.

8.2 Description of skin architecture and radiation-induced side effects

The skin is the largest human organ, with an overall area of $\sim 1.8 \text{ m}^2$ [323]. The skin performs fundamental functions such as protection against external threats, endocrine and

exocrine activities, thermo-regulation, and sensory perception. In addition, its peculiarity is that the skin undergoes constant metabolic renewal. Skin damage is one of the most common side effects in patients undergoing RT, especially in the case of head and neck cancer, breast cancer, or skin cancer. Furthermore, the development of skin injuries is particularly influenced by the presence of ROS [184].

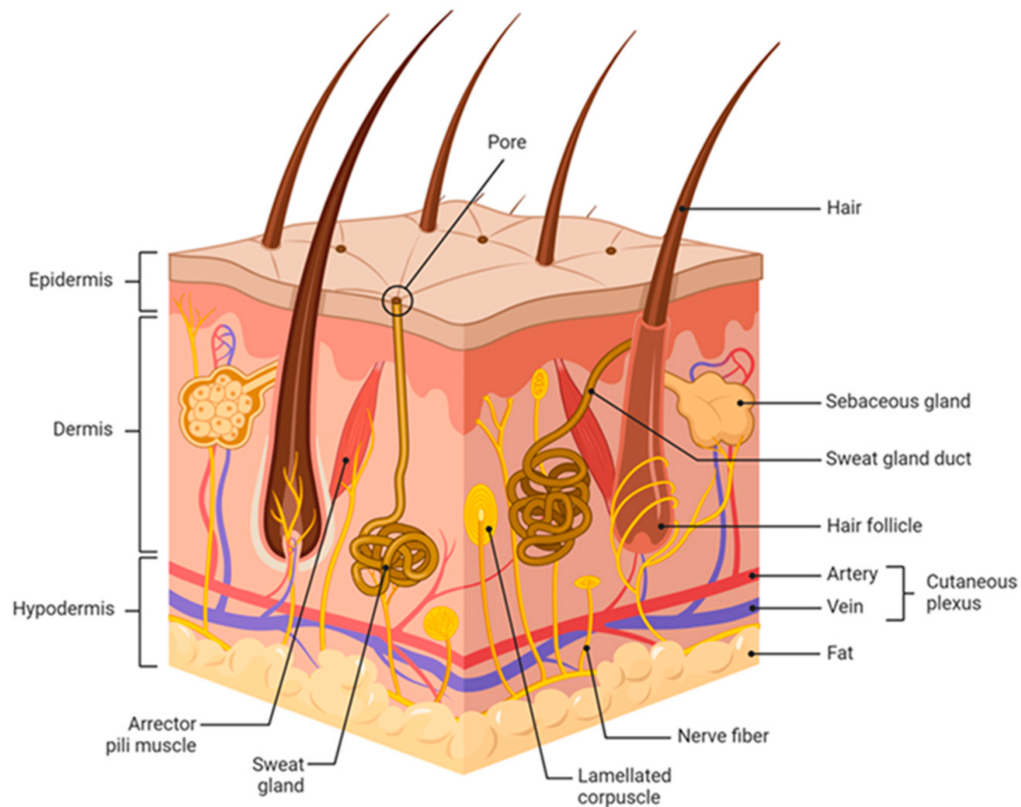
The study of skin responses to radiation has been extensively investigated, not only in the context of FLASH RT, as discussed in Section 3.4, where numerous experiments have been carried out, particularly in mice, and even the first human patient has been treated, but also with Conv IR, notably using low-energy electrons. Consequently, the development of specific mathematical models to investigate radiation-induced skin reactions can provide valuable support for *in-silico* studies, complementing *in-vivo* experiments.

From a structural point of view, the skin is characterized by a very complex layered architecture: each of these layers has specific properties and functions, as illustrated in Figure 8.4. In detail, we can group these layers into three main tiers, that is,

1. The epidermis, which is the outer layer and mainly contains keratinocytes, namely progenitor cells generated by epidermal stem cells, and that can undergo differentiation processes. In detail, they generate the suprabasal stratum spinosum, the stratum granulosum, and the terminally differentiated stratum corneum. In fact, as keratinocytes develop, they move toward the skin's surface, become flattened, and ultimately shed in the horny layer, producing the characteristic stratification of the epidermis. In addition, this layer is non-vascularized and nutrients arrive via diffusion from the dermis. The main functions are waterproof and protective covering;
2. The dermis, which is the middle layer, and is divided into the papillary layer, which is mainly made by areolar connective tissue, and reticular layer, which is characterized by dense connective tissue with collagen, elastic and reticular fibers. In addition, this layer contains nerve endings, glands, and a capillarized vessel system. This layer plays a crucial role in sensing heat and touch, as well as in hormone production;
3. The hypodermis or subcutaneous tissue, which is the innermost layer, and contains connective tissue, where the main cell type is fibroblast, adipose tissue, with adipose cells, and blood vessels, with macrophages. The main functions are to act as a connection to muscle and bones, and as an insulator from cold.

The skin, following exposure to ionizing radiation, can develop a plethora of skin injuries, as illustrated in Figure 8.5, which can be grouped into [324]:

1. Acute effects, which emerge within hours to weeks after IR exposure. They are associated with DNA damage and have dose-dependent onset thresholds influenced by radiation quality and skin area sensitivity. The main diseases are erythema, desquamation, and ulceration;
2. Late effect, which occur between weeks and years after IR. They are chronic and progressive diseases associated with inflammatory processes. The main pathologies are necrosis, fibrosis, and telangiectasia.



(a) Skin architecture.

Epidermis (stratified squamous epithelium)Stratum basale:

- Epidermal stem cells: generation of progenitor cells

Stratum spinosum:

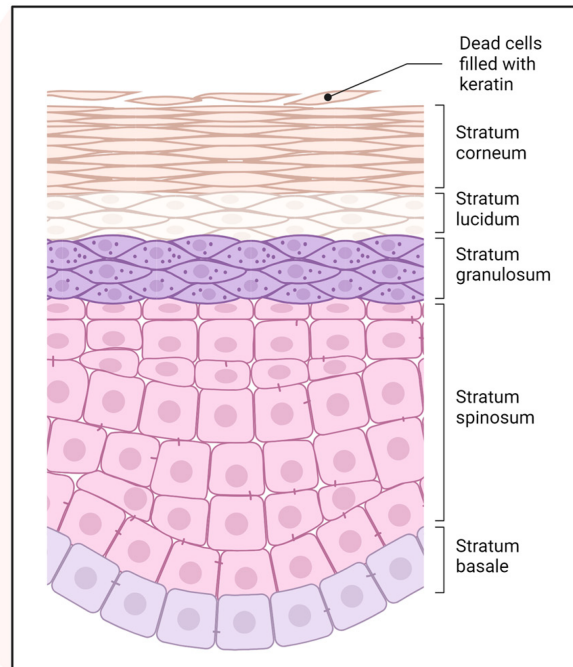
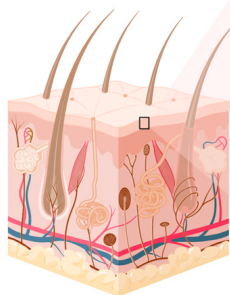
- keratinocytes: sequential differentiation

Stratum granulosum & lucidum:

- keratinocytes: terminal differentiation

Stratum corneum:

- corneocytes: desquamation in horny layer



(b) Epidermis layers.

Figure 8.4: Illustration of the biological structure of the human skin. From [323].

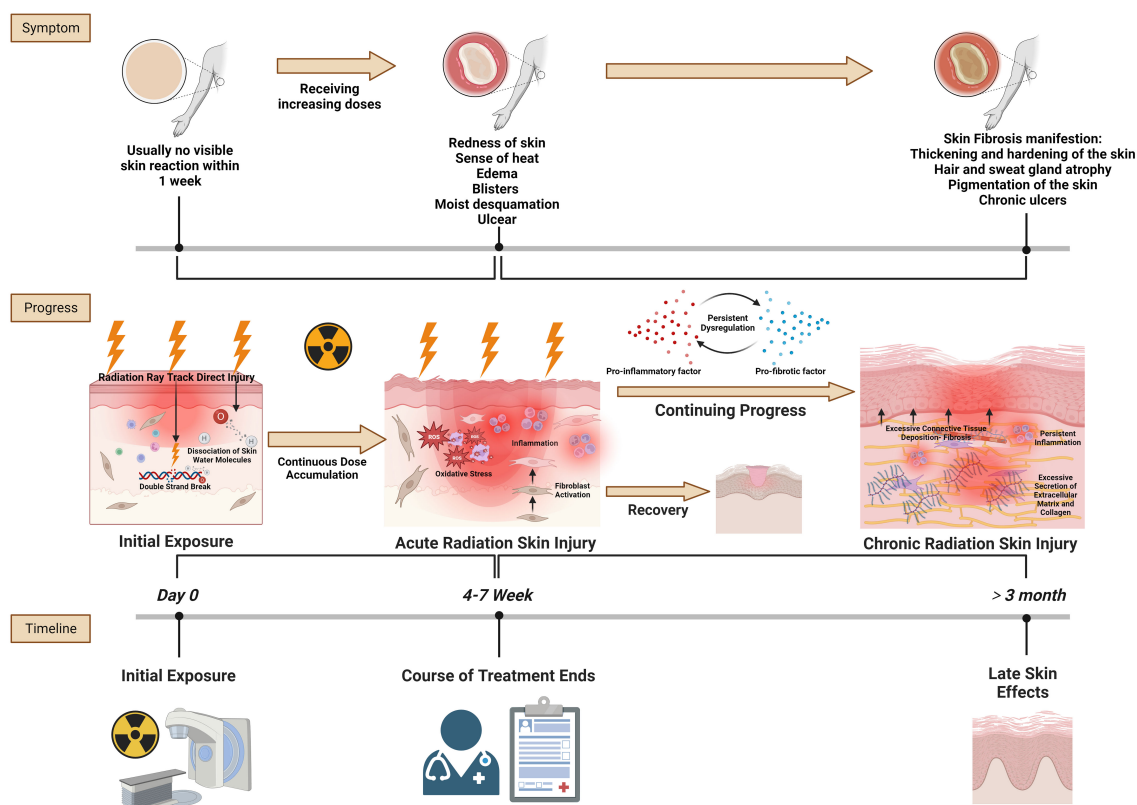


Figure 8.5: Illustration of the radiation-induced acute and late effects in the human skin. From [324].

Therefore, our primary goal is to develop a model of the layered structure of the skin, for example considering different cell lines and specific concentrations of chemical species, which will allow us to study side effects using the NTCP models established in Chapters 5 and 6. In addition, starting from the discussion in Section 8.1, our aim is to further expand the chemical system of the MS-GMS² model from Chapter 4 to incorporate alternative cellular death processes, such as apoptosis, senescence, and ferroptosis, which are important in the context of skin injuries, and thus to describe more complex radiation-induced side effects.

8.3 Towards FLASH biological treatment planning system (TPS)

In conventional radiation therapy, a fundamental step in treatment planning is the plan optimization procedure. In particular, in particle therapy, this is done via the so-called inverse planning, whose aim is to achieve an acceptable treatment plan through an optimization process, i.e. a minimization of the overall deviations of the prescribed doses to the delivered ones, which determines the optimal number of particles for each pencil-beam

to generate a 3D dose distribution that meets the required dosimetric constraints. The starting point of this procedure is the definition of a cost function that mathematically represents the treatment goals. Typically, the primary goals of radiation treatment include ensuring adequate tumor coverage, in order to control the tumor growth, and minimizing exposure to healthy tissues, to reduce the probability of occurrence of radiation-induced complications. Thus, in a realistic clinical setting, this procedure must be accurate and fast, and require efficient radiobiological models. The inclusion of a specific radiobiological effect is at the basis of the so-called biological treatment planning where the effect is evaluated beyond a pure dosimetric profile. The resulting treatment plans can ultimately be evaluated using both dosimetric indices and predictive models to estimate the probability of complications for healthy tissue and control of tumor volume growth, such as those discussed in Section 2.4 and those developed in Chapter 5.

The clinical translation of FLASH radiotherapy requires the development of a dedicated radiobiological mechanistic model for outcome prediction. In this context, as we discussed in Chapter 4, we developed the MS-GSM², a multi-stage radiobiological model capable of replicating all the principal data of cellular response to UHDR irradiations, taking into account a wide range of types of irradiation and characteristics of the tissue environment. However, a fully mechanistic approach, while fundamental for basic understanding, is computationally expensive and, as a result, is currently impractical for biological optimization of treatment plans at FLASH dose rates. Indeed, a traditional approach requires predicting biological outcomes for a huge number of data points, across various doses, dose rates, LET and oxygenation levels, typically in the order of 10^5 .

Therefore, we developed the above discussed AI-enhanced mechanistic model, i.e. a surrogate model of the MS-GSM², for a fast study of the FLASH effect in preclinical and clinical scenarios, as shown in Chapter 7. The AI-based surrogate model is trained to replicate the predictions of the MS-GSM², combining the speed and the predictive capabilities of AI approaches, with the fundamental underpinnings of the mechanistic model. So, this AI-enhanced MS-GSM² is ideal for both fast prediction of possible healthy tissue complications, coupled with a NTCP model, as presented in Chapters 5 and 6, and for the biological optimization of UHDR treatment plans, also including volume effects via a NTCP-driven optimizer, such as that proposed in [80].

In addition, our setting is general enough to account for different environmental conditions, i.e., oxygenation and scavenger concentration, dose-delivery time structures, and radiation qualities. Thus, in principle, we can calculate a dose distribution in a proton treatment plan using a MC tool, such as OpenTOPAS [45, 46] or FRED [48], which is a GPU-accelerated MC code for fast dose calculation, and by combining it with temporal information of dose delivery, we can analyze several dose-delivery time structures and scanning pathways, on the emergence of the FLASH effect, considering different environmental conditions of the irradiated tissue. Another example of future applications is the investigation of different dose rate metrics, as discussed in Section 3.3, such as in [152], but applying the MS-GSM² model. The flexibility of MS-GSM² will allow us to study how different scanning patterns affect the dose rate and the resulting biological effect, for example those reported in Figure 8.6. Calculating a look-up table in dose rate, LET,

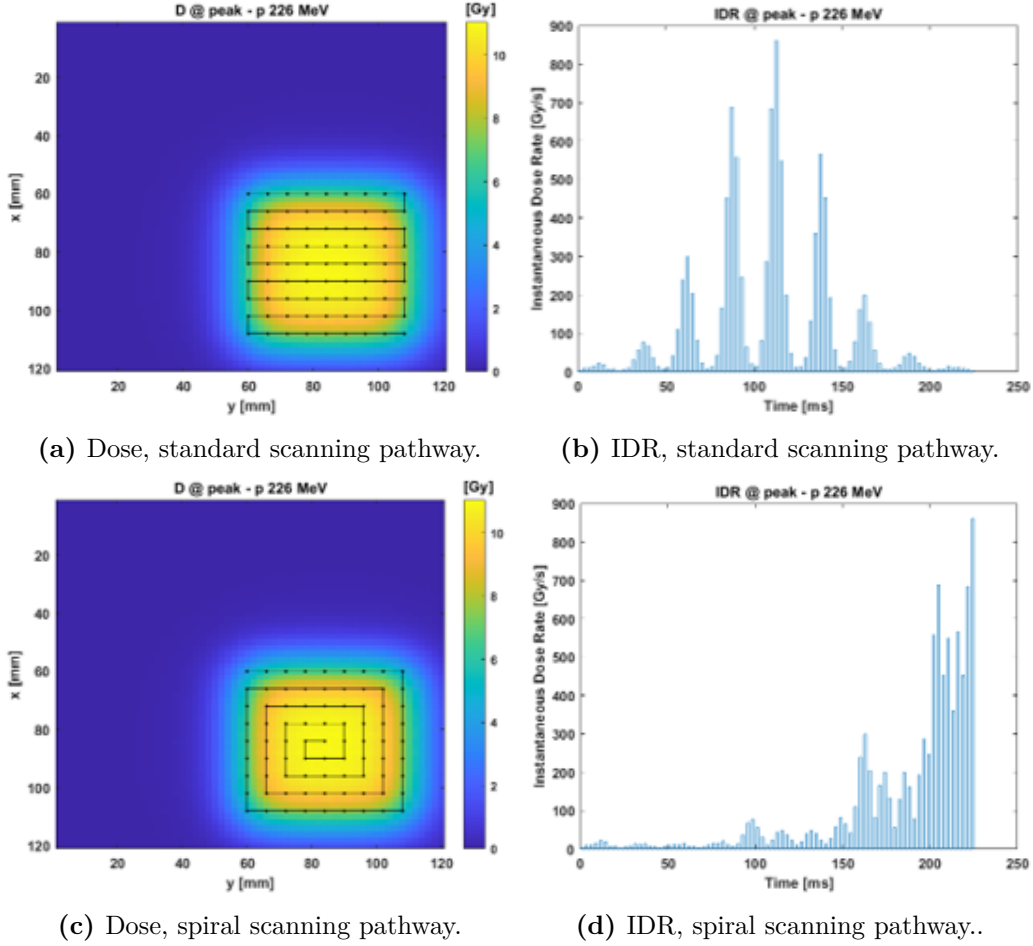


Figure 8.6: **a, c)** Example of dose distributions calculated with TOPAS, considering different dose-delivery scanning pathways, and **b, d)** the corresponding instantaneous dose rate (IDR) patterns, as defined in Eq. 3.5, for the voxel in the center of a cubic irradiated with protons of 226 MeV. We can analyze these different situations applying the MS-GSM² in each voxel, considering the real dose-delivery time structure. We can also extend this approach, e.g., considering regions with different oxygen conditions, different irradiated geometries, several radiation qualities, etc.

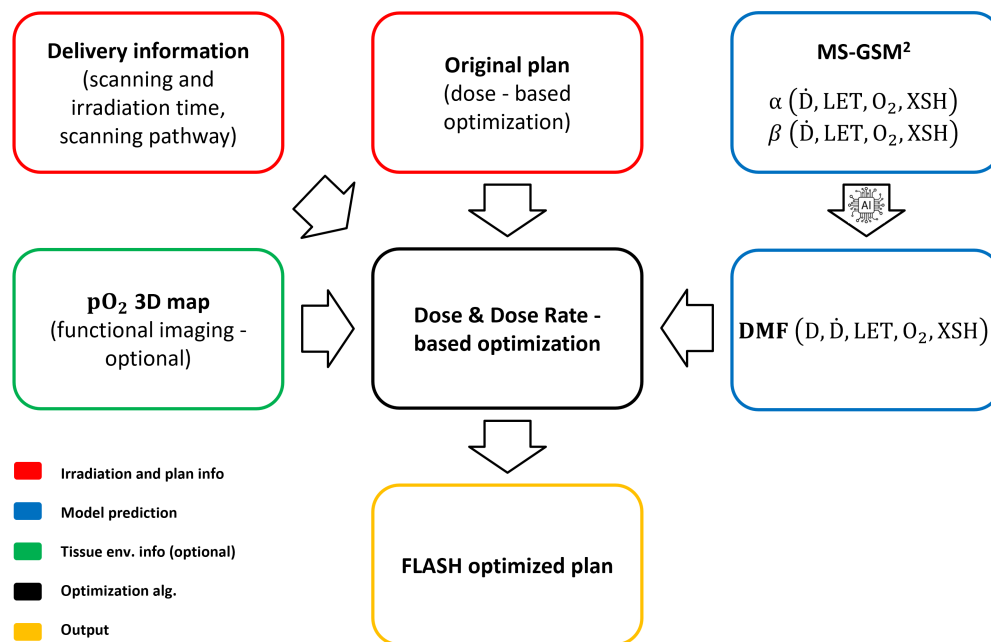


Figure 8.7: Schematic representation of the workflow for enabling FLASH treatment planning with protons. The red boxes represent the information from the original plan, the blue boxes describe how the MS-GSM² is included in the treatment planning system (TPS), while the green box shows possible additional information, e.g. environmental conditions. The black box is the optimizer and yellow box is the biological-driven optimized plan for UHDR irradiation.

and oxygenation through the MS-GSM² or using the AI-enhanced MS-GSM² model for faster prediction, we can investigate the biological effect of different physical parameters for UHDR radiation.

Lastly, the main idea is to optimize the biological dose through the AI-enhanced MS-GSM² model, including it in a dedicated TPS for FLASH RT, as we can from the optimization framework proposed in Figure 8.7. In particular, the basic idea is to optimize the biological effect, predicted by the MS-GSM² model (blue boxes in the figure), which is enhanced by AI, obtaining the best dose and dose rate distributions that maximize the FLASH sparing effect, leading to the FLASH optimized plan (yellow box). The optimizer (black box) received as input the original plan for Conv RT and the delivery information such as the scanning and irradiation time, and the scanning pathway (red boxes). Furthermore, the optimizer, in principle, can receive additional environmental information (green box), such as a 3D map of oxygenation obtained through functional imaging, e.g. FMISO-PET [325]. The original plan is also intended for model calibration in the Conv regime, with the goal of predicting the relative biological effect in the UHDR regime.

Therefore, the MS-GSM² model and its extensions have the potential to drive significant advancements in the clinical application of electron and ion irradiations in the UHDR regime.

Chapter 9

Summary and Conclusions

In this dissertation, we presented a novel biophysical model to investigate the intricate mechanism behind the FLASH effect. The MS-GSM² [1, 2] is a multi-scale radiobiological model in the sense that it combines different stages of radiation-induced biological damage. In particular, we showed how the model includes these stages, from the fastest physical processes, which is characterized mainly by the radiation quality of the primary particle and by the prescribed dose and dose rates of the irradiation beam, to the slowest biological pathways, enabling the description of the cell survival probability under various scenarios. The key aspect is the inclusion in the model of the homogeneous chemical stage, which describes the reactions that involve organic substrate and environmental chemical species, such as oxygen and scavengers. In addition, the developed model is probabilistic in the sense that it can take into account different levels of spatio-temporal stochasticity in physics, chemistry, and biology. In detail, it can describe the stochasticity in energy deposition by ionizing radiation, the impact of it on the chemistry evolution, and in the formation and time evolution of DNA damages.

We proved the feasibility of the MS-GSM² model to describe UHDR radiation under different experimental scenarios. In detail, we showed how the model can predict the cell survival probability for different particle types, energies, LETs, doses, mean dose rates and dose-delivery time structures of the radiation beam, and for various environmental oxygenation levels and cell lines. In addition, we described the impact of different physical, chemical, and biological parameters on the manifestation of the FLASH sparing effect. Finally, we proposed a consistent novel hypothesis supported by *in-silico* evidence to describe the FLASH differential effect: we showed how the combined action of oxygenation level, scavenging activity, and macroscopic dose can modulate the protective effect that occurs after UHDR irradiation. This study has shown how the FLASH sparing effect is attenuated or leveled out with increasing antioxidant rate and oxygen concentration or with decreasing absorbed dose. Therefore, what we can conclude from these findings is that the mechanism underlying the FLASH effect cannot be confined within a rigid classification, but instead involves multiple scales and combined mechanisms that trigger and modulate its occurrence. Our findings suggest an intricate dynamic between competing reactions of the homogeneous chemistry, which influence the formation of DNA lesions.

This complex interplay is modulated by physical factors, such as radiation quality, dose, and dose rate, as well as by physiological conditions of the biological tissue considered, including cell type, oxygenation level, redox environment, and antioxidant activity.

The need to translate the initial findings from *in-vitro* to *in-vivo* outcomes, motivated the development of a novel, mechanistically grounded NTCP model, achieved by integrating the MS-GSM² framework into a NTCP formulation [12]. Firstly, the MS-GSM²-driven NTCP model was able to remarkably reproduce conventional data of different experimental scenarios, considering several radiation qualities, different prescribed dose and dose rates, and a wide range of fractionation schemes. In addition, the model can exploit different bio-chemical effects, such as DNA damage fixation due to environmental oxygen, volume effects [80], and biological endpoints, such as spinal cord tolerance and skin desquamation. Besides that, the model was also applicable to FLASH regime. This study has specifically demonstrated that a reduction in DNA damage, even if significant, resulting from changes in the chemical environment induced by UHDR radiation, can account for key experimental observations following proton-FLASH irradiation. These findings also support speculation that, in complex macroscopic biological systems, alternative cell-death pathways, beyond those mediated by DNA damage, such as cell membrane damage, may contribute to the onset of the FLASH effect.

The mechanistic framework developed in this thesis for UHDR irradiation can, at least in part, be adapted to explore other radiobiological phenomena that remain poorly understood, while accounting for their specific differences. For instance, the implementation of the volume effect could offer valuable insights into the mini-beam effect. Although the mini-beam effect likely involves distinct mechanisms compared to FLASH irradiation, both share a significant influence of the spatio-temporal pattern of dose delivery.

Finally, we proposed an innovative strategy to substantially accelerate the MS-GSM² model while preserving its mechanistic foundation. We showed how a mechanistic learning approach can be pivotal for the development of additional extensions of the MS-GSM² model, particularly to investigate the biological response of complex systems or to develop biological optimizers of treatment plans for FLASH therapy. In addition, this study highlights the importance of a key characteristic of surrogate models, namely the explainability. The AI-enhanced MS-GSM² model developed here proved particularly valuable for exploring how physical and physiological parameters influence the induction of the FLASH effect.

In conclusion, this thesis explored several approaches to extending a conventional radiobiological model, incorporating additional temporal and spatial scales, such as the effects of homogeneous chemistry and the treatment of macroscopic biological systems. The findings presented in this thesis demonstrated the feasibility of describing the FLASH effect assuming a reduction of DNA damage due to a modification of the chemical environment of the cell, whose intensity depends on many physical and physiological factors. The hope of the author is that the developments presented in this dissertation represent a small but meaningful step toward a deeper understanding of the FLASH effect, helping to illuminate the elusive mechanisms triggered by UHDR radiation, and providing fundamental tools for further investigations and applications.

Appendix

This Section reports in Algorithm 1 how to construct and simulate stepwise the solution of the MS-GSM² discussed in Section 4.2. Such construction is based on the joint usage of a standard stochastic simulation algorithm (SSA), together with a solver of ODEs for the chemical system.

Algorithm 1 MS-GSM²

1: Notation:

$$\begin{aligned} h_{1+3(d-1)}(x) &= rX^{(c,d)}, \quad h_{2+3(d-1)}(x) = aX^{(c,d)}, \\ h_{3+3(d-1)}(x) &= bX^{(c,d)}(X^{(c,d)} - 1), \quad h_{1+3N_d}(x) = q\dot{d}, \\ h_{2+3N_d}(x) &= (1-q)\dot{d}, \quad h_0(x) := \sum_{i=1}^{2+3N_d} h_i(x). \end{aligned}$$

2: start with an initial chemical environment $\xi(t) = \xi_0$, biological system $X(t) = Y(t) = 0$ and initial time $t = 0$;

3: set $T_i = 0$, for $i = 1, \dots, 2 + 3N_d$;

4: **while** $t < T$ **do**

5: generate two random numbers $R_1, R_2 \sim U(0, 1)$;

6: compute $\tau = \frac{1}{h_0(x)} \log \frac{1}{R_1}$;

7: solve the chemical system for ξ in the time interval $[t, t + \tau]$;

8: select the minimum reaction index j such that $\sum_{i=1}^j h_i(x) > R_2 h_0(x)$;

9: update $t = t + \tau$;

10: **if** $j = 1 + 3(d-1)$, for some $d = 1, \dots, N_d$, **then**

11: update $X^{(c,d)}(t) = X^{(c,d)}(t) - 1$, $Y^{(c,d)}(t) = Y^{(c,d)}(t)$ and $\xi^d(t) = \xi^d(t)$;

12: **else if** $j = 2 + 3(d-1)$, for some $d = 1, \dots, N_d$, **then**

13: update $X^{(c,d)}(t) = X^{(c,d)}(t) - 1$, $Y^{(c,d)}(t) = Y^{(c,d)}(t) + 1$ and $\xi^d(t) = \xi^d(t)$;

14: **else if** $j = 3 + 3(d-1)$, for some $d = 1, \dots, N_d$, **then**

15: update $X^{(c,d)}(t) = X^{(c,d)}(t) - 2$, $Y^{(c,d)}(t) = Y^{(c,d)}(t) + 1$ and $\xi^d(t) = \xi^d(t)$;

16: **else if** $j = 1 + 3N_d$, **then**

17: simulate the position where the track hits;

18: **for** $d = 1$ to N_d **do**

19: compute the specific energy deposition z^d according to an amorphous track model;

20: update $\xi^d(t) = \xi^d(t) + G_\xi \rho z^d$;

21: simulate $Z_{Y;indir}^{(c,d)}$ from a Poisson random variable of average $\lambda(\text{LET}, [\text{O}_2]) \varrho \int_0^t [\text{R}\ddot{\text{O}}\text{O}_d^\bullet](s) ds$;

22: simulate $Z_{X;indir}^{(c,d)}$ from a Poisson random variable of average $\kappa(\text{LET}, [\text{O}_2]) \varrho \int_0^t [\text{R}\ddot{\text{O}}\text{O}_d^\bullet](s) ds$;

23: update $X^{(c,d)}(t) = X^{(c,d)}(t) + Z_{X;indir}^{(c,d)}$, $Y^{(c,d)}(t) = Y^{(c,d)}(t) + Z_{Y;indir}^{(c,d)}$;

24: **else if** $j = 2 + 3N_d$, **then**

25: **for** $d = 1$ to N_d **do**

26: compute the specific energy deposition z_d according to an amorphous track model;

27: update $\xi^d(t) = \xi^d(t) + G_\xi \rho z^d$;

28: simulate $Z_{Y;dir}^{(c,d)}$ from a Poisson random variable of average $\lambda(\text{LET}, [\text{O}_2])$;

29: simulate $Z_{X;dir}^{(c,d)}$ from a Poisson random variable of average $\kappa(\text{LET}, [\text{O}_2])$;

30: update $X^{(c,d)}(t) = X^{(c,d)}(t) + Z_{X;dir}^{(c,d)}$, $Y^{(c,d)}(t) = Y^{(c,d)}(t) + Z_{Y;dir}^{(c,d)}$;

31: update $h_i(x)$, for $i = 0, \dots, 2 + 3N_d$.

32: **end the system.**

List of Abbreviations and Acronyms

The main abbreviations and acronyms used in this thesis are introduced below:

ADR Average Dose Rate	DNA DeoxyriboNucleic Acid
AI Artificial Intelligence	FMF FLASH-Mmodifying Factor
AT Amorphous Track	FSU Functional-Sub-Unit
AUC Area-Under-the-Curve	gEUD Generalized Equivalent Uniform Dose
BED Biologically Effective Dose	GPx Glutathione Peroxidase
Conv Conventional dose rate	GSH Glutathione
DADR Dose-Averaged Dose Rate	GSM² Generalized Stochastic Microdosimetric Model
DMF Dose Mmodifying Factor	HDR High Dose Rate
DNN Deep Neural Network	HRF Hypoxia Reduction Factor
DPP Dose-Per-Pulse	IDR Instantaneous Dose Rate
DR Dose Rate	IR Ionising Radiation
DREF Dose-Rate Effectiveness Factor	IRT Independent Reaction Time
DSB Double-Strand Break	IT Inter-Track
DT Decision Tree	
DTDR Dose-Threshold Dose Rate	

LEM Local Effect Model	PRF Pulse Repetition Frequency
LET Linear Energy Transfer	RBE Relative Biological Effectiveness
LKB Lyman-Kutcher-Burman	ROD Radiolytic Oxygen Depletion
LINAC LINear ACcelerator	ROS Reactive Oxygen Species
LQ Linear-Quadratic	RR Relative Radiosensitivity
LUT Look-Up Table	RS RadioSensitivity
MC Monte Carlo	RSE Relative Sparing Effect
MKM Microdosimetric Kinetic Model	RT RadioTherapy
MME Microdosimetric Master Equation	SDE Stochastic Differential Equation
MS-GSM² MultiScale-Generalized Stochastic Microdosimetric Model	SSA Stochastic Simulation Algorithm
NMSE Normalized Mean Square Error	SOBP Spread-Out Bragg Peak
NN Neural Network	SSB Single-Strand Break
NTCP Normal Tissue Complication Probability	TCP Tumor Ccontrol Probability
ODE Ordinary Differential Equation	TPS Treatment Planning System
OER Oxygen Enhancemen Ratio	UHDR Ultra-High Dose Rate
ORR Organic Radical Recombination	VE Volume Effect
PBS Pencil Beam Scanning	VHEE Very-High Energy Electron
PI Partial Irradiation	XAI Explainable Artificial Intelligence
	XSH Antioxidant

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