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CHUV - Département d'Oncologie
Laboratoire de Radio-Oncologie

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Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine de l'Université de Lausanne
en cotutelle internationale avec
l'Université Paris-Saclay Paris-Sud

par

Pierre Montay-Gruel

Élève Normalien
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et de l'Université Paris-Saclay Paris-Sud

Jury

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Pr Nicole Deglon, experte
Dre Nathalie Gault, experte

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Normal brain, neural stem cells and glioblastoma responses to FLASH irradiation

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pour le Doyen
de la Faculté de biologie et de médecine

Prof. Luc Pellerin

université PARIS-SACLAY

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Thèse de doctorat

NORMAL BRAIN, NEURAL STEM CELLS AND GLIOBLASTOMA RESPONSES TO FLASH IRRADIATION

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SUMMARY

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ABSTRACT

Nowadays, more than 50% of cancer patients can benefit from a radiation-therapy treatment. Despite important technological advance and dose delivery precision, encephalic radiation-therapy still induces large and irreversible side effects in pediatric and adult cancer patients, justifying the urge to develop new radiation-therapy techniques. Preclinical studies on FLASH irradiation (FLASH-RT) showed a possibility to efficiently treat the tumors, without inducing drastic side-effects on the normal tissue, by increasing the dose-rate over 40 Gy/s. This so called “FLASH effect” set off an important interest in this new irradiation technology to increase the therapeutic ratio of radiation-therapy.

This PhD work aimed at investigating the anti-tumor effect of FLASH-RT on brain tumor models along with the assessment of the ultra-high dose-rate irradiation effects on the normal brain tissue. In this context, subcutaneous, orthotopic and transgenic glioblastoma murine models were used to investigate the curative effect of FLASH irradiation delivered with an experimental LINAC available at the CHUV, and able to deliver both conventional and FLASH irradiation. Moreover, murine models of whole brain irradiation were developed to investigate the radiation-induced cellular and functional alterations at early and late time-points post-FLASH-RT. These models were used to decipher the cellular effectors involved in the brain’s radiation response including hippocampal cell-division and neuronal responses but also more physiopathological aspects as radiation-induced reactive astrogliosis and neuroinflammation. A panel of well-defined cognitive tests was also developed to investigate the radiation-induced cognitive alterations. Eventually, the physico-chemical events induced by FLASH-RT, and particularly the role of dioxygen consumption, were investigated to decipher the mechanisms that underlie the FLASH effect.

In all investigated tumor models, FLASH-RT displayed an efficient anti-tumor effect at least similar to the conventional irradiation. The whole brain irradiation models showed an innocuousness of FLASH-RT on the normal brain tissue, with an absence of cognitive deficit several months after irradiation at dose-rates above 100 Gy/s, coupled with a preservation of hippocampal cell division and neuronal structure. This protection was also observed at the physiopathological level with an absence of astrogliosis and neuroinflammation. Moreover, these results were reproduced with ultra-high dose-rate X-rays delivered with a synchrotron light source. On the mechanistic side, the reversion of the protective effects of FLASH-RT by hyperoxia, and the absence of effect of anoxia on the anti-tumor effect, along with a decreased ROS production underlie the primary role of dioxygen consumption during ultra-high dose-rate irradiation.

Altogether, these unique results depict the possibility to increase the therapeutic index of radiation-therapy by the use of FLASH-RT. Indeed, this new irradiation technology preserves the normal brain tissue from radiation-induced toxicities by increasing the dose-rate over 100 Gy/s, while keeping an anti-tumor effect equivalent to the conventional dose-rate irradiation. According to these preclinical results and an upcoming clinical translation, FLASH-RT might become a major contributor to the cancer treatment by radiation therapy.

RESUMÉ EN FRANCAIS

De nos jours, plus de 50% des patients porteurs de tumeur bénéficient d'un traitement de radiothérapie. Malgré des avancées technologiques récentes augmentant la précision des traitements, la radiothérapie encéphalique induit toujours des effets secondaires invalidants et irréversibles. Ce constat justifie le développement de nouvelles techniques de radiothérapie. Des études précliniques réalisées sur l'irradiation FLASH ont montré la possibilité de maintenir un effet anti-tumoral tout en réduisant drastiquement les effets secondaires sur le tissu sain. Cet effet a été appelé « l'effet FLASH ». Cette technologie consistant à délivrer des doses à des débits supérieurs à 40 Gy/s a généré un intérêt important pour l'augmentation de l'index thérapeutique de la radiothérapie.

Ce travail de thèse vise à étudier l'effet anti-tumoral de l'irradiation FLASH sur des modèles précliniques de glioblastome, tout en évaluant ses effets sur le tissu cérébral sain. Des modèles murins de glioblastome sous-cutané, orthotopique et transgénique ont été développés et irradiés grâce à un prototype d'accélérateur linéaire d'électrons délivrant une irradiation FLASH ou conventionnelle. De plus, des modèles murins d'irradiation encéphalique ont été mis au point afin d'investiguer les effets cellulaires et les altérations fonctionnelles induites par l'irradiation FLASH. La division cellulaire et la structure neuronale dans l'hippocampe ont été évaluées, ainsi que des aspects plus physiopathologiques comme la neuroinflammation ou l'astrogliose. Un panel de tests cognitifs a également été utilisé afin d'étudier les altérations cognitives induites par l'irradiation encéphalique. Enfin, les évènements physico-chimiques engendrés par l'irradiation FLASH et plus particulièrement le rôle de la consommation de dioxygène lors de l'irradiation, ont été analysés afin d'élucider les mécanismes qui supportent l'effet FLASH.

Dans tous les modèles étudiés, l'irradiation FLASH a présenté un effet anti-tumoral au minimum similaire à celui de l'irradiation conventionnelle. Les modèles d'irradiation encéphalique ont montré une innocuité de l'irradiation FLASH sur le tissu cérébral sain, avec une absence de déficits cognitifs pour des débits de dose supérieurs à 100 Gy/s, couplée à une absence d'altération de la division cellulaire et de la structure neuronale dans l'hippocampe, une absence de neuroinflammation et d'astrogliose. De plus, des résultats similaires ont été observés avec l'utilisation de rayons X délivrés à ultra-haut débit par un rayonnement synchrotron. Sur le plan mécanistique, la réversion des effets protecteurs de l'irradiation FLASH par l'induction d'une hyperoxie, l'absence d'effet de l'anoxie sur l'effet anti-tumoral et la production de moins de radicaux libres souligne le rôle primaire du dioxygène dans l'effet FLASH.

L'ensemble de ces résultats illustre la possibilité d'augmenter l'index thérapeutique de la radiothérapie en utilisant l'irradiation FLASH. En effet, cette nouvelle technologie permet de préserver le tissu sain contre les toxicités radio-induites lorsque l'irradiation est délivrée à des débits supérieurs à 100 Gy/s, tout en conservant un effet anti-tumoral équivalent à l'irradiation conventionnelle. D'après ces résultats précliniques et grâce à un transfert clinique envisagé dans un futur proche, l'irradiation FLASH pourrait devenir une technique de choix dans le traitement des tumeurs par radiothérapie.

RÉSUMÉ DE VULGARISATION

De nos jours, plus de 50% des patients porteurs de tumeurs bénéficient d'un traitement de radiothérapie, couramment appelé « rayons ». Malgré de récentes avancées technologiques augmentant la précision de ces traitements, l'utilisation de la radiothérapie pour traiter les tumeurs du cerveau induit toujours des effets secondaires invalidants et irréversibles comme des pertes de mémoire ou des inflammations du cerveau. Ce constat justifie le développement de nouvelles techniques de radiothérapie. Des études réalisées sur des souris avec une irradiation appelée FLASH ont montré la possibilité de traiter les tumeurs tout en réduisant drastiquement les effets secondaires sur le tissu sain qui les entoure : cet effet a été appelé l'effet FLASH. Cette technologie consistant à délivrer des doses élevées de radiothérapie dans un temps court (quelques millisecondes) pourrait améliorer l'efficacité du traitement des tumeurs du cerveau et la qualité de vie des patients à long terme.

Ce travail de thèse vise à étudier sur des modèles de souris la possibilité de traiter des tumeurs du cerveau par l'irradiation FLASH, tout en évaluant ses effets sur les parties saines du cerveau. Différents modèles de souris ont été traités avec un prototype d'irradiateur permettant d'irradier de manière conventionnelle (durée d'irradiation de plusieurs minutes), ou de manière FLASH avec une durée d'irradiation de l'ordre de la milliseconde. Enfin, le rôle de l'oxygène lors de l'irradiation FLASH a été analysé.

Dans tous les modèles de tumeurs du cerveau étudiés, l'irradiation FLASH a présenté une efficacité de traitement au minimum similaire à celui de l'irradiation conventionnelle. Les modèles d'irradiation du cerveau ont montré une innocuité de l'irradiation FLASH sur le tissu sain, avec une absence de déficits cognitifs à long-terme. Nous avons aussi montré que l'irradiation FLASH réduisait la production de radicaux libres, ceci expliquant vraisemblablement son effet protecteur.

L'ensemble de ces résultats illustre la possibilité d'augmenter l'efficacité de la radiothérapie en utilisant l'irradiation FLASH. En effet, pouvoir délivrer l'irradiation dans un temps très court permet de préserver le tissu sain contre les toxicités induites par les rayons tout en gardant la possibilité d'éliminer les tumeurs. Avec ces résultats précliniques et grâce à une application chez les patients envisagée dans un futur proche, l'irradiation FLASH pourrait devenir une technique de choix dans le traitement des tumeurs par radiothérapie.

FOREWORD

The concept of ionizing radiation arose in 1895 when Wilhelm Röntgen identified for the first time a particular form of "rays" produced by vacuum tubes. He observed their characteristics to penetrate through the matter and described them as "a new kind of invisible light […] clearly something new, something unrecorded." (W. C. Röntgen 1898). Röntgen subsequently performed several experiments involving his discovery, including the famous photograph of his spouse's hand. Temporarily named "X-rays" according to their unknown characteristics and composition, this appellation stayed, and X-rays started to be widely used in all scientific fields. The discovery of this technologically produced ionizing radiation pre-dated the description in 1886 by Henri Becquerel of similar rays naturally emitted by uranium salts. A few years later, Becquerel's thesis student Maria Sklodowska-Curie and her husband Pierre Curie identified the radium as another natural source of radiation and named this phenomenon: "radioactivity".

The first physiological effects of ionizing radiations were described in 1901 by P. Curie and H. Becquerel who observed similar actions of radium and X-rays on the biological tissues (P. Curie et al. 1901). Their precise observations were made after the accidental but also intentional exposition of Becquerel's, Giesel's and P. Curie's skin to radium ionization. Firstly, a few hours after the exposition, a light redness was observed on the irradiated skin. Two to three weeks later the redness increased, followed by the formation of a scab and a wound. Interestingly, the authors described different long-term evolutions depending on the time of exposition and the activity of the source. In the best cases, normal healing was observed whereas, for longer expositions, deeper and necrotic wounds were described. These first experiments and observations following radioactive sources handling allowed to identify the dangers but also the possible benefits of ionizing radiations.

Immediately after their discovery by Röntgen, X-rays were used in 1896 by Despeignes in France to treat the gastrointestinal cancer of his neighbor (V. Despeignes 1896). This clinical case is nowadays considered as the first radiation therapy treatment ever delivered. The same year, Ludlam and Gilman used a Crookes tube developed by Grubbé to treat a breast carcinoma in Chicago (E. H. Grubbé 1933), and Freund treated the first melanocytic nevus-bearing pediatric patient with X-rays in Vienna (E. Schiff et al. 1898). These three patients treated the same year but independently from each other and in three different countries, are the first recorded cases of radiation-therapy treatments. Interestingly, they were treated before the description of the biological effects of ionizing radiations by Becquerel and Curie. During the early 20th century, many studies described the possibility to treat skin malignancies with radium radiations, benefiting from their highly toxic effects on the cells and low penetration into tissues. Moreover, higher energy X-rays devices were developed by Coolidge around 1910 and gave access to

deeper tumors (E. O. Lawrence et al. 1932). Nevertheless, despite their obvious benefits on the cancer treatment, the use of ionizing radiations was highly limited by their important side effects on normal tissues, mainly due to a non-regulated use and a lack of knowledge on their characteristics and mechanisms of action. During the following decades, the scientists, aware of the unique cancer-cure possibilities provided by radiation-therapy, developed methods, tools and devices to extensively characterize the ionizing radiations and use them in the most safely and efficient way in radiation-therapy.

The French scientists, such as P. and M. Curie, Bergonié, Tribondeau or Régaud have been important pioneers in radiobiology and radiation-therapy. Improving the differential effect of radiation-therapy in increasing the tumor control while reducing the normal tissue injury has always been the main goal of the research in radiobiology, exemplified by the development of the fractionated radiation-therapy by Claudius Régaud in 1920 (C. Régaud 1920). With the advent of new technologies at the end of the 20th century, radiation-therapy devices became more and more reliable, allowing the delivery of extremely precise doses on the tumors with the possibility to spare the surrounding normal tissues.

Within slightly more than a century, radiation-therapy became, along with surgery and chemotherapy, a major contributor to the treatment of cancer. Nowadays, more than 50% of the cancer patients benefit from safe and efficient radiation-therapy treatments. Nevertheless, and despite efficient therapies, cancer cure is not always achievable due to highly resistant tumor types. Moreover, the occurrence of radiation-induced side-effects on the normal tissues is still high, especially in very radiation sensitive organs. In this context, it is of an utmost interest to orient the research toward an increase of the radiation-therapy's therapeutic index.

In line with the French school of radiobiology, and with the goal to improve the differential effect of radiation-therapy, the work carried out in the framework of this PhD thesis aimed at investigating a new technology of radiation-therapy delivered at ultra-high dose-rate and called "FLASH-RT" in the context of the brain tumor treatment. On one hand, preclinical studies have been performed to evaluate the anti-tumor effects of FLASH-RT on glioblastoma tumors along with the assessment of the normal tissue toxicities. On the other hand, the biological events involved after irradiation have been investigated in order to decipher the mechanisms of FLASH-RT interaction with the biological matter.

INTRODUCTION

1. Brain tumors and their treatments

Brain tumors can be divided into two main subtypes: malignant, i.e.: cancerous tumors, or benign tumors. Primary cancerous brain tumors directly develop within the brain and are, in almost all cases, limited to this organ (P. Beauchesne 2011). Moreover, in nearly all cases, the causes of primary brain tumors development are mostly unknown and few genetic or environment-associated factors are identified (J. L. Fisher et al. 2007). On the contrary, metastatic brain malignancies derive from a distant primary tumor that spreads out and forms brain metastases, mainly from lung, breast, melanoma and colorectal cancers (Carsten Nieder et al. 2011). Brain tumors symptoms vary depending on their localization but include mainly headaches, vomiting or seizures along with functional alterations such as visual deficits, motor and sensation disorders (E. Davies et al. 2004; A. Perkins et al. 2016). For evident reasons, this introduction will not discuss the whole brain tumor diversity but will rather develop the main features, epidemiology and treatments of the most prevalent ones, mostly according to their relevance in this thesis work. Medulloblastoma, glioblastoma and brain metastases will particularly be developed because of their high prevalence in childhood and adulthood respectively. The high frequency occurrence of radiation-induced brain toxicities in pediatric and adult brain-tumor patients following radiation-therapy treatments justifies the interest in developing new technologies to prevent these impairments. Moreover, the bad prognoses associated with glioblastoma and brain metastases make them ideal candidates to investigate innovative treatments.

1.1. Childhood brain tumors and medulloblastoma

As for many other tumor types, brain tumors in children differ from the adult malignancies, due to the ongoing development of the brain during the childhood. With an incidence rate of 1-5 over 100’000 people, pediatric brain tumors are rare diseases. Nevertheless, they remain the most common form of solid tumors among children under the age of 15 and represent the second cause of death in this population (K. J. Johnson et al. 2014). Pediatric brain tumors comprise a myriad of different tumor types that are classically classified on the basis of their histological features and presumed site of origin. Nevertheless, the increasing use of biological parameters to describe these tumors tend to implement the classification with molecular features, rendering it less confusing and more informative for potential therapeutic strategies. This introduction focuses on the most common pediatric brain tumor: medulloblastoma and its current therapeutic management.

1.1.1. General features of medulloblastoma

Medulloblastoma is the most frequent malignant brain tumor in childhood, comprising 40% of all childhood posterior-fossa tumors and with an incidence of 1.5 per million people (N. R. Smoll et al. 2012). They occur all throughout childhood with two identified peaks of incidence between 3-4 and 7-10 years of age. Nevertheless, 15-20% of medulloblastomas are developed under 2 years of age and complicates the therapeutic management due to the highly sensitivity of the developing central nervous system (CNS). (D. N. Louis et al. 2016). In terms of symptoms and diagnosis, medulloblastomas often develop in the fourth ventricle region and rapidly result in cerebellar deficits, cranial neuropathies and obstruction of the cerebrospinal fluid (CSF) flow resulting in hydrocephalus in 80% of the patients.

The recent molecular characterization of medulloblastoma has allowed a classification into 4 biological subtypes (Table 1): Wnt mutated, SHH mutated, Group 3 with MYC amplifications and Group 4 with NMYC and CDK6 amplifications (M. Kool et al. 2012; M. D. Taylor et al. 2012). This molecular classification is currently being integrated into the treatment planning since it has been associated with patients' outcomes and metastatic progression (O. Klein et al. 2015).

Table 1: Medulloblastoma classification by molecular biology. Adapted from the Neurosurgical Encyclopedia (*Encyclopedia Neurochirurgica*). CTNNB1: Bêta catenin gene ; CDK6: Cyclin Dependent Kinase 6 ; Gli2: Zinc Finger Protein GLI2 gene; PTCH1: Protein Pateched Homolog 1; SUFU: Suppressor of Fused Homolog.

FEATURES	Wnt MUTATED (15%)	Shh MUTATED (25%)	GROUP 3	GROUP 4
Histology	Classical, rare large cells / anaplasia	Classical, large cells, anaplasia, desmoplasia, nodule	Classical, large cells, anaplasia	Classical, large cells, anaplasia
Metastases	Rare	Not frequent	Highly frequent	Frequent
Prognosis	Very good	Good for infants, Intermediate for older children	Bad	Intermediate
Genetics	CTNNB1 mutation	PPTCH1, Smo and SUFU mutations Gli2 and NMYC amplifications	MYC amplification	NMYC and CDK6 amplifications

1.1.2. Medulloblastoma treatment

Currently, the treatment management, consisting in surgery and postsurgical adjuvant radiation-therapy (RT) and chemotherapy for children above 3 years of age has been stratified based on two major risk-groups of patients. Patients with “average-risk disease” have undergone a total or near-total surgical resection, without any evidence of dissemination at the time of the diagnosis. Patients with “high-risk disease” display tumors with sub-total resection or have evidence of dissemination (R. J. Packer et al. 2003).

Following surgery, patients with average-risk medulloblastoma are treated with craniospinal and tumor site boost radiation-therapy and chemotherapy, during and after radiation-therapy. Interestingly, the dose of craniospinal radiation-therapy has been reduced over the past decade for patients with non-disseminated disease (around 24 Gy) and has not resulted in a higher incidence of disease relapse (A. Gajjar et al. 2006; R. J. Packer et al. 2003). A variety of different chemotherapy regimens have been utilized, including vincristine during radiation-therapy and drugs such as vincristine, cisplatin, CCNU (lomustine), and cyclophosphamide following radiation (A. Gajjar et al. 2006; R. J. Packer et al. 2003). Randomized studies are presently attempting to determine if the dose of craniospinal radiation-therapy can be further reduced. With the current treatments, children with an average-risk disease have an 80% to 90% likelihood of 5-year disease control, the majority of patients being cured of their tumor (A. Gajjar et al. 2006; R. J. Packer et al. 2003). However, because of the tumor development, the surgery, the use of craniospinal and tumor-bed radiation-therapy and chemotherapy, survivors are at risk to develop significant long-term sequelae, including neurocognitive damage, cerebrovascular complications, endocrinological deficiencies or secondary brain tumors (A. O. Von Bueren et al. 2011; D. N. Louis et al. 2016; M. D. Ris et al. 2001).

For children with high-risks medulloblastoma, the therapeutic management is less settled. Higher doses of craniospinal radiation-therapy are usually delivered (36-40 Gy), and more aggressive chemotherapy is given during and following radiation. However, even within this poorer-risk group of patients, 50% to 60% of children can be expected to be alive and free of disease 5 years after treatment, again with many cured of their disease (A. Gajjar et al. 2006; R. J. Komotar et al. 2009). Because higher doses of radiation-therapy are delivered, children are at even higher risks of long-term sequelae development.

1.1.3. The use of proton-therapy in pediatric patients

Considering the long-term sequelae induced by the conventional post-operative X-rays irradiation, the use of proton-therapy has been seriously considered due to its particular in-depth penetration, allowing the reduction of the dose delivered to the normal tissue. Initial dosimetry studies showed a

reduced dose-delivery to the normal brain (R. Miralbell et al. 1997), suggesting a potential benefit in terms of second cancer development and cognitive function (T. E. Merchant et al. 2008; X. Mu et al. 2005; R. Zhang et al. 2014).

Clinical data comparing the secondary malignancy incidence in long-term survivors following proton and photon therapy are limited given the significant follow-up time necessary to effectively evaluate radiation-induced malignancy occurrence. Nevertheless, follow-up studies of medulloblastoma patients treated with proton-therapy tend to show a very low incidence of secondary malignancies compared to conventional X-rays radiation-therapy (C. S. Chung et al. 2013; B. R. Eaton et al. 2015; T. I. Yock et al. 2016).

Early-toxicity studies have shown that proton-therapy induced low toxicity rates compared to photon radiation-therapy (B. J. Moeller et al. 2011), suggesting a better long-term toxicity outcome and a better patient's quality of life (QOL) (K. A. Kuhlthau et al. 2012). The evaluation of early cognitive toxicity after proton-therapy showed no significant alteration of the intellectual quotient (IQ), verbal comprehension, perceptual reasoning, or working memory, 2.5 years post-irradiation, suggesting encouraging outcomes compared to photon radiation-therapy (M. B. Pulsifer et al. 2015). Moreover, no IQ decline was observed in children treated with proton-therapy up to 7 years post-irradiation, when X-rays RT induced a lower mean IQ (12.5 points lower) and a decline of 1.57 points per year (L. S. Kahalley et al. 2016).

Even if more long-term follow-up studies are needed to fully conclude on the advantages of proton-therapy *versus* conventional X-rays RT, these results tend to show a protection of the normal tissue by enhancing the dose delivery geometry. The recent development of proton-therapy for the treatment of pediatric brain tumors shows how a technological advance in radiation-therapy techniques can increase the therapeutic index by decreasing the deleterious effects on the normal tissue.

1.2. Glioblastoma multiforme: the most common and aggressive adult CNS tumor

Each year, 5-6 out of 100'000 adult people are diagnosed with a primary malignant brain tumor, with a vast predominance of gliomas (J. A. Schwartzbaum et al. 2006). Gliomas are defined as a type of brain tumors developed from neoplastic glial cells (M. L. Goodenberger et al. 2012). The World Health Organization (WHO) classifies gliomas in grades, mostly depending on certain pathological features, such as nuclear atypia, mitotic activity, vascular proliferation, necrosis, proliferative potential, and features of clinical course and treatment outcome. Malignant gliomas are subcategorized into grade III tumors (anaplastic astrocytomas, oligodendrogliomas or ependymomas) and grade IV (out of IV) tumors: glioblastoma multiforme (D. N. Louis et al. 2016). Given to their relatively low incidence, some glioma sub-types cause disproportionate mortality and morbidity compared to other tumor types like breast or

prostate cancers (Q. T. Ostrom et al. 2015). This is particularly the case for the glioblastoma multiforme (GBM).

1.2.1. General features of glioblastoma multiforme

With an incidence around 3 out of 100'000 people, GBM is the most common primary brain tumor in adults: 80% of all primary CNS malignant tumors and 55% of the gliomas. The mean diagnosis age is 64 years and it is 1.5 more common in men than in women with a predominance in the population of Caucasian origin (Q. T. Ostrom et al. 2015). As for many other tumor types, its incidence has slightly increased within the last decades, mostly due to an improvement of the radiologic diagnosis and an increase in life expectancy (J. L. Fisher et al. 2007). As for other brain tumors, GBM patients present with headaches, neurologic and cognitive deficits or seizures. Diagnosis and follow-up are usually realized by magnetic resonance imaging (MRI), and can be reinforced by the use of functional MRI and positron-emission tomography (PET) (P. Y. Wen et al. 2006).

The etiology of GBM identifies several risk factors that lead to its development. Environmental risk factors include a former exposition to ionizing radiations or toxic substances such as vinyl chloride, petroleum-associated materials, pesticides and smoking (M. Wrensch et al. 2002). Other controversial environmental factors like formaldehyde, residential electromagnetic fields, diagnostic radiation exposure and cell-phone use are still under investigation. Concerning genetic alterations, EGRF amplifications and mutations, IDH1 mutations, amplifications of CDK4 and MDM2 oncogenes, and mutations or deletions of TP53, RB or PTEN cancer suppressor genes have been documented (M. Wrensch et al. 2002). Nevertheless, no risk factor or particular genetic alteration has been found to be accounting for a large percentage of GBM.

The pathogenesis and the different available and experimental GBM treatments are reviewed in the following paragraphs.

1.2.2. Glioblastoma multiforme pathogenesis

The large majority of the GBM patients are associated with a sporadic tumor development, whether in only 5% of them, hereditary syndromes such as neurofibromatosis can be identified (C. J. Farrell et al. 2007). The important aspect of GBM pathogenesis and malignant transformation is the accumulation of genetic alterations and an abnormal upregulation of cell-cycle-associated genes and pathways. The aberrant cell-proliferation has been identified as mediated by several growth factors such as: Vascular Endothelial Growth Factor (VEGF), Epidermal Growth Factor (EGF), Platelet-derived Growth Factor (PDGF) or Hepatocyte Growth Factor (HGF). Moreover, alterations in cell-cycle pathways, including Phosphatase and Tensin homolog (PTEN) and PI3K/AKT, have been described (P. Y. Wen et al. 2008). When low-grade

gliomas with these genetic features recur, a higher histologic grade is most frequently described, supporting a tumor progression rather than a new primary tumor development (D. N. Louis 2006). For example, low-grade gliomas are often associated with p53 mutations and PDGF-α and PDGFR overexpression, while their transitions to higher grades and GBM is characterized by RB, p16 and 19q tumor-suppressor genes disruption (D. N. Louis 2006).

GBM is classically subcategorized into primary (95%) and secondary (5%) GBM, depending on whether it originates from a *de-novo* tumorigenesis or from the recurrence of a former low-grade glioma. Interestingly, even if the carcinogenesis process might be different, the same molecular pathways are often affected and the patient's response to the treatments are similar (P. Kleihues et al. 1999). Based on the growing understanding of the molecular heterogeneity in GBM, the Cancer Genome Atlas (TCGA) has divided GBM in molecular subclasses based on genetic alterations and expression profiles especially of PDGFRA, EGFR, Neurofibromin (NF1) and Isocitrate Dehydrogenase (IDH1) genes.

In order to better understand the GBM tumors' biology and to provide efficient treatments, the research on GBM has led to the identification of target cell-types. The recent discovery of adult stem cells in the CNS, able of self-renewal, proliferation and differentiation, suggested the hypothesis of the existence of tumor initiating cells (TICs) also called glioma stem-like cells (GSCs) (S. Facchino et al. 2011). These so called "stem-like" cells have been shown to express the controversial CD133 surface marker associated with a cell resistance to radiation-therapy and chemotherapy (P. Brescia et al. 2013; D. V. Brown et al. 2017; S. K. Singh et al. 2003). Nevertheless, other studies have shown that $CD133^-$ cells also have stem-like properties and can participate to the tumor initiation (E. Irollo et al. 2013). Moreover, GSCs have the potential promote tumoral angiogenesis *via* the expression of VEGF and pericyte differentiation, thus enhancing the tumor environment and the tumor growth (L. Cheng et al. 2013). Interestingly, mutations in the adult neural stem cells (NSCs) have been recently observed (H. Koso et al. 2012). The transmission of these mutations to downstream glia precursors might partially initiate the GBM tumorigenesis, acting *via* the deregulation of the cell-growth signaling and potentially Sonic Hedgehog (SHH) pathway that participates to the NSCs renewal and is thought to be involved in the treatment resistance of GBM (V. Clement et al. 2007). Directly targeting the GSCs *via* the modulation of oncogenes and cancer suppressor genes expression might then be a potential treatment strategy to overcome GBM treatment resistance.

1.2.3. Current treatments of glioblastoma multiforme and perspectives

The current standard treatment for GBM consists in combining surgery, radiation-therapy and chemotherapy. The highly infiltrative features of GBM make the complete tumor resection almost impossible and a combined radio-chemotherapy is essential to provide a good tumor control.

1.2.3.1. Radiation-therapy and concomitant temozolomide

Temozolomide (TMZ) is an alkylating agent that pass the blood brain barrier (BBB) with an excellent bioavailability, around 95%. The TMZ toxicity on cells is mediated *via* the DNA alkylation / methylation of the DNA, mainly on the O^6 position of the guanine, and to a lesser extent on N^7 guanine and N^3 adenine. It is usually administrated at a daily dose of 75mg.m^{-2} concomitantly to 6 weeks of daily fractionated radiation-therapy, usually consisting in 30 times 2 Gy to the surgical cavity. An adjuvant TMZ treatment is given within 6 cycles post-RT with a maintenance dose of 150mg.m^{-2} during the first week, and if well tolerated, scaled up to 200 mg.m^{-2}.day^{-1} for 5 days in 28 days cycle (O. L. Chinot et al. 2001; J. L. Villano et al. 2009). Concomitant TMZ and RT result in a median survival of 14.6 months post-diagnosis and a two-year survival rate of 26.5% compared to respectively 12.1 months and 10.4% with RT alone. Patients with O^6-methylguanine-DNA-methyltransferase (MGMT) methylated promoter exhibit a better prognosis with an overall survival rate at 2 years of 46% *versus* 14% for patients with non-methylated MGMT promoter (M. E. Hegi et al. 2005; Roger Stupp et al. 2005, 2009).

1.2.3.2. Resistance of glioblastoma multiforme to radiation and chemotherapy

GBM tumors often exhibit an intrinsic or acquired resistance to chemotherapy. The silencing of MGMT gene expression *via* the methylation of its promoter contributes to the TMZ sensitivity by inhibiting the O^6 guanine alkylation repair (M. Esteller et al. 2000; M. E. Hegi et al. 2005). Absence of MGMT methylation has been associated with a lower median survival. A possibility to overcome this resistance is to increase the doses of TMZ in order to enhance the DNA alkylation and/or administrate O^6-benzylguanine to saturate the repair mechanisms (J. L. Clarke et al. 2009; J. A. Quinn et al. 2009). Poly(ADP-ribose) polymerase-1 (PARP-1)-associated resistance has also been described. Its role in N^7 and N^3-purine base excision repair counteracts the TMZ toxicities, particularly in the cases when O^6-methylguanine adducts are repaired. PARP-1 inhibitors have shown promising results in combination to TMZ (P. Y. Wen et al. 2008; J. Zhang et al. 2012).

Several preclinical studies have proven that GSCs highly contribute to GBM resistance to chemotherapy and radiation-therapy. Expression of multidrug resistance protein MDRP-1 and P-glycoprotein (Pgp) was observed in gliomas. Interestingly, MDRP-1 expression was found in a higher percentage grade IV gliomas compared to the Pgp that has been associated to lower-grade gliomas (G. P. De Faria et al. 2008). EGFR and EGFRvIII expression increase has also been identified and might explain the high resistance of GBM to cancer treatments by an exacerbated GSCs proliferation (J. L. Munoz et al. 2014).

These treatment resistance mechanisms show the importance of the development of new therapeutical strategies to provide a better tumor control and prognosis.

1.2.3.3. New therapeutic targets to treat glioblastoma multiforme

The increasing investigations led on GBM genetic features have promoted a rational development of new targeted therapies. Many studies have investigated the administration of tyrosine-kinase receptors antagonists such as EGF-R, PDGF-R, VEGF-R or IGF-R inhibitors. Unfortunately, neither monotherapy nor combined therapy has reported a highly enhanced clinical benefit (S. Sathornsumetee et al. 2007; T. Wilson et al. 2014). Other studies, have and are currently investigating potential other molecular targets (c-MET, mTOR, PI3K, SHH, HDAC, Hsp, JAK/STAT…) to develop new GBM targeted treatments, but none of them has shown promising results yet. The potential therapeutic benefits of monoclonal antibodies (mAb) against EGF-R, VEGF-R, PDFG-R, have also been widely investigated. The VEGFR-targeting mAb Bevacizumab, which has shown interesting benefits in other cancer types, has been administered concomitantly to TMZ and RT in a phase III clinical trial. Again, no improvement in the overall survival (OS) has been observed (15.7 vs 16.1 months), and even if a slight improvement in progression-free survival (PFS) was described (10.7 vs. 7.3 months), Bevacizumab was associated with a decrease in patient's QOL (M. R. Gilbert et al. 2014). The uncountable number of preclinical and clinical studies that have or are currently investigating potential interest in inhibiting molecular pathways in the case of GBM without outstanding results, highlights the medical urgent need to develop new treatment strategies.

1.2.4. Preclinical glioblastoma multiforme murine models

The need to develop new treatments for the therapeutic management of GBM in clinics is translated by an important research on the set-up of preclinical GBM models. While *in vitro* cultured GBM cell lines can be useful for drug-screening or pilot studies, the translation of new treatments to the clinics requires the use of animal models. GBM animal models include non-mammalian animals such as drosophila and zebrafishes (R. D. Read 2011; M. Vittori et al. 2015), murine experimental models (E. Laurenti et al. 2009) and pet dogs involved in veterinary clinical trials (J. Hicks et al. 2017). This paragraph focuses on the murine models, which are the most common GBM animal models.

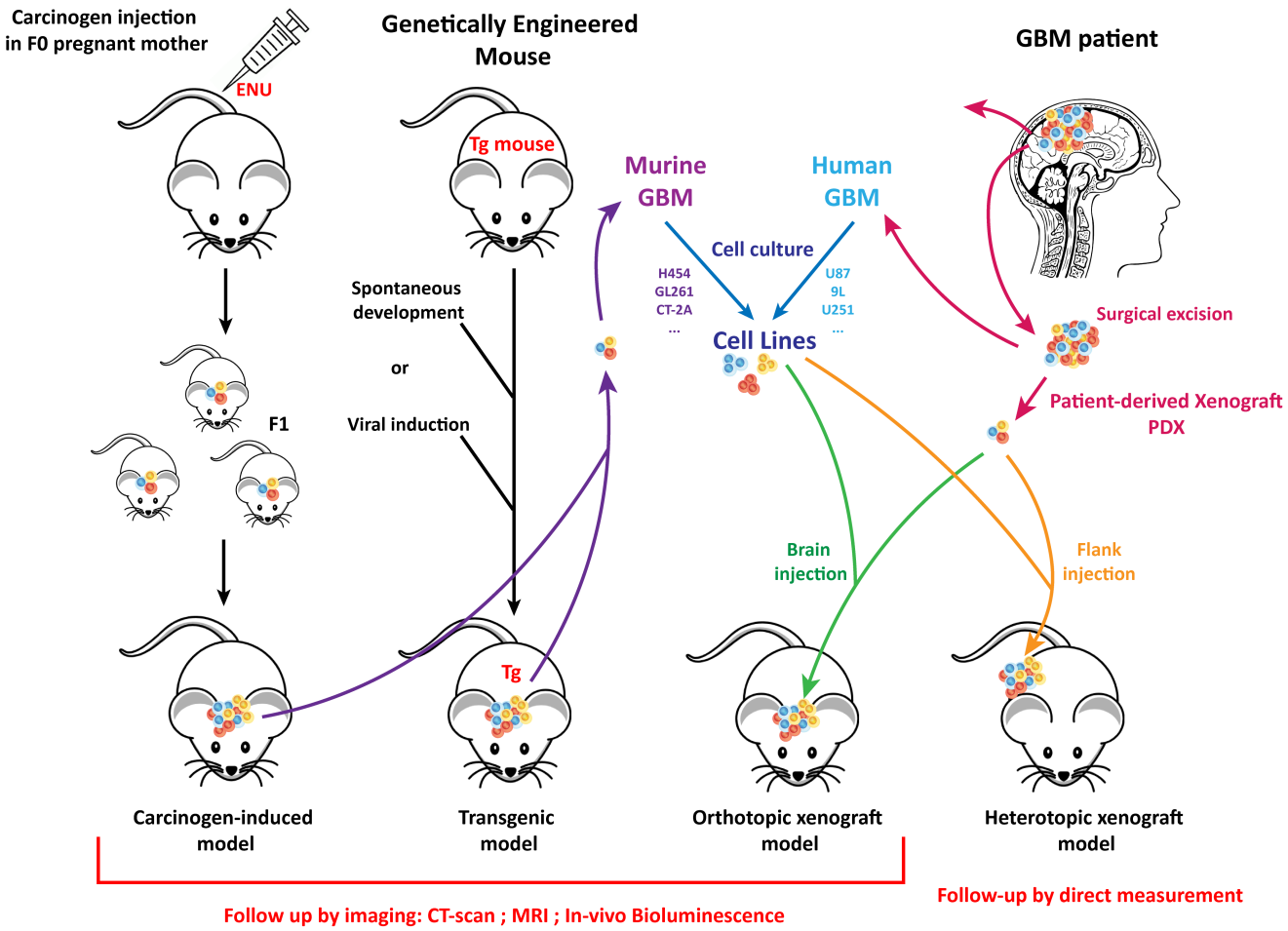

Figure 1: Preclinical models of glioblastoma multiforme. None of this model can faithfully reproduce the spontaneous human carcinogenesis, but the use of multiple models in a study allows to be as closer as possible to the GBM pathogenesis and treatment response.

Ideally, a preclinical glioma model has to meet different requirements: 1) its genetic background must be as close as possible to the human GBM (taking in consideration the different subsets of human GBMs); 2) it has to reproduce the tumoral heterogeneity in its genetic and phenotypic features; 3) it has to provide an adequate microenvironment including cell-cell interactions, vascular compartment, immune system; 4) it needs to be reproducible within the different experiments and can be followed by the experimenter. The different preclinical glioma models can be subdivided into separate groups (Figure 1): carcinogen induced murine model, genetical engineered murine model, orthotopic and heterotopic xenografted murine models.

The relevance of each model regarding the requirements listed above is summarized in Table 2. In an ideal preclinical study, the investigation of different models is necessary to answer the hypotheses.

Table 2: Relevance of the different preclinical murine glioblastoma models regarding the requirements needed to mimic the human GBM pathogenesis. Adapted from Lenting *et al.* (K. Lenting et al. 2017). BBB: Blood Brain Barrier; PDX: Patient's Derived Xenograft.

MODEL	(Epi)GENETIC BACKGROUND	HETEROGENEITY	IMMUNE SYSTEM	MICRO ENVIRONMENT	BBB	STABILITY FOLLOW-UP
ENU induced model	Partly relevant (p53, bRAF, PDGFR) No IDH mutation.	Heterogeneous. Different cell-types initiate the tumor.	Yes	Relevant	Yes	No Imaging (CT; MRI; IVIS)
GEM Transgenic mice	Depends on the model. p53; KRas; IDH1…	Homogeneous. Initiation depends on the promoter	Yes	Relevant	Yes	Yes Imaging (CT; MRI; IVIS)
PDX Subcut.	Partly relevant	Intra-tumoral heterogeneity	No	Non-relevant	No	Yes Direct measurement
PDX Orthotopic	Relevant	Intra-tumoral heterogeneity	No	Relevant	Yes, partly	Yes Imaging (CT; MRI; IVIS)
Cell lines neuro-spheres Orthotopic	Possibly relevant	Homogeneous	Yes/no depends on the model	Relevant	Yes, partly	Yes Imaging (CT; MRI; IVIS)
Cell lines monolayer Subcut.	Less relevant	Homogeneous	Yes/no depends on the model	Non-relevant	No	Yes Direct measurement

1.3. Brain metastases

Brain metastases (BM) are the most common intracranial malignancies in adults and display a significant cause of morbidity and mortality in cancer patients. The incidence of BM is difficult to assess due to the high heterogeneity in methodology. Nevertheless, in the US, an estimated range of 150'000 to 200'000 cases per-year has been reported, representing 25-30% of all the cancer patients (B. D. Fox et al. 2011). The frequency of diagnosis appears to increase over time due to longer survival resulting from more effective systemic treatment, the aging population and improved imaging techniques. Most BM originate from lung, breast cancer or melanoma, but renal cell carcinoma, colon cancer, and gynecologic malignancies also make up a significant fraction. While lung cancer accounts for the majority of brain metastases, melanoma has the highest propensity to disseminate to the brain; 50% of patients with

advanced melanoma eventually develop metastatic brain disease (I. T. Gavrilovic et al. 2005; J. H. Sampson et al. 1998). Interestingly, the localization of BM in the brain reflects the cerebral blood flow and volume. Approximately 80% of metastases occur in the cerebral hemispheres, 15% in the cerebellum, and 5% in the brainstem (J. Y. Delattre et al. 1988).

1.3.1. Physiopathology, clinical features and prognostic factors of brain metastases

Metastatic spread involves a series of sequential steps, beginning with the escape of cancer cells from the primary tumor site, followed by invasion of surrounding tissue into the bloodstream or lymphatics, and finally the extravasation, survival, and proliferation in a secondary site. It is believed that only a subpopulation of cells has the required genetic and epigenetic alterations necessary to escape, to invade and to disseminate (R. Bernards et al. 2002; C. L. Chaffer et al. 2011)

Several theories have been postulated to explain the propensity of tumor types to disseminate to specific organs, including the brain. The classic “seed and soil” hypothesis, originated by Paget in 1889, states that a tumor’s organ-specific spread is dependent on pro-tumorigenic interactions between the tumor cell and its microenvironment (S. Paget et al. 2006). Alternatively, the pathologist Ewing believed that circulatory patterns between the primary tumor and targeted secondary organs were sufficient to explain the specific pattern of metastatic spread (E. D. Munz 2017). Today, attention has been directed to the role of cancer stem cells as the “seed” and the effects of specific stromal components, or the “soil,” on malignant progression and resistance to therapy (R. R. Langley et al. 2011).

Brain metastases are typically associated with a poor prognosis: most studies show a median survival of less than 6 months. Important prognostic factors include age, functional status (designated by Karnofsky Performance Scale - KPS), number of brain metastases, primary tumor type, extent of active systemic disease and the time elapsed between the primary cancer diagnosis and the development of the brain disease. The number of brain metastases appears to be important in the survival; across multiple studies, patients with single metastases consistently survive longer than those with multiple metastases, even after adjusting for other prognostic factors (J. Lutterbach et al. 2002). Retrospective data analyzing the molecular alterations in primary sites suggest that ERBB2-positive breast cancer patients or those with NSCLC caused by mutations in EGFR, survive longer than comparable patients with wild-type tumors (April F. Eichler et al. 2008, 2010).

1.3.2. Treatment of brain metastases

The therapeutic management of brain metastases has improved over the past decades with advances in surgical and radiation techniques, a better understanding of the underlying biology, and an awareness of prognostic factors leading to better patient selection for invasive treatments. Surgical resection of brain

metastases is a treatment option considered primarily in patients with single large tumors. Although uncommon *prior* to the 1980s, the resection of brain metastases has now become a standard treatment option in patients with surgically accessible single lesions, good performance status, and controlled or absent extracranial disease.

1.3.2.1. Whole-Brain radiation-therapy (WBRT)

Traditionally, radiation-therapy has constituted the backbone of treatment for brain metastases. WBRT is administered to address both visible disease and presumed micro depositions of tumor cells in the brain. A total dose of 30 Gy to 40 Gy is typically delivered to the patient in daily fractions of 2 Gy to 3 Gy. Increase of either total dose or daily fraction dose raises the risk of neurotoxicity. WBRT can be given alone or as adjunctive therapy, although monotherapy is usually reserved for patients with multiple brain metastases not amenable to surgery, poor performance status, or active systemic disease.

Nonrandomized studies have found that WBRT impacts survival only modestly, increasing median survival from 1 to 2 months to 3 to 6 months (D. Khuntia et al. 2006). About 60% of patients experience a complete response or partial response on follow-up imaging. Tumor histology also plays a role in the effectiveness of WBRT: some tumors, such as small cell lung cancer, breast cancer, and germ cell tumors, tend to be highly sensitive to fractionated radiation, whereas melanoma, sarcoma, and renal cell carcinoma are relatively unresponsive.

One of the greatest concerns about WBRT is the risk of late neurocognitive effects, which can range from mild cognitive impairment to overt dementia. Thus, the current practice is to limit daily WBRT fractions to 3 Gy or less. Even with these schedules, however, it is clear that some patients develop significant problems with short-term memory and cognition that cannot be explained by tumor progression or other insults and that negatively impact their quality of life. The second part of this introduction further investigates this point.

1.3.2.2. Stereotactic radiosurgery (SRS)

Because of the concerns of late neurotoxicity associated with WBRT, attention has been directed toward more focal treatments, including stereotactic radiosurgery (SRS). SRS uses multiple convergent beams to deliver a single high dose of radiation to a discrete target volume. There are a variety of devices that can be used, including Gamma Knife (Elekta AB, Stockholm, Sweden), Cyberknife (Accuray, Sunnyvale, CA, USA), gantry–based linear accelerator (LINAC) systems (Novalis TX, BrainLab) and less commonly proton beam-based systems. All systems have a rapid fall-off dose at the margin of the tumor volume, resulting in delivery of a very low radiation dose to the surrounding normal tissue (L. Halasz et al. 2013). Because brain metastases are distinct lesions with discrete pathologic and radiographic margins, they are

attractive targets for SRS that displays the advantage to treat locations that are surgically inaccessible, such as the brainstem or basal ganglia. Local tumor control with SRS ranges from 70% to 90% at 1 year in various studies and tends to be higher when combined with WBRT (L. J. Hazard et al. 2005). In the RTOG 95-08.28 study, 333 patients with one to three brain metastases were randomized to receive either WBRT with SRS to all tumors or WBRT alone. Overall, no significant difference was seen in median overall survival. However, in patients with a single metastasis, median survival was increased in the WBRT plus SRS group. Patients with one to three lesions experienced superior local control.

1.3.2.3. Chemotherapy

Several factors have traditionally limited the role of chemotherapy in the treatment of brain metastases, notably because of the blood brain barrier presence. Another limitation regarding the efficacy of chemotherapy for brain metastases is the exposition of the patients to a primary line of chemotherapy, that can induce the selection of resistant clones, responsible for tumor dissemination. The few chemotherapy treatments tested or used to target BM are summarized in Table 3.

For non-small cell lung-cancer (NSCLC) patients, a variety of agents with activity against NSCLC have been studied for patients with brain metastases, and response rates tend to be higher in patients who have not received prior systemic chemotherapy (A. F. Eichler et al. 2007).

Breast cancer is generally more chemo-sensitive than NSCLC, and consequently many of the traditional drugs with activity in breast cancers and reasonable BBB penetration have utility in patients with brain metastases.

Metastatic melanoma typically has a poor response rate to systemic chemotherapy given both intracranially and extracranially. Only a few trials have evaluated the role of chemotherapy in melanoma patients with brain metastases, and most have focused on fotemustine and TMZ.

Table 3: Chemotherapy for the treatment of BM. CTL-4: Cytotoxic T-lymphocyte associated antigen 4; CycloP: cyclophosphamide; EGFR: Epithelial Growth Factor Receptor; ERBB2: Erythroblastosis oncogene B2; mAb: monoclonal antibody; metho: methotrexate; pred.: prednisone; vinc: vincristine; 5-FU: 5-fluorouracile.

BM ORIGIN	DRUG	EFFECTS	REFERENCES
Non-small-cell lung cancer (NSCLC)	Temozolomide	No effect (because no effect on the NSCLC?)	(R. . Dziadziuszko et al. 2003)
	EGFR inhibitors	Better response in EGFR mutated patients	(G. L. Ceresoli 2004)
Breast tumors	cycloP/5-FU/pred.; 5FU/pred./metho./vinc.; 5-FU/metho.	Better response rates (42 to 59%)	(W. Boogerd et al. 1992; D. Rosner et al. 1986)
	Trastuzumab (anti ERBB2 mAb)	Poor effect on the BM due to BBB penetration Increase the BM occurrence (increase local tumor control and survival)	(H. J. Burstein et al. 2005)
	Lapatinib (EGFR & ERBB2 inhibitor)	Phase II on Trastuzumab + RT patients Increased response rate	(N. U. Lin et al. 2009, 2011)
Melanoma	Fotemustine	Modest activity when combined to WBRT	(M. F. Avril et al. 2004; F. Mornex et al. 2003)
	Temozolomide	Increase in the objective response rate Stable disease in 26% of the patients	(M. Hofmann et al. 2006)
	Ipilimumab (anti CTL-4 mAb)	Objective response Durable disease control	(G. Feldmann et al. 2013; F. S. Hodi et al. 2008)

2. Effects of radiations on the normal brain tissue

Radiation-induced brain injuries have been largely described after cranial exposure to ionizing radiations. *Via* their direct and indirect toxicities on the brain cells, ionizing radiations induce an alteration of the brain homeostasis by molecular and cellular modifications that lead to functional alterations (Figure 3). Since no single cell-type can explain the response of an organ to a particular stress, the radiation-induced brain toxicities are nowadays considered to occur in a dynamic environment made out of multiple brain cell-types (P. J. Tofilon et al. 2016).

2.1. Effects of radiations on the glial cells

Glial cells include four different cell-types: astrocytes, microglia, oligodendrocytes and ependymal cells. They highly contribute to the brain's homeostasis and stress response by their numerous roles including physical support, nutrients and oxygen supply, or immune functions (S. Jäkel et al. 2017).

2.1.1. Oligodendrocytes

Oligodendrocytes are responsible for the formation of the myelin sheet in the brain. Their role in the CNS initially focused the research on the radiation-induced brain toxicity due to the major contribution of myelin to the neurotransmission. Acute decrease in oligodendrocytes type-2 astrocytes (O-2A) population and loss of their proliferation abilities have been described as soon as 24h post WBI above 3 Gy in murine models (M. Bellinzona et al. 1996; H. Kurita et al. 2001; M. C. Raff et al. 1983; C. Shinohara et al. 1997). The radiation-induced loss of O-2A cells results in an acute brain demyelination that cannot be counterbalanced by the surviving fraction of oligodendrocytes (D. M. Chari et al. 2003). Interestingly, the acute decrease in the number of oligodendrocytes has been attributed to a p53 dependent radiation-induced apoptosis in specific regions of the brain such as the sub-ventricular zone (SVZ) and the sub-granular zone of the hippocampus dentate gyrus (SGZ) that both support neurogenesis (B. M. Chow et al. 2000; H. Kurita et al. 2001; K. Sano et al. 1997; R. Sasaki et al. 2000). Nevertheless, no decrease in oligodendrocytes number nor in myelin distribution has been observed one year post-fractionated irradiation in rats with a total delivered dose of 45 Gy, despite an important decline in cognitive functions (L. Shi et al. 2009). Even if these results tend to attribute to oligodendrocytes an acute role in the brain radiation-induced toxicity, their involvement in long-term changes remains unclear.

2.1.2. Astrocytes

Astrocytes represent about 50% of the total glial cell population and play diverse functions including synaptic transmission modulation, nutrient supply and secretion of growth factors. Their implication in

the blood brain barrier by tight interactions with endothelial cells and neurons triggers a protective role against oxidative or physical stresses. In the context of a brain injury, morphological changes are observed along with an increase in proliferation and expression of Glial Fibrillary Acidic Protein (GFAP). These modifications known as reactive astrogliosis have been described after brain exposure to ionizing radiations. Increase in GFAP expression has been observed acutely as soon as 24 hours in the irradiated brain, but also chronically up to 180 days post whole brain irradiation (WBI) above 7 Gy (C. S. Chiang et al. 1993; J. H. Hong et al. 1995). Moreover, these reactive astrocytes secrete pro-inflammatory molecules such as COX-2 or ICAM-1 that may induce neuroinflammation and peripheral immune cell recruitment. Even if their exact role in the radiation-induced brain pathogenesis is mostly unknown, the interactions of astrocytes with microglial and endothelial cells surely contribute to the brain's response to ionizing radiations.

2.1.3. Microglia

Microglia cells account for 10 to 15% of the total brain cells (P. J. Gebicke-Haerter 2001; L. J. Lawson et al. 1992) and are described as the brain's innate immune cells due to their role in monitoring the brain environment and their high similarity to the macrophages upon activation (G. Stollg et al. 1999). Microglia activation following injury consists in a loss of their processes and an increase in proliferation, production of reactive oxygen species (ROS), and release of cytokines and chemokines that mediate the neuroinflammation (P. J. Gebicke-Haerter 2001; S. Y. Hwang et al. 2006; W. H. Lee et al. 2010; G. Stollg et al. 1999). Even though their phagocytosis role might be important for dead cells and debris clearance following irradiation (R. Fu et al. 2014), microglial activation has been identified as a major contributor to chronic neuroinflammation. *In-vitro* experiments on microglia cultures showed a radiation-induced production of pro-inflammatory molecules such as TNFα, IL-1β, IL-6, IL-18, INFγ, MCP-1 or Cox-2. Interestingly, an increase in the expression of these factors has been described in the murine brain following whole brain irradiation with a dose of 10 Gy (C. S. Chiang et al. 1993; S. Y. Hwang et al. 2006; S. Kyrkanides et al. 1999; W. H. Lee et al. 2010), along with an augmentation of microglial activation characterized by an increase in CD11b, CD68 and Iba1 expression (C. S. Chiang et al. 1997; R. Ladeby et al. 2005; M. Mildenberger et al. 1990; M. L. Monje et al. 2003). Moreover, radiation-induced microglial activation has been associated with a chronic neuroinflammation. Indeed, elevated expression of TNFα was measured in the mouse brain up to 6 months post-irradiation (J. H. Hong et al. 1995), along with an elevation of the CCR2 receptor expression level 9 months post-exposure (R. Rola et al. 2005) and a persistent microglial activation (K. R. Conner et al. 2010; S. Ramanan et al. 2008; M. K. Schindler et al. 2008). This chronic neuroinflammation has been linked to neurogenesis deficits and loss of cognitive functions in rodent models (M. L. Monje et al. 2002, 2007; R. Rola et al. 2004).

2.2. Effects of radiations on the brain's vascular compartment

The brain's vasculature differs from the systemic network by the presence of tight junctions between endothelial cells, which restricts the flow of molecules from the blood to the brain compartment. This so called "Blood Brain Barrier" (BBB) makes the brain a particularly isolated organ and allows the precise control of homeostasis to ensure a proper cerebral function (R. Daneman et al. 2015). The structure of the BBB also depends on the presence of astrocytic processes or "feet" that ensure a physical and biochemical support to endothelial cells (N. J. Abbott et al. 2006). Many studies have focused on the impact of irradiation on the endothelial cells and on the BBB integrity since breakages in this physical protection can lead to major brain dysfunctions.

Long-term blood vessel dilatation, blood vessel wall thickening and endothelial cell nuclear enlargement have been extensively described following whole brain irradiation in rats at doses ranging from 17-25 Gy (W. Calvo et al. 1988; H. S. Reinhold et al. 1990). Moreover, these results correspond to the description of a dose-dependent decrease in CD31+ endothelial cells along with a loss of brain blood vessel density and length (W. R. Brown et al. 2005; H. S. Reinhold et al. 1990). Ionizing radiation exposure induces ceramide-mediated endothelial cells apoptosis within 24h post-irradiation *via* the activation of the acid sphingomyelinase (Y.-Q. Li et al. 2004; Y. Q. Li et al. 2003; L. A. Peña et al. 2000; P. Santana et al. 1996). The radiation-induced blood brain barrier disruption and the underlying mechanisms have also been investigated. The use of permeability tracers such as FITC-dextran, albumin or diethylenetriaminepentaacetic acid (DTPA), allowed the observation of a radiation-induced BBB disruption as soon as 24 hours post-irradiation with single-doses ranging from 2 to 50 Gy. These alterations have been described as long-lasting and associated with white matter necrosis (D. d'Avella et al. 1998; T. E. Schultheiss et al. 1992; H. Yuan et al. 2006). Alteration in the metalloproteases MMP-2, MMP-9 and TIMP-1 expressions balance, along with a degradation of the type IV collagen in the BBB extracellular matrix have been described 24 hours post single-dose and fractionated whole brain irradiation (W. H. Lee et al. 2012). These modifications in the extracellular compartment are associated to a significant decrease in VEGF, Ang-1 and Tie-2 expression and an increase in Ang-2 mRNAs amount (W. H. Lee et al. 2011). An increase in VEGF expression by the astrocytes after the delivery of 8 to 22 Gy to the rat's brain has also been linked to an increase in vascular permeability (M. N. Tsao et al. 1999). In the specific region of the hippocampus, a rarefaction of blood capillaries along with tissue hypoxia has been identified months after the delivery of 36 Gy in a fractionated regimen (J. P. Warrington et al. 2011, 2012). Altogether, the loss of endothelial cells, decrease in vessel density, increase in the vessels permeability, loss of endothelial cells tight junctions and destruction of the extracellular matrix have been associated to breakages in the BBB accompanied by an increase in leukocyte adhesion (T. E. Schultheiss et al. 1992; H. Yuan et al. 2003). This peripheral immune cell infiltration participates to the establishment

of a radiation-induced chronic neuroinflammation along with the activation of the microglia, and thus increases the response of the other brain cell-types in a downward circle.

2.3. Effects of radiations on neurons and neurogenesis

2.3.1. Neurons

Due to their former classification as post-mitotic cells, neurons have been long considered as a radiation-resistant cell population. Nevertheless, an important number of studies have described molecular, morphological and functional radiation-induced alterations in neurons and in their progenitors. In 1960, experiments performed on cat's brain identified electrical activity changes in the EEG following whole brain irradiations at doses of 20 and 40 Gy (H. Gangloff et al. 1960). Further investigations performed with microelectrodes after a 45 Gy total body irradiation in rabbits showed important disturbances in the hippocampal cellular activity. These modifications were concomitant with a decrease in the neuronal discharge rate and deficits in synaptic efficiency and spike generation (M. H. Bassant et al. 1978). Neuronal gene expression changes have also been identified post-irradiation. A significant reduction in both mRNA and protein levels of Arc (activity-regulated cytoskeleton-associated protein) has been described 2 months post 10 Gy WBI (S. Rosi et al. 2008). Increase in the level of expression of Arc have been related to cognitive stimuli, supporting a critical role in memory consolidation (J. F. Guzowski et al. 2000). Moreover, changes in the expression of receptors involved in neurotransmission have been described. A persistent decrease in the expression of the glutamatergic NDMA receptor was observed up to 180 days post-irradiation in the rat brain (M. Machida et al. 2010). Similarly and in the same model, glutaminergic, glutamatergic and GABAergic transmissions were affected by whole brain irradiation (B. H. Rohde et al. 1979). The increase in NMDA receptor at the cell surface, coupled with an increase in GABA receptors expression induced an inhibition of long term potentiation in the dentate gyrus (P. H. Wu et al. 2012) Moreover, increased expression of PC-2 gene, involved in the processing of neuro-endocrine peptides, has been measured as soon as 6 hours post-irradiation at 10 Gy (F. Noel et al. 1998). Interestingly, these radiation-induced modifications occur without any change in the total number of neurons, or in the number of myelinated axons (L. Shi et al. 2008), suggesting an importance of neuronal network and communication between neurons and other brain cells. Changes in dendritic structure has also been observed in hippocampal neurons 2 months post-WBI, including a decrease in dendritic spines, dendritic length and branches, and a decrease in PSD-95 expression (V. K. Parihar et al. 2015)

2.3.2. Neurogenesis

The large majority of the neurons in the mammalian brain are produced before birth and during childhood, whereas adult *de-novo* neurogenesis has long been controversial. Nevertheless, it is nowadays admitted that the adult mammalian brain contains highly active regions of neurogenesis. Indeed, neural stem cells (NSCs) have been identified in two distinct regions: the subventricular zone (SVZ), and the sub-granular zone (SGZ) of the hippocampus dentate gyrus (DG) (Figure 2) (A. Alvarez-Buylla et al. 2004; F Doetsch et al. 1997; G. Kempermann 2002) and associated with a particular niche environment (Fiona Doetsch 2003). These NCSs, originating from multipotent neuronal precursors, are able to differentiate into new neurons, astrocytes and oligodendrocytes (D. N. Abrous 2005; A. Carleton et al. 2003; P. M. Lledo et al. 2006). Neurogenesis is finely regulated in a specific microenvironment defined by the presence of vascular endothelial cells and astrocytes (Fiona Doetsch 2003; T. D. Palmer et al. 2000; H. Song et al. 2002).

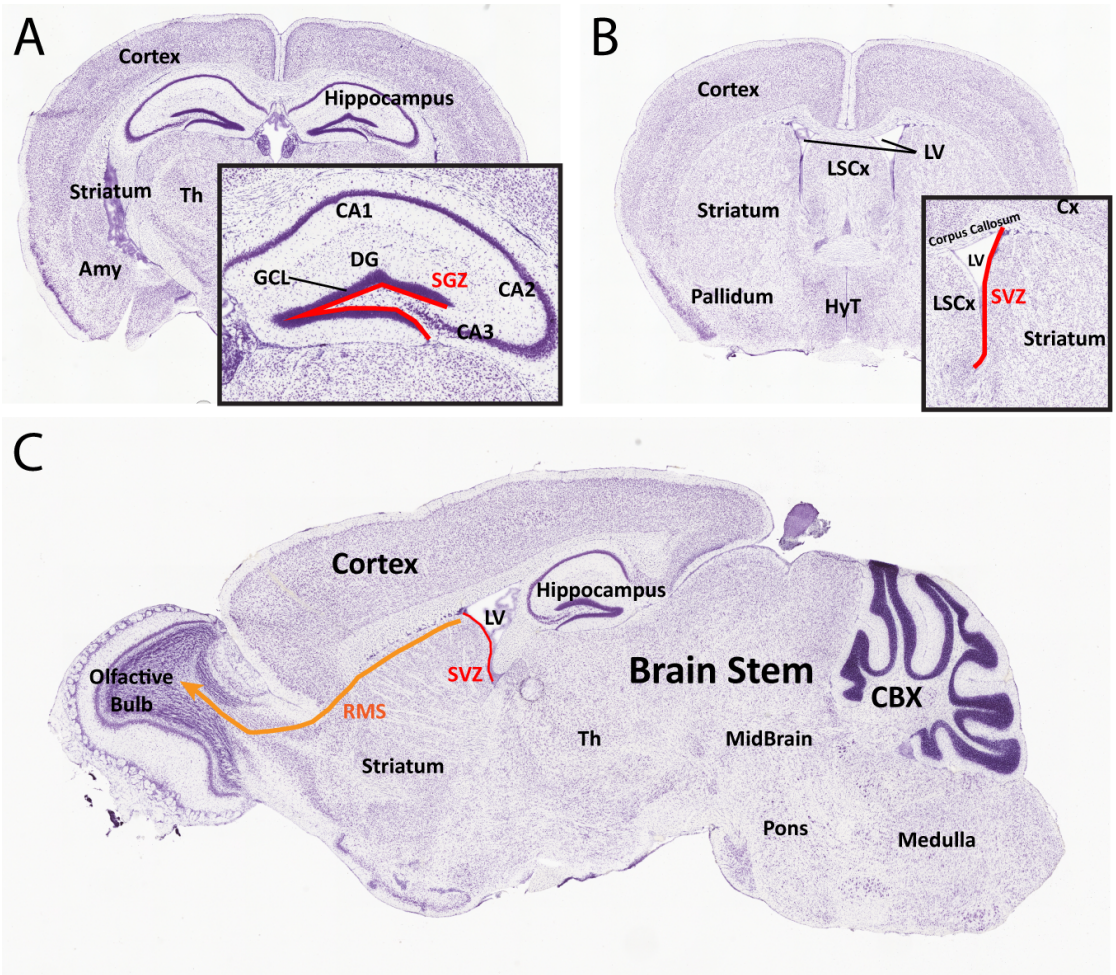

Figure 2: The neurogenesis niches. A: Coronal section of a mouse brain in the region of the hippocampus and zoom on the hippocampus. B: Coronal section of a mouse brain in the region of the lateral ventricles and zoom on a lateral ventricle. C: Sagittal section of a mouse brain.
Amy: amygdala; CA: *cornu ammonis* areas; CBX: cerebellum; Cx: cortex; DG: dentate gyrus; GCL: granule cell layer; HyT: hypothalamus; LSCx: lateral septal complex; LV: lateral ventricles; RMS: rostral migratory stream; SVZ: sub-ventricular zone; SGZ: sub-granular zone; Th: thalamus.

Due to their ongoing activity of cell division and neurogenesis, both SVZ and SGZ regions are sensitive to ionizing radiations. A radiation-induced increase in hippocampal apoptosis evaluated by TUNEL assays and DNA laddering has been described after the delivery of 10-18 Gy to the whole brain of rodents, peaking between 6-9 hours post-irradiation (I. Ferrer et al. 1993; R. Nagai et al. 2000; W. Peißner et al. 1999). Moreover, single and fractionated doses of radiation-therapy delivered to the whole brain have been described as responsible for a decrease in NSCs division and generation of new neurons in the hippocampal DG (M. L. Monje et al. 2002; J. Raber et al. 2004; R. Rola et al. 2004). A dose-dependent decrease in NSCs proliferation and differentiation into neurons have also been observed in the irradiated SVZ of adult rats with doses between 1 and 30 Gy at both acute and late time-points (T. Amano et al. 2002; M. Bellinzona et al. 1996; C. Shinohara et al. 1997). The migration of newly born neurons from the SVZ to the olfactive bulb (OB) through the rostral migratory stream (RMS) (Figure 2) has also been studied post-irradiation. An increase in the number of doublecortin (DCX) labelled cells identified as immature neurons has been found in the SVZ and in the RMS 7 days post-irradiation (S. Balentova et al. 2015). Nevertheless, a decrease in the number of the DCX-labelled cells has been described in the same region of the rat's brain 1, 2 and 3 months post-fractionated irradiation at 3 to 5 Gy (S. Balentova et al. 2014). The acute increase in immature neurons might be explained by a recruitment of quiescent stem cells (qNSCs) observed shortly after brain irradiation (S. Mizumatsu et al. 2003; C. Shinohara et al. 1997). The identification of markers able to discriminate qNSCs from activated NSCs (aNSCs) allowed to verify this hypothesis and described a Lex(bright);EGFR- cell population able to enter proliferation *via* Hedgehog and GABA receptor signaling following irradiation (M. Daynac et al. 2013, 2016). Nevertheless, the extensive number of markers recently used to identify NSCs underlies an important heterogeneity that questions the *in-vivo* dynamics of stem cells in both homeostasis and stress-associated contexts (Z. Chaker et al. 2016).

Interestingly, the role of NSCs in the adult brain has been largely studied within the last decades and neurogenesis has been linked to cognitive functions in both rodent and human brains (L. A. Christie et al. 2012; D. L. Clarke 2003; K. L. Spalding et al. 2013). The radiation-induced impact on cognitive skills will be further developed in this introduction.

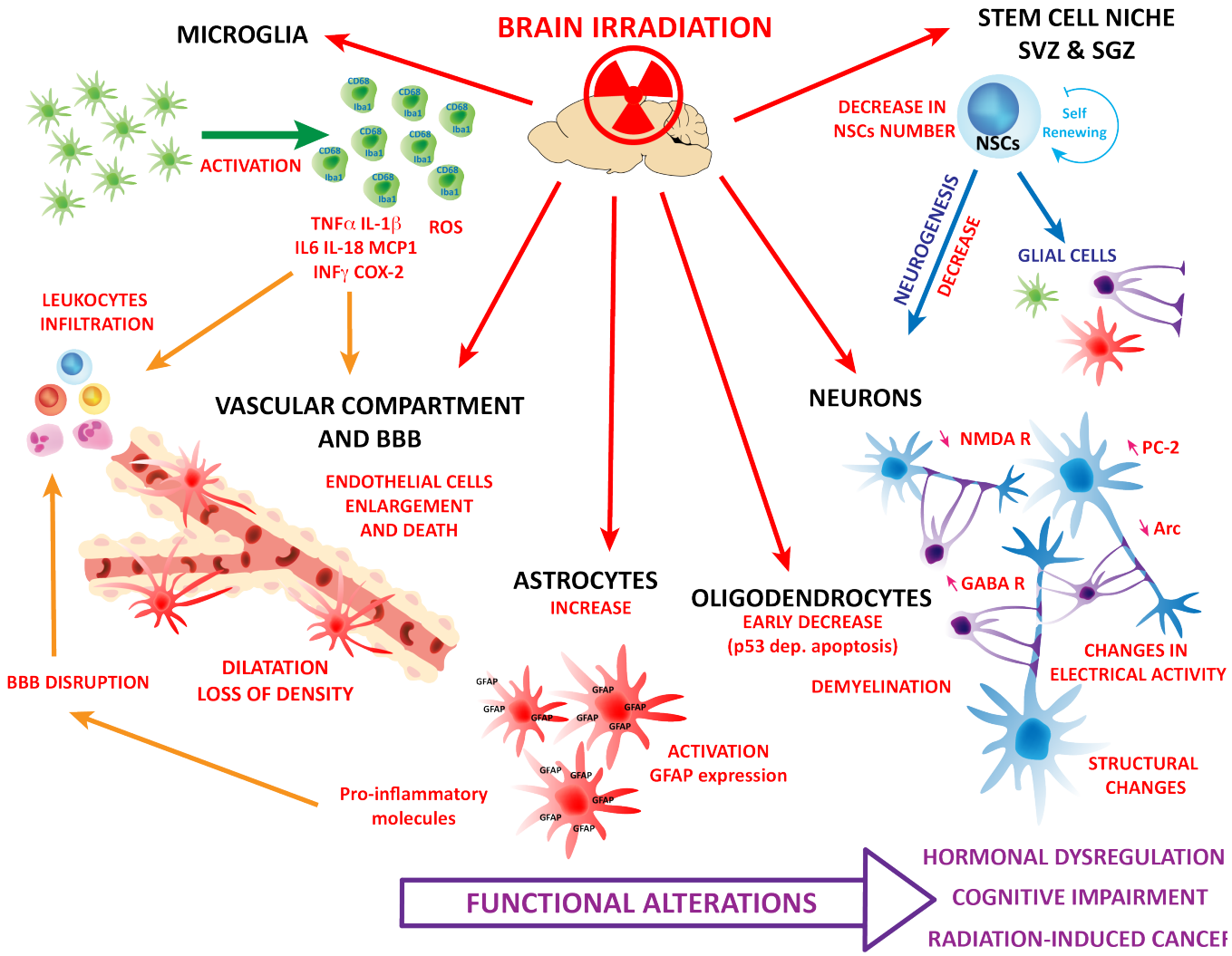

Figure 3: Radiation-induced alterations on the normal brain. Irradiation of the healthy brain induces early and late time-point modifications in all cellular compartments and leads to functional alterations.

2.4. Radiation-induced functional alterations in the brain

Extensive molecular and cellular changes have been described in all cell-types after brain exposure to ionizing radiations. These modifications in cell phenotypes and functions have been associated to functional alterations and particularly to cognitive impairment.

2.4.1. Cognitive impairment in brain tumor patients

As survival among children treated for brain cancer continues to improve, more attention is being focused on the chronic neurocognitive effects of cranial radiation-therapy. Deficits in cognitive development have been described most thoroughly among children treated for posterior-fossa tumors, and specifically medulloblastomas. Many studies have shown a decrease in IQ up to 4.3 points per year post-RT, with a greater deficit with younger age at radiotherapy and a dose-dependent decline (J. Grill et al. 1999; R. K. Mulhern et al. 2001; S. L. Palmer et al. 2003; M. D. Ris et al. 2001). The main cognitive deficits were found in visual attention, verbal and spatial memories (D. R. Copeland et al. 1999; E. Hoppe-

Hirsch et al. 1990, 1995; R. K. Mulhern et al. 2001; Raymond K. Mulhern et al. 2004), with sometimes the necessity to enroll children in schools dedicated to children in educational difficulties (A. W. Glaser et al. 1997).

Radiation-induced cognitive deficits, from slight memory loss to dementia, have been reported to occur in 50 to 90% of adult brain tumor patients who survive more than 6 months post-irradiation (J. R. Crossen et al. 1994; K. Edelstein et al. 2017; A. R. Giovagnoli et al. 1994; T. B. Johannesen et al. 2003; C. A. Meyers et al. 2006). These impairments can be characterized by loss of attention and/or a decrease in verbal, spatial and working memories identified by difficulties in problem-solving abilities (F. H. Hochberg et al. 1980; E. Laukkanen et al. 1988; D. D. Roman et al. 1995; A. Twijnstra et al. 1987). Cognitive impairment incidence has also been described as increased over time and with a progressive severity that can lead to dementia (C Nieder et al. 1999; J. N. Scott et al. 1999; M. C. Vigliani et al. 1999), which is however less frequent in patients treated by radiosurgery or fractionated radiotherapy with fractions under 3 Gy (E. L. Chang et al. 2009; L. M. DeAngelis et al. 1989; M. Klein et al. 2002).

All of these cognitive impairments have an impact on the patient's quality of life, regardless of their severity. Indeed, studies showed that cognitive dysfunctions assessed by neurocognitive testing significantly correlated with the patient's performance on the Functional Assessment of Cancer Therapy -Brain Specific test (FACT-Br) aiming at evaluating the patient's QOL (J. F. Gleason et al. 2007; J. Li et al. 2008). Similarly, Karnofsky Performance Status was found declined in 20% of the patients treated with fractionated radiation-therapy and affected by cognitive deficiencies (C Nieder et al. 1999). The impact of brain radiotherapy treatments on the patient's QOL is of major importance since current therapies are more and more efficient and brain cancer patients survive longer. The development of preclinical models to prevent, mitigate or reverse the radiation-induced cognitive deficits is thus essential to provide better clinical treatments.

2.4.2. Radiation-induced cognitive impairment in preclinical models

Preclinical studies are crucial to assess the different types of cognitive impairments following the brain exposure to ionizing radiations, and to decipher the mechanisms involved in this pathogeny to further implement techniques aiming at avoiding these dysfunctions. Given the importance of the hippocampus in the short-term and long term memory, and its implication in the spatial orientation and learning (N. S. Burghardt et al. 2012; E. Butti et al. 2014; P. M. Lledo et al. 2006; G. Winocur et al. 2006), most of the investigations conducted on murine models have focused on this brain region and have been recently reviewed by Tomé and collaborators (W. A. Tomé et al. 2016).

These impairments have been assessed using several behavioral tests, showing impaired performances following brain irradiation (Table 4). Reduction in the hippocampal neurogenesis, assessed

by Ki67+, BrdU+ and/or DCX+ cells quantification of the SGZ has been broadly correlated with learning and spatial memory deficits 1 to 9 months after single and fractionated doses between 2 and 25 Gy (K. Akiyama et al. 2001; T. M. Madsen et al. 2003; A. A. Nageswara Rao et al. 2011; J. Raber et al. 2004; R. Rola et al. 2004; J. S. Snyder et al. 2005; W. A. Tome et al. 2015; W. A. Tomé et al. 2016). Interestingly, the radiation-induced deficit in neurogenesis that has been linked to a loss of cognitive skills seems to be age-dependent and was not observed in old irradiated rats (I. Lamproglou et al. 1995; M. K. Schindler et al. 2008) in which a higher inflammatory response was identified (S. Rosi et al. 2008; M. K. Schindler et al. 2008).

Table 4: Different behavioral tests used in preclinical models to assess the radiation-induced cognitive impairments.

TEST	ENDPOINT	NEURONAL PATHWAYS	REFERENCES
Novel Object Recognition (NOR) and associated tests	Visual / Working memory	Hippocampus Pre-frontal cortex Perirhinal cortex	(M. M. Acharya et al. 2009; Munjal M. Acharya et al. 2011, 2016; J. E. Baulch et al. 2016; D. Greene-Schloesser et al. 2014)
Temporal Order (TO)	Visual / Working / Temporal memory	Hippocampus Pre-frontal cortex Perirhinal cortex	
Object in Place (OiP)	Visual / Working / Spatial memory	Hippocampus Pre-frontal cortex Perirhinal cortex	
Passive avoidance (PA)	Contextual memory Emotional memory	Hippocampus Amygdala	(K. Akiyama et al. 2001; G. Baydas et al. 2008; J. Raber et al. 2004; W. A. Tome et al. 2015)
Barnes Maze (BM)	Visual / Working / Spatial memory	Hippocampus Pre-frontal cortex Perirhinal cortex	(P. M. Lledo et al. 2006; M. Machida et al. 2010; J. P. Warrington et al. 2012)
Morris Maze	Visual / Working / Spatial memory Anxiety	Hippocampus Pre-frontal cortex Amygdala	(X. Dong et al. 2015; R. A. Rice et al. 2015; R. Rola et al. 2004)
Elevated Plus Maze (EPM)	Anxiety	Hippocampus Pre-frontal cortex Amygdala	(S. Pellow et al. 1985; R. Rola et al. 2004)
Forced Swim Test (FST)	Behavioral despair Depression	Hippocampus Pre-frontal cortex Amygdala	(A. Can et al. 2011)
Fear Extinction (FE)	Conditioned learning Anxiety Fear suppression Dissociation learning	Hippocampus Pre-frontal cortex Amygdala	(P. Achanta et al. 2009; L. Villasana et al. 2010)

Nevertheless, the hippocampus is not the only region that seems to be involved in the radiation-induced cognitive dysfunction. The amygdala and hippocampus regions both contribute to fear, anxiety and depression-like responses to stress (R. G. Phillips et al. 1992) that can also be evaluated using different

tests (Table 3). Fear conditioning assays showed a decrease in test performances as soon as 2 weeks post 4 Gy delivery (R. H. J. Olsen et al. 2017, 2014). The prefrontal and perirhinal cortices-dependent functions, evaluated by adapted NOR tests were also found impaired in rodents models post-irradiation (Munjal M. Acharya et al. 2016; D. Greene-Schloesser et al. 2014; T. C. Lee et al. 2012; V. K. Parihar et al. 2015; K. T. Wheeler et al. 2014). The cellular implication in olfaction of the new neurons originating from the SVZ and localized in the olfactive bulb after their migration through the rostral migratory stream is still controversial (K. G. Bath et al. 2008; G. Gheusi et al. 2000; W. R. Kim et al. 2007). Nevertheless, long-term olfactory memory was found affected by SVZ irradiation at 15 Gy, whereas short-term memory, odorant molecules discrimination and social-guided olfactive behaviors were not affected (F. Lazarini et al. 2009).

2.5. Strategies to prevent or reverse the radiation-induced toxicities in the brain

Many preclinical and clinical studies have focused on strategies to prevent, mitigate or reverse the radiation-induced brain toxicities, compatible with the difficulty to access to the brain through the BBB. Different approaches have been developed including the administration of pharmacologic agents, physical avoidance of radiation sensitive brain regions or stem-cells therapies aiming at decreasing the radiation-induced pathogeny. Cell-death, neurogenesis reduction, neuroinflammation, vascular damage and cognitive deficits were equally investigated.

2.5.1. Prevention against oxidative stress and neuroinflammation

Radiation-induced oxidative stress is thought to be responsible for - and result from - the neuroinflammatory response, linked to the microglia activation, the ROS production and the peripheral immune cell infiltration. Nevertheless, ROS production at both acute and late time-points post-irradiation is difficult to measure and the inflammatory response is often used as a surrogate to oxidative stress assessment.

The use of Peroxisome Proliferator-Activated (PPAR) agonists such as pioglitazone or fenofibrate, already administrated in clinics to treat type 2 diabetes or dyslipidemia (G. Derosa 2010; K. McKeage et al. 2011) have been widely studied to improve the radiation-induced brain toxicities. PPARγ, α and δ, *via* their transcription factor activity have been shown to trigger anti-inflammatory and neuroprotective effects on CNS disorders including multiple sclerosis, Alzheimer's and Parkinson's diseases and after traumatic brain injuries (J. J. Bright et al. 2008; P. F. Stahel et al. 2008), including ionizing radiation exposure (S. Ramanan et al. 2010). Interestingly, the administration of a PPARγ agonist 3 days prior, during and 52 weeks after the delivery of a total 40 Gy dose of fractionated brain irradiation in rats significantly improved cognition skills compared to non-treated animals and animals treated after the irradiation only

(W. Zhao et al. 2007). Treatment with PPARα agonists one week before, during and up to 30 weeks after a 10 Gy single-dose WBI was shown to preserve the hippocampal neurogenesis and the survival of immature neurons while inhibiting the microglia activation (S. Ramanan et al. 2009). Similarly, PPARδ administration 14 days before, during and after a 10 Gy single-dose WBI induced an acute decrease in IL-1β expression and ERK phosphorylation along with a prevention of microglia activation and astrogliosis. Nevertheless, no long-term cognitive improvement was observed compared to the non-treated irradiated animals (C. I. Schnegg et al. 2013).

Other strategies have directly targeted microglial cells. Microglia have recently been identified as CSF-1 dependent for their survival (M. R. P. Elmore et al. 2014) and CSF-1 receptor inhibitors have been successfully used to reversely deplete microglia in adult rodents without inducing persistent abnormalities (M. R. P. Elmore et al. 2015). Moreover, administration of CSF-1R inhibitors was found to prevent glioblastoma recurrence by inhibiting the myeloid cells recruitment into the brain, leading to a successful clinical trial (N. Butowski et al. 2016; J. H. Stafford et al. 2016). Interestingly, the same pharmaceutical agent provided a significant cognitive benefit 3 to 6 weeks post 9 Gy single-dose WBI with a near complete elimination of microglia investigated by Iba-1 and CD68 markers (Munjal M. Acharya et al. 2016).

2.5.2. Other pharmaceutical strategies

Other pharmaceutical strategies are summarized in Table 5. A particularly promising approach consists in blocking the renin-angiotensin system (RAS) with angiotensin-converting enzyme inhibitors or angiotensin type-I receptors blockers (ARB), that are already routinely use in clinics to treat hypertension-associated nephropathies and pneumopathies (a Molteni et al. 2000; J. E. Moulder et al. 2003). Moreover, hypoxia modulation and neurogenic niche protection have been investigated.

Table 5: Pharmaceutical strategies to prevent, mitigate or reverse the radiation-induced brain injuries

AIM	MOLECULE	ADMINISTRATION	EFFECTS	REFERENCE
RAS BLOCKING	ARB L158,809	3 days before, during and after fWBI	Protection against neurocognitive impairment 26 to 52 weeks post-RT. No late histopathology changes.	(M. E. Robbins et al. 2009)
	Ramipril	3 days before, during and after fWBI.	Decrease in microglial activation Protection against perirhinal cortex-dependent cognitive deficits. No improvement of neurogenesis	(T. C. Lee et al. 2012)
		24h before and 12 weeks after a 10 or 15 Gy single dose WBI.	Slight preservation of neurogenesis No modulation of the microglia activation and neuroinflammation	(K. A. Jenrow et al. 2010)
HYPOXIA MODULATION	Hypoxia Induction	Exposition of mice to 11% of O_2 initiated one month post-WBI.	Reversion of the radiation-induced cognitive impairments (contextual learning) Persistent increase in blood-vessel density	(J. P. Warrington et al. 2011, 2012)
	EPO	Intracranial administration of EPO analogue in irradiated rats	Protection against radiation-induced neuronal dysfunction	(J. P. Knisely et al. 2004)
NEUROGENESIS PRESERVATION	Lithium	Oral administration before WBI	Protection of hippocampal neurons against radiation-induced apoptosis Increase in Akt and Bcl-2 Improved cognitive performance	(E. M. Yazlovitskaya et al. 2006)
	NSI-189	Oral administration after fWBI	Preservation of hippocampal and perirhinal cortex-dependent cognitive function at acute and late time-point Increase in neurogenesis Decrease in microglia activation	(B. Allen et al. 2018)

2.5.3. Stem-cell therapies and radiation-induced brain injuries

In addition to pharmaceutical treatments, a particular interest has been sustained to stem cell therapies aiming at protecting or restoring neurogenesis and improving neurocognition. Several cellular sources of NSCs have been used, including embryonic stem cells (ESCs), adult NSCs and neural progenitors. In 1990, one of the first study evaluating the possibility to rescue radiation-induced cognitive impairment by cell transplantation was realized using fetal neurons and neural precursors injected either in the cortex or in the hippocampus of rats, 22 weeks post WBI at 13Gy. Interestingly, both cortex and hippocampal transplants induced a differential enhancement on behavioral tests performances (G. A. Mickley et al. 1990). Moreover, the transplantation of human stem cells into irradiated murine brains has povided promising results. Intra-hippocampal injection of human ESCs (hESCs) two days after brain irradiation in rats induced a preserved neurogenesis and immature neuron migration in the hippocampus, along with

the expression of the memory-associated Arc protein. These results correlated with a protection against radiation-induced cognitive impairment at early and late time-points post-irradiation (M. M. Acharya et al. 2009, 2011; Munjal M. Acharya et al. 2011). Interestingly, the implantation of hESCs-derived oligodendrocyte progenitors in the forebrain and cerebellum of irradiated rats induced a remyelination and a functional preservation of the brain (J. Piao et al. 2015). These studies show that the radiation-induced drop in neurogenesis coupled with cognitive deficits can be reversed by stem cells transplantation, and the first guidelines for a potential clinical trial have been published (H. Huang et al. 2018)

2.5.4. New technologies of radiation-therapy

In order to directly prevent the occurrence of radiation-induced brain toxicities, new irradiation techniques have been developed and investigated in preclinical and clinical trials.

One strategy is to avoid the brain structures associated with the cognitive function, and especially the hippocampus region. A preclinical study on mice evaluated in 2015 described the benefits of hippocampal sparing WBI *versus* classical WBI after the delivery of a single dose of 10 Gy. Interestingly, this study showed a significant improvement of hippocampal-associated behavior in the animals that received hippocampal sparing WBI *versus* the WBI group (W. A. Tome et al. 2015). This preclinical model was supposed to biologically support an ongoing phase II clinical trial (RTOG-0933) aiming at evaluating the benefits on cognition of hippocampal avoidance during whole brain irradiation to treat brain metastases (V. Gondi et al. 2014). An improvement in cognition and in patient's QOL was reported in 42 patients 4 months post fractionated WBRT (3x10 Gy). However, less than 45% of the radiation-oncologists in the US would change their clinical practice to include hippocampus avoidance and further validations in a phase III trial was supported by 76% of them (A. N. Slade et al. 2016). This reluctance might be due to the fact that technology has evolved to potentially avoid WBRT with radiosurgery techniques, but also that other regions than the hippocampus are involved in cognition, and that no long-term cognitive improvement has been shown yet (D. Greene-Schloesser et al. 2012). The risk to miss or protect tumor cells by such brain region avoidance must also be addressed. Moreover, other studies have recently assessed the feasibility of hippocampus sparing with several radiation-therapy techniques, both in preclinical models and in clinical brain tumor cases, but without further *in-vivo* or clinical application (C. Di Carlo et al. 2018; C. K. Cramer et al. 2015; K. Thippu Jayaprakash et al. 2017; S. W. Yoon et al. 2017).

Highly innovative irradiation techniques have also been investigated. Microbeam Radiation-Therapy (MRT) uses highly collimated, quasi-parallel arrays of X-rays microbeams, produced by 3rd generation synchrotron sources. It consists in delivering X-rays on 25-75 μm coplanar beams spaced from each other by 100-400 μm (J. A. Laissue et al. 1999). MRT research has been widely developed and has engendered

many preclinical results on different animal models, including brain tumors and radiation-induced brain injuries. Interestingly, MRT showed the possibility to highly increase the delivered dose (measure at the entry point) without inducing any severe injury. Indeed, only 12% of glioma-bearing rats developed abnormal clinical signs after the delivery of 625 Gy with beams spaced by 200 μm, whereas the same dose with beams spaced by 100 μm induced injuries in more than 70% of the animals. Moreover no histological lesions were observed in the first group (P. Regnard et al. 2008). In addition, cognition sparing has been described in mice 8 months after the delivery of a 10 Gy equivalent dose to the whole brain with MRT, without any histological change (S. Bazyar et al. 2017). A preservation of the rat sensory-motor cortex, evaluated by motor performance and cortical architecture, was also observed after the delivery of 150 or 360 Gy to the whole brain with MRT (E. Fardone et al. 2017). Recent studies tend to show that MRT does not affect the vascular compartment of the irradiated normal brain (A. Bouchet et al. 2015), preventing the occurrence of radiation-induced edema (R. Serduc et al. 2008). These promising results trigger the possibility to apply MRT in clinics for brain tumor treatment (M. A. Grotzer et al. 2015).

Despite an important number of preclinical studies develop with the aim to reduce the radiation-induced brain injury, only a few of them are translated to clinical trials and toxicities induced by radiation-therapy treatments are still affecting the brain tumor patient's quality of life. It is thus of major importance to develop new pharmaceutical and technological strategies to increase the therapeutical index of radiation-therapy and propose better and innovative treatments in clinics. It is nevertheless crucial to fully investigate the radiobiological processes involved in the development of these side effects in order to ideally adapt the therapeutical strategies.

3. FLASH irradiation

The present work has focused on a new irradiation technology called FLASH Radiation-Therapy (FLASH-RT) that consists in the delivery of ultra-high dose-rate irradiations (> 100 Gy/s) when conventional radiation-therapy (CONV) delivers dose-rates around 0.1 Gy/s. Recently identified as a possibility to increase the therapeutic index of radiation-therapy by decreasing the normal tissue injury while keeping an efficient anti-tumor effect, FLASH-RT has become a point of interest in the new irradiation technologies development.

3.1. The FLASH-RT technology and dosimetry

Opportunities to improve the efficacy of radiation-therapy may have been under explored. Today, modern radiation-therapy devices still use the same technology of electron acceleration in waveguides as half a century ago. However, the recent development of proton-therapy facilities and the use of high linear-energy transfer (LET) ions exemplify some of the possibilities that are currently opened. Previous experiments conducted with short pulses of X-rays on lymphocytes (T. Prempree et al. 1969) or more recently conducted with protons on human-hamster hybrid cells and skin cells (S. Auer et al. 2011; T. E. Schmid et al. 2010, 2011) including micro-channel radiotherapy that operates at a dose-rate of 200 $Gy.s^{-1}$ (O. Zlobinskaya et al. 2013) showed less cytogenetic damages and markedly protect normal tissue for radiation-induced acute and long-term damages.

Nowadays, only few devices are able to deliver ultra-high dose-rate irradiation on large volumes of tissue. One of them is the experimental electron eRT6 Oriatron 6MeV LINAC (Figure 4) that has been installed at the CHUV, Lausanne, Switzerland and that was built by a French company (PMB-Alcen) (M. Jaccard et al. 2018). This LINAC was built up based on the Kinetron 4.5MeV LINAC, available in the Institut Curie, Orsay, France, and built by CGR-MeV. These LINACs have been designed to deliver a pulsed electron beam at variable dose-rates: from a few Gy/min (CONV) up to thousands of Gy/s (FLASH).

Figure 4: Photograph of the eRT6 Oriatron LINAC installed at the CHUV, Lausanne, Switzerland. This LINAC prototype delivers a pulsed electron beam at conventional or ultra-high (FLASH) dose-rates.

The use of such high mean dose-rates but also high dose-rate per-pulse, raised the challenge of performing a reliable dosimetry in unusual irradiation conditions. Ionization chambers, which are in general the instruments of choice for reference dosimetry, cannot be used directly because of strong saturation effects induced by the intense beam of the LINAC prototype, which cannot be corrected in a satisfactory way by existing saturation models (J. W. Boag et al. 1996; D. T. Burns et al. 1998; F. Di Martino et al. 2005). An important work on dosimetry has been realized in Lausanne to assess the application of classical dosimeters to FLASH-RT and ensure a proper-dose delivery for the biological investigations. EBT3 Gafchromic™ films usability with ultra-high dose-rates has been investigated due to their easy handling (M. Jaccard et al. 2017). The dependence as a function of dose-rate per pulse has been studied with the eRT6 LINAC from 7.10^3 to 8.10^6 Gy/s and showed that EBT3 Gafchromic™ films dosimetry was independent from the dose-rate, with an excellent consistency between films and thermo-luminescent dosimeters (TLDs), already described as dose-rate independent (L. Karsch et al. 2012). Nevertheless, both TLDs and EBT3 Gafchromic™ films are offline dosimeters that do not allow to get a dosimetry at the moment of the irradiation. However, online dosimetry in high dose-rate and high dose-rate per-pulse beams is challenging because current radiotherapy dosimetry protocols are not designed for such conditions and because the detectors available for online measurements (i.e., ionization chambers, diodes, and diamond detectors) start to exhibit a non-negligible ion recombination when the dose-rate and/or the dose-per-pulse is increased beyond what is used in conventional radiotherapy. The possibility to correct the ion-recombination of the Advanced Markus ionization chamber has been investigated to

potentially use this technology as an online dosimeter for the eRT6 (K. Petersson et al. 2016). Measurements performed in electron beams with varying mean dose-rates, dose-rate within the pulse, and dose-per-pulse (10^{-2} ≤ mean dose-rate ≤ 10^{3} Gy/s, 10^{2} ≤ mean dose-rate within the pulse ≤ 10^{7} Gy/s, 10^{-4} ≤ dose-per-pulse ≤ 10^{1} Gy), allowed the establishment of a correction factor, making possible the use of the Advanced Markus ionization chamber as an online dosimeter for FLASH-RT studies.

3.2. Interaction with the biological tissue

3.2.1. Ultra-high dose-rate irradiation triggers a radiation-induced injury protection

Early evidences that ultra-high dose-rate irradiation had a particular protective effect against radiation-induced damage were obtained in the 60s. Experiments performed on *Serratia marcescens* demonstrated that prokaryotic cells irradiated using an intense electron beam (100 Gy/s) at doses between 100 to 200 Gy have a lower radiation-sensitivity (D. L. Dewey 1969; D. L. Dewey et al. 1959). Similar results were obtained on mammalian cells like HeLa cell-type and Chinese hamster ovary cells (R. J. Berry et al. 1969; E. R. Epp et al. 1972; C. D. Town 1967). Investigation of the DNA damage induced by ultra-high dose-rate irradiation has shown that a same dose delivered with short pulses of X-rays or protons produces 35% fewer dicentric chromosomes than conventional dose-rate irradiation in human blood lymphocytes (T. Prempree et al. 1969). More recently, similar results were observed with pulsed protons on human-hamster hybrid cells, with less dicentrics, centric rings and excess acentric chromosomes observed after high dose-rate irradiation (T. E. Schmid et al. 2011). In a reconstructed skin model, 10% less micronuclei were observed in human keratinocytes after high dose-rate pulsed protons irradiation (T. E. Schmid et al. 2010). In terms of cell-cycle, these differences tend to induce less cell-cycle arrest, with the observation of a differential G2 arrest reported after ultra-high dose-rate irradiation compared to conventional dose-rate irradiation (S. Auer et al. 2011).

Concerning *in-vivo* data, a protection of radiation-induced skin injury triggered by ultra-high dose-rate irradiations has also been described. Radiation-induced skin reactions and late deformities were compared after the irradiation of rats' feet with 7 MeV electrons between 0.03 and 80 Gy/s. Interestingly, a 30-40% increase in dose was necessary to induce similar skin reactions with high dose-rate irradiation compared to conventional dose-rate (S. B. Field et al. 1974). Moreover, a lower skin reaction injury and a faster recovery was observed on mice model after skin irradiation with 30 Gy at 6000 Gy/s compared to conventional dose-rate irradiation (T. Inada et al. 1980). Similar results were assessed on a tail radio-necrosis mouse model, with an increase of 30% of the Necrosis Dose 50 (ND50) induced by 10 MeV electrons delivered at a dose-rate per-pulse above 10^{4} Gy/s (J. H. Hendry et al. 1982).

More recently, our group has published the first study combining normal tissue injury and anti-tumor efficacy evaluation after ultra-high dose-rate irradiation (Vincent Favaudon et al. 2014). Histopathology of lungs irradiated with conventional dose-rate γ-rays and electrons showed the occurrence of lung fibrosis as soon as 24 weeks after 17 Gy whole-lung delivery, with an increase in fibrosis development 36 weeks post-irradiation. None of these fibrotic patterns were observed after the delivery of the same dose with ultra-high dose-rate "FLASH" electrons, and an increase in the dose up to 30 Gy was necessary to observe the occurrence of lung fibrosis 36 weeks post-FLASH-RT. This tissue response was associated with a decrease in apoptosis in vascular and bronchial smooth muscle cells after FLASH-RT compared to conventional dose-rate irradiation, evaluated by caspase 3 cleavage and TUNEL staining 1 hour post-irradiation. This protection triggered by FLASH-RT on the normal lung tissue could suggest an absence of tumoral response. Nevertheless, FLASH-RT and conventional dose-rate irradiation triggered a similar growth delay on HBCx-12A breast cancer cells and Hep-12 H&N cancer cells xenografted in nude mice. Moreover, to complete the data obtained on the normal lung tissue, the radiation response of a TC-1 orthotopic syngeneic lung-tumor model was evaluated. Both FLASH and conventional dose-rate irradiations induced an increase survival as compared to non-treated mice but, interestingly, no difference in survival was observed between FLASH and conventional dose-rate irradiation at isodose. Moreover, the absence of normal tissue injury allowed a dose escalation and a total tumor cure of 70% of the mice after the delivery of 28 Gy with FLASH-RT. This study was the first to provide a preclinical evidence that FLASH-RT is able to increase the therapeutic index of radiation-therapy by decreasing the radiation-induced normal lung toxicity while maintaining a similar anti-tumor effect compared to conventional dose-rate irradiation. These unique properties were called the "FLASH effect".

3.2.2. FLASH-RT: What are the important parameters of the interaction between ionizing radiations and the biological matter?

The interaction of the ionizing radiation with the biological matter follows a precise chronology through physical, chemical and biological steps (Figure 5). The radiation-induced atomic ionizations and excitations within the irradiated matter are followed by molecular dissociations in less than 10^{-12} seconds after the beginning of the exposure. These physical interactions are followed by chemical reactions and diffusions happening within seconds after the beginning of the irradiation. The biological steps then take over with the activation of the DNA Damage Response (DDR) pathways and the cellular and tissue responses, occurring several minutes and hours post-irradiation.

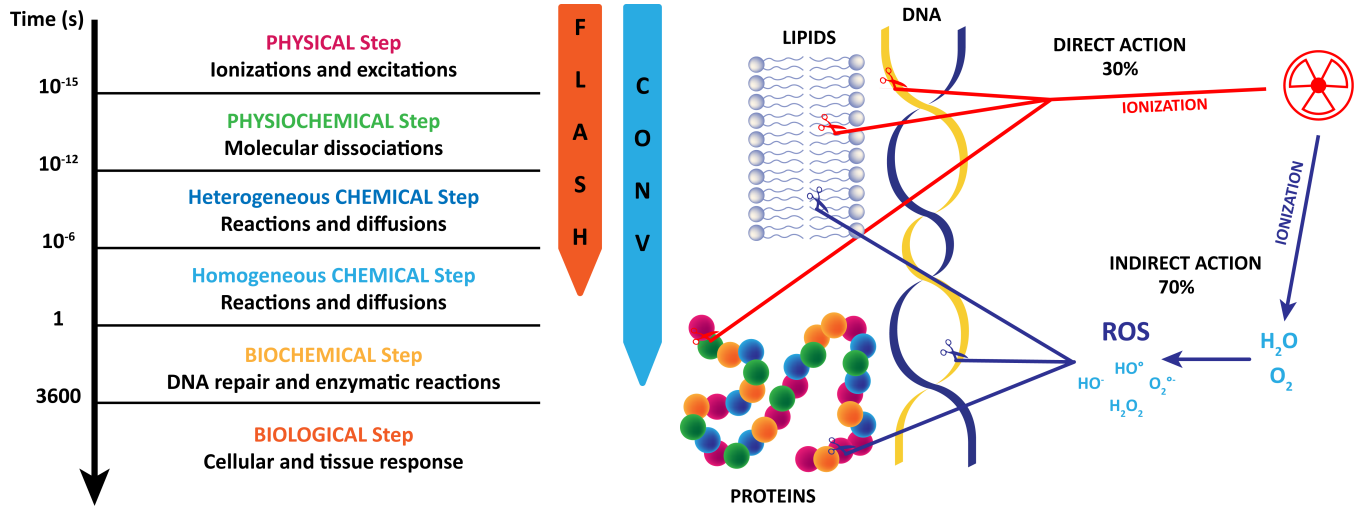

Figure 5: Left: physico-chemical and biological interactions between ionizing radiations and the biological matter. FLASH-RT and conventional dose-rate irradiation differ in the time of exposure. FLASH-RT does not interfere with the homogeneous chemical step and the biological steps due to the very short time of irradiation. Right: direct and indirect actions of the ionizing radiations with the biological macromolecules. Direct action is due to the direct ionizations on the macromolecules whereas indirect action is mediated by free radicals produced by water and dioxygen radiolysis.

These chronological interactions with the biological matter explain the generation of biological damage *via* direct and indirect actions of the ionizing radiations. The direct effects consist in the direct energy deposition on the biological macromolecules (DNA, RNA, proteins, lipids…) inducing molecule ionizations and structural alterations leading to cellular damage. In the indirect action of ionizing radiations, the energy is deposited on the water and dioxygen molecules present in the cells, inducing water radiolysis and oxygen ionization leading to the production of ROS. Free radicals are characterized by an unpaired electron in the structure, which is very reactive, and therefore reacts with the biological molecules to cause molecular structural damage. Hydrogen peroxide, H_2O_2, produced by the ROS recombination is also toxic for the biological macromolecules. The indirect action of radiation on molecules results in cell death or impairment of function. The number of free radicals produced by ionizing radiation depends on the total dose. It has been found that the majority of radiation-induced damage results from the indirect action mechanism, due to the fact that water constitutes nearly 70% of the cell composition (O. Desouky et al. 2015). In addition to the ROS production, other cellular damage can be induced by the formation of Reactive Nitrogen Species (RNS) as a result of the ionization of nitrogen-containing molecules.

All these physical and chemical interactions between ionizing radiations and the biological matter, leading to cellular damages are dose and radiation-dependent, i.e.: for a given ionizing particle, the reactions kinetics depend on the energy deposition at the moment of the irradiation. In the case of FLASH-RT, one could question how the time of irradiation might interfere with these interactions. Indeed, for a similar dose, neither the quantity of deposited energy nor the ionizing particle differs between

conventional and FLASH-RT. Nevertheless, the time of exposition to the radiation is highly decreased, and the energy deposition stops before the end of the chemical reactions and the beginning of the biological step, whereas conventional irradiation induces an exposition to the radiations for several minutes. The investigation of a potential difference in the radiation-induced cascade of events between FLASH and conventional irradiation might be essential to understand the physico-chemical bases leading to the radiobiological properties of the FLASH effect.

4. Objectives of the thesis work

This thesis work, based on the previous results obtained with the FLASH-RT, has aimed at investigating the FLASH effect in the context of the brain in order to provide the biological rational for clinical trials. Several murine models have been developed to investigate the effects of FLASH-RT on brain tumors and on the normal brain tissue. Moreover, these models, along with *in vitro* techniques have been used to investigate the physico-chemical mechanisms underlying the radiobiological properties of FLASH-RT

4.1. Assessment of the FLASH-RT efficacy on brain tumors

Brain tumors, and especially GBM, harbor the particularity to be highly resistant to the radio-chemotherapy treatments, and to develop in a very radiation-sensitive organ. The current treatments are not optimal, and the GBM-associated prognosis remains poor compared to other more frequent tumor-types. The assessment of the FLASH-RT efficacy on brain tumors *via* the use of murine models is essential to consider a potential clinical application and to ensure a significant anti-tumor effect, at least as potent as conventional radiation-therapy. In this thesis work, subcutaneous xenografted GBM, orthotopic and transgenic GBM models were developed to study the tumor response to several FLASH-RT regimens. Murine GBM models can be treated with the experimental LINAC available at the CHUV. The tumor localization in the brain is ideal since 6MeV electrons cannot penetrate deeper than 1.5-2 cm, which is sufficient to ensure a proper whole-brain irradiation of preclinical GBM models.

4.2. Evaluation of the radiation-induced normal brain injury after FLASH-RT

The radiation-induced brain injury has been largely studied and described in the context of the conventional dose-rate irradiation. The molecular, cellular and functional alterations induced by the exposition of this particularly radiation-sensitive organ to ionizing radiations represent the main limitation in the brain tumor treatment by radiation-therapy. The normal tissue protection triggered by FLASH-RT as previously observed in the lung might also apply for normal brain tissue and might thus help to improve the brain tumors treatment. In line with this hypothesis, we have used wild-type and transgenic mice models in order to investigate the cellular and functional alterations induced by FLASH-RT after the delivery of whole brain irradiation in the frame of a very precise irradiation and dosimetry set-up.

4.3. Physico-chemical and biological mechanisms involved in the FLASH effect

The delivery of ultra-high dose-rate irradiation highly decreases the time of exposure of the biological matter to the electrons. This characteristic has been thought to be responsible for the FLASH effect, without further knowledge concerning the involved mechanism. This thesis work has thus aimed at

deciphering the physico-chemical and radiobiological mechanisms that trigger the FLASH effect using the tumoral and the normal brain tissue models, along with *in vitro* studies and zebrafish models.

RESULTS

1. Scientific publication 1: Irradiation in a FLASH: Unique sparing of memory in mice after whole brain irradiation with dose-rates above 100 Gy/s

Published in Radiotherapy and Oncology (2017)

This interdisciplinary study has aimed at developing a relevant mouse model in order to investigate the effect of whole brain FLASH irradiation on the cognitive functions in the context of a precise physical dosimetry to ensure a proper and homogenous dose-delivery in FLASH and conventional dose-rate.

In-silico dose-measurements were realized by the physicist team with different off-line and on-line dosimeters: Gafchromic™ EBT3 films, alanine pellets, TLDs and Advanced Markus ionizing chamber. These measurements allowed to characterize the homogeneity of the delivery of a 10 Gy dose at dose-rates ranging from 0.1Gy/s to 10 Gy delivered in a single 1.8 μs pulse. Moreover, measurements realized with Gafchromic™ EBT3 films allowed to define the in-depth dose distribution and the beam profile of the delivery of 10 Gy at FLASH and conventional dose-rates. To complete the dosimetry characterization, *ex-vivo* measurements were realized using TLDs placed in the brain of a mouse cadaver, to confirm the homogeneous distribution of the 10 Gy dose delivered to the whole brain.

Cognitive function following 10 Gy whole brain irradiation was evaluated using a Novel Object Recognition test (NOR), set up in our lab with the collaboration of Dr Raphaël Doenlen and consisting in measuring the discrimination of two different objects. This NOR test evaluated the hippocampal-associated working memory 2 months post-irradiation of control non-irradiated animals and of 10 Gy whole-brain irradiated mice at different dose-rates: 0.1 (CONV; 1; 3; 10; 30; 100; 500 Gy/s as well as 10 Gy delivered in a single pulse of 1.8 μs (FLASH-RT).

The absorbed doses measured by the different dosimeters showed an accurate delivery of the 10 Gy dose for all the tested dose-rates. Moreover, a homogenous distribution of the 10 Gy dose was measured *ex-vivo* with the TLDs placed in the mouse brain for both FLASH-RT and conventional dose-rates irradiations. These results validate the whole brain irradiation model for a 10 Gy dose delivery at dose-rates from 0.1 Gy/s to 10 Gy delivered in a single 1.8 μs pulse.

NOR realized 2 months post-irradiation showed a significant impairment in the memory function of mice irradiated at conventional dose-rate compared to the non-irradiated animals. For the same dose, no alteration in memory was found in the FLASH-RT group. Interestingly, this memory preservation was found in all groups irradiated with dose-rates above 100Gy/s. Moreover, the cell division was found

relatively preserved in the hippocampus of FLASH-RT irradiated animals as compared to the conventional dose-rate group.

All in all, in this study, the association of a precise dosimetry and of a robust behavioral test allowed the set-up of a well-defined mouse model of whole brain irradiation useful to study the functional effects of FLASH-RT. Our results show a preservation of the cognitive function two-months after the delivery of 10 Gy FLASH-RT for dose-rates above 100Gy/s, defining for the first time a threshold for the FLASH effect.

IRRADIATION IN A FLASH:

UNIQUE SPARING OF MEMORY IN MICE AFTER WHOLE BRAIN IRRADIATION WITH DOSE-RATES ABOVE 100 GY/S

Pierre Montay-Gruel, Kristoffer Petersson, Maud Jaccard, Gaël Boivin, Jean-François Germond, Benoit Petit, Raphaël Doenlen, Vincent Favaudon, François Bochud, Claude Bailat, Jean Bourhis, Marie-Catherine Vozenin.

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Flash irradiation

Irradiation in a flash: Unique sparing of memory in mice after whole brain irradiation with dose rates above 100 Gy/s

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ABSTRACT

This study shows for the first time that normal brain tissue toxicities after WBI can be reduced with increased dose rate. Spatial memory is preserved after WBI with mean dose rates above 100 Gy/s, whereas 10 Gy WBI at a conventional radiotherapy dose rate (0.1 Gy/s) totally impairs spatial memory.
 Radiotherapy and Oncology 124 (2017) 365–369

Our recent publications have shown that irradiation at an ultra-high dose rate was able to protect normal tissue from radiation-induced toxicity. When compared to radiotherapy delivered at conventional dose rates (1–4 Gy/min), this so called "Flash" radiotherapy (>40 Gy/s; Flash-RT) was shown to enhance the differential effect between normal tissue and tumor in lung models [1,2] and consequently allowed for dose escalation. The biological interest of Flash-RT seems to rely essentially on a specific, yet undefined, response occurring in normal cells and tissues. We initially hypothesized that the protective effect of Flash was related to the high dose rate delivery, in other words related to the very short time of exposure. In order to further explore Flash-RT and to validate its protective effect on normal tissues, we decided to extend our observation from the lung to other organs. We decided to investigate brain response to Flash-RT as it is a well-defined and robust model in radiobiology [3–5].

When dealing with unexpected biological results, such as the ones previously described with Flash-RT, accurate dosimetry of the delivered irradiation is essential. However, dosimetry at (an ultra-)high dose rate in high dose-per-pulse beams is non-trivial as current radiotherapy dosimetry protocols are not designed for such conditions and because the detectors available for online measurements (i.e. ionization chambers, diodes, and diamond detectors) start to saturate when the dose rate/dose-per-pulse is increased beyond what is used in conventional radiotherapy [6–8]. Therefore, we needed to rely on dosimeters that had been previously validated to function accurately at more extreme irradiation conditions, i.e. mainly passive dosimeters. Among these options, we selected thermo-luminescent dosimeter (TLD) chips because of their small size ($3.2 \times 3.2 \times 0.9$ mm^3) so that they could be used for measuring dose in the brain of mice. By positioning the TLD inside the skull of a sacrificed mouse, we were able to validate the dose delivered to the brain during whole brain irradiation (WBI).

Brain injuries after WBI at sub-lethal doses delivered at conventional radiotherapy dose rates are well described [5,9,10]. They include functional alterations, neuronal [11], glial [12,13] and vasculature toxicities [14,15]. Cognitive impairments are the most described functional defects observed in mice and humans following WBI [4,16]. They are caused by an alteration of hippocampal neurogenesis, which can occur as early as one month post 10 Gy single fraction WBI [17]. These cognitive impairments can be evaluated using the "Novel Object Recognition test" [18] on WBI murine models [19]. Therefore, we used this assay to investigate the functional effect of Flash-RT on the normal brain of irradiated mice.

Using a combination of accurate dosimetry measurements and robust biological tests, we first aimed to investigate the potential neuroprotective effect of Flash-RT and indeed found memory preservation in mice after 10 Gy WBI with Flash-RT (delivered in

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0167-8140/

a single 1.8 μs electron pulse), whereas 10 Gy WBI delivered with a dose rate similar to what is conventionally used in radiotherapy (0.1 Gy/s) impaired mice memory. Then, we decided to further investigate the dose rate limits for Flash-induced neuropreservation. Using systematic dose rate escalation, 100 Gy/s was found to be the lower limit for full preservation of memory functions after 10 Gy WBI.

Materials and methods

Irradiation device

Irradiation was performed using prototype electron beam linear accelerators (LINACs) of type Oriatron 6e (6 MeV) and Kinetron (4.5 MeV) (PMB-Alcen, Peynier, France). This LINAC is able to produce electron beams at a mean dose rate ranging from 0.1 Gy/s (=6 Gy/min, i.e. similar to dose rates conventionally used for radiotherapy) to 1000 Gy/s, corresponding to a dose, in each electron pulse, ranging between 0.01 and 10 Gy. This wide range of dose rate is made possible by varying the linac gun grid tension, the pulse repetition frequency, pulse width, and the source-to-surface distance (SSD).

Dose prescription and measurement

The standard prescription dose for cognition assay is 10 Gy. Therefore, 10 Gy was used in this study as the prescription dose for the WBI. The irradiation settings, corresponding to the prescription dose, were defined according to surface dose measurement in a 30 x 30 cm^2 solid water phantom positioned behind a 1.7 cm in diameter aperture of a graphite applicator ($13.0 \times 13.0 \times 2.5$ cm^3). Beam profiles and percentage depth dose curves of the beam behind the applicator are presented in Fig. S1 in Sup. The measurements were performed for different linac set-ups (LINAC gun grid tension, pulse repetition frequency, number of pulses, and SSD) corresponding to different dose rates used in this study, i.e. 0.1, 1, 3, 10, 30, 100, and 500 Gy/s, as well as 10 Gy in a single 1.8 μs electron pulse (5.6 MGy/s). The pulse repetition frequency was kept at 100 Hz except for the lowest dose rate setting, for which a pulse repetition frequency of 10 Hz was used. The pulse width was kept at 1.8 μs except for the lowest dose rate setting, for which a pulse width of 1.0 μs was used. The absorbed dose at the surface of the solid water phantom was measured for the different dose rate settings with an ionization chamber (Advanced Markus, PTW-Freiburg GmbH, Freiburg, Germany) corrected for chamber saturation [20], with radiochromic film (Gafchromic™ EBT3, Ashland Inc., Covington, Kentucky, USA), with TLD (type: LiF-100), and with Alanine pellets. These different dosimeters, with appropriate correction factors, have all previously been reported to function correctly at the various dose rates used in this study [8,21–25].

Validation of the absorbed dose in the mouse brain

In order to validate that the dose measured at the surface of the solid water phantom actually corresponds to the absorbed dose in the mouse brain, TLD chips were positioned in the brain of one mouse, which had been sacrificed just prior to the experiment. The TLD chips (in vacuum sealed plastic bags) were positioned in the proximal part of the brain, between the two cerebral hemispheres, and in the lateral parts of the brain (left and right sides).

Mice irradiation

95 Female C57BL/6J mice (n = 5–13) were purchased from CRL at the age of eight weeks. Animal experiments were approved by the Ethics Committee for Animal Experimentation of France and Switzerland and performed within institutional guidelines.

Cognitive tests

Dose rate effect on neuroprotection was evaluated by "Novel Object Recognition test" [18], performed on the mice two months post-irradiation, as described by Acharya et al. [19]. All the experiments were video-recorded. Analysis was performed blindedly and the time spent on each object was measured in order to calculate the Recognition Ratio (RR) such as: $RR = \left(\frac{\text{time spent investigating the novel object}}{\text{time spent investigating the two objects}}\right)$.

Statistical Analysis

The statistical analyses of the Novel Object Recognition test and the BrdU data were performed using unpaired t-tests. Results were expressed as mean values ± standard deviations and the significance level chosen was 5%, with Bonferroni correction for multiple comparisons.

Results

Dose prescription and validation measurements

The absorbed dose measurements carried out at the surface of the solid water phantom for the various types of dosimeters showed that 10 Gy was accurately delivered, at the different dose rates used (Fig. S2 in Sup.). The TLD measurements in the brain of the mouse cadaver validated that 10 Gy was actually the dose delivered to the brain for the prescription of 10 Gy WBI, for the highest (10 Gy in a single 1.8 μs pulse) and the lowest dose rate (0.1 Gy/s) used in this study (Fig. 1a and b). The measured absorbed dose in the brain was 10 Gy (10.06 and 9.90 ± 8.2%, k = 2) in the center of the brain (proximal measurements) and slightly below 10 Gy (lateral left: 9.62 and 9.29 ± 8.2%, lateral right 9.56 and 9.72 ± 8.2%, k = 2) at the edge of the brain (lateral measurements in Fig. 1b).

Flash WBI preserves memory and neurogenesis in the hippocampus

A first set of *in vivo* assessments were conducted on mice following 10 Gy WBI with a conventional radiotherapy dose rate (0.1 Gy/s) or with Flash-RT (10 Gy in a single 1.8 μs pulse). Novel Object Recognition tests performed two months post-irradiation showed a significant drop in RR in mice irradiated with 10 Gy at a conventional radiotherapy dose rate, compared to the non-irradiated control group (53.0 ± 1.7% vs. 78.3 ± 2.6%). Interestingly, mice irradiated with 10 Gy in a single pulse did not show any change in RR compared to the control (75.9 ± 4.0% vs. 78.3 ± 2.6%) (Fig. 1c).

BrdU incorporation in the SGZ of the hippocampus was investigated in order to evaluate *de novo* neurogenesis. Multiple neurogenesis sites were found all over the non-irradiated SGZ with a mean (±standard deviation) of 771 ± 188 BrdU positive clusters (Fig. 2) Surprisingly, more than 37% (292 ± 80) of these clusters were preserved in brains irradiated with 10 Gy in a single pulse, whereas, as expected, mice irradiated with 10 Gy at 0.1 Gy/s only showed a low and significantly different preservation of 14% (108 ± 19) BrdU positive clusters. These results highlight a relative preservation of neurogenesis after Flash-RT WBI compared to conventional dose rate WBI. This *de novo* neurogenesis could partially explain the functional preservation described above.

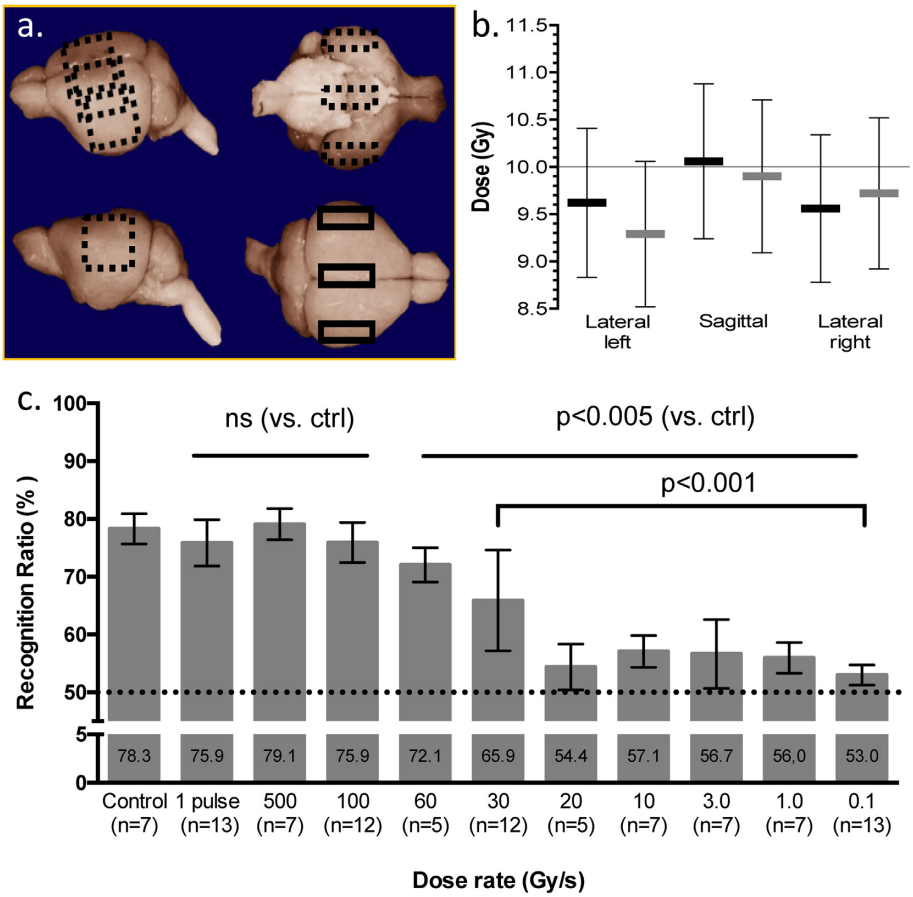

Fig. 1. TLD measurements in the brain of a mouse cadaver, a: TLD chips positions at the center of the brain (sagittal) and at either side of the brain (Lateral left and right); b: measurement results for a 10 Gy WBI delivery with a single 1.8 μs electron pulse (filled markers) and at a 0.1 Gy/s dose rate (open markers). Error bars represent the (expanded, $k = 2$) uncertainty in the absorbed dose measurements with the TLD; c: Evaluation of the Recognition Ratio (RR) two months post irradiation for groups of mice that received sham irradiation (Control) and 10 Gy WBI with a dose rate of 0.1, 1.0, 3, 10, 20, 30, 60, 100, or 500 Gy/s, or with a single 1.8 μs electron pulse (1 Pulse). Bars represent mean values and whiskers the standard deviations.

Flash-RT neuroprotective effect is lost below 30 Gy/s but fully preserved above 100 Gy/s

In order to further investigate the dose rate limits of the Flash-induced neuro-preservation, the experiment was repeated for intermediate dose rates. Interestingly, no memory alteration was observed in the groups irradiated with dose rates of 100 Gy/s or higher (RR were comparable to the ones of the control group), whereas a significant drop in the RR was observed for the group irradiated at 30 Gy/s (Fig. 1c). For the groups irradiated at dose rates below 30 Gy/s, the drop became even slightly larger as the dose rate was further lowered.

Discussion

We have for the first time been able to show that the damage to normal brain tissue, for a given absorbed dose of 10 Gy, can be reduced simply by increasing the dose rate to values 1000 times above what is used in conventional radiotherapy treatments. These unique results show a preservation of memory two months after a 10 Gy WBI with dose rates above 100 Gy/s, whereas 10 Gy WBI at a conventional radiotherapy dose rate (0.1 Gy/s) totally impaired memory.

TLD measurements of the absorbed dose in the mouse brain showed that 10 Gy (9.90–10.06 ± 8.2%, $k = 2$) was truly delivered to the brain center, and slightly below 10 Gy (9.29–9.72 ± 8.2%, $k = 2$) to its lateral parts. This slightly lower lateral dose was expected as the beam profiles (Fig. S1 in Sup.) clearly show a slight decrease in dose with distance from the beam center. As the dose prescription, which was based on surface measurements of a solid water phantom, was validated for the two extreme dose rate settings used in this study, we assumed its validity also for the intermediate dose rate settings.

Brain exposure to ionizing radiation is known to be responsible for long lasting and hardly reversible impairment of cognitive skills. Our results focus on hippocampal related memorization impairment two months post-WBI. Our results show relative hippocampal neurogenesis preservation after Flash-RT WBI assessed by BrdU incorporation, when irradiation at a conventional radiotherapy dose rate is known to directly impair neurogenesis. Neural Stem Cells (NSCs) have been identified in the adult brain as responsible for *de novo* neurogenesis [26]. Therefore, we suggest that the

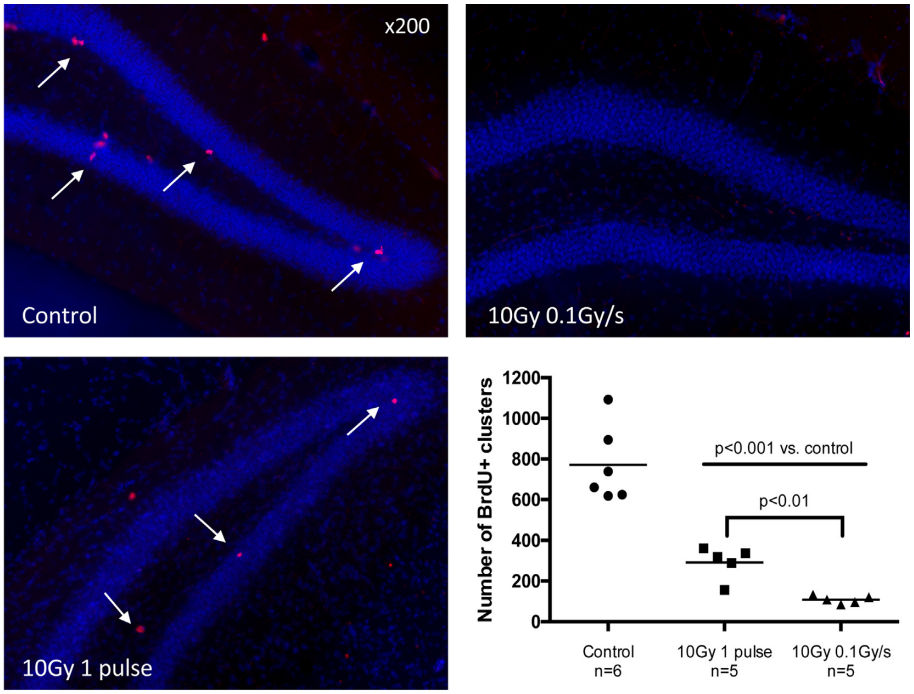

Fig. 2. BrdU immunostaining on brain hippocampal sections of non-irradiated mice (Control) and mice irradiated with 10 Gy at 0.1 Gy/s and mice irradiated with a single 1.8 μs pulse (1 Pulse). Arrows point at BrdU positive clusters in the SGZ. Blue: DAPI; Red: BrdU. Quantification was realized all over the hippocampal sections.

Flash-RT protective effect on neurogenesis relies partly on NSCs preservation. Nevertheless, the memory skills preservation certainly relies on other radiation-induced effects. Both neuroinflammation [27] and synaptic changes [28] are known to interfere with cognitive functions after WBI and could be differentially induced after Flash WBI.

The observed dose rate range of the Flash protective effect on normal tissue gives some physical and biological indications for further investigation regarding the antitumor effect. Despite recent technological developments in radiotherapy, radiation-induced neurotoxicity remains severe in both adult and pediatric patients treated for brain tumors. Our previous results show that Flash-RT demonstrates an antitumor effect similar to conventional radiotherapy [1] in various tumor types, including glioblastoma (preliminary *in vitro* and *in vivo* data). In this context, considering the use of a high dose rate in clinics could be an efficient way to increase the therapeutic ratio of radiation therapy. This radiobiological advantage, together with other practical considerations that benefit from rapid radiotherapy treatment delivery, such as minimizing intra-fractional motion, increased patient comfort, and improved treatment efficiency, makes Flash-RT a promising treatment modality.

Conflict of interest statement

None of the authors have any conflict of interests.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.radonc.2017.05.003.

References

[1] Favaudon V, Caplier L, Monceau V, et al. Ultrahigh dose-rate FLASH irradiation increases the differential response between normal and tumor tissue in mice. Science Transl Med 2014;6:245ra93.
[2] Favaudon V, Fouillade C. Vozenin MC [Ultrahigh dose-rate, "flash" irradiation minimizes the side-effects of radiotherapy]. Cancer Radiother 2015;19:526–31.
[3] Tada E, Parent JM, Lowenstein DH, Fike JR. X-irradiation causes a prolonged reduction in cell proliferation in the dentate gyrus of adult rats. Neuroscience 2000;99:33–41.
[4] Rola R, Raber J, Rizk A, et al. Radiation-induced impairment of hippocampal neurogenesis is associated with cognitive deficits in young mice. Exp Neurol 2004;188:316–30.
[5] Balentova S, Adamkov M. Molecular, Cellular and Functional Effects of Radiation-Induced Brain Injury: A Review. Int J Mol Sci. 2015;16:27796–815.
[6] Technical Reports Series No. 398. International Atomic Energy Agency; 2000.
[7] Almond PR, Biggs PJ, Coursey BM, et al. AAPM's TG-51 protocol for clinical reference dosimetry of high-energy photon and electron beams. Med Phys 1999;26:1847–70.
[8] Karsch L, Beyreuther E, Burris-Mog T, et al. Dose rate dependence for different dosimeters and detectors: TLD, OSL, EBT films, and diamond detectors. Med Phys 2012;39:2447–55.
[9] Mizumatsu S, Monje ML, Morhardt DR, Rola R, Palmer TD, Fike JR. Extreme sensitivity of adult neurogenesis to low doses of X-irradiation. Cancer Res 2003;63:4021–7.

[10] Silasi G, Diaz-Heijtz R, Besplug J, et al. Selective brain responses to acute and chronic low-dose X-ray irradiation in males and females. Biochem Biophys Res Commun 2004;325:1223–35.
[11] Gobbel GT, Bellinzona M, Vogt AR, Gupta N, Fike JR, Chan PH. Response of postmitotic neurons to X-irradiation: implications for the role of DNA damage in neuronal apoptosis. J Neurosci 1998;18:147–55.
[12] Chiang CS, McBride WH, Withers HR. Radiation-induced astrocytic and microglial responses in mouse brain. Radiother Oncol 1993;29:60–8.
[13] Hwang SY, Jung JS, Kim TH, et al. Ionizing radiation induces astrocyte gliosis through microglia activation. Neurobiol Dis 2006;21:457–67.
[14] Brown WR, Thore CR, Moody DM, Robbins ME, Wheeler KT. Vascular damage after fractionated whole-brain irradiation in rats. Radiat Res 2005;164:662–8.
[15] Yuan H, Gaber MW, Boyd K, Wilson CM, Kiani MF, Merchant TE. Effects of fractionated radiation on the brain vasculature in a murine model: blood-brain barrier permeability, astrocyte proliferation, and ultrastructural changes. Int J Radiat Oncol Biol Phys 2006;66:860–6.
[16] Winocur G, Wojtowicz JM, Sekeres M, Snyder JS, Wang S. Inhibition of neurogenesis interferes with hippocampus-dependent memory function. Hippocampus 2006;16:296–304.
[17] Tome WA, Gokhan S, Brodin NP, et al. A mouse model replicating hippocampal sparing cranial irradiation in humans: A tool for identifying new strategies to limit neurocognitive decline. Sci Rep 2015;5:14384.
[18] Antunes M, Biala G. The novel object recognition memory: neurobiology, test procedure, and its modifications. Cogn Process 2012;13:93–110.
[19] Acharya MM, Christie LA, Lan ML, et al. Human neural stem cell transplantation ameliorates radiation-induced cognitive dysfunction. Cancer Res 2011;71:4834–45.
[20] Petersson K, Jaccard M, Germond JF, et al. High dose-per-pulse electron beam dosimetry - A model to correct for the ion recombination in the Advanced Markus ionization chamber. Med Phys 2017.
[21] Desrosiers MF, Puhl JM, Cooper SL. An Absorbed-Dose/Dose-Rate Dependence for the Alanine-EPR Dosimetry System and Its Implications in High-Dose Ionizing Radiation Metrology. J Res Natl Inst Stand Technol 2008;113:79–95.
[22] Hansen JW, Olsen KJ, Wille M. The Alanine Radiation Detector for High and Low-LET Dosimetry. Radiat Prot Dosimetry 1987;19:43–7.
[23] Jaccard M, Petersson K, Buchillier T, et al. High dose-per-pulse electron beam dosimetry: Usability and dose-rate independence of EBT3 Gafchromic films. Med Phys 2017;44:725–35.
[24] Olsen KJ, Hansen JW, Wille M. Response of the alanine radiation dosemeter to high-energy photon and electron beams. Phys Med Biol 1990;35:43.
[25] Tochilin E, Goldstein N. Dose Rate and Spectral Measurements from Pulsed X-ray Generators. Health Phys 1966;12:1705–14.
[26] Price J, Williams BP. Neural stem cells. Curr Opin Neurobiol 2001;11:564–7.
[27] Cherry JD, Olschowka JA, O'Banion MK. Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. J Neuroinflammation 2014;11:98.
[28] Parihar VK, Allen B, Tran KK, et al. What happens to your brain on the way to Mars. Sci Adv 2015;1.

Supplementary material and methods

Validation of the absorbed dose in the mouse brain

The mouse cadaver with the TLDs inserted in the brain was irradiated with 10 Gy WBI (according to surface measurements of the solid water phantom) at the highest dose-rate (10 Gy in a single 1.8 μs pulse), these first TLDs were removed and replaced with new ones and the irradiation was repeated for a total of five measurements at the same dose-rate. The procedure was then repeated for the lowest dose-rate (0.1 Gy/s, i.e. conventional radiotherapy dose-rate).

Irradiation of mice

Mice were irradiated under Isoflurane anesthesia. The head was positioned behind and in contact with the aperture of the graphite applicator in order to irradiate the whole encephalon region while limiting the dose to eyes, mouth and the rest of the body. Each group of mice received 10 Gy WBI at various dose-rate: 0.1, 1, 3, 10, 20, 30, 60, 100, and 500 Gy/s and 10 Gy in a single 1.8 μs pulse.

Sampling, histology and Immunofluorescence

After cognitive evaluation and two hours before sampling, mice were injected with a BrdU solution (150 mg/kg i.p.), sacrificied in a CO_2 chamber and immediately intracardiacally perfused with a 4% PFA solution for the brain fixation. The brain was removed and stored in 30% sucrose 0.1% azide at 4°C. Serial sections (35 μm) were cut through the entire hippocampus with a cryostat and stored in phosphate buffer saline (PBS) with 0.1% azide. Neurogenesis clusters were quantified on serial brain sections using anti-BrdU monoclonal antibody (1:100; BD 347580), from mice irradiated with the two extreme dose-rates (0.1 Gy/s and 10 Gy in a single pulse) and non-irradiated mice (Control). The sections were then incubated for one hour with a goat anti-mouse secondary antibody (1:200; Life Technologies A11005). The number of BrdU clusters was counted all over the irradiated sub-granular zone (SGZ) using a stereological method described by David et al. [1].

[1] D.J. David, J. Wang, B.A. Samuels, Q. Rainer, I. David, A.M. Gardier, R. Hen, Implications of the functional integration of adult-born hippocampal neurons in anxiety-depression disorders, Neuroscientist 16(5) (2010) 578-91.

Supplementary Figures

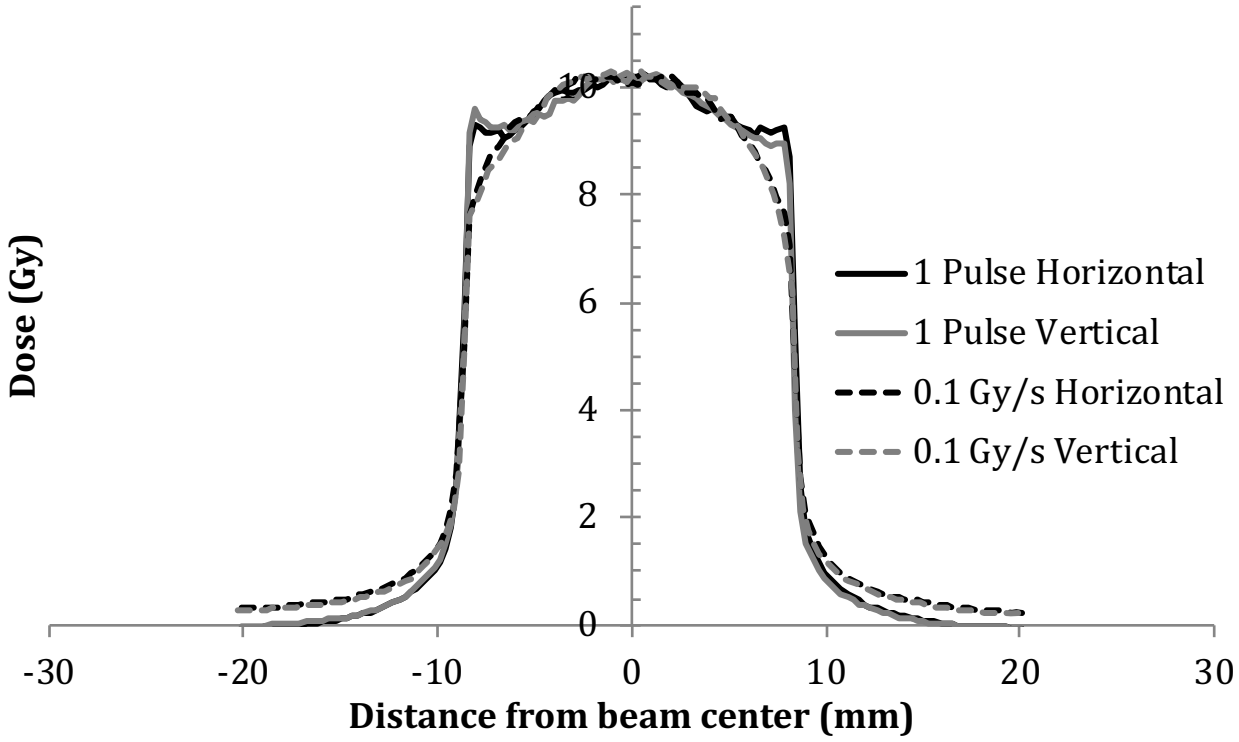

Figure S1: The beam profiles (above) and percentage depth dose curves (below) produced by the prototype LINAC and measured using radiochromic film (Gafchromic™™ EBT3) in a solid water phantom (profiles measured at 0 mm depth) behind the 1.7 cm in diameter aperture of the graphite applicator, for the highest (10 Gy in a single 1.8 µs pulse; 1 Pulse) and lowest dose-rate (0.1 Gy/s) used in the study. dy.

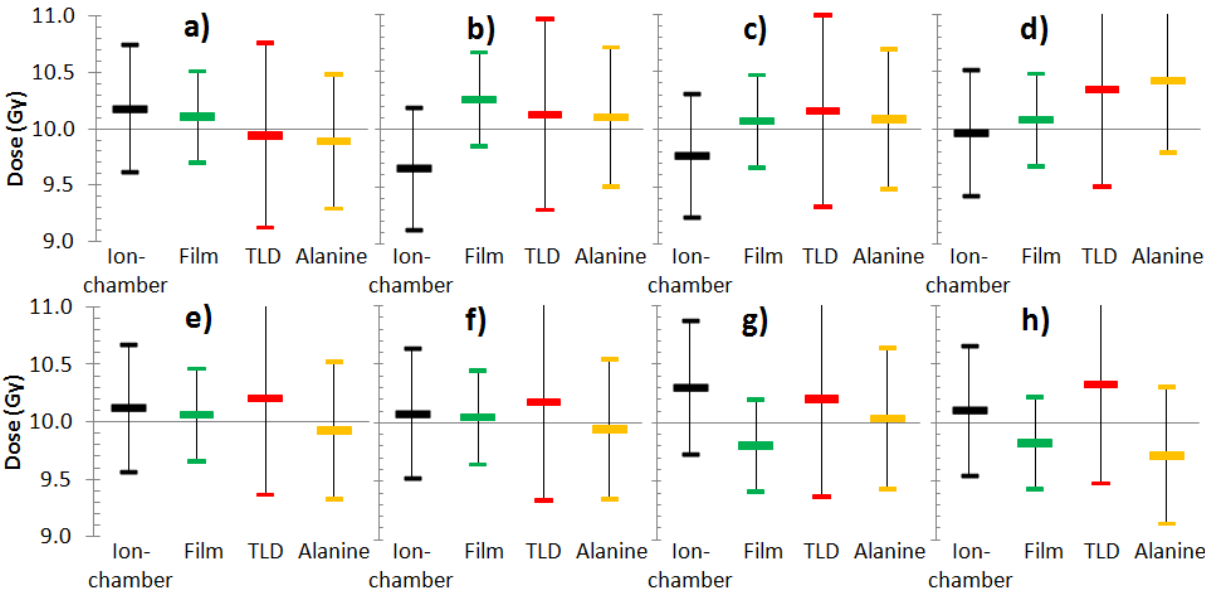

Figure S2: Absorbed dose measurements with the Advanced Markus ionization chamber (Ion-chamber), radiochromic film (Film), thermo-luminescent dosimeters (TLD), and Alanine pellets (Alanine) for various dose-rates used in this study: a) 10 Gy in a single 1.8 µs pulse, b) 500 Gy/s, c) 100 Gy/s, d) 30 Gy/s, e) 10 Gy/s, f) 3 Gy/s, g) 1 Gy/s, h) 0.1 Gy/s. Error bars represent the (expanded, k=2) uncertainties in the absorbed dose measurements, which are different for the various dosimeters.

2. Scientific publication 2: X-rays can trigger the FLASH effect: Ultra-high dose-rate synchrotron light source prevents normal brain injury after whole brain irradiation in mice.

Submitted to *Radiotherapy and Oncology,* June 2018.

With the aim to speed up the clinical application of FLASH-RT, our mouse model of whole brain irradiation was used to investigate whether the FLASH effect observed with a pulsed-electron beam could be reproduced using X-rays.

FLASH-X-rays whole brain irradiation at 10 Gy was delivered by a synchrotron light source operated broad-beam at the ESRF, Grenoble, France. The use of synchrotron-generated X-rays allowed to reach a mean dose-rate around 12000 Gy/s, above the threshold defined previously (P. Montay-Gruel et al. 2017). Cognitive function of mice irradiated with FLASH-X-rays was evaluated with the NOR test 2 and 6 months post-WBI and compared to non-irradiated mice and animals irradiated with conventional dose-rate X-rays. Hippocampal cell division was investigated 2 months post-RT by BrdU incorporation in all groups. Cellular toxicity was assessed by astrocytes staining to quantify the radiation-induced astrogliosis.

NOR test performed 2 months post 10 Gy WBI showed no difference in memory skills between FLASH-X-rays irradiated and non-irradiated animals, whereas mice irradiated with the same dose at conventional dose-rate had a completely impaired memory. Similar results were observed 6 months post-RT, suggesting a long-lasting memory preservation triggered by FLASH-X-rays. BrdU incorporation in the hippocampus highlighted a relative preservation of the hippocampal cellular division in the animals irradiated with FLASH-X-rays compared to conventional dose-rate irradiation, consistent with the conservation of hippocampal-associated memory in the same group. Moreover, astrocytes staining by GFAP immunofluorescence showed the occurrence of a radiation-induced astrogliosis 2 months post-irradiation in the brain of animals irradiated with conventional dose-rate X-rays. This radiation-induced brain injury signature was not observed in the brain of animals irradiated with FLASH-X-rays, confirming a protection against cellular injury by X-rays delivered at ultra-high dose-rate.

This study represents a proof of concept that the FLASH effect previously described in the lung and in the brain after FLASH-electrons irradiation is also triggered by ultra-high dose-rates X-rays generated by a synchrotron light-source. Indeed, we described here a long-lasting functional protection along with a relative preservation of the hippocampal cell-division and the absence of cellular toxicity after FLASH-X-rays whole brain irradiation. These results are of major importance for a potential clinical transfer, due to

the physical advantage of X-rays compared to electrons, especially in terms of depth penetration. Moreover, the observation of the FLASH effect triggered by another irradiation particle suggest that this effect is particle-independent and caused by the increase in the dose-rate.

X-RAYS CAN TRIGGER THE FLASH EFFECT:

ULTRA-HIGH DOSE-RATE SYNCHROTRON LIGHT SOURCE PREVENTS NORMAL BRAIN INJURY AFTER WHOLE BRAIN IRRADIATION IN MICE.

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Running title: Memory cognition sparing by ultra-high dose-rate synchrotron X-ray radiation

Key-words: Ultra-high dose-rate X-ray radiation, synchrotron radiation, cognitive dysfunction.

Summary

This study is the first proof of concept that the FLASH effect can be triggered by X-rays. Our results show that a 10 Gy whole-brain irradiation delivered at ultra-high dose-rate with synchrotron generated X-rays does not induce memory deficit; it reduces hippocampal cell-division impairment and induces less reactive astrogliosis.

Introduction

Research into ultra-high dose-rate irradiation has been increasing over the last few years [1–5]. Interest in this new irradiation concept arises from a goal to improve current radiation therapy treatments and enhance tumor control, especially since any reduction of the radiation-induced toxicities on normal tissues enables a dose escalation. In this context, we are the first group to investigate the biological effects triggered by this novel modality of irradiation named FLASH-radiotherapy (FLASH-RT). FLASH-RT involves delivering large single doses of radiation (10-20 Gy) at mean dose-rates above 100 Gy/s. This has previously been performed using electron beams of 4-6 MeV, produced with experimental LINACs. The reduction of irradiation exposure to a few milliseconds induced differential biological effects between normal and tumoral tissues. Indeed, a similar antitumor effect compared to conventional radiation therapy was observed, along with a reduction of the radiation-induced side effects. This sparing effect, called the “FLASH effect” has been found in several pre-clinical models, showing that electrons, delivered at ultra-high dose-rates were able to protect normal lung [6] and brain tissues [7,8]. More recently, the FLASH effect has been extended to pig skin and to SCC-bearing cat patients irradiated within a phase I veterinary clinical trial [9].

Nowadays, proton therapy facilities operating with pencil beam scanning (PBS) are also able to produce very high instantaneous dose-rates in the range of 200 Gy/s. However, the duration of the scanning process drops the actual mean dose-rate (1-2 Gy/min) and limits the investigation of the FLASH effect with Protons/PBS over large irradiation fields (cm^3). Synchrotron facilities are a potential source of ultra-high dose-rate X-rays. For instance, the European Synchrotron Radiation Facility (ESRF) storage ring in Grenoble, France, produces X-rays at instantaneous dose-rates of up to 18,000 Gy/s. The ID17 Biomedical beamline is dedicated to medical research, mainly developing imaging methods and novel radiotherapy techniques. The ID17 wiggler source produces highly coherent and non-diverging X-rays at a mean energy of 102 keV, enabling the treatment of deep-seated lesions (half value layer of 5.5 cm). Thanks to these unique physical properties, Microbeam Radiation Therapy (MRT) has been developed at the ESRF in close collaboration with various user groups into a very mature technique. MRT is based on the spatial fractionation of the incident X-ray beam into wafers of parallel microbeams which are a few

tens of µm wide and separated by a few hundred µm [10]. This approach has an interesting anti-tumor effect [11] and a protection of normal vessels [12–14] by the possible combination of both spatial fractionation and/or ultra-high dose-rate irradiation. In this context, the protective vascular effect could either be due to spatial fractionation of the irradiation and/or ultra-high dose-rate delivery.

In the current study, our aim was to investigate whether the FLASH effect observed after WBI in mice [7] with a pulsed electron beam (eRT6, [15]) could be reproduced with X-rays. Previous FLASH effect results were obtained with a pulsed electron beam delivering a dose-rate in the pulse of 4.5 10^5 Gy/s, corresponding to a mean dose-rate of 200 Gy/s [15]. Therefore, with the possibility to deliver a dose-rate in the slice of 12 000 Gy/s corresponding to a mean dose-rate of 37 Gy/s (FLASH-X-rays), the ESRF synchrotron facility was the ideal candidate to test this hypothesis. We used a broad beam, i.e. a flat beam of 50 µm without microbeam patterns. A dose of 10 Gy FLASH-X-rays was delivered to the whole brain of C57Bl/6J mice (<1 cm^3) with strict dosimetry recordings [16] by moving the head of the mice through the beam. Cognitive function and cellular brain toxicity were evaluated. Using a robust novel object recognition test and immunofluorescence assays, we observed an absence of radiation-induced memory-loss up to 6 months after irradiation, along with a better preservation of hippocampal cell-division and less radiation-induced scar astrogliosis compared to X-ray irradiation performed at a conventional dose-rate (0.05 Gy/s, Pxi Precision X-Ray), something which also irreversibly altered memory cognition in mice. These results were fully comparable with our previous results obtained with FLASH-electrons [7].

Materials and Methods

Irradiation devices

Irradiations were performed at the ID17 Biomedical Beamline of the ESRF (Grenoble, France). Conventional dose-rate irradiations were performed using a XRad 225Cx (Pxi Precision X-Ray) at the Lausanne University Hospital.

Dose prescription and measurement

The absorbed doses for both irradiation methods were measured with radiochromic films (Gafchromic™ EBT3, Ashland Inc., Covington, Kentucky, USA). The standard prescription dose for cognition assay is 10 Gy. Therefore, 10 Gy was used in this study as the prescription dose for the WBI. The irradiation settings for conventional dose-rate irradiations, corresponding to the prescription dose, were defined at a 5 mm depth for a 10x10 mm^2 field according to depth dose measurement in a solid water phantom. The measurements were performed at 225 keV, 13 mA, with a 0.3 mm copper filter.

FLASH-X-rays irradiations were performed using the synchrotron beam on ID17 (ESRF, Grenoble, France). The ID17 wiggler gap was set to 24.8 mm in order to benefit from the maximal photon flux, and aluminum and copper attenuators were inserted to produce the standard MRT spectrum with its maximum intensity at 102 keV [17]. The dose-rate was measured for broad beam conditions in solid water plates (Goettingen White Water; 30 x 30 x 12 cm3) using a Pinpoint ionization chamber (PTW, Ref. 31014). The chamber was calibrated with TH200 beam quality using an X-ray generator at a mean energy of 109 keV, which is very close to the MRT filtered spectrum resulting in a mean energy of 102 keV [17]. The measured dose-rate under reference conditions was entered in the MRT Graphical User Interface and an adequate speed for the vertical translation (moving the head of the mice through the beam) was automatically calculated while considering the machine current in the storage ring. A pre-calculated Monte Carlo beam model was used to find the settings which would enable a 10 Gy delivery at 5 mm depth.

Mice irradiation

Twenty-nine Female C57Bl/6 J mice (n=5-10 animals per group) were purchased from CRL at the age of eight weeks. Animal experiments were approved by the Ethics Committee for Animal Experimentation of France and Switzerland and performed within institutional guidelines. All irradiations were performed under isoflurane anesthesia.

We delivered 10 Gy absorbed dose to water whole brain irradiation (WBI) at conventional dose-rate (CONV-X-rays, 0.05 Gy/s) using a 10 x 10 mm^2 field size, after fluoroscan imaging to position the mouse in order to avoid irradiating their eyes, mouth cavity, esophagus and trachea. Two horizontal opposed beams each delivering 5 Gy at 5 mm depth were irradiating the brain.

For 10 Gy WBI with FLASH-X-rays, the mice were anesthetized under isoflurane inhalation and irradiated under broad beam conditions. A horizontal slit height of 50 μm was selected to be able to adapt the speed of the MRT goniometer to scan the mouse vertically through the beam at speeds around 62 mm/s to cover a total field height of 17 mm diameter defined by a conformal mask placed 1 m upstream from the animals. A dose-rate in each 50 μm slice of 67 Gy/(s.mA) was measured at 2 cm depth using a Pinpoint ionization chamber (PTW, Ref. 31014). During the experiment, the machine current was 178 mA, leading to a dose-rate in the slice of about 12,000 Gy/s, corresponding to a mean dose-rate of 37 Gy/s for the delivery of 10 Gy to the whole mouse brain with the duration of 0.27 s.

Despite a difference in irradiation geometry between conventional dose-rate X-rays (10 x 10 mm^2 field size) and FLASH-X-rays (17 mm diameter), imaging performed before the irradiation ensured a proper mouse-positioning and the actual irradiation of the entire brain in both configurations.

Cognitive tests

Cognitive skills, evaluated using a "Novel Object Recognition test" [18], were performed on the mice two and six months post-irradiation, as described by Acharya *et al.* [19]. All the experiments were video-recorded. Analysis was performed blindly, and the time spent on each object was measured in order to calculate the Recognition Ratio (RR) such as: RR = [(time spent on the novel object – time spent on the old object) / (total exploration time) x 100].

Sampling and Histology

After cognitive evaluation two months post-irradiation and two hours before sampling, mice were injected with a BrdU solution (150 mg/kg i.p.). They were euthanized in a CO_2 chamber and immediately perfused with a 4% PFA solution to allow brain fixation. The brains were removed and stored in 30% sucrose, 0.1% azide at 4°C. Serial sections (35 μm) were cryo-cut through the entire hippocampus and stored as floating sections in phosphate buffer saline (PBS) with 0.1% azide.

Immunofluorescence and Microscopy

Neurogenesis clusters were quantified on serial brain sections using mouse anti-BrdU monoclonal antibody (1:100; BD 347580) incubated overnight at 4°C. The sections were then incubated for one hour with a goat anti-mouse AF594 secondary antibody (1:200; Life Technologies A11005). BrdU clusters were counted all over the irradiated subgranular zone (SGZ) using the stereological method described by David *et al*. [20].

GFAP expression in the striatum was assessed using GFAP immunofluorescence. Floating brain sections were incubated with an anti-GFAP primary antibody (1:500; clone GA5; MAB360) overnight at 4°C. The sections were then incubated for one hour with a donkey anti-mouse AF555 secondary antibody (1:200; Life Technologies A11005). Image acquisition was performed using an upright Zeiss Axiovison microscope. GFAP expression was quantified on 15-18 fields of view (X200) using a homemade software MoreHisto. Briefly, GFAP positive structures were isolated from background using sets of filters and threshold and fluorescence intensities (as grey levels) were integrated on the different slices, pooled and plotted as an occurrence of pixel.

Statistical analysis

The statistical analyses of the Novel Object Recognition test and the BrdU immunofluorescence data were performed using unpaired non-parametric Mann-Whitney tests. GFAP expressions were compared using a paired non-parametric Wilcoxon test. Results were expressed as mean values ± standard deviations and the significance level chosen was 5% ($p < 0.05$).

Results

Long-lasting memory skills preservation

In order to compare the effects of FLASH-X-rays and conventional dose-rate X-rays (CONV-X-rays) WBI on cognition skills, Novel Object Recognition tests were performed 2 and 6 months post-irradiation. Mice irradiated with a single dose of 10 Gy at conventional dose-rate showed a significant drop in their Recognition Ratio (RR) 2 months post-RT compared to control non-irradiated mice (51.9 ± 6.5% vs. 74.7 ± 5.1%, p<0.001) (Figure 1a). For the same delivered dose and at the same time-point post-irradiation, mice irradiated with FLASH-X-rays did not show any decrease in RR compared to the control group (72.0 ± 4.1% vs. 74.7 ± 5.1%, ns). Furthermore, mice irradiated with conventional dose-rate showed no real improvement of their RR 6 months post-RT (56.0 ± 6.1% vs. 74.6 ± 5.2%, p=0.003) (Figure 1b), whereas RR of the FLASH-X-rays WBI group was still comparable to the non-irradiated animals (73.8 ± 4.9% vs. 76.7 ± 4.3%, ns). These results suggest a long-lasting preservation of the cognitive memory skills of mice irradiated with a single dose of 10 Gy WBI at ultra-high dose-rate synchrotron X-rays, while the same dose delivered at a conventional dose-rate induces irreversible memory alteration.

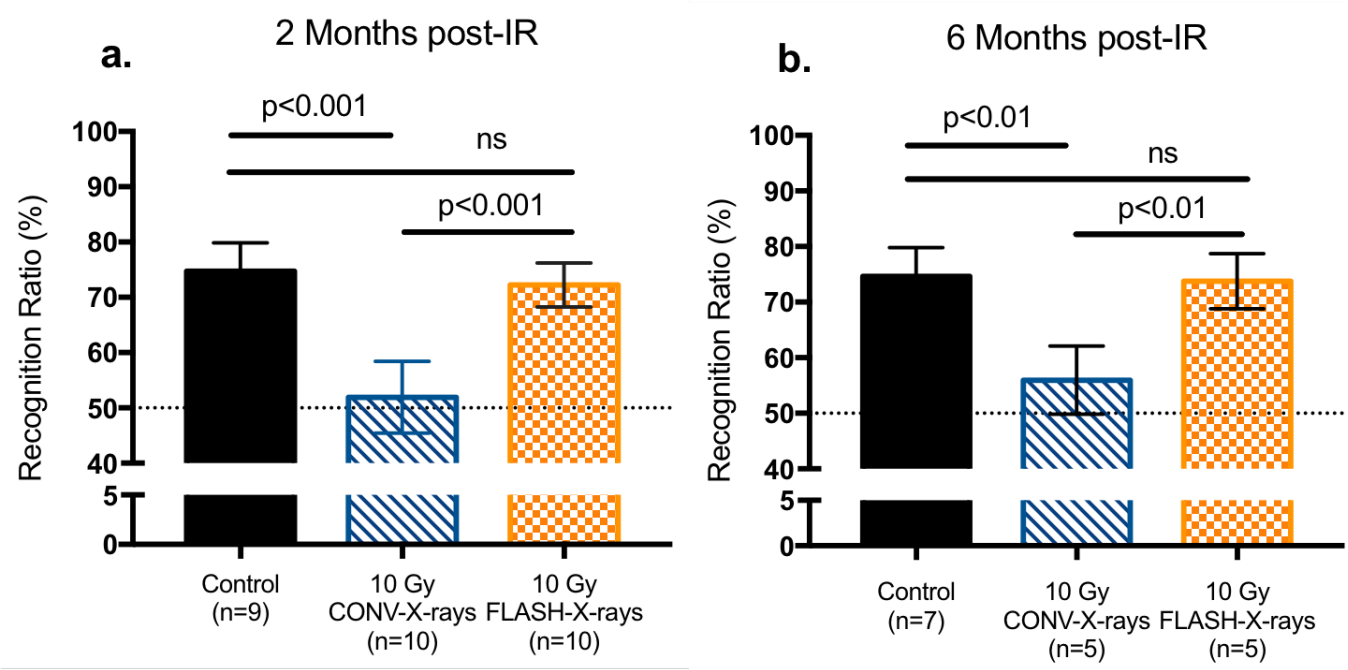

Figure 1: Evaluation of the Recognition Ratio (RR) two (a) and six months (b) post-irradiation for groups of mice that received sham irradiation (Control) and 10 Gy WBI with FLASH-X-rays or with X-rays delivered at conventional dose-rate (CONV-X-rays). Bars represent mean values and whiskers the standard deviations.

Preservation of the cellular division in the hippocampus after FLASH-X-rays WBI

Cellular division was investigated 2 months post-irradiation in the hippocampus subgranular zone (SGZ) of FLASH and conventional dose-rate X-rays irradiated mice by BrdU incorporation (Figure 2). A large number of dividing cells clusters were found in the SGZ of non-irradiated animals with a mean of 938 ± 63

BrdU+ clusters. In all irradiated animals, a significant decrease in BrdU positive clusters was observed. However, more clusters were found in mice irradiated with FLASH-X-rays as compared to mice irradiated with conventional dose-rate X-rays (248 ± 78 vs. 115 ± 34, p=0.032). This result shows a relative preservation of the cellular division in the SGZ of FLASH-X-rays irradiated mice, suggesting a preservation of the neurogenesis in this memory-involved brain region.

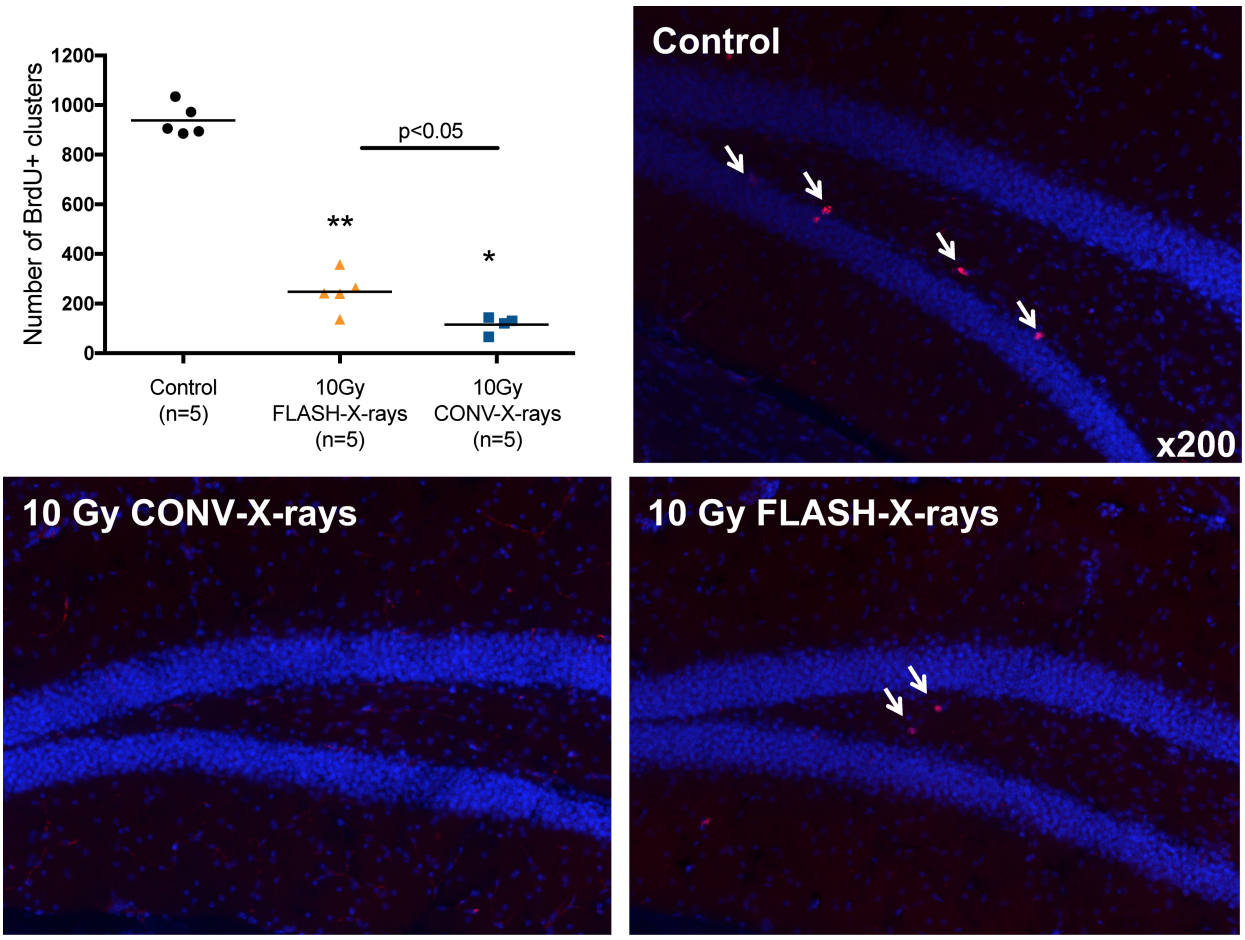

Figure 2: BrdU immunostaining on brain hippocampal sections of non-irradiated mice (Control) and mice irradiated with 10 Gy WBI with FLASH-X-rays or with X-rays delivered at conventional dose-rate (CONV-X-rays). Arrows point at BrdU positive clusters in the SGZ. Blue: DAPI; Red: BrdU. Quantification was realized all over the hippocampal sections. Statistical analysis performed with unpaired non-parametric Mann-Whitney test. *: $p < 0.05$ vs. control; **: $p < 0.01$ vs. control.

FLASH-X-rays induces less astrogliosis in the irradiated brain

Reactive astrogliosis consists of an abnormal stress-induced increase in the number of astrocytes, frequently observed after brain irradiation, which leads to cellular dysfunctions. GFAP expression in the striatum of non-irradiated and irradiated mice was quantified by immunofluorescence to assess the occurrence of radiation-induced astrogliosis 2 months post-RT (Figure 3). A low GFAP staining was observed in the striatum of non-irradiated animals, suggesting the presence of astrocytes, with a classical

star-shaped morphology and extended processes. The GFAP staining was highly and significantly increased in the striatum of mice irradiated at 10 Gy conventional dose-rate, with an increase in astrocyte number, mainly organized in patches. For a same irradiation dose, significantly less GFAP staining intensity was quantified after FLASH-X-rays irradiation, with a localization pattern and a cell morphology similar to the non-irradiated group. This result suggests that FLASH-X-rays induce less radiation-induced reactive astrogliosis compared to conventional dose-rate X-rays. As the astrocytes are highly involved in brain homeostasis, this reduced toxicity is consistent with the preservation of cognitive functions and hippocampal cell-division.

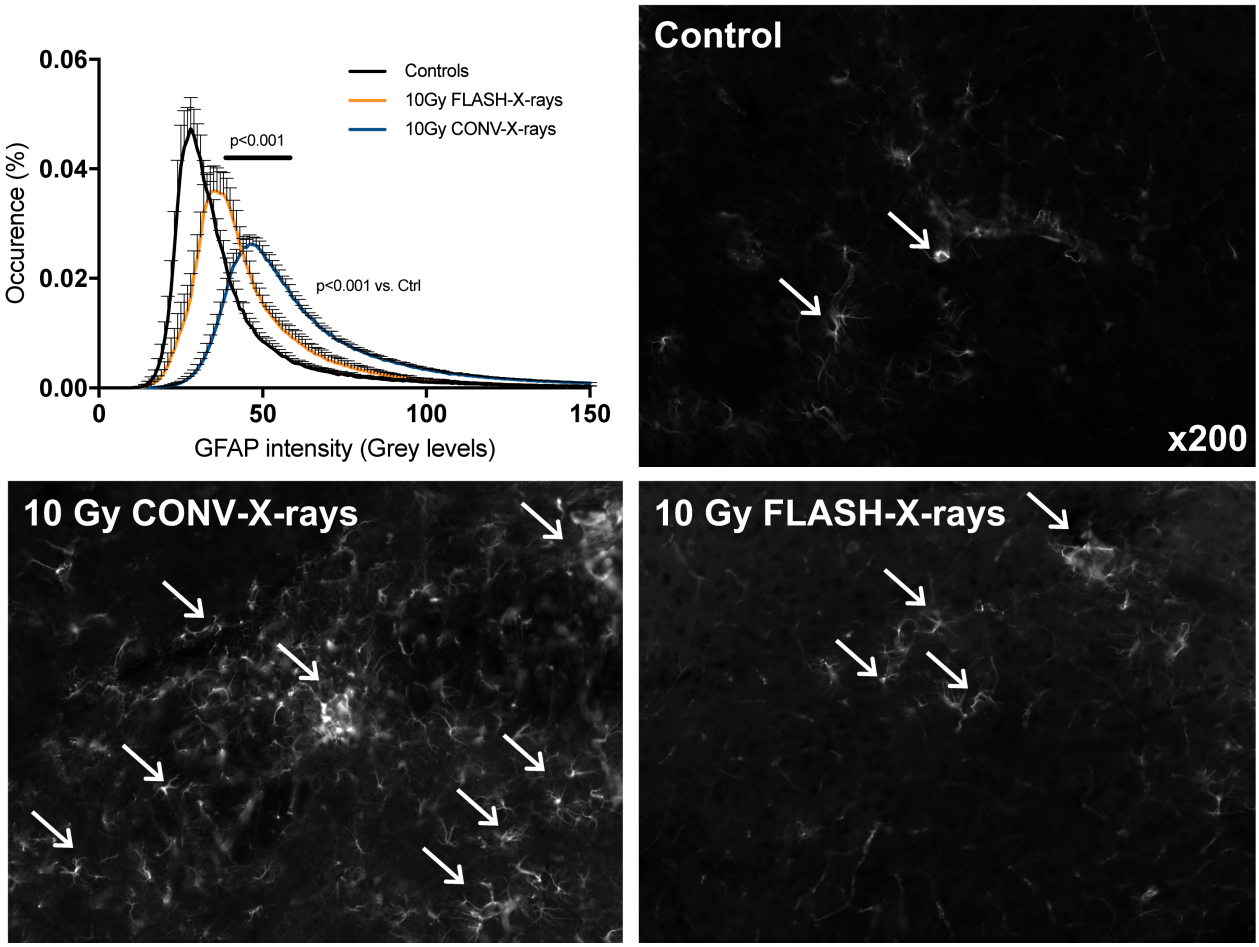

Figure 3: Reactive astrogliosis assessment by GFAP immunostaining on brain striatum sections of non-irradiated mice (Control) and mice irradiated with 10 Gy WBI with FLASH-X-rays or with X-rays delivered at conventional dose-rate (CONV-X-rays). Arrows point at GFAP positive cells in the striatum. Quantification realized over striatum sections with MoreHisto software. Statistical analysis performed with non-parametric Wilcoxon test.

Discussion

The current experiments performed at the ESRF resulted in a first proof of concept that the FLASH effect occurs after X-ray irradiation using synchrotron sources. These results confirm the preservation of memory two and six months after a 10 Gy single dose FLASH-X-rays WBI delivered at a mean dose-rate of 37 Gy/s (i.e. dose-rate in the slice of 12000 Gy/s), and previously described after Flash-electrons above 100 Gy/s [7] (i.e. dose-rate in the pulse of 4.5 10^5 Gy/s). Furthermore, a preservation of hippocampal cell division and a reduced induction of reactive astrogliosis were also shown. In comparison, 10 Gy WBI delivered at a conventional dose-rate (0.05 Gy/s) irreversibly impaired memory skills and induced a large decrease in hippocampal cell division along with the development of a reactive astrogliosis.

Interestingly, our results reported here on preservation of cognition when using FLASH-X-rays delivered by synchrotron light open optimistic venues for further investigations. The fact that both electrons and photons delivered at ultra-high dose-rates do not affect cognition supports the hypothesis that the time of exposure has a major impact on the biological responses. One hypothesis is that the kinetics of heterogeneous and homogeneous chemical reactions are modified by the brief exposure to the radiation and subsequently modify the downstream biological cascades. In addition, a careful examination of dose delivery modalities obtained with the synchrotron light source (ESRF) and the pulsed electron beam (eRT6) shows a major difference. Indeed, with the pulsed electron beam, the dose is temporally fractionated in microsecond pulses (eRT6). In these conditions, the threshold for cognitive protection was identified around a mean dose-rate of 30 Gy/s and a total absence of toxicity was observed above 100 Gy/s[7]. With the ESRF synchrotron, the dose is spatially fractionated in slices of 50 μm, delivered at a mean dose-rate of 37 Gy/s and induces cognitive protection. This last result suggests that the mean dose rate is not the parameter of importance but that the dose rate in the pulse or in the slice is paramount. In addition, the combination of both spatial and temporal delivery of the dose might bring an additional benefit for normal tissue protection.

The results obtained so far on the impact of ultra-high dose-rate irradiation on biological tissues are challenging and spark intense discussions in the radio-oncology and radiobiology fields. Reports published more than 40 years ago [21–23] already described a protective effect on mammalian cells and rodents exposed to a pulsed electron beam. However, these investigations were not continued until recently, when we published a provocative paper showing that ultra-high dose-rate “FLASH” irradiation induces less toxicity on normal tissues with a similar anti-tumor effect on lung tumors compared to conventional dose-rate irradiation [6]. We further confirmed the protective effect of FLASH-RT on normal brain tissues [7] while another team observed protective effects of FLASH-RT in the gut of mice after whole-abdominal

irradiation [24] using a pulsed electron beam obtained with a modified clinical LINAC [4]. Today, there are only a few experimental devices available to deliver ultra-high dose-rate irradiations across large volumes of tissue. Moreover, their usefulness is limited to experimental settings either because of the beam energy (Kinetron, Orsay, France; Oriatron, Lausanne, Switzerland) or because of the positioning of the target [4] (Modified clinical accelerator, Stanford, USA), which limits the irradiations to superficial targets or small animals. Despite these technological limitations, clinical applications are promising. A first successful phase I veterinary clinical trial was conducted in cat patients [9] and confirmed the minimal toxicity induced by FLASH-RT delivered with electrons, associated with an efficient tumor control in the context of non-curable SCCs. These results support the development of devices suitable for clinical applications. A first option is the implementation of the FLASH technology into Intra-Operative Radiation-Therapy (IORT) as the current IORT protocols consist in delivering a single high dose of radiation to the tumor bed during surgery with 10 MeV electron beams. According to our results, IORT-FLASH should minimize normal tissue toxicity and should be suitable for treatment optimization by dose-rate escalation in non-curable tumors such as pancreatic, Head and Neck and brain cancers. Another possible solution is the use of Very-High Energy Electrons (VHEE) devices [25] that will probably be possible in the future pending major technological improvement. Given the distribution profile of photons in matter, the production of ultra-high dose-rate X-rays (FLASH-X-rays) is a good option to accelerate the clinical transfer. Nowadays, 3rd generation synchrotrons are the only facilities enabling the translation of FLASH-X-rays into clinics with practical limitations related to the size and cost of synchrotron facilities. This issue might be overcome by using compact synchrotron sources (ThomX, MAP) that are able to produce high brilliance X-rays, and to deliver dose-rates above 100 Gy/sec [26,27]. Indeed, our results show that FLASH-X-ray minimizes radiation-induced toxicity making it very significant from a therapeutic perspective.

The benefit of FLASH-RT, delivered either with electrons or X-rays [28], could be important for cancer patients for whom normal tissue toxicities limit the therapeutic management. This could be especially interesting for brain tumor patients, including Glioblastoma (GBM), medulloblastoma as well as brain metastases [29–31]. GBM is one of the most common primary malignant brain tumors in adults with a very poor prognosis and a median survival around 14.6 months after diagnosis [32]. The standard treatments for GBM consist of surgical resection whenever possible, followed by radiotherapy (total dose of 60 Gy in 2 Gy fraction) +/- concomitant chemotherapy based on Temozolomide [29,30,33]. This type of aggressive protocol induces irreversible neurocognitive complications including memory deficits with learning impairments and loss of attention [34,35] and FLASH-RT might provide an opportunity to safely escalate the dose to improve tumor control.

In conclusion, our study shows that FLASH-X-rays produced using a synchrotron light source reduces the normal brain toxicity following whole brain irradiation compared to irradiation at conventional dose-rates. Therefore, this technique could improve the therapeutic management of brain cancer. Additional investigations are needed to assess whether spatial fractionation of the beam (MRT), achievable with synchrotron light sources, could further improve the normal tissue protection without compromising tumor control.

Conflict of interest statement

None of the authors have any conflicts of interest.

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References

[1] Favaudon V, Fouillade C, Vozenin MC. Radiothérapie « flash » à très haut débit de dose: Un moyen d'augmenter l'indice thérapeutique par minimisation des dommages aux tissus sains ? Cancer/Radiotherapie 2015;19:526–31. doi:10.1016/j.canrad.2015.04.006.

[2] Favaudon V, Fouillade C, Vozenin MC. La radiothérapie FLASH pour épargner les tissus sains. Medecine/Sciences 2015;31:121–3. doi:10.1051/medsci/20153102002.

[3] Fouillade C, Favaudon V, Vozenin MC, Romeo PH, Bourhis J, Verrelle P, et al. Les promesses du haut débit de dose en radiothérapie. Bull Cancer 2017;104:380–4. doi:10.1016/j.bulcan.2017.01.012.

[4] Schüler E, Trovati S, King G, Lartey F, Rafat M, Villegas M, et al. Experimental Platform for Ultra-high Dose Rate FLASH Irradiation of Small Animals Using a Clinical Linear Accelerator. Int J Radiat Oncol Biol Phys 2017;97:195–203. doi:10.1016/j.ijrobp.2016.09.018.

[5] Montay-Gruel P, Meziani L, Yakkala C, Vozenin M-C. Expanding the therapeutic index of radiation therapy by normal tissue protection. Br J Radiol 2018:20180008. doi:10.1259/bjr.20180008.

[6] Favaudon V, Caplier L, Monceau V, Pouzoulet F, Sayarath M, Fouillade C, et al. Ultrahigh dose-rate FLASH irradiation increases the differential response between normal and tumor tissue in mice. Sci Transl Med 2014;6. doi:10.1126/scitranslmed.3008973.

[7] Montay-Gruel P, Petersson K, Jaccard M, Boivin G, Germond JF, Petit B, et al. Irradiation in a flash: Unique sparing of memory in mice after whole brain irradiation with dose rates above 100 Gy/s. Radiother Oncol 2017;124:365–9. doi:10.1016/j.radonc.2017.05.003.

[8] Montay-Gruel P, Acharya MM, Petersson K, Alikhani L, Yakkala C, Allen BD, et al. Spare the brain but not the tumor, oxygen-dependent benefits of FLASH-radiotherapy. Prep 2018.

[9] Vozenin MC, De Fornel P, Petersson K, Favaudon V, Jaccard M, Germond JF, et al. The adavatge of FLASH radiotherapy confirmed in mini-pig and cat-cancer patients. Clin Cancer Res 2018;In press.

[10] Bräuer-Krisch E, Serduc R, Siegbahn EA, Le Duc G, Prezado Y, Bravin A, et al. Effects of pulsed, spatially fractionated, microscopic synchrotron X-ray beams on normal and tumoral brain tissue. Mutat Res - Rev Mutat Res 2010;704:160–6. doi:10.1016/j.mrrev.2009.12.003.

[11] Bouchet A, Bräuer-Krisch E, Prezado Y, El Atifi M, Rogalev L, Le Clec'h C, et al. Better Efficacy of Synchrotron Spatially Microfractionated Radiation Therapy Than Uniform Radiation Therapy on Glioma. Int J Radiat Oncol Biol Phys 2016;95:1485–94. doi:10.1016/j.ijrobp.2016.03.040.

[12] Bouchet A, Serduc R, Laissue JA, Djonov V. Effects of microbeam radiation therapy on normal and tumoral blood vessels. Phys Medica 2015;31:634–41. doi:10.1016/j.ejmp.2015.04.014.

[13] Serduc R, Vérant P, Vial JC, Farion R, Rocas L, Rémy C, et al. In vivo two-photon microscopy study of short-term effects of microbeam irradiation on normal mouse brain microvasculature. Int J

Radiat Oncol Biol Phys 2006;64:1519–27. doi:10.1016/j.ijrobp.2005.11.047.

[14] Bouchet A, Lemasson B, Le Duc G, Maisin C, Bräuer-Krisch E, Siegbahn EA, et al. Preferential effect of synchrotron microbeam radiation therapy on intracerebral 9l gliosarcoma vascular networks. Int J Radiat Oncol Biol Phys 2010;78:1503–12. doi:10.1016/j.ijrobp.2010.06.021.

[15] Jaccard M, Durán MT, Petersson K, Germond JF, Liger P, Vozenin MC, et al. High dose-per-pulse electron beam dosimetry: Commissioning of the Oriatron eRT6 prototype linear accelerator for preclinical use: Commissioning. Med Phys 2018;45:863–74. doi:10.1002/mp.12713.

[16] Fournier P, Crosbie JC, Cornelius I, Berkvens P, Donzelli M, Clavel AH, et al. Absorbed dose-to-water protocol applied to synchrotron-generated x-rays at very high dose rates. Phys Med Biol 2016;61:N349–61. doi:10.1088/0031-9155/61/14/N349.

[17] Crosbie JC, Fournier P, Bartzsch S, Donzelli M, Cornelius I, Stevenson AW, et al. Energy spectra considerations for synchrotron radiotherapy trials on the ID17 bio-medical beamline at the European Synchrotron Radiation Facility. J Synchrotron Radiat 2015;22:1035–41. doi:10.1107/S1600577515008115.

[18] Antunes M, Biala G. The novel object recognition memory: Neurobiology, test procedure, and its modifications. Cogn Process 2012;13:93–110. doi:10.1007/s10339-011-0430-z.

[19] Acharya MM, Christie L-A, Lan ML, Donovan PJ, Cotman CW, Fike JR, et al. Rescue of radiation-induced cognitive impairment through cranial transplantation of human embryonic stem cells. Proc Natl Acad Sci 2009;106:19150–5. doi:10.1073/pnas.0909293106.

[20] David DJ, Wang J, Samuels BA, Rainer Q, David I, Gardier AM, et al. Implications of the functional integration of adult-born hippocampal neurons in anxiety-depression disorders. Neuroscientist 2010;16:578–91. doi:10.1177/1073858409360281.

[21] Hendry JH, Moore J V, Hodgson BW, Keene JP. The constant low oxygen concentration in all the target cells for mouse tail radionecrosis. Radiat Res 1982;92:172–81. doi:10.2307/3575852.

[22] Weiss H, Epp ER, Heslin JM, Ling CC, Santomasso A. Oxygen depletion in cells irradiated at ultra-high dose-rates and at conventional dose-rates. Int J Radiat Biol 1974;26:17–29. doi:10.1080/09553007414550901.

[23] Epp ER, Weiss H, Djordjevic B, Santomasso A. The Radiosensitivity of Cultured Mammalian Cells Exposed to Single High Intensity Pulses of Electrons in Various Concentrations of Oxygen. Radiat Res 1972;52:324. doi:10.2307/3573572.

[24] Loo BW, Schuler E, Lartey FM, Rafat M, King GJ, Trovati S, et al. (P003) Delivery of Ultra-Rapid Flash Radiation Therapy and Demonstration of Normal Tissue Sparing After Abdominal Irradiation of Mice. Int J Radiat Oncol 2017;98:E16. doi:10.1016/j.ijrobp.2017.02.101.

[25] Malka V, Faure J, Gauduel YA. Ultra-short electron beams based spatio-temporal radiation biology

and radiotherapy. Mutat Res - Rev Mutat Res 2010;704:142–51. doi:10.1016/j.mrrev.2010.01.006.

[26] Jacquet M, Suortti P. Radiation therapy at compact Compton sources. Phys Medica 2015;31:596–600. doi:10.1016/j.ejmp.2015.02.010.

[27] Jacquet M. Potential of compact Compton sources in the medical field. Phys Medica 2016;32:1790–4. doi:10.1016/j.ejmp.2016.11.003.

[28] Bartzsch S, Oelfke U. Line focus x-ray tubes - A new concept to produce high brilliance x-rays. Phys Med Biol 2017;62:8600–15. doi:10.1088/1361-6560/aa910b.

[29] Stupp R, Brada M, van den Bent MJ, Tonn JC, Pentheroudakis G. High-grade glioma: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Ann Oncol 2014;25:93–101. doi:10.1093/annonc/mdu050.

[30] Tsao MN, Rades D, Wirth A, Lo SS, Danielson BL, Gaspar LE, et al. Radiotherapeutic and surgical management for newly diagnosed brain metastasis(es): An American Society for Radiation Oncology evidence-based guideline. Pract Radiat Oncol 2012;2:210–25. doi:10.1016/j.prro.2011.12.004.

[31] Martin AM, Raabe E, Eberhart C, Cohen KJ. Management of Pediatric and Adult Patients with Medulloblastoma. Curr Treat Options Oncol 2014;15:581–94. doi:10.1007/s11864-014-0306-4.

[32] Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol 2009;10:459–66. doi:10.1016/S1470-2045(09)70025-7.

[33] Stupp R, Mason W, van den Bent MJ, Weller M, Fisher BM, Taphoorn MJB, et al. Radiotherapy plus Concomitant\nand Adjuvant Temozolomide for Glioblastoma. N Engl J Med 2005;352:987–96. doi:10.1056/NEJMoa043330.

[34] Glauser TA, Packer RJ. Cognitive deficits in long-term survivors of childhood brain tumors. Childs Nerv Syst 1991;7:2–12. doi:10.1007/BF00263824.

[35] Gehrke AK, Baisley MC, Sonck ALB, Wronski SL, Feuerstein M. Neurocognitive deficits following primary brain tumor treatment: systematic review of a decade of comparative studies. J Neurooncol 2013;115:135–42. doi:10.1007/s11060-013-1215-2.

3. Scientific publication 3: Spare the Brain but not the Tumor, Oxygen-Dependent Benefits of FLASH-Radiotherapy

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This study was realized in collaboration with the team of C. Limoli in the Universtiy of California Irvine, USA. Murine models of normal brain irradiation and murine glioblastoma models were developed to investigate the FLASH-effect on the normal brain tissue at both cellular and functional levels, along with the characterization of the anti-tumor effect triggered by FLASH-RT. Moreover, both *in-vivo* and *in vitro* experiments were led to identify the crucial role played by the dioxygen in the FLASH-effect.

For GBM studies, subcutaneously xenografted U87 GBM tumors, orthotopic H454 GBM tumors and transgenic mice developing spontaneous GBM were irradiated with the eRT6 prototype at FLASH or conventional dose-rate to investigate the FLASH-RT anti-tumor effect. NOR test was performed on orthotopic GBM-bearing mice one month post 10 Gy irradiation to evaluate the memory function.

For normal tissue response, WT and *Thy1-eGFP* transgenic mice were irradiated to the whole brain with 10 Gy FLASH-RT or conventional dose-rate according to the set-up described previously (P. Montay-Gruel et al. 2017). A series of behavioral tests were performed in UCI 1, 2 and 6 months post-irradiation to evaluate the hippocampal, amygdala and perirhinal cortex-associated cognitive functions. Radiation-induced neuroinflammation, astrogliosis and hippocampal neuronal structure modifications were also investigated.

ROS production after FLASH and conventional dose-rate irradiation was assessed *in vitro* by water radiolysis products measurements and *in vitro* investigation of the cell clonogenic potential was realized for different oxic conditions. A total-body irradiation model of zebrafish was also used to investigate the effect of antioxidant molecules on the radiation-induced toxicities triggered by both dose-rates. Both normal brain tissue and glioblastoma models were used to decipher the role of dioxygen in the FLASH effect. Tumor irradiation at both FLASH and conventional dose-rates was performed in hypoxic and hyperoxic conditions on xenografted and orthotopic GBM models. Moreover, NOR test was performed on animals irradiated in hyperoxic conditions to assess the impact of an increase in dioxygen concentration in the brain on the cognitive functions.

Tumor growth delay, tumor burden measurement and animal survival following the delivery of FLASH and conventional dose-rate revealed no difference in the anti-tumor effect between both dose-rates in all tested animal models.

The cognitive function investigation 1 and 6 months post-irradiation revealed a better performance in all tests for the animals irradiated to the whole brain at 10 Gy with FLASH-RT, whereas animals irradiated at conventional dose-rate exhibited a significant impairment in cognition. These results suggest a preservation of hippocampal, amygdala and perirhinal cortex-associated cognitive functions triggered by FLASH-RT. These results correlated with lower levels of neuroinflammation and astrogliosis post-FLASH-RT compared to conventional dose-rate. Moreover, no alteration in neuronal morphology and structure was found in the hippocampus of FLASH irradiated mice whereas conventional dose-rate irradiation was associated with modifications in the dendritic structure.

The measurement of ROS production by water radiolysis after FLASH and conventional dose-rates showed higher H_2O_2 concentrations after conventional dose-rate irradiation compared to FLASH-RT and for a similar delivered dose. An increased clonogenic potential was also observed after FLASH-RT compared to conventional dose-rate irradiation for low dioxygen concentration. Moreover, zebrafish morphology after total-body irradiation was less altered by FLASH-RT than by conventional dose-rate and antioxidant treatment did not modify the FLASH-induced injury. Memory protection triggered by FLASH was reversed when the irradiation was delivered in hyperoxic conditions, and no effect of hypoxia was found on the tumor growth delay induced by FLASH-RT.

This study confirmed the anti-tumor efficacy of FLASH-RT on different GBM models along with a normal tissue protection demonstrated by an absence of functional impairment and cellular alteration on multiple behavioral tests and histology analyses.

Our results suggest that dioxygen concentration in the tissue at the moment of irradiation is crucial for the FLASH effect occurrence, and that lower ROS production could explain the differential effect observed in the normal tissue, while the concentration of dioxygen in the hypoxic tumors do not affect the tumor growth delay. We showed for the first time that the differential dioxygen consumption and ROS production at the moment of irradiation, induced by a large decrease in the irradiation time, is a major primary event involved in the FLASH effect occurrence.

All irradiations, tumor studies, NOR tests, astrogliosis immunofluorescence, *in vitro* and zebrafish experiments were performed in Lausanne. All other behavioral tests, neuroinflammation and neuronal structure assessments were performed in UCI. This collaboration was concretized in December 2016 by a 4-week visit in UCI to perform behavioral tests and neuronal structure experiments, funded by an ESTRO Mobility grant.

SPARE THE BRAIN BUT NOT THE TUMOR, OXYGEN-DEPENDENT BENEFITS OF FLASH-RADIOTHERAPY

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Running title: Neurocognitive sparing by FLASH radiotherapy

Key words: ultra-high dose rate irradiation, cognitive dysfunction, glioblastoma, neuronal morphology, neuroinflammation, oxygen toxicity, reactive oxygen species

Abstract

Exploiting the differences between normal and tumor tissues lies at the heart of every oncology treatment, and in this context, we describe a potentially paradigm shifting technology, termed FLASH, involving the delivery of ionizing radiation at ultra-high dose rates (100 Gy/s and above). Compared to conventional dose rate modalities (CONV), we showed that FLASH was iso-efficient at controlling GBM growth in three different experimental models. Importantly, only FLASH was found to spare radiation-induced cognitive deficits in learning and memory in tumor bearing and tumor free animals. Moreover, at 6 months after exposure, FLASH attenuated anxiety- and depression-like behaviors and improved significantly fear extinction, whereas CONV caused permanent alterations in cognitive functionality. The long-lasting neurocognitive benefits provided by FLASH were also associated with significant reductions in neuroinflammation and a marked preservation of neuronal morphology and dendritic spine density. Lastly, we provide compelling evidence that the beneficial effects of FLASH are mediated through a mechanism involving the rapid consumption of oxygen and resultant induction of transient hypoxia. With the capability to significantly exploit differences in oxygen tension between normal tissue and tumors comes genuine therapeutic gain, and the exciting capabilities that FLASH afford promise to hasten the translation of this groundbreaking modality of irradiation into clinical practice.

Introduction

Radiation therapy (RT) remains an essential part of cancer treatment and more than half of all cancer patients are treated with RT for tumor control. Over the decades multiple strategies have focused on increasing the amount of toxicity imparted to the tumor while minimizing such effects in the surrounding normal tissue and today, the benefit of RT would increase dramatically if normal tissues surrounding the tumor could tolerate higher doses of radiation [1-3]. In the last decade, major advances in high precision treatment delivery and multimodal imaging have improved tolerance to RT, leading to an increased proportion of patients tumor free with fewer side effects [4]. Despite this progress, selective protection of normal tissue remains a significant clinical challenge and radiation-induced toxicities as a consequence of tumor eradication still cause complications that adversely impact quality of life. This latter fact largely remains an unmet medical, and points to an urgent need to develop improved RT modalities for combating those cancers refractory to state-of-the- art protocols.

This issue is especially critical for those afflicted with brain tumors including glioblastoma (GBM), medulloblastoma as well as brain metastases [5-7]. GBM is one of the most common primary malignant brain tumors in adults with a very poor prognosis, having a median survival around 14.6 months after diagnosis [8]. Standard treatment of GBM consists in surgical resection followed by RT and concomitant chemotherapy (Temolozomide). Typical protocols for GBM consist in 60 Gy total doses delivered in 2 Gy fractions, 5 days a week. In this clinical context, progressive and debilitating neurocognitive complications inevitably arise, where survivors routinely exhibit impairments in learning and memory, attention, executive function and exhibit a range of mood disorders that severely compromise their quality of life [9-13]. Past work from us has linked adverse neurocognitive outcomes following cranial irradiation to a range of associated pathologies including, reductions in dendritic complexity and spine density [14-17], reductions in microvascular density [18-20], reduced myelination and synapse density [21] and increased neuroinflammation [22,23]. In addition, large clinical trials report disappointing results and poor responses of GBM to chemotherapy, where patients with recurrent disease show a median survival of 25 weeks [24] with no added benefit of Bevacizumab [25]. Given the paucity of promising data for these patient cohorts major improvements are clearly needed to improve the efficacy and tolerance of RT in managing this deadly disease.

Rationally in view of protecting at maximum the organs at risks, manufacturers of electron acceleration have directed their developments toward improving the conformational delivery of the beam. On the other hand, some advancement has come at the implementation of particle accelerators, where proton and heavier ion therapy facilities highlight some of the more promising current modalities.

Previous experiments conducted with short pulses of X-rays on lymphocytes [26] or more recently conducted with protons on human-hamster hybrid cells and skin cells [27-29] including micro-channel radiotherapy that operates at 200 Gy/s dose-rate [30] have shown reduced levels of cytogenetic damage, along with a marked protection of normal tissue from acute and long-term radiation injury. In part based on these findings, and in efforts to more fully develop a truly innovative approach to RT, we have been the first to conceptualize and implement a novel modality of irradiation, named FLASH radiotherapy (FLASH-RT) [31] [32]. In the brain, cognitive sparing was demonstrated when single doses of 10 Gy were delivered at dose rates exceeding 100 Gy/s with an apparent threshold when dose rates fell below ~30 Gy/s [32]. While this impressive FLASH effect seems likely to enhance significantly the therapeutic ratio of this unique cancer treatment, more detailed studies into the potential impact on brain tumor control as well as the mechanistic basis of neurocognitive sparing remained to be elucidated.

Based on these recent developments we first investigated the response of brain tumors to FLASH-RT. Using three different models of GBM, we found that FLASH-RT was iso-efficient to radiation therapy delivered at conventional dose rates for GBM control. Interestingly, FLASH-irradiated mice did not exhibit neurocognitive decline, prompting the current series of studies aimed at elucidating the underlying mechanisms. Therefore, we investigated the impact of FLASH-RT on a variety of neurocognitive outcomes using robust experimental models and found significant short (1 month) and long-term (6 months) benefits on cognition and a host of associated pathologies. Further experimentation explored the physico-chemical basis of the FLASH effect [33]. Based on the ultra-high dose rates inherent to the FLASH irradiation (i.e 1000 times faster), we postulated that FLASH exposure could induce a rapid consumption of localized oxygen, precluding typical reoxygenation kinetics. This is in distinct contrast to irradiation at conventional dose rate where an equilibrium is established between vascular oxygen delivery, diffusion and the consumption of free oxygen available in the tissue. To critically test this tenet, we modulated oxygen tension in the brain and found that FLASH-RT immediately consumed local O_2 leading to rapid normal tissue hypoxia, thereby pointing to a fundamental mechanism for the protective effect of FLASH-RT. Here we report our findings elucidating the mechanisms of neuroprotection in the FLASH irradiated brain, thereby promoting the translational potential of this promising new modality for the treatment of brain cancer.

Results

FLASH and conventional dose-rate irradiations are iso-efficient in the control of GBM but only FLASH-RT preserves neurocognitive function.

We evaluated the antitumor effect of FLASH irradiation in 3 different tumor models.

First, a tumor growth delay assay was performed on subcutaneous U87 human glioblastoma engrafted in the flank of female nude mice. A mean tumor growth delay of 16 days was observed in the group irradiated with 20 Gy FLASH single dose compared to the control. Interestingly, this tumor growth delay was similar to the one induced by a single dose of 20 Gy at conventional dose-rate, showing an equivalent anti-tumor effect, independent of the dose-rate (**Fig. 1A**).

Second, to validate this result, the antitumor effects of FLASH and conventional dose-rate irradiation modalities were compared on genetically modified mice *GFAP-HRas^{V12}; GFAP-CRE; p53$^{flox/wt}$* that spontaneously develop GBM between 8 to 35 weeks of age. A prophylactic treatment of 15 Gy single-dose delivered with FLASH-RT or conventional dose-rate irradiation, was given to 5-week-old mice before the onset of GBM. The follow-up of these animals showed that radiotherapy significantly increased survival but showed no difference between the irradiation modalities (**Fig. 1B**).

The foregoing established that dose rate modulation inherent to each modality did not compromise tumor control, and validated a critically important feature of FLASH-RT. However, to demonstrate the distinguishing characteristic that sets FLASH-RT apart from any other current modalities, it was necessary to analyze its capability to protect normal tissue in the presence of the tumor. Tumors typically confound neurocognitive assessment due to a variety of factors, so we focused our initial measurements on a time (1 month post-IR) preceding the development of major neurological complications. To assess cortical and hippocampal memory function, novel object recognition (NOR) was performed on orthotopic glioblastoma bearing mice whole-brain irradiated with a single dose of 10 Gy delivered by FLASH-RT or conventional dose-rate irradiation. Importantly, we observed that deficits in the NOR tasks were not affected by the tumor *per-se* since non-irradiated animals showed a discrimination index (DI, means ± SEM, n=8-10) of 54.80 ± 8.10. However, a drastic and significant drop in the DI was observed for the conventional dose-rate irradiation group compared to controls (11.32 ± 5.71 vs 54.80 ± 8.10; P=0.0043). Remarkably, animals subjected to FLASH-RT exhibited no such decrements and were statistically similar to controls (45.67 ± 2.87 vs 54.80 ± 8.10; P=0.2403) (**Fig. 2E**). This encouraging finding marks the first demonstration of an intervention of any kind, capable of preventing radiation-induced neurocognitive function in the presence of an orthotopic tumor, thereby paving the way for the following mechanistic studies.

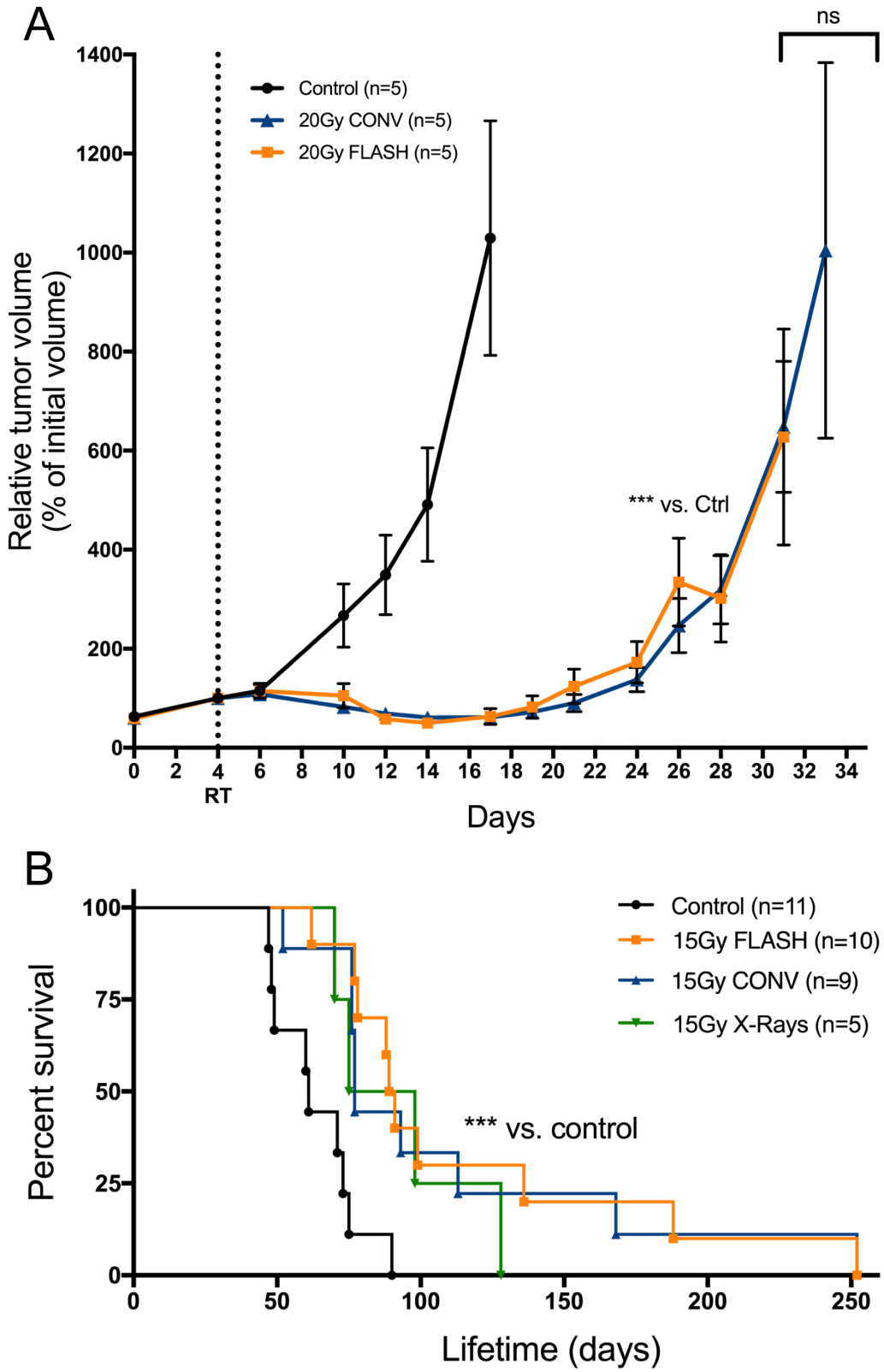

Figure 1: FLASH and conventional dose-rate irradiations display a similar anti-tumor effect. Iso-efficacy of FLASH-RT and conventional dose-rate irradiation was observed by tumor-growth delay assessment of U87 human GBM xenografted tumors irradiated at 0 or 20 Gy with FLASH-RT (FLASH) and conventional dose-rate irradiation (CONV) (**A**). Transgenic *GFAP-HRas^{V12}; GFAP-CRE; p53$^{flox/wt}$* mice developing spontaneous GBM were prophylactically whole-brain irradiated at 0 or 15 Gy with FLASH-RT (FLASH), conventional dose-rate irradiation (CONV) or X-rays (**B**). For tumor growth volumes, results are given in mean-value ± SEM. P values are derived from unpaired t-tests performed after Gaussian distribution assessment with Shapiro test. Survival curves analyzed with Mantel-Cox test: ****P*<0.001

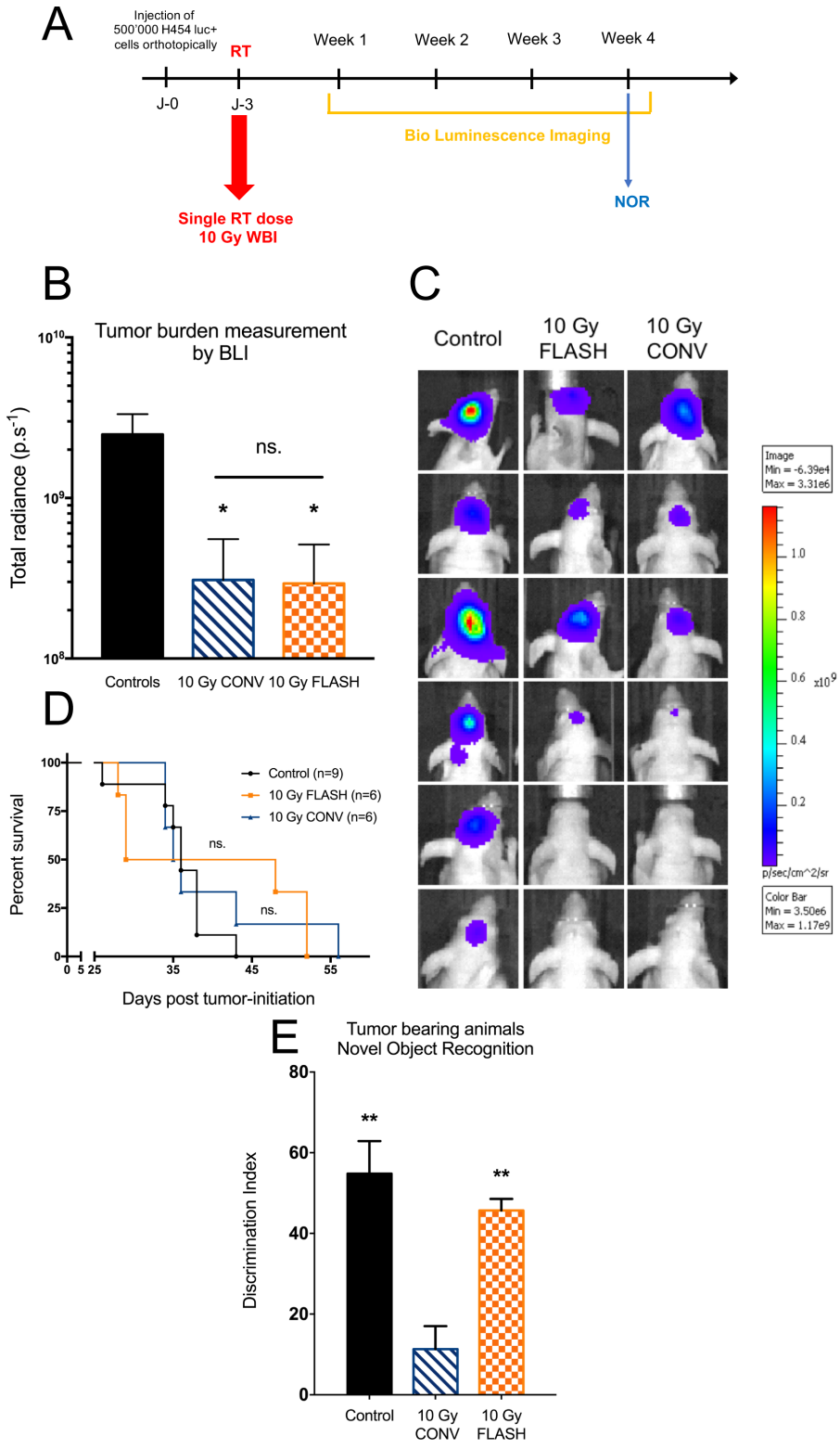

Figure 2: FLASH-RT can treat an orthotopic GBM model and protects the neurocognitive unction of GBM-bearing mice. Mice orthotopically implanted with H454 murine GBM cells were treated with a single-dose whole brain irradiation of 0 (n=11) or 10 Gy with FLASH-RT (FLASH, n=6) or conventional dose-rate irradiation (CONV, n=6) **(A)**. Tumor burden evaluation was realized with BLI quantification 4 weeks post-irradiation (**B** & **C**), along with survival follow-up showing no difference between FLASH and CONV groups (**D**). Results are given in mean values ± SD. P values are derived from unpaired non-parametric Mann-Whitney tests: *P<0.05 vs. controls. Statistics on survival curves were performed with Mantel-Cox test against the control group (**B**). Neurocognitive function was assessed 4 weeks post-irradiation in all groups of mice by novel object recognition and showed a preservation of the memory in the FLASH group. Results are given in mean values ± SD. P values are derived from unpaired non-parametric Mann-Whitney tests: **P<0.01 vs. CONV group. (**E**).

FLASH-RT minimizes radiation-induced cognitive dysfunction

To further elucidate the many possible neurobiological mechanisms responsible for the beneficial cognitive effects of FLASH-RT, it was necessary to move to tumor free mice, where short and long-term studies could be conducted in the absence of confounding disease. Therefore, to explore the capability of FLASH irradiation to minimize neurocognitive decrements caused by cranial irradiation, animals were subjected to a series of spontaneous exploration tasks known to interrogate hippocampal and cortical learning and memory. The first cohort of animals was first tested using the Novel Object Recognition (NOR) task. In the test phase, a significant overall group difference was found between the three cohorts for the DI ($F_{(2,33)} = 7.94$, $P=0.0015$). After a 5-minute retention interval between the familiarization and test phases, control mice showed a preference for the novel object (**Fig. 3A**) that was abolished by conventional dose-rate irradiation ($P<0.05$). Animals given FLASH irradiation were statistically indistinguishable from controls, and the FLASH cohorts were significantly different than the CONV cohort ($P<0.001$).

Following NOR testing, mice were habituated and tested on the Object in Place (OIP) task. All cohorts exhibited the same trends as in the NOR task, but variability within the groups precluded the data from reaching statistical significance (**Fig. 3B**).

Lastly, animals were subjected to the Temporal Order (TO) task, where animals were familiarized with two sets of objects, 4 hours apart (**Fig. 3C**). For this task, there was a significant difference between the cohorts for the DI during the test phase ($F_{(2,20)} = 12.9$, $P=0.0003$). Controls showed preference for the prior object compared to animals subjected to conventional dose-rate irradiation ($P<0.0001$), whereas the FLASH cohort was statistically indistinguishable from controls and differs significantly from the CONV cohort ($P<0.05$).

Mice from all cohorts subjected to spontaneous exploration tasks exhibited normal motor function and exploration, where the total exploration time (time spent exploring both novel and familiar objects) was unchanged following either irradiation modalities. These findings argue that results were not confounded by any radiation-induced motor decrements.

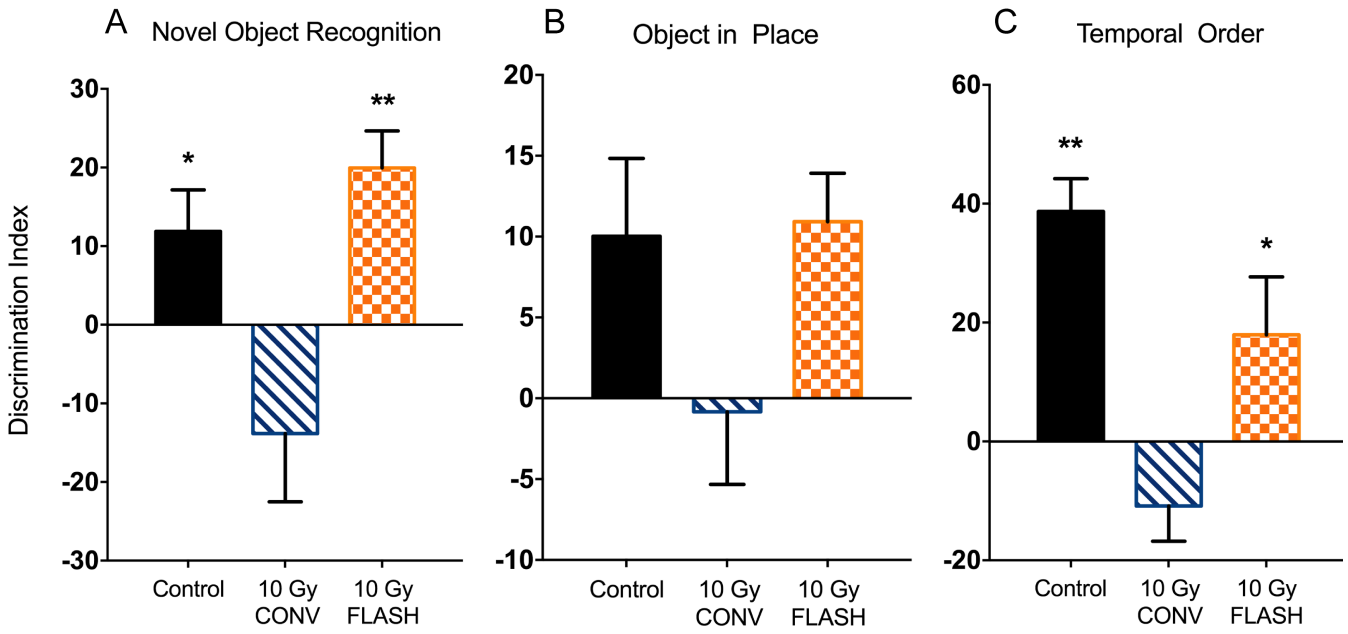

Figure 3: FLASH-RT minimizes radiation-induced learning and memory deficits caused by conventional dose-rate irradiation 1 month after exposure. Wild type mice were irradiated (0 or 10 Gy, head only) under conventional or FLASH dose-rate irradiation modalities and 1 month after exposure were subjected tested on spatial, episodic and temporal order memory retention using the novel object recognition (NOR, **A**), the object in place (OIP, **B**) and temporal order tasks (TO, **C).** The tendency to explore novel objects and/or location(s) can be assessed through a Discrimination Index (DI), calculated as ([Novel location exploration time/Total exploration time] – [Familiar location exploration time/Total exploration time]) × 100. Cranial irradiation delivered under conventional dose-rate conditions caused significant reductions in DI on the NOR and TO tasks and similar trends on the OIP task compared to controls. In each instance, FLASH-RT prevented radiation-induced cognitive deficits evaluated with the NOR, TO and OIP tasks. Data are presented as mean ± SEM (*N*=10-12 mice/group). *P* values are derived from ANOVA and Bonferroni's multiple comparisons test. **P*<0.05; ***P*<0.01, compared to the 10 Gy CONV group.

To determine whether the level of anxiety and/or depression differed between our groups, our second cohort of animals were subjected to an elevated plus maze (EPM) and a light-dark box (LDB) test to quantify anxiety-like behavior [34], along with a forced swim test (FST) used to quantify depression-like behavior 6 months after exposure. The EPM task provides a choice of remaining in either "open" or more protected, "closed", arms of the maze, while the LDB test quantifies the number of transitions between protected "dark" and less protected "light" regions of the apparatus. The FST test measures immobility or floating and is used as an indication of despair or "giving up".

Significant overall group effects between the cohorts for the time spent in either arm of the EPM task were found ($F_{(2,22)}$ = 6.77, *P*=0.005). Multiple comparison analysis revealed that controls spend significantly more time in the open arms compared to the animals irradiated at conventional dose-rate (P<0.005), while the FLASH cohort trended toward increased time in the open arms but was not significantly different than the CONV group (**Fig. 4A**). For the LDB test, significant overall group effects were again found between the cohorts ($F_{(2,22)}$ = 3.77, *P*=0.05). In this instance, similar analyses showed

that controls trended toward an increased number of transitions compared to CONV cohorts while the FLASH cohort exhibited a significantly higher number of transitions between light and dark regions of the apparatus compared to the CONV cohort (P<0.05) (**Fig. 4B**). In no instance did the FLASH cohorts exhibit elevated anxiety-like behavior compared to the CONV cohort. For the FST, analysis of time spent floating revealed a statistically significant overall group effect ($F_{(2,22)}$ = 7.48, *P*=0.005). Multiple comparisons analysis revealed that mice subjected to conventional dose-rate irradiation exhibited significantly increased time spent floating compared to controls (P<0.01) or FLASH irradiated animals (P<0.01) (**Fig. 4C)**. Collectively, these data demonstrate that FLASH irradiation reduced both anxiety- and depression-like behavior that persist 6 months following conventional dose-rate exposures, representing a significant benefit of FLASH-RT in preventing radiation-induced mood disorders.

The finding that FLASH-RT could attenuate anxiety and depression suggested it might have an impact on extinction memory. The inability to dissociate certain events and unpleasant outcomes can often lead to stress and anxiety, particularly if the process of fear extinction or inhibitory learning is impaired. The capability to engage this active process is critical for cognitive health, as it facilitates how stressful situations are managed [35,36]. To establish whether FLASH irradiation might facilitate fear extinction (FE) 6 months after exposure, mice were subjected to a rigorous protocol designed to determine whether mice could unlearn the association between a tone and mild foot shock. Data showed that irradiation had no impact on associative learning (T1-T3), as all cohorts exhibited robust freezing in response to the tone-foot shock pairing (**Fig. 4D**). In contrast, two-way RM ANOVA across three testing sessions (Day 1-3) revealed a significant overall group effect for the radiation effect ($F_{(2,1473)}$ = 31, *P*<0.00001) and extinction training ($F_{(14,1473)}$ = 25, *P*<0.00001), but not for the radiation × extinction training interaction ($F_{(28,1473)}$ = 1.25, *P*=0.18) (**Fig. 4D**). In control and FLASH irradiated mice, freezing progressively decreased over extinction training (20 trials over 3 days), while mice irradiated at conventional dose-rate showed significantly higher freezing (*P* < 0.05-0.0001) compared to the other cohorts during this same training interval (note: asterisks indicate significant differences between CONV vs FLASH) (**Fig. 4D**). Importantly, these differences persisted, as animals subjected to the fear extinction test 72h after the cessation of the extinction trials showed significant overall group differences ($F_{(2,25)}$ = 7.27, *P*<0.01) (**Fig. 4d1**). Multiple comparisons analysis showed that control and FLASH irradiated mice exhibited significant extinction behavior, while mice irradiated at conventional dose-rate could not, having significantly higher freezing compared to control (*P*<0.01) and FLASH cohorts (*P*<0.005) when the tone was presented in the extinction context (**Fig. 4d1)**. Collectively, cognitive data derived 6 months following exposure, provides compelling evidence for the neurocognitive sparing of FLASH-RT compared to conventional dose-rate irradiation.

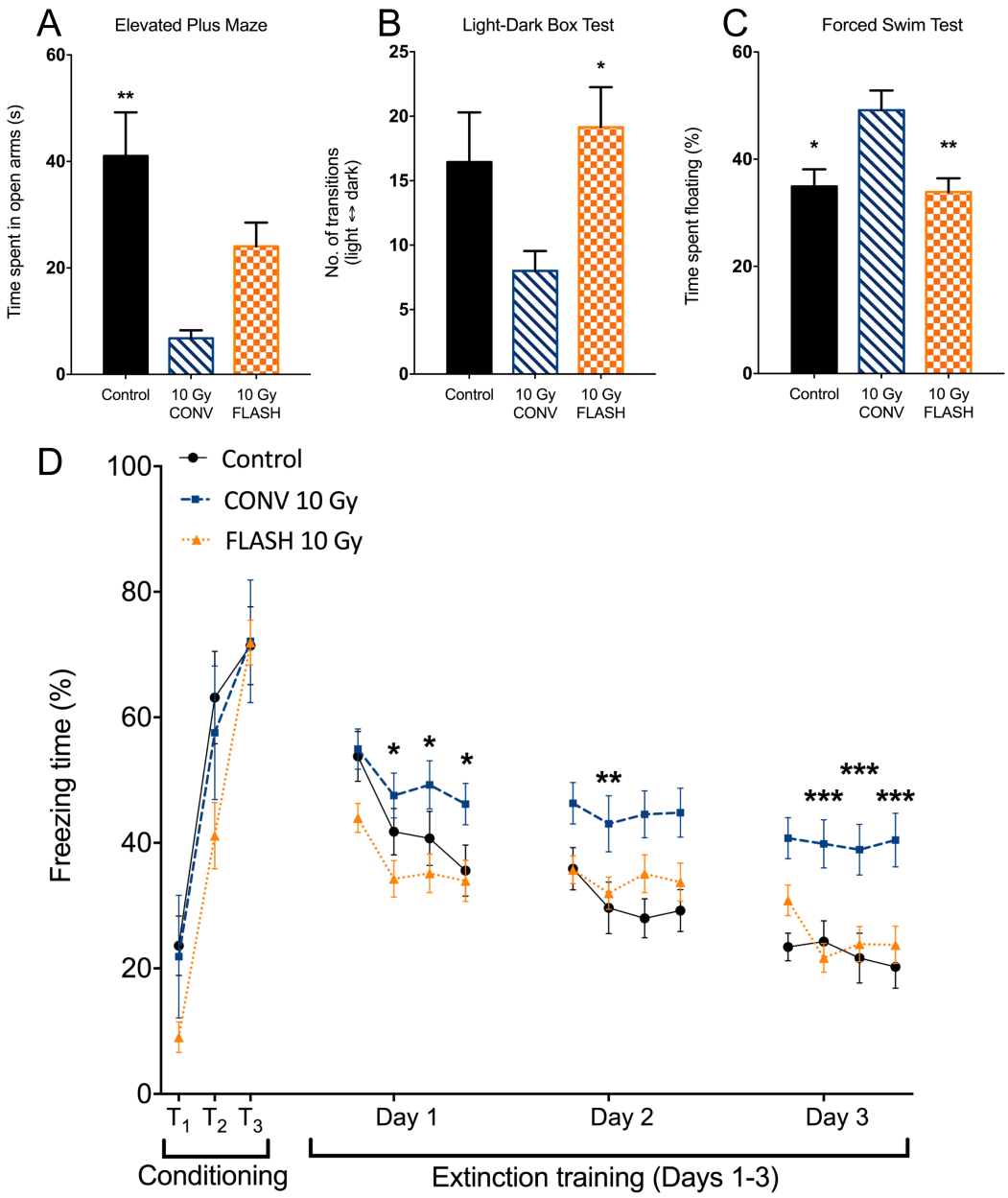

Figure 4: FLASH-RT reduces mood disorders and improves fear extinction compared to conventional dose-rate irradiation 6 months after exposure. Mice were subjected to the elevated plus maze (EPM, A) and the light dark box (B) to measure anxiety-like behavior, followed by the forced swim test (FST, C) to quantify depression-like behavior. Mice subjected to conventional dose-rate irradiation spent significantly less time in the open arms of the EPM and exhibited significantly fewer transitions between the light and dark regions of the box compared to controls. FLASH cohorts showed significant increase in the number of transitions on the light dark box compared to CONV. Mice exposed to conventional dose-rate irradiation spent significantly more time floating compared to either controls or FLASH, indicating the capability of the FLASH-RT to reduce depression. Data are presented as mean ± SEM (*N*=10-12 mice/group). *P* values are derived from ANOVA and Bonferroni's multiple comparisons test. **P*<0.05; ***P*<0.01, compared to the 10 Gy CONV group. Following these mood disorder tests, mice received 3 mild tone-foot shock pairings (2-s, 0.7mA) to establish fear, and were subjected to extinction training (tone alone) administered 24 h later to determine whether animals could unlearn the prior association within the same context. Exposure to either irradiation modalities did not impair the acquisition of conditioned fear as demonstrated by similar freezing times during the 3 tone-foot shock tri als for both cohorts (D). All mice showed a gradual decrease in freezing behavior over the 20 extinction trials however, the time spent freezing was significantly greater for the mice irradiated with conventional dose-rate modality as compared to controls or the FLASH cohort (D). Control and FLASH mice successfully abolished fear memory when compared to CONV group (d1). All cohorts n=8-12/group. Data are expressed as the mean ± SEM. **P* < 0.05; ***P* < 0.01, ****P* < 0.001; Two-way repeated ANOVA followed by Bonferroni post-hoc test.

Attenuation of neuroinflammation by FLASH-RT

Reactive astrogliosis consists of an abnormal stress-induced increase in the number of astrocytes, known to be responsible for cellular dysfunction. GFAP expression in the striatum of non-irradiated and irradiated mice was quantified by immunofluorescence to assess the occurrence of radiation-induced astrogliosis at different time-points. Following FLASH irradiation (2 weeks), very few GFAP positive astrocytes around CD31+ cells were observed around major striatum vessels (**Fig. 5**). A single dose of 10 Gy delivered at conventional dose-rate induced a significant 3.6-fold increase in GFAP immunoreactivity around CD31+ cells (P=0.0077), with the presence of numerous astrocytes in close proximity to blood vessels. Interestingly, only a non-significant 1.7-fold increase in GFAP immunoreactivity was observed after FLASH-RT (P=0.1573), with a cell distribution similar to that found in the non-irradiated controls. Two months post-irradiation, GFAP staining was minimal in the striatum of controls, suggesting the relative absence of reactive astrocytes, with a classical star-shaped morphology and extended processes (**Fig. 5)**. On the contrary, in animals subjected to 10 Gy delivered at conventional dose-rate, a significant 2.6-fold increase in GFAP immunoreactivity (P=0.0034) was observed in the striatum of mice at this latter post-irradiation time. At this same dose and time, GFAP immunoreactivity was non-significantly elevated by 1.2-fold after FLASH-RT (P=0.5335). This result suggests FLASH-RT minimizes activation of astrogliosis around blood vessels 2 weeks post-irradiation. Similarly, FLASH-RT significantly minimized radiation-induced scar astrogliosis 2 months post-irradiation compared to conventional dose-rate irradiation, thereby demonstrating long-lasting protection against inflammatory processes that are consistent with the preservation of cognitive function.

To ascertain the impact of each irradiation modality on microglia, we quantified the number of IBA-1$^+$ (resting or total) and CD68$^+$ (activated) microglia in the hippocampus at 1 and 6 months after exposure (**Fig. 6**). Representative images show typical staining patterns obtained for resting microglia (IBA-1$^+$ cells) in the hippocampus 1 month following each irradiation modality (**Fig. 6A**). At one month, little change is noted in the total number of resting microglia (**Fig. 6B).** At 6 months, significant group differences were found ($F_{(2,15)} = 12.2$, $P<0.001$), where conventional dose-rate irradiation was found to reduce the yield of resting microglia significantly compared to control ($P<0.005$) and FLASH ($P<0.001$) cohorts (**Fig. 6B**). Data indicates that resting microglial levels were relatively unresponsive to the different irradiation modalities used in this study.

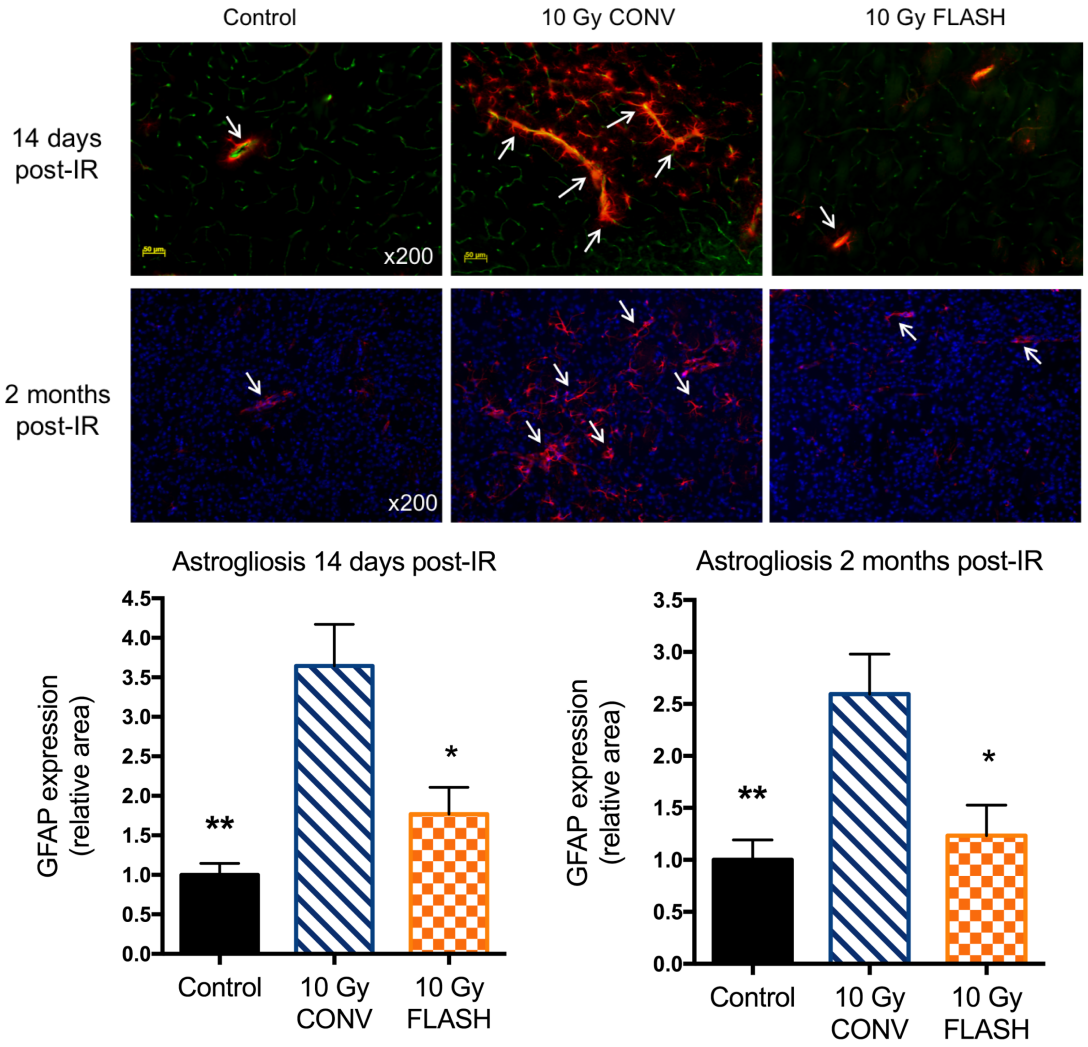

Figure 5: FLASH-RT reduces reactive gliosis over the course of 2 months in the striatum compared to conventional dose-rate irradiation. Immunofluorescence staining and upright fluorescence microcopy was performed on representative brain sections from each irradiated cohort. Representative high-resolution (200×) micrographs show GFAP$^+$ astrocytes (Red) in the vicinity of CD31$^+$ endothelial cells (Green). Evident from these images is that conventional dose-rate irradiation leads to a marked rise in GFAP$^+$ cells, indicating an increase in reactive gliosis. FLASH-RT did not elicit such increased levels of reactive gliosis and was comparable to controls. Quantification of these data at 2 weeks and 2 months post-irradiation reveals qualitatively similar yet significant effects. For each post-irradiation time, conventional dose-rate irradiation increased reactive gliosis significantly, whereas FLASH-RT did not, statistically similar to controls. Data are presented as mean ± SD (*N*=5 animals/group. *P* values derived from non-parametric Mann-Whitney unpaired test.)

The response of activated microglia was then investigated, where images show typical staining patterns obtained for activated microglia (CD68+ cells) in the hippocampus 1 month following each irradiation modality (**Fig. 6C**). Significant group differences in the yields of activated microglia were found 1 month after irradiation ($F_{(2,15)}$ = 155, P<0.0001). Consistent with past results, conventional dose-rate irradiation caused a significant increase in the number of CD68$^+$ cells compared to controls (P<0.0001) and importantly, FLASH irradiation reduced significantly (P<0.0001) the yield of activated microglia to control levels (**Fig. 6D**). Remarkably, similar protective effects were found to persist 6 months following

irradiation, where significant group differences were again found for the yields of activated microglia persisting in the irradiated hippocampus ($F_{(2,15)}$ = 30.1, P<0.0001). Compared to controls, significant (P<0.0001) increases in $CD68^+$ cells were observed 6 months after conventional dose-rate irradiation, an effect that was attenuated significantly (P<0.0001) by FLASH irradiation (**Fig. 6D**). Since the activation of microglia has been correlated with radiation-induced cognitive dysfunction, it is plausible that one mechanism by which FLASH-RT spares neurocognitive decline is by preventing the early activation of microglia and limiting their transition to a chronically activated state.

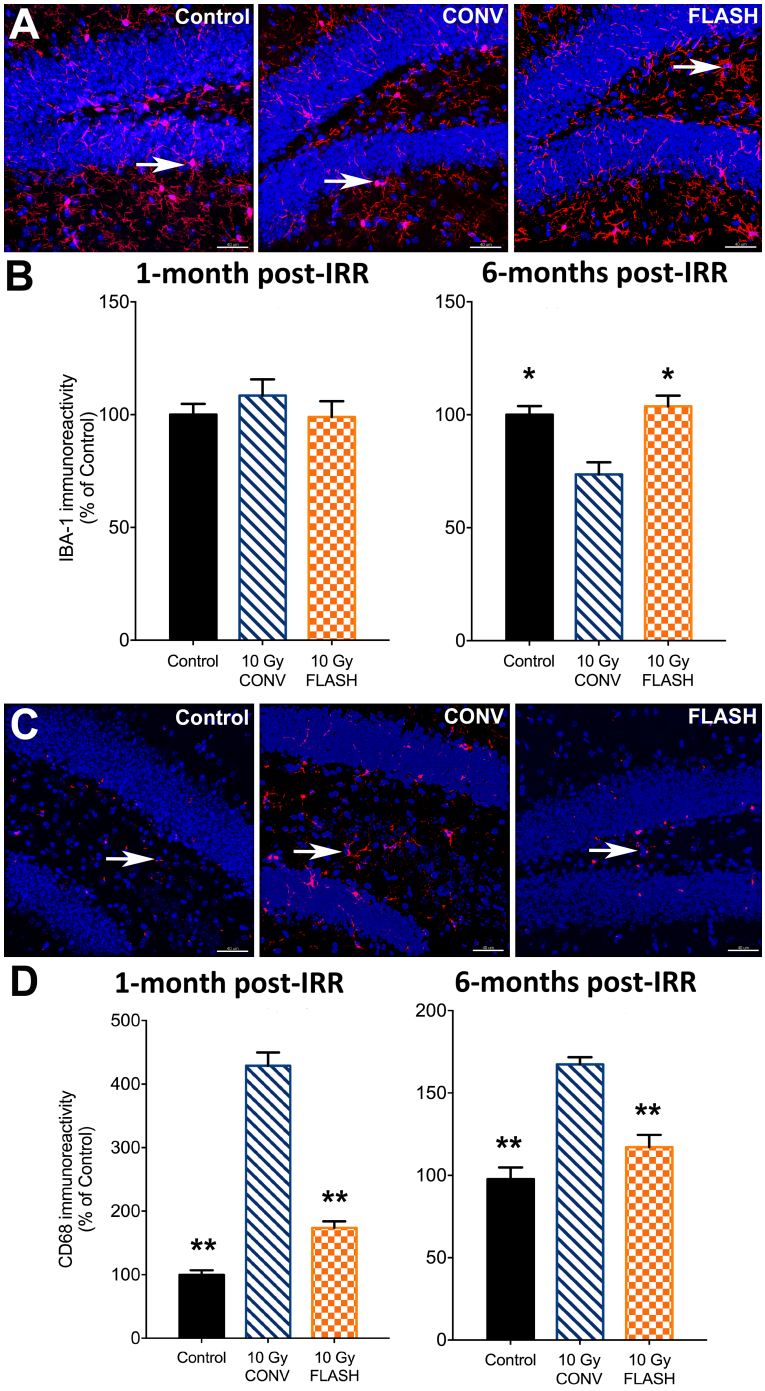

Figure 6: FLASH irradiation attenuates neuroinflammation compared to conventional dose-rate irradiation. Immunofluorescence staining and laser scanning confocal microcopy was performed on representative brain sections from each irradiated cohort. Representative high-resolution (60×) confocal micrographs from the hippocampal dentate hilus (DH) and granule cell layer (GCL) show IBA-1$^+$ microglial cell bodies (red) against the background of granule cell neurons (blue) for each of the experimental cohorts (**A**). Quantification of IBA-1$^+$ microglia show little effect at 1 month, but a reduction at 6 months after conventional dose-rate irradiation (**B**). For resting microglia, FLASH cohort was statistically indistinguishable from controls at each of these time points (**B**). Similar representative images were obtained for CD68$^+$ activated microglia (red) against the granule cell neurons (Blue) for each of the experimental cohorts (**C**). Quantification of CD68$^+$ cells show a marked increase in activated microglia at both 1 and 6 months following conventional dose-rate irradiation compared to controls (**D**). For each time-point, FLASH-RT prevented the increase in activated microglia, and was statistically indistinguishable from controls (**D**). Importantly, these data demonstrate the capability of the FLASH-RT to significantly attenuate neuroinflammation over protracted post-irradiation intervals. Data are presented as mean ± SEM (*N*=4 animals/group. *P* values derived from ANOVA and Bonferroni's multiple comparisons test. *$P < 0.05$; **$P < 0.01$, compared to the 10 Gy CONV group.

Preservation of host neuronal structure by FLASH-RT

Significant past data has found irradiation to significantly compromise the structure of multiple mature neuronal subtypes throughout various regions of the brain [37]. Reductions in dendritic complexity and spine density persist for weeks to months following exposure to a variety of radiation modalities and the resultant structural changes have been linked to functional decrements in cognition. Furthermore, microglia have been shown to re-shape the dendritic tree, through active pruning and trimming of unwanted or damaged dendritic arbors and spines to promote the structural plasticity needed for remodeling the synaptic landscape. Therefore, based on the capability of FLASH-RT to minimize radiation-induced cognitive dysfunction and attenuate the activation of microglia, another plausible mechanism underlying the neuroprotective properties of FLASH-RT may involve the preservation of host neuronal morphology.

To determine whether FLASH-RT had a differential impact on sparing host neuronal morphology compared to conventional dose-rate irradiation, mice were analyzed for changes in hippocampal granule cell neurons 1 and 6 months after exposure. Reconstructed images reveal the loss of dendritic complexity in the CONV cohorts compared to controls, an effect that was not evident in the FLASH irradiated group (**Fig. 7A**) 1 month after exposure. Higher resolution imaging provided the capability to quantify dendritic spines, and revealed that compared to controls, conventional dose-rate irradiation caused a substantial reduction in the number of dendritic spines (**Fig. 7B**, red), whereas no significant effect was observed in the FLASH irradiated cohort 1 month after exposure.

Quantification and analysis of dendritic parameters 1 month following irradiation indicated significant group differences for dendritic area ($F_{(2,9)} = 7.4$, $P<0.01$), length ($F_{(2,9)} = 4.5$, $P<0.05$) and branches ($F_{(2,9)} = 7.7$, $P<0.01$), and *posthoc* tests confirmed that CONV cohorts showed significantly reduced dendritic area ($P<0.05$) compared to controls with trends in length and branching (**Fig. 8A**). Importantly, FLASH irradiated cohorts were found to exhibit significant improvements in each of these morphologic parameters, showing increased dendritic area ($P<0.01$), length ($P<0.05$) and branches ($P<0.01$) compared to the CONV cohorts (**Fig. 8A**). Further analysis of dendritic spines revealed significant group differences for spine number ($F_{(2,9)} = 7.4$, $P<0.01$), density ($F_{(2,9)} = 29.4$, $P<0.0001$) and volume ($F_{(2,9)} = 8.1$, $P<0.01$) (**Fig. 8B**). *Posthoc* tests again confirmed that CONV cohorts showed significantly reduced dendritic spine numbers ($P<0.01$), density ($P<0.0001$) and volume ($P<0.05$) compared to controls. Interestingly, FLASH irradiated cohorts were found to prevent the radiation-induced reductions dendritic spine parameters typical of conventional dose-rate irradiation modalities, leading to significant increase in dendritic spine numbers ($P<0.05$), density ($P<0.0001$) and volume ($P<0.01$) compared to CONV cohorts (**Fig. 8B**). Morphologic data

collected 1 month following irradiation provide conclusive evidence that FLASH irradiation preserves host neuronal morphology compared to conventional dose-rate irradiation.

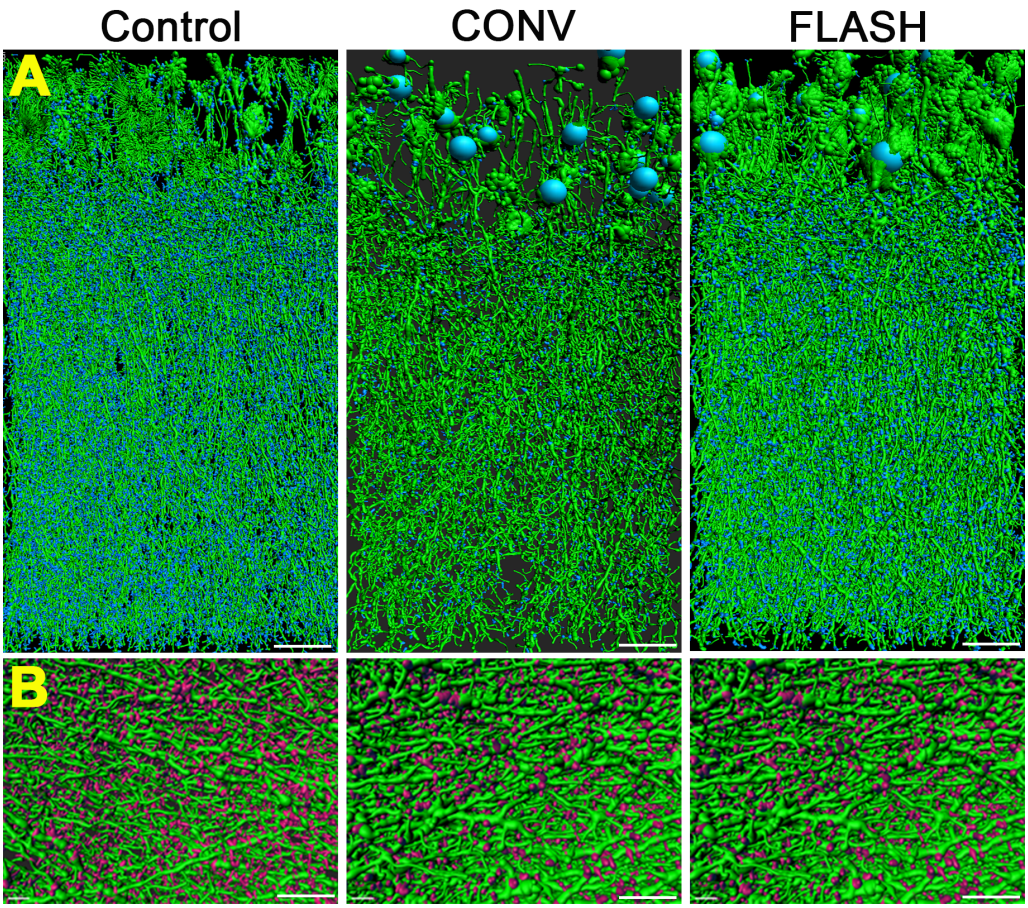

Figure 7: FLASH-RT preserves host neuronal morphology compared to conventional dose-rate irradiation 1 and 6 months following irradiation. Representative digital images derived from deconvoluted z-stacks depict granule cell neurons from the hippocampal dentate gyrus. Dendrites (green) are shown for each radiation modality along with major branch points (blue)(**A**). Evident from these images is that the arborization in the granule cell layer is reduced by conventional dose-rate irradiation compared to controls, an effect not apparent in the FLASH irradiated brain. Higher magnification images reveal dendritic spines (red) against the dendritic tree following similar irradiation modality (**B**). Apparent from these images is that dendritic spines numbers are reduced following conventional dose-rate irradiation compared to controls, an effect again not evident in the FLASH irradiated brain. Scale bar 20 µm (A) and 5 µm (B).

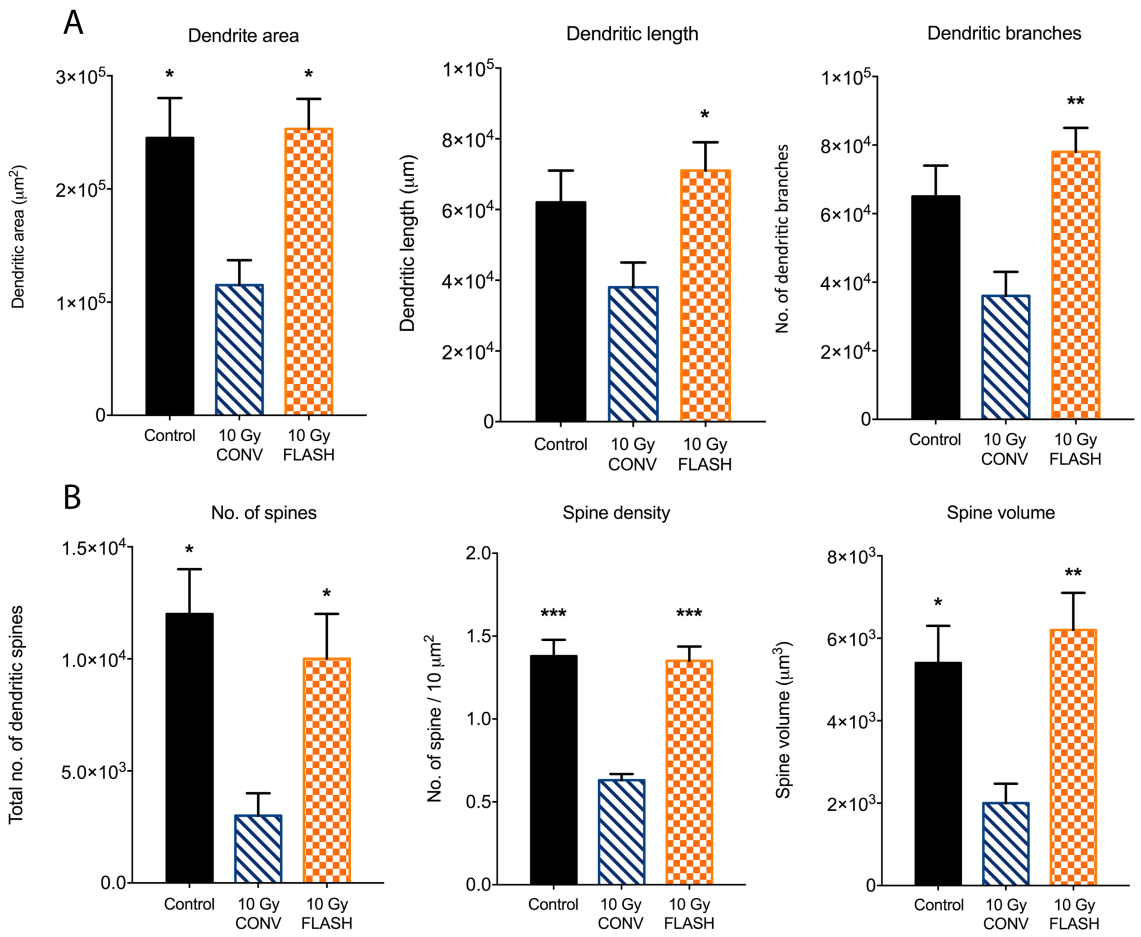

Figure 8: Morphological parameters of granule cell neurons are preserved 1 month after FLASH-RT compared to conventional dose-rate irradiation. Analysis of the granule cell neuron dendritic tree reveals reductions in dendritic area, length and branching following conventional dose-rate irradiation compared to controls, effects that were all significantly preserved in the FLASH irradiated brain (**A**). Similar findings were evident following quantification of dendritic spines, where reductions in spine numbers, density and volume were found after conventional dose-rate irradiation compared to controls (**B**). In each of these instances, FLASH-RT preserved dendritic spine parameters significantly (**B**). Data are expressed as mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; one-way ANOVA followed by Bonferroni's multiple comparison post hoc analysis.

Long-term analyses were conducted 6-months following exposure to determine the persistence of any structural changes caused by either irradiation modalities. Quantification and analysis of dendritic parameters at this protracted endpoint revealed significant group differences for dendritic area ($F_{(2,9)}$ = 36.4, *P*<0.0001), length ($F_{(2,9)}$ = 158, *P*<0.0001) and branches ($F_{(2,9)}$ = 10.6, *P*<0.005) (**Fig. 9A**). In each instance, *posthoc* analysis revealed that conventional dose-rate irradiation caused persistent and significant reductions in dendritic area (*P*<0.0001), length (*P*<0.0001) and branches (*P*<0.01) compared to controls. These effects were not found in the FLASH irradiated cohorts, and compared to CONV cohorts, FLASH irradiation spared significantly dendritic area (*P*<0.0001), length (*P*<0.0001) and branches (**Fig. 9A**).

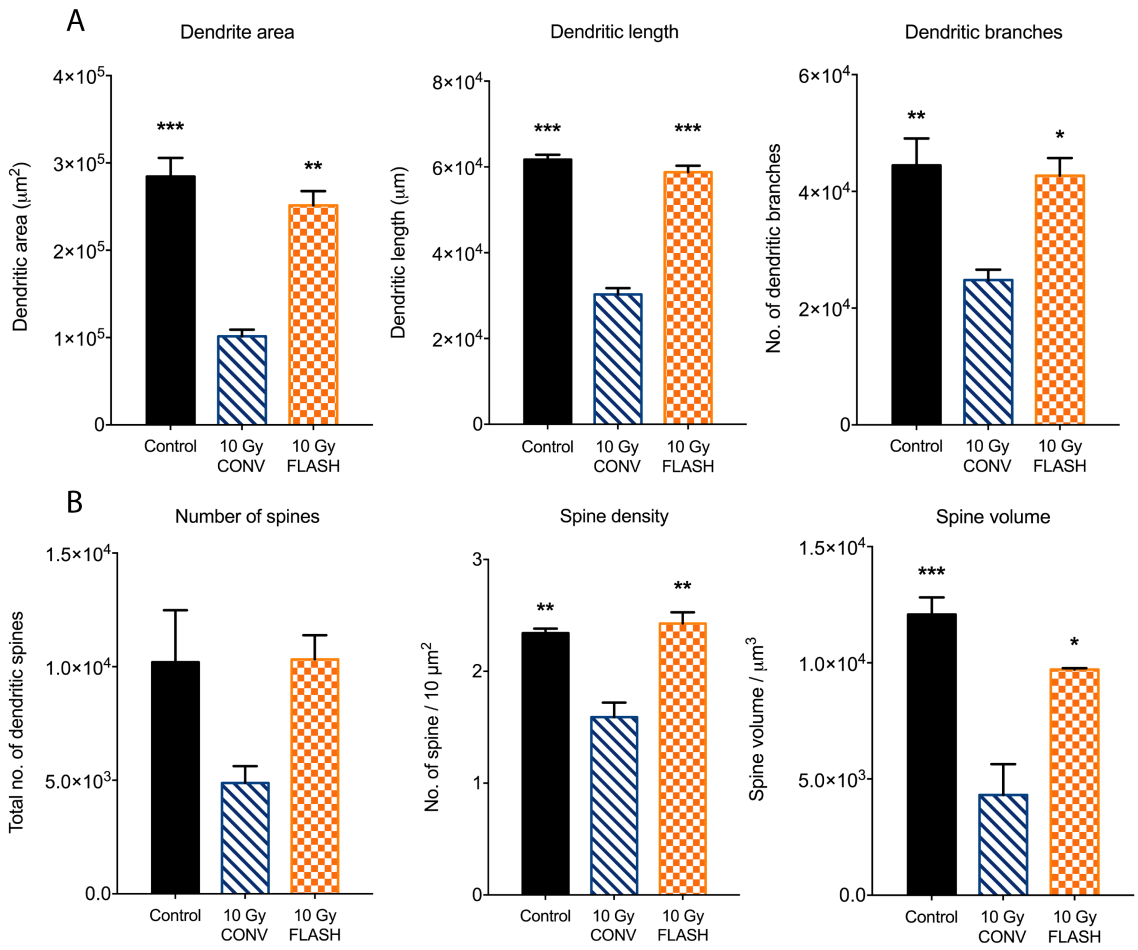

Figure 9: Morphological parameters of granule cell neurons are preserved 6 months after FLASH-RT compared to conventional dose-rate irradiation. Similar analyses of granule cell neurons at a protracted post-irradiation time reveals persistent reductions in dendritic area, length and branching following conventional dose-rate irradiation compared to controls, effects that were all significantly preserved in the FLASH irradiated brain (**A**). Similar findings were again evident following quantification of dendritic spines, where reductions in spine numbers, density and volume were found after conventional dose-rate irradiation compared to controls (**B**). With the exception of spine numbers, FLASH-RT again preserved dendritic spine parameters significantly (**B**). Data are expressed as mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; one-way ANOVA followed by Bonferroni's multiple comparison post hoc analysis.

Additional analyses of dendritic spine parameters revealed the persistent benefits of FLASH irradiation. At this protracted post-irradiation time point, significant group differences were found for spine density ($F_{(2,9)} = 22.4$, $P<0.0001$) and volume ($F_{(2,9)} = 20.1$, $P<0.0005$) (**Fig. 9B**). Cohorts subjected to conventional dose-rate irradiation exhibited significant reductions in dendritic spine density ($P<0.001$) and volume ($P<0.0005$), with trends for reduced spine numbers (**Fig. 9B**). While positive trends were found for increased spine numbers after FLASH-RT, multiple comparison analyses indicated that FLASH cohorts exhibited significant increases in spine density ($P<0.001$) and volume ($P<0.005$) compared to CONV cohorts (**Fig. 9B**). These data again point to the neuroprotective properties of FLASH-RT, where significant long-term sparing of radiation-induced structural degradation to mature neurons is likely to underlie a certain fraction of the functional improvements in cognition reported here.

To investigate further the mechanisms by which FLASH-RT might differentially impact the synaptic landscape in the brain compared to conventional dose-rate irradiation, we analyzed postsynaptic density protein 95 (PSD-95). Past work has shown conventional dose-rate irradiation to alter the expression of PSD-95 foci in certain hippocampal and cortical regions of the brain [14,37], and current studies sought to determine the response of this critical synaptic protein to FLASH irradiation. Representative images show the expression of fluorescent PSD-95 foci (red puncta) in the hippocampus for each cohort (**Fig. 10A**). Interestingly, 1 month following irradiation, significant group differences were found for PSD-95 foci on granule cell neurons in the dentate gyrus ($F_{(2,9)} = 10.3$, $P<0.005$). Animals subjected to conventional dose-rate irradiation exhibited significantly lower yields of PSD-95 compared to control ($P<0.005$) or FLASH ($P<0.01$) cohorts (**Fig. 10B**). These changes were not found at this time when PSD-95 was analyzed along dendrites of CA1 pyramidal neurons (**Fig. 10C**). Analysis at the 6 month time-point revealed similar changes in both brain regions where significant group differences were found in the dentate gyrus ($F_{(2,11)} = 8.44$, $P<0.005$) and hippocampal CA1 ($F_{(2,13)} = 15.7$, $P<0.001$). Cohorts subjected to conventional dose-rate irradiation were significantly lower than controls in the dentate gyrus ($P<0.005$, **Fig. 10D**) and CA1 region ($P<0.001$, **Fig. 10E**). While PSD-95 levels were not significantly different than controls at this latter time, data from each region do show trends that approach controls. It is noteworthy that for each of the foregoing situations, PSD-95 levels found after FLASH irradiation more closely paralleled those levels found in controls than after conventional dose-rate irradiation. These data suggest that the high dose rate FLASH-RT maintained a synaptic landscape more similar to a non-irradiated brain.

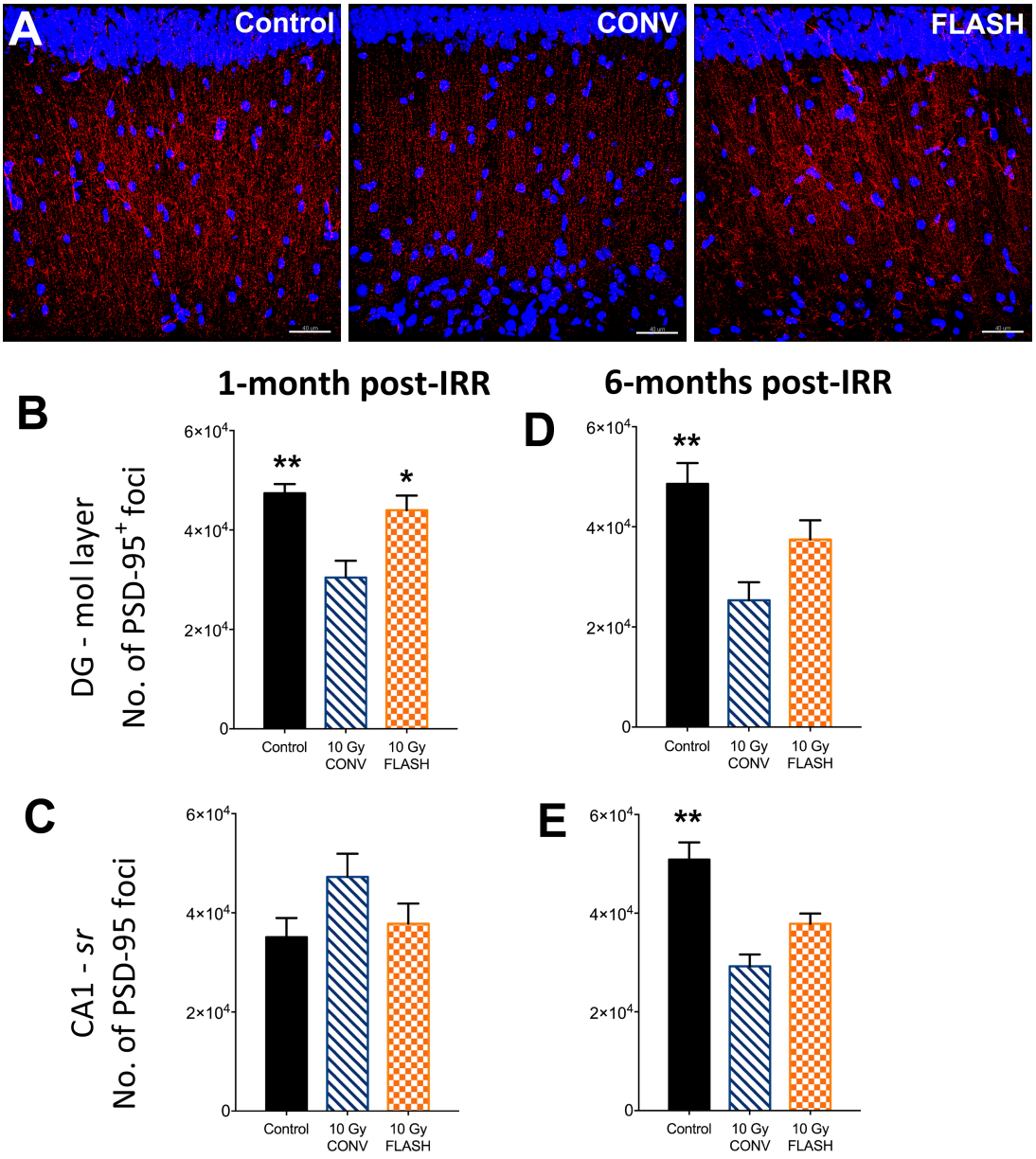

Figure 10: FLASH-RT does not perturb PSD-95 levels compared to the brains irradiated with conventional dose-rate. Representative fluorescence micrographs showing PSD-95 puncta (red) against the soma (blue) of granule cell neurons following each irradiation modality (**A**). Quantitative analyses of fluorescent PSD-95 foci show that exposure to conventional dose-rate reduces PSD-95 levels at both 1 (**B**) and 6 months (**D**) following exposure compared to controls, and effect not found in the FLASH irradiated brain. Analysis of CA1 pyramidal cell neurons reveals different trends in PSD-95 levels after irradiation, but after 1 month (**C**) or 6 month (**E**), the FLASH irradiated brain was similar to controls, and did not show the types of changes evident after conventional dose-rate irradiation. Data are expressed as the mean ± SEM. **P* < 0.05; ***P* < 0.01; Two-way repeated ANOVA followed by Bonferroni post-hoc test.

Physico-chemical basis of FLASH effect – minimizing oxygen toxicity

The partial pressure of oxygen in irradiated tissue is responsible for reactive oxygen species (ROS) production and leads to a large majority of radiation-induced damage. To evaluate its contribution in the FLASH effect, we induced an increase in oxygen concentration in the brain via carbogen breathing before and during the 10 Gy WBI (**Fig. 11A**). Carbogen breathing had no impact on the discrimination index (DI,

means ± SEM, n=8-10) of non-irradiated mice under both partial oxygen pressure (58.34 ± 2.63 vs. 60.11 ± 2.28; P=0.7990). A drop in DI was observed in carbogen breathing mice irradiated with conventional dose-rate irradiation, at levels comparable with the DI of air-breathing mice (14.05 ± 5.61 vs. 8.95 ± 2.29; P=0.2234). Nevertheless, a significant drop in DI was observed in the carbogen-breathing mice irradiated with FLASH-RT when compared to both controls and the air-breathing cohort treated with FLASH-RT (28.53 ± 4.27 vs. 60.11 ± 2.28; P=0.0003 and 28.53 ± 4.27 vs 52.99 ± 1.99; P=0.0079). These results show that increasing the oxygen concentration in the brain before and during FLASH-RT reverses the neurocognitive sparing observed under ambient air-breathing conditions. Therefore, the variation of oxygen concentration in the brain has a significant impact on the FLASH effect, where the differential consumption of oxygen and resultant production of toxic ROS in the brain may in part, explain why only FLASH-RT imparts normal tissue sparing under normoxia.

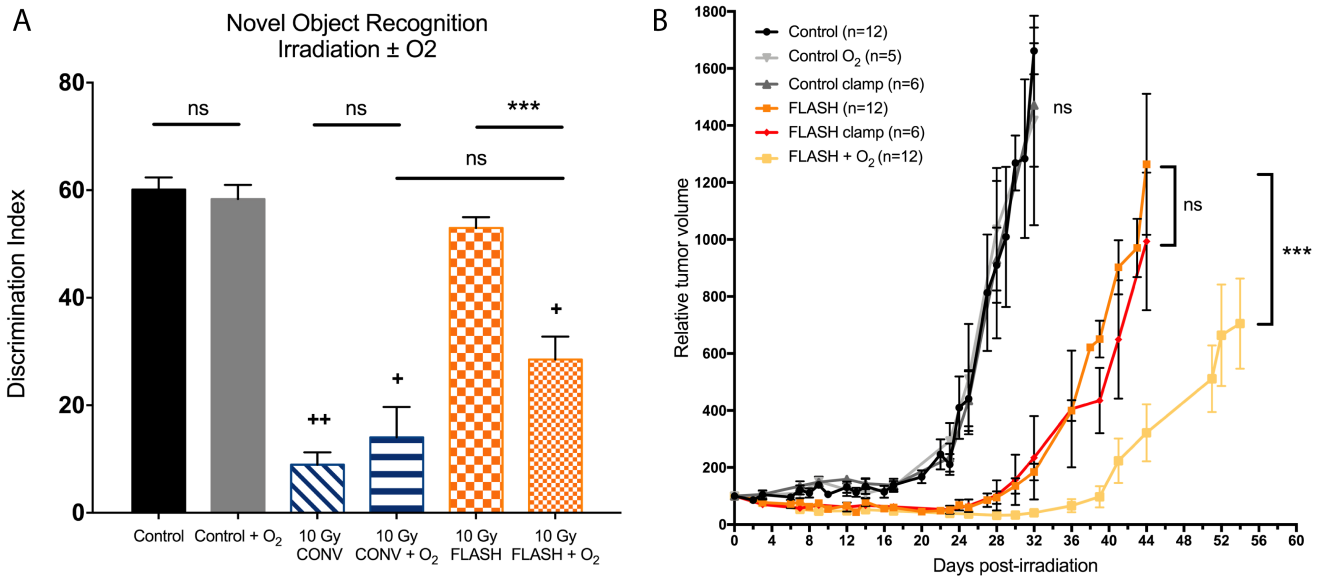

Figure 11: Intratumoral and normal brain pO_2 is critical for the FLASH effect. Increase in normal brain pO_2 by carbogen breathing before and during the delivery of 0 or 10 Gy WBI with FLASH-RT or conventional dose-rate irradiation showed a loss of the neurocognitive preservation induced by FLASH-RT in normoxic conditions (**A**). Results are given as mean ± SD and statistical analyses were performed with an unpaired non-parametric Mann-Whitney test. The effects of tumor-clamping induced hypoxia and carbogen-breathing induced hyperoxia on the tumor growth delay of a U87 human GBM xenografted tumors after the delivery of 20 Gy FLASH or conventional dose-rate irradiations showed that hypoxia does not impact the anti-tumor effect of FLASH-RT (**B**). Results are given as mean ± SEM and statistical analyses were performed with an unpaired Student t-test after Gaussian distribution assessment by Shapiro-Wilk normality: *P<0.05; **P<0.01***P<0.001.

As tumor tissue is typically more hypoxic, dose-rate induced changes in oxygen tension would be predicted to be minimal, while elevations in tumor oxygen levels should elicit enhanced kill. To formally test these possibilities, nude mice bearing U87 human GBM sub-cutaneous tumors were locally irradiated with a single 20 Gy dose of FLASH-RT. Oxygen depletion and supplementation were performed by tumor

clamping or carbogen breathing before and during the irradiation (**Fig. 11B**). No impact of the transient vascular clamps or carbogen breathing were observed on non-irradiated tumor growth over the course of this study. Compared to the air breathing and irradiated cohorts, a significant increase in tumor growth delay was observed in the FLASH-RT group, subjected to carbogen breathing, validating the expected increase in radiosensitization at elevated oxygen levels. On the contrary, no significant difference in the tumor growth delay was observed between clamped (hypoxic) and normoxic cohorts subjected to FLASH-RT. This result demonstrates how the FLASH effect depends on ambient oxygen levels, where FLASH-induced tumor control on hypoxic tumors such as subcutaneous engrafted GBM is oxygen-independent, increasing only when oxygen levels are experimentally elevated under carbogen breathing.

FLASH irradiation produces less H_2O_2

Our results lead us to postulate that for a given iso-dose, FLASH-RT leads to lower ROS production compared to conventional dose-rate irradiation. H2O2 is the only end-product of water radiolysis partial reactions that can be easily quantified, therefore H2O2 concentration was measured using a cell free radiochemical assay with AmplexRed after FLASH or conventional dose-rate irradiation **(Fig. 12A**). This measurement was used as a surrogate to evaluate the radiation-induced production of ROS by water radiolysis. An oxygen concentration of 4% in the water was used as mimetic of physiological oxygen tension. Interestingly, and for all doses above 10 Gy, a significantly lower concentration of H2O2 was observed in the FLASH irradiated aqueous solution ($P<0.001$), supporting our hypothesis that FLASH-RT reduces the production of toxic ROS.

Moving from a cell-free system, we then focused on the biological consequences of differential ROS production generated by both irradiation modalities. First, we investigated the modulation of intrinsic radiation sensitivity *in vitro* (**Fig. 12B**) using H454 murine glioblastoma cells irradiated at 21% and 4% of oxygen. Consistent with previous observations [31], for all delivered doses under 10 Gy, no significant difference in clonogenic survival was observed between FLASH and conventional dose-rate irradiations when cells were irradiated at an oxygen concentration of 21% or 4%. Nevertheless, a significantly better clonogenic survival was observed after the delivery of 20 Gy with FLASH-RT compared to conventional dose-rate irradiation. Decreasing the oxygen concentration to 4% during the irradiation enhanced the clonogenic survival of cells irradiated with FLASH-RT for doses above 15 Gy, suggesting a protection of these cells from radiation-induced damage when irradiated at a physiological oxygen partial pressure.

Moving then to a more complex model system, we performed ROS scavenging experiments *in-vivo* on zebrafish embryos using N-acetyl cysteine (NAC) and amisfostine (**Fig. 12C**). We validated the protective

effect of FLASH-RT on the development of zebrafish embryos. When 4-hour post-fertilization embryos were irradiated at 8 Gy, both conventional and FLASH dose-rate irradiations led to significant development alteration assessed by body length measurement. Nevertheless, FLASH-irradiated embryos were significantly less altered in their development than embryos irradiated with conventional dose-rate irradiation. To assess the importance of radiation-induced ROS production in the development damage induced by both irradiation modalities, zebrafish embryos were pre-incubated with antioxidant molecules (amisfostine or NAC). Embryos treated with antioxidants were protected from conventional dose-rate irradiation injury following irradiation at 8 Gy whereas no further protection was observed in the FLASH-RT groups. Moreover, even with the antioxidant treatments, the body length of embryos irradiated with conventional dose-rate irradiation was still lower than the FLASH-irradiated animals without any treatment. These results support the hypothesis that FLASH-RT leads to a lower production of ROS, and thus to a lower level of radiation-induced damages to the normal tissues.

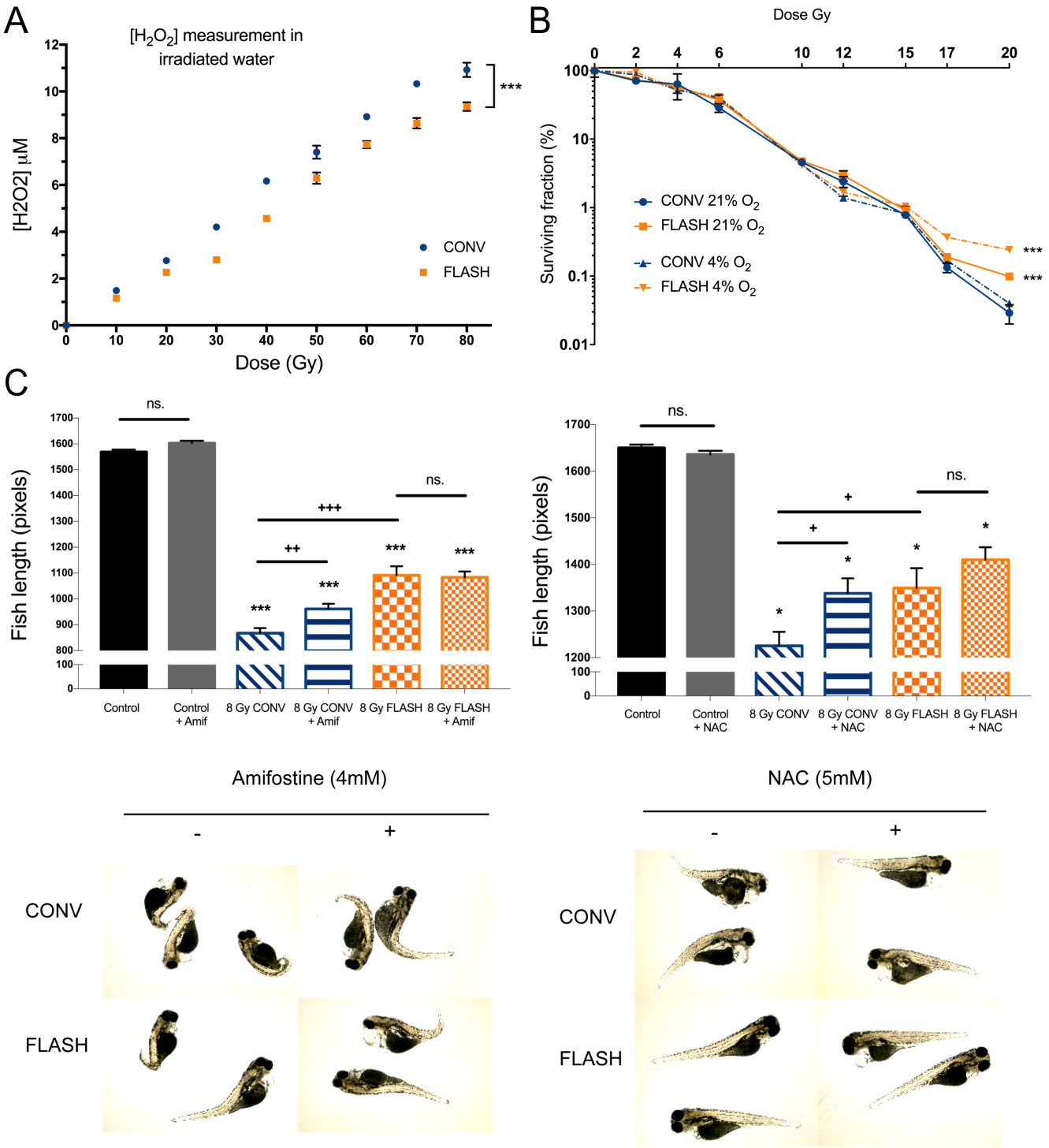

Figure 12: FLASH-RT produces less H_2O_2 and oxidative stress. ROS production by water radiolysis was evaluated via H_2O_2 measurement with AmplexRed method in water with FLASH or conventional dose-rate irradiations and showed that FLASH-RT produces less H_2O_2 than conventional dose-rate irradiation for a similar delivered dose (**A**). pO_2 impact on clonogenic survival of murine GBM H454 cell line was studied by irradiation at 21% and 1% of dioxygen with FLASH-RT and conventional dose-rate irradiation up to 20 Gy (**B**). Radio-induced alteration of the zebrafish morphology was assessed by body length measurement following total body irradiation with 8Gy FLASH-RT or conventional dose-rate irradiation and an antioxidant treatment by 5mM of NAC or 4mM of Amifostine FLASH-RT induces less morphological alterations than all other irradiated groups which are not reversed by antioxidant treatments (**C**). Results are given as mean ± SD and statistical analyses were performed with an unpaired non-parametric Mann-Whitney test: *P<0.05; ** P<0.01; ***P<0.001.

Discussion

Significance and rationale of the FLASH effect

Radiobiologists have sought to improve the differential sensitivity of tumor and normal tissue for decades for the enhancement of therapeutic efficacy. Chemical and biologic modifiers have been at the forefront of past and current research efforts designed to capitalize on different metabolic and signaling pathways that distinguish cancer cells from their normal tissue counterparts. Major advancements outside of these broader disciplines have come largely from conformational improvements in targeted beam delivery, where image guided pencil beam irradiations from a variety of stereotactic modalities provides improved tumor coverage while minimizing collateral dose outside the margins to the surrounding normal tissue. While beneficial, advancements in targeted beam delivery have been relatively incremental and costly and have failed to provide significant conceptual and innovative advancements able to capitalize on the differential radiation response between malignant and normal tissue.

Normal tissue sparing by FLASH irradiation has caught the field by surprise, representing a rather unexpected outcome that repudiates many of the traditional tenets of radiobiology. This potentially paradigm shifting technology, antagonizes a wealth of data documenting normal (and cancer) tissue sparing achieved by reducing the dose rate (~ 0.0017Gy/s). The fact that lowering the dose rate can prevent nearly every adverse radiobiological endpoint has long been attributed to DNA repair transpiring during dose delivery and contributes to the counterintuitive nature of achieving normal tissue sparing via FLASH irradiation [38]. Data presented now provides compelling evidence that FLASH irradiation can attenuate neurocognitive complications and associated pathology, without compromising tumor kill, through a mechanism involving oxygen consumption. These findings corroborate very recent work from our labs on the brain, lung, skin and gut [31,32], and point to the general applicability of FLASH irradiation, to exploit differences in oxygen tension between tumors and normal tissue for therapeutic gain.

FLASH-RT does not compromise tumor cure

Given the dire prognosis of brain tumor patients, novel strategies are in desperate need for improving the radiotherapeutic management of medulloblastoma, glioblastoma and brain metastases. These tumors are known to be highly resistant to the current treatments consisting of standard radiation and chemotherapy regimens. Subsequently, the prevalence of radiation-induced brain toxicities in pediatric and adult brain-tumor patients has negatively impacted patient care and quality of life of survivors for decades [9,10]. Given this rather dubious backdrop, brain tumors were logical targets for the assessment of FLASH-RT efficacy. The use of murine models was selected for reliable and quantitative comparisons

between FLASH-RT and conventional dose-rate irradiation efficacy. The feasibility of treating such murine tumors was also possible with the experimental LINAC available at the CHUV since the localization of the tumor in the brain was suitable for the depth dose penetration of the electron beam (1.5-2 cm). Three different models were used from the simple subcutaneous xenografted human GBM to the more complex orthotopic engrafted murine GBM and transgenic spontaneous GBM in mice. In all 3 cases, doses were administered as single fractions of 10, 15 and 20 Gy to achieve tumor control. Subcutaneous tumors given 20 Gy by either FLASH-RT or conventional dose-rate irradiation induced a growth delay of 16 days. FLASH or conventional dose-rate irradiations used to deliver prophylactic WBI at 15 Gy, before the onset of any symptoms in transgenic animals with spontaneous GBM, enhanced survival of mice by 2.5-fold. Similar treatments using 10 Gy delivered by either radiation modality in the orthotopic model achieved tumor growth delay of 12 days. In all 3 models, the anti-tumor efficacy of FLASH-RT and conventional dose-rate irradiation were equivalent and induced significant tumor growth delay without cure. These anti-tumor effects using multiple GBM models corroborate our previous observations on lung, breast and H&N tumor models [31]. However, present findings highlight the significant benefit of neurocognitive sparing provided by FLASH-RT in orthotopic tumor-bearing mice irradiated at 10 Gy.

These results open prospects on the possibility to improve the tumor control. As current GBM treatments consist of concomitant and adjuvant Temozolomide along with fractionated radiation-therapy, it will be necessary to further investigate the benefits of such combined treatments with FLASH-RT. Moreover, recently developed brain metastases mice models [39] or lentivirus-activated glioblastoma models [40] would provide additional opportunities to evaluate preclinical efficacy of the FLASH-RT modality. In addition, there is a growing interest in the study of GBM immune infiltration and its role in modulating treatment response and outcome. Recently, the use of CSF1 receptor inhibitors has been shown to modify the tumor immune microenvironment and abrogate the therapeutic resistance of GBM [41]. As FLASH-RT is preventing activation of immunosuppressive signals (i.e. TGF-β1 [31]), the investigation of the immune infiltration in the tumors treated with FLASH-RT represents an important next step toward the optimization of immuno-therapy protocols in combination with FLASH-RT.

Neuroprotective effects of FLASH-RT in tumor free mice

The provocative data showing that neurocognitive sparing can be achieved in tumor bearing, FLASH irradiated animals without compromising tumor control points to the potential promise of increasing the dose-rate to improve patient outcomes. Moreover, this significant finding represents the first demonstration of an intervention capable of improving neurocognitive outcome in an orthotopic tumor bearing mouse model without compromising radiocurability of the tumor itself. To further understand

the scope of the cognitive benefits and to interrogate the neuroprotective mechanisms of the FLASH effect paradigm, it was necessary to evaluate tumor free animals subjected to FLASH or conventional dose-rate irradiations so as to eliminate any potential confounding effects of the tumor on the normal brain. Each of these tasks revealed qualitatively similar trends, and most showed that compared to conventional dose-rate irradiation, FLASH-RT did not cause hippocampal and cortical based deficits in learning and memory. These findings demonstrate that impairments in spatial, episodic and recognition memory in the normal, disease free brain can be spared by FLASH-RT, and importantly, provided the means for evaluating the long-term consequences of irradiation on CNS functionality, where animals would otherwise succumb to tumor progression.

The comprehensive array of behavioral tasks presented in this study revealed significant cognitive deficits encompassing multiple forms of learning and memory and showed that relatively early changes in cognition extended to longer post-conventional dose-rate irradiation intervals. Animals subjected to FLASH-RT were preserved from radiation-induced increased anxiety- and depression-like behavior. The inability to dissociate unpleasant events is an active process of unlearning "extinction" involving the medial prefrontal cortex among other brain regions. The finding that conventional dose-rate irradiation elicits such marked and long-term deficits in fear extinction points to the similarities that the irradiated brain shares with other stress-induced disorders such as post-traumatic stress disorder (PTSD) [42,43]. The emergence and persistence of these aberrant behaviors post-treatment highlight the types of mood disorders that can plague the recovery and quality of life of brain tumor and cancer survivors alike, particularly pediatric brain cancer survivors [44,45]. Remarkably, FLASH irradiation eliminated each of these late neurocognitive complications, reducing anxiety as measured by the EPM and light dark box, reducing depression as measured by the FST, and improving extinction. These findings represent the first demonstration that such long-term, functional CNS endpoints can be resolved by any intervention (biological, chemical or physical), again highlighting the potential clinical utility of the FLASH effect.

To investigate the potential neurobiological mechanisms that might account for such impressive cognitive benefits, we investigated how each irradiation modality might differentially impact neuroinflammation. Reactive gliosis can disrupt CNS functionality by promoting proinflammatory processes that can disrupt stromal and parenchymal cell compartments [46]. Astrogliosis is frequently observed after brain irradiation [47] and can be linked to radiation-induced vascular damage. The capability to minimize astrocytic and microglial activation would have important long-term implications for the irradiated brain, by ameliorating the chronic footprint of radiation-injury. Data indicates that while conventional dose-rate irradiation elevated reactive gliosis, FLASH-RT did not, pointing to the capability

of FLASH-RT to minimize astrogliosis adjacent to blood vessels, possibly by preserving the vascular endothelium. Similarly, the analysis of microglia showed that FLASH reduced significantly the yields of activated (CD68+) microglia. These findings corroborate a wealth of past data showing conventional dose-rate cranial irradiation to elicit a robust neuroinflammatory response involving activated microglia [23,48-50], but also highlights a major benefit of the FLASH modality. The attenuation of chronic inflammation by FLASH-RT has far reaching implications and suggests that many of the resultant adverse effects on the structural and functional integrity of the CNS may be preventable by implementing this novel irradiation modality.

Recent data from our laboratories has demonstrated that an acute and long-term consequence of conventional dose-rate cranial irradiation is a marked and persistent reduction in dendritic complexity and spine density [37]. The capability of FLASH-RT to minimize secondary reactive processes in the irradiated brain suggests that the structural degradation of neurons, in part mediated by the pruning activities of activated microglia, may also be attenuated. Analysis of hippocampal granule cell neurons 1 month following exposure to conventional dose-rate irradiation reveals reductions in dendritic area, length and branching, effects that are all resolved significantly by FLASH-RT. The sparing effects of FLASH-RT on dendritic arborization extended to dendritic spines, as the number, density and volume of spines was again preserved significantly after FLASH-RT compared to conventional dose-rate irradiation. Remarkably, these same protective effects on the structural integrity of mature neurons were found 6 months after FLASH-RT exposure, demonstrating that compared to conventional dose-rate irradiation, FLASH-RT preserved host neuronal morphology at multiple dendritic levels over extended post-irradiation intervals. Similar to the overt protective effects on neuronal structure, FLASH irradiation also paralleled controls in the number of PSD-95 synaptic foci found 1 and 6 months following. This critical synaptic protein has been shown to organize the composition of proteins and receptors at the synaptic cleft [51,52], and changes induced by conventional dose-rate irradiation were not found after FLASH-RT, suggesting again, that FLASH-RT prevents more traditional radiation-induced changes in synaptic protein expression and/or relocalization capable of disrupting neurotransmission. While the precise mechanism/s underlying the prolonged structural preservation of host neurons and stabilization of synaptic proteins in the FLASH irradiated brain remain to be elucidated, reductions in microglial activation represent a plausible explanation.

Physico-chemical mechanism of FLASH-RT

The foregoing data has demonstrated a potentially paradigm shifting radiation modality, capable of minimizing normal tissue toxicity without compromising tumor cure. To explore the radiochemical basis

of this differential effect on normal and tumor tissue, we sought to analyze whether differences in oxygen tension might provide a clue. Oxygen has long been known to be a potent radiosensitizer, and significant past effort has been devoted to a variety of approaches aimed at modulating oxygen tension between normal and tumor tissue for therapeutic gain [53-59]. Importantly, normal tissue is already maximally sensitized to ionizing radiation under normoxic conditions (oxygen levels ~4-6%), and only becomes meaningfully resistant under conditions where oxygen levels approach ≤1.5%. Previous old studies showed that the survival of anoxic bacteria after radiation exposure was independent of dose-rate, whereas aerobic bacteria at very low oxygen tensions were less sensitive to radiation at high dose-rates than fully aerobic bacteria [60,61]. Similar results were obtained on mammalian cells clonogenic survival, where increased clonogenicity was found in cells irradiated with high dose-rates under hypoxia (~1% of O_2) [33,62-65]. Both type of studies concluded that the protective effect on clonogenic survival might be due to the transient depletion of oxygen during high dose-rate irradiation that lowers the concentration of oxygen in the cellular environment. These data are fully consistent with our clonogenic survival assays under hypoxia and water radiolysis experiments demonstrating lower quantities of hydrogen peroxide produced after FLASH-RT, suggesting a differential production of ROS between conventional and ultra-high dose-rate irradiation. These results suggest that the duration of irradiation is the variable that is absolutely critical to induce the differential effect between ultra-high and conventional dose-rate irradiation. Thus, if FLASH irradiation depletes oxygen levels from normoxic to hypoxic levels, then normal tissue sparing would be evident, while a normally hypoxic tumor would exhibit relatively minimal response to such dramatic changes in dose rate. Here we posit that the relative instantaneous depletion of oxygen by FLASH represents a plausible mechanism for the differential effects of FLASH-RT between normoxic and hypoxic tissues.

To formally test this hypothesis, two series of experiments were performed. ROS scavenging studies implementing millimolar concentration of antioxidants to zebrafish embryos showed that amifostine or NAC had no effect on FLASH-irradiated embryos whereas the same treatments significantly improved embryo morphology after conventional dose-rate irradiation. Furthermore, dysmorphisms were more severe in the embryos irradiated by conventional dose-rate irradiation, suggesting FLASH-RT induced lower levels of toxic ROS. The presence of either antioxidant had a predictably muted effect after FLASH-RT compared to conventional dose-rate irradiation, again pointing to the capability of FLASH-RT to minimize oxygen toxicity, thereby limiting the efficacy of the antioxidants to scavenge oxygen free radicals existing at reduced levels.

In addition, mice were subjected to carbogen breathing before and during conventional dose-rate irradiation or FLASH-RT, able to double the ambient oxygen concentration in the brain [66]. Following irradiation, animals were subjected to a NOR task used before to quantify neurocognitive functionality 2 months after exposure. This mode of oxygen supplementation had no impact on unirradiated cohorts, or those subjected to conventional dose-rate irradiation. However, for animals subjected to FLASH-RT, the neurocognitive benefits found after ambient air breathing were eradicated under excess oxygen, demonstrating that carbogen breathing could functionally reverse the FLASH effect on cognition. While a number of explanations are certainly possible, the simplest one suggests that under the given carbogen breathing conditions, FLASH-RT was unable to deplete oxygen to the levels necessary to achieve neurocognitive sparing. Similar experiments conducted on a tumor flank model confirmed expectations, where carbogen breathing had no impact on tumor control in unirradiated cohorts or on those subjected to FLASH-RT with transient hypoxia. Increased oxygen tension in the tumor did promote control, validating the radiosensitizing effects of oxygen on hypoxic tissue. These data provide convincing support for the idea of an oxygen-concentration dependent mechanism driving the differential response of normal tissue from that of tumors subjected to FLASH-RT. Our data are fully supported by previous studies, where the absence of skin toxicity observed in rats irradiated with high dose-rates was associated with an oxygen partial pressure of 5-10mmHg, protection that was lost under anoxic conditions [67]. Moreover, the protection against radiation-induced tail necrosis triggered by high dose-rate irradiation was reversed by an increase in oxygen concentration [68].

In each instance, cell free, cell-based, zebra fish and mouse models provide fundamentally consistent results compatible with the idea that normal tissue sparing resulting from FLASH-RT is at least in part, based on an oxygen-dependent mechanism leading to lower levels of ROS and normal tissue toxicity. Due to tissue hypoxia in tumors, similar effects do not operate, so that tumor cure is not impacted, leading to the unexpected protective effects in normal tissue of delivering radiation at ultra-high dose rates.

Conclusions

Recent and current data make a compelling case for FLASH technology being at the cusp of changing radiotherapeutic protocols worldwide. Many past strategies have long sought to enhance therapeutic gain by modulating oxygen tension to increase tumor radiosensitivity (higher pO_2) while protecting normal tissue (lower pO_2) with mixed success over the years. Noteworthy too is the surprising realization that the FLASH advancement achieves such marked and persistent normal tissue sparing while transpiring within microseconds, a timeframe simply not obtainable from the majority of efforts focused on biological interventions, typically able to intervene on processes operating on a relative timescale 6 orders of

magnitude slower (**Fig. 13A**). This single fact is perhaps why this technology stands to change the landscape of radiotherapy, in that no special drugs, reagents, patient preparation or overly expensive equipment are required for its clinical implementation. Here we describe a potentially revolutionary way to sterilize tumors while greatly minimizing the adverse side effects associated with normal tissue damage. This is accomplished through a mechanism involving reduced oxygen toxicity, where the FLASH irradiation effectively exploits the oxygen differential between normal tissue and tumors instantaneously (**Fig. 13B**) to elicit normal tissue sparing. The resultant increases in normal tissue tolerance afford tremendous potential for dose escalation while avoiding severe late effects and toxicities associated with previous chemical modifiers and/or altered fractionation protocols. Further experimentation is clearly needed to substantiate the enormous potential of this burgeoning technology, but based on current data, rapid implementation of this promising new cancer treatment seems just a matter of time.

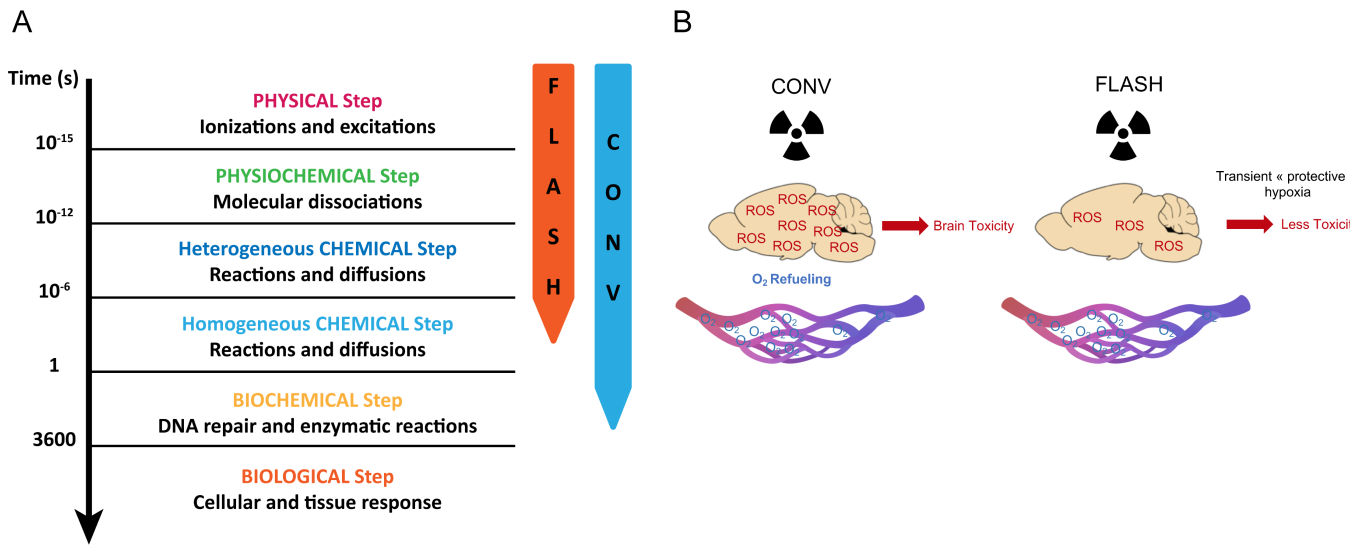

Figure 13: The very short time of dose-delivery induces differential physico-chemical events that explain the FLASH effect. A- FLASH-RT is more than 1000 times quicker than conventional dose-rate irradiation to deliver a similar dose. While conventional dose-rate irradiation is still ongoing when chemical and biological steps happen, FLASH-RT does not interact with these primary radiation-induced steps. B- FLASH-RT induces transient oxygen consumption and local hypoxia. Reduces the level of ROS produced and normal brain toxicity as compared with irradiation at conventional dose rate.

Material and methods

Animal experiments

Animal experiments were approved by Swiss (VD2920) and American (Institutional Animal Care and Use Committee, IACUC) Ethics Committee for Animal Experimentation and performed within institutional guidelines.

Irradiation devices

Irradiation was performed using a prototype 6MeV electron beam linear accelerator (LINAC) of type Oriatron 6e (eRT6; PMB-Alcen, Peynier, France), available at Lausanne University Hospital and described previously [69]. This LINAC is able to produce pulsed electron beams at a mean dose rate ranging from 0.1 Gy/s (i.e. comparable to conventional dose rates used in radiation-therapy) up to 1000 Gy/s, corresponding to a dose, in each electron pulse, ranging from 0.01 up to 10 Gy. All irradiations were performed at dose rate above 100 Gy/s FLASH and the parameters used are included in supplementary Table 1. The dose prescriptions for FLASH irradiations were determined by surface dose measurements on a 30 x 30 cm2 solid water slab positioned behind a 1.7 cm in diameter aperture of a graphite applicator (13.0 x 13.0 x 2.5 cm3), as previously described [32]. The mouse head was positioned behind and in contact with the aperture of the 1.7cm diameter graphite applicator in order to irradiate the whole encephalon region while limiting the dose to the eyes, the mouth and the rest of the body. Subcutaneous tumors were positioned in sandwich between the aperture of the 1.7cm diameter graphite applicator and a solid water slab. All FLASH irradiations were delivered in less than 0.2 s.

X-rays irradiations at conventional dose-rates were performed using a XRad 225Cx (Pxi Precision X-Ray). The dose prescriptions were determined at 5 mm depth for a 10x10 mm2 field according to previous depth dose measurement in solid water phantom. The irradiations were performed at 225 keV, 13 mA, with a 0.3 mm copper filter. Irradiations were delivered after fluoroscan imaging to position the mouse in order to avoid irradiating their eyes, mouth cavity, esophagus and trachea. Whole brain irradiation was performed with two horizontal opposed beams delivering each 7.5 Gy at 5 mm depth.

Tumor models and irradiation

For subcutaneous glioblastoma models, 10M U87 human GBM cells were engrafted in the flank of female nude mice under isoflurane anesthesia. Tumors were irradiated at 20 Gy as described above when their volume reached of 60mm3 (57± 17 mm3) using a 1.7cm round collimator. For hyperoxic conditions, mice were anesthetized under carbogen (95% O_2, 5% CO_2) and isoflurane for 15 minutes, including the irradiation time. Hypoxic conditions were performed using a vascular clamp placed on the tumor for 5

minutes, including the irradiation time. Tumor growth was evaluated three times a week by caliper measurement.

For orthotopic glioblastoma models, 500'000 H454 Luc+ murine GBM cells (D. Hanahan, EPFL, Switzerland) were orthotopically injected in the striatum of female nude mice with the coordinates: (+1; +1; -3). Whole brain irradiations at 10 Gy were performed as described above 3 days post tumor implantation. Tumoral development was assessed by bioluminescence imaging the day before irradiation and weekly post-irradiation. Image acquisition was performed under isoflurane anesthesia using a Xenogen and 10 minutes after an ip. injection of 15mg/kg of luciferin and bioluminescence was quantified (Living image).

A transgenic GBM model *GFAP-HRas^{V12}; GFAP-CRE; p53$^{flox/wt}$* (D. Hanahan, EPFL, Switzerland) was used for prophylactic irradiation treatment. The mice were irradiated to the whole brain with a single dose of 15Gy FLASH, or at conventional dose rate (electrons and X-rays) as described above, before the development of the tumor at 4 weeks of age. Tumor symptoms and survival was assessed.

Whole brain irradiations

Female C57Bl6/J mice (n=5-16 animals per group) and female Nude mice (n=5-12 animals per group) were purchased from CRL at the age of eight weeks. Transgenic *Thy1-eGFP* mice (n=10-12 animals per group) were bred in UCI animal facility. Transgenic *GFAP-HRas^{V12}; GFAP-CRE; p53$^{flox/wt}$* (n=5-11 animals per group) were obtained from D. Hanahan's laboratory in EPFL (Lausanne, Switzerland) and bred in EPFL animal facility. Whole brain irradiations were performed under isoflurane anesthesia as described above. For irradiations in hyperoxic conditions, mice were anesthetized with isoflurane and carbogen (95% O_2, 5% CO_2) for 20 minutes, including the irradiation time.

Cognitive testing

To determine the effects of conventional and FLASH dose-rate irradiations on cognitive function, mice were subjected to behavioral testing 1 and 6 months after irradiation. Early testing (1 month) was conducted over 2 weeks and included three open field, spontaneous exploration tasks following our previously described protocols [16,32,47,70]. Data analysis was conducted independently and blind and is presented as the average of all trials scored for each task. Female nude mice bearing orthotopic GBM tumors were administered the novel object recognition (NOR) task to assess neurocognitive functionality. Tumor free transgenic mice [strain *Thy1-eGFP*, MJrsJ, stock no. 007788, The Jackson Laboratory, Sacramento, CA] harboring the *Thy1- eGFP* transgene were subjected to a more extensive neurocognitive

battery to eliminate any confounding effects of disease. These animals were first administered the NOR, followed by the object in place (OIP) and lastly the temporal order (TO) task.

Novel Object Recognition (NOR) task involved a sequence of habituation (no objects), familiarization (2 distinct objects) and lastly a test phase, in which one of the prior objects is switched with a new one. Animals have a tendency to explore the novel object, and successful performance on this task is reliant on intact perirhinal cortex function [71,72].

For the Object in Place (OIP) task, animals were habituated then familiarized with 4 different objects at discrete locations. Following familiarization, the location of 2 objects is switched and animals are reintroduced to the arena for the test phase, and their ability to discriminate the novel object locations. Performance on the OIP task is dependent on intact hippocampal function in addition to the prefrontal and perirhinal cortices [71,72].

For the Temporal Order (TO) task, animals were familiarized with two sets of objects, 4 hours apart. In this instance, mice with functional connectivity between the hippocampus, mPFC and PRC show a preference for exploring the prior, rather than the more recent object [71,72].

The NOR, OiP and TO tasks rely on intact hippocampal, medial prefrontal cortex (mPFC) and perirhinal cortex function. While the NOR task measures the preference for novelty, the OiP task is a test of associative recognition memory and the TO task provides a measure of temporal order memory, that depend on interactions between the hippocampus, mPFC and perirhinal cortices. Time spent exploring both familiar and novel object or object was counted when the nose of the mouse was within 1 cm and pointed in the direction of the object. Mice did not show object climbing or neophobic behavior. NOR, OIP and TO data are presented as a discrimination index (DI) and calculated as ([Novel location exploration time/Total exploration time] – [Familiar location exploration time/Total exploration time]) × 100. A positive index indicates that a mouse spent more time exploring novelty (i.e. switched objects or locations), while a negative score indicates little or no preference for exploring novelty.

Radiation-induced cognitive impairments typically present several months following the cessation of treatment and often manifest as a variety of mood disorders [73] [10-13,74,75]. Longer-term (6 month) assessments of behavior necessitated the use of tumor free mice and were designed to assess potential radiation-induced changes in anxiety, depression and extinction. The EPM, LDB and FST provide indirect measures of anxiety- and depression-like behavior respectively [16,70]. The former tasks measures an

animal's confidence for exploring the open rather than the closed arms of a maze or the tendency to move between dark and light areas, while the latter task provides a measure of despair, as animals suffering from depression tend to float more often. These tasks are quantified by calculating the amount of time spent in the open versus closed arms of the EPM, the number of transitions between light and dark regions of the LDB or by the amount of time floating versus swimming during the FST, behaviors that can each be linked to the amygdala (among other regions).

Fear extinction follows a modified fear conditioning protocol [76] in which repeated trials dissociating the tone-shock pairing can be used to measure the rate of reduced freezing or fear extinction. Deficits in this behavior have been linked to the infralimbic region of the mPFC and require active learning, thereby provide a measure of cognitive flexibility. Briefly, mice received three conditioning trials (tone-foot shock pairings) to establish fear. Mice were placed in a conditioning chamber with Plexiglas walls and a metal grid bottom. They were left to acclimate for 5 min and were given the tone followed by a mild foot-shock (2s, 0.7 mA constant current). Freezing was used to measure the conditional fear response during the fear conditioning phase, extinction training phase and testing phase. At 24 hours following conditioning, animals were trained to "unlearn" the association by repeatedly playing the tone without the shock. Mice were given a total of 15 extinction trials (tone alone) in 3 days (3x5 trials) to test their ability to extinguish conditioned fear in same context. The inability to unlearn the association reinforced during conditioning with the tone-foot shock pairing during the extinction and test phases indicates impairment in extinction memory. Deficits in this behavior have been linked to the infralimbic region of the mPFC and require active unlearning, thereby providing another measure of cognitive flexibility – or the ability to adapt to a changing environment[16,35,36,70].

Immunohistochemistry, confocal microscopy, and quantification

At select times post-irradiation animals were deeply anesthetized with isoflurane and euthanized with saline with heparin (10 U/ml, Sigma-Aldrich) followed by 4% paraformaldehyde (intracardiac perfusion). Brains were cryoprotected (30% sucrose) and sectioned coronally (30 µm thick) using a cryostat (Leica Microsystems, Germany).

For the assessment of reactive gliosis, immunofluorescence was performed on floating brain sections. Sections were incubated overnight at 4°C with the primary antibodies, washed with PBS and incubated with the secondary antibody at room temperature for 1h. Sections were mounted on microscope slides with Vectashield + Dapi (Vector Lab H-1500). GFAP and CD31 expressions were assessed in the striatum of control and irradiated mice. Floating brain sections were incubated with an anti-GFAP (1:500; clone

GA5; MAB360) and an anti-CD31 (1:150; BD Bioscience, 553370) primary antibodies. Alexa Fluor 568-labeled Goat anti-mouse (1/200) (Thermofisher, A21124) and Alexa 488-labeled Donkey anti-rat (1/200) (Thermofisher, A11070) secondary antibodies were used. Image acquisition was performed using an upright Zeiss Axiovison microscope. GFAP expression area was quantified using ImageJ.

For the assessment of microglia, the following primary and secondary antibodies were used: rabbit anti-IBA-1 (1:500, Wako), rat anti-mouse CD68 (1:500, AbD Serotec), donkey anti-rabbit or anti-mouse conjugated with Alexa Fluor 488 or 594 (Life Technologies/Invitrogen) and DAPI nuclear counterstain (Sigma-Aldrich). Representative sections (3-4 sections/animal, 4-6 animals/group) through the middle of the hippocampus were selected and immunofluorescence staining followed procedures described in detail previously [23,77]. IBA-1 or CD68 positive cells were visualized under fluorescence as green against DAPI stained nuclei (blue). Immunofluorescent sections were imaged using Nikon Eclipse Ti C2 microscope to obtain 20 to 30 z stacks (1024 × 1024 pixels, 1 µm each) using 10 and 60× PlanApo oil-immersion lens (Nikon). For quantification of IBA-1^{+} and CD68^{+} cells, 3D deconvolution and reconstruction was carried out using the AutoQuantX3 algorithm (MediaCybernetics). Deconvolution combined with 3D reconstruction yields higher spatial resolution images for the immunofluorescent cell bodies and stellae. Quantification was facilitated using Imaris spot tool (v8.0, Bit Plane Inc., Switzerland) that detect immunostained puncta within 3D deconvoluted image stacks based on a predefined diameter and red/green channel intensity threshold. IBA-1 and CD68 data are expressed as mean immunoreactivity (percentage) relative to unirradiated (0 Gy) controls.

The assessment of PSD-95 foci has been described previously [14,37]. Briefly, serial 30 µm thick sections (3/animals) from the anterior to posterior hippocampus were selected, and three different fields in each section were imaged from the dentate gyrus. Images were collected using a Nikon Eclipse TE 2000-U microscope with 0.5 l m-interval high-resolution Z-stacks (1024x1024 pixel). Analysis of PSD-95 was performed using the IMARIS spot tool, and puncta satisfying pre-defined criteria (verified visually for accuracy) were converted to spots for quantification under preset parameters kept constant throughout subsequent analyses.

Morphometric assessments of neurons

The strong signal-to-noise ratio of fluorescent neurons in *Thy1-eGFP* mice provides for the high-resolution micromorphometric analyses of specific neuronal subsets. Details regarding the reconstruction of neurons and the morphologic classification of spines have been described [14,37]. Briefly, an algorithm is used for the tracing of dendritic filaments to reconstruct the entire dendritic tree, where tracing originates

from the soma and terminates near terminal dendritic diameter thresholds. Reconstructed dendritic segments can be analyzed under higher magnification for dendritic spines that can be labeled, manually verified, morphologically categorized, and quantified. All morphometric parameters were validated from an independent series of pilot reconstructions in both manual and semiautomatic modes. Images were then compared for accuracy and consistency to ensure that selected parameters represented actual variations in dendritic structure.

For dendritic analyses, 100 mm thick hippocampal sections were prepared for confocal imaging. Three sections per animal were used to generate Z-stacks from four animals using a Nikon Eclipse TE 2000-U microscope (Nikon, Japan). Images comprising each Z-stack (1024 x 1024 pixels) were acquired at (60x) over the entire dendrite tree at 0.5 mm increments. Quantification of dendritic parameters was derived from Z-stacks reconstructed in 3D from deconvoluted images using the AutoQuantX3 algorithm (MediaCybernetics, MD, USA). Deconvoluted 3D reconstructions yielded high spatial resolution images for detailed dendritic tracing and spine classification using the IMARIS software suite (Bitplane Inc.) as described previously [14,37]. For spines to be included in our analyses a maximum spine length and minimum spine end diameter were set at 2.5 and 0.4 mm, respectively. Parameters of neuronal structure that were identified and quantified through image reconstruction and deconvolution using the IMARIS software suite (Bitplane Inc.) included the cell body, dendritic and axonal length, branching and branch points, dendritic complexity, spines, and boutons.

H_2O_2 production measurements (Amplex Red)

H_2O_2 production measurements were performed using Amplex Red staining. MilliQ water (6.9 < pH < 7.1 ; 21°C < T°C < 22°C) was equilibrated in a hypoxia hood for 24h at 4% O2. Water was irradiated in airtight Eppendorf polypropylene tubes at 0, 10, 20, 30, 40, 50, 60, 70 or 80 Gy at FLASH or conventional dose-rate in a water tank. AmplexRed was added to the irradiated water (v/v) exactly 195s after the beginning of irradiation at a final concentration of 16.67uM (previously defined as optimal) and incubated for 90min protected from light. H_2O_2 solutions from 0.007 to 10uM were used as standards. Fluorescence quantification was performed using a plate reader 90 minutes post-irradiation (Excitation: 530nm; Emission: 590nm). Measurements were realized in triplicates.

Clonogenic assays

Murine Glioblastoma cells H454 (D. Hanahan, EPFL, Switzerland) were cultured in DMEM + 10% FBS (ThermoFischer) at 37°C and under different dioxygen concentrations: 4 or 21%. The day of irradiation, cells were harvested with trypsin + EDTA 0.25% (ThermoFisher), counted and placed in airtight Eppendorf

tubes for cell suspension irradiations. Tubes were irradiated in a water-tank at 0, 2, 4, 6, 10, 12, 15, 17 or 20Gy at FLASH or conventional dose-rate. Cells were then plated at a concentration of 200 to 100'000 cells in six-wells plates or petri dishes and incubated at 37°C; 5%CO_2, 21%O_2. 7 days post-irradiation, colonies were fixed and stained using crystal-violet (Sigma). Colonies over 50 cells were counted and plating efficiency and survival fractions were determined.

Oxidative stress measurement in Zebra fishes

For *in vivo* oxidative stress studies, WT Zebra fishes were bred in our fish facility (CHUV, Lausanne, Switzerland). All in-vivo experiments on zebra fishes were performed on embryos under 5dpf. Fertilized WT zebra fishes' eggs were incubated at 28°C until 5dpf. Anesthesia was performed with 168mg/L of tricaine and 10 to 20 embryos were transferred in 2mL Eppendorf tubes. Water + tricaine was then removed and replaced by pure H2O + 60mg/L of ocean salt. For antioxidant treatments on WT animals, NAC (5mM, pH=7.5; Sigma) or Amifostine (4mM; Sigma) were added to the water for 1h before irradiation. Irradiation was performed 4hpf at 8 Gy FLASH and conventional dose-rate. Embryos were fixed 5 dpf with a solution of PFA 4% final concentration before microscopic analysis (Evos XL Core Cell Imaging System; ThermoFisher). Fish length was measured using ImageJ 1.X. software.

Statistics

Statistical analyses were carried out using GraphPad Prism (v6) software. One-way ANOVA was used to assess significance between control and irradiated groups, and when overall group effects were found to be statistically significant, a Bonferroni's multiple comparisons test was used to compare the control and FLASH groups against the CONV cohort. In addition, the unpaired non-parametric Mann-Whitney test or unpaired t-test after Gaussian distribution assessment by Shapiro-Wilk normality test was used. For survival studies, Mantel-Cox test was realized. Results were expressed as mean values ± SD or mean values ± SEM and all analyses considered a value of $P \leq 0.05$ to be statistically significant.

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Author contribution

PMG, MMA, CLL, MCV designed the experiments and wrote the manuscript; KP, JFG FB, CB designed the irradiation procedure and dosimetry; KP, PJG, RM performed all irradiation; PMG, MMA, LA, CY, BDA, JO, BP, ARS, THN, ADB, CL, PS, PF performed the experiments; PMG, MMA, KP, LFG, PF, CB, JB, CLL, MCV analyzed the data; JB, CLL and MCV secured funding.

Supplementary Table S1: Irradiation parameters

Subcutaneous GBM in Nude +/- clamp ; +/- carbogen (Fig. 1A and Fig. 11B)		Beam parameters				
Mode	Prescribed dose	Frequency (Hz)	SSD (mm)	Pulse width (µs)	Number of pulses	Treatment time (s)
CONV	20	10	800	1.0	1810-1920	180.9-191.9
FLASH	20	100	925	1.8	20	0.19

Spontaneous GBM model (Fig. 1B)		Beam parameters				
Mode	Prescribed dose	Frequency (Hz)	SSD (mm)	Pulse width (µs)	Number of pulses	Treatment time (s)
CONV	15	10	800	1.0	1320-1560	132.9-155.9
FLASH	15	100	780	1.8	10	0.09

Orthotopic GBM (Fig. 1B) Normal tissue toxicity +/- carbogen (Fig. 3-11)		Beam parameters				
Mode	Prescribed dose	Frequency (Hz)	SSD (mm)	Pulse width (µs)	Number of pulses	Treatment time (s)
CONV	10	10	612-800	1.0	639-1180	63.8-117.6
FLASH	10	100	350	1.8	1	$1.8 \cdot 10^{-6}$

Pure water (Fig. 12A)		Beam parameters				
Mode	Prescribed dose	Frequency (Hz)	SSD (mm)	Pulse width (µs)	Number of pulses	Treatment time (s)
CONV	10	10	400	1.0	350	349.9
	20				696	69.5
	30				1047	104.6
	40				1390	138.8
	50				1730	172.9
	60				2075	207.4
	70				2440	243.9
	80				2800	279.9
FLASH	10	100	460	1.75	2	0.01
	20			1.8	4	0.03
	30			1.84	6	0.05
	40			1.87	8	0.07
	50			1.89	10	0.09
	60			1.9	12	0.11
	70			1.87	14	0.13
	80			1.87	16	0.15

Clonogenic cell survival (Fig. 12B)		Beam parameters				
Mode	Prescribed dose	Frequency (Hz)	SSD (mm)	Pulse width (µs)	Number of pulses	Treatment time (s)
CONV	2	10	400	1.0	101	10
	4				202	20.1
	6				303	30.2
	10				505	50.4
	12				605	60.4
	15				755	75.4
	17				855	85.4
	20				1000	99.9
FLASH	2	100	700	2.0	1	$2 \cdot 10^{-6}$
	4		500	1.85	1	$1.85 \cdot 10^{-6}$
	6		426	1.9	1	$1.9 \cdot 10^{-6}$
	10		335	1.98	2	0.01
	12		416	1.83	2	0.01
	15		370	1.73	2	0.01
	17		370	2.01	2	0.01
	20		388	1.48	3	0.02

Fish eggs +/- NAC ; +/- amifostine (Fig. 12C)		Beam parameters				
Mode	Prescribed dose	Frequency (Hz)	SSD (mm)	Pulse width (µs)	Number of pulses	Treatment time (s)
CONV	8	10	808	1.0	1262	126.1
FLASH	8	100	350	1.49	1	$1.49 \cdot 10^{-6}$

References

1. Bentzen, S.M. Preventing or reducing late side effects of radiation therapy: radiobiology meets molecular pathology. Nature reviews. Cancer 6, 702-713 (2006).
2. Yarnold, J. & Brotons, M.C. Pathogenetic mechanisms in radiation fibrosis. Radiother Oncol 97, 149-161 (2010).
3. Begg, A.C., Stewart, F.A. & Vens, C. Strategies to improve radiotherapy with targeted drugs. Nature reviews. Cancer 11, 239-253 (2011).
4. Bortfeld, T. IMRT: a review and preview. Phys Med Biol 51, R363-379 (2006).
5. Stupp, R., et al. High-grade glioma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 25 Suppl 3, iii93-101 (2014).
6. Tsao, M.N., et al. Radiotherapeutic and surgical management for newly diagnosed brain metastasis(es): An American Society for Radiation Oncology evidence-based guideline. Pract Radiat Oncol 2, 210-225 (2012).
7. Martin, A.M., Raabe, E., Eberhart, C. & Cohen, K.J. Management of pediatric and adult patients with medulloblastoma. Curr Treat Options Oncol 15, 581-594 (2014).
8. Stupp, R., et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol 10, 459-466 (2009).
9. Meyers, C.A. Neurocognitive dysfunction in cancer patients. Oncology (Williston Park) 14, 75-79; discussion 79, 81-72, 85 (2000).
10. Butler, J.M., Rapp, S.R. & Shaw, E.G. Managing the cognitive effects of brain tumor radiation therapy. Curr Treat Options Oncol 7, 517-523 (2006).
11. Wellisch, D.K., Kaleita, T.A., Freeman, D., Cloughesy, T. & Goldman, J. Predicting major depression in brain tumor patients. Psychooncology 11, 230-238 (2002).
12. Tang, Y., Luo, D., Rong, X., Shi, X. & Peng, Y. Psychological disorders, cognitive dysfunction and quality of life in nasopharyngeal carcinoma patients with radiation-induced brain injury. PLoS One 7, e36529 (2012).
13. Caceres, L.G., et al. Pharmacological alterations that could underlie radiation-induced changes in associative memory and anxiety. Pharmacol Biochem Behav 111, 37-43 (2013).
14. Parihar, V.K. & Limoli, C.L. Cranial irradiation compromises neuronal architecture in the hippocampus. Proc Natl Acad Sci U S A 110, 12822-12827 (2013).
15. Parihar, V.K., et al. Targeted overexpression of mitochondrial catalase prevents radiation-induced cognitive dysfunction. Antioxid Redox Signal 22, 78-91 (2015).

16. Parihar, V.K., et al. Cosmic radiation exposure and persistent cognitive dysfunction. Sci Rep 6, 34774 (2016).

17. Baulch, J.E., et al. Cranial grafting of stem cell-derived microvesicles improves cognition and reduces neuropathology in the irradiated brain. Proc Natl Acad Sci U S A 113, 4836-4841 (2016).

18. Warrington, J.P., et al. Cerebral microvascular rarefaction induced by whole brain radiation is reversible by systemic hypoxia in mice. Am J Physiol Heart Circ Physiol 300, H736-744 (2011).

19. Lee, W.H., Cho, H.J., Sonntag, W.E. & Lee, Y.W. Radiation attenuates physiological angiogenesis by differential expression of VEGF, Ang-1, tie-2 and Ang-2 in rat brain. Radiat Res 176, 753-760 (2011).

20. Craver, B.M., et al. 3D surface analysis of hippocampal microvasculature in the irradiated brain. Environ Mol Mutagen 57, 341-349 (2016).

21. Atkinson, S., Li, Y.Q. & Wong, C.S. Changes in oligodendrocytes and myelin gene expression after radiation in the rodent spinal cord. Int J Radiat Oncol Biol Phys 57, 1093-1100 (2003).

22. Moravan, M.J., Olschowka, J.A., Williams, J.P. & O'Banion, M.K. Cranial irradiation leads to acute and persistent neuroinflammation with delayed increases in T-cell infiltration and CD11c expression in C57BL/6 mouse brain. Radiat Res 176, 459-473 (2011).

23. Acharya, M.M., et al. Elimination of microglia improves cognitive function following cranial irradiation. Sci Rep 6, 31545 (2016).

24. Wong, E.T., et al. Outcomes and prognostic factors in recurrent glioma patients enrolled onto phase II clinical trials. J Clin Oncol 17, 2572-2578 (1999).

25. Gilbert, M.R., et al. Dose-dense temozolomide for newly diagnosed glioblastoma: a randomized phase III clinical trial. J Clin Oncol 31, 4085-4091 (2013).

26. Prempree, T., Michelsen, A. & Merz, T. The repair time of chromosome breaks induced by pulsed x-rays on ultra-high dose-rate. Int J Radiat Biol Relat Stud Phys Chem Med 15, 571-574 (1969).

27. Auer, S., et al. Survival of tumor cells after proton irradiation with ultra-high dose rates. Radiat Oncol 6, 139 (2011).

28. Schmid, T.E., et al. Differences in the kinetics of gamma-H2AX fluorescence decay after exposure to low and high LET radiation. Int J Radiat Biol 86, 682-691 (2010).

29. Schmid, T.E., et al. The effectiveness of 20 mev protons at nanosecond pulse lengths in producing chromosome aberrations in human-hamster hybrid cells. Radiat Res 175, 719-727 (2011).

30. Zlobinskaya, O., et al. Reduced side effects by proton microchannel radiotherapy: study in a human skin model. Radiat Environ Biophys 52, 123-133 (2013).

31. Favaudon, V., et al. Ultrahigh dose-rate FLASH irradiation increases the differential response between normal and tumor tissue in mice. Sci Transl Med 6, 245ra293 (2014).

32. Montay-Gruel, P., et al. Irradiation in a flash: Unique sparing of memory in mice after whole brain irradiation with dose rates above 100Gy/s. Radiother Oncol (2017).

33. Weiss, H., Epp, E.R., Heslin, J.M., Ling, C.C. & Santomasso, A. Oxygen depletion in cells irradiated at ultra-high dose-rates and at conventional dose-rates. Int J Radiat Biol Relat Stud Phys Chem Med 26, 17-29 (1974).

34. Pellow, S., Chopin, P., File, S.E. & Briley, M. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 14, 149-167 (1985).

35. Milad, M.R. & Quirk, G.J. Neurons in medial prefrontal cortex signal memory for fear extinction. Nature 420, 70-74 (2002).

36. Sotres-Bayon, F., Sierra-Mercado, D., Pardilla-Delgado, E. & Quirk, G.J. Gating of fear in prelimbic cortex by hippocampal and amygdala inputs. Neuron 76, 804-812 (2012).

37. Parihar, V.K., et al. Persistent changes in neuronal structure and synaptic plasticity caused by proton irradiation. Brain Struct Funct 220, 1161-1171 (2015).

38. Hall, E.J. & Giaccia, A.J. Radiobiology for the radiologist. (Wolters Kluwer Health/Lippincott Williams & Wilkins,, Philadelphia, 2012).

39. Sevenich, L., et al. Analysis of tumour- and stroma-supplied proteolytic networks reveals a brain-metastasis-promoting role for cathepsin S. Nat Cell Biol 16, 876-888 (2014).

40. Marumoto, T., et al. Development of a novel mouse glioma model using lentiviral vectors. Nat Med 15, 110-116 (2009).

41. Yan, D., et al. Inhibition of colony stimulating factor-1 receptor abrogates microenvironment-mediated therapeutic resistance in gliomas. Oncogene 36, 6049-6058 (2017).

42. Wessa, M. & Karl, A. Stress sensitization and fear learning in posttraumatic stress disorder. Psychophysiology 44, S8-S8 (2007).

43. Kaplan, G.B., et al. Pathophysiological Bases of Comorbidity: Traumatic Brain Injury and Post-Traumatic Stress Disorder. J Neurotrauma 35, 210-225 (2018).

44. Saury, J.M.G. & Emanuelson, I. Cognitive Consequences of the Treatment of Medulloblastoma Among Children. Pediatr Neurol 44, 21-30 (2011).

45. Butler, R.W., et al. Intellectual functioning and multi-dimensional attentional processes in long-term survivors of a central nervous system related pediatric malignancy. Life sciences 93, 611-616 (2013).

46. Liddelow, S.A., et al. Neurotoxic reactive astrocytes are induced by activated microglia. Nature 541, 481-487 (2017).

47. Acharya, M.M., et al. Adenosine Kinase Inhibition Protects against Cranial Radiation-Induced Cognitive Dysfunction. Front Mol Neurosci 9, 42 (2016).

48. Chiang, C.S., McBride, W.H. & Withers, H.R. Radiation-induced astrocytic and microglial responses in mouse brain. Radiother Oncol 29, 60-68 (1993).

49. Hwang, S.Y., et al. Ionizing radiation induces astrocyte gliosis through microglia activation. Neurobiology of Disease 21, 457-467 (2006).

50. Kalm, M., et al. Transient inflammation in neurogenic regions after irradiation of the developing brain. Radiat Res 171, 66-76 (2009).

51. Keith, D. & El-Husseini, A. Excitation Control: Balancing PSD-95 Function at the Synapse. Front Mol Neurosci 1, 4 (2008).

52. Preissmann, D., et al. Increased postsynaptic density protein-95 expression in the frontal cortex of aged cognitively impaired rats. Exp Biol Med (Maywood) 237, 1331-1340 (2012).

53. Brady, L.W., et al. Hyperbaric oxygen therapy for carcinoma of the cervix--stages IIB, IIIA, IIIB and IVA: results of a randomized study by the Radiation Therapy Oncology Group. Int J Radiat Oncol Biol Phys 7, 991-998 (1981).

54. Chaplin, D.J. & Acker, B. The effect of hydralazine on the tumor cytotoxicity of the hypoxic cell cytotoxin RSU-1069: evidence for therapeutic gain. Int J Radiat Oncol Biol Phys 13, 579-585 (1987).

55. Rockwell, S., Mate, T.P., Irvin, C.G. & Nierenburg, M. Reactions of tumors and normal tissues in mice to irradiation in the presence and absence of a perfluorochemical emulsion. Int J Radiat Oncol Biol Phys 12, 1315-1318 (1986).

56. Song, C.W., Lee, I., Hasegawa, T., Rhee, J.G. & Levitt, S.H. Increase in pO2 and radiosensitivity of tumors by Fluosol-DA (20%) and carbogen. Cancer research 47, 442-446 (1987).

57. Overgaard, J. & Horsman, M.R. Modification of hypoxia-induced radioresistance in tumors by the use of oxygen and sensitizers. Seminars in Radiation Oncology 6, 10-21 (1996).

58. Hoskin, P.J., Rojas, A.M., Bentzen, S.M. & Saunders, M.I. Radiotherapy with concurrent carbogen and nicotinamide in bladder carcinoma. J Clin Oncol 28, 4912-4918 (2010).

59. Janssens, G.O., et al. Accelerated radiotherapy with carbogen and nicotinamide for laryngeal cancer: results of a phase III randomized trial. J Clin Oncol 30, 1777-1783 (2012).

60. Dewey, D.L. & Boag, J.W. Modification of the oxygen effect when bacteria are given large pulses of radiation. Nature 183, 1450-1451 (1959).

61. Dewey, D.L. & Michael, B.D. Pulse-irradiated bacterial survival in the presence of nitric oxide. Radiat Res 39, 82-89 (1969).

62. Epp, E.R., Weiss, H., Djordjevic, B. & Santomasso, A. The radiosensitivity of cultured mammalian cells exposed to single high intensity pulses of electrons in various concentrations of oxygen. Radiat Res 52, 324-332 (1972).

63. Ling, C.C., Michaels, H.B., Epp, E.R. & Peterson, E.C. Oxygen diffusion into mammalian cells following ultrahigh dose rate irradiation and lifetime estimates of oxygen-sensitive species. Radiat Res 76, 522-532 (1978).

64. Michaels, H.B., Epp, E.R., Ling, C.C. & Peterson, E.C. Oxygen sensitization of CHO cells at ultrahigh dose rates: prelude to oxygen diffusion studies. Radiat Res 76, 510-521 (1978).

65. Michaels, H.B. & Hunt, J.W. A model for radiation damage in cells by direct effect and by indirect effect: a radiation chemistry approach. Radiat Res 74, 23-34 (1978).

66. Khan, N., et al. Tissue pO2 of orthotopic 9L and C6 gliomas and tumor-specific response to radiotherapy and hyperoxygenation. Int J Radiat Oncol Biol Phys 73, 878-885 (2009).

67. Field, S.B. & Bewley, D.K. Effects of dose-rate on the radiation response of rat skin. Int J Radiat Biol Relat Stud Phys Chem Med 26, 259-267 (1974).

68. Hendry, J.H., Moore, J.V., Hodgson, B.W. & Keene, J.P. The constant low oxygen concentration in all the target cells for mouse tail radionecrosis. Radiat Res 92, 172-181 (1982).

69. Jaccard, M., et al. High dose-per-pulse electron beam dosimetry: Commissioning of the Oriatron eRT6 prototype linear accelerator for preclinical use. Med Phys 45, 863-874 (2018).

70. Parihar, V.K., et al. Persistent nature of alterations in cognition and neuronal circuit excitability after exposure to simulated cosmic radiation in mice. Exp Neurol 305, 44-55 (2018).

71. Barker, G.R., Bird, F., Alexander, V. & Warburton, E.C. Recognition memory for objects, place, and temporal order: a disconnection analysis of the role of the medial prefrontal cortex and perirhinal cortex. J Neurosci 27, 2948-2957 (2007).

72. Barker, G.R. & Warburton, E.C. When is the hippocampus involved in recognition memory? J Neurosci 31, 10721-10731 (2011).

73. Andersen, B.L. & Tewfik, H.H. Psychological reactions to radiation therapy: reconsideration of the adaptive aspects of anxiety. J Pers Soc Psychol 48, 1024-1032 (1985).

74. Kaleita, T.A., et al. Pilot study of modafinil for treatment of neurobehavioral dysfunction and fatigue in adult patients with brain tumors. J Clin Oncol 24, 1503 (2006).

75. Yen, P.N., et al. Risk factors of depression after prolonged low-dose rate environmental radiation exposure. Int J Radiat Biol 90, 859-866 (2014).

76. Leanza, L., et al. Clofazimine, Psora-4 and PAP-1, inhibitors of the potassium channel Kv1.3, as a new and selective therapeutic strategy in chronic lymphocytic leukemia. Leukemia 27, 1782-1785 (2013).

77. Acharya, M.M., et al. Consequences of low dose ionizing radiation exposure on the hippocampal microenvironment. PLoS One 10, e0128316 (2015).

4. Supplementary Results

4.1. Material and methods

4.1.1. Clonogenic assays

For clonogenic assays, U87, HeLa and HaCaT cell lines were cultured in monolayer with DMEM + 5% FBS (ThermoFisher) medium. The day of irradiation, cells were harvested, counted and placed in cryotubes for irradiations. Tubes were irradiated in the water at 0, 2, 4 or 6 Gy at FLASH or conventional dose-rate using eRT6 LINAC. Cells were then plated at a concentration of 100 to 1000 cells per well in six-wells plates and incubated at 37°C; 5%CO_2. 7 to 14 days post-irradiation, colonies were fixes and stained using crystal-violet (Sigma). Colonies over 50 cells were counted and plating efficiency and survival fractions were determined. Clonogenic survival curves were modeled using a linear quadratic model in GraphPad Prism.

4.1.2. Mice irradiation and tumor engraftment

For normal brain studies, C57Bl6 female mice were irradiated at the age of 8 weeks or 1 year with a single dose of 10Gy. Whole brain irradiations wit FLASH or conventional dose-rate were performed under anesthesia using the eRT6 LINAC as previously described.

For orthotropic GBM model, 500'000 H454 luc+ murine GBM tumor cells (D. Hanahan, EPFL) were cultured, injected in the brain of Nude mice as described before. Mice received total brain irradiation using the eRT6 LINAC 3 days post-injection with 10, 13 or at 15 Gy FLASH or conventional dose-rate for single dose experiments. For fractionation experiments, 5x5 Gy or 3x8 Gy at FLASH or conventional dose-rate were delivered with 24h between each fraction. Irradiation set up was realized as described before. Tumor growth evaluation was realized weekly by bioluminescence and survival follow-up was realized.

4.1.3. Novel Object Recognition

Novel Object Recognition tests were performed on conventional irradiated, FLASH irradiated and non-irradiated control mice two, six and nine months post-irradiation as previously described. Analysis was performed blindly, and the time spent on each object was measured in order to calculate the Recognition Ratio (RR) such as: [(time spent on the novel object – time spent on the old object) / (Total exploration time) x 100].

4.1.4. Sampling and immunofluorescence assays

Mice brains were fixed, sampled, stored and immunofluorescence assays for astrogliosis quantification were performed on striatum sections 3, 14 and 60 days post- irradiation as previously described. TUNEL assays were performed on hippocampal sections 3 days post-irradiation with an

ApopTag® Fluorescein in-situ apoptosis detection kit (Merck S7110). The slides were analyzed using an epifluorescence microscope (Zeiss, Axio Imager Z1). Quantification of GFAP-staining and TUNEL assays were performed by area measurement or spot quantification with ImageJ 1x.

4.1.5. Statistics

The statistical analyses of the Novel Object Recognition test and immunofluorescence quantitation were performed using unpaired non-parametric Mann-Whitney tests. Results were expressed as mean values ± standard deviations and the significance level chosen was 5%. Survival curves analysis was performed using a Mantel-Cox test. * $p<0.05$, ** $p<0.01$, *** $p< 0.001$.

4.2. Results

4.2.1. Clonogenic survival is not affected by the dose-rate at doses under 6Gy

To first investigate the biological effects of FLASH irradiation, clonogenic survival was investigated on both tumor and immortalized cells *in vitro*. Interestingly, no difference was observed in terms of surviving fraction on all the different cell types after an irradiation at 2, 4 or 6 Gy with FLASH or CONV irradiation (Figure S1). These results suggest that increasing the dose-rate does not modifies the intrinsic radio sensitivity of cells *in vitro* for doses below 6 Gy.

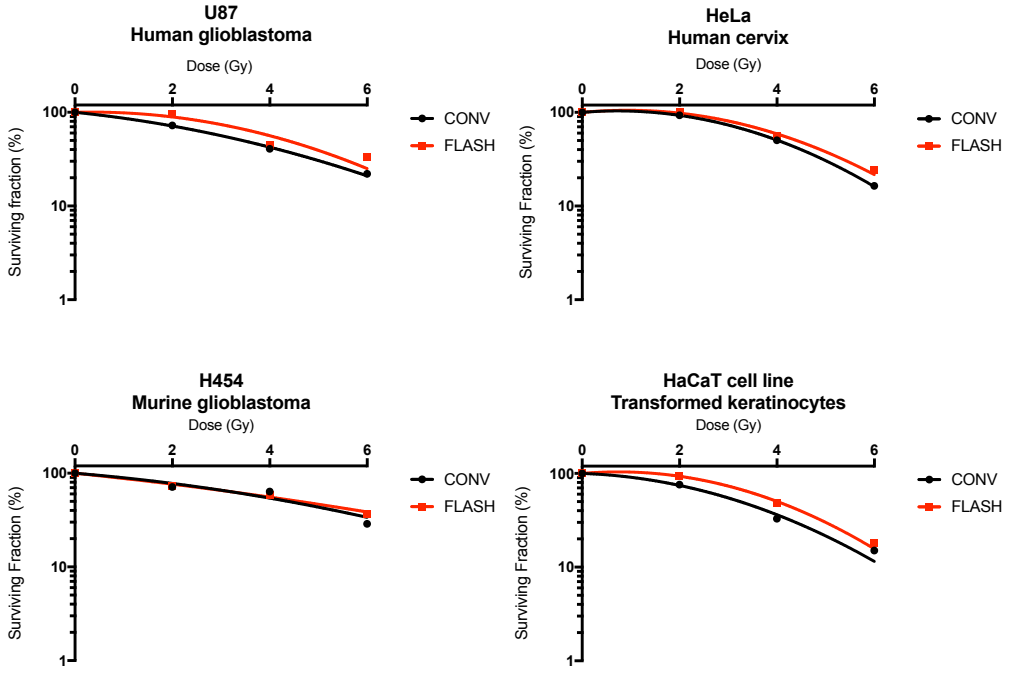

Figure S1: Clonogenic survival of different cancer (U87, H454, HeLa) and immortalized (HaCaT) cells after FLASH (red curves) and CONV (black curves) irradiation.

4.2.2. Glioblastoma treatment

To complete the evaluation of FLASH irradiation effectiveness to treat GBM (Montay-Gruel et al. 2018; in prep), the H454 orthotopic GBM mouse model was used to perform a dose escalation and dose fractionation studies (Figure S2). After the development of the disease in the brain, tumor-bearing mice were whole-brain irradiated with a single dose of 10, 13 or 15 Gy FLASH or at conventional dose-rate. Four weeks post-irradiation, all irradiated groups displayed a significant anti-tumor response quantified by bioluminescence compared to the control non-irradiated animals. All doses with both irradiation techniques induced a significant tumor growth retardation compared to the non-irradiated animals measured by bioluminescence 4 weeks post-irradiation (respectively $2.9x10^8$ and $2.7x10^8$ and $4.6x10^7$ vs. $2.5x10^9$ photons.s^{-1}).

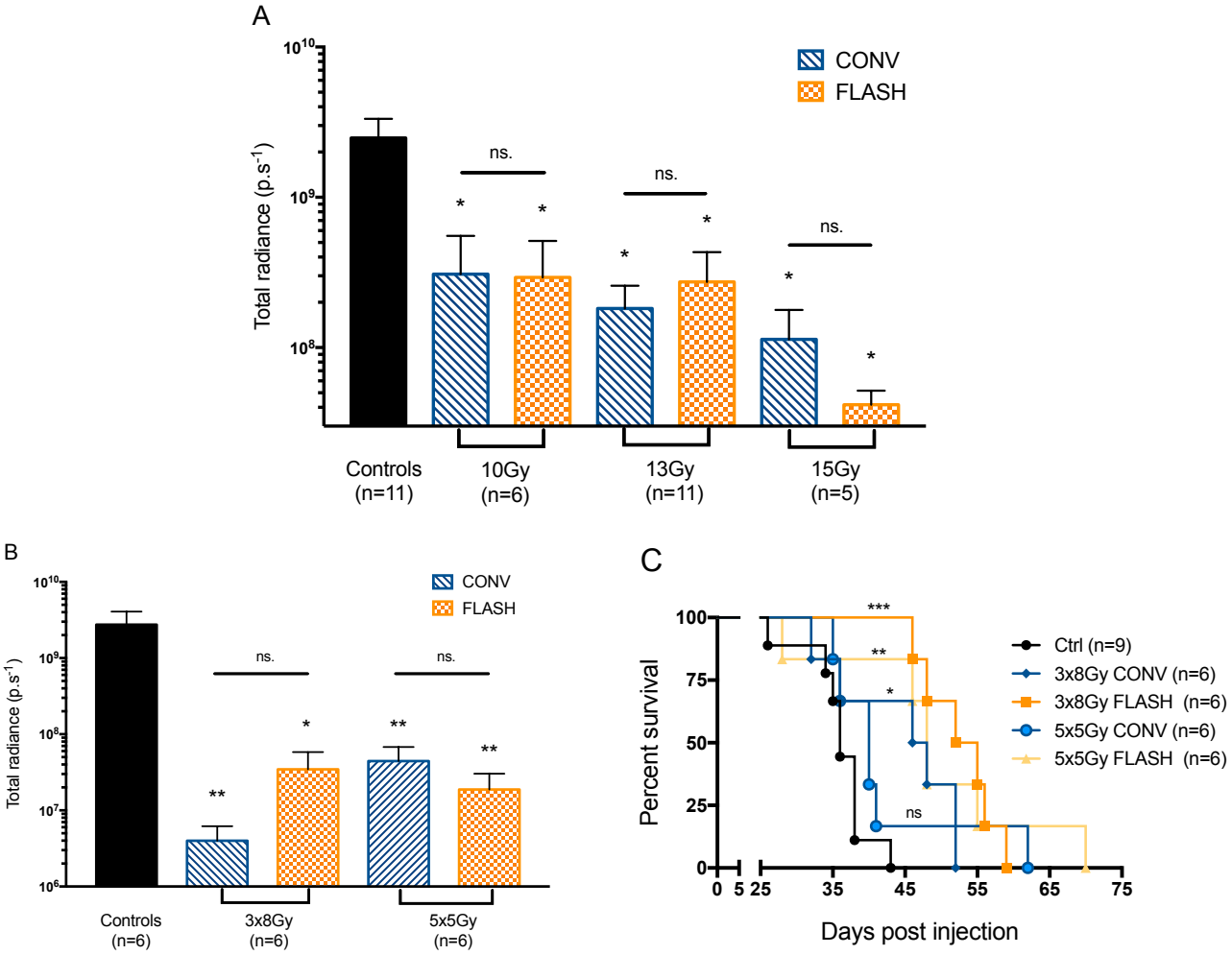

Figure S2: Evaluation of FLASH anti-tumor response on orthotopic H454 murine glioblastoma model. Tumor burden measurement was performed weekly by IVIS on animals irradiated with (a) a single dose or (b) a fractionated regimen with FLASH or conventional dose-rate radiation-therapy (CONV). Bars represent mean total radiance and whiskers represent standard deviations. Statistical analyses were performed with Mann-Whitney test. (c) Survival of animals irradiated with a fractionation regiment FLASH or conventional dose-rate radiation-therapy. Statistical analyses were performed with Mantel-Cox test and * = p<0.05; ** = p<0.01; *** = p<0.001.

Once again, no difference was observed between FLASH and conventional dose-rate anti-tumor effect at isodose, confirming with an orthotopic model that both techniques trigger the same anti-tumor effect. Dose fractionation is suitable with FLASH radiotherapy. As most of the radiation-therapy treatments are based on fractionation regimens, it was necessary to evaluate the feasibility of dose fractionation for the tumor treatment with FLASH irradiation. Two different daily fractionation regimens were evaluated on orthotopic glioblastoma bearing mice.

Whole brain irradiation was delivered in 3 times 8 Gy or 5 times 5 Gy with FLASH or conventional dose-rate irradiation. Tumor burden evaluation by bioluminescence 4 weeks post-irradiation showed a significant anti-tumor effect of both fractionation regimen with FLASH irradiation compared to the non-irradiated animals ($3.8x10^7$ and $1.9x10^7$ vs. $2.7x10^9$ photons.s^{-1}). Moreover, no statistical difference was observed between FLASH and conventional dose-rate irradiated groups. These results are in correlation with survival studies, suggesting that fractionated radiation-therapy regimen is suitable with FLASH irradiation and induce a similar anti-tumor effect on glioblastoma model compared to conventional dose-rate fractionated radiation-therapy.

4.2.3. Long-lasting cognitive protection triggered by FLASH-RT

To investigate whether the memory preservation triggered by FLASH irradiation one and two months post-RT (P. Montay-Gruel et al. 2017) (Montay-Gruel et al. 2018; in prep.) was long-lasting, NOR tests were performed on animals six and nine months post-WBI at 10 Gy (Figure S3). Even if a higher heterogeneity was observed in the control groups at later time points, no significant difference was observed between non-irradiated and FLASH irradiated animals 6 and 9 months post-WBI. On the contrary, the significant drop in RR observed in animals irradiated with conventional dose-rate was still observed 6 and 9 months post-WBI. These results show that conventional dose-rate induces irreversible memory alterations when, for a same dose, a long-lasting memory preservation is observed in animals irradiated with FLASH-RT.

To assess the dose limit to protect the functional skills after FLASH-WBI, NOR tests were performed on animals after the delivery of a dose-escalation up to 14 Gy single dose WBI (Figure S4A). No difference in RR was observed for animals irradiated at 10 and 12 Gy FLASH-WBI compared to the controls. Nevertheless, a single dose of 14 Gy FLASH-WBI induced a significant drop in RR, with values comparable to the ones of animals irradiated with 10 Gy conventional dose-rate. We conclude that FLASH irradiation protects the mice's memory up to minimum 12 Gy single-dose, with a total loss of memory skills after a single dose of 14 Gy WBI two months post-RT.

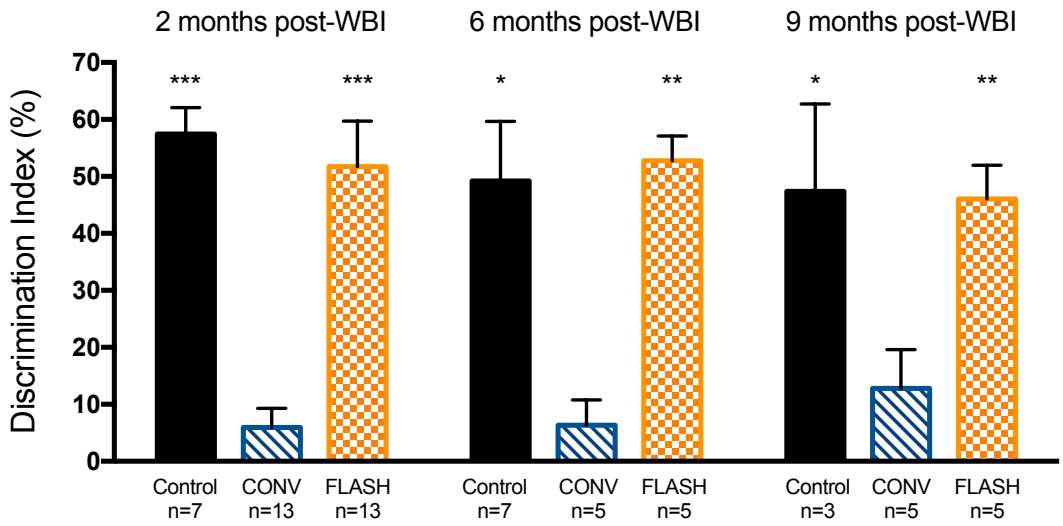

Figure S3: Evaluation of the Recognition Ratio (RR) two, six and nine months post irradiation for groups of mice that received sham irradiation (Control), and 10 Gy CONV or FLASH. Bars represent mean values and whiskers standard deviations. Statistical analysis is realized with Mann-Whitney test.

As all the previous experiments were done on animals irradiated before 10 weeks of age, it was necessary to assess the memory cognition of animals irradiated at older ages. For this experiment, one-year-old mice received a single dose of 10 Gy WBI and NOR test was performed two months post-irradiation (Figure S4B). All groups of older mice showed higher SDs compared to the young mice groups, suggesting more heterogenous results. Nevertheless, no influence of the age on the RR was observed in non-irradiated animals. As for young animals, a significant drop was observed after WBI at conventional dose-rate. On the contrary, no significant difference was observed in the RR of old animals irradiated with FLASH-WBI compared to the control groups. Nevertheless, this result was significantly lower than the one obtained on young mice irradiated with the same dose-rate. With a higher heterogeneity in all groups and a significantly lower RR in the FLASH irradiated group, animals irradiated at an older age tend to be more sensitive to FLASH irradiation than younger mice. Nevertheless, FLASH irradiation still triggers a memory preservation on older animals, depicting an advantage compared to conventional dose-rate irradiation.

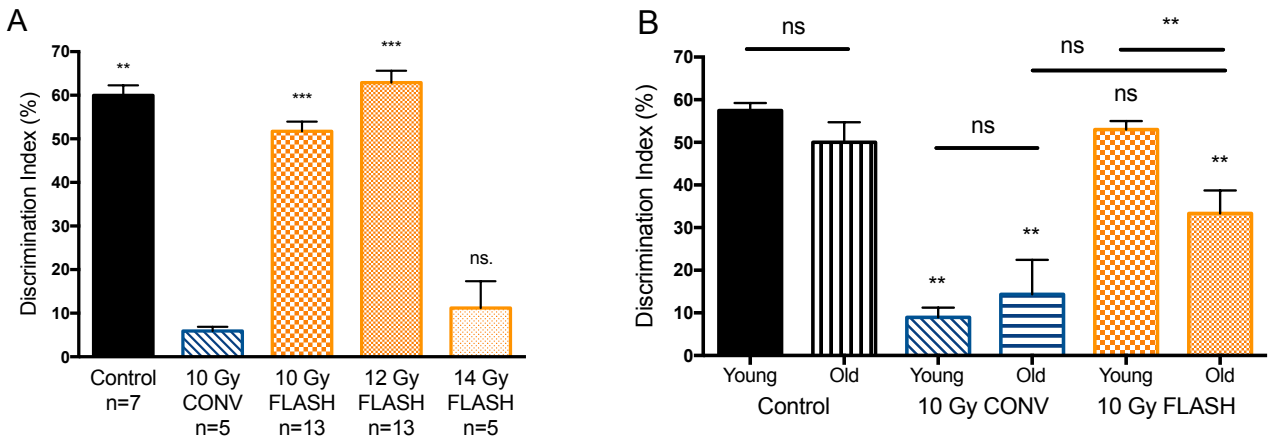

Figure S4: (A) Evaluation of the Recognition Ratio (RR) two months post irradiation for groups of mice that (a) received sham irradiation (Control), 10 Gy Conv, and 10, 12 or 14 Gy FLASH. (B) Evaluation of RR in mice that received sham irradiation (Control) or 10 Gy WBI with FLASH or conventional dose-rate at 8 weeks (Young) or 1 year (Old) of age. Bars represent mean values and whiskers standard deviations. Statistical analysis is realized with Mann-Whitey test.

4.2.4. Hippocampal apoptosis

To have a first insight of a differential radiation-induced cell toxicity after FLASH and conventional dose-rate, apoptotic cells were quantified in the hippocampi of mice irradiated at 10 Gy WBI with FLASH or conventional dose-rate 3 days post-RT (Figure S5). The basal level of apoptotic cells was quantified in non-irradiated animals. A significant increase of more than 6 folds in the mean number of TUNEL+ cells was observed in the hippocampi of mice irradiated with conventional dose-rate compared to the control group 3 days post-irradiation. Interestingly, only a two-fold-increase was observed in the FLASH irradiated group. These results show an increase in the radiation-induced remaining apoptosis 3 days post-irradiation with conventional dose-rate, when for a same delivered dose and at the same time-point, significantly less remaining apoptosis is observed. This difference suggests a protection against cell toxicity and cell death triggered by FLASH compared to conventional dose-rate irradiation.

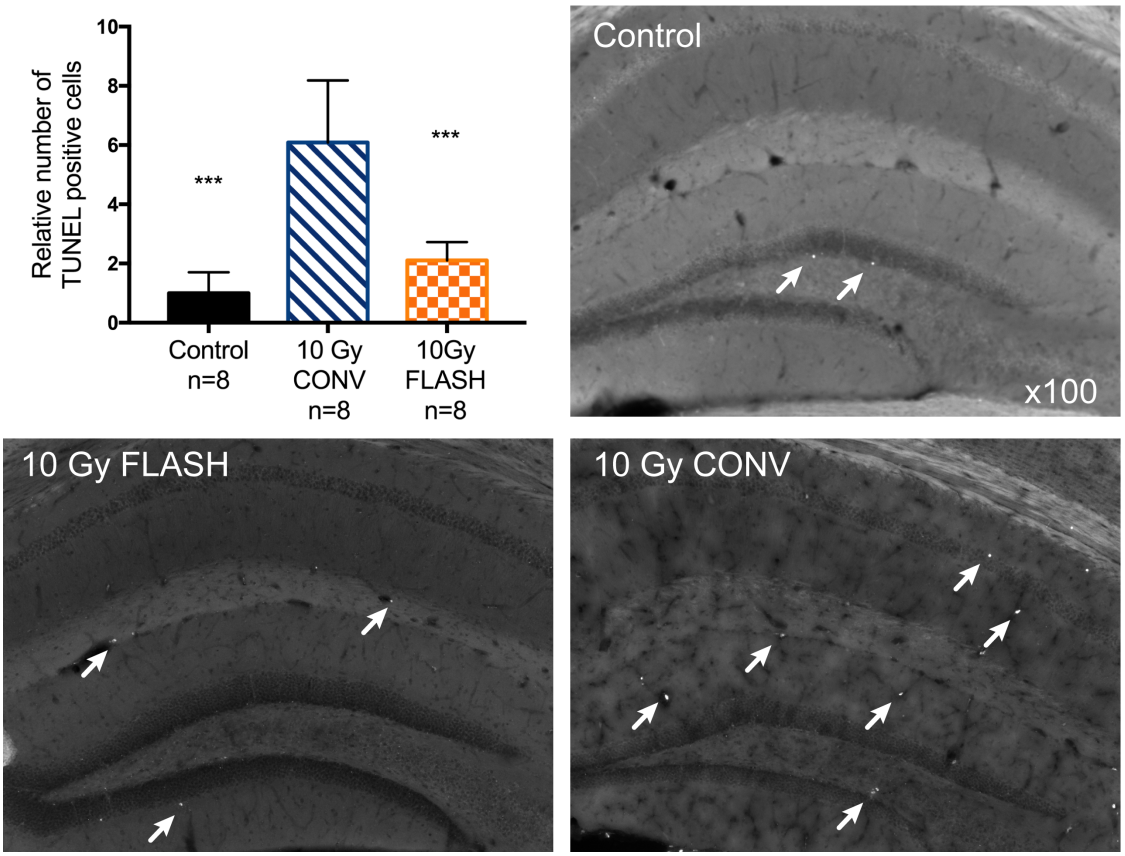

Figure S5: Relative number of TUNEL+ cells in the hippocampi of mice irradiated with 10 Gy WBI at FLASH or conventional dose-rate 3 days post-irradiation. Results are normalized against TUNEL+ cell number of control non-irradiated animals. Bars represent mean values and whiskers standard deviations. Statistical analysis is realized with Mann-Whitey test.

4.2.5. Reactive astrogliosis assessment

To assess the reactive astrogliosis occurrence in the brain after FLASH and conventional dose-rate irradiation, GFAP expression was observed and quantified in 10 Gy irradiated brain at 3, 14 and 60 days time-points (Figure S6). Three days post-irradiation, no difference in astrocytes number was observed between control mice and irradiated groups. Fourteen days post-irradiation, GFAP expression was observed in clusters in the striatum of mice irradiated with conventional dose-rate whereas no increase in the GFAP expression was observed in the FLASH group. Two months post-irradiation, an increase of more than 2.5 folds in GFAP expression was observed all over the striatum of the conventional irradiation group (Figure S6) suggesting, as expected, an important increase in astrocyte number. Nevertheless, no difference in terms of GFAP staining area was observed between FLASH and control groups suggesting the absence of radiation-induced reactive astrogliosis.

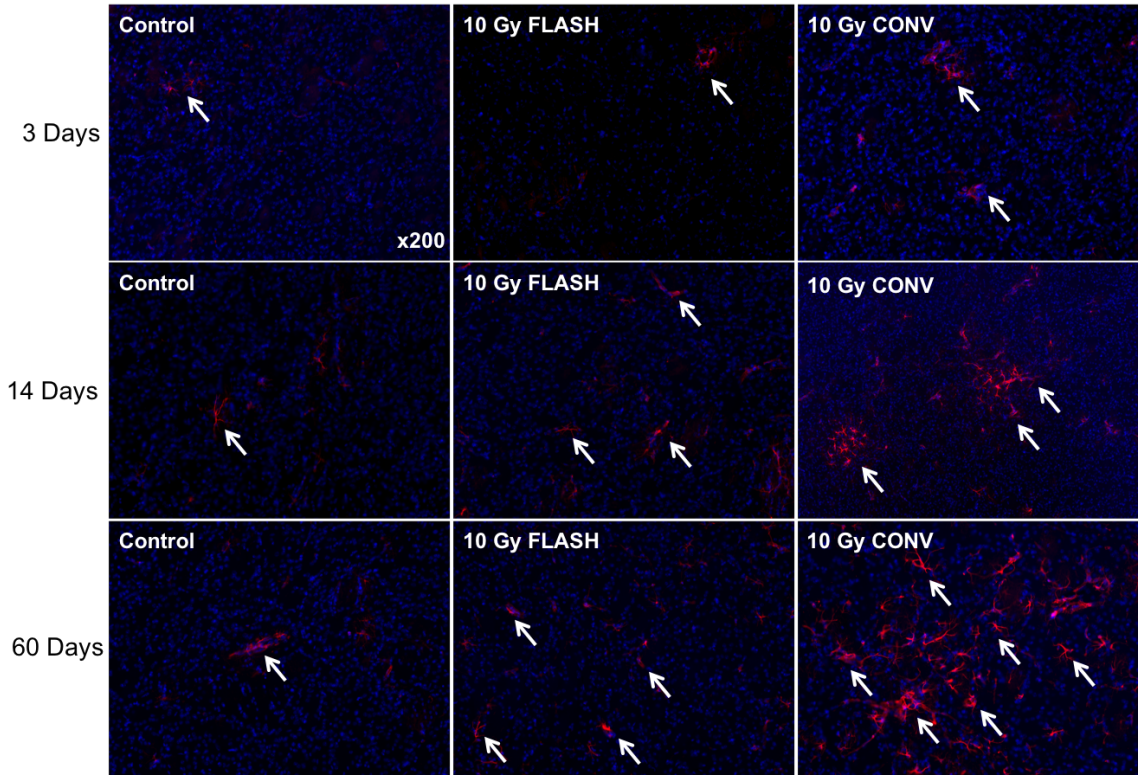

Figure S6: Reactive astrogliosis evaluation by GFAP immunostaining and quantitation on brain striatum sections of non-irradiated mice (Control) and mice irradiated with 10 Gy CONV or FLASH to identify radiation-induced astrogliosis. Arrows point at GFAP expressing astrocytes. Red: GFAP, Blue: DAPI.

GENERAL DISCUSSION

1. Discussion

This PhD work focused on the study of the glioblastoma and the normal brain tissue responses to the delivery of ultra-high dose-rate irradiation called “FLASH-RT” and delivered by an experimental LINAC.

The investigation of the tumor growth delay after the delivery of FLASH-RT on subcutaneous xenografted human GBM, orthotopic xenografted murine GBM and transgenic spontaneous GBM mice models showed a similar anti-tumor effect of ultra-high (FLASH) and conventional dose-rate irradiations. The equivalent anti-tumor effects provided by FLASH-RT and conventional dose-rate irradiation on the GBM models are in line with the observations made on the lung, breast and H&N tumor models studied previously (Vincent Favaudon et al. 2014).

At the normal brain level, the investigation of the cell toxicity revealed a lower level of radiation-induced delayed apoptosis after FLASH-RT compared to conventional dose-rate irradiation. This observation correlated with the absence of astrogliosis development when conventional dose-rate irradiation is known to be responsible for pathogenic reactive astrogliosis (C. S. Chiang et al. 1993; S. Y. Hwang et al. 2006; L. Yang et al. 2017). Moreover, the absence of microglial activation in the brain of FLASH-irradiated animals suggests that FLASH-RT triggers a long-term protection against radiation-induced neuroinflammation which has been described as associated to neurogenesis and cognitive impairments (J. D. Cherry et al. 2014; X. Dong et al. 2015; C. T. Ekdahl et al. 2009; W. J. Streit et al. 2004; P. Su et al. 2014). Interestingly, our results on hippocampal cell-division and neuronal structure preservation are consistent with those reported in these studies.

The protection of the cellular compartment from radiation-induced toxicity triggered by FLASH-RT evoked a functional preservation, especially concerning the cognitive skills. The behavioral tests performed 1 to 9 months post-irradiation showed a protection of the memory and an absence of anxiety and stress disorders in animals irradiated with FLASH-RT above 100 Gy/s. Importantly, the cognitive skills of orthotopic GBM-bearing animals treated with FLASH-WBRT were similar to the non-treated animals, whereas mice treated with conventional irradiation showed a significant decrease in their memory functions. This unique result shows that FLASH-RT can prevent from the radiation-induced cognitive impairment, even in GBM-bearing animals.

All the results obtained on the normal tissue fit with similar preservations recently described in a lung fibrosis model (Vincent Favaudon et al. 2014) and in a gastro-intestinal toxicity model (B. W. Loo et al. 2017).

Eventually, normal brain toxicity studies after the delivery of ultra-high dose-rate X-rays irradiation showed that FLASH-X-rays produced by a synchrotron light-source induce the same protection pattern than FLASH-electrons. The demonstration of a particle-independent FLASH-effect is coherent with the observations of similar results obtained with protons (S. Auer et al. 2011; T. Prempree et al. 1969; T. E. Schmid et al. 2010, 2011). The observation of the FLASH effect obtained with different irradiation modalities will surely accelerate the clinical transfer.

In-vitro, when no difference in clonogenic survival was observed following FLASH and conventional dose-rate irradiations at doses under 10 Gy, the same experiments performed with higher doses in physiological dioxygen concentration (1-4%) showed a radioprotection and a better clonogenic potential in cells irradiated with FLASH-RT. Previous studies showed that the survival of anoxic bacteria after radiation exposure was independent on the dose-rate, whereas aerobic bacteria at very low oxygen tensions were less sensitive to radiation at high dose-rates than the same bacteria placed in high oxygen tensions (D. L. Dewey 1969; D. L. Dewey et al. 1959). Similar results were obtained on mammalian cells regarding clonogenic survival, with the observation of an increased clonogenic potential in cells irradiated with high dose-rates in hypoxic conditions around 1% of O_2 (E. R. Epp et al. 1972; H. Weiss et al. 1974). Both studies concluded that this protective effect on clonogenic survival might be due to a transitory dioxygen depletion during the delivery of high dose-rate irradiation, due to the low concentration in dioxygen in the cell environment and the very rapid delivery of the dose. Nevertheless, none of these studies addressed the production of ROS by the different irradiation modalities.

In the present work, water radiolysis experiments demonstrated lower quantities of hydrogen peroxide produced after FLASH-RT, suggesting a differential production of ROS between conventional and ultra-high dose-rate irradiation. This result obtained in a non-biological model suggests that the time of irradiation, which is the only variable, is crucial to induce the differential effect between ultra-high and conventional dose-rate irradiation. In addition, ROS scavenging study *via* the use of an antioxidant delivery to a zebrafish embryo model of total body irradiation demonstrated that the use of amifostine or NAC had no effect on FLASH-irradiated embryos whereas the same treatments significantly improved the embryos morphology after conventional dose-rate irradiation. Nevertheless, even with the antioxidant treatment, these dysmorphisms were more severe in the embryos irradiated at conventional dose-rate, suggesting a higher innocuousness of FLASH-RT induced by lower levels of ROS production.

Finally, oxygen-dependence studies were performed on both tumoral and normal tissues in murine models. Of note, irradiation of subcutaneous GBM in hypoxic and hyperoxic conditions revealed that the tumor growth delay was not altered by hypoxia in the FLASH-irradiated group, whereas the anti-tumor effect induced by conventional dose-rate irradiation was totally altered by hypoxia. Nevertheless,

hyperoxia induced an increase in the tumor growth delay for both dose-rate irradiations. The identification of dioxygen concentration as a primary mediator of the FLASH effect *via* a differential production of ROS depending on the irradiation time, is consistent with previous *in vitro* and *in-vivo* studies. Other investigations realized *in-vivo* led to similar conclusions. The absence of skin toxicity observed on rats irradiated with high dose-rates was observed at an oxygen partial pressure of 5-10mmHg, and this protection was not observed in complete anoxic conditions (S. B. Field et al. 1974). Moreover, the protection against radiation-induced tail necrosis triggered by high dose-rate irradiation was reversed by an increase in dioxygen concentration (J. H. Hendry et al. 1982).

Interestingly, the reversion of the FLASH-associated cognitive protection induced by an increase in dioxygen concentration is consistent with the hypothesis of a transient hypoxia induced by ultra-high dose-rate irradiation. Indeed, the delivery of the dose in a very short time is thought to consume the dioxygen present in the tissue at the time of irradiation, without allowing the refueling of dioxygen by diffusion from the vascular compartment. This transient hypoxia without re-oxygenation before the end of radiation delivery would thus induce a lower ROS production that could explain the lower tissue toxicity compared to conventional dose-rate irradiation. By contrast, in a very hypoxic or anoxic condition as the tumor environment, the indirect action of radiations is minimal, and the time of dose-delivery does no influence the radiation-induced damage, leading to similar effects. This hypothesis would explain the absence of difference in clonogenic survival observed in ambient dioxygen concentrations with doses that are not high enough to induce a transient hypoxia in the cells.

Altogether, these results suggest that FLASH-RT has the potential to improve the therapeutic index of radiation-therapy by decreasing the radiation-induced brain toxicity while keeping a similar anti-tumor effect compared to conventional dose-rate irradiation. Moreover, *in vitro* and *in-vivo* models, including water-radiolysis experiments, cell lines irradiation, zebrafish models and normal tissue and GBM murine models, allowed to identify the dioxygen concentration as a primary mediator of the FLASH effect.

2. Perspectives

The data generated in this work raise several questions, hypotheses and perspectives summarized in the Figure 5.

The results obtained on the different tumor models open new avenues on the possibility to improve the tumor control. As the current GBM treatment consists in the administration of concomitant and adjuvant TMZ and fractionated radiation-therapy, it will be necessary to further investigate such

combined treatments with FLASH-RT. Moreover, the use of recently developed brain metastases mice models (L. Sevenich et al. 2014) or lentivirus-activated glioblastoma models (T. Marumoto et al. 2008) would complete the investigation of the FLASH-RT anti-tumor effect. In addition, there is a growing interest in the study of GBM immune infiltration and its role in the treatment response and outcome. Studies on the GBM microenvironment, recently and elegantly reviewed by Quail and Joyce, showed the importance of the resident glial cells along with the peripheral immune cells, including dendritic cells, neutrophils and lymphocytes, in the cancer progression and therapeutic response of GBM (D. F. Quail et al. 2017). Recently, the use of CSF1-R inhibitors has been demonstrated capable to modify the tumor immune microenvironment and to abrogate the therapeutic resistance of GBM (D. Yan et al. 2017). As we showed that FLASH-RT does not induce neuroinflammation assessed by microglial activation in the normal brain, the investigation of the immune infiltration in the tumors treated with FLASH-RT is essential to consider the use of combined therapeutic agents.

The investigation of the hippocampal cell division in the FLASH irradiated mouse brain showed a relative preservation as compared to conventional dose-rate, which might explain the absence of radiation-induced cognitive decline. A further investigation of the NSCs fate is currently ongoing, with the set-up of a cytometry panel aiming at identifying the quiescent and activated NSCs along with neural precursors (M. Daynac et al. 2015). This particular qNSCs population differs from other proliferative populations such as neuroblasts, which are usually depleted after irradiation (M. Daynac et al. 2013; C. Shinohara et al. 1997; E. Tada et al. 1999). Indeed, qNSCs have been identified as able to re-enter cell cycle and to induce *de novo* neurogenesis (F. Doetsch et al. 1999; C. M. Morshead et al. 1994). Flow cytometry experiments will thus be performed soon to further investigate the phenotype modifications and to test the hypothesis that an increase in qNSCs population could explain the neurogenesis and functional preservation linked to FLASH irradiation. For this purpose, qNSCs will be sorted and their functionalities will be investigated *in vitro* (P. Codega et al. 2014; M. Daynac et al. 2016) along with single cell mRNAseq (P. Codega et al. 2014) with the aim to identify potential target genes differentially activated after FLASH irradiation that might be involved in this particular protective phenotype balance.

The cognitive preservation observed after FLASH irradiation most probably relies on other radiation-induced cellular and molecular effects. Many different types of intracellular signaling such as NOS/ROS, TGFβ and TNFα can be secreted following irradiation and have been identified as astrogliosis inducing factors (M. V. Sofroniew 2009). Thus, differential astrogliosis induction following FLASH and conventional irradiation will be further investigated using a *GFAP-eGFP* transgenic mouse model. These mice will be used to precisely radiation-induced reactive astrogliosis quantitation in several brain regions by flow

cytometry. Moreover, it is described that ionizing radiations induce reactive astrogliosis *via* microglial activation and neuroinflammation (S. Y. Hwang et al. 2006) and that this neuroinflammation participates to the cognitive dysfunctions and neurogenesis impairment (J. D. Cherry et al. 2014; X. Dong et al. 2015; C. T. Ekdahl et al. 2009; W. J. Streit et al. 2004; P. Su et al. 2014). In this context, we have observed that, by contrast to conventional irradiation, FLASH-RT does not induce microglial activation *via* CD68 expression. In order to confirm this result and also to explore further the mechanisms behind it, we will analyze, through flow cytometry studies, the infiltration of immune cells in the brain including bone-marrow derived macrophages, lymphocytes and monocytes. This might probably allow us to identify a potential difference in chronic neuroinflammation following FLASH-RT. Moreover, cytokine expression in the whole brain and in specific brain regions such as the hippocampus, will be investigated by protein arrays and RNAseq.

Dioxygen consumption at the moment of irradiation has been identified as an important parameter to trigger the FLASH effect. This work supports the hypothesis of the induction of a transient O_2 depletion by FLASH-RT that would induce less damage to the normal tissue in normoxic conditions by less production of ROS. To further investigate this hypothesis, *in vitro* models will be reproduced in normo-, hypo- and anoxic conditions. Moreover, a transgenic zebrafish model *Hyper*, allowing to evaluate *in-vivo* the ROS production will be used (P. Niethammer et al. 2009; M. Oparka et al. 2016), along with *in vitro* models of ROS quantification by CM-H2DCFDA oxidative stress indicator and *in-silico* models developed by chemical experiments and computer scientists.

The hydroxyl radical has the particularity to be highly reactive and to induce a large amount of damage to the cell. Nevertheless, its instability makes it difficult to measure its formation. As FLASH-RT produces lower H_2O_2 concentration, and given that the hydroxyl radicals react to form H_2O_2 molecules, we hypothesize that FLASH-RT might lead to lower secondary hydroxyl radical production. The use of an electron paramagnetic resonance (EPR) apparatus available at the CHUV, will allow for spin-trapping measurements. Spin-trapping is a technique which permits the determination of very reactive radicals, such as the hydroxyl radical, by stabilization of the spin through the formation of an adduct with other molecules (e.g. PBN or DMPO). The irradiation of solutions containing spin-trapping molecules by both FLASH and conventional irradiation techniques will be realized and will allow us to identify the different ROS along with the measurement of the total radical concentration by EPR spectrum generation. We are also interested in determining the differences in production of the superoxide anion ($O_2^{\bullet -}$) between FLASH and conventional irradiation. Indeed, the production of the superoxide anion is a function of the O_2 concentration, which is an essential parameter of the observed difference in biological effect between FLASH and CONV. We will thus try to measure the differences in the production of the superoxide ion by

using the superoxide dismutase enzyme (SOD). SOD will inhibit the formation of the superoxide-adduct, lowering the overall EPR signal. In addition, kinetic competition experiments will be performed to establish the existence of the free hydroxyl radicals and quantified them. Using such experiments, we expect to be able to determine the concentration of primary radicals formed during water radiolysis upon both irradiation modalities. ROS are not the only damaging molecules produced by radiations. The effect of various free Fe^{2+} concentrations mimicking biological free $Fe2^{+}$ concentration on the overall H_2O_2 production will be investigated by irradiating solutions containing Fe^{2+}.

Experiences performed in the 70's on *in vitro* irradiation of bacteria and mammalian cells by ultra-high dose-rate irradiation have revealed that the measured fractional survival curves exhibit a break at large dose beyond which their exponential slope become independent of the molecular oxygen concentration. This particular behavior has been successfully explained by assuming that ultra-high dose-rate irradiation depletes the oxygen concentration within the cells and modifies accordingly the Alper and Howard-Flanders single target model of cell radiation-sensitivity to oxygen (H. Weiss et al. 1974). This model has the advantage of downscaling the biological mechanisms of radiation-sensitivity to three phenomenological parameters: K, the rate of increased sensitivity to the increase of oxygen partial pressure; m, the maximum relative sensitivity and g, the oxygen depletion dose factor. When fitted to the 70's data, the model also predicts a larger radiation-sensitivity of cells to oxygen (large parameter K) at ultra-high dose-rate. In a first time, we will confirm that the measured fractional survival curves of mammalian cells irradiated by our prototype eRT6 LINAC with different oxygen concentrations and different dose-rates can adequately be reproduced by Weiss depleting oxygen concentration model. Repeating the measurements for various pulse configurations will further provide the dependence of the parameters K, m and g on the time delivery structure. In a second time, we intend to explore the possibility of expanding the Alper and Howard-Flanders model to predict the dose-rate dependence of selected indicators of biological response to *in-vivo* irradiation in the zebrafish and normal brain tissue models developed previously. If successful, we will further investigate if we can expand the model to take into account the use of radioprotectors such amifostine by mimicking the oxygen depletion model of Weiss.

In conclusion, the description of this unique preservation of the normal brain tissue with an efficient anti-tumor effect on glioblastoma models and the identification of physico-chemical events associated with the FLASH-effect is encouraging and might lead to the implementation of FLASH-RT in the clinics in a near future. Nowadays, only a few devices are able to deliver ultra-high dose-rate irradiation to large treatment fields. The potential of low-energy electrons for FLASH-RT delivery has now been well characterized, in this work and in previous studies, including a veterinary clinical trial on H&N cancer-bearing pet cats (Vozenin et al, 2018; in press). The current development of a 10 MeV LINAC will allow the

delivery of FLASH-electrons and a clinical transfer *via* the use of intra-operative radiation-therapy (IORT) in order to overcome the limited in-depth penetration of electrons. Moreover, this work showed that the FLASH effect is also triggered by ultra-high dose-rate X-rays produced by a 3rd generation synchrotron light-source. This additional benefit should accelerate the clinical application, as X-rays-based radiation-therapy has a broader applicability given the distribution profile of photons *versus* electrons. Thus, the ability to deliver radiation-therapy treatments in a very short time also represents a clinical improvement by potentially overriding the tumor-motion challenge but also an economical advantage by increasing the number of treated patients per day. Added to the increase in the therapeutic index of radiation therapy, all the advantages of FLASH-RT will undeniably accelerate it transfer to clinics to allow the addition of a new tool in the cancer treatment management, providing a better tumor treatment and a better quality of life for the patients.

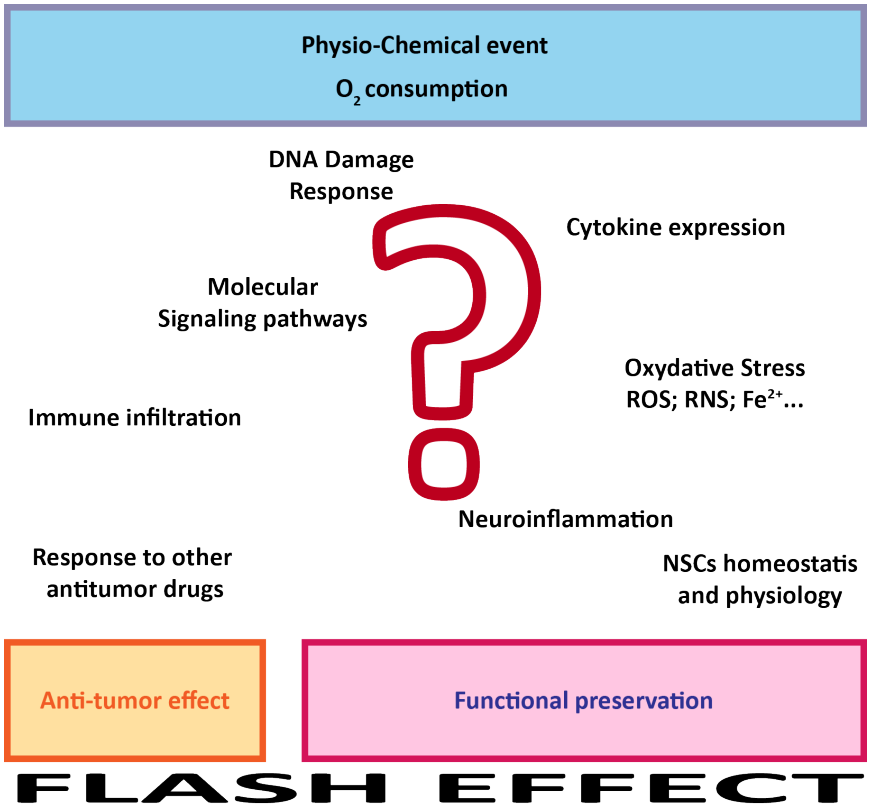

Figure 5: The description of a physico-chemical event triggering the FLASH-effect raises several questions, hypotheses and work perspectives.

APPENDICES

1. **Book chapter: Novel strategies to prevent, mitigate or reverse radiation injury and fibrosis**

NOVEL STRATEGIES TO PREVENT, MITIGATE OR REVERSE RADIATION INJURY AND FIBROSIS

IN: STRATEGIES TO ENHANCE THE THERAPEUTIC RATIO OF RADIATION AS A CANCER TREATMENT.

Pierre Montay-Gruel, Gaël Boivin, and Marie-Catherine Vozenin.

Editors: Mitchell S. Anscher & Kristoffer Valerie

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Chapter 4
Novel Strategies to Prevent, Mitigate or Reverse Radiation Injury and Fibrosis

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Abstract Despite recent advances in Radiation Oncology with treatment planning and delivery of image-guided radiation therapy, acute tissue toxicity is still a dose-limiting factor for optimal local tumor control. Additionally, as the number of long-term cancer survivors is increasing, unacceptable complications emerge and dramatically impair the patients' quality of life. This means patients and clinicians expect therapeutic management of radiation-induced complications. Over the past four decades, research has enhanced our understanding of the pathophysiological, cellular and molecular processes governing normal tissue toxicity. This knowledge has provided us with tools to improve the therapeutic ratio of radiation therapy by enhancing its tumoricidal effect and protecting normal tissue. In this chapter, we review biology-driven efforts to develop translatable therapeutic approaches to prevent, mitigate or reverse radiation injury based upon cellular and signalling pathways targeting. We also highlight innovative approaches based upon manipulating external contributors such as the microbiota and applying novel radiotherapy delivery procedures.

Keywords Normal tissue complication • Fibrosis • Therapeutic strategies • Stem cells • Stroma • Inflammation • Immune response • Microbiome • Novel radiotherapy procedure

4.1 Introduction

The incidence of cancer is increasing worldwide with more than 14 million new cases per year. About 50 % of cancer patients are treated with radiation therapy (RT), making it, after surgery, the most important contributor to cancer cure. In the era of targeted therapies, RT is one of the best examples of a precise and

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powerful targeted treatment. Thanks to major advancements in physics, imaging and ballistics, high-precision dose delivery has succeeded in safely reducing the volume of irradiated normal tissues. New and very appealing RT approaches using high or very high dose per fraction (hypofractionation) such as stereotactic body radiotherapy (SBRT) also called stereotactic ablative radiotherapy (SABR) are increasingly used, both in early stages cancers and in some oligo-metastatic patients. In parallel, the particular dose distribution of protons and heavy ions has been therapeutically exploited with the aim to efficiently spare sensitive organs and enhance tumor cure.

At the biological level, the molecular response of cells and normal tissues to ionizing radiation involves a complex series of events that leads to the loss of tissue homeostasis caused by a direct killing of cells and an indirect stimulation of inflammatory mediators, as well as vascular alteration and release of thrombotic factors, recruitment of immune cells, remodeling of the extracellular matrix and stromal compartment associated with fibrosis initiation and maintenance. These phenomena may lead to genomic instability, persistent modulation of gene expression and alteration of the cellular phenotype leading to organ dysfunctions. This kind of modification of normal tissue homeostasis is the origin of disabling radiation-induced side effects which can have a huge impact on the patient's quality of life.

Therefore, increasing tumor sensitivity to radiation or increasing normal tissue tolerance to radiation are the two major paths toward improving the therapeutic index of radiotherapy. In this chapter, we will discuss the management of normal tissue complications, a research topic initiated decades ago by pioneer researchers in the field (including [1–5]). We will review the current status and future opportunities for clinical implementation of novel strategies to prevent, mitigate, and cure radiation injuries based upon the molecular understanding of cell and tissue responses to ionizing radiation.

4.2 Protection of Stem Cells

Recent studies have highlighted the importance of adult stem cells in restoring tissue homeostasis after radiation injury (reviewed in [6]). Because of their unique properties of self-renewal, pluripotency and the ability to differentiate into organ-specific functional cells, stem cells are fundamentally relevant in terms of maintaining life-long tissue homeostasis.

Radiation exposure may directly kill adult stem cells or induce degenerative-mutations leading to stem cell depletion. Therefore the protection, stimulation, recruitment or replacement of stem cells with intact functional properties facilitate tissue regeneration, and wound healing. Novel technological, pharmaceutical, or biological strategies to spare adult stem cells have been actively explored as well as replacement strategies based on stem therapy (Fig. 4.1).

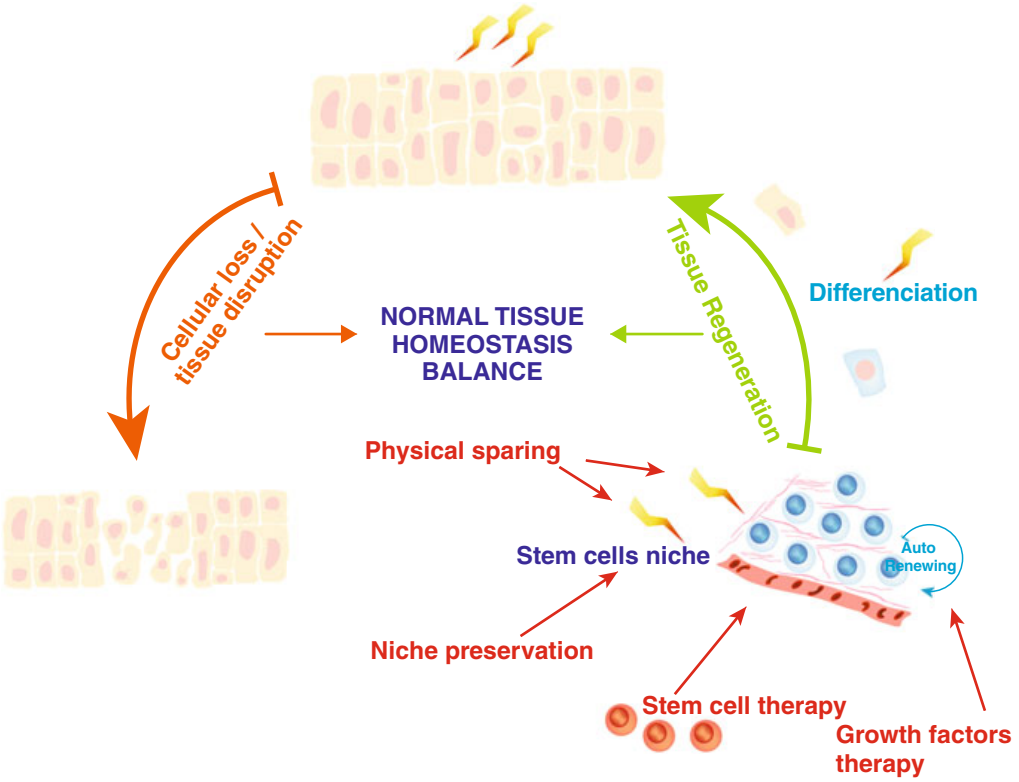

Fig. 4.1 A proper balance between tissue regeneration and disruption is key to normal tissue homeostasis through the stem cell pool. Radiation modifies this balance and different prophylactic, therapeutic agents, and technologies have been developed to preserve and restore it

4.2.1 Preventing the Depletion of Stem Cell Pools and Improvement of Tissue Regeneration

Recent studies have shown that adult stem cells are not evenly distributed in tissues but rather located in specific niches able to trigger regeneration and differentiation. The recognition of the stem cell niche and its relevance to stem cell function has prompted extensive research into the possibility of ballistic protection of stem cells. For instance, in the brain, Neural Stem Cells (NSCs) are mainly localized in the subventricular and subgranular zones (SVZ/SGZ). SGZ-NSCs are of major importance for cognitive skills as both retrospective and prospective trials have demonstrated radio-induced neurocognitive impairment upon hippocampal irradiation [7, 8]. Interestingly, recent technological advances (IMRT, Tomotherapy, proton) have shown it is possible to spare the hippocampus (or at least reduce the dose) with a good preservation of functional NSCs in the SGZ (preclinical model from [9]) as well as encouraging results in terms of verbal memory (phase II clinical trial from [8]). The major limitation of this strategy is tumor control or relapse in the radiation spared field.

Therapeutic agents have also been tested to protect stem cells from radiation injury especially by stimulating the stem cell pool. For instance, radiation-induced xerostomia can be counteracted by administrating Keratinocyte Growth Factor before and just after irradiation [10]. Along the same line, the trophic factor GLP-2 [11, 12] and the peptide TP508 prevent GI crypts ulceration. TP508 is able to up-regulate the expression of GI stem cell markers such as DCLK1 and LGR5 [13], increase the stemness potential of tissues and restore their integrity. A third method of stem cell preservation is via niche-mediated protection. One study, for instance, reported that the use of pharmacological inhibitors of Prolyl-hydroxylase Domains proteins (PHD) before and after abdominal irradiation of mice showed an HIF-mediated increase in crypt survival, enhancement of crypt regeneration, and increase in mice survival [14].

4.2.2 Stem Cell Therapy to Counteract Radio-Induced Toxicities

Restoration of the stem cell pool and function can also be achieved by stem cell transplantation from syngenic or xenogenic origin; impressive positive results have been reported in most organs (see Table 4.1). The transplanted stem cells repopulate the injured tissue leading to cellular differentiation and cell proliferation However, in most cases paracrine stimulation is also involved. Tissue restoration correlates with a decrease in local inflammation, apoptosis and microvasculature damage, altogether resolving the niche injury. Modifications in protein expression also drive the niche restoration such as TGF-β, CTGF, col1α2/col3α2 and MMP/TIMP balance in the case of skin fibrosis treatment.

4.3 Protection of Resident Cells

Besides the impact triggered by ionizing radiation on the fate and function of stem cells, irradiation also dramatically alters the immediate and long-term function of differentiated cells. The complex interplay and the various cross-communication that occur between the different cellular compartments, *i.e*. epithelial, endothelial, mesenchymal, and immune cells of a given organ after irradiation can initiate, amplify, and maintain tissue injury [34–40]. It is now clear that the complexity of these interactions induces a heterogeneous response, which can only be assessed *in vivo* or using sophisticated 3D-models.

Today, studies in stem cell biology (see Sect. 4.1) and microbiota (see Sect. 4.6) show how acute ulceration and epithelial apoptosis/anoikis function can initiate and amplify radiation injury. In addition, endothelial radiation sensitivity [41] and

Table 4.1 Preclinical and clinical trials using stem cell therapies for treatment of radiation induced normal tissue injury

Organ system	Endpoint	Toxicity	Preclinical studies (stem cell type)	Clinical trials (stem cell type)	References
Skin	Fibrosis, Radionecrosis	Stem cell depletion, Inflammation, Fibroblast death, Epidermis	MSC, ADSC, EPC	MSC	[15]
					[16]
					[17]
					[18]
					[19]
Brain	Cognitive dysfunction, Radionecrosis	NSCs depletion, niche destruction, inflammation	hESC, hNSC	–	[20]
Bone marrow	Aplasia	HSCs depletion, niche destruction	BMDC, HSC, MSC	BM	[21]
H&N	Xerostomia	Stem cell depletion	BMDC, MSC, SGSC	–	[22]
					[23]
					[24]
					[25]
					[26]
GI	Rectitis, Proctitis	Epithelial stem cells depletion, inflammation	MSC	MSC	[27]
					[28]
					[29]
					[30]
Bone	Bone growth	Niche destruction	BMDC, MSC	BM	[24]
	Radionecrosis				[31]
Liver	Liver disease	Hepatocyte cell death	Hepatocyte	Hepatocyte	[32]
			hMSCs		[33]

MSC mesenchymal stem cells, *BMDC* bone marrow-derived cells, *EPC* endothelial progenitor cells, *hESC* human embryonic stem cells, *hNSC* human neural stem cells, *NSC* neural stem cells, *HSC* hematopoietic stem cells, *SGSC* salivary gland stem cells

thrombogenic activation [42] has been extensively studied. The extravasation of blood fluids and leukocytes into the extracellular milieu generates a wounded area prone to long-term endothelium remodeling such as endothelial-mesenchymal transition (EMT). This chronic environment ultimately causes endothelium wall thickening, muscular media replacement by connective tissue, and the activation of myofibroblasts. Defined as the principal cellular effector of radiation-induced fibrosis [36, 43], myofibroblasts can arise from a variety of sources [44] such as trans-differentiated local fibroblasts or mesenchymal cells [45, 46], as well as from epithelial or endothelial cells *via* EMT. Tissue exposure to ionizing radiation induces phenotypic alteration of all resident cells orchestrated by TGF-β1 and a growing list of growth factors including CTGF/lysophosphatidic acid (LPA) and

Rho/ROCK axis, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) [47] as well proinflammatory mediators, cytokines, interleukin (IL)-6 [48] and reactive oxygen species (see Sects. 4.3, 4.4 and 4.5).

4.3.1 Cytoprotective Therapies

Several cytoprotective therapies [49] based upon administering trophic growth factors have been utilized to protect stem (see Sect. 4.1) as well as differentiated epithelial and endothelial cells [50]. Epithelial cells [11, 12] from the gut have been shown to be protected by the trophic factor GLP-2 which stimulates proliferation and integrity of the intestinal barrier [11, 12]. GLP-2 protects mice from radiation-induced mucosal ulceration prone to bacterial translocation and sepsis (see Sect. 4.6). Similarly, KGF has been shown to stimulate cell proliferation and promote epithelial cell survival and differentiation of oral mucosa both in pre-clinical and clinical trials [51, 52] as well as displayed an off-target effect by decreasing ROS levels and stimulating DNA repair [51, 52].

Endothelial cell apoptosis can be inhibited by the transient blockade of p53 and the exogenous administration of basic fibroblast growth factor (bFGF). Both approaches protect the gastrointestinal tract from radiation injury [53, 54]. Similarly, ceramide-targeting antibody [55], and the Flagellin-derivative, CBLB502 [56, 57], protect microvascular endothelial cells of the gut from radiation-induced apoptosis by activation of NF-κB. Finally, enhancing endothelial cell radiation resistance has also been achieved by blocking the TSP1/CD47 pathway [58] in addition to stimulating M1 macrophage infiltration known to be prone to wound resolution and restoration of tissue homeostasis (see Sect. 4.5).

Whether epithelial [59] or endothelial [60] cell death is the primary inducer of acute toxicity has been a long-term dispute within the radiobiology community; however, given the complexity of the pathogenic process it is today obvious that effective therapeutic strategies cannot target only one cell type or pathway but must rely upon coordinated stimulation of stem cell function, resident cell phenotype immunity, and reducing inflammation.

4.3.2 Phenotypical Modulators

Radiation exposure induces a persistent phenotypic activation of endothelial cells and fibroblasts. In endothelial cells this activated phenotype is composed of the expression of thrombogenic and adhesive markers. In fibroblasts, radiation results in the trans-differentiation of myofibroblasts that then synthetize fibrogenic molecules and oversecrete extracellular matrix. Antioxidant therapies including SOD [61–64], pentoxifylline-tocol combination [65–70], and anti-inflammatory agents such as statins [71–75] have been shown to reverse

these activated phenotypes by inhibiting specific signaling mediators such as ROS, thrombogenic factors i.e. Thrombin or fibrogenic pathways such as TGF-β, Protein C, and CTGF.

4.4 Modulation of Signaling Cascade That Regulates Resident Cell Fate Upon Radiation Injury

Of the many signaling cascades governing normal tissue response to radiation injury, we have selected some recently described pathways for their relevance and clinical implication. Most of these pathways are involved in multiple radiation response processes, such as vascular/microvascular damages as well as inflammatory and fibrogenic responses. This means that the drugs that target these pathways are effective in mitigating toxicity to normal tissues by their combined action on these multiple pathogenic processes.

4.4.1 Protein C Pathway

Microvascular injury is a prominent feature of normal tissue radiation injury and plays a critical role in both acute/inflammatory and chronic/fibrotic radiation responses. The dysfunction of the Thrombomodulin (TM)-protein C (PC) system is involved in the pathogenic process (Fig. 4.2). Acute radiation-induced ROS release inactivates the TM, its transcription and release into the circulation. TM alteration in endothelial cells causes loss of local vascular thrombo-resistance, excessive activation of protease-activated receptor-1 by thrombin, and insufficient activation of protein C. When they persist, these acute alterations are also involved in the fibrogenesis and maintenance of fibrogenic signals.

4.4.1.1 Inhibition of Coagulation

Direct inhibition of coagulation using anti-coagulant strategies such as Hirudin and Octreotide have demonstrated efficacy in experimental models when administered before irradiation [76–78]. Activated PC is one other potent anti-coagulant and cyto-protectant that inhibits blood clotting (through the proteolysis of factors V and VII), promotes fibrinolysis and exerts potent anti-inflammatory and cytoprotective effects on endothelial cells, neurons and innate immune cell populations [79]. It has shown considerable promise as a radiation mitigator as seen in a study in which the systemic administration of soluble TM or activated PC to lethally irradiated wild-type mice resulted in an accelerated recovery of hematopoietic progenitor activity in bone marrow [80].

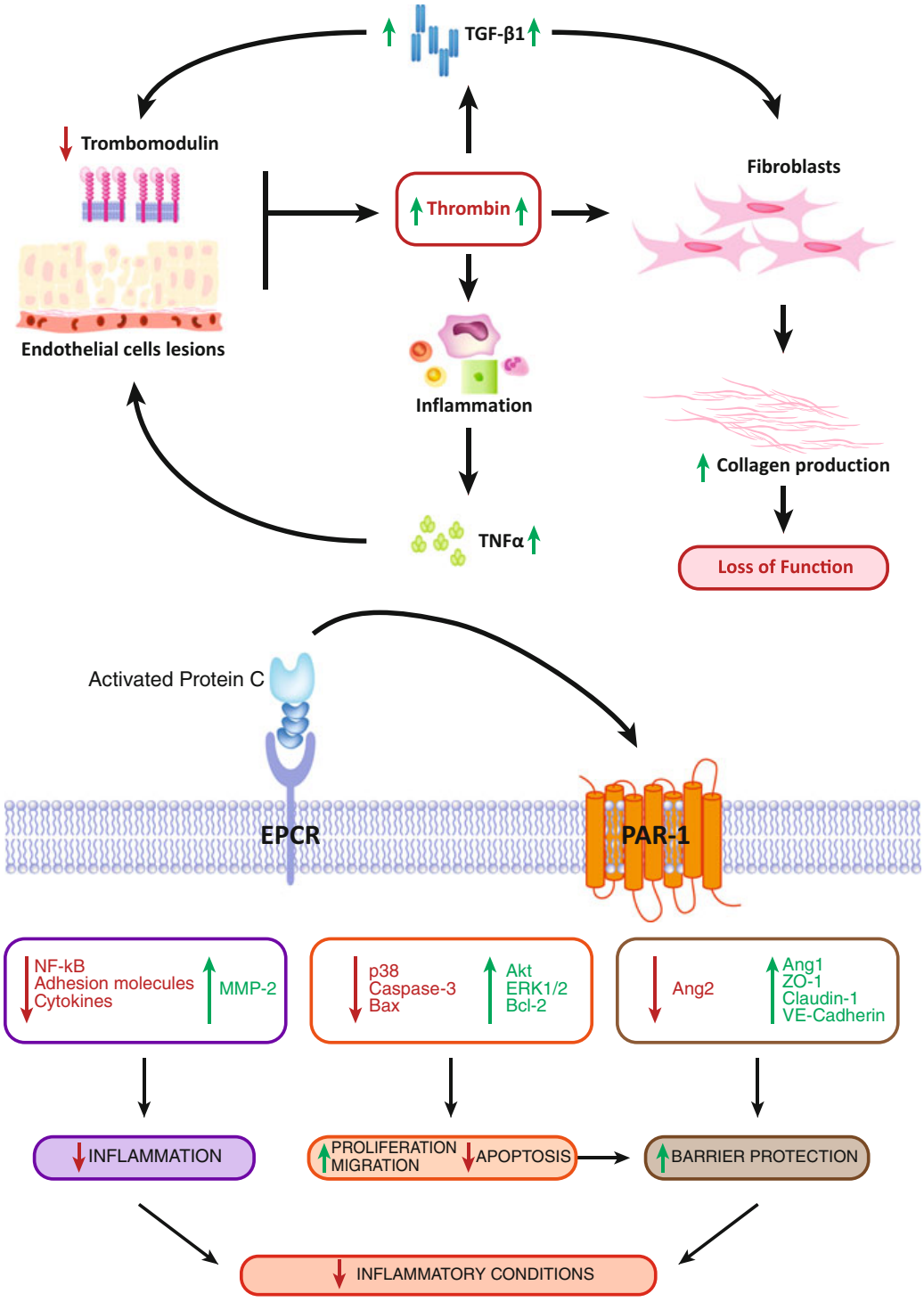

Fig. 4.2 *Radiation-induced alteration of the Thrombomodulin—protein C system.* Radiation exposure of microvessels induces a deficiency in thrombomodulin (TM) and leads to enhanced coagulation status via the accumulation of thrombin and deposition of fibrin. Thrombin also has powerful inflammatory, mitotic and pro-fibrogenic effects via TGFβ- activation. In addition TM deficiency prevents PC activation and anticoagulant activity of APC. The anti-inflammatory, anti-apoptotic activities of APC along with its protective effect on endothelial barrier function, require the cellular receptors EPCR and PAR-1

4.4.2 Transforming Growth Factor (TGF-β1)

The transforming growth factor-β (TGFβ) pathway contribution to radiation injury has been extensively studied and reviewed in many articles [36, 81–85], therefore we will shortly summarize its function and will focus on some of its less described properties.

Transforming growth factor is secreted as a large latent complex that must be released by proteolysis for full activity. Its signal transduction is mediated via two serine/threonine kinase receptors [86] that recruit and phosphorylate Smad proteins which are considered as the canonical TGFβ mediated signal transduction pathway [87] (Fig. 4.3). However, non-Smad mediated transduction also occurs via Erk, p38, and c-Jun N-terminal (JNK) MAP kinases, PI3K-Akt, and small GTPase pathways [86]. The TGFβ receptor can also—through as yet unknown intermediates—engage the Rho-ROCK1 signaling module [88] as well as the Cdc42/Rac1-PAK2 complex [89]. These molecular pathways are transactivating thrombogenic and fibrogenic genes: More recently, TGFβ has been shown to protect cells from radiation through activation of the NHEJ repair pathway [90].

A remarkable but less-explored feature of TGFβ-activated Smad2/3 is its ability to bind p68, a component of the microRNA (miRNA) processing complex DROSHA. First described to target the primary transcript of miR-21 (pre-miR-21) in vascular smooth muscle cells [91] where it regulates the contractile phenotype of the cells, this mechanism has been now extended to the regulation of cardiogenesis [92] and myocardial remodelling [93]. This new mechanism of selective microRNAs maturation mediated by TGFβ could be of great interest in the field of normal tissue injury since miRNA can be either biomarkers or mediators of normal tissue injury, as highlighted in recent publications that identified miR-21, -29 and 101 in fibrotic tissue [94, 95] and miR-210 as a possible anti-fibrotic target in radiation enteropathy [96].

4.4.2.1 Inhibition of TGF-β Using Antibodies and Pirfenidone

One of the earliest therapeutic studies targeting TGFβ was conducted with a neutralising antibody against TGFβ and was effective in a model of rat lung fractionated irradiation. A reduction in alveolar septal wall thickness, macrophage activation, TGFβ and its downstream signal transduction proteins was seen [97]. Subsequently, a small molecule inhibitor, SM16, targeting TGFβ type 1 receptor kinase was shown to be effective in a similar model [98]. Other studies used a human recombinant adenoviral vector carrying the gene for a TGFβ type II receptor, which acted as a plasmatic competitor trapping TGFβ and leading to an improvement of radiation pulmonary and intestinal toxicity [99, 100]. There are some limitations to interpretating these pre-clinical studies: the use of treatment schedules not fully representative of clinical settings and the fact that these interventional therapies have predominantly been tested during the early phase of the disease. However, some compounds, including Pirfenidone, described as a selective regulator of most fibrogenic molecules including TGFβ and PDGF, β-FGF,

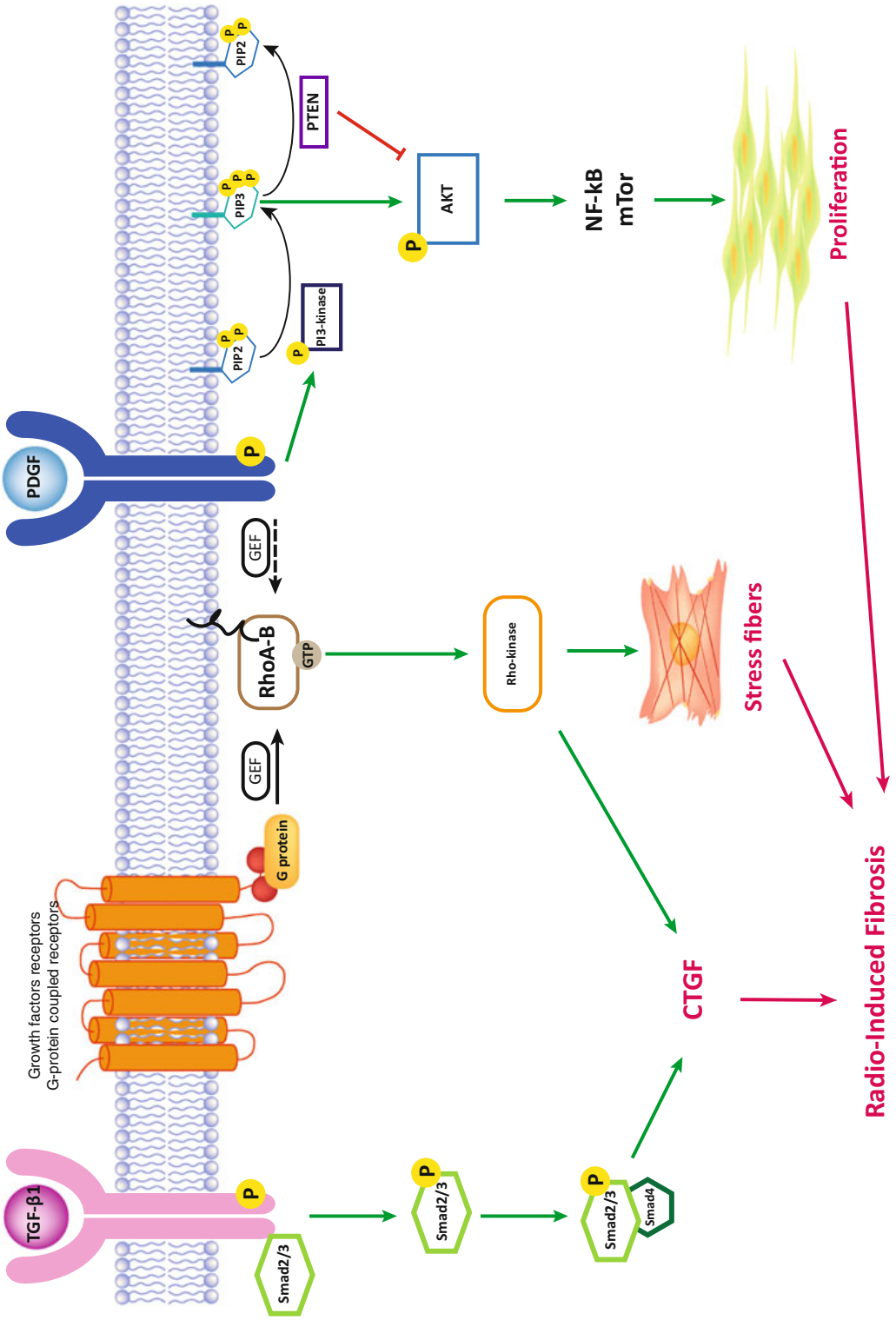

Fig. 4.3 *Signaling cascades that regulate radiation-induced fibrogenesis*. Fibrogenic growth factor including TGFß and Platelet-derived growth factor-BB (PDGF-BB) activate signaling cascade and lead to the development and maintenance of fibrosis mediated by the differentiation of fibroblasts and smooth muscle cells, enhanced CTGF expression and extracellular matrix synthesis and accumulation

EGF, TNF-α, have been used with a certain success in humans with IPF [101]. One pilot trial published in 2007 reported the stabilization of radiation-induced lung fibrosis [102] but a proper randomized trial is still missing and the efficacy of Pirfenidone disputed. In addition, clinical trials using TEW-7197, LY2157299, associated or not with anti-cancer treatment are ongoing, but to our knowledge none of them is associated with radiotherapy and the pleiotropic effects of TGFβ probably remain the main limitation for the clinical application of TGFβ inhibition.

4.4.3 RHO/ROCK Signaling Pathway

Guanosine triphosphatases (GTPases) from the Rho family (from "Ras homologous") are fundamental regulatory molecules in cells [103]. Post-translational modification by prenylation (geranylgeranylation) is required for Rho activation which is determined by the ratio of GTP/GDP-bound forms, and mediated by various activators: the guanosine nucleotide exchange factors (GEFs); and inactivators: the guanine dissociation inhibitors (GDIs). The biological effects of Rho are mediated by a number of downstream effector proteins, including the Rho-associated kinase (ROCK) (Fig. 4.3). Alterations in the expression of the genes coding for proteins of the Rho family have been reported both in human samples and mice models of delayed radiation injury affecting various organs including the gut, lung and heart [104–107] and can be modulated using pharmacological agents [108].

4.4.3.1 Modulation of Rho/ROCK Using Statins and ROCK Inhibitors

Regulation of Rho/ROCK pathway can be achieved using the approved drugs called statins which work inhibiting HMG-CoA reductase, the rate-limiting enzyme in mevalonate synthesis needed to produce isoprenoid intermediates. Several pre-clinical studies have shown that statins were able to modulate fibrogenic and thrombogenic differentiation of myofibroblasts and endothelial cells; they also reduce the expression of CTGF/CCN2, TGFβ, and Col Iα2 genes [71, 72, 74, 109–111] and help to restore the "gatekeeper" function of the endothelium after irradiation [112] without decreasing the tumor's radiation sensitivity [73]. These interesting pre-clinical findings were supported by retrospective trial conducted on statin users with rectal cancer [113] and further confirmed in a prospective trial that included 308 patients undergoing radiotherapy for the treatment of pelvic cancer [114]. In this study, the use of a statin in combination or not with ACEi medication reduced acute gastrointestinal symptom scores and also appears to have provided longer-term sustained protection. A second trial is currently ongoing to confirm this beneficial effect in Head&Neck cancer and make it available for patients.

4.4.4 The Connective Tissue Growth Factor (CTGF/CCN2)

CTGF/CCN2 is a matri-cellular protein with heparin-binding activity. Composed of four modules, it is susceptible to protease cleavage and can be found in its cleaved form in various biological fluids where they play distinct functions [115]. Its synthesis is stimulated by various fibrogenic mediators, such as endothelin-1 and TGFβ [116, 117], environmental changes such as hypoxia and biochemical stimuli such as stretch [118]. CTGF/CCN2 is overexpressed in radiation-induced fibrotic diseases [43, 105, 106, 119–123]. Despite many efforts, a specific CTGF/CCN2 receptor has yet to be identified; CTGF/CCN2 appears to perform many of its functions through integrins, heparin sulfate-containing proteoglycans, and the LPA axis [124, 125]. The effects of CTGF/CCN2 seem to mirror TGFβ's fibrogenic functions [126] but is a more attractive anti-fibrotic target as it does not display pleiotropic function but rather an almost selective action on mesenchymal cells.

4.4.4.1 Inhibition of CTGF/CCN2

In pulmonary fibrosis, the LPA–LPAR1/3 axis has been described as a potent modulator of CTGF/CCN2 expression. Its inhibition using VPC 12249 has demonstrated anti-CTGF/CCN2 action associated with decreased fibroblast proliferation, improvement of histological structures and pulmonary function [127]. More specific inhibition of CTGF/CCN2 using the monoclonal anti-CTGF antibody FG-3019 has been reported [128] to prevent and reverse lung radiation-induced lung fibrosis. This anti-CTGF/CCN2 antibody is being currently tested in the context of IPF, but to our knowledge no studies are ongoing in the context of radiation-induced fibrosis.

4.4.5 The Platelet Derived Growth Factor (PDGF)

Like TGFβ, the PDGF is released from platelets upon radiation exposure and binds to a tyrosine kinase receptor to transduce a mitogenic and fibrogenic signal that stimulates the transdifferentiation of fibroblasts into myofibroblasts (Fig. 4.3). PDGF is mainly synthesized by platelets and stored in their alpha granules; however numerous cells such as activated macrophages, endothelial cells and smooth muscle cells, have been shown to produce PDGF.

4.4.5.1 Inhibition of PDGF

Imatinib, desotinib and nilotinib are amongst the tyrosine kinase inhibitors that suppress the PDGF receptor signaling. Imatinib anti-fibrotic efficacy was proved more than 10 years ago in preclinical experiments [129] and is currently being assessed

in clinical trials. Nindetanib is another tyrosine kinase inhibitor that has shown encouraging results in the management of idiopathic pulmonary fibrosis and is being tested in lung cancer and neuroblastoma patients undergoing radiotherapy with assessment of normal tissue complications as secondary endpoints.

4.4.6 Blockade of Other Growth Factors (EGF, FGF2 and IGF) and Heparanase

Many other signaling cascades regulated by EGF, FGF and IGF are involved in the acute and delayed radiation response of normal tissue. Broad range molecules such as Suramin, a polysulfonated naphthylurea that acts as a potent competitive inhibitor of reverse transcriptase, have been described to block the activity of these growth factors. Their inhibitory action seems mediated via heparanase inhibition [130] and physical sequestration of the fibrogenic factors. Suramin has been combined with RT [131], but the outcome in terms of toxicities remains to be investigated.

4.4.7 Modulation of Redox Status

Exposure to ionizing radiation produces a burst of free radicals resulting from the ionization of water molecules. This is followed by a persistent and prolonged increase in both Reactive Oxygen and Nitrogen Species (ROS/RNS). Upon injury, if the initial increase in ROS is relatively small, the antioxidative response may be sufficient to compensate for the increase in ROS and to reset the original balance between ROS production and ROS scavenging capacity. However, when high and persistent ROS production occurs, following exposure to high radiotherapy doses for example, the antioxidant response is not sufficient to reset the system to the original level of redox homeostasis. This new steady state is called chronic oxidative stress. The radiation-induced vascular cell damage [53, 60] contributes to the redox imbalance with alternate sequences of hyper- and hypoperfusion-lead ROS burst and tissue hypoxia [132], leading to HIFs stabilisation, transativation of proangiogenic (VEGF) and pro-wounding (TGFβ) genes, all of which perpetuates the vicious circle.

4.4.7.1 Therapeutic Modulation of the Redox Status and Antioxidant Strategies

Treatment with hyperbaric oxygen (HBO) [133] and antioxidant therapy [64, 134, 135] were both successfully used despite their apparent antagonistic mechanism of action. HBO induces transient tissue hyperoxia (typically ~2 h/day) that should not overcome natural antioxidant defenses [136] but may help to remobilize tissue remodelling by activating signaling molecules in transduction cascades

(see the review [137]). Antioxydant therapies scavange ROS. Initial studies with Amifostine [138–140] and bovine liposomal Cu/Zn superoxide dismutase showed anti-fibrotic efficacy associated with TGFβ inhibition [61]. More recent trials investigated the benefits of tocol isoforms (Vitamin E analogs) such as high-dose alpha-tocopherol combined with pentoxifylline and Clodronate [66, 141], and γ-tocotrienol (GT3) [142]. In addition to their antioxidant action, both strategies have displayed off-target benefits with protective endothelial activity [69, 143] and miRNA regulation [96]. Interestingly, the efficacy of GT3 is enhanced when combined with pentoxifylline [68]. Lastly, hypoxia-regulating molecules such as 2-methoxyestradiol (2-ME) have been shown to downregulate HIF1α-mediated Smad activation and inhibit radiation-induced lung fibrosis in mice [144].

4.5 Modulation of Inflammation

Acute normal tissue response to radiation exposure is characterized by the orchestrated release of numerous pro-inflammatory mediators such as tumor-necrosis factor (TNF)-α, cytokines and chemokines. This early inflammatory phase is characterized by the rapid resolution of the vascular changes, oedema and neutrophil infiltration and can be followed either by a regenerative phase or by a chronic inflammation that persists over weeks and months. This chronic inflammation is today recognized as the main contribution to fibrosis, in which persistent immune responses occur alongside tissue remodeling and repair processes [145, 146] and the results obtained using anti-inflammatory interventions suggest that both processes do feed off each other (Fig. 4.4).

4.5.1 Corticosteroid to Reduce Inflammation

Corticosteroids have a long-standing history of use in patients with severe radiation complications after radiotherapy to inhibit inflammation; however their anti-fibrotic properties remain uncertain. Hirota et al. [147] noted that patients in their series who received corticosteroids as part of chemotherapy regimens had significantly lower incidences of severe fibrosis. However, well-controlled randomized clinical trials are lacking and similarly, experimental results are inconsistent [148, 149] which further supports their use as anti-inflammatory agents to be administered initially but not for their anti-fibrotic effects.

4.5.2 Blockade of TNF-α

TNF-α deficient mice have been described to be radioresistant [150] and TNFα inhibition with chitosan/DsiRNA nanoparticles [151] and ambroxol [152] has been shown to protect mice from acute inflammation. TNFα overexpression has also been reported in radiation fibrosis but its inhibition does not trigger an anti-fibrotic

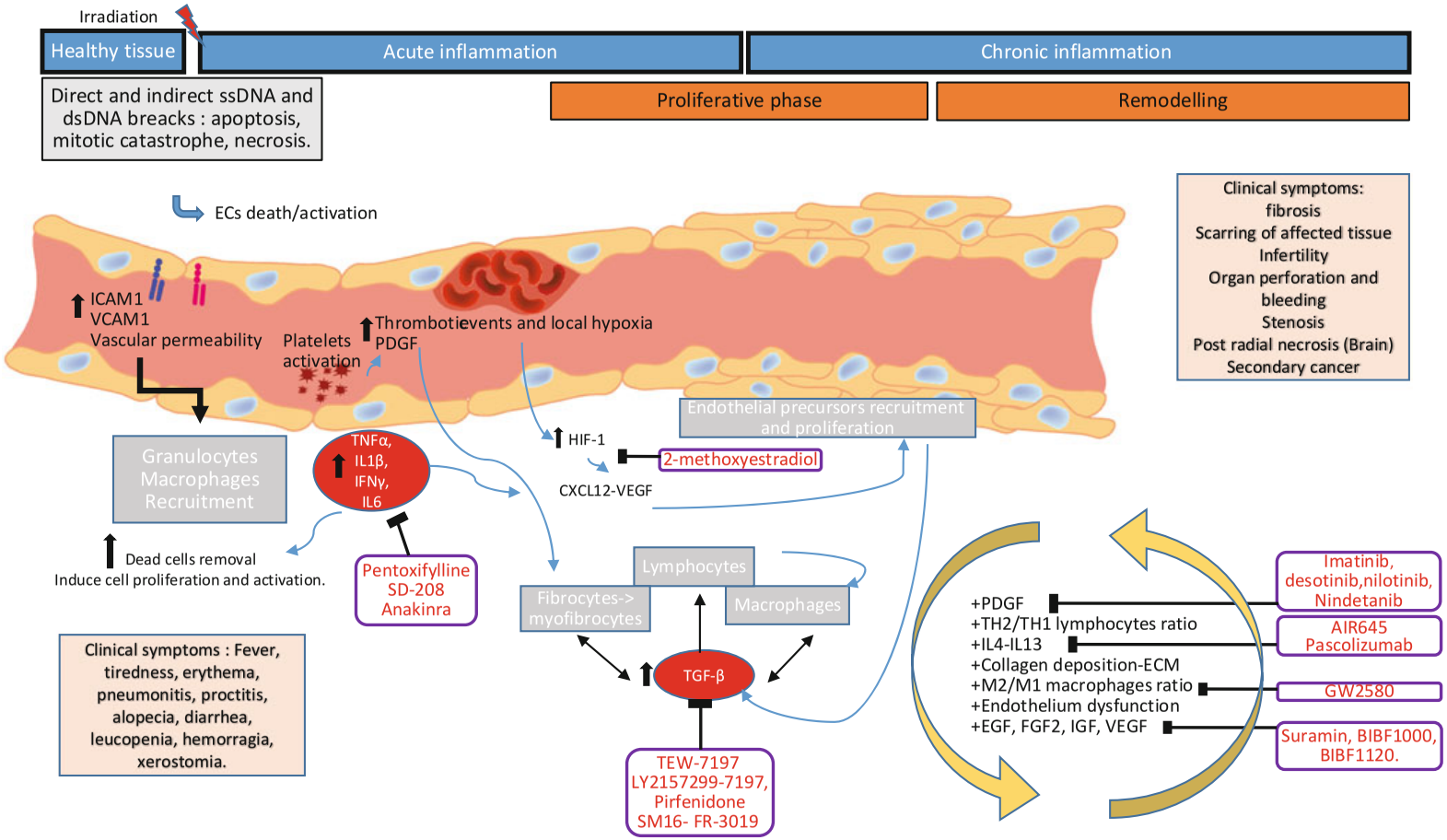

Fig. 4.4 Inflammatory and immune contribution to acute and chronic radiation response. *ICAM* intercellular adhesion molecule 1, *VCAM1* vascular cell adhesion protein, *ssDNA* single strand DNA, *dsDNA* double strand DNA, *Ecs* endothelial cells, *PDGF* platelet derived growth factor, *TNF α* tumor necrosis factor alpha, *IL1* interleukine 1, *INF γ* interferon gamma, *HIF-1* hypoxia inducible factor 1, *TH1/TH2* helper T cell 1 &2, *ECM* extra cellular matrix, *EGF* endothelial growth factor, *FGF* fibroblast growth factor, *IGF* insulin like growth factor, *VEGF* vascular endothelial growth factor, *TGFβ*

effect but can potentiate the anti-fibrotic action when combined with other molecules—one such example is the combination of pentoxifylline (PTX), a well-known anti-TNFα drug, with an antioxidant, the alpha-tocopherol (vitamin E) and an anti-macrophage, Clodronate [65, 153].

4.5.3 *Blockade of Pro-inflammatory Cytokines*

Strategies to inhibit pro-inflammatory cytokines have been developed to treat inflammatory diseases. Recent molecules such as the IL-1R antagonist (Anakinra-Kineret®) are currently used to treat rheumatoid polyarthritis [154] and might be of interest to treat radiation-induced fibrosis as the inhibition of IL-1β attenuates fibrosis [155]. IL-4 and IL-13 are other potential targets with common functional activities and a common receptor (IL-4Rα) which activates STAT6-dependant signalling pathway [156]. In vivo, blocking studies were successfully conducted and confirm the fibrogenic role of IL-4 and IL-13 in various fibrosis models including skin [157], liver [158], and lung [159, 160]. IL-4 inhibitors have been consistently used for managing airway inflammatory disease (AIR645, pascolizumab). This new compound may be of great interest for avoiding or reducing radiation-induced toxicities, but it has not yet been validated for radiation injuries and the sequence of administration needs to be accurately assessed to avoid protecting the tumor and impairing the start of wound healing.

4.5.4 *Blockade of Chemokines*

Tissue homeostasis is tightly controlled by chemokine balance. CCL3 (also known as macrophage inflammatory protein 1α, MIP1α) and CCL2 (also known as monocyte chemoattractant protein 1, MCP1) have been identified as key chemotactic molecules for recruiting mononuclear phagocytes. Anti-CCL3 and CCL2 antibodies prevent the development of bleomycin-induced fibrosis [161–163]. On the other hand, CXCL10 and CXCL11 are natural inhibitors of fibroblast recruitment and neoangiogenesis via the production of the antifibrotic cytokine IFN-ɣ, [164, 165]. Thus, modulating specific chemokine signaling pathways in order to restore the natural balance between profibrotic and anti-fibrotic signals is theoretically achievable, but the fine tuning required seems hardly compatible with a clinical application.

4.6 Modulation of the Immune System

The composition of the immune cell compartment is organ-specific and organized in a fragile balance to react against stress and restore homeostasis. However, in certain conditions of non-self-resolutive immune activation like wounds (ex: cheloids),

infections (ex: tuberculosis) or inflammatory diseases (ex: familial Mediterranean fever) involving severe tissue injury can occur. In the same way, tissue exposure to radiotherapy induces a dramatic remodeling of the tissue microenvironment. This means that elucidating the impact of radiotherapy on the immune compartment and subsequent immunomodulation is currently one of the most promising strategies for enhancing the differential effect of radiotherapy.

4.6.1 Modulation of the Pool of Adaptive Immune Cells

Studies have suggested that regulating the adaptive immune cell balance would reduce both acute and chronic injury of normal tissue. Radiation is known to modulate the polarization of CD4+ cells within normal tissues and prime tissue response towards fibrogenesis when TH2 polarization occurs [157, 159], whereas TH1 polarization would be anti-fibrotic *via* INF-γ secretion. Similarly, the role of FoxP3+ Tregs could be either anti-inflammatory/anti-fibrotic [166] or pro-fibrotic via secretion of the fibrogenic growth factor TGFβ [167, 168]. CD4+ Th17 seems pro-fibrotic via the secretion of IL-1β, IL-23 and TGFβ [169, 170] and the recruitment of neutrophil and MMP-1 [171].

Pharmacological interventions have been performed to eradicate or reprogram adaptive immune cells [172] but with limited success. Recent studies, however, performed in blood samples of whole body irradiated mice have shown differential radiosensitivity of subtypes of immune cells. Persistent changes in immune phenotype [173] with a permanent TH1 drop associated with an increase in the percentage of blood TH17+ or FoxP3+ T cells have been observed. This recent observation suggests that circulating cells may trigger a fibrogenic effect, something which has never been investigated and is worth future attention.

4.6.2 Modulation of the Pool of Innate Immune Cells

Researchers have recently given a lot of attention to the role of macrophage reprogramming occurring during radiotherapy. Their relevance has been demonstrated in both tumor and normal tissue response to radiotherapy with potential therapeutic implications [174, 175]. Macrophage phenotype is highly dependent upon the micro-environment and at least two functionally distinct populations—"classical, M1 macrophages" and "alternative, M2 macrophages"—have been described upon exposure to Th1 or Th2 cytokines, respectively [176]. A hybrid phenotype has also been reported [177], illustrating the high plasticity of these cells and corroborating their function as a sensor and rheostat of tissue homeostasis (Table 4.2). In fact, macrophage polarization seems to drive the balance between the exacerbation of tissue damage (M1 polarization) and tissue recovery and fibrosis (M2 polarization) [178–180]. M2 polarized macrophages are especially relevant to fibrosis as they display immunosuppressive properties, secrete large amounts of the fibrogenic mediator TGFβ [181], go on to activate the Smad pathway and stimulate fibrogenic

Table 4.2 Functional impact of macrophage phenotypes

M1 macrophages	M2 macrophages	Hybrid macrophages
Induced by Th1 cytokines including IFN-γ	Induced by Th2 cytokines including IL-13 and IL-4	
Produce TNF-α, IL-12 and IL-6 and increase inducible nitric oxide synthase (iNOS), superoxide anions ($O_2^{-\bullet}$) and oxygen radical	Produce PDGF, TGF-β1, arginase type 1 (arg-1)	
Arginase and iNOS		Arginase and iNOS to limit T cell function
CD40, ICAM-1, MHC class II, CD80, CD25	CD206, Dectin1, CD71, CD163 and chemokine receptors including CXCR1, CXCR2 and CCR2	
MCR1 low	MCR1 high	
CD11c high	CD11c low	

genes such as CTGF and PAI-1 [182]. The macrophages isolated from broncho-alveolar fluid from patients undergoing thoracic irradiation spontaneously released PDGF, another important fibrogenic growth factor [129] (Table 4.2).

Recent studies have suggested that depending on the dose administered, radiotherapy could induce Th1/M1 or Th2 /M2 polarization [183]. High doses of ionizing radiation induce immunogenic cell death and normalize tumor vasculature, thereby improving the recruitment of tumor-specific cytotoxic T cells [184, 185]. However, the balance is tight and in B16F10 melanoma, high doses of radiation promote M2 polarization and inhibit TNF-α expression, supporting tumor-induced anergy [186]. In a recent study, Klug et al. used a lower range of radiation doses (down to 2 Gy) in combination with immunotherapy to induce the reprogramming of M2 macrophages into M1 macrophages and subsequent elimination of the tumor [187]. Interestingly, tumor-associated macrophages and fibrotic tissue-infiltrating macrophages display similar M2-oriented phenotypes, suggesting that the modulation of macrophage polarization could improve radiotherapy outcomes by enhancing anti-tumor efficacy and preventing radiation-induced fibrosis.

4.6.2.1 Macrophage Reprogramming

Clodronate liposomes were used to deplete macrophages in several studies [188]. The reduction of the number of macrophages by clodronate in wounded tissue indeed reduced excessive scar formation and delayed cutaneous wound healing [189]. Froom and colleagues [190] showed that oral administration of clodronate (bisphosphonate) significantly reduced bone marrow fibrosis. Delanian and Lefaix proposed clodronate administration in combination with the pentoxifylline-vitamin E (PE) treatment, and showed improved efficacy in the treatment of radiation-induced fibronecrosis [153,

191]. The very targeted depletion of M2 macrophages by inhibiting CSF1/CSF1R signalling [192] seems to be even more promising. CSF1R inhibition using a neutralizing mAb (AFS98) showed a decrease in macrophage accumulation in atherosclerotic lesions of ApoE-deficient mice [193], in renal allografts [194] and damaged skeletal muscle [195]. Its effect on fibrosis is more disputed, as it may increase renal fibrosis [196–198] but may be beneficial in other fibrosis. Interestingly, a combination of CSF-1R inhibition using GW2580 with radiotherapy suppressed tumor growth more effectively than irradiation alone in a mouse prostate cancer model by TAM blockade, suggesting that CSF-1R inhibitor should enhance radiotherapy's differential effect [199].

4.6.2.2 Targeting Neutrophils, DCs and Other Immune cells

Neutrophils and DCs are also relevant to radiation injury. The recruitment of neutrophils at the injury site is important for removing tissue debris and killing invading pathogens. They also, however, secrete ROS/NOS that may exacerbate tissue damage and induce scarring [166]. Because of this, neutrophils have been described as either pro-fibrotic (bleomycin, hypersensitivity pneumonitis–induced fibrosis) [200] or anti-fibrotic via extracellular matrix clearance [201].

DCs are professional antigen-presenting cells (APCs) able to migrate into secondary lymphoid organs to activate T helper cells for pathogen control and clearance, but in pathological inflammation and autoimmune disease, DCs can contribute to local tissue injury [166]. Like neutrophils, the role of DCs in fibrosis is dual with high infiltration described in Hepatic and lung fibrosis [202, 203], but not in cardiac fibrosis [204].

Other innate immune cells, such as mast cells, eosinophils and basophils have also been implicated in the pathogenesis of fibrosis in multiple organ systems and are viewed as potential therapeutic targets. Indeed, mast cells have been described to promote fibrosis by recruiting inflammatory leukocytes and by producing pro-fibrotic mediators [205]. Eosinophils are important sources of TGF-β1 and IL-13 [206] and have been found to be associated with the development of pulmonary fibrosis [207], skin, liver and idiopathic retroperitoneal fibrosis [206, 208]. The role of basophils has not been explored in the context of radiation injury but they are an important source of type 2 cytokines such as IL-4– and/or IL-13.

4.7 Contribution of the Microbiome: An Emerging Contributor and a Possible Target?

There has not been much exploration, until recently, of the possible role of the microbiome in regulating susceptibility/resistance to radiotherapy. Yet, bacterial translocation induced by the disruption of the epithelial/mucosal barrier is one of the main consequences of radiotherapy. The gastrointestinal tract from oral

mucosa to rectum is an ideal model to study the contribution of the flora to normal tissue damage induced by radiation therapy and define possible innovative prevention and/or mitigating strategies [209]. The recent interest in flora is partly driven by technological advances, particularly metagenomic sequencing and marker gene-based phylotyping. These novel approaches have helped to understand that the microbiota is far more diverse than previously thought [210, 211]. The complex interactions that occur in between epithelial cells and microbiota is the guarantee for their respective homeostasis and constitute the hormesis concept. Host factors are known to influence the microbiota composition [212] and anticancer treatment, including radiotherapy, may alter this makeup. The alteration in the microbiota composition is named dysbiosis.

Recent studies have investigated the impact of radiation-induced damaging signals coming from host cells that can modulate microbiota composition. One of the primary effects of radiation therapy is ROS-mediated. The strong oxidative milieu generated upon irradiation interferes with many cellular functions, such as cell cycle progression and pro-apoptotic pathways. This causes ulceration that can be modulated using anti-oxidants including SOD, Amifostine and Vitamin E. The long-term breakage of the epithelial barrier is the first point of entry for bacteria and is mainly caused by the loss of adult stem cells. In the gut, the stem cell response is mediated by p53 activation, which in turn induces PUMA as a signal triggering progenitor and stem cell death via intrinsic apoptosis [213, 214]. But the mechanism is not as simplistic, because, at the same time, p53 induces p21, thereby facilitating cell-cycle arrest and DNA repair in progenitor cells, consequently increasing cell survival and tissue regeneration [215].

A direct role for the microbiota in regulating epithelial homeostasis has been described. In the gut, the regulation has been shown to be mediated through activation of Toll-like receptors [216]. The immuno-modulatory activity of the gut microbiome has been investigated by Zitvogel et al. who showed that gut flora elicited innate and adaptive immune responses [217]. Long-lasting dysbiosis has indeed been associated with cancer [218]; it may promote low-grade inflammation [219], and increase cell transformation [220]. Recent studies also suggest that the presence of crypt-associated flora bacteria could act as “gate keepers” and help in the protection against colonization by pathogenic bacteria, thus maintaining the homeostasis of the regenerative apparatus [221].

4.7.1 Therapeutic Modulation of the Microbiome

Studies focusing on the relevance of the microbiota to the pathogenesis of radiation-induced normal tissue complications are just emerging. Some bacteria, such as *Roseburia* or *Eubacterium,* seem to have beneficial effects by producing molecules such as butyrate. Whether it is the absolute composition or the relative changes in the microbiota that is relevant to understand and modulate the pathogenic process is another question. Some clinical studies profiled the intestinal [222–226] and the

oral [227–229] microbiome after radiotherapy. Andreyev et al. described gram-negative bacterial overgrowth in patients with radiation enteropathy, both in the acute and late settings [230, 231]. In a further study they assessed faecal microbial populations and reported an overall increase in *Bacilli* and *Actinobacteria*, and a decrease in *Clostridia* [227]. De Rick et al. measured shifts in the oral microbial community during radiotherapy with a decrease in the richness and presence of a small fraction of species. These shifts correlated with a poor functional outcome including pain and nutrition problems. However, these studies included only a small number of patients and were only associative, making it difficult to discern cause and effect. The use of probiotics has also been developed and preclinical studies with *Lactobacillus spp.* were able to partially treat proctitis in rats while preserving intestinal morphology [232, 233]. Similar clinical trials have been developed using *Lactobacillus spp.* as a probiotic treatment to mitigate gastrointestinal injury after radiation therapy. Prophylactic treatments also seem to be efficient [234, 235]. Nevertheless, no unique microorganism strain or product has been described in clinical trials and further studies are required to address this promising question.

4.8 Conclusion

The aim of modern targeted radiotherapy is to kill a maximum of cancer cells while reducing normal tissue injury and decreasing morbidity. To achieve that aim, selective protection of normal tissue function is a powerful approach to improve cure rates and simultaneously improve the quality of life of long-term cancer survivors. The development of complex models of radiation injury based upon the use of transgenic animals and targeted irradiation procedures with Image Guided Radiotherapy devices dedicated to small animals has lead to a better understanding of the normal tissue response to radiation injury. The complexity of the phenomena has been dissected and an interconnected series of processes has been deciphered. These series include inflammation, alteration of the vascularisation which leads to alternative sequences of perfusion and hypoxia within tissues, alteration of the immune cell composition and infiltration, remodeling of the extracellular matrix and tissue fibrosis that may ultimately lead to irreversible organ failure.

The therapeutic challenge is now driven by the complexity of radiation-induced processes. Combination strategies that target distinct pathogenic pathways with several "old" or existing molecules—such as the combination of anti-inflammatory agents, vascular protectors, antioxidants and immunomodulators—have given good pre-clinical and clinical results. However, dosage and administration sequences require a personalized and fine-tuned follow-up for each patient. More recent targeted therapies using specific pathway inhibitors or biological agents such as antibodies can now be foreseen as the next approach in modulating radiation injury. Lastly, fascinating clinical questions are being raised, aiming to study organ ecosystem and how it might be exploited in the future with direct and "natural" therapeutic agents to treat radiotherapy complications and restore the fine equilibrium altered by anti-cancer therapies.

Interestingly, the changes described at the normal tissue level also occur in the tumor's microenvironment. Numerous factors activated in response to irradiation in normal tissue such as TGF-β, CTGF and PDGF, cytokines, TNF-α and Interleukins, are similarly altering cellular phenotype in tumors *i.e.* CAFs display myofibroblastic differentiation; TAM display M2 polarization. Consequently, the next challenge will be to develop rational radiotherapy-drug combinations to target tumors and avoid normal tissue toxicity.

References

1. Quastler H, Lanzl EF, Keller ME, Osborne JW. Acute intestinal radiation death. Studies on roentgen death in mice, III. Am J Physiol. 1951;164:546–56.
2. Rubin P, Casarett G. Clinical radiation pathology. In: Co WS, editor. Philadelphia; 1968. pp. 193–240.
3. Fowler JF, Bewley DK, Morgan RL, Silvester JA, Alper T, Hornsey S. Dose-effect relationships for radiation damage to organized tissues. Nature. 1963;199:253–5.
4. Stewart FA, Michael BD, Denekamp J. Late radiation damage in the mouse bladder as measured by increased urination frequency. Radiat Res. 1978;75:649–59.
5. Fajardo LG. Pathology of radiation injury. In: Sternberg SS, editor. Masson monographs in diagnostic pathology. Chicago: Year Book Medical publisher; 1982. p. 47–76.
6. Benderitter M, Caviggioli F, Chapel A, Coppes RP, Guha C, Klinger M, Malard O, Stewart F, Tamarat R, Luijk PV, Limoli CL. Stem cell therapies for the treatment of radiation-induced normal tissue side effects. Antioxid Redox Signal. 2014;21:338–55.
7. Rola R, Raber J, Rizk A, Otsuka S, VandenBerg SR, Morhardt DR, Fike JR. Radiation-induced impairment of hippocampal neurogenesis is associated with cognitive deficits in young mice. Exp Neurol. 2004;188:316–30.
8. Gondi V, Pugh SL, Tome WA, Caine C, Corn B, Kanner A, Rowley H, Kundapur V, DeNittis A, Greenspoon JN, Konski AA, Bauman GS, Shah S, Shi W, Wendland M, Kachnic L, Mehta MP. Preservation of memory with conformal avoidance of the hippocampal neural stem-cell compartment during whole-brain radiotherapy for brain metastases (RTOG 0933): a phase II multi-institutional trial. J Clin Oncol Off J Am Soc Clin Oncol. 2014;32:3810–6.
9. Cramer CK, Yoon SW, Reinsvold M, Joo KM, Norris H, Hood RC, Adamson JD, Klein RC, Kirsch DG, Oldham M. Treatment planning and delivery of whole brain irradiation with hippocampal avoidance in rats. PLoS One. 2015;10, e0143208.
10. Lombaert IM, Brunsting JF, Wierenga PK, Faber H, Stokman MA, Kok T, Visser WH, Kampinga HH, de Haan G, Coppes RP. Rescue of salivary gland function after stem cell transplantation in irradiated glands. PLoS One. 2008;3, e2063.
11. Booth C, Booth D, Williamson S, Demchyshyn LL, Potten CS. Teduglutide ([gly2]glp-2) protects small intestinal stem cells from radiation damage. Cell Prolif. 2004;37:385–400.
12. Torres S, Thim L, Milliat F, Vozenin-Brotons MC, Olsen UB, Ahnfelt-Ronne I, Bourhis J, Benderitter M, Francois A. Glucagon-like peptide-2 improves both acute and late experimental radiation enteritis in the rat. Int J Radiat Oncol Biol Phys. 2007;69:1563–71.
13. Kantara C, Moya SM, Houchen CW, Umar S, Ullrich RL, Singh P, Carney DH. Novel regenerative peptide tp508 mitigates radiation-induced gastrointestinal damage by activating stem cells and preserving crypt integrity. Lab Invest. 2015;95:1222–33.
14. Taniguchi CM, Miao YR, Diep AN, Wu C, Rankin EB, Atwood TF, Xing L, Giaccia AJ. PhD inhibition mitigates and protects against radiation-induced gastrointestinal toxicity via hif2. Sci Transl Med. 2014;6:236ra264.
15. Akita S, Akino K, Hirano A, Ohtsuru A, Yamashita S. Noncultured autologous adipose-derived stem cells therapy for chronic radiation injury. Stem Cells Int. 2010;2010:532704.

16. Benderitter M, Gourmelon P, Bey E, Chapel A, Clairand I, Prat M, Lataillade JJ. New emerging concepts in the medical management of local radiation injury. Health Phys. 2010;98:851–7.
17. Caviggioli F, Maione L, Forcellini D, Klinger F, Klinger M. Autologous fat graft in postmastectomy pain syndrome. Plast Reconstr Surg. 2011;128:349–52.
18. Ebrahimian TG, Pouzoulet F, Squiban C, Buard V, Andre M, Cousin B, Gourmelon P, Benderitter M, Casteilla L, Tamarat R. Cell therapy based on adipose tissue-derived stromal cells promotes physiological and pathological wound healing. Arterioscler Thromb Vasc Biol. 2009;29:503–10.
19. Klinger M, Caviggioli F, Vinci V, Salval A, Villani F. Treatment of chronic posttraumatic ulcers using autologous fat graft. Plast Reconstr Surg. 2010;126:154–5.
20. Acharya MM, Christie LA, Lan ML, Limoli CL. Comparing the functional consequences of human stem cell transplantation in the irradiated rat brain. Cell Transplant. 2013;22:55–64.
21. Gorin NC. [Bone marrow autograft. Current status and perspectives]. Nouv Rev Fr Hematol. 1983;25:391–9.
22. Banh A, Xiao N, Cao H, Chen CH, Kuo P, Krakow T, Bavan B, Khong B, Yao M, Ha C, Kaplan MJ, Sirjani D, Jensen K, Kong CS, Mochly-Rosen D, Koong AC, Le QT. A novel aldehyde dehydrogenase-3 activator leads to adult salivary stem cell enrichment in vivo. Clin Cancer Res. 2011;17:7265–72.
23. Coppes RP, Stokman MA. Stem cells and the repair of radiation-induced salivary gland damage. Oral Dis. 2011;17:143–53.
24. Espitalier F, Vinatier C, Lerouxel E, Guicheux J, Pilet P, Moreau F, Daculsi G, Weiss P, Malard O. A comparison between bone reconstruction following the use of mesenchymal stem cells and total bone marrow in association with calcium phosphate scaffold in irradiated bone. Biomaterials. 2009;30:763–9.
25. Lombaert IM, Knox SM, Hoffman MP. Salivary gland progenitor cell biology provides a rationale for therapeutic salivary gland regeneration. Oral Dis. 2011;17:445–9.
26. Pringle S, Van Os R, Coppes RP. Concise review: adult salivary gland stem cells and a potential therapy for xerostomia. Stem Cells. 2013;31:613–9.
27. Gao Z, Zhang Q, Han Y, Cheng X, Lu Y, Fan L, Wu Z. Mesenchymal stromal cell-conditioned medium prevents radiation-induced small intestine injury in mice. Cytotherapy. 2012;14:267–73.
28. Kudo K, Liu Y, Takahashi K, Tarusawa K, Osanai M, Hu DL, Kashiwakura I, Kijima H, Nakane A. Transplantation of mesenchymal stem cells to prevent radiation-induced intestinal injury in mice. J Radiat Res. 2010;51:73–9.
29. Semont A, Mouiseddine M, Francois A, Demarquay C, Mathieu N, Chapel A, Sache A, Thierry D, Laloi P, Gourmelon P. Mesenchymal stem cells improve small intestinal integrity through regulation of endogenous epithelial cell homeostasis. Cell Death Differ. 2010;17:952–61.
30. Linard C, Busson E, Holler V, Strup-Perrot C, Lacave-Lapalun JV, Lhomme B, Prat M, Devauchelle P, Sabourin JC, Simon JM, Bonneau M, Lataillade JJ, Benderitter M. Repeated autologous bone marrow-derived mesenchymal stem cell injections improve radiation-induced proctitis in pigs. Stem Cells Transl Med. 2013;2:916–27.
31. Olivier MP. Curative treatment of osteoradionecrosis (orn) with hybrid bone substitution. NCT01147315: NCT01147315; 2010–2015.
32. IJ F. Hepatocyte transplantation for liver based meta- bolic diosorders. NCT01345578: NCT01345578; 2011.
33. Francois S, Bensidhoum M, Mouiseddine M, Mazurier C, Allenet B, Semont A, Frick J, Sache A, Bouchet S, Thierry D, Gourmelon P, Gorin NC, Chapel A. Local irradiation not only induces homing of human mesenchymal stem cells at exposed sites but promotes their widespread engraftment to multiple organs: A study of their quantitative distribution after irradiation damage. Stem Cells. 2006;24:1020–9.
34. Tsoutsou PG, Koukourakis MI. Radiation pneumonitis and fibrosis: mechanisms underlying its pathogenesis and implications for future research. Int J Radiat Oncol Biol Phys. 2006;66:1281–93.

35. Gervaz P, Morel P, Vozenin-Brotons MC. Molecular aspects of intestinal radiation-induced fibrosis. Curr Mol Med. 2009;9:273–80.
36. Yarnold J, Brotons MC. Pathogenetic mechanisms in radiation fibrosis. Radiother Oncol. 2010;97:149–61.
37. Fajardo LF. The endothelial cell is a unique target of radiation: an overview. In: Rubin DB, editor. The radiation biology of the vascular endothelium. Boca Raton: CRC; 1998. p. 1–13.
38. Trott KR, Doerr W, Facoetti A, Hopewell J, Langendijk J, van Luijk P, Ottolenghi A, Smyth V. Biological mechanisms of normal tissue damage: importance for the design of ntcp models. Radiother Oncol. 2012;105:79–85.
39. Authors on behalf of I, Stewart FA, Akleyev AV, Hauer-Jensen M, Hendry JH, Kleiman NJ, Macvittie TJ, Aleman BM, Edgar AB, Mabuchi K, Muirhead CR, Shore RE, Wallace WH. ICRP publication 118: ICRP statement on tissue reactions and early and late effects of radiation in normal tissues and organs—threshold doses for tissue reactions in a radiation protection context. Ann ICRP 2012;41:1–322.
40. Hauer-Jensen M, Kumar KS. Targets of potential radioprotective drugs. Curr Drug Targets. 2010;11:1351.
41. Kolesnick R, Fuks Z. Radiation and ceramide-induced apoptosis. Oncogene. 2003;22:5897–906.
42. Wang J, Boerma M, Fu Q, Hauer-Jensen M. Significance of endothelial dysfunction in the pathogenesis of early and delayed radiation enteropathy. World J Gastroenterol. 2007;13:3047–55.
43. Haydont V, Riser BL, Aigueperse J, Vozenin-Brotons MC. Specific signals involved in the long-term maintenance of radiation-induced fibrogenic differentiation: a role for CCN2 and low concentration of TGF-beta1. Am J Physiol Cell Physiol. 2008;294:C1332–41.
44. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechanoregulation of connective tissue remodelling. Nat Rev Mol Cell Biol. 2002;3:349–63.
45. Guyot C, Lepreux S, Combe C, Doudnikoff E, Bioulac-Sage P, Balabaud C, Desmouliere A. Hepatic fibrosis and cirrhosis: the (myo)fibroblastic cell subpopulations involved. Int J Biochem Cell Biol. 2006;38:135–51.
46. Epperly MW, Guo H, Gretton JE, Greenberger JS. Bone marrow origin of myofibroblasts in irradiation pulmonary fibrosis. Am J Respir Cell Mol Biol. 2003;29:213–24.
47. Perl AK, Gale E. FGF signaling is required for myofibroblast differentiation during alveolar regeneration. Am J Physiol Lung Cell Mol Physiol. 2009;297:L299–308.
48. Gallucci RM, Lee EG, Tomasek JJ. Il-6 modulates alpha-smooth muscle actin expression in dermal fibroblasts from il-6-deficient mice. J Invest Dermatol. 2006;126:561–8.
49. Winczura P, Jassem J. Combined treatment with cytoprotective agents and radiotherapy. Cancer Treat Rev. 2010;36:268–75.
50. Booth D, Potten CS. Protection against mucosal injury by growth factors and cytokines. J Natl Cancer Inst Monogr. 2001:16–20.
51. Dorr W, Spekl K, Farrell CL. Amelioration of acute oral mucositis by keratinocyte growth factor: fractionated irradiation. Int J Radiat Oncol Biol Phys. 2002;54:245–51.
52. Dorr W, Heider K, Spekl K. Reduction of oral mucositis by palifermin (rhukgf): dose-effect of rhukgf. Int J Radiat Biol. 2005;81:557–65.
53. Pena LA, Fuks Z, Kolesnick RN. Radiation-induced apoptosis of endothelial cells in the murine central nervous system: protection by fibroblast growth factor and sphingomyelinase deficiency. Cancer Res. 2000;60:321–7.
54. Mangoni M, Vozenin MC, Biti G, Deutsch E. Normal tissues toxicities triggered by combined anti-angiogenic and radiation therapies: hurdles might be ahead. Br J Cancer. 2012;107:308–14.
55. Rotolo J, Stancevic B, Zhang J, Hua G, Fuller J, Yin X, Haimovitz-Friedman A, Kim K, Qian M, Cardo-Vila M, Fuks Z, Pasqualini R, Arap W, Kolesnick R. Anti-ceramide antibody prevents the radiation gastrointestinal syndrome in mice. J Clin Invest. 2012;122:1786–90.
56. Burdelya LG, Gleiberman AS, Toshkov I, Aygun-Sunar S, Bapardekar M, Manderscheid-Kern P, Bellnier D, Krivokrysenko VI, Feinstein E, Gudkov AV. Toll-like receptor 5 agonist protects mice from dermatitis and oral mucositis caused by local radiation: implications for head-and-neck cancer radiotherapy. Int J Radiat Oncol Biol Phys. 2012;83:228–34.

57. Burdelya LG, Krivokrysenko VI, Tallant TC, Strom E, Gleiberman AS, Gupta D, Kurnasov OV, Fort FL, Osterman AL, Didonato JA, Feinstein E, Gudkov AV. An agonist of toll-like receptor 5 has radioprotective activity in mouse and primate models. Science. 2008;320:226–30.
58. Maxhimer JB, Soto-Pantoja DR, Ridnour LA, Shih HB, Degraff WG, Tsokos M, Wink DA, Isenberg JS, Roberts DD. Radioprotection in normal tissue and delayed tumor growth by blockade of cd47 signaling. Sci Trans Med. 2009;1:3ra7.
59. Potten CS. Radiation, the ideal cytotoxic agent for studying the cell biology of tissues such as the small intestine. Radiat Res. 2004;161:123–36.
60. Paris F, Fuks Z, Kang A, Capodieci P, Juan G, Ehleiter D, Haimovitz-Friedman A, Cordon-Cardo C, Kolesnick R. Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice. Science. 2001;293:293–7.
61. Vozenin-Brotons MC, Sivan V, Gault N, Renard C, Geffrotin C, Delanian S, Lefaix JL, Martin M. Antifibrotic action of cu/zn sod is mediated by TGF-beta1 repression and phenotypic reversion of myofibroblasts. Free Radic Biol Med. 2001;30:30–42.
62. Epperly M, Bray J, Kraeger S, Zwacka R, Engelhardt J, Travis E, Greenberger J. Prevention of late effects of irradiation lung damage by manganese superoxide dismutase gene therapy. Gene Ther. 1998;5:196–208.
63. Niu Y, Epperly MW, Shen H, Smith T, Wang H, Greenberger JS. Intraesophageal mnsod-plasmid liposome enhances engraftment and self-renewal of bone marrow derived progenitors of esophageal squamous epithelium. Gene Ther. 2007;15(5):347–56.
64. Lefaix JL, Delanian S, Leplat JJ, Tricaud Y, Martin M, Nimrod A, Baillet F, Daburon F. Successful treatment of radiation-induced fibrosis using cu/zn-sod and mn-sod: an experimental study. Int J Radiat Oncol Biol Phys. 1996;35:305–12.
65. Delanian S, Lefaix JL. Current management for late normal tissue injury: radiation-induced fibrosis and necrosis. Semin Radiat Oncol. 2007;17:99–107.
66. Delanian S, Balla-Mekias S, Lefaix JL. Striking regression of chronic radiotherapy damage in a clinical trial of combined pentoxifylline and tocopherol. J Clin Oncol Off J Am Soc Clin Oncol. 1999;17:3283–90.
67. Hamama S, Gilbert-Sirieix M, Vozenin MC, Delanian S. Radiation-induced enteropathy: molecular basis of pentoxifylline-vitamin E anti-fibrotic effect involved TGF-beta1 cascade inhibition. Radiother Oncol. 2012;105:305–12.
68. Berbee M, Fu Q, Garg S, Kulkarni S, Kumar KS, Hauer-Jensen M. Pentoxifylline enhances the radioprotective properties of gamma-tocotrienol: differential effects on the hematopoietic, gastrointestinal and vascular systems. Radiat Res. 2011;175:297–306.
69. Berbee M, Fu Q, Boerma M, Sree Kumar K, Loose DS, Hauer-Jensen M. Mechanisms underlying the radioprotective properties of gamma-tocotrienol: comparative gene expression profiling in tocol-treated endothelial cells. Genes Nutr. 2012;7:75–81.
70. Sridharan V, Tripathi P, Sharma S, Corry PM, Moros EG, Singh A, Compadre CM, Hauer-Jensen M, Boerma M. Effects of late administration of pentoxifylline and tocotrienols in an image-guided rat model of localized heart irradiation. PLoS One. 2013;8, e68762.
71. Williams JP, Hernady E, Johnston CJ, Reed CM, Fenton B, Okunieff P, Finkelstein JN. Effect of administration of lovastatin on the development of late pulmonary effects after whole-lung irradiation in a murine model. Radiat Res. 2004;161:560–7.
72. Haydont V, Bourgier C, Pocard M, Lusinchi A, Aigueperse J, Mathe D, Bourhis J, Vozenin-Brotons MC. Pravastatin inhibits the rho/ccn2/extracellular matrix cascade in human fibrosis explants and improves radiation-induced intestinal fibrosis in rats. Clin Cancer Res. 2007;13:5331–40.
73. Haydont V, Gilliot O, Rivera S, Bourgier C, Francois A, Aigueperse J, Bourhis J, Vozenin-Brotons MC. Successful mitigation of delayed intestinal radiation injury using pravastatin is not associated with acute injury improvement or tumor protection. Int J Radiat Oncol Biol Phys. 2007;68:1471–82.
74. Wang J, Boerma M, Fu Q, Kulkarni A, Fink LM, Hauer-Jensen M. Simvastatin ameliorates radiation enteropathy development after localized, fractionated irradiation by a protein c-independent mechanism. Int J Radiat Oncol Biol Phys. 2007;68:1483–90.

75. Holler V, Buard V, Gaugler MH, Guipaud O, Baudelin C, Sache A, Perez Mdel R, Squiban C, Tamarat R, Milliat F, Benderitter M. Pravastatin limits radiation-induced vascular dysfunction in the skin. J Invest Dermatol. 2009;129:1280–91.
76. Wang J, Zheng H, Ou X, Albertson CM, Fink LM, Herbert JM, Hauer-Jensen M. Hirudin ameliorates intestinal radiation toxicity in the rat: support for thrombin inhibition as strategy to minimize side-effects after radiation therapy and as countermeasure against radiation exposure. J Thromb Haemost. 2004;2:2027–35.
77. Wang J, Zheng H, Hauer-Jensen M. Influence of short-term octreotide administration on chronic tissue injury, transforming growth factor beta (TGF-beta) overexpression, and collagen accumulation in irradiated rat intestine. J Pharmacol Exp Ther. 2001;297:35–42.
78. Wang J, Zheng H, Sung CC, Hauer-Jensen M. The synthetic somatostatin analogue, octreotide, ameliorates acute and delayed intestinal radiation injury. Int J Radiat Oncol Biol Phys. 1999;45:1289–96.
79. Hauer-Jensen M, Fink LM, Wang J. Radiation injury and the protein C pathway. Crit Care Med. 2004;32:S325–30.
80. Geiger H, Pawar SA, Kerschen EJ, Nattamai KJ, Hernandez I, Liang HP, Fernandez JA, Cancelas JA, Ryan MA, Kustikova O, Schambach A, Fu Q, Wang J, Fink LM, Petersen KU, Zhou D, Griffin JH, Baum C, Weiler H, Hauer-Jensen M. Pharmacological targeting of the thrombomodulin-activated protein c pathway mitigates radiation toxicity. Nat Med. 2012;18:1123–9.
81. Begg AC, Stewart FA, Vens C. Strategies to improve radiotherapy with targeted drugs. Nat Rev Cancer. 2011;11:239–53.
82. Moding EJ, Kastan MB, Kirsch DG. Strategies for optimizing the response of cancer and normal tissues to radiation. Nat Rev Drug Discov. 2013;12:526–42.
83. Du S, Barcellos-Hoff MH. Tumors as organs: biologically augmenting radiation therapy by inhibiting transforming growth factor beta activity in carcinomas. Semin Radiat Oncol. 2013;23:242–51.
84. Barcellos-Hoff MH, Cucinotta FA. New tricks for an old fox: impact of TGFbeta on the DNA damage response and genomic stability. Sci Signal. 2014;7:re5.
85. Barker HE, Paget JT, Khan AA, Harrington KJ. The tumour microenvironment after radiotherapy: mechanisms of resistance and recurrence. Nat Rev Cancer. 2015;15:409–25.
86. Derynck R, Feng XH. TGF-beta receptor signaling. Biochim Biophys Acta. 1997;1333:F105–50.
87. Shi Y, Massague J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell. 2003;113:685–700.
88. Bhowmick NA, Ghiassi M, Bakin A, Aakre M, Lundquist CA, Engel ME, Arteaga CL, Moses HL. Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a rhoa-dependent mechanism. Mol Biol Cell. 2001;12:27–36.
89. Suzuki K, Wilkes MC, Garamszegi N, Edens M, Leof EB. Transforming growth factor beta signaling via ras in mesenchymal cells requires p21-activated kinase 2 for extracellular signal-regulated kinase-dependent transcriptional responses. Cancer Res. 2007;67:3673–82.
90. Kim MR, Lee J, An YS, Jin YB, Park IC, Chung E, Shin I, Barcellos-Hoff MH, Yi JY. TGFbeta1 protects cells from gamma-ir by enhancing the activity of the NHEJ repair pathway. Mol Cancer Res. 2015;13:319–29.
91. Davis BN, Hilyard AC, Lagna G, Hata A. Smad proteins control drosha-mediated microrna maturation. Nature. 2008;454:56–61.
92. Peng Y, Song L, Zhao M, Harmelink C, Debenedittis P, Cui X, Wang Q, Jiao K. Critical roles of mirna-mediated regulation of TGFbeta signalling during mouse cardiogenesis. Cardiovasc Res. 2014;103:258–67.
93. Garcia R, Nistal JF, Merino D, Price NL, Fernandez-Hernando C, Beaumont J, Gonzalez A, Hurle MA, Villar AV. P-smad2/3 and dicer promote pre-mir-21 processing during pressure overload-associated myocardial remodeling. Biochim Biophys Acta. 1852;2015:1520–30.
94. Pan Z, Sun X, Shan H, Wang N, Wang J, Ren J, Feng S, Xie L, Lu C, Yuan Y, Zhang Y, Wang Y, Lu Y, Yang B. Microrna-101 inhibited postinfarct cardiac fibrosis and improved left ventricular compliance via the FBJ osteosarcoma oncogene/transforming growth factor-beta1 pathway. Circulation. 2012;126:840–50.

95. Thum T, Gross C, Fiedler J, Fischer T, Kissler S, Bussen M, Galuppo P, Just S, Rottbauer W, Frantz S, Castoldi M, Soutschek J, Koteliansky V, Rosenwald A, Basson MA, Licht JD, Pena JT, Rouhanifard SH, Muckenthaler MU, Tuschl T, Martin GR, Bauersachs J, Engelhardt S. Microrna-21 contributes to myocardial disease by stimulating map kinase signalling in fibroblasts. Nature. 2008;456:980–4.
96. Hamama S, Noman MZ, Gervaz P, Delanian S, Vozenin MC. Mir-210: a potential therapeutic target against radiation-induced enteropathy. Radiother Oncol. 2014;111:219–21.
97. Anscher MS, Thrasher B, Rabbani Z, Teicher B, Vujaskovic Z. Antitransforming growth factor-beta antibody 1d11 ameliorates normal tissue damage caused by high-dose radiation. Int J Radiat Oncol Biol Phys. 2006;65:876–81.
98. Anscher MS, Thrasher B, Zgonjanin L, Rabbani ZN, Corbley MJ, Fu K, Sun L, Lee WC, Ling LE, Vujaskovic Z. Small molecular inhibitor of transforming growth factor-beta protects against development of radiation-induced lung injury. Int J Radiat Oncol Biol Phys. 2008;71:829–37.
99. Zheng H, Wang J, Koteliansky VE, Gotwals PJ, Hauer-Jensen M. Recombinant soluble transforming growth factor beta type II receptor ameliorates radiation enteropathy in mice. Gastroenterology. 2000;119:1286–96.
100. Rabbani ZN, Anscher MS, Zhang X, Chen L, Samulski TV, Li CY, Vujaskovic Z. Soluble TGFbeta type II receptor gene therapy ameliorates acute radiation-induced pulmonary injury in rats. Int J Radiat Oncol Biol Phys. 2003;57:563–72.
101. Noble PW, Albera C, Bradford WZ, Costabel U, Glassberg MK, Kardatzke D, King Jr TE, Lancaster L, Sahn SA, Szwarcberg J, Valeyre D, du Bois RM. Pirfenidone in patients with idiopathic pulmonary fibrosis (capacity): two randomised trials. Lancet. 2011;377:1760–9.
102. Simone NL, Soule BP, Gerber L, Augustine E, Smith S, Altemus RM, Mitchell JB, Camphausen KA. Oral pirfenidone in patients with chronic fibrosis resulting from radiotherapy: a pilot study. Radiat Oncol. 2007;2:19.
103. Ridley AJ. Rho family proteins: coordinating cell responses. Trends Cell Biol. 2001;11:471–7.
104. Vozenin-Brotons MC, Milliat F, Linard C, Strup C, Francois A, Sabourin JC, Lasser P, Lusinchi A, Deutsch E, Girinsky T, Aigueperse J, Bourhis J, Mathe D. Gene expression profile in human late radiation enteritis obtained by high-density cdna array hybridization. Radiat Res. 2004;161:299–311.
105. Haydont V, Mathe D, Bourgier C, Abdelali J, Aigueperse J, Bourhis J, Vozenin-Brotons MC. Induction of cTGF by TGF-beta1 in normal and radiation enteritis human smooth muscle cells: Smad/rho balance and therapeutic perspectives. Radiother Oncol. 2005;76:219–25.
106. Monceau V, Pasinetti N, Schupp C, Pouzoulet F, Opolon P, Vozenin MC. Modulation of the rho/rock pathway in heart and lung after thorax irradiation reveals targets to improve normal tissue toxicity. Curr Drug Targets. 2010;11:1395–404.
107. Monceau V, Llach A, Azria D, Bridier A, Petit B, Mazevet M, Strup-Perrot C, To TH, Calmels L, Germaini MM, Gourgou S, Fenoglietto P, Bourgier C, Gomez AM, Escoubet B, Dorr W, Haagen J, Deutsch E, Morel E, Vozenin MC. Epac contributes to cardiac hypertrophy and amyloidosis induced by radiotherapy but not fibrosis. Radiother Oncol. 2014;111:63–71.
108. Bourgier C, Monceau V, Bourhis J, Deutsch E, Vozenin MC. [Pharmacological modulation of late radio-induced side effects]. Cancer Radiother. 2011;15:383–9.
109. Bourgier C, Haydont V, Milliat F, Francois A, Holler V, Lasser P, Bourhis J, Mathe D, Vozenin-Brotons MC. Inhibition of rho kinase modulates radiation induced fibrogenic phenotype in intestinal smooth muscle cells through alteration of the cytoskeleton and connective tissue growth factor expression. Gut. 2005;54:336–43.
110. Haydont V, Bourgier C, Vozenin-Brotons MC. Rho/rock pathway as a molecular target for modulation of intestinal radiation-induced toxicity. Br J Radiol. 2007;80(Spec No 1):S32–40.
111. Ostrau C, Hulsenbeck J, Herzog M, Schad A, Torzewski M, Lackner KJ, Fritz G. Lovastatin attenuates ionizing radiation-induced normal tissue damage in vivo. Radiother Oncol. 2009;92:492–9.
112. Boerma M, Fu Q, Wang J, Loose DS, Bartolozzi A, Ellis JL, McGonigle S, Paradise E, Sweetnam P, Fink LM, Vozenin-Brotons MC, Hauer-Jensen M. Comparative gene expression

profiling in three primary human cell lines after treatment with a novel inhibitor of rho kinase or atorvastatin. Blood Coagul Fibrinolysis. 2008;19:709–18.
113. Katz MS, Minsky BD, Saltz LB, Riedel E, Chessin DB, Guillem JG. Association of statin use with a pathologic complete response to neoadjuvant chemoradiation for rectal cancer. Int J Radiat Oncol Biol Phys. 2005;62:1363–70.
114. Wedlake LJ, Silia F, Benton B, Lalji A, Thomas K, Dearnaley DP, Blake P, Tait D, Khoo VS, Andreyev HJ. Evaluating the efficacy of statins and ace-inhibitors in reducing gastrointestinal toxicity in patients receiving radiotherapy for pelvic malignancies. Eur J Cancer. 2012;48:2117–24.
115. Brigstock DR, Steffen CL, Kim GY, Vegunta RK, Diehl JR, Harding PA. Purification and characterization of novel heparin-binding growth factors in uterine secretory fluids. Identification as heparin-regulated mr 10,000 forms of connective tissue growth factor. J Biol Chem. 1997;272:20275–82.
116. Shi-wen X, Pennington D, Holmes A, Leask A, Bradham D, Beauchamp JR, Fonseca C, du Bois RM, Martin GR, Black CM, Abraham DJ. Autocrine overexpression of cTGF maintains fibrosis: Rda analysis of fibrosis genes in systemic sclerosis. Exp Cell Res. 2000;259:213–24.
117. Holmes A, Abraham DJ, Sa S, Shiwen X, Black CM, Leask A. CTGF and smads, maintenance of scleroderma phenotype is independent of smad signaling. J Biol Chem. 2001;276:10594–601.
118. Grotendorst GR, Okochi H, Hayashi N. A novel transforming growth factor beta response element controls the expression of the connective tissue growth factor gene. Cell Growth Differ. 1996;7:469–80.
119. Flanders KC, Major CD, Arabshahi A, Aburime EE, Okada MH, Fujii M, Blalock TD, Schultz GS, Sowers A, Anzano MA, Mitchell JB, Russo A, Roberts AB. Interference with transforming growth factor-beta/smad3 signaling results in accelerated healing of wounds in previously irradiated skin. Am J Pathol. 2003;163:2247–57.
120. Xue J, Li X, Lu Y, Gan L, Zhou L, Wang Y, Lan J, Liu S, Sun L, Jia L, Mo X, Li J. Gene-modified mesenchymal stem cells protect against radiation-induced lung injury. Mol Ther. 2013;21:456–65.
121. Vozenin-Brotons MC, Milliat F, Sabourin JC, de Gouville AC, Francois A, Lasser P, Morice P, Haie-Meder C, Lusinchi A, Antoun S, Bourhis J, Mathe D, Girinsky T, Aigueperse J. Fibrogenic signals in patients with radiation enteritis are associated with increased connective tissue growth factor expression. Int J Radiat Oncol Biol Phys. 2003;56:561–72.
122. Haydont V, Vozenin-Brotons MC. Maintenance of radiation-induced intestinal fibrosis: cellular and molecular features. World J Gastroenterol. 2007;13:2675–83.
123. Zhang P, Cui W, Hankey KG, Gibbs AM, Smith CP, Taylor-Howell C, Kearney SR, MacVittie TJ. Increased expression of connective tissue growth factor (cTGF) in multiple organs after exposure of non-human primates (nhp) to lethal doses of radiation. Health Phys. 2015;109:374–90.
124. Chen CC, Chen N, Lau LF. The angiogenic factors cyr61 and connective tissue growth factor induce adhesive signaling in primary human skin fibroblasts. J Biol Chem. 2001;276:10443–52.
125. Segarini PR, Nesbitt JE, Li D, Hays LG, Yates 3rd JR, Carmichael DF. The low density lipoprotein receptor-related protein/alpha2-macroglobulin receptor is a receptor for connective tissue growth factor. J Biol Chem. 2001;276:40659–67.
126. Grotendorst GR. Connective tissue growth factor: a mediator of TGF-beta action on fibroblasts. Cytokine Growth Factor Rev. 1997;8:171–9.
127. Xue J, Gan L, Li X, Li J, Qi G, Wu Y, Fu X, Mao Q, Ao R, Lang J, Lu Y. Effects of lysophosphatidic acid and its receptors lpa(1/3) on radiation pneumonitis. Oncol Rep. 2010;24:1515–20.
128. Bickelhaupt S, Peschke P, Erbel C, Tietz A, Timke C, Wirkner U, Debus J, Lipson K, Huber PE. Attenuation and reversal of radiation-induced pulmonary fibrosis in a murine model by an anti-cTGF monoclonal antibody. Int J Radiat Oncol. 2010;78:abstract number 84.
129. Abdollahi A, Li M, Ping G, Plathow C, Domhan S, Kiessling F, Lee LB, McMahon G, Grone HJ, Lipson KE, Huber PE. Inhibition of platelet-derived growth factor signaling attenuates pulmonary fibrosis. J Exp Med. 2005;201:925–35.

130. Tayel A, Abd El Galil KH, Ebrahim MA, Ibrahim AS, El-Gayar AM, Al-Gayyar MM. Suramin inhibits hepatic tissue damage in hepatocellular carcinoma through deactivation of heparanase enzyme. Eur J Pharmacol. 2014;728:151–60.
131. McGeary RP, Bennett AJ, Tran QB, Cosgrove KL, Ross BP. Suramin: clinical uses and structure-activity relationships. Mini Rev Med Chem. 2008;8:1384–94.
132. Vujaskovic Z, Anscher MS, Feng QF, Rabbani ZN, Amin K, Samulski TS, Dewhirst MW, Haroon ZA. Radiation-induced hypoxia may perpetuate late normal tissue injury. Int J Radiat Oncol Biol Phys. 2001;50:851–5.
133. Pasquier D, Hoelscher T, Schmutz J, Dische S, Mathieu D, Baumann M, Lartigau E. Hyperbaric oxygen therapy in the treatment of radio-induced lesions in normal tissues: a literature review. Radiother Oncol. 2004;72:1–13.
134. Benhyahia BCF, Perdereau B, Gez E, Fourquet A, Magdelena H. Effects of sod topical treatment on human skin radiofibrosis: a pathological study. Breast. 1996;5:75–81.
135. Liu R, Oberley TD, Oberley LW. Transfection and expression of mnsod cdna decreases tumor malignancy of human oral squamous carcinoma scc-25 cells. Hum Gene Ther. 1997;8:585–95.
136. Dennog C, Radermacher P, Barnett YA, Speit G. Antioxidant status in humans after exposure to hyperbaric oxygen. Mutat Res. 1999;428:83–9.
137. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. 2007;39:44–84.
138. Vujaskovic Z, Feng QF, Rabbani ZN, Anscher MS, Samulski TV, Brizel DM. Radioprotection of lungs by amifostine is associated with reduction in profibrogenic cytokine activity. Radiat Res. 2002;157:656–60.
139. Fleischer G, Dorr W. Amelioration of early radiation effects in oral mucosa (mouse) by intravenous or subcutaneous administration of amifostine. Strahlenther Onkol. 2006;182:567–75.
140. Koukourakis MI, Tsoutsou PG, Abatzoglou IM, Sismanidou K, Giatromanolaki A, Sivridis E. Hypofractionated and accelerated radiotherapy with subcutaneous amifostine cytoprotection as short adjuvant regimen after breast-conserving surgery: interim report. Int J Radiat Oncol Biol Phys. 2009;74:1173–80.
141. Boerma M, Roberto KA, Hauer-Jensen M. Prevention and treatment of functional and structural radiation injury in the rat heart by pentoxifylline and alpha-tocopherol. Int J Radiat Oncol Biol Phys. 2008;72:170–7.
142. Berbee M, Fu Q, Boerma M, Wang J, Kumar KS, Hauer-Jensen M. Gamma-tocotrienol ameliorates intestinal radiation injury and reduces vascular oxidative stress after total-body irradiation by an hmg-coa reductase-dependent mechanism. Radiat Res. 2009;171:596–605.
143. Berbee M, Fu Q, Boerma M, Pathak R, Zhou D, Kumar KS, Hauer-Jensen M. Reduction of radiation-induced vascular nitrosative stress by the vitamin E analog gamma-tocotrienol: evidence of a role for tetrahydrobiopterin. Int J Radiat Oncol Biol Phys. 2011;79:884–91.
144. Choi SH, Hong ZY, Nam JK, Lee HJ, Jang J, Yoo RJ, Lee YJ, Lee CY, Kim KH, Park S, Ji YH, Lee YS, Cho J, Lee YJ. A hypoxia-induced vascular endothelial-to-mesenchymal transition in development of radiation-induced pulmonary fibrosis. Clin Cancer Res. 2015;21:3716–26.
145. Wynn TA. Fibrotic disease and the t(h)1/t(h)2 paradigm. Nat Rev Immunol. 2004;4:583–94.
146. Wynn TA. Integrating mechanisms of pulmonary fibrosis. J Exp Med. 2011;208:1339–50.
147. Hirota K, Hashimoto H, Tsubo T, Ishihara H, Matsuki A. Quantification and comparison of pulmonary emboli formation after pneumatic tourniquet release in patients undergoing reconstruction of anterior cruciate ligament and total knee arthroplasty. Anesth Analg. 2002;94:1633–8.
148. Evans ML, Graham MM, Mahler PA, Rasey JS. Use of steroids to suppress vascular response to radiation. Int J Radiat Oncol Biol Phys. 1987;13:563–7.
149. Ward HE, Kemsley L, Davies L, Holecek M, Berend N. The effect of steroids on radiation-induced lung disease in the rat. Radiat Res. 1993;136:22–8.
150. Hill RP, Zaidi A, Mahmood J, Jelveh S. Investigations into the role of inflammation in normal tissue response to irradiation. Radiother Oncol. 2011;101:73–9.

151. Nawroth I, Alsner J, Behlke MA, Besenbacher F, Overgaard J, Howard KA, Kjems J. Intraperitoneal administration of chitosan/dsirna nanoparticles targeting tnfalpha prevents radiation-induced fibrosis. Radiother Oncol. 2010;97:143–8.
152. Xia DH, Xi L, Xv C, Mao WD, Shen WS, Shu ZQ, Yang HZ, Dai M. The protective effects of ambroxol on radiation lung injury and influence on production of transforming growth factor beta1 and tumor necrosis factor alpha. Med Oncol. 2010;27:697–701.
153. Delanian S, Lefaix JL. Complete healing of severe osteoradionecrosis with treatment combining pentoxifylline, tocopherol and clodronate. Br J Radiol. 2002;75:467–9.
154. Fleischmann RM, Schechtman J, Bennett R, Handel ML, Burmester GR, Tesser J, Modafferi D, Poulakos J, Sun G. Anakinra, a recombinant human interleukin-1 receptor antagonist (r-methuil-1ra), in patients with rheumatoid arthritis: a large, international, multicenter, placebo-controlled trial. Arthritis Rheum. 2003;48:927–34.
155. Piguet PF, Vesin C, Grau GE, Thompson RC. Interleukin 1 receptor antagonist (il-1ra) prevents or cures pulmonary fibrosis elicited in mice by bleomycin or silica. Cytokine. 1993;5:57–61.
156. Zurawski SM, Vega Jr F, Huyghe B, Zurawski G. Receptors for interleukin-13 and interleukin-4 are complex and share a novel component that functions in signal transduction. EMBO J. 1993;12:2663–70.
157. Ong C, Wong C, Roberts CR, Teh HS, Jirik FR. Anti-il-4 treatment prevents dermal collagen deposition in the tight-skin mouse model of scleroderma. Eur J Immunol. 1998;28:2619–29.
158. Cheever AW, Williams ME, Wynn TA, Finkelman FD, Seder RA, Cox TM, Hieny S, Caspar P, Sher A. Anti-il-4 treatment of schistosoma mansoni-infected mice inhibits development of t cells and non-b, non-t cells expressing th2 cytokines while decreasing egg-induced hepatic fibrosis. J Immunol. 1994;153:753–9.
159. Chiaramonte MG, Donaldson DD, Cheever AW, Wynn TA. An il-13 inhibitor blocks the development of hepatic fibrosis during a t-helper type 2-dominated inflammatory response. J Clin Invest. 1999;104:777–85.
160. Kumar RK, Herbert C, Yang M, Koskinen AM, McKenzie AN, Foster PS. Role of interleukin-13 in eosinophil accumulation and airway remodelling in a mouse model of chronic asthma. Clin Exp Allergy. 2002;32:1104–11.
161. Smith RE. Chemotactic cytokines mediate leukocyte recruitment in fibrotic lung disease. Biol Signals. 1996;5:223–31.
162. Smith RE, Strieter RM, Phan SH, Kunkel SL. C-c chemokines: novel mediators of the profibrotic inflammatory response to bleomycin challenge. Am J Respir Cell Mol Biol. 1996;15:693–702.
163. Belperio JA, Dy M, Murray L, Burdick MD, Xue YY, Strieter RM, Keane MP. The role of the th2 cc chemokine ligand ccl17 in pulmonary fibrosis. J Immunol. 2004;173:4692–8.
164. Jiang D, Liang J, Campanella GS, Guo R, Yu S, Xie T, Liu N, Jung Y, Homer R, Meltzer EB, Li Y, Tager AM, Goetinck PF, Luster AD, Noble PW. Inhibition of pulmonary fibrosis in mice by cxcl10 requires glycosaminoglycan binding and syndecan-4. J Clin Invest. 2010;120:2049–57.
165. Burdick MD, Murray LA, Keane MP, Xue YY, Zisman DA, Belperio JA, Strieter RM. Cxcl11 attenuates bleomycin-induced pulmonary fibrosis via inhibition of vascular remodeling. Am J Respir Crit Care Med. 2005;171:261–8.
166. Wynn TA, Ramalingam TR. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. Nat Med. 2012;18:1028–40.
167. Estes JD, Wietgrefe S, Schacker T, Southern P, Beilman G, Reilly C, Milush JM, Lifson JD, Sodora DL, Carlis JV, Haase AT. Simian immunodeficiency virus-induced lymphatic tissue fibrosis is mediated by transforming growth factor β1-positive regulatory t cells and begins in early infection. J Infect Dis. 2007;195(4):551–61.
168. Liu X, Hu H, Yin JQ. Therapeutic strategies against TGF-beta signaling pathway in hepatic fibrosis. Liver Int. 2006;26:8–22.
169. Wilson IA, Madala SK, Ramalingam TR, Gochuico BR, Rosas IO, Cheever AW, Wynn TA. Bleomycin and il-1β–mediated pulmonary fibrosis is il-17a dependent. J Exp Med. 2010;207(3):535–52.

170. Gasse P, Riteau N, Vacher R, Michel ML, Fautrel A, di Padova F, Fick L, Charron S, Lagente V, Eberl G, Le Bert M, Quesniaux VF, Huaux F, Leite-de-Moraes M, Ryffel B, Couillin I. Il-1 and il-23 mediate early il-17a production in pulmonary inflammation leading to late fibrosis. PLoS One. 2011;6, e23185.
171. Cortez DM, Feldman MD, Mummidi S, Valente AJ, Steffensen B, Vincenti M, Barnes JL, Chandrasekar B. Il-17 stimulates mmp-1 expression in primary human cardiac fibroblasts via p38 mapk- and erk1/2-dependent c/ebp-beta, nf-kappab, and ap-1 activation. Am J Physiol Heart Circ Physiol. 2007;293:H3356–65.
172. Cao H, Zhou X, Zhang J, Huang X, Zhai Y, Zhang X, Chu L. Hydrogen sulfide protects against bleomycin-induced pulmonary fibrosis in rats by inhibiting nf-kappab expression and regulating th1/th2 balance. Toxicol Lett. 2014;224:387–94.
173. Zheng X, Guo Y, Wang L, Zhang H, Wang S, Wang L, An L, Zhou X, Li X, Yao C. Recovery profiles of t-cell subsets following low-dose total body irradiation and improvement with cinnamon. Int J Radiat Oncol Biol Phys. 2015;93:1118–26.
174. De Palma M, Coukos G, Hanahan D. A new twist on radiation oncology: low-dose irradiation elicits immunostimulatory macrophages that unlock barriers to tumor immunotherapy. Cancer Cell. 2013;24:559–61.
175. Groves AM, Johnston CJ, Misra RS, Williams JP, Finkelstein JN. Whole-lung irradiation results in pulmonary macrophage alterations that are subpopulation and strain specific. Radiat Res. 2015;184:639–49.
176. Gordon S. Alternative activation of macrophages. Nat Rev Immunol. 2003;3:23–35.
177. Morris Jr SM, Kepka-Lenhart D, Chen LC. Differential regulation of arginases and inducible nitric oxide synthase in murine macrophage cells. Am J Physiol. 1998;275:E740–7.
178. Troidl C, Mollmann H, Nef H, Masseli F, Voss S, Szardien S, Willmer M, Rolf A, Rixe J, Troidl K, Kostin S, Hamm C, Elsasser A. Classically and alternatively activated macrophages contribute to tissue remodelling after myocardial infarction. J Cell Mol Med. 2009;13:3485–96.
179. Hu Y, Zhang H, Lu Y, Bai H, Xu Y, Zhu X, Zhou R, Ben J, Xu Y, Chen Q. Class a scavenger receptor attenuates myocardial infarction-induced cardiomyocyte necrosis through suppressing m1 macrophage subset polarization. Basic Res Cardiol. 2011;106(6):1311–28.
180. Usher MG, Duan SZ, Ivaschenko CY, Frieler RA, Berger S, Schutz G, Lumeng CN, Mortensen RM. Myeloid mineralocorticoid receptor controls macrophage polarization and cardiovascular hypertrophy and remodeling in mice. J Clin Invest. 2010;120:3350–64.
181. Roberts Jr NJ, Prill AH, Mann TN. Interleukin 1 and interleukin 1 inhibitor production by human macrophages exposed to influenza virus or respiratory syncytial virus. Respiratory syncytial virus is a potent inducer of inhibitor activity. J Exp Med. 1986;163:511–9.
182. Ikushima H, Miyazono K. TGFbeta signalling: a complex web in cancer progression. Nat Rev Cancer. 2010;10:415–24.
183. De Palma M, Lewis CE. Macrophage regulation of tumor responses to anticancer therapies. Cancer Cell. 2013;23:277–86.
184. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, Mignot G, Maiuri MC, Ullrich E, Saulnier P, Yang H, Amigorena S, Ryffel B, Barrat FJ, Saftig P, Levi F, Lidereau R, Nogues C, Mira JP, Chompret A, Joulin V, Clavel-Chapelon F, Bourhis J, Andre F, Delaloge S, Tursz T, Kroemer G, Zitvogel L. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med. 2007;13:1050–9.
185. Burnette B, Weichselbaum RR. The immunology of ablative radiation. Semin Radiat Oncol. 2015;25:40–5.
186. Meng YR, Beckett MA, Liang H, Mauceri HJ, van Rooijen N, Cohen KS, Weichselbaum RR. Blockade of tumor necrosis factor alpha signaling in tumor-associated macrophages as a radiosensitizing strategy. Cancer Res. 2010;70:1534–43.
187. Klug F, Prakash H, Huber PE, Seibel T, Bender N, Halama N, Pfirschke C, Voss RH, Timke C, Umansky L, Klapproth K, Schakel K, Garbi N, Jager D, Weitz J, Schmitz-Winnenthal H, Hammerling GJ, Beckhove P. Low-dose irradiation programs macrophage differentiation to an inos(+)/m1 phenotype that orchestrates effective t cell immunotherapy. Cancer Cell. 2013;24:589–602.

188. Thepen T, Van Rooijen N, Kraal G. Alveolar macrophage elimination in vivo is associated with an increase in pulmonary immune response in mice. J Exp Med. 1989;170:499–509.
189. Lu SW, Zhang XM, Luo HM, Fu YC, Xu MY, Tang SJ. Clodronate liposomes reduce excessive scar formation in a mouse model of burn injury by reducing collagen deposition and TGF-beta1 expression. Mol Biol Rep. 2014;41:2143–9.
190. Froom P, Elmalah I, Braester A, Aghai E, Quitt M. Clodronate in myelofibrosis: a case report. Am J Med Sci. 2002;323:115–6.
191. Delanian S, Lefaix JL, Maisonobe T, Salachas F, Pradat PF. Significant clinical improvement in radiation-induced lumbosacral polyradiculopathy by a treatment combining pentoxifylline, tocopherol, and clodronate (pentoclo). J Neurol Sci. 2008;275:164–6.
192. Hume DA, MacDonald KP. Therapeutic applications of macrophage colony-stimulating factor-1 (csf-1) and antagonists of csf-1 receptor (csf-1r) signaling. Blood. 2012;119:1810–20.
193. Murayama T, Yokode M, Kataoka H, Imabayashi T, Yoshida H, Sano H, Nishikawa S, Nishikawa S, Kita T. Intraperitoneal administration of anti-c-fms monoclonal antibody prevents initial events of atherogenesis but does not reduce the size of advanced lesions in apolipoprotein e-deficient mice. Circulation. 1999;99:1740–6.
194. Jose MD, Le Meur Y, Atkins RC, Chadban SJ. Blockade of macrophage colony-stimulating factor reduces macrophage proliferation and accumulation in renal allograft rejection. Am J Transplant. 2003;3:294–300.
195. Segawa M, Fukada S, Yamamoto Y, Yahagi H, Kanematsu M, Sato M, Ito T, Uezumi A, Hayashi S, Miyagoe-Suzuki Y, Takeda S, Tsujikawa K, Yamamoto H. Suppression of macrophage functions impairs skeletal muscle regeneration with severe fibrosis. Exp Cell Res. 2008;314:3232–44.
196. Menke J, Iwata Y, Rabacal WA, Basu R, Yeung YG, Humphreys BD, Wada T, Schwarting A, Stanley ER, Kelley VR. Csf-1 signals directly to renal tubular epithelial cells to mediate repair in mice. J Clin Invest. 2009;119:2330–42.
197. Menke J, Kriegsmann J, Schimanski CC, Schwartz MM, Schwarting A, Kelley VR. Autocrine csf-1 and csf-1 receptor coexpression promotes renal cell carcinoma growth. Cancer Res. 2012;72:187–200.
198. Menke J, Rabacal WA, Byrne KT, Iwata Y, Schwartz MM, Stanley ER, Schwarting A, Kelley VR. Circulating csf-1 promotes monocyte and macrophage phenotypes that enhance lupus nephritis. J Am Soc Nephrol. 2009;20:2581–92.
199. Xu J, Escamilla J, Mok S, David J, Priceman SJ, West BL, Bollag G, McBride WH, Wu L. Abrogating the protumorigenic influences of tumor-infiltrating myeloid cells bycsf1r signaling blockade improves the efficacy of radiotherapy in prostatecancer. Cancer Res. 2013;73:2782–94.
200. Jones HA, Schofield JB, Krausz T, Boobis AR, Haslett C. Pulmonary fibrosis correlates with duration of tissue neutrophil activation. Am J Respir Crit Care Med. 1998;158:620–8.
201. Borthwick LA, Suwara MI, Carnell SC, Green NJ, Mahida R, Dixon D, Gillespie CS, Cartwright TN, Horabin J, Walker A, Olin E, Rangar M, Gardner A, Mann J, Corris PA, Mann DA, Fisher AJ. Pseudomonas aeruginosa induced airway epithelial injury drives fibroblast activation: a mechanism in chronic lung allograft dysfunction. Am J Transplant. 2016;16(6):1751–65.
202. Connolly MK, Bedrosian AS, Mallen-St Clair J, Mitchell AP, Ibrahim J, Stroud A, Pachter HL, Bar-Sagi D, Frey AB, Miller G. In liver fibrosis, dendritic cells govern hepatic inflammation in mice via tnf-alpha. J Clin Invest. 2009;119:3213–25.
203. Greer AM, Matthay MA, Kukreja J, Bhakta NR, Nguyen CP, Wolters PJ, Woodruff PG, Fahy JV, Shin JS. Accumulation of bdca1(+) dendritic cells in interstitial fibrotic lung diseases and th2-high asthma. PLoS One. 2014;9, e99084.
204. Nagai T, Honda S, Sugano Y, Matsuyama TA, Ohta-Ogo K, Asaumi Y, Ikeda Y, Kusano K, Ishihara M, Yasuda S, Ogawa H, Ishibashi-Ueda H, Anzai T. Decreased myocardial dendritic cells is associated with impaired reparative fibrosis and development of cardiac rupture after myocardial infarction in humans. J Am Heart Assoc. 2014;3, e000839.

205. Levick SP, McLarty JL, Murray DB, Freeman RM, Carver WE, Brower GL. Cardiac mast cells mediate left ventricular fibrosis in the hypertensive rat heart. Hypertension. 2009;53:1041–7.
206. Reiman RM, Thompson RW, Feng CG, Hari D, Knight R, Cheever AW, Rosenberg HF, Wynn TA. Interleukin-5 (il-5) augments the progression of liver fibrosis by regulating il-13 activity. Infect Immun. 2006;74:1471–9.
207. Humbles AA, Lloyd CM, McMillan SJ, Friend DS, Xanthou G, McKenna EE, Ghiran S, Gerard NP, Yu C, Orkin SH, Gerard C. A critical role for eosinophils in allergic airways remodeling. Science. 2004;305:1776–9.
208. Levi-Schaffer F, Garbuzenko E, Rubin A, Reich R, Pickholz D, Gillery P, Emonard H, Nagler A, Maquart FA. Human eosinophils regulate human lung- and skin-derived fibroblast properties in vitro: a role for transforming growth factor beta (TGF-beta). Proc Natl Acad Sci U S A. 1999;96:9660–5.
209. Taur Y, Pamer EG. The intestinal microbiota and susceptibility to infection in immunocompromised patients. Curr Opin Infect Dis. 2013;26:332–7.
210. Logan AC, Katzman MA, Balanza-Martinez V. Natural environments, ancestral diets, and microbial ecology: is there a modern "paleo-deficit disorder"? Part II. J Physiol Anthropol. 2015;34:9.
211. Logan AC, Katzman MA, Balanza-Martinez V. Natural environments, ancestral diets, and microbial ecology: is there a modern "paleo-deficit disorder"? Part I. J Physiol Anthropol. 2015;34:1.
212. Smith P, Siddharth J, Pearson R, Holway N, Shaxted M, Butler M, Clark N, Jamontt J, Watson RP, Sanmugalingam D, Parkinson SJ. Host genetics and environmental factors regulate ecological succession of the mouse colon tissue-associated microbiota. PLoS One. 2012;7, e30273.
213. Qiu W, Carson-Walter EB, Liu H, Epperly M, Greenberger JS, Zambetti GP, Zhang L, Yu J. Puma regulates intestinal progenitor cell radiosensitivity and gastrointestinal syndrome. Cell Stem Cell. 2008;2:576–83.
214. Wang X, Wei L, Cramer JM, Leibowitz BJ, Judge C, Epperly M, Greenberger J, Wang F, Li L, Stelzner MG, Dunn JC, Martin MG, Lagasse E, Zhang L, Yu J. Pharmacologically blocking p53-dependent apoptosis protects intestinal stem cells and mice from radiation. Sci Rep. 2015;5:8566.
215. Leibowitz BJ, Qiu W, Liu H, Cheng T, Zhang L, Yu J. Uncoupling p53 functions in radiation-induced intestinal damage via puma and p21. Mol Cancer Res. 2011;9:616–25.
216. Rakoff-Nahoum S, Medzhitov R. Role of the innate immune system and host-commensal mutualism. Curr Top Microbiol Immunol. 2006;308:1–18.
217. Viaud S, Saccheri F, Mignot G, Yamazaki T, Daillere R, Hannani D, Enot DP, Pfirschke C, Engblom C, Pittet MJ, Schlitzer A, Ginhoux F, Apetoh L, Chachaty E, Woerther PL, Eberl G, Berard M, Ecobichon C, Clermont D, Bizet C, Gaboriau-Routhiau V, Cerf-Bensussan N, Opolon P, Yessaad N, Vivier E, Ryffel B, Elson CO, Dore J, Kroemer G, Lepage P, Boneca IG, Ghiringhelli F, Zitvogel L. The intestinal microbiota modulates the anticancer immune effects of cyclophosphamide. Science. 2013;342:971–6.
218. Kostic AD, Gevers D, Pedamallu CS, Michaud M, Duke F, Earl AM, Ojesina AI, Jung J, Bass AJ, Tabernero J, Baselga J, Liu C, Shivdasani RA, Ogino S, Birren BW, Huttenhower C, Garrett WS, Meyerson M. Genomic analysis identifies association of fusobacterium with colorectal carcinoma. Genome Res. 2012;22:292–8.
219. Grivennikov SI, Wang K, Mucida D, Stewart CA, Schnabl B, Jauch D, Taniguchi K, Yu GY, Osterreicher CH, Hung KE, Datz C, Feng Y, Fearon ER, Oukka M, Tessarollo L, Coppola V, Yarovinsky F, Cheroutre H, Eckmann L, Trinchieri G, Karin M. Adenoma-linked barrier defects and microbial products drive il-23/il-17-mediated tumour growth. Nature. 2012;491:254–8.
220. Arthur JC, Perez-Chanona E, Muhlbauer M, Tomkovich S, Uronis JM, Fan TJ, Campbell BJ, Abujamel T, Dogan B, Rogers AB, Rhodes JM, Stintzi A, Simpson KW, Hansen JJ, Keku TO, Fodor AA, Jobin C. Intestinal inflammation targets cancer-inducing activity of the microbiota. Science. 2012;338:120–3.

221. Farin HF, Van Es JH, Clevers H. Redundant sources of wnt regulate intestinal stem cells and promote formation of paneth cells. Gastroenterology. 2012;143:1518–29. e1517.
222. Ferreira MR, Andreyev HJ. Editorial: gut microbiota and chemotherapy- or radiation-induced gastrointestinal mucositis. Aliment Pharmacol Ther. 2014;40:733–4.
223. Ferreira MR, Muls A, Dearnaley DP, Andreyev HJ. Microbiota and radiation-induced bowel toxicity: lessons from inflammatory bowel disease for the radiation oncologist. Lancet Oncol. 2014;15:e139–47.
224. Wedlake L, Slack N, Andreyev HJ, Whelan K. Fiber in the treatment and maintenance of inflammatory bowel disease: a systematic review of randomized controlled trials. Inflamm Bowel Dis. 2014;20:576–86.
225. Eloe-Fadrosh EA, Brady A, Crabtree J, Drabek EF, Ma B, Mahurkar A, Ravel J, Haverkamp M, Fiorino AM, Botelho C, Andreyeva I, Hibberd PL, Fraser CM. Functional dynamics of the gut microbiome in elderly people during probiotic consumption. MBio 2015;6.
226. Hibberd PL, Kleimola L, Fiorino AM, Botelho C, Haverkamp M, Andreyeva I, Poutsiaka D, Fraser C, Solano-Aguilar G, Snydman DR. No evidence of harms of probiotic lactobacillus rhamnosus gg atcc 53103 in healthy elderly-a phase i open label study to assess safety, tolerability and cytokine responses. PLoS One. 2014;9, e113456.
227. De Ryck T, Vanlancker E, Grootaert C, Roman BI, De Coen LM, Vandenberghe I, Stevens CV, Bracke M, Van de Wiele T, Vanhoecke B. Microbial inhibition of oral epithelial wound recovery: potential role for quorum sensing molecules? AMB Express. 2015;5:27.
228. Vanhoecke B, De Ryck T, Stringer A, Van de Wiele T, Keefe D. Microbiota and their role in the pathogenesis of oral mucositis. Oral Dis. 2015;21:17–30.
229. Vanhoecke BW, De Ryck TR, De Boel K, Wiles S, Boterberg T, Van de Wiele T, Swift S. Low-dose irradiation affects the functional behavior of oral microbiota in the context of mucositis. Exp Biol Med. 2016;241:60–70.
230. Andreyev J. Gastrointestinal symptoms after pelvic radiotherapy: a new understanding to improve management of symptomatic patients. Lancet Oncol. 2007;8:1007–17.
231. Hauer-Jensen M, Denham JW, Andreyev HJ. Radiation enteropathy—pathogenesis, treatment and prevention. Nat Rev Gastroenterol Hepatol. 2014;11:470–9.
232. Dandin O, Akin ML, Balta AZ, Yucel E, Karakas DO, Demirbas S, Ozdemir S, Haholu A. The efficacy of probiotic (lactobacillus rhamnosus gg) and 5-asa (aminosalicylic acid) in the treatment of experimental radiation proctitis in rats. Indian J Surg. 2015;77:563–9.
233. Ki Y, Kim W, Cho H, Ahn K, Choi Y, Kim D. The effect of probiotics for preventing radiation-induced morphological changes in intestinal mucosa of rats. J Korean Med Sci. 2014;29: 1372–8.
234. Demers M, Dagnault A, Desjardins J. A randomized double-blind controlled trial: impact of probiotics on diarrhea in patients treated with pelvic radiation. Clin Nutr. 2014;33:761–7.
235. Chitapanarux I, Chitapanarux T, Traisathit P, Kudumpee S, Tharavichitkul E, Lorvidhaya V. Randomized controlled trial of live lactobacillus acidophilus plus bifidobacterium bifidum in prophylaxis of diarrhea during radiotherapy in cervical cancer patients. Radiat Oncol. 2010;5:31.

2. Review of the literature: Expanding the therapeutic index of radiation-therapy by normal tissue protection.

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EXPANDING THE THERAPEUTIC INDEX OF RADIATION-THERAPY BY NORMAL TISSUE PROTECTION.

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PUSHING THE FRONTIERS OF RADIOBIOLOGY: A SPECIAL FEATURE IN MEMORY OF SIR OLIVER SCOTT AND PROFESSOR JACK FOWLER: REVIEW ARTICLE

Expanding the therapeutic index of radiation therapy by normal tissue protection

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ABSTRACT

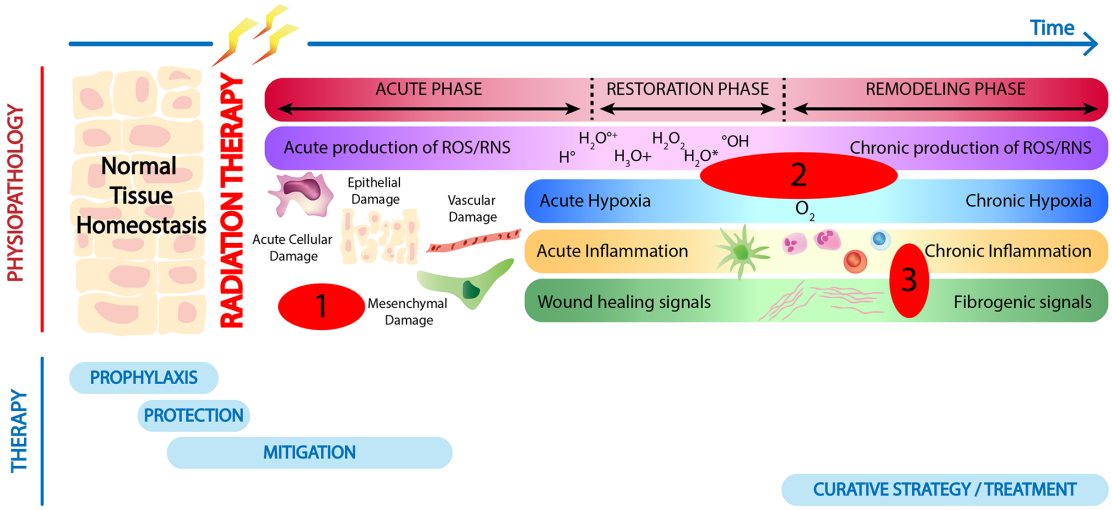
Normal tissue damages induced by radiation therapy remain dose-limiting factors in radiation oncology and this is still true despite recent advances in treatment planning and delivery of image-guided radiation therapy. Additionally, as the number of long-term cancer survivors increases, unacceptable complications emerge and dramatically reduce the patients' quality of life. This means that patients and clinicians expect discovery of new options for the therapeutic management of radiation-induced complications. Over the past four decades, research has enhanced our understanding of the pathophysiological, cellular and molecular processes governing normal tissue toxicity. Those processes are complex and involve the cross-talk between the various cells of a tissue, including fibroblasts, endothelial, immune and epithelial cells as well as soluble paracrine factors including growth factors and proteases. We will review the translatable pharmacological approaches that have been developed to prevent, mitigate, or reverse radiation injuries based upon the targeting of cellular and signalling pathways. We will summarize the different steps of the research strategy, from the definition of initial biological hypotheses to preclinical studies and clinical translation. We will also see how novel research and therapeutic hypotheses emerge along the way as well as briefly highlight innovative approaches based upon novel radiotherapy delivery procedures.

INTRODUCTION

Cancer causes 8.2 million deaths each year globally. Today, most of the anti-cancer therapeutic improvements are achieved by combined treatment modalities, in which radiation therapy remains a cornerstone and is delivered with curative intent in 50% of cancer patients. During the past 20 years, ballistics and imaging improvements have enabled the individualized treatment of patients with a precise and conformal delivery of the dose to the tumor. These technological improvements have greatly enhanced the irradiation therapeutic index. Nevertheless, the level of dose that can be delivered, and accordingly, the possibility of achieving local control over the tumor, is still limited by the toxicity induced to normal (surrounding) tissues. In addition, the combined treatment protocols currently used against cancer are associated with an enhanced risk of toxicity. As the number of cancer survivors increase, preventing and reducing the treatment's side effects is a priority. The present review provides a list of validated strategies as exhaustive as possible that have been validated to prevent, mitigate, or reverse radiation-induced toxicity in preclinical and clinical studies, together with several new options provided by novel types of radiation therapy.

Cancer causes 8.2 million deaths each year globally. Today, most of the anticancer therapeutic improvements are achieved by combined treatment modalities, in which radiation therapy remains a cornerstone and is delivered with curative intent in 50% of cancer patients. During the past twenty years, ballistics and imaging improvements have enabled the individualised treatment of patients with a precise and conformal delivery of the dose to the tumor. These technological improvements have greatly enhanced the irradiation therapeutic index. Nevertheless, the level of dose that can be delivered, and, accordingly, the possibility of achieving local control over the tumour, is still limited by the toxicity induced to normal (surrounding) tissues.

Figure 1. Biology-driven strategy to identify therapeutic approaches. RNS, reactive nitrogen species; ROS, reactive oxygen species.



1- Early inhibition of radiation-induced acute cell death and depletion is presented in part 1.
2- Regulation of the redox imbalance is presented in part 2 .
3- Regulation of inflammatory and fibrogenic signals are presented in part 3.

In addition, the combined treatment protocols currently used against cancer are associated with an enhanced risk of toxicity. As the number of cancer survivors increase, preventing and reducing the treatment's side effects is a priority. The present review provides a list of validated strategies as exhaustive as possible that have been validated to prevent, mitigate, or reverse radiation-induced toxicity in preclinical and clinical studies, together with several new options provided by novel types of radiation therapy.

POST-RADIOTHERAPEUTIC NORMAL TISSUE INJURY

Typical side effects are systemic in the case of drug therapies, whereas radiation-induced normal tissue damages are local or locoregional and can be divided into early and late side-effects. Typically, in the clinic, early effects occur during the time-course of the treatment or within a few weeks of the completion of a fractionated radiotherapy schedule. These effects include skin erythema, dry or moist desquamation of the skin, mucositis, nausea, diarrhea, edema or headaches. Late effects are expressed after latent periods of months to years, and include radiation-induced fibrosis, atrophy, and vascular damage (Figure 1). Likewise, in pre-clinical models, radiation-induced normal tissue injury can be dichotomized into an acute inflammatory phase followed by a late chronic phase characterized by both chronic inflammation and fibrosis. The complications varies from undetectable to highly disabling levels for the patient, inducing a loss of function of the altered organ[1] depending upon extrinsic factors such as variations in the dose delivered, changes in the treatment volume or dose fractionation as well as intrinsic factors such as individual radiation sensitivity and the presence of comorbidity factors.[2–5]

In order to reduce toxicities, it is important to understand the mechanisms underlying the radiation injury of normal tissues. While cell death is the primary and intended effect of ionizing radiation on tumor cells, the deleterious effects of irradiation on normal tissues comprise a cascade of molecular, cellular, and tissue events that spans for a long time after exposure,[6] and has been compared to a "complex wound".[7] However, whereas wound repair has three distinct stages that include a clotting/coagulation phase, a restoration phase with fibroblast migration/proliferation, and a final remodeling phase where the normal tissue architecture is restored (Figure 1), this cascade of finely-tuned processes is disrupted post-radiation therapy. Tissue response to irradiation depends upon the intrinsic sensitivity of the various cellular compartments (direct cell death) that compose the organ and the complex crosstalk established in between all these compartments (indirect functional effects). For instance, rapid renewing compartments, like the epithelial layers and bone marrow, show an acute response to irradiation. This is due to a severe depletion of actively dividing upstream progenitor compartments, ultimately resulting in a loss of replenishment of downstream mature cell pools.[8,9]

In contrast, in connective tissues where cellular turnover is low, radiation injury may be expressed months or even years after exposure if cell death occurs when the cellular division is attempted. The functional consequences are a result of non-lethal effects on different intra- and extracellular molecules and

changes in gene expression in irradiated cells, synthesis of paracrine factors leading, *e.g.* to direct inactivation of anticoagulant molecules, activation of latent growth factors such as transforming growth factor, TGFβ_1, and activation of proteases. The various steps involved in this cascade can constitute valuable therapeutic targets to modify normal tissue injury and enhance the outcome of radiation therapy. In the following sections of this review, we will focus our attention on selected targets, explain their pathophysiological relevance and report the therapeutic strategies that have been used over the past decades. We also discuss how the selection of the timing of administration depends upon the pathway targeted and precludes the selection of the appropriate drug.

Treatment of normal tissue injury- terminology

Before reviewing available treatment options developed to modulate radiation-induced toxicity, it is important to precisely define the possible types of intervention that depend on the time of administration relative to the time of radiation exposure and the appearance of symptoms.[6] Prophylactic agents or protectors are administered before radiation exposure, mitigators are administered shortly after exposure, but before symptoms arise, while curative treatments are given after the appearance of symptoms as shown in Figure 1.

INHIBITING RADIATION-INDUCED ACUTE CELL DEATH AND DEPLETION

To prevent and inhibit radiation-induced toxicity, the early prevention of epithelial, endothelial, and stem cell deaths is one of the primary options that can be proposed. These interventions imply the administration of drugs delivered prophylactically (Table 1) including inhibitors of pro-apoptotic molecules, such as the transient blockade of p53 (Pifithrin);[10,11] the inhibition of p-53 upregulated modulator of apoptosis (Puma Inhibitors) or the inhibition of the ceramide pathway in endothelial cells.[16,17] Alternatively, stimulation of antiapoptotic molecules such as NF-κB by the flagellin-derivative, CBLB502[18,19] protects microvascular endothelial cells from radiation-induced death. Similarly, the enhancement of endothelial cell radiation resistance can be achieved by blocking the TSP1/CD47 pathway.[20] Other cytoprotective therapies such as keratinocyte growth factor, fibroblast growth factor and glucagon-like peptide-2 treatment[15,21,22] also have proven efficacy, and prevent the development of normal tissue toxicity. For instance, keratinocyte growth factor administration has been validated and shown to stimulate cell proliferation and promote epithelial cell survival along with the differentiation of oral mucosa, both in pre-clinical and clinical trials.[23,24] Furthermore, it displayed a beneficial off-target effect by decreasing reactiveoxygen species (ROS) levels and stimulating DNA repair.[23,24]

These strategies prevent the alteration of an organ's structure and function, and/or enable the rapid restoration of the tissue, markedly when stem cells are protected and activated. Activation of deleterious cascades will be interrupted, avoiding the excessive release of inflammatory mediators and dramatic tissue injury. However, two major drawbacks are associated with these strategies: primarily, there is a high risk of tumor protection and secondly, the acute rescue of heavily damaged cells could have a delayed detrimental impact on normal tissue structure and function, including the occurrence of a secondary cancers.

RESTORING THE REDOX EQUILIBRIUM IN TISSUE AFTER RADIOTHERAPY

Another important strategy that has been extensively explored to counteract radiation injury involves antioxidant molecules and scavengers aiming at restoring the redox equilibrium of the tissue, immediately after irradiation or at later time points (Table 2).

Direct interactions of ionizing radiation with biological matter induces excitations and ionizations resulting in the ejection of electrons from biomolecules. In addition, indirect interactions occur through ionization of the water and represent the major part of the radiation's effects on the biological matter. Both effects lead to free radical formation. In addition to this rapid burst of free radicals that occurs immediately following radiation, persistent and prolonged increase in reactive oxygen species/reactive nitrogen species (ROS/RNS) is also observed after irradiation. While ROS/RNS in physiological conditions do perform useful functions such as cell proliferation and differentiation[41,42] and are involved in homeostatis processes such as wound healing,[43] when ROS production escalates beyond a certain threshold and becomes persistent, the antioxidant response is not sufficient to reset the system to the original level of redox homeostasis. These high levels of ROS/RNS result in pathological stress to tissues and cells[2,44] by acting as messenger molecules in cytoplasmic signalling pathways, and by direct effects on transcription.[45] These elevated concentrations of ROS/RNS not only cause DNA damage, but also alter proteins, lipids, carbohydrates, and complex molecules.

At acute time point post-irradiation, early hypoperfusion occurs due to vascular changes, endothelial cell damages[16,46] and escalated oxygen consumption, a consequence of increased cellular metabolism. It generates tissue hypoxia, which further exacerbates the injury.[47] Then, as time passes, the system may still reach an equilibrium associated with higher ROS concentrations called chronic oxidative stress,[48] which does not really involve a loss of homeostasis but rather a chronic shift in the level of homeostasis. The high level of ROS/RNS are immediate activators of the fibrogenic signals including transcriptional activation of one of the most potent fibrogenic growth factor TGFβ_1,[49,50] that subsequently upregulates collagen synthesis and perpetuates self-induction and autocrine induction of another potent fibrogenic growth factor, connective tissue growth factor (CTGF).[51] Activation of inflammation mediators has also been described in the case of a high ROS level status, leading to deleterious chronic inflammation in the irradiated tissue. These observations support the fact that radiation-induced late effects are partially propelled by a chronic oxidative stress[48] induced at late stages by the redox imbalance that occurs in the tissue as a result of intrinsic hypoxia[52] which further enhance the redox imbalance.

Given the central and persistent role played by the loss of redox equilibrium in the tissue response to irradiation, targeting redox

Table 1. Therapeutic strategies that inhibit acute cell death and depletion

Substance	Intervention/ administration route	Function	Pre-clinical results/ clinical use	References
Pifithrin-α pifithrin-μ	Prophylaxis/protection Oral application of drugs	Inhibition of p53-induced apoptosis	Radioprophylaxis/protection in mice after total body irradiation	10,11
KGF-1 (palifermin), KGF-2 (repifermin), FGF-20 (velafermin)	Prophylaxis/protection Oral application of recombinant proteins	Suppression of apoptosis	Reduced mucositis by KGF-1 in patients after whole body irradiation, failure of KGF-2 and FGF-20 in clinical studies	10
Synthetic triterpenoids (bardoxolone methyl (BARD) and 2-cyano-3,12-dioxooleana-1,9 (11)-dien-28-oic acid-ethylamide)	Prophylaxis/protection Oral application	Reduced apoptosis by activation of transcription factor NRF2	Radioprophylaxis/protection in mice after total body irradiation	10,12
PUMA inhibitors	Prophylaxis/protection Lentiviral vector	Suppression of apoptosis by inhibition of PUMA	Radioprophylaxis/protection *in vitro* in germ cell tumor line NCCIT, murine hematopoietic progenitor cell line 32D cl 3, and human lymphoblast cell line TK6	10,13
Recombinant MDR1	Prophylaxis/Protection Lentiviral vector	Suppression of apoptosis	Radioprophylaxis/protection *in vitro* in human hematopoietic stem cells	10,14
CBLB502/entolimod	Prophylaxis/protection Oral application	Reduced apoptosis by activation of transcription factor NF-κB	Radioprophylaxis/protection in mice and rhesus macaques; clinical study terminated by financial sponsor	10
GLP-2	Prophylaxis/protection	Trophic factor	RIF gut in rats	15

FGF, fibroblast growth factor; GLP, glucagon-like peptide; KGF, keratinocyte growth factor; MDR, multiple drug resistance; NRF, nuclearre spiratory factor; PUMA, p-53 upregulated modulator of apoptosis; RIF, radiation-induced fibrosis.

Table 2. Therapeutic strategies that prevent, mitigate, and reverse normal tissue injury by modulation of the redox equilibrium

Substance	Intervention/ administration route	Mechanism	Pre-clinical results/clinical use	References
Amifostine	Prophylaxis/protection Oral application of prodrug, mainly activated in normal cells	Scavenger of free radicals	Prophylaxis/protection against xerostomia during radiotherapy of head and neck cancer	25–27
Curcumin, ellagic acid, and bixin	Prophylaxis/protection, mitigation	ROS scavenger	RIF lung in rats and mice	28
Dietary flaxseed	Prophylaxis/protection, mitigation	ROS scavenger	RIF lung in rats and mice	29,30
Glutathione (GSH)	Prophylaxis/protection Oral application of GSH esters or reduced GSH	Scavenger of free radicals (hydroxyl)	Conflicting results in animal models regarding radioprotective effects	10
Genistein	Mitigation	ROS scavenger	RIF lung in rats	31,32
Soy Isoflavone (83.3% genistein, 14.6% daidzein and 0.26% glycitein)	Mitigation	ROS scavenger	RIF lung in mice	33
SOD therapy 1. Recombinant protein 2. Gene therapy 3. SOD mimetics AEOL10150, Eukarion compounds, JP4–039	Mitigation treatment	Detoxification of superoxide	1. Clinical trials recombinant protein not available anymore 2. Radioprophylaxis/protection by viral delivery of SOD2 in preclinical models; validation of plasmid delivery of SOD2 in clinical studies (suspended) 3. RIF lung in rats	5,31,34,35
Pentoxyfilline + vitamin E+/ clodronatePentoxyfilline +γ-tocotrienol	Treatment	Antioxidants, improves blood flow, anti-inflammatory, TNF-α and TGF-β1 inhibition	Clinical evidence but lack of randomized trialClinical trials starting (*e.g.* NCT02230800)	36–40

RIF, radiation-induced fibrosis; ROS, reactive oxygen species; SOD, super oxide dismutase; TGF, transforming growth factor; TNF, tumor necrosis factor.

imbalance, ROS/RNS, and hypoxia is an obvious therapeutic option with applications during any of the steps of the cascade as shown in Table 2. Treatment with hyperbaric oxygen (HBO)[53] and antioxidant therapy[54–56] were both successfully used despite their apparent antagonistic mechanism of action. HBO induces transient tissue hyperoxia (typically ~2 h day^{-1}) that should not overcome natural antioxidant defenses,[57] but may help to remobilize tissue remodelling by activating signaling molecules in transduction cascades (see the review)[58] and stimulate angiogenesis.[59] In addition, the results from recent clinical trial show no benefice of HBO on lymphoedema.[60,61] Antioxidant therapies are based on a different mode of action and aim at scavenging ROS. Initial studies with amifostine[25–27] and bovine liposomal Cu/Zn superoxide dismutase showed antifibrotic efficacy associated with TGFβ inhibition.[62] More recent trials investigated the benefits of tocol isoforms (Vitamin E analogs) such as high-dose alpha-tocopherol combined with pentoxifylline and clodronate,[36,37] and γ-tocotrienol (GT3).[39] In addition to their antioxidant action, both strategies have displayed off-target benefits with protective endothelial activity[63,64] and miRNA regulation.[65] Interestingly, the efficacy of GT3 is enhanced when combined with pentoxifylline.[66] Lastly, hypoxia-regulating molecules such as 2-methoxyestradiol have been shown to downregulate HIF1α-mediated Smad activation and inhibit radiation-induced lung fibrosis in mice.[67]

TARGETING INFLAMMATORY AND FIBROGENIC SIGNALS INDUCED BY RADIOTHERAPY

The third type of strategy is based upon the understanding of the pathophysiological processes (cellular and molecular) governing normal tissue toxicity. This knowledge has provided us with tools to improve the therapeutic ratio of radiation therapy, and biology-driven efforts have enabled the development of translatable therapeutic approaches to prevent, mitigate, and even reverse radiation injury based upon the targeting of signalling pathways. The relevance of these various signalling pathways to the pathogenesis and maintenance of radiation injury has been extensively and recently reviewed in several articles.[40,51,68,69] Therefore, we will focus on recent results that highlight the relevance of immune cells in response to irradiation. Elucidating the impact of radiotherapy on the immune compartment and subsequent immunomodulation is nowadays one of the most promising strategies for improving anticancer treatment,[70] and recent studies suggest that it may also enhance the differential effect of radiotherapy.

The importance of myeloid cells in the radiation-induced response has been proposed and the role of macrophage reprogramming by radiotherapy has been demonstrated.[71–73] Macrophage phenotypes are highly dependent upon the

microenvironment and recent publications have revealed their complexity.[74] In fibrotic tissue, macrophages do display immunosuppressive properties, secrete large amounts of the fibrogenic mediator TGF-β1[75] that activates the Smad pathway, and stimulate downstream fibrogenic genes such as CTGF and PAI-1.[76] The macrophages isolated from bronchoalveolar fluid from patients undergoing thoracic irradiation spontaneously released platelet-derived growth factor, another important fibrogenic growth factor.[77] Several older studies have suggested possible benefits of macrophage depletion using clodronate liposomes.[78] The reduction of the number of macrophages by clodronate in wounded tissue indeed reduced excessive scar formation and delayed cutaneous wound healing.[79] Froom and colleagues[80] showed that the oral administration of clodronate (bisphosphonate) significantly reduced bone marrow fibrosis, and in the early 2000's Delanian and Lefaix successfully administered clodronate in combination with a pentoxifylline–vitamin E treatment, and showed improved efficacy in the treatment of radiation-induced fibronecrosis.[81,82]

More recent data validate and refine this strategy bringing molecular highlights and a biological rationale for macrophage targeting in the management of radiation-induced normal tissue complications.[83,84] Recently, the P Huber group showed that blocking CTGF with a specific antibody (FG-3019) was able to attenuate radiation-induced pulmonary remodeling and reverse fibrosis. Interestingly, they showed that this treatment was associated with the abrogation of M2-like macrophages influx.[83] We extended these findings and recently characterized the contribution of pulmonary macrophages to radiation-induced pulmonary fibrosis.[84] We showed that the populations of pulmonary macrophages are heterogeneous and their contribution to fibrosis is complex. A differential phenotype for alveolar and interstitial macrophages was indeed shown along with a specific fibrogenic contribution of interstitial macrophages but not alveolar macrophages. Ultimately, selective targeting of interstitial macrophages with CSF1R mAb was shown to display antifibrotic action.

Lastly, an overview of the drugs that have been used to prevent and mitigate radiation damages as well as the drugs that have been successfully used to reverse radiation-induced complications, fibrosis in particular, are provided in Tables 3 and 4. The impressive list of compounds shows the vitality of the research in this field with an impressive rate of translational/clinical studies (Table 4) using curative strategies. Three parameters have probably fostered this progress: first, the irreversibility of fibrosis was challenged by many cellular

Table 3. Therapeutic strategies that prevent and mitigate normal tissue injury by modulation of inflammatory and fibrogenic signals

Substance	Intervention/administration route	Mechanisms	Pre-clinical results/clinical use	References
Ambroxol	Prophylaxis/protection	TNF-α and TGF-β1 inhibition	Clinical trial	85
Taurine	Prophylaxis/protection	TGF-β1 and collagen inhibition	RIF lung in mice	86,87
IL-11 (targeted administration)	Prophylaxis/protection	TGF-β1 and collagen inhibition	RIF gut in mice	88
Hirudine	Prophylaxis/protection	Thrombine inhibition	RIF gut in rats	89
Halofunginone	Prophylaxis/protection	TGF-β1 inhibition	RIF skin in mice	90
Octreotide	Prophylaxis/protection	Somatostatin analogue	RIF gut in rats	91
Soluble TGF-β type II receptor	Prophylaxis/protection	TGF-β1 inhibition	RIF gut in mice	92
ACE inhibitors angiotensin II blockers	Prophylaxis/protection and mitigation	Angiotensin II modulator and inhibition of TGF-β	Clinical trial	93
Methylprednisolone, dexamethasone, ibuprofen)	Prophylaxis/protection and mitigation	Anti-inflammatory	RIF kidney and heart in rats and rabbits	94–96
Gefinitinib	Mitigation	EGFR inhibition-TKi	RIF lung in rats nhances inflammation but decreases fibrosis	97
LY2109761	Mitigation	TGF-βR1 inhibition-S/TKi	RIF lung in mice Reduces inflammation and fibrosis	98
Chitosan/DsiRNA targeting TNF-α	Mitigation	TNF-α inhibition	RIF subcutaneous in mice	99

ACE, angiotensin converting enzyme; EGFR, epidermal growth factor; RIF, radiation-induced fibrosis; TGF, transforming growth factor;TKi, tyrosine kinase inhibitor; TNF, tumor necrosis factor.

Table 4. Therapeutic strategies that reverse normal tissue injury by modulation of inflammatory and fibrogenic signals

Substance	Intervention/administration route	Mechanisms	Pre-clinical results/ clinical use	References
All –trans-retinoic acid	Prophylaxis/Protection and treatment	TGF-β1, IL-6 and collagen inhibition	RIF lung and gut in mice	100,101
Angelica sinensis	Prophylaxis/Protection and treatment	TGF-β1 inhibition	RIF lung in mice	102
Antibody against CTGF	Prophylaxis/protection and treatment	CTGF inhibition and macrophages depletion	RIF lung in mice	83
CSFR1 inhibition	Treatment	Macrophages depletion	RIF lung in mice	84
Interferon gamma (low dose)	Treatment	Collagen production inhibition	Small Clinical trial	103
Pirfenidone	Treatment	TGF-β1, PDGF, b-FGF, EGF, TNF-α inhibition	Clinical trial open	104
Heparine and Wwarfarine	Treatment	Anticoagulant	Small clinical trial open	105
Colchicine	Treatment	Collagen production inhibition	Clinical trial	106
Statins	Treatment	Vascular protector, anti-inflammatory, TGF-β1 inhibition	Clinical evidence but lack of randomized trial	93,107
Pentoxyfilline + vitamin E+/- clodronatePentoxyfilline +γ-tocotrienol	Treatment	Antioxidants, improves blood flow, anti-inflammatory, TNF-α and TGF-β1 inhibition Macrophages depletion	Clinical evidence but no randomized trialClinical trials starting(e.g., NCT02230800)	36–40

CSF, colony stimulating factor; CTGF, connective tissue growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; RIF, radiation-induced fibrosis; TGF, transforming growth factor; TNF, tumor necrosis factor.

and experimental studies; second, curative strategies are clinically interesting as they do not interfere with anticancer treatments through possible tumor protection; third, they can be delivered to the targeted population of patients that need it.

USE OF NOVEL RT APPROACHES TO PROTECT NORMAL TISSUES

Aside from novel biological interventions, improvements in physics have been critical for protecting normal tissue, enhancing differential effects, and making progress on tumor control. Novel technologies based upon sophisticated instrumentation used in conjunction with imaging, such as stereotactic body radiotherapy, have helped to protect normal tissue by reducing the irradiated volume and more accurately targeting the tumor. More complex and expensive technologies, such as proton and carbon ion therapies, take advantage of their specific pattern of energy deposition in the biological matter and are already implemented in clinical practice. Other novel ideas and approaches have also been recently proposed such as dose rate escalation.[108,109] This last approach should be translatable into clinical application soon, which illustrates the broad range of opportunities that exist in radiation therapy, and highlight the need for interdisciplinary working teams composed of biologists, physicists, and physicians.

IGRT and Stereotactic body radiotherapy

Image-guided radiation therapy (IGRT) has been a major advancement in radiotherapy and makes it possible to visualize a target volume (tumor + margins) and the surrounding organs at risk before treatment and during the treatment course. Modifications in the targeted volume as well as movements are taken into consideration, and can be compensated for and even halted in the case of percussion-assisted RT (PART) recently described by Péguret et al.[110] This innovative technique induces a long-lasting apnea-like suppression of respiratory motion for up to 10–11 min without inducing any physiological side effect.[111] The pilot clinical study reported an interesting advantage of percussion-assisted RT compared to free-breathing or maximal-inspiration techniques coupled with three-dimensional conformal RT, SBRT or VMAT irradiations, in breast cancer, lung cancer, and lung metastases patients. IGRT combined with motion management is associated with the prescription of highly conformational doses (intensity modulatedradiotherapy, IMRT) which are meant to drastically decrease the irradiated volume and the dose delivered to the normal tissue. Different techniques may be used to follow the anatomical structures, with or without the addition of fiducial markers, starting from fluoroscopy, to CT, MRI, PET-CT, ultrasounds or optical tracking.

Studies have shown an improvement in toxicity prevention on several tumor sites[112] and especially in prostate cancer patients,[113] even if these results can sometimes be controversial.[114] It is difficult, however, to differentiate the IGRT from the IMRT effects,

and several studies have exposed this limitation[115,116] through their explanation of the reduced occurrence of xerostomia due to PTV margin reductions.

Proton therapy

Ballistic features of protons prevent normal tissue toxicities

Due to the protons' in-depth dose deposition curves, one can expect to prevent normal tissue toxicity and therefore, decrease normal tissue injury. By now, more than 100,000 patients have been treated worldwide with proton beam therapy (PBT) in approximately 20 centers. This means that evaluating PBT superiority in terms of normal tissue protection is now feasible.[117]

Proton therapy has been largely used to treat ocular tumors such as uveal carcinoma, making it an alternative to enucleation or ocular brachytherapy thus sparing visual acuity.[118–121] Concerning skull base tumor treatments, different Swiss and American studies have shown that the percentage of patients treated with PBT and suffering from temporal lobe injury is reduced compared to conventional X-Ray radiation therapy and IMRT.[122–124]

In pediatrics, the large volume of tissue exposed to low-doses induces severe and non-acceptable long-term injuries such as neurocognitive impairments, hearing loss, hormonal dysfunctions, infertility and secondary malignancies. Therefore, PBT seems to be ideal for treating pediatric patients. Dosimetry reconstruction comparing X-Rays, IMRT and PBT treatment plans show an improved dose distribution[125] with PBT which, according to modeling, could be responsible for at least a 2-fold, and up to a 15-fold reduction in secondary malignancies because of normal tissue irradiation.[126] Interestingly, a Chinese study estimated that IMRT is the technique that displays the highest risk of secondary malignancy (30%), while PBT was linked to only a 4% risk of developing a radio-induced cancer.[127]

PBT has also shown encouraging results concerning the neurocognitive toxicity of whole and partial brain irradiation in children. The reduction of the irradiated normal brain volume enables a drastic reduction of the deleterious effects in the early cognitive outcome 1 year post-RT compared to photon therapy. These results were observed for IQ, verbal comprehension, and working memory.[128]

Lung cancer treatments with PBT show a possibility of increasing dose and subsequent anti-tumor efficacy with reduced adverse effects—especially dermatitis, esophagitis, and pneumonitis—compared to classical X-ray treatments. For breast cancer treatments, PBT plans decrease by 71–81% and 75–99% of lung and heart irradiation respectively. Moreover, dose to the contralateral breast and dose to the whole body were also drastically reduced.[129–134] All of these results suggest a decrease in radio-induced lung dysfunctions and cardiopathies, but also for the occurrence of secondary malignancies, showing that proton therapy, by decreasing dose to the normal tissue, reduces the occurrence of radiation-induced injury, and can improve the therapeutic ratio of radiation therapy.

Carbon ion therapy

Increase the relative biological effectiveness and the therapeutic ratio

The efficacy of high linear energy transfer (LET) particles in radiation therapy has been investigated since the late 70s.[135] The first clinical trial was conducted in Japan in 1994,[136] and since then, more than 13,000 patients have been treated with carbon ion radiation therapy.[137] Carbon ions display the same pattern of dose deposition as protons, with a Bragg peak deposition of the dose, low entry and exit doses as well as a fall-off after the Bragg peak that is significantly steeper compared to protons. The dose deposited beyond the Bragg peak is higher compared to protons because of the nuclear interaction and fragmentation. One other additional interesting property of Carbon ions is their high LET, which is directly linked to their RBE that ranges between 2.0 and 3.5. The high RBE is certainly beneficial for tumor control, but can be detrimental to the normal tissue and therefore increase complications. Still, exploring the differential effects of Carbon ions is of great interest, and pioneering work performed more than 20 years ago by the Denekamp team has shown that reducing the number of fractions lowers the RBE for both normal tissue but not for the tumor.[138] Hypofractionation in carbon ion radiation therapy would increase the therapeutic ratio, and provides a strong biological rationale for Carbon ion use.[138–142]

FLASH-RT

Opportunities to improve the efficacy of radiation therapy via the development of new irradiation techniques may have been under explored. Today, modern radiation therapy devices still use the same technology of electron acceleration in waveguides as half a century ago. However, the recent development of proton therapy facilities and the use of high LET ions exemplify some of the possibilities that are currently opened. Previous experiments conducted with short pulses of X-rays on lymphocytes,[143] or more recently, conducted with protons on human–hamster hybrid cells and skin cells,[144–146] including microchannel radiotherapy that operates at 200 $Gy.s^{-1}$ dose-rate,[147] showed fewer cytogenetic damages and significantly protected normal tissue from radiation-induced acute and long-term damages.

In line with these experiments and with the objective of fostering innovation in radiation therapy, we have been the first to propose a completely novel modality of irradiation, named FLASH radiotherapy. It markedly increases the differential effect between tumors and normal tissues, and is able to destroy tumors with the same efficiency while providing better protection to the normal tissues and preventing side effects. Indeed, using several pre-clinical models,[108,109] we have shown that an ultrahigh dose rate delivery of irradiation was able to protect normal tissues in mice (lung, brain, gut and skin), in pigs (skin), and in a clinical trial performed in cats bearing spontaneous cancers for whom a major protection of normal tissues was observed, while maintaining a strong anti-tumor efficacy[108] (Vozenin et al, under revision; Montay-Gruel et al. in prep This effect has been called the Flash effect. The Flash effect has been confirmed by another team from Stanford University (USA)[148] and we found similar observations reported more than 40 years ago.[149,150] In addition to this radiobiological advantage, the ultrashort duration

of dose deposition overcomes the potential problems associated with tumor motion, and can then enhance RT delivery accuracy. Currently, only a few devices are able to deliver an ultrahigh dose rate irradiation across a large volume of tissue; experimental electron Linacs of 4/6 MeV have limited therapeutic applications due to their energy profile and limited in-depth dose deposition (Kinetron, Orsay, Fr, PMB-Alcen, Pegnier, France; Oriatron, Lausanne, CH, PMB-Alcen, Pegnier, France; modified clinical accelerator, Stanford, USA).[109,151] However, several technological improvements are ongoing to upgrade those systems and develop clinical transfer.[152,153]

CONCLUSION

The selective protection of normal tissue function using modern targeted radiotherapy is a powerful approach to improve cure rates and simultaneously enhance the quality of life of long-term cancer survivors. Nowadays, high precision radiation therapy induces a drop in the rate of complications. In parallel, advancements in radiobiology have deciphered the complexity of the biological response induced by tissue exposure to ionizing radiation, and enabled the identification of therapeutic targets. These processes include profound microenvironment remodeling with alteration of the vascularization, perfusion/hypoxia, inflammation, modulation of immune compartments and stromal remodeling. Therefore, currently, well-selected combination strategies that target distinct pathogenic pathways induced by irradiation at specific time points of the pathogenic process can be proposed, and the next challenge will be to develop rational radiotherapy–drug combinations to maximize the therapeutic impact. The management of RT complication has also reach the era of personalized medicine and in many centers in Europe (France, UK, CH for instance), patients presenting with complications are managed in the frame of multidisciplinary consultations to adapt the best therapeutic answer for each specific situation. Simultaneously, novel radiotherapy approaches such as the ultra-high dose rate, Flash RT, have been developed, offering the potential to radically change the way radiation therapy is employed and delivered over the next few years.

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REFERENCES

1. Bentzen SM. Preventing or reducing late side effects of radiation therapy: radiobiology meets molecular pathology. *Nat Rev Cancer* 2006; **6**: 702–13. doi: https://doi.org/10.1038/nrc1950
2. Delanian S, Lefaix J-L. The radiation-induced fibroatrophic process: therapeutic perspective via the antioxidant pathway. *Radiother Oncol* 2004; **73**: 119–31. doi: https://doi.org/10.1016/j.radonc.2004.08.021
3. Alsner J, Andreassen CN, Overgaard J. Genetic markers for prediction of normal tissue toxicity after radiotherapy. *Semin Radiat Oncol* 2008; **18**: 126–35. doi: https://doi.org/10.1016/j.semradonc.2007.10.004
4. Begg AC, Stewart FA, Vens C. Strategies to improve radiotherapy with targeted drugs. *Nat Rev Cancer* 2011; **11**: 239–53. doi: https://doi.org/10.1038/nrc3007
5. Bourgier C, Levy A, Vozenin M-C, Deutsch E. Pharmacological strategies to spare normal tissues from radiation damage: useless or overlooked therapeutics? *Cancer Metastasis Rev* 2012; **31**: 699–712. doi: https://doi.org/10.1007/s10555-012-9381-9
6. Denham JW, Hauer-Jensen M, Peters LJ. Is it time for a new formalism to categorize normal tissue radiation injury? *Int J Radiat Oncol Biol Phys* 2001; **50**: 1105–6. doi: https://doi.org/10.1016/S0360-3016(01)01556-5
7. Denham JW, Hauer-Jensen M. The radiotherapeutic injury – a complex 'wound'. *Radiother Oncol* 2002; **63**: 129–45. doi: https://doi.org/10.1016/S0167-8140(02)00060-9
8. Hall EJ. *Radiobiology for the radiobiologist*. Fifth Edition. Philadelphia: Lippincott Williams & Wilkins; 2001.
9. Joiner MCE, Van der Kogel AE. *Basic clinical radiobiology*. 4th edition. London; 2009.
10. Maier P, Wenz F, Herskind C. Radioprotection of normal tissue cells. *Strahlenther Onkol* 2014; **190**: 745–52. doi: https://doi.org/10.1007/s00066-014-0637-x
11. Sinn B, Schulze J, Schroeder G, Konschak R, Freyer D, Budach V, et al. Pifithrin-α as a potential cytoprotective agent in radiotherapy: protection of normal tissue without decreasing therapeutic efficacy in glioma cells. *Radiat Res* 2010; **174**: 601–10. doi: https://doi.org/10.1667/RR2147.1
12. Kim SB, Pandita RK, Eskiocak U, Ly P, Kaisani A, Kumar R, et al. Targeting of Nrf2 induces DNA damage signaling and protects colonic epithelial cells from ionizing radiation. *Proc Natl Acad Sci U S A* 2012; **109**: E2949–E2955. doi: https://doi.org/10.1073/pnas.1207718109
13. Maier P, Herskind C, Barzan D, Zeller WJ, Wenz F. SNAI2 as a novel radioprotector of normal tissue by gene transfer using a lentiviral bicistronic SIN vector. *Radiat Res* 2010; **173**: 612–9. doi: https://doi.org/10.1667/RR1952.1
14. Maier P, Herskind C, Fleckenstein K, Spier I, Laufs S, Zeller WJ, et al. *MDR1* gene transfer using a lentiviral SIN vector confers radioprotection to human CD34 $^{+}$hematopoietic progenitor cells. *Radiat Res* 2008; **169**: 301–10. doi: https://doi.org/10.1667/RR1067.1
15. Torres S, Thim L, Milliat F, Vozenin-Brotons M-C, Olsen UB, Ahnfelt-Rønne I, et al. Glucagon-like peptide-2 improves both acute and late experimental radiation enteritis in the rat. *Int J Radiat Oncol Biol Phys* 2007; **69**: 1563–71. doi: https://doi.org/10.1016/j.ijrobp.2007.08.051
16. Pena LA, Fuks Z, Kolesnick RN. Radiation-induced apoptosis of endothelial cells in the murine central nervous system: protection by fibroblast growth factor and sphingomyelinase deficiency. *Cancer Res* 2000; **60**: 321–7.

17. Rotolo J, Stancevic B, Zhang J, Hua G, Fuller J, Yin X, et al. Anti-ceramide antibody prevents the radiation gastrointestinal syndrome in mice. *J Clin Invest* 2012; **122**: 1786–90. doi: https://doi.org/10.1172/JCI59920
18. Burdelya LG, Krivokrysenko VI, Tallant TC, Strom E, Gleiberman AS, Gupta D, et al. An agonist of toll-like receptor 5 has radioprotective activity in mouse and primate models. *Science* 2008; **320**: 226–30. doi: https://doi.org/10.1126/science.1154986
19. Burdelya LG, Gleiberman AS, Toshkov I, Aygun-Sunar S, Bapardekar M, Manderscheid-Kern P, et al. Toll-like receptor 5 agonist protects mice from dermatitis and oral mucositis caused by local radiation: implications for head-and-neck cancer radiotherapy. *Int J Radiat Oncol Biol Phys* 2012; **83**: 228–34. doi: https://doi.org/10.1016/j.ijrobp.2011.05.055
20. Maxhimer JB, Soto-Pantoja DR, Ridnour LA, Shih HB, Degraff WG, Tsokos M, et al. Radioprotection in normal tissue and delayed tumor growth by blockade of CD47 signaling. *Sci Transl Med* 2009; **13**: ra7. doi: https://doi.org/10.1126/scitranslmed.3000139
21. Booth C, Booth D, Williamson S, Demchyshyn LL, Potten CS, Teduglutide PCS. Teduglutide ([Gly2]GLP-2) protects small intestinal stem cells from radiation damage. *Cell Prolif* 2004; **37**: 385–400. doi: https://doi.org/10.1111/j.1365-2184.2004.00320.x
22. Winczura P, Jassem J. Combined treatment with cytoprotective agents and radiotherapy. *Cancer Treat Rev* 2010; **36**: 268–75. doi: https://doi.org/10.1016/j.ctrv.2009.12.001
23. Dörr W, Heider K, Spekl K. Reduction of oral mucositis by palifermin (rHuKGF): dose-effect of rHuKGF. *Int J Radiat Biol* 2005; **81**: 557–65. doi: https://doi.org/10.1080/09553000500196136
24. Dörr W, Spekl K, Farrell CL. Amelioration of acute oral mucositis by keratinocyte growth factor: fractionated irradiation. *Int J Radiat Oncol Biol Phys* 2002; **54**: 245–51. doi: https://doi.org/10.1016/S0360-3016(02)02918-8
25. Vujaskovic Z, Anscher MS, Feng Q-F, Rabbani ZN, Amin K, Samulski TS, et al. Radiation-induced hypoxia may perpetuate late normal tissue injury. *Int J Radiat Oncol Biol Phys* 2001; **50**: 851–5. doi: https://doi.org/10.1016/S0360-3016(01)01593-0
26. Fleischer G, Dörr W. Amelioration of early radiation effects in oral mucosa (mouse) by intravenous or subcutaneous administration of amifostine. *Strahlenther Onkol* 2006; **182**: 567–75. doi: https://doi.org/10.1007/s00066-006-1587-8
27. Koukourakis MI, Tsoutsou PG, Abatzoglou IM, Sismanidou K, Giatromanolaki A, Sivridis E. Hypofractionated and accelerated radiotherapy with subcutaneous amifostine cytoprotection as short adjuvant regimen after breast-conserving surgery: interim report. *Int J Radiat Oncol Biol Phys* 2009; **74**: 1173–80. doi: https://doi.org/10.1016/j.ijrobp.2008.09.016
28. Lee JC, Kinniry PA, Arguiri E, Serota M, Kanterakis S, Chatterjee S, et al. Dietary curcumin increases antioxidant defenses in lung, ameliorates radiation-induced pulmonary fibrosis, and improves survival in mice. *Radiat Res* 2010; **173**: 590–601. doi: https://doi.org/10.1667/RR1522.1
29. Christofidou-Solomidou M, Tyagi S, Tan K-S, Hagan S, Pietrofesa R, Dukes F, et al. Dietary flaxseed administered post thoracic radiation treatment improves survival and mitigates radiation-induced pneumonopathy in mice. *BMC Cancer* 2011; **11**: 269. doi: https://doi.org/10.1186/1471-2407-11-269
30. Lee JC, Krochak R, Blouin A, Kanterakis S, Chatterjee S, Arguiri E, et al. Dietary flaxseed prevents radiation-induced oxidative lung damage, inflammation and fibrosis in a mouse model of thoracic radiation injury. *Cancer Biol Ther* 2009; **8**: 47–53. doi: https://doi.org/10.4161/cbt.8.1.7092
31. Mahmood J, Jelveh S, Calveley V, Zaidi A, Doctrow SR, Hill RP. Mitigation of lung injury after accidental exposure to radiation. *Radiat Res* 2011; **176**: 770–80. doi: https://doi.org/10.1667/RR2562.1
32. Mahmood J, Jelveh S, Calveley V, Zaidi A, Doctrow SR, Hill RP. Mitigation of radiation-induced lung injury by genistein and EUK-207. *Int J Radiat Biol* 2011; **87**: 889–901. doi: https://doi.org/10.3109/09553002.2011.583315
33. Hillman GG, Singh-Gupta V, Runyan L, Yunker CK, Rakowski JT, Sarkar FH, et al. Soy isoflavones radiosensitize lung cancer while mitigating normal tissue injury. *Radiother Oncol* 2011; **101**: 329–36. doi: https://doi.org/10.1016/j.radonc.2011.10.020
34. Delanian S, Baillet F, Huart J, Lefaix J-L, Maulard C, Housset M. Successful treatment of radiation-induced fibrosis using liposomal Cu/Zn superoxide dismutase: clinical trial. *Radiother Oncol* 1994; **32**: 12–20. doi: https://doi.org/10.1016/0167-8140(94)90444-8
35. Epperly M, Bray J, Kraeger S, Zwacka R, Engelhardt J, Travis E, et al. Prevention of late effects of irradiation lung damage by manganese superoxide dismutase gene therapy. *Gene Ther* 1998; **5**: 196–208. doi: https://doi.org/10.1038/sj.gt.3300580
36. Delanian S, Balla-Mekias S, Lefaix J-L. Striking regression of chronic radiotherapy damage in a clinical trial of combined pentoxifylline and tocopherol. *J Clin Oncol* 1999; **17**: 3283–90. doi: https://doi.org/10.1200/JCO.1999.17.10.3283
37. Boerma M, Roberto KA, Hauer-Jensen M. Prevention and treatment of functional and structural radiation injury in the rat heart by pentoxifylline and alpha-tocopherol. *Int J Radiat Oncol Biol Phys* 2008; **72**: 170–7. doi: https://doi.org/10.1016/j.ijrobp.2008.04.042
38. Delanian S, Lefaix J-L. Current management for late normal tissue injury: radiation-induced fibrosis and necrosis. *Semin Radiat Oncol* 2007; **17**: 99–107. doi: https://doi.org/10.1016/j.semradonc.2006.11.006
39. Berbée M, Fu Q, Boerma M, Wang J, Kumar KS, Hauer-Jensen M. γ-Tocotrienol ameliorates intestinal radiation injury and reduces vascular oxidative stress after total-body irradiation by an HMG-CoA reductase-dependent mechanism. *Radiat Res* 2009; **171**: 596–605. doi: https://doi.org/10.1667/RR1632.1
40. Berbee M, Hauer-Jensen M. Novel drugs to ameliorate gastrointestinal normal tissue radiation toxicity in clinical practice: what is emerging from the laboratory? *Curr Opin Support Palliat Care* 2012; **6**: 54–9.
41. Murrell GAC, Francis MJO, Bromley L. Modulation of fibroblast proliferation by oxygen free radicals. *Biochem J* 1990; **265**: 659–65. doi: https://doi.org/10.1042/bj2650659
42. Allen RG. Oxygen-reactive species and antioxidant responses during development: the metabolic paradox of cellular differentiation. *Proc Soc Exp Biol Med* 1991; **196**: 117–29. doi: https://doi.org/10.3181/00379727-196-43171A
43. Gordillo GM, Sen CK. Revisiting the essential role of oxygen in wound healing. *Am J Surg* 2003; **186**: 259–63. doi: https://doi.org/10.1016/S0002-9610(03)00211-3
44. Andersen JK. Oxidative stress in neurodegeneration: cause or consequence? *Nat Med* 2004; **10 Suppl**: S18–S25. doi: https://doi.org/10.1038/nrn1434
45. Bedard K, Krause K-H. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* 2007; **87**: 245–313. doi: https://doi.org/10.1152/physrev.00044.2005
46. Paris F, Fuks Z, Kang A, Capodieci P, Juan G, Ehleiter D, et al. Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice. *Science* 2001; **293**:

293–7. doi: https://doi.org/10.1126/science.1060191
47. Fleckenstein K, Zgonjanin L, Chen L, Rabbani Z, Jackson IL, Thrasher B, et al. Temporal onset of hypoxia and oxidative stress after pulmonary irradiation. *Int J Radiat Oncol Biol Phys* 2007; **68**: 196–204. doi: https://doi.org/10.1016/j.ijrobp.2006.12.056
48. Zhao W, Diz DI, Robbins ME. Oxidative damage pathways in relation to normal tissue injury. *Br J Radiol* 2007; **80** : S23–S31. doi: https://doi.org/10.1259/bjr/18237646
49. Barcellos-Hoff MH, Dix TA. Redox-mediated activation of latent transforming growth factor-beta 1. *Mol Endocrinol* 1996; **10**: 1077–83.
50. Jobling MF, Mott JD, Finnegan MT, Jurukovski V, Erickson AC, Walian PJ, et al. Isoform-specific activation of latent transforming growth factor β (LTGF-β) by reactive oxygen species. *Radiat Res* 2006; **166**: 839–48. doi: https://doi.org/10.1667/RR0695.1
51. Yarnold J, Vozenin Brotons M-C, Brotons MC. Pathogenetic mechanisms in radiation fibrosis. *Radiother Oncol* 2010; **97**: 149–61. doi: https://doi.org/10.1016/j.radonc.2010.09.002
52. Vujaskovic Z, Feng Q-F, Rabbani ZN, Anscher MS, Samulski TV, Brizel DM. Radioprotection of lungs by amifostine is associated with reduction in profibrogenic cytokine activity. *Radiat Res* 2002; **157**: 656–60. doi: https://doi.org/10.1667/0033-7587(2002)157[0656:ROLBAI]2.0.CO;2
53. Pasquier D, Hoelscher T, Schmutz J, Dische S, Mathieu D, Baumann M, et al. Hyperbaric oxygen therapy in the treatment of radio-induced lesions in normal tissues: a literature review. *Radiother Oncol* 2004; **72**: 1–13. doi: https://doi.org/10.1016/j.radonc.2004.04.005
54. Benyahia B, Campana F, Perdereau B, Gez E, Fourquet A, Magdelenat H. Effects of SOD topical treatment on human skin radiofibrosis: a pathological study. *The Breast* 1996; **5**: 75–81. doi: https://doi.org/10.1016/S0960-9776(96)90125-3
55. Lefaix J-L, Delanian S, Leplat J-J, Tricaud Y, Martin M, Nimrod A, et al. Successful treatment of radiation-induced fibrosis using Cu/Zn-SOD and Mn-SOD: an experimental study. *Int J Radiat Oncol Biol Phys* 1996; **35**: 305–12. doi: https://doi.org/10.1016/0360-3016(96)00061-2
56. Liu R, Oberley TD, Oberley LW. Transfection and expression of MnSOD cDNA decreases tumor malignancy of human oral squamous carcinoma SCC-25 cells. *Hum Gene Ther* 1997; **8**: 585–95. doi: https://doi.org/10.1089/hum.1997.8.5-585
57. Dennog C, Radermacher P, Barnett YA, Speit G. Antioxidant status in humans after exposure to hyperbaric oxygen. *Mutat Res* 1999; **428**: 83–9. doi: https://doi.org/10.1016/S1383-5742(99)00034-4
58. Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 2007; **39**: 44–84. doi: https://doi.org/10.1016/j.biocel.2006.07.001
59. O'Sullivan B, Levin W. Late radiation-related fibrosis: pathogenesis, manifestations, and current management. *Semin Radiat Oncol* 2003; **13**: 274–89. doi: https://doi.org/10.1016/S1053-4296(03)00037-7
60. Gerlach NL, Barkhuysen R, Kaanders JHAM, Janssens GORJ, Sterk W, Merkx MAW. The effect of hyperbaric oxygen therapy on quality of life in oral and oropharyngeal cancer patients treated with radiotherapy. *Int J Oral Maxillofac Surg* 2008; **37**: 255–9. doi: https://doi.org/10.1016/j.ijom.2007.11.013
61. Gothard L, Haviland J, Bryson P, Laden G, Glover M, Harrison S, et al. Randomised phase II trial of hyperbaric oxygen therapy in patients with chronic arm lymphoedema after radiotherapy for cancer. *Radiother Oncol* 2010; **97**: 101–7. doi: https://doi.org/10.1016/j.radonc.2010.04.026
62. Vozenin-Brotons M-C, Sivan V, Gault N, Renard C, Geffrotin C, Delanian S, et al. Antifibrotic action of Cu/Zn SOD is mediated by TGF-β1 repression and phenotypic reversion of myofibroblasts. *Free Radic Biol Med* 2001; **30**: 30–42. doi: https://doi.org/10.1016/S0891-5849(00)00431-7
63. Berbee M, Fu Q, Boerma M, Pathak R, Zhou D, Kumar KS, et al. Reduction of radiation-induced vascular nitrosative stress by the vitamin E analog gamma-tocotrienol: evidence of a role for Tetrahydrobiopterin. *Int J Radiat Oncol Biol Phys* 2011; **79**: 884–91. doi: https://doi.org/10.1016/j.ijrobp.2010.08.032
64. Berbée M, Fu Q, Boerma M, Sree Kumar K, Loose DS, Hauer-Jensen M. Mechanisms underlying the radioprotective properties of γ-tocotrienol: comparative gene expression profiling in tocol-treated endothelial cells. *Genes Nutr* 2012; **7**: 75–81. doi: https://doi.org/10.1007/s12263-011-0228-8
65. Hamama S, Noman MZ, Gervaz P, Delanian S, Vozenin M-C. MiR-210: a potential therapeutic target against radiation-induced enteropathy. *Radiother Oncol* 2014; **111**: 219–21. doi: https://doi.org/10.1016/j.radonc.2013.10.030
66. Berbée M, Fu Q, Garg S, Kulkarni S, Kumar KS, Hauer-Jensen M. Pentoxifylline enhances the radioprotective properties of γ-tocotrienol: differential effects on the hematopoietic, gastrointestinal and vascular systems. *Radiat Res* 2011; **175**: 297–306. doi: https://doi.org/10.1667/RR2399.1
67. Choi S-H, Hong Z-Y, Nam J-K, Lee H-J, Jang J, Yoo RJ, et al. A hypoxia-induced vascular endothelial-to-mesenchymal transition in development of radiation-induced pulmonary fibrosis. *Clin Cancer Res* 2015; **21**: 3716–26. doi: https://doi.org/10.1158/1078-0432.CCR-14-3193
68. Hauer-Jensen M, Denham JW, Andreyev HJN. Radiation enteropathy—pathogenesis, treatment and prevention. *Nat Rev Gastroenterol Hepatol* 2014; **11**: 470–9. doi: https://doi.org/10.1038/nrgastro.2014.46
69. Montay-Gruel P, Boivin G, Vozenin MC. Novel strategies to prevent, mitigate or reverse radiation injury and fibrosis. In: Anscher MSV, ed. *Strategies to enhance the therapeutic ratio of radiation as a cancer treatment*. Berlin: Springers; 2016.
70. Herrera FG, Bourhis J, Coukos G. Radiotherapy combination opportunities leveraging immunity for the next oncology practice. *CA Cancer J Clin* 2017; **67**: 65–85. doi: https://doi.org/10.3322/caac.21358
71. De Palma M, Coukos G, Hanahan D. A new twist on radiation oncology: low-dose irradiation elicits immunostimulatory macrophages that unlock barriers to tumor immunotherapy. *Cancer Cell* 2013; **24**: 559–61. doi: https://doi.org/10.1016/j.ccr.2013.10.019
72. Klug F, Prakash H, Huber PE, Seibel T, Bender N, Halama N, et al. Low-dose irradiation programs macrophage differentiation to an iNOS+/M1 phenotype that orchestrates effective T cell immunotherapy. *Cancer Cell* 2013; **24**: 589–602. doi: https://doi.org/10.1016/j.ccr.2013.09.014
73. Groves AM, Johnston CJ, Misra RS, Williams JP, Finkelstein JN. Whole-lung irradiation results in pulmonary macrophage alterations that are subpopulation and strain specific. *Radiat Res* 2015; **184**: 639–49. doi: https://doi.org/10.1667/RR14178.1
74. Chevrier S, Levine JH, Zanotelli VRT, Silina K, Schulz D, Bacac M, et al. An immune atlas of clear cell renal cell carcinoma. *Cell* 2017; **169**: 736–49. doi: https://doi.org/10.1016/j.cell.2017.04.016
75. Roberts NJ, Prill AH, Mann TN. Interleukin 1 and interleukin 1 inhibitor production by human macrophages exposed to influenza virus or respiratory syncytial virus. Respiratory syncytial virus is a potent inducer of inhibitor activity. *J Exp Med* 1986; **163**: 511–9. doi: https://doi.org/10.1084/jem.163.3.511

76. Ikushima H, Miyazono K. TGFβ signalling: a complex web in cancer progression. *Nat Rev Cancer* 2010; **10**: 415–24. doi: https://doi.org/10.1038/nrc2853
77. Abdollahi A, Li M, Ping G, Plathow C, Domhan S, Kiessling F, et al. Inhibition of platelet-derived growth factor signaling attenuates pulmonary fibrosis. *J Exp Med* 2005; **201**: 925–35. doi: https://doi.org/10.1084/jem.20041393
78. Thepen T, Van Rooijen N, Kraal G. Alveolar macrophage elimination in vivo is associated with an increase in pulmonary immune response in mice. *J Exp Med* 1989; **170**: 499–509. doi: https://doi.org/10.1084/jem.170.2.499
79. SW L, Zhang XM, Luo HM, YC F, MY X, Tang SJ. Clodronate liposomes reduce excessive scar formation in a mouse model of burn injury by reducing collagen deposition and TGF-beta1 expression. *Mol Biol Rep* 2014; **41**: 2143–9.
80. Froom P, Braester A, Aghai E, Quitt M, Elmalah I. Clodronate in myelofibrosis: a case report. *Am J Med Sci* 2002; **323**: 115–6. doi: https://doi.org/10.1097/00000441-200202000-00012
81. Delanian S, Lefaix J-L. Complete healing of severe osteoradionecrosis with treatment combining pentoxifylline, tocopherol and clodronate. *Br J Radiol* 2002; **75**: 467–9. doi: https://doi.org/10.1259/bjr.75.893.750467
82. Delanian S, Lefaix J-L, Maisonobe T, Salachas F, Pradat P-F. Significant clinical improvement in radiation-induced lumbosacral polyradiculopathy by a treatment combining pentoxifylline, tocopherol, and clodronate (Pentoclo). *J Neurol Sci* 2008; **275**: 164–6. doi: https://doi.org/10.1016/j.jns.2008.08.004
83. Bickelhaupt S, Erbel C, Timke C, Wirkner U, Dadrich M, Flechsig P, et al. Effects of CTGF blockade on attenuation and reversal of radiation-Induced pulmonary fibrosis. *J Natl Cancer Inst* 2017; **109**. doi: https://doi.org/10.1093/jnci/djw339
84. Meziani L, Mondini M, Petit B, Boissonnas A, Thomas de Montpreville V, Mercier O, et al. CSF1R inhibition prevents radiation pulmonary fibrosis by depletion of interstitial macrophages. *Eur Respir J* 2018; **51**: 1702120. doi: https://doi.org/10.1183/13993003.02120-2017
85. Xia D-H, Xi L, Xv C, Mao W-D, Shen W-S, Shu Z-Q, et al. The protective effects of ambroxol on radiation lung injury and influence on production of transforming growth factor β1 and tumor necrosis factor α. *Med Oncol* 2010; **27**: 697–701. doi: https://doi.org/10.1007/s12032-009-9271-3
86. Robb WB, Condron C, Moriarty M, Walsh TN, Bouchier-Hayes DJ. Taurine attenuates radiation-induced lung fibrosis in C57/Bl6 fibrosis prone mice. *Ir J Med Sci* 2010; **179**: 99–105. doi: https://doi.org/10.1007/s11845-009-0389-2
87. Song L, Wang D, Cui X, Hu W. The protective action of taurine and L-arginine in radiation pulmonary fibrosis. *J Environ Pathol Toxicol Oncol* 1998; **17**: 151–7.
88. Boerma M, Wang J, Burnett AF, Santin AD, Roman JJ, Hauer-Jensen M. Local administration of interleukin-11 ameliorates intestinal radiation injury in rats. *Cancer Res* 2007; **67**: 9501–6. doi: https://doi.org/10.1158/0008-5472.CAN-07-0810
89. Xavier S, Pike E, Fujii M, Javelaud D, Mauviel A, Flanders KC, et al. Amelioration of radiation-induced fibrosis: inhibtion of transforming growth factor-b signaling by halofuginone. *J Biol Chem* 2004;.
90. Wang J, Zheng H, Ou X, Albertson CM, Fink LM, Herbert J-M, et al. Hirudin ameliorates intestinal radiation toxicity in the rat: support for thrombin inhibition as strategy to minimize side-effects after radiation therapy and as countermeasure against radiation exposure. *J Thromb Haemost* 2004; **2**: 2027–35. doi: https://doi.org/10.1111/j.1538-7836.2004.00960.x
91. Wang J, Zheng H, Hauer-Jensen M. Influence of short-term octreotide administration on chronic tissue injury, transforming growth factor beta (TGF-beta) overexpression, and collagen accumulation in irradiated rat intestine. *J Pharmacol Exp Ther* 2001; **297**: 35–42.
92. Zheng H, Wang J, Koteliansky VE, Gotwals PJ, Hauer–Jensen M. Recombinant soluble transforming growth factor β type II receptor ameliorates radiation enteropathy in mice. *Gastroenterology* 2000; **119**: 1286–96. doi: https://doi.org/10.1053/gast.2000.19282
93. Wedlake LJ, Silia F, Benton B, Lalji A, Thomas K, Dearnaley DP, et al. Evaluating the efficacy of statins and ACE-inhibitors in reducing gastrointestinal toxicity in patients receiving radiotherapy for pelvic malignancies. *Eur J Cancer* 2012; **48**: 2117–24. doi: https://doi.org/10.1016/j.ejca.2011.12.034
94. Geraci JP, Mariano MS, Jackson KL. Amelioration of radiation nephropathy in rats by dexamethasone treatment after irradiation. *Radiat Res* 1993; **134**: 86–93. doi: https://doi.org/10.2307/3578505
95. Reeves WC, Stryker JA, Abt AB, Chung CK, Whitesell L, Zelis R. Early corticosteroid administration in experimental radiation-induced heart disease. *Radiology* 1980; **134**: 533–5. doi: https://doi.org/10.1148/radiology.134.2.7352245
96. Reeves WC, Cunningham D, Schwiter EJ, Abt A, Skarlatos S, Wood MA, et al. Myocardial hydroxyproline reduced by early administration of methylprednisolone or ibuprofen to rabbits with radiation-induced heart disease. *Circulation* 1982; **65**: 924–7. doi: https://doi.org/10.1161/01.CIR.65.5.924
97. Miyake K, Tani K, Kakiuchi S, Suzuka C, Toyoda Y, Kishi J, et al. Epidermal growth factor receptor-tyrosine kinase inhibitor (gefitinib) augments pneumonitis, but attenuates lung fibrosis in response to radiation injury in rats. *J Med Invest* 2012; **59**: 174–85. doi: https://doi.org/10.2152/jmi.59.174
98. Flechsig P, Dadrich M, Bickelhaupt S, Jenne J, Hauser K, Timke C, et al. LY2109761 attenuates radiation-induced pulmonary murine fibrosis via reversal of TGF-beta and BMP-associated proinflammatory and proangiogenic signals. *Clin Cancer Res* 2012; **18**: 3616–27. doi: https://doi.org/10.1158/1078-0432.CCR-11-2855
99. Nawroth I, Alsner J, Behlke MA, Besenbacher F, Overgaard J, Howard KA, et al. Intraperitoneal administration of chitosan/DsiRNA nanoparticles targeting TNFα prevents radiation-induced fibrosis. *Radiother Oncol* 2010; **97**: 143–8. doi: https://doi.org/10.1016/j.radonc.2010.09.010
100. Tabata C, Kadokawa Y, Tabata R, Takahashi M, Okoshi K, Sakai Y, et al. All- *trans* -retinoic acid prevents radiation- or bleomycin-induced pulmonary fibrosis. *Am J Respir Crit Care Med* 2006; **174**: 1352–60. doi: https://doi.org/10.1164/rccm.200606-862OC
101. Okoshi K, Kubo H, Nagayama S, Tabata C, Kadokawa Y, Hisamori S, et al. All-trans-retinoic acid attenuates radiation-induced intestinal fibrosis in mice. *J Surg Res* 2008; **150**: 53–9. doi: https://doi.org/10.1016/j.jss.2007.12.762
102. Han G, Zhou YF, Zhang MS, Cao Z, Xie CH, Zhou FX, et al. Angelica sinensis down-regulates hydroxyproline and Tgfb1 and provides protection in mice with radiation-induced pulmonary fibrosis. *Radiat Res* 2006; **165**: 546–52. doi: https://doi.org/10.1667/RR3543.1
103. Gottlöber P, Steinert M, Bähren W, Weber L, Gerngross H, Peter RU. Interferon-gamma in 5 patients with cutaneous radiation syndrome after radiation therapy. *Int J Radiat Oncol Biol Phys* 2001; **50**: 159–66. doi: https://doi.org/10.1016/S0360-3016(00)01542-X
104. Simone NL, Soule BP, Gerber L, Augustine E, Smith S, Altemus RM, et al. Oral pirfenidone in patients with chronic fibrosis

resulting from radiotherapy: a pilot study. *Radiat Oncol* 2007; **2**: 19. doi: https://doi.org/10.1186/1748-717X-2-19

105. Matsushita S, Ando K, Koike S, Grdina DJ, Furukawa S. Radioprotection by WR-151327 against the late normal tissue damage in mouse hind legs from gamma ray radiation. *Int J Radiat Oncol Biol Phys* 1994; **30**: 867–72. doi: https://doi.org/10.1016/0360-3016(94)90362-X
106. Percarpio B, Fischer JJ. Beta-aminopropionitrile as a radiation reaction preventive agent. *Radiology* 1976; **121**: 737–40. doi: https://doi.org/10.1148/121.3.737
107. Bourgier C, Rivera S, Vozenin MC, Boisselier P, Azria D, Lassau N, et al. Pravastatin reverses established radiation-induced cutaneous and subcutaneous fibrosis in head and neck cancer patients: results of a biology-driven clinical trial, pravacur phase 2. *Int J Radiat Oncol Biol Phys* 2017; **99**: S74–S. doi: https://doi.org/10.1016/j.ijrobp.2017.06.180
108. Favaudon V, Caplier L, Monceau V, Pouzoulet F, Sayarath M, Fouillade C, et al. Ultrahigh dose-rate FLASH irradiation increases the differential response between normal and tumor tissue in mice. *Sci Transl Med* 2014; **6**: 245ra93. doi: https://doi.org/10.1126/scitranslmed.3008973
109. Montay-Gruel P, Petersson K, Jaccard M, Boivin G, Germond JF, Petit B, et al. Irradiation in a flash: Unique sparing of memory in mice after whole brain irradiation with dose rates above 100Gy/s. *Radiother Oncol* 2017; **124**: 365–9. doi: https://doi.org/10.1016/j.radonc.2017.05.003
110. Péguret N, Ozsahin M, Zeverino M, Belmondo B, Durham AD, Lovis A, et al. Apnea-like suppression of respiratory motion: First evaluation in radiotherapy. *Radiother Oncol* 2016; **118**: 220–6. doi: https://doi.org/10.1016/j.radonc.2015.10.011
111. Ogna A, Bernasconi M, Belmondo B, Long O, Simons J, Peguret N, et al. Prolonged apnea supported by high-frequency noninvasive ventilation: a pilot study. *Am J Respir Crit Care Med* 2017; **195**: 958–60. doi: https://doi.org/10.1164/rccm.201608-1572LE
112. Jaffray DA. Image-guided radiotherapy: from current concept to future perspectives. *Nat Rev Clin Oncol* 2012; **9**: 688–99. doi: https://doi.org/10.1038/nrclinonc.2012.194
113. Zelefsky MJ, Kollmeier M, Cox B, Fidaleo A, Sperling D, Pei X, et al. Improved clinical outcomes with high-dose image guided radiotherapy compared with non-IGRT for the treatment of clinically localized prostate cancer. *Int J Radiat Oncol Biol Phys* 2012; **84**: 125–9. doi: https://doi.org/10.1016/j.ijrobp.2011.11.047
114. Créhange G, Martin E, Supiot S, Chapet O, Mazoyer F, Naudy S, et al. Image-guided radiotherapy in prostate cancer: concepts and implications. *Cancer Radiother* 2012; **16**: 430–8. doi: https://doi.org/10.1016/j.canrad.2012.07.183
115. Nutting CM, Morden JP, Harrington KJ, Urbano TG, Bhide SA, Clark C, et al. Parotid-sparing intensity modulated versus conventional radiotherapy in head and neck cancer (PARSPORT): a phase 3 multicentre randomised controlled trial. *Lancet Oncol* 2011; **12**: 127–36. doi: https://doi.org/10.1016/S1470-2045(10)70290-4
116. Chen AM, Li BQ, Farwell DG, Marsano J, Vijayakumar S, Purdy JA. Improved dosimetric and clinical outcomes with intensity-modulated radiotherapy for head-and-neck cancer of unknown primary origin. *Int J Radiat Oncol Biol Phys* 2011; **79**: 756–62. doi: https://doi.org/10.1016/j.ijrobp.2009.11.020
117. Jermann M. Particle therapy statistics in 2014. *International Journal of Particle Therapy* 2015; **2**: 50–4. doi: https://doi.org/10.14338/IJPT-15-00013
118. Rajendran R, Weinberg V, Daftari I, Castro J, Quivey J, Char D, et al. Decreased incidence of neovascular glaucoma by sparing anterior structures of the eye for proton beam therapy of ocular melanoma. *Int J Radiat Oncol Biol Phys* 2004; **60**: S311–S312. doi: https://doi.org/10.1016/S0360-3016(04)01391-4
119. Munzenrider JE. Uveal melanomas. Conservation treatment. *Hematol Oncol Clin North Am* 2001; **15**: 389–402.
120. Dendale R, Lumbroso-Le Rouic L, Noel G, Feuvret L, Levy C, Delacroix S, et al. Proton beam radiotherapy for uveal melanoma: results of curie institut-orsay proton therapy center (ICPO). *Int J Radiat Oncol Biol Phys* 2006; **65**: 780–7. doi: https://doi.org/10.1016/j.ijrobp.2006.01.020
121. Egger E, Schalenbourg A, Zografos L, Bercher L, Boehringer T, Chamot L, et al. Maximizing local tumor control and survival after proton beam radiotherapy of uveal melanoma. *Int J Radiat Oncol Biol Phys* 2001; **51**: 138–47. doi: https://doi.org/10.1016/S0360-3016(01)01560-7
122. Krishnan S, Foote RL, Brown PD, Pollock BE, Link MJ, Garces YI. Radiosurgery for cranial base chordomas and chondrosarcomas. *Neurosurgery* 2005; **56**: 777–84. doi: https://doi.org/10.1227/01.NEU.0000156789.10394.F5
123. Ares C, Hug EB, Lomax AJ, Bolsi A, Timmermann B, Rutz HP, et al. Effectiveness and safety of spot scanning proton radiation therapy for chordomas and chondrosarcomas of the skull base: first long-term report. *Int J Radiat Oncol Biol Phys* 2009; **75**: 1111–8. doi: https://doi.org/10.1016/j.ijrobp.2008.12.055
124. Munzenrider JE. Proton therapy for uveal melanomas and other eye lesions. *Strahlenther Onkol* 1999; **175**(Suppl 2): 68–73. doi: https://doi.org/10.1007/BF03038893
125. Miralbell R, Lomax A, Russo M. Potential role of proton therapy in the treatment of pediatric medulloblastoma/primitive neuro-ectodermal tumors: spinal theca irradiation. *Int J Radiat Oncol Biol Phys* 1997; **38**: 805–11. doi: https://doi.org/10.1016/S0360-3016(97)00005-9
126. Miralbell R, Lomax A, Cella L, Schneider U. Potential reduction of the incidence of radiation-induced second cancers by using proton beams in the treatment of pediatric tumors. *Int J Radiat Oncol Biol Phys* 2002; **54**: 824–9. doi: https://doi.org/10.1016/S0360-3016(02)02982-6
127. Mu X, Björk-Eriksson T, Nill S, Oelfke U, Johansson KA, Gagliardi G, et al. Does electron and proton therapy reduce the risk of radiation induced cancer after spinal irradiation for childhood medulloblastoma? A comparative treatment planning study. *Acta Oncol* 2005; **44**: 554–62. doi: https://doi.org/10.1080/02841860500218819
128. Pulsifer MB, Sethi RV, Kuhlthau KA, MacDonald SM, Tarbell NJ, Yock TI. Early cognitive outcomes following proton radiation in pediatric patients with brain and central nervous system tumors. *Int J Radiat Oncol Biol Phys* 2015; **93**: 400–7. doi: https://doi.org/10.1016/j.ijrobp.2015.06.012
129. Clarke M, Collins R, Darby S, Davies C, Elphinstone P, Evans V, et al. Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 2005; **366**: 2087–106. doi: https://doi.org/10.1016/S0140-6736(05)67887-7
130. Lomax AJ, Cella L, Weber D, Kurtz JM, Miralbell R. Potential role of intensity-modulated photons and protons in the treatment of the breast and regional nodes. *Int J Radiat Oncol Biol Phys* 2003; **55**: 785–92. doi: https://doi.org/10.1016/S0360-3016(02)04210-4
131. Johansson J, Isacsson U, Lindman H, Montelius A, Glimelius B. Node-positive left-sided breast cancer patients after breast-conserving surgery: potential outcomes of radiotherapy modalities and techniques. *Radiother Oncol* 2002; **65**: 89–98. doi: https://doi.org/10.1016/S0167-8140(02)00266-9

132. Weber DC, Ares C, Lomax AJ, Kurtz JM. Radiation therapy planning with photons and protons for early and advanced breast cancer: an overview. *Radiat Oncol* 2006; **1**: 22. doi: https://doi.org/10.1186/1748-717X-1-22
133. Taghian AG, Kozak KR, Katz A, Adams J, Lu HM, Powell SN, et al. Accelerated partial breast irradiation using proton beams: Initial dosimetric experience. *Int J Radiat Oncol Biol Phys* 2006; **65**: 1404–10. doi: https://doi.org/10.1016/j.ijrobp.2006.03.017
134. Kozak KR, Smith BL, Adams J, Kornmehl E, Katz A, Gadd M, et al. Accelerated partial-breast irradiation using proton beams: initial clinical experience. *Int J Radiat Oncol Biol Phys* 2006; **66**: 691–8. doi: https://doi.org/10.1016/j.ijrobp.2006.06.041
135. Tsuji T, Ibaragi S, Shima K, Hu MG, Katsurano M, Sasaki A, et al. Epithelial-mesenchymal transition induced by growth suppressor p12CDK2-AP1 promotes tumor cell local invasion but suppresses distant colony growth. *Cancer Res* 2008; **68**: 10377–86. doi: https://doi.org/10.1158/0008-5472.CAN-08-1444
136. Okada T, Kamada T, Tsuji H, Mizoe JE, Baba M, Kato S, et al. Carbon ion radiotherapy: clinical experiences at National Institute of Radiological Science (NIRS). *J Radiat Res* 2010; **51**: 355–64. doi: https://doi.org/10.1269/jrr.10016
137. Kramer D. Carbon-ion cancer therapy shows promise. *Phys Today* 2015; **68**: 24–5. doi: https://doi.org/10.1063/PT.3.2812
138. Denekamp J, Waites T, Fowler JF. Predicting realistic RBE values for clinically relevant radiotherapy schedules. *Int J Radiat Biol* 1997; **71**: 681–94. doi: https://doi.org/10.1080/095530097143699
139. Ando K, Kase Y. Biological characteristics of carbon-ion therapy. *Int J Radiat Biol* 2009; **85**: 715–28. doi: https://doi.org/10.1080/09553000903072470
140. Ando K, Koike S, Uzawa A, Takai N, Fukawa T, Furusawa Y, et al. Biological gain of carbon-ion radiotherapy for the early response of tumor growth delay and against early response of skin reaction in mice. *J Radiat Res* 2005; **46**: 51–7. doi: https://doi.org/10.1269/jrr.46.51
141. Battermann JJ, Mijnheer BJ. The Amsterdam fast neutron therapy project: a final report. *Int J Radiat Oncol Biol Phys* 1986; **12**: 2093–9. doi: https://doi.org/10.1016/0360-3016(86)90007-6
142. Laramore GE. The use of neutrons in cancer therapy: a historical perspective through the modern era. *Semin Oncol* 1997; **24**: 672–85.
143. Prempree T, Michelsen A, Merz T. The repair time of chromosome breaks induced by pulsed x-rays on ultra-high dose-rate. *Int J Radiat Biol Relat Stud Phys Chem Med* 1969; **15**: 571–4. doi: https://doi.org/10.1080/09553006914550871
144. Auer S, Hable V, Greubel C, Drexler GA, Schmid TE, Belka C, et al. Survival of tumor cells after proton irradiation with ultra-high dose rates. *Radiat Oncol* 2011; **6**: 139. doi: https://doi.org/10.1186/1748-717X-6-139
145. Schmid TE, Dollinger G, Beisker W, Hable V, Greubel C, Auer S, et al. Differences in the kinetics of gamma-H2AX fluorescence decay after exposure to low and high LET radiation. *Int J Radiat Biol* 2010; **86**: 682–91. doi: https://doi.org/10.3109/09553001003734543
146. Schmid TE, Dollinger G, Hable V, Greubel C, Zlobinskaya O, Michalski D, et al. The effectiveness of 20 mev protons at nanosecond pulse lengths in producing chromosome aberrations in human-hamster hybrid cells. *Radiat Res* 2011; **175**: 719–27. doi: https://doi.org/10.1667/RR2465.1
147. Zlobinskaya O, Girst S, Greubel C, Hable V, Siebenwirth C, Walsh DW, et al. Reduced side effects by proton microchannel radiotherapy: study in a human skin model. *Radiat Environ Biophys* 2013; **52**: 123–33. doi: https://doi.org/10.1007/s00411-012-0450-9
148. Loo BW, Schuler E, Lartey FM, Rafat M, King GJ, Trovati S, et al. Delivery of ultra-rapid flash radiation therapy and demonstration of Normal tissue sparing after abdominal irradiation of mice. *Int J Radiat Oncol Biol Phys* 2017; **98**(2 suppl): E16. doi: https://doi.org/10.1016/j.ijrobp.2017.02.101
149. Hendry JH, Moore JV, Hodgson BW, Keene JP. The constant low oxygen concentration in all the target cells for mouse tail radionecrosis. *Radiat Res* 1982; **92**: 172–81. doi: https://doi.org/10.2307/3575852
150. Weiss H, Epp ER, Heslin JM, Ling CC, Santomasso A. Oxygen depletion in cells irradiated at ultra-high dose-rates and at conventional dose-rates. *Int J Radiat Biol Relat Stud Phys Chem Med* 1974; **26**: 17–29. doi: https://doi.org/10.1080/09553007414550901
151. Schüler E, Trovati S, King G, Lartey F, Rafat M, Villegas M, et al. Experimental platform for ultra-high dose rate FLASH irradiation of small animals using a clinical linear accelerator. *Int J Radiat Oncol Biol Phys* 2017; **97**: 195–203. doi: https://doi.org/10.1016/j.ijrobp.2016.09.018
152. Schüler E, Eriksson K, Hynning E, Hancock SL, Hiniker SM, Bazalova-Carter M, et al. Very high-energy electron (VHEE) beams in radiation therapy; treatment plan comparison between VHEE, VMAT, and PPBS. *Med Phys* 2017; **44**: 2544–55. doi: https://doi.org/10.1002/mp.12233
153. Glinec Y, Faure J, Malka V, Fuchs T, Szymanowski H, Oelfke U. Radiotherapy with laser-plasma accelerators: monte carlo simulation of dose deposited by an experimental quasimonoenergetic electron beam. *Med Phys* 2006; **33**: 155–62. doi: https://doi.org/10.1118/1.2140115

3. Curriculum-vitae and publication list

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PhD Student
Student of the Ecole Normale Supérieure
Biochemistry and Biology Teacher

French citizen, Swiss Residency
28yo, 21.05.1990
Single

SCIENTIFIC EXPERIENCE

- **August 2014 – Nowadays** – PhD studies. Radiation Oncology department, CHUV, Lausanne, Switzerland. **« Normal brain tissue, Neural Stem Cells and Glioblastoma response to ultra-high dose-rate Flash radiotherapy »** Supervised by Dr. PhD. Marie-Catherine Vozenin, Dr. PhD Vincent Favaudon and Pr. MD PhD Jean Bourhis.
- **December 2016** – Visiting Scientist. Radiobiology laboratory, UC Irvine, California, USA. Supervised by Dr. PhD. C. Limoli.
- **January – June 2014** – MSc internship. Radiation Oncology, CHUV, Lausanne, Switzerland. **« Innovative approach for brain tumors treatment with Flash radiation therapy »** Supervised by Dr. PhD. Marie-Catherine Vozenin and Pr. MD PhD Jean Bourhis.
- **October – November 2013** – Internship in a University Hospital for translational research. Department of Radiation Oncology of Gustave Roussy, Villejuif, France. Supervised by Pr. MD PhD Eric Deutsch.
- **July – August 2012** – Translational research internship, Radiotherapy INSERM UMR1030, Gustave Roussy, Villejuif, France. **« Radiation Induced Autophagy in Lung Cancer Cells »** Supervised by Pr. MD PhD Eric Deutsch and Pr. MD PhD Jean Bourhis.

EDUCATION

- **2014 – Nowadays** – **PHD STUDIES** – University of Lausanne Biology and Medicine Faculty, Switzerland. Oncology Doctoral School of Paris, France. Swiss-French International supervision.
- **2014** – **M.SC. THESIS** in Oncology and Radiobiology – ENS de Cachan, University Paris Saclay, Gustave Roussy.
- **2014** – **GRADUATED BY THE ECOLE NORMALE SUPERIEURE DE CACHAN** – Department of Biology.
- **2013** – **COMPETITIVE EXAMINATION OF THE AGREGATION** (Highschool and University teachers) Biochemistry, Physiology. Ranked 7/324.
- **2013** – **M.ED. THESIS** – « Biochemistry, Microbiology, Physiology » – ENS de Cachan.
- **2012** – **1ST YEAR OF M.SC.** – Health and Biology, Oncology – ENS Cachan, University Paris Saclay.

UNIVERSITY AND HIGH SCHOOL SERVICE

- **2015 – Nowadays** – Radiobiology courses for students in Medical Radiation Technology, CHUV, HESAV, Lausanne, Switzerland.
- **2014 – Nowadays** – Biochemistry practical-work for medical students, University of Lausanne, Switzerland.
- **2012** – Biochemistry practical-work to high school students in biotechnologies, Lycée Vallée de Chevreuse, Gif-sur-Yvette, France.

GENERAL SKILLS

- **French** – Native
- **English** – Fluent C2 - 930 TOEIC (May 2011)
- **German** – B1
- **Spanish** – A2
- **Lab Technical skills** – Cell Culture, Animal Experimentation, Immunohistochemistry, Stereological Brain Implantation and Surgery, Irradiation and CT imaging procedures, Behavioural experimentation, General Molecular Biology, General Biochemistry.
- **Software** – Word, Excel, Powerpoint, Prism GraphPad, EndNote, Adobe Illustrator, Photoshop, ImageJ, FlowJo

SUPERVISION

- **May – December 2018** – M.Sc. thesis supervision of Philippe Fuchs, University of Lausanne, Switzerland.
- **May – December 2018** – M.Sc. thesis supervision of Shing-Chi Liu , University of Lausanne, Switzerland.
- **October – December 2017** –1st step M.Sc. thesis supervision of Céline Godfroid, University of Lausanne, Switzerland.
- **October – December 2016** – 1st step M.Sc. thesis supervision of Aurélie De Vallière, University of Lausanne, Switzerland.

GRANT SUPPORT

- **2017 – 2018** – PhD funding by the ISREC Foundation, thanks to a Biltema donation.
- **December 2016** – Mobility Grant for Scientific Training, ESTRO. Visit of C. Limoli's lab. UC Irvine, California, USA.
- **2014 – 2017** – Specific PhD grant for ENS Student, French Ministry of Education and Research.
- **2014 – 2016** – PhD funding, Swiss National Funding for the Scientific Research, 31003a_156892.
- **2011 – 2014** – University Grant for ENS students by the French Ministry of Education and Research.

SCIENTIFIC PUBLICATIONS

- P. G. Montay-Gruel, R. Serduc, M. Jaccard, K. Petersson, D. Patin, B. Petit, F. Bochud, C. Bailat, E. Brauer-Kirsch, J. Bourhis and M. C. Vozenin. « X-Rays can trigger the FLASH effect: Ultra-high dose-rate synchrotron light source prevents normal brain injury after whole brain irradiation in mice » **Submitted to Radiotherapy and Oncology**, June 2018

- P. G. Montay-Gruel, Acharya, M., […] C. Bailat, J. Bourhis, C. Limoli and M. C. Vozenin. « Spare the Brain but not the Tumor, Oxygen-Dependent Benefits of FLASH-Radiotherapy » **Submitted to Nature Medicine**, June 2018.

- P. G. Montay-Gruel, L. Meziani, C. Yakkala, M.C. Vozenin « Expanding the therapeutic index of radiation therapy by normal tissue protection » **The British Journal of Radiology.** 2018.

- P.G. Montay-Gruel, K. Petersson, M. Jaccard, G. Boivin, J. F. Germond, B. Petit, R. Doenlen, F. Bochud, C. Bailat, J. Bourhis and M. C. Vozenin. « Irradiation in a Flash: Unique Sparing of Spatial Memory in Mice after Whole Brain Irradiation with Dose-Rates above 100Gy/s » **Radiotherapy and Oncology.** 2017.

- P.G. Montay-Gruel, G. Boivin and M. C. Vozenin. Novel Strategies to Prevent, Mitigate or Reverse Radiation Injury and Fibrosis. In « Strategies to Enhance the Therapeutic Ratio of Radiation as a Cancer Treatment », **Springer.** 2016; 75-108.

- G. Boivin, P. Kalambaden, J. Faget, S. Rusakiewicz, P.G. Montay-Gruel, E. Meylan, J. Bourhis, G. Lesec and M.C. Vozenin. « Cellular Composition and Contribution of TLS to tumour immune infiltration and modulation by Radiatoin-Therapy », **Frontiers in Oncology.** 2018.
-
- P. Tsoutsou, P. G. Montay-Gruel and M.C. Vozenin. « The Era of Modern Radiation Therapy: Innovations to Spare Normal Tissues », **Under review, Springer Nature**, 2018

ORAL COMMUNICATIONS

- P. G. Montay-Gruel, K. Petersson, C. Yakkala, M. Jaccard, J. F. Germond, B. Petit, F. Bochud, C. Bailat, J. Bourhis and M. C. Vozenin. « *Identification of dioxygen as a primary key event involved in the normal brain protection induced by Flash-RT.* » **Lausanne Cancer Research Retreat,** 15th November 2017, Ecole Hôtelière de Lausanne, Switzerland.

- P. G. Montay-Gruel, K. Petersson, C. Yakkala, M. Jaccard, J. F. Germond, B. Petit, F. Bochud, C. Bailat, J. Bourhis and M. C. Vozenin. « *Brain irradiation in a Flash: From cognition sparing to possible mechanisms of action.* » **Ludwig Institute for Cancer Research Trainee Meeting,** 5th May 2017, University of Lausanne, Switzerland.

- P. G. Montay-Gruel, K. Petersson, M. Jaccard, G. Boivin, J. F. Germond, B. Petit , C. Yakkala, F. Bochud, C. Bailat, J. Bourhis and M. C. Vozenin. « *Brain irradiation in a Flash: Cognition sparing and antitumor effect on glioblastoma.* » **1st workshop on ultra-high dose-rate irradiation,** 1st December 2016, Institut Curie, Orsay, France.

- P. G. Montay-Gruel, K. Petersson, M. Jaccard, G. Boivin, J. F. Germond, B. Petit, F. Bochud, C. Bailat, J. Bourhis and M. C. Vozenin. « *Normal brain, neural stem cells and glioblastoma response to Flash irradiation* », **Ludwig Institute for Cancer Research Trainee Meeting,** 17th February 2016, University of Lausanne, Switzerland.

SCIENTIFIC POSTERS

- P. G. Montay-Gruel, K. Petersson, M. Jaccard, J. F. Germond, B. Petit, C. Yakkala, R. Doenlen, F. Bochud, C. Bailat, J. Bourhis, M. C. Vozenin. «*Unique sparing of spatial memory and control of glioblastoma tumorigenesis in mice models after whole brain irradiation.* » **Lausanne Cancer Research Retreat,** 14-15 November 2016, Ecole Hôtelière de Lausanne, Switzerland.

- P. G. Montay-Gruel, K. Petersson, B. Petit, J. Bourhis, V. Favaudon, M. C. Vozenin. « *Normal brain, neural stem cells and glioblastoma response to Flash irradiation.* » **Cancerology Doctoral School PhD Retreat,** 2-4 May 2016, Station Scientifique de Roscoff, France.

- P. G. Montay-Gruel, K. Petersson, B. Petit, J. Bourhis, V. Favaudon, M. C. Vozenin. « *Induction of quiescence in NSCs and preservation of neurogenesis in mouse adult brain after Flash whole brain irradiation*. » **ICTR-PHE,** 15-19 February 2016, Geneva, Switzerland.

- P. G. Montay-Gruel, K. Petersson, B. Petit, J. Bourhis, V. Favaudon, M. C. Vozenin. « *Induction of quiescence in NSCs and preservation of neurogenesis in mouse adult brain after Flash whole brain irradiation.* » **Lausanne Cancer Research Retreat,** 10-11 November 2015, Ecole Hôtelière de Lausanne, Switzerland.

- P. G. Montay-Gruel, B. Petit, F. Bochud, V. Favaudon, J. Bourhis, M. C. Vozenin. « *Normal brain, neural stem cells and glioblastoma response to Flash radiotherapy.* » **Wolfsberg Radiobiology Meeting,** 20-22 June 2015, Wolfsberg Castle, Switzerland.

- P. G. Montay-Gruel, B. Petit, F. Bochud, V. Favaudon, J. Bourhis, M. C. Vozenin. « *Normal brain, neural stem cells and glioblastoma response to Flash radiotherapy.* » **ESTRO FORUM,** 24-28 April 2015, Barcelona, Spain.

- P. G. Montay-Gruel, B. Petit, F. Bochud, V. Favaudon, J. Bourhis, M. C. Vozenin. « *Normal brain, neural stem cells and glioblastoma response to Flash radiotherapy.* » **Lausanne Cancer Research Retreat,** 11-12 November 2014, Ecole Hôtelière de Lausanne, Switzerland.

BIBLIOGRAPHY

Abbott, Rönnbäck and Hansson. 2006. “Astrocyte-Endothelial Interactions at the Blood-Brain Barrier.” *Nature Reviews Neuroscience* 7(1): 41–53.

Abrous. 2005. “Adult Neurogenesis: From Precursors to Network and Physiology.” *Physiological Reviews* 85(2): 523–69.

Achanta, Fuss and Martinez. 2009. “Ionizing Radiation Impairs the Formation of Trace Fear Memories and Reduces Hippocampal Neurogenesis.” *Behavioral Neuroscience* 123(5): 1036–45.

Acharya, M. M., Christie, Lan, Donovan, Cotman, Fike and Limoli. 2009. “Rescue of Radiation-Induced Cognitive Impairment through Cranial Transplantation of Human Embryonic Stem Cells.” *Proceedings of the National Academy of Sciences* 106(45): 19150–55.

Acharya, M. M., Christie, Lan, Giedzinski, Fike, Rosi and Limoli. 2011. “Human Neural Stem Cell Transplantation Ameliorates Radiation-Induced Cognitive Dysfunction.” *Cancer Research* 71(14): 4834–45.

Acharya, Munjal M., Roa, Bosch, Lan and Limoli. 2011. “Stem Cell Transplantation Strategies for the Restoration of Cognitive Dysfunction Caused by Cranial Radiotherapy.” *Journal of Visualized Experiments* (56).

Acharya, Munjal M., Green, Allen, Najafi, Syage, Minasyan, Le, Kawashita, Giedzinski, Parihar, West, Baulch and Limoli. 2016. “Elimination of Microglia Improves Cognitive Function Following Cranial Irradiation.” *Scientific Reports* 6: 31545.

Akiyama, Tanaka, Sato and Takeda. 2001. “Cognitive Dysfunction and Histological Findings in Adult Rats One Year after Whole Brain Irradiation.” *Neurologia medico-chirurgica* 41(12): 590–98.

Allen, Acharya, Lu, Giedzinski, Chmielewski, Quach, Hefferan, Johe and Limoli. 2018. “Remediation of Radiation-Induced Cognitive Dysfunction through Oral Administration of the Neuroprotective Compound NSI-189.” *Radiation Research* 189(4): RR14879.1.

Alvarez-Buylla and Lim. 2004. “For the Long Run: Maintaining Germinal Niches in the Adult Brain.” *Neuron* 41(5): 683–86.

Amano, Inamura, Wu, Kura, Nakamizo, Inoha, Miyazono and Ikezaki. 2002. “Effects of Single Low Dose Irradiation on Subventricular Zone Cells in Juvenile Rat Brain.” *Neurological research* 24(8): 809–16.

Antunes and Biala. 2012. “The Novel Object Recognition Memory: Neurobiology, Test Procedure, and Its Modifications.” *Cognitive Processing* 13(2): 93–110.

Auer, Hable, Greubel, Drexler, Schmid, Belka, Dollinger and Friedl. 2011. “Survival of Tumor Cells after Proton Irradiation with Ultra-High Dose Rates.” *Radiation Oncology* 6(1): 139.

Avril … Menu. 2004. “Fotemustine Compared with Dacarbazine in Patients with Disseminated Malignant Melanoma: A Phase III Study.” *Journal of Clinical Oncology* 22(6): 1118–25.

Balentova and Adamkov. 2015. “Molecular, Cellular and Functional Effects of Radiation-Induced Brain

Injury: A Review." *International Journal of Molecular Sciences* 16(11): 27796–815.

Balentova, Hajtmanova, Trylcova, Adamkov and Lehotsky. 2014. "Ionizing Radiation Induced Long-Term Alterations in the Adult Rat Rostral Migratory Stream." *Acta Histochemica* 116(1): 265–71.

Bassant and Court. 1978. "Effects of Whole-Body Gamma Irradiation on the Activity of Rabbit Hippocampal Neurons." *Radiat.Res.* 75(3): 593–606.

Bath, Mandairon, Jing, Rajagopal, Kapoor, Chen, Khan, Proenca, Kraemer, Cleland, Hempstead, Chao and Lee. 2008. "Variant Brain-Derived Neurotrophic Factor (Val66Met) Alters Adult Olfactory Bulb Neurogenesis and Spontaneous Olfactory Discrimination." *Journal of Neuroscience* 28(10): 2383–93.

Baulch, Acharya, Allen, Ru, Chmielewski, Martirosian, Giedzinski, Syage, Park, Benke, Parihar and Limoli. 2016. "Cranial Grafting of Stem Cell-Derived Microvesicles Improves Cognition and Reduces Neuropathology in the Irradiated Brain." *Proceedings of the National Academy of Sciences* 113(17): 4836–41.

Baydas, Nedzvetskii, Kirichenko and Nerush. 2008. "Astrogliosis in the Hippocampus and Cortex and Cognitive Deficits in Rats with Streptozotocin-Induced Diabetes: Effects of Melatonin." *Neurophysiology* 40(2): 91–97.

Bazyar, Inscoe, Benefield, Zhang, Lu, Zhou and Lee. 2017. "Neurocognitive Sparing of Desktop Microbeam Irradiation." *Radiation Oncology* 12(1): 127.

Beauchesne. 2011. "Extra-Neural Metastases of Malignant Gliomas: Myth or Reality?" *Cancers* 3(1): 461–77.

Begg, Stewart and Vens. 2011. "Strategies to Improve Radiotherapy with Targeted Drugs." *Nature Reviews Cancer* 11(4): 239–53.

Bellinzona, Gobbel, Shinohara and Fike. 1996. "Apoptosis Is Induced in the Subependyma of Young Adult Rats by Ionizing Irradiation." *Neuroscience Letters* 208(3): 163–66.

Bentzen. 2006. "Preventing or Reducing Late Side Effects of Radiation Therapy: Radiobiology Meets Molecular Pathology." *Nature Reviews Cancer* 6(9): 702–13.

Bernards and Weinberg. 2002. "A Progression Puzzle." *Nature* 418(6900): 823.

Berry, Hall, Forster, Storr and Goodman. 1969. "Survival of Mammalian Cells Exposed to x Rays at Ultra-High Dose-Rates." *The British journal of radiology* 42(494): 102–7.

Boag, Hochhäuser and Balk. 1996. "The Effect of Free-Electron Collection on the Recombination Correction to Ionization Measurements of Pulsed Radiation." *Physics in Medicine and Biology* 41(5): 885–97.

Bonner, Harari, Giralt, Azarnia, Shin, Cohen, Jones, Sur, Raben, Jassem, Ove, Kies, Baselga, Youssoufian, Amellal, Rowinsky and Ang. 2006. "Radiotherapy plus Cetuximab for Squamous-Cell Carcinoma of the Head and Neck." *New England Journal of Medicine* 354(6): 567–78.

Boogerd, Dalesio, Bais and van der Sande. 1992. “Response of Brain Metastases From Breast Cancer to Systemic Chemotherapy.” *Cancer* 69(4): 972–80.

Bortfeld. 2006. “IMRT: A Review and Preview.” *Physics in Medicine and Biology* 51(13).

Bouchet, Lemasson, Le Duc, Maisin, Bräuer-Krisch, Siegbahn, Renaud, Khalil, Rémy, Poillot, Bravin, Laissue, Barbier and Serduc. 2010. “Preferential Effect of Synchrotron Microbeam Radiation Therapy on Intracerebral 9l Gliosarcoma Vascular Networks.” *International Journal of Radiation Oncology Biology Physics* 78(5): 1503–12.

Bouchet, Serduc, Laissue and Djonov. 2015. “Effects of Microbeam Radiation Therapy on Normal and Tumoral Blood Vessels.” *Physica Medica* 31(6): 634–41.

Bräuer-Krisch, Serduc, Siegbahn, Le Duc, Prezado, Bravin, Blattmann and Laissue. 2010. “Effects of Pulsed, Spatially Fractionated, Microscopic Synchrotron X-Ray Beams on Normal and Tumoral Brain Tissue.” *Mutation Research - Reviews in Mutation Research* 704(1–3): 160–66.

Brescia, Ortensi, Fornasari, Levi, Broggi and Pelicci. 2013. “CD133 Is Essential for Glioblastoma Stem Cell Maintenance.” *STEM CELLS* 31(5): 857–69.

Bright, Kanakasabai, Chearwae and Chakraborty. 2008. “PPAR Regulation of Inflammatory Signaling in CNS Diseases.” *PPAR Research* 2008: 658520.

Brown, Daniel V., Filiz, Daniel, Hollande, Dworkin, Amiridis, Kountouri, Ng, Morokoff and Mantamadiotis. 2017. “Expression of CD133 and CD44 in Glioblastoma Stem Cells Correlates with Cell Proliferation, Phenotype Stability and Intra-Tumor Heterogeneity” ed. Jeffrey K. Harrison. *PLOS ONE* 12(2): e0172791.

Brown, William R, Thore, Moody, Robbins and Wheeler. 2005. “Vascular Damage after Fractionated Whole-Brain Irradiation in Rats.” *Radiation research* 164(5): 662–68.

Von Bueren, Von Hoff, Pietsch, Gerber, Warmuth-Metz, Deinlein, Zwiener, Faldum, Fleischhack, Benesch, Krauss, Kuehl, Kortmann and Rutkowski. 2011. “Treatment of Young Children with Localized Medulloblastoma by Chemotherapy Alone: Results of the Prospective, Multicenter Trial HIT 2000 Confirming the Prognostic Impact of Histology.” *Neuro-Oncology* 13(6): 669–79.

Burghardt, Park, Hen and Fenton. 2012. “Adult-Born Hippocampal Neurons Promote Cognitive Flexibility in Mice.” *Hippocampus* 22(9): 1795–1808.

Burns and McEwen. 1998. “Ion Recombination Corrections for the NACP Parallel-Plate Chamber in a Pulsed Electron Beam.” *Physics in Medicine and Biology* 43(8): 2033–45.

Burstein, Lieberman, Slamon, Winer and Klein. 2005. “Isolated Central Nervous System Metastases in Patients with HER2-Overexpressing Advanced Breast Cancer Treated with First-Line Trastuzumab-Based Therapy.” *Annals of Oncology* 16(11): 1772–77.

Butowski, Colman, De Groot, Omuro, Nayak, Wen, Cloughesy, Marimuthu, Haidar, Perry, Huse, Phillips,

West, Nolop, Hsu, Ligon, Molinaro and Prados. 2016. “Orally Administered Colony Stimulating Factor 1 Receptor Inhibitor PLX3397 in Recurrent Glioblastoma: An Ivy Foundation Early Phase Clinical Trials Consortium Phase II Study.” *Neuro-Oncology* 18(4): 557–64.

Butti, Cusimano, Bacigaluppi and Martino. 2014. “Neurogenic and Non-Neurogenic Functions of Endogenous Neural Stem Cells.” *Frontiers in Neuroscience* 8(8 APR): 92.

Calvo, Hopewell, Reinhold and Yeung. 1988. “Time- and Dose-Related Changes in the White Matter of the Rat Brain after Single Doses of X Rays.” *British Journal of Radiology* 61(731): 1043–52.

Can, Dao, Arad, Terrillion, Piantadosi and Gould. 2011. “The Mouse Forced Swim Test.” *Journal of Visualized Experiments* (58): e3638.

Carleton, Petreanu, Lansford, Alvarez-Buylla and Lledo. 2003. “Becoming a New Neuron in the Adult Olfactory Bulb.” *Nature Neuroscience* 6(5): 507–18.

Di Carlo, Trignani, Caravatta, Vinciguerra, Augurio, Perrotti, Di Tommaso, Nuzzo, Giancaterino, Falco and Genovesi. 2018. “Épargne De L’Hippocampe Lors De La Radiothérapie En Conditions Stéréotaxiques Des Métastases Cérébrales: Faut-Il Délinéer Ou Non L’Hippocampe ?” *Cancer/Radiotherapie*.

Ceresoli. 2004. “Gefitinib in Patients with Brain Metastases from Non-Small-Cell Lung Cancer: A Prospective Trial.” *Annals of Oncology* 15(7): 1042–47.

Chaffer and Weinberg. 2011. “A Perspective on Cancer Cell Metastasis.” *Science* 331(6024): 1559–64.

Chaker, Codega and Doetsch. 2016. “A Mosaic World: Puzzles Revealed by Adult Neural Stem Cell Heterogeneity.” *Wiley Interdisciplinary Reviews: Developmental Biology* 5(6): 640–58.

Chang, Wefel, Hess, Allen, Lang, Kornguth, Arbuckle, Swint, Shiu, Maor and Meyers. 2009. “Neurocognition in Patients with Brain Metastases Treated with Radiosurgery or Radiosurgery plus Whole-Brain Irradiation: A Randomised Controlled Trial.” *The Lancet Oncology* 10(11): 1037–44.

Chari, Crang and Blakemore. 2003. “Decline in Rate of Colonization of Oligodendrocyte Progenitor Cell (OPC)-Depleted Tissue by Adult OPCs with Age.” *Journal of Neuropathology and Experimental Neurology* 62(9): 908–16.

Cheng, Huang, Zhou, Wu, Donnola, Liu, Fang, Sloan, Mao, Lathia, Min, McLendon, Rich and Bao. 2013. “Glioblastoma Stem Cells Generate Vascular Pericytes to Support Vessel Function and Tumor Growth.” *Cell* 153(1): 139–52.

Cherry, Olschowka and O’Banion. 2014. “Neuroinflammation and M2 Microglia: The Good, the Bad, and the Inflamed.” *Journal of Neuroinflammation* 11: 98.

Chiang, Hong, Stalder, Sun, Withers and McBride. 1997. “Delayed Molecular Responses to Brain Irradiation.” *International Journal of Radiation Biology* 72(1): 45–53.

Chiang, McBride and Withers. 1993. “Radiation-Induced Astrocytic and Microglial Responses in Mouse Brain.” *Radiotherapy and Oncology* 29(1): 60–68.

Chinot, Honore, Dufour, Barrie, Figarella-Branger, Muracciole, Braguer, Martin and Grisoli. 2001. “Safety and Efficacy of Temozolomide in Patients with Recurrent Anaplastic Oligodendrogliomas after Standard Radiotherapy and Chemotherapy.” *Journal of Clinical Oncology* 19(9): 2449–55.

Chow, Li and Wong. 2000. “Radiation-Induced Apoptosis in the Adult Central Nervous System Is P53-Dependent.” *Cell death and differentiation* 7(8): 712–20.

Christie, Acharya, Parihar, Nguyen, Martirosian and Limoli. 2012. “Impaired Cognitive Function and Hippocampal Neurogenesis Following Cancer Chemotherapy.” *Clinical Cancer Research* 18(7): 1954–65.

Chung, Yock, Nelson, Xu, Keating and Tarbell. 2013. “Incidence of Second Malignancies among Patients Treated with Proton versus Photon Radiation.” *International Journal of Radiation Oncology Biology Physics* 87(1): 46–52.

Clarke, D L. 2003. “Neural Stem Cells.” *Bone Marrow Transplant* 32 Suppl 1(5): S13-7.

Clarke, Jennifer L., Iwamoto, Sul, Panageas, Lassman, DeAngelis, Hormigo, Nolan, Gavrilovic, Karimi and Abrey. 2009. “Randomized Phase II Trial of Chemoradiotherapy Followed by Either Dose-Dense or Metronomic Temozolomide for Newly Diagnosed Glioblastoma.” *Journal of Clinical Oncology* 27(23): 3861–67.

Clement, Sanchez, de Tribolet, Radovanovic and Ruiz i Altaba. 2007. “HEDGEHOG-GLI1 Signaling Regulates Human Glioma Growth, Cancer Stem Cell Self-Renewal, and Tumorigenicity.” *Current Biology* 17(2): 165–72.

Codega, Silva-Vargas, Paul, Maldonado-Soto, DeLeo, Pastrana and Doetsch. 2014. “Prospective Identification and Purification of Quiescent Adult Neural Stem Cells from Their In Vivo Niche.” *Neuron* 82(3): 545–59.

Conner, Payne, Forbes, Robbins and Riddle. 2010. “Effects of the AT_1 Receptor Antagonist L-158,809 on Microglia and Neurogenesis after Fractionated Whole-Brain Irradiation.” *Radiation Research* 173(1): 49–61.

Copeland, DeMoor, Moore and Ater. 1999. “Neurocognitive Development of Children after a Cerebellar Tumor in Infancy: A Longitudinal Study.” *Journal of Clinical Oncology* 17(11): 3476–86.

Cramer, Yoon, Reinsvold, Joo, Norris, Hood, Adamson, Klein, Kirsch and Oldham. 2015. “Treatment Planning and Delivery of Whole Brain Irradiation with Hippocampal Avoidance in Rats.” *PLoS ONE* 10(12): e0143208.

Crosbie, Fournier, Bartzsch, Donzelli, Cornelius, Stevenson, Requardt and Bräuer-Krisch. 2015. “Energy Spectra Considerations for Synchrotron Radiotherapy Trials on the ID17 Bio-Medical Beamline at the European Synchrotron Radiation Facility.” *Journal of Synchrotron Radiation* 22(4): 1035–41.

Crossen, Garwood, Glatstein and Neuwelt. 1994. “Neurobehavioral Sequelae of Cranial Irradiation in

Adults: A Review of Radiation-Induced Encephalopathy." *Journal of Clinical Oncology* 12(3): 627–42.

Curie and Becquerel. 1901. "Action Physiologique Des Rayons Du Radium." *CR Acad Sci Gen* 132: 1289–91.

d'Avella, Cicciarello, Angileri, Lucerna, La Torre and Tomasello. 1998. "Radiation-Induced Blood-Brain Barrier Changes: Pathophysiological Mechanisms and Clinical Implications." *Acta neurochirurgica. Supplement* 71: 282–84.

Daneman and Prat. 2015. "The Blood–Brain Barrier." *Cold Spring Harbor Perspectives in Biology* 7(1): a020412.

Davies and Clarke. 2004. "Early Symptoms of Brain Tumours." *Journal of Neurology, Neurosurgery and Psychiatry* 75(8): 1205–6.

Daynac, Morizur, Kortulewski, Gauthier, Ruat, Mouthon and Boussin. 2015. "Cell Sorting of Neural Stem and Progenitor Cells from the Adult Mouse Subventricular Zone and Live-Imaging of Their Cell Cycle Dynamics." *Journal of Visualized Experiments* (103).

Daynac, Chicheportiche, Pineda, Gauthier, Boussin and Mouthon. 2013. "Quiescent Neural Stem Cells Exit Dormancy upon Alteration of GABAAR Signaling Following Radiation Damage." *Stem Cell Research* 11(1): 516–28.

Daynac, Tirou, Faure, Mouthon, Gauthier, Hahn, Boussin and Ruat. 2016. "Hedgehog Controls Quiescence and Activation of Neural Stem Cells in the Adult Ventricular-Subventricular Zone." *Stem Cell Reports* 7(4): 735–48.

DeAngelis, Delattre and Posner. 1989. "Radiation-Induced Dementia in Patients Cured of Brain Metastases." *Neurology* 39(6): 789–96.

Delattre, Krol, Thaler and Posner. 1988. "Distribution of Brain Metastases." *Archives of Neurology* 45(7): 741–44.

Derosa. 2010. "Efficacy and Tolerability of Pioglitazone in Patients with Type 2 Diabetes Mellitus: Comparison with Other Oral Antihyperglycaemic Agents." *Drugs* 70(15): 1945–61.

Desouky, Ding and Zhou. 2015. "Targeted and Non-Targeted Effects of Ionizing Radiation." *Journal of Radiation Research and Applied Sciences* 8(2): 247–54.

Despeignes. 1896. "Observation Concernant Un Cas de Cancer de l'estomac Traite Par Les Rayons Roentgen." *Lyon Med.* 82: 428–30.

Dewey. 1969. "An Oxygen-Dependent X-Ray Dose-Rate Effect in Serratia Marcescens." *Radiation Research* 38(3): 467.

Dewey and Boag. 1959. "Modification of the Oxygen Effect When Bacteria Are given Large Pulses of Radiation." *Nature* 183(4673): 1450–51.

Doetsch, F., Garcia-Verdugo and Alvarez-Buylla. 1999. "Regeneration of a Germinal Layer in the Adult

Mammalian Brain." *Proceedings of the National Academy of Sciences* 96(20): 11619–24.

Doetsch, F, García-Verdugo and Alvarez-Buylla. 1997. "Cellular Composition and Three-Dimensional Organization of the Subventricular Germinal Zone in the Adult Mammalian Brain." *The Journal of neuroscience : the official journal of the Society for Neuroscience* 17(13)(13): 5046–61.

Doetsch, Fiona. 2003. "A Niche for Adult Neural Stem Cells." *Current Opinion in Genetics and Development* 13(5): 543–50.

Dong, Luo, Huang, Zhang, Tong, Cheng, Cai, Dong, Wu and Cheng. 2015. "Relationship between Irradiation-Induced Neuro-Inflammatory Environments and Impaired Cognitive Function in the Developing Brain of Mice." *International Journal of Radiation Biology* 91(3): 224–39.

Dziadziuszko, Ardizzoni, Postmus, Smit, Price, Debruyne, Legrand and Giaccone. 2003. "Temozolomide in Patients with Advanced Non-Small Cell Lung Cancer with and without Brain Metastases: A Phase II Study of the EORTC Lung Cancer Group (08965)." *European Journal of Cancer* 39(9): 1271–76.

Eaton, MacDonald, Yock and Tarbell. 2015. "Secondary Malignancy Risk Following Proton Radiation Therapy." *Frontiers in Oncology* 5: 261.

Edelstein, Richard and Bernstein. 2017. "Neurocognitive Impact of Cranial Radiation in Adults with Cancer: An Update of Recent Findings." *Current Opinion in Supportive and Palliative Care* 11(1): 32–37.

Eichler, A. F. and Loeffler. 2007. "Multidisciplinary Management of Brain Metastases." *The Oncologist* 12(7): 884–98.

Eichler, April F., Kuter, Ryan, Schapira, Younger and Henson. 2008. "Survival in Patients with Brain Metastases from Breast Cancer: The Importance of HER-2 Status." *Cancer* 112(11): 2359–67.

Eichler, April F., Kahle, Wang, Joshi, Willers, Engelman, Lynch and Sequist. 2010. "EGFR Mutation Status and Survival after Diagnosis of Brain Metastasis in Nonsmall Cell Lung Cancer." *Neuro-Oncology* 12(11): 1193–99.

Ekdahl, Kokaia and Lindvall. 2009. "Brain Inflammation and Adult Neurogenesis: The Dual Role of Microglia." *Neuroscience* 158(3): 1021–29.

Elmore, Najafi, Koike, Dagher, Spangenberg, Rice, Kitazawa, Matusow, Nguyen, West and Green. 2014. "Colony-Stimulating Factor 1 Receptor Signaling Is Necessary for Microglia Viability, Unmasking a Microglia Progenitor Cell in the Adult Brain." *Neuron* 82(2): 380–97.

Elmore, Lee, West and Green. 2015. "Characterizing Newly Repopulated Microglia in the Adult Mouse: Impacts on Animal Behavior, Cell Morphology, and Neuroinflammation" ed. Thomas Langmann. *PLoS ONE* 10(4): e0122912.

Epp, Weiss, Djordjevic and Santomasso. 1972. "The Radiosensitivity of Cultured Mammalian Cells Exposed to Single High Intensity Pulses of Electrons in Various Concentrations of Oxygen." *Radiation Research* 52(2): 324.

Esteller, Garcia-Foncillas, Andion, Goodman, Hidalgo, Vanaclocha, Baylin and Herman. 2000. “Inactivation of the DNA-Repair Gene *MGMT* and the Clinical Response of Gliomas to Alkylating Agents.” *New England Journal of Medicine* 343(19): 1350–54.

Facchino, Abdouh and Bernier. 2011. “Brain Cancer Stem Cells: Current Status on Glioblastoma Multiforme.” *Cancers* 3(2): 1777–97.

Fardone, Bravin, Conti, Bräuer-Krisch, Requardt, Bucci, Le Duc, Battaglia and Romanelli. 2017. “Rat Sensorimotor Cortex Tolerance to Parallel Transections Induced by Synchrotron-Generated X-Ray Microbeams.” *Scientific Reports* 7(1): 14290.

De Faria, De Oliveira, De Oliveira, Romano, Neto and Maia. 2008. “Differences in the Expression Pattern of P-Glycoprotein and MRP1 in Low-Grade and High-Grade Gliomas.” *Cancer Investigation* 26(9): 883–89.

Farrell and Plotkin. 2007. “Genetic Causes of Brain Tumors: Neurofibromatosis, Tuberous Sclerosis, von Hippel-Lindau, and Other Syndromes.” *Neurologic Clinics* 25(4): 925–46.

Favaudon, V., Fouillade and Vozenin. 2015. “Radiothérapie « flash » à Très Haut Débit de Dose: Un Moyen d’augmenter l’indice Thérapeutique Par Minimisation Des Dommages Aux Tissus Sains ?” *Cancer/Radiotherapie* 19(6–7): 526–31.

Favaudon, Vincent, Fouillade and Vozenin. 2015. “La Radiothérapie FLASH Pour Épargner Les Tissus Sains.” *Medecine/Sciences* 31(2): 121–23.

Favaudon, Vincent, Caplier, Monceau, Pouzoulet, Sayarath, Fouillade, Poupon, Brito, Hupé, Bourhis, Hall, Fontaine and Vozenin. 2014. “Ultrahigh Dose-Rate FLASH Irradiation Increases the Differential Response between Normal and Tumor Tissue in Mice.” *Science Translational Medicine* 6(245).

Feldmann, Brossart, Zipfel and Von Lilienfeld-Toal. 2013. “Mixed Response to Ipilimumab in a Melanoma Patient with Brain Metastases: Case Report and Review of the Literature.” *Case Reports in Oncology* 6(1): 229–35.

Ferrer, Serrano, Alcantara, Tortosa and Graus. 1993. “X-Ray-Induced Cell Death in the Developing Hippocampal Complex Involves Neurons and Requires Protein Synthesis.” *J Neuropathol Exp Neurol* 52(4): 370–78.

Field and Bewley. 1974. “Effects of Dose-Rate on the Radiation Response of Rat Skin.” *International journal of radiation biology and related studies in physics, chemistry, and medicine* 26(3): 259–67.

Fisher, Schwartzbaum, Wrensch and Wiemels. 2007. “Epidemiology of Brain Tumors.” *Neurologic Clinics* 25(4): 867–90.

Fouillade, Favaudon, Vozenin, Romeo, Bourhis, Verrelle, Devauchelle, Patriarca, Heinrich, Mazal and Dutreix. 2017. “Les Promesses Du Haut Débit de Dose En Radiothérapie.” *Bulletin du Cancer* 104(4): 380–84.

Fournier, Crosbie, Cornelius, Berkvens, Donzelli, Clavel, Rosenfeld, Petasecca, Lerch and Bräuer-Krisch. 2016. “Absorbed Dose-to-Water Protocol Applied to Synchrotron-Generated x-Rays at Very High Dose Rates.” *Physics in Medicine and Biology* 61(14): N349–61.

Fox, Cheung, Patel, Suki and Rao. 2011. “Epidemiology of Metastatic Brain Tumors.” *Neurosurgery Clinics of North America* 22(1): 1–6.

Fu, Shen, Xu, Luo and Tang. 2014. “Phagocytosis of Microglia in the Central Nervous System Diseases.” *Molecular Neurobiology* 49(3): 1422–34.

Gajjar … Gilbertson. 2006. “Risk-Adapted Craniospinal Radiotherapy Followed by High-Dose Chemotherapy and Stem-Cell Rescue in Children with Newly Diagnosed Medulloblastoma (St Jude Medulloblastoma-96): Long-Term Results from a Prospective, Multicentre Trial.” *Lancet Oncology* 7(10): 813–20.

Gangloff and Haley. 1960. “Effects of X-Irradiation on Spontaneous and Evoked Brain Electrical Activity in Cats.” *Radiat Res* 12(6): 694–704.

Gavrilovic and Posner. 2005. “Brain Metastases: Epidemiology and Pathophysiology.” *Journal of Neuro-Oncology* 75(1): 5–14.

Gebicke-Haerter. 2001. “Microglia in Neurodegeneration: Molecular Aspects.” *Microscopy Research and Technique* 54(1): 47–58.

Gehrke, Baisley, Sonck, Wronski and Feuerstein. 2013. “Neurocognitive Deficits Following Primary Brain Tumor Treatment: Systematic Review of a Decade of Comparative Studies.” *Journal of Neuro-Oncology* 115(2): 135–42.

Gheusi, Cremer, McLean, Chazal, Vincent and Lledo. 2000. “Importance of Newly Generated Neurons in the Adult Olfactory Bulb for Odor Discrimination.” *Proceedings of the National Academy of Sciences of the United States of America* 97(4): 1823–28.

Gilbert … Mehta. 2014. “A Randomized Trial of Bevacizumab for Newly Diagnosed Glioblastoma.” *New England Journal of Medicine* 370(8): 699–708.

Giovagnoli and Boiardi. 1994. “Cognitive Impairment and Quality of Life in Long-Term Survivors of Malignant Brain Tumors.” *The Italian Journal of Neurological Sciences* 15(9): 481–88.

Glaser, Abdul Rashid, U and Walker. 1997. “School Behaviour and Health Status after Central Nervous System Tumours in Childhood.” *British journal of cancer* 76(5): 643–50.

Glauser and Packer. 1991. “Cognitive Deficits in Long-Term Survivors of Childhood Brain Tumors.” *Childs Nervous System* 7(1): 2–12.

Gleason, Case, Rapp, Ip, Naughton, Butler, McMullen, Stieber, Saconn and Shaw. 2007. “Symptom Clusters in Patients with Newly-Diagnosed Brain Tumors.” *The journal of supportive oncology* 5(9): 427–33, 436.

Gondi, Pugh, Tome, Caine, Corn, Kanner, Rowley, Kundapur, DeNittis, Greenspoon, Konski, Bauman, Shah, Shi, Wendland, Kachnic and Mehta. 2014. “Preservation of Memory with Conformal Avoidance of the Hippocampal Neural Stem-Cell Compartment during Whole-Brain Radiotherapy for Brain Metastases (RTOG 0933): A Phase II Multi-Institutional Trial.” *Journal of Clinical Oncology* 32(34): 3810–16.

Goodenberger and Jenkins. 2012. “Genetics of Adult Glioma.” *Cancer Genetics* 205(12): 613–21.

Greene-Schloesser, Robbins, Peiffer, Shaw, Wheeler and Chan. 2012. “Radiation-Induced Brain Injury: A Review.” *Frontiers in Oncology* 2(July): 73.

Greene-Schloesser, Payne, Peiffer, Hsu, Riddle, Zhao, Chan, Metheny-Barlow and Robbins. 2014. “The Peroxisomal Proliferator-Activated Receptor (PPAR) α Agonist, Fenofibrate, Prevents Fractionated Whole-Brain Irradiation-Induced Cognitive Impairment.” *Radiation Research* 181(1): 33–44.

Grill, Renaux, Bulteau, Viguier, Levy-Piebois, Sainte-Rose, Dellatolas, Raquin, Jambaqué and Kalifa. 1999. “Long-Term Intellectual Outcome in Children with Posterior Fossa Tumors According to Radiation Doses and Volumes.” *International Journal of Radiation Oncology Biology Physics* 45(1): 137–45.

Grotzer, Schültke, Bräuer-Krisch and Laissue. 2015. “Microbeam Radiation Therapy: Clinical Perspectives.” *Physica Medica* 31(6): 564–67.

Grubbé. 1933. “Priority in the Therapeutic Use of X-rays.” *Radiology* 21(2): 156–62.

Guzowski, Lyford, Stevenson, Houston, McGaugh, Worley and Barnes. 2000. “Inhibition of Activity-Dependent Arc Protein Expression in the Rat Hippocampus Impairs the Maintenance of Long-Term Potentiation and the Consolidation of Long-Term Memory.” *The Journal of neuroscience : the official journal of the Society for Neuroscience* 20(11): 3993–4001.

Halasz and Rockhill. 2013. “Stereotactic Radiosurgery and Stereotactic Radiotherapy for Brain Metastases.” *Surgical Neurology International* 4(5): 185.

Hazard, Jensen and Shrieve. 2005. “Role of Stereotactic Radiosurgery in the Treatment of Brain Metastases.” *Am J Clin Oncol* 28(4): 403–10.

Hegi, Diserens, Gorlia, Hamou, de Tribolet, Weller, Kros, Hainfellner, Mason, Mariani, Bromberg, Hau, Mirimanoff, Cairncross, Janzer and Stupp. 2005. “*MGMT* Gene Silencing and Benefit from Temozolomide in Glioblastoma.” *New England Journal of Medicine* 352(10): 997–1003.

Hendry, Moore, Hodgson and Keene. 1982. “The Constant Low Oxygen Concentration in All the Target Cells for Mouse Tail Radionecrosis.” *Radiation research* 92(1): 172–81.

Herrera, Bourhis and Coukos. 2017. “Radiotherapy Combination Opportunities Leveraging Immunity for the next Oncology Practice.” *CA: A Cancer Journal for Clinicians* 67(1): 65–85.

Hicks, Platt, Kent and Haley. 2017. “Canine Brain Tumours: A Model for the Human Disease?” *Veterinary and Comparative Oncology* 15(1): 252–72.

Hochberg and Slotnick. 1980. “Neuropsychologic Impairment in Astrocytoma Survivors.” *Neurology* 30(2): 172–77.

Hodi, Oble, Drappatz, Velazquez, Ramaiya, Ramakrishna, Day, Kruse, Rae, Hoos and Mihm. 2008. “CTLA-4 Blockade with Ipilimumab Induces Significant Clinical Benefit in a Female with Melanoma Metastases to the CNS.” *Nature Clinical Practice Oncology* 5(9): 557–61.

Hofmann, Kiecker, Wurm, Schlenger, Budach, Sterry and Trefzer. 2006. “Temozolomide with or without Radiotherapy in Melanoma with Unresectable Brain Metastases.” *Journal of Neuro-Oncology* 76(1): 59–64.

Hong, Chiang, Campbell, Sun, Withers and McBride. 1995. “Induction of Acute Phase Gene Expression by Brain Irradiation.” *International Journal of Radiation Oncology, Biology, Physics* 33(3): 619–26.

Hoppe-Hirsch, Renier, Lellouch-Tubiana, Sainte-Rose, Pierre-Kahn and Hirsch. 1990. “Medulloblastoma in Childhood: Progressive Intellectual Deterioration.” *Child’s Nervous System* 6(2): 60–65.

Hoppe-Hirsch, Brunet, Laroussinie, Cinalli, Pierre-Kahn, Rénier, Sainte-Rose and Hirsch. 1995. “Intellectual Outcome in Children with Malignant Tumors of the Posterior Fossa: Influence of the Field of Irradiation and Quality of Surgery.” *Child’s Nervous System* 11(6): 340–45.

Huang … Li. 2018. “Clinical Cell Therapy Guidelines for Neurorestoration (IANR/CANR 2017).” *Cell Transplantation* 27(2): 310–24.

Hwang, Jung, Kim, Lim, Oh, Kim, Ji, Joe, Cho and Han. 2006. “Ionizing Radiation Induces Astrocyte Gliosis through Microglia Activation.” *Neurobiology of Disease* 21(3): 457–67.

Inada, Nishio, Amino, Abe and Saito. 1980. “High Dose-Rate Dependence of Early Skin Reaction in Mouse.” *International Journal of Radiation Biology and Related Studies in Physics, Chemistry and Medicine* 38(2): 139–45.

Irollo and Pirozzi. 2013. “CD133: To Be or Not to Be, Is This the Real Question?” *American journal of translational research* 5(6): 563–81.

Jaccard, Petersson, Buchillier, Germond, Durán, Vozenin, Bourhis, Bochud and Bailat. 2017. “High Dose-per-Pulse Electron Beam Dosimetry: Usability and Dose-Rate Independence of EBT3 Gafchromic Films: Usability.” *Medical Physics* 44(2): 725–35.

Jaccard, Durán, Petersson, Germond, Liger, Vozenin, Bourhis, Bochud and Bailat. 2018. “High Dose-per-Pulse Electron Beam Dosimetry: Commissioning of the Oriatron ERT6 Prototype Linear Accelerator for Preclinical Use: Commissioning.” *Medical Physics* 45(2): 863–74.

Jacquet. 2016. “Potential of Compact Compton Sources in the Medical Field.” *Physica Medica* 32(12): 1790–94.

Jacquet and Suortti. 2015. “Radiation Therapy at Compact Compton Sources.” *Physica Medica* 31(6): 596–600.

Jäkel and Dimou. 2017. “Glial Cells and Their Function in the Adult Brain: A Journey through the History of Their Ablation.” *Frontiers in Cellular Neuroscience* 11: 24.

Jenrow, Brown, Liu, Kolozsvary, Lapanowski and Kim. 2010. “Ramipril Mitigates Radiation-Induced Impairment of Neurogenesis in the Rat Dentate Gyrus.” *Radiation Oncology* 5(1): 6.

Johannesen, Lien, Hole and Lote. 2003. “Radiological and Clinical Assessment of Long-Term Brain Tumour Survivors after Radiotherapy.” *Radiotherapy and Oncology* 69(2): 169–76.

Johnson, Cullen, Barnholtz-Sloan, Ostrom, Langer, Turner, McKean-Cowdin, Fisher, Lupo, Partap, Schwartzbaum and Scheurer. 2014. “Childhood Brain Tumor Epidemiology: A Brain Tumor Epidemiology Consortium Review.” *Cancer Epidemiology Biomarkers and Prevention* 23(12): 2716–36.

Kahalley, Ris, Grosshans, Okcu, Paulino, Chintagumpala, Moore, Guffey, Minard, Stancel and Mahajan. 2016. “Comparing Intelligence Quotient Change after Treatment with Proton versus Photon Radiation Therapy for Pediatric Brain Tumors.” *Journal of Clinical Oncology* 34(10): 1043–49.

Karsch, Beyreuther, Burris-Mog, Kraft, Richter, Zeil and Pawelke. 2012. “Dose Rate Dependence for Different Dosimeters and Detectors: TLD, OSL, EBT Films, and Diamond Detectors.” *Medical Physics* 39(5): 2447–55.

Kempermann. 2002. “Why New Neurons ? Possible Functions for Adult Hippocampal.” *The Journal of Neuroscience* 22(3): 635–38.

Khuntia, Brown, Li and Mehta. 2006. “Whole-Brain Radiotherapy in the Management of Brain Metastasis.” *Journal of Clinical Oncology* 24(8): 1295–1304.

Kim, Kim, Eun, Park, Kim, Kim, Park, Vinsant, Oppenheim and Sun. 2007. “Impaired Migration in the Rostral Migratory Stream But Spared Olfactory Function after the Elimination of Programmed Cell Death in Bax Knock-Out Mice.” *Journal of Neuroscience* 27(52): 14392–403.

Kleihues and Ohgaki. 1999. “Primary and Secondary Glioblastomas: From Concept to Clinical Diagnosis.” *Neuro-Oncology* 1(1): 44–51.

Klein, M., Heimans, Aaronson, Van Der Ploeg, Grit, Muller, Postma, Mooij, Boerman, Beute, Ossenkoppele, Van Imhoff, Dekker, Jolles, Slotman, Struikmans and Taphoorn. 2002. “Effect of Radiotherapy and Other Treatment-Related Factors on Mid-Term to Long-Term Cognitive Sequelae in Low-Grade Gliomas: A Comparative Study.” *Lancet* 360(9343): 1361–68.

Klein, Olivier, Chastagner, Pretat, Bernier-Chastagner, Joud and Marchal. 2015. *Médulloblastomes de l’enfant - L’encyclopédie Neurochirurgicale*.

Knisely, de Lotbiniere, De Lanerolle and Brines. 2004. “Randomized Trial of Erythropoietin as a Central Nervous System Radioprotectant.” *International Journal of Radiation Oncology*Biology*Physics* 60(1): S343–44.

Komotar, Otten, Garrett and Anderson. 2009. “Treatment of Early Childhood Medulloblastoma by Postoperative Chemotherapy Alone-a Critical Review.” *Clinical medicine. Oncology* 3: 13–14.

Kool … Pfister. 2012. “Molecular Subgroups of Medulloblastoma: An International Meta-Analysis of Transcriptome, Genetic Aberrations, and Clinical Data of WNT, SHH, Group 3, and Group 4 Medulloblastomas.” *Acta Neuropathologica* 123(4): 473–84.

Koso, Takeda, Yew, Ward, Nariai, Ueno, Nagasaki, Watanabe, Rust, Adams, Copeland and Jenkins. 2012. “Transposon Mutagenesis Identifies Genes That Transform Neural Stem Cells into Glioma-Initiating Cells.” *Proceedings of the National Academy of Sciences of the United States of America* 109(44): E2998-3007.

Kuhlthau, Pulsifer, Yeap, Morales, Delahaye, Hill, Ebb, Abrams, MacDonald, Tarbell and Yock. 2012. “Prospective Study of Health-Related Quality of Life for Children with Brain Tumors Treated with Proton Radiotherapy.” *Journal of Clinical Oncology* 30(17): 2079–86.

Kurita, Kawahara, Asai, Ueki, Shin and Kirino. 2001. “Radiation-Induced Apoptosis of Oligodendrocytes in the Adult Rat Brain.” *Neurological Research* 23(8): 869–74.

Kyrkanides, Olschowka, Williams, Hansen and O’Banion. 1999. “TNFα and IL-1β Mediate Intercellular Adhesion Molecule-1 Induction via Microglia-Astrocyte Interaction in CNS Radiation Injury.” *Journal of Neuroimmunology* 95(1–2): 95–106.

Ladeby, Wirenfeldt, Dalmau, Gregersen, García-Ovejero, Babcock, Owens and Finsen. 2005. “Proliferating Resident Microglia Express the Stem Cell Antigen CD34 in Response to Acute Neural Injury.” *Glia* 50(2): 121–31.

Laissue, Lyubimova, Wagner, Archer, Slatkin, Michiel, Nemoz, Renier, Bräuer-Krisch, Spanne, Gebbers, Dixon and Blattmann. 1999. “Microbeam Radiation Therapy.” In *Proceedings of SPIE*, eds. H. Bradford Barber and Hans Roehrig. International Society for Optics and Photonics, 38–45.

Lamproglou, Chen, Boisserie, Mazeron, Poisson, Baillet, Le Poncin and Delattre. 1995. “Radiation-Induced Cognitive Dysfunction: An Experimental Model in the Old Rat.” *International Journal of Radiation Oncology, Biology, Physics* 31(1): 65–70.

Langley and Fidler. 2011. “The Seed and Soil Hypothesis Revisited-The Role of Tumor-Stroma Interactions in Metastasis to Different Organs.” *International Journal of Cancer* 128(11): 2527–35.

Laukkanen, Klonoff, Allan, Graeb and Murray. 1988. “The Role of Prophylactic Brain Irradiation in Limited Stage Small Cell Lung Cancer: Clinical, Neuropsychologic, and Ct Sequelae.” *International Journal of Radiation Oncology, Biology, Physics* 14(6): 1109–17.

Laurenti, Wilson and Trumpp. 2009. “Myc’s Other Life: Stem Cells and Beyond.” *Current Opinion in Cell Biology* 21(6): 844–54.

Lawrence and Livingston. 1932. “The Production of High Speed Light Ions without the Use of High

Voltages." *Physical Review* 40(1): 19–35.

Lawson, Perry and Gordon. 1992. "Turnover of Resident Microglia in the Normal Adult Mouse Brain." *Neuroscience* 48(2): 405–15.

Lazarini, Mouthon, Gheusi, de Chaumont, Olivo-Marin, Lamarque, Abrous, Boussin and Lledo. 2009. "Cellular and Behavioral Effects of Cranial Irradiation of the Subventricular Zone in Adult Mice." *PLoS ONE* 4(9): e7017.

Lee, Tammy C, Greene-Schloesser, Payne, Diz, Hsu, Kooshki, Mustafa, Riddle, Zhao, Chan and Robbins. 2012. "Chronic Administration of the Angiotensin-Converting Enzyme Inhibitor, Ramipril, Prevents Fractionated Whole-Brain Irradiation-Induced Perirhinal Cortex-Dependent Cognitive Impairment." *Radiation research* 178(1): 46–56.

Lee, Won Hee, Cho, Sonntag and Lee. 2011. "Radiation Attenuates Physiological Angiogenesis by Differential Expression of VEGF, Ang-1, Tie-2 and Ang-2 in Rat Brain." *Radiation research* 176(6): 753–60.

Lee, Won Hee, Sonntag, Mitschelen, Yan and Lee. 2010. "Irradiation Induces Regionally Specific Alterations in Pro-Inflammatory Environments in Rat Brain." *International Journal of Radiation Biology* 86(2): 132–44.

Lee, Won Hee, Warrington, Sonntag and Lee. 2012. "Irradiation Alters MMP-2/TIMP-2 System and Collagen Type IV Degradation in Brain." *International Journal of Radiation Oncology Biology Physics* 82(5): 1559–66.

Lenting, Verhaak, ter Laan, Wesseling and Leenders. 2017. "Glioma: Experimental Models and Reality." *Acta Neuropathologica* 133(2): 263–82.

Li, Jing, Bentzen, Li, Renschler and Mehta. 2008. "Relationship Between Neurocognitive Function and Quality of Life After Whole-Brain Radiotherapy in Patients With Brain Metastasis." *International Journal of Radiation Oncology Biology Physics* 71(1): 64–70.

Li, Yu-Qing, Chen, Jain, Reilly and Wong. 2004. "Early Radiation-Induced Endothelial Cell Loss and Blood-Spinal Cord Barrier Breakdown in the Rat Spinal Cord." *Radiation research* 161(2): 143–52.

Li, Yu Qing, Chen, Haimovitz-Friedman, Reilly and Wong. 2003. "Endothelial Apoptosis Initiates Acute Blood-Brain Barrier Disruption after Ionizing Radiation." *Cancer Research* 63(18): 5950–56.

Lin … Winer. 2009. "Multicenter Phase II Study of Lapatinib in Patients with Brain Metastases from HER2-Positive Breast Cancer." *Clinical Cancer Research* 15(4): 1452–59.

Lin, Eierman, Greil, Campone, Kaufman, Steplewski, Lane, Zembryki, Rubin and Winer. 2011. "Randomized Phase II Study of Lapatinib plus Capecitabine or Lapatinib plus Topotecan for Patients with HER2-Positive Breast Cancer Brain Metastases." *Journal of Neuro-Oncology* 105(3): 613–20.

Lledo, Alonso and Grubb. 2006. "Adult Neurogenesis and Functional Plasticity in Neuronal Circuits."

Nature Reviews Neuroscience 7(3): 179–93.

Loo, Schuler, Lartey, Rafat, King, Trovati, Koong and Maxim. 2017. "(P003) Delivery of Ultra-Rapid Flash Radiation Therapy and Demonstration of Normal Tissue Sparing After Abdominal Irradiation of Mice." *International Journal of Radiation Oncology*Biology*Physics* 98(2): E16.

Louis. 2006. "Molecular Pathology of Malignant Gliomas." *Annual Review of Pathology: Mechanisms of Disease* 1(1): 97–117.

Louis, Perry, Reifenberger, von Deimling, Figarella-Branger, Cavenee, Ohgaki, Wiestler, Kleihues and Ellison. 2016. "The 2016 World Health Organization Classification of Tumors of the Central Nervous System: A Summary." *Acta Neuropathologica* 131(6): 803–20.

Lutterbach, Bartelt and Ostertag. 2002. "Long-Term Survival in Patients with Brain Metastases." *Journal of Cancer Research and Clinical Oncology* 128(8): 417–25.

Machida, Lonart and Britten. 2010. "Low (60 CGy) Doses of ^{56} Fe HZE-Particle Radiation Lead to a Persistent Reduction in the Glutamatergic Readily Releasable Pool in Rat Hippocampal Synaptosomes." *Radiation Research* 174(5): 618–23.

Madsen, Kristjansen, Bolwig and Wörtwein. 2003. "Arrested Neuronal Proliferation and Impaired Hippocampal Function Following Fractionated Brain Irradiation in the Adult Rat." *Neuroscience* 119(3): 635–42.

Malka, Faure and Gauduel. 2010. "Ultra-Short Electron Beams Based Spatio-Temporal Radiation Biology and Radiotherapy." *Mutation Research - Reviews in Mutation Research* 704(1–3): 142–51.

Di Martino, Giannelli, Traino and Lazzeri. 2005. "Ion Recombination Correction for Very High Dose-per-Pulse High-Energy Electron Beams." *Medical Physics* 32(7): 2204–10.

Marumoto, Friedmann-Morvinski, Yang, Hoffman and Verma. 2008. "Novel Mouse Glioma Model: Cell-Type and Region Specific Activation of Oncogenes Using Lentiviral Vectors." *Protocol Exchange*.

McKeage, Keating, Bhatnagar, Elam, Farnier and Mohiuddin. 2011. "Fenofibrate: A Review of Its Use in Dyslipidaemia." *Drugs* 71(14): 1917–46.

Merchant, Hua, Shukla, Ying, Nill and Oelfke. 2008. "Proton versus Photon Radiotherapy for Common Pediatric Brain Tumors: Comparison of Models of Dose Characteristics and Their Relationship to Cognitive Function." *Pediatric Blood and Cancer* 51(1): 110–17.

Meyers and Brown. 2006. "Role and Relevance of Neurocognitive Assessment in Clinical Trials of Patients with CNS Tumors." *Journal of Clinical Oncology* 24(8): 1305–9.

Mickley, Ferguson, Nemeth and Mulvihill. 1990. "Neural Grafts Attenuate Behavioral Deficits Produced by Early Radiation-Induced Hypoplasia of Fascia Dentata Granule Cells." *Brain Research* 509(2): 280–92.

Mildenberger, Beach, McGeer and Ludgate. 1990. "An Animal Model of Prophylactic Cranial Irradiation: Histologic Effects at Acute, Early and Delayed Stages." *International Journal of Radiation Oncology,*

Biology, Physics 18(5): 1051–60.

Miralbell, Lomax and Russo. 1997. “Potential Role of Proton Therapy in the Treatment of Pediatric Medulloblastoma/Primitive Neuro-Ectodermal Tumors: Spinal Theca Irradiation.” *International Journal of Radiation Oncology Biology Physics* 38(4): 805–11.

Mizumatsu, Monje, Morhardt, Rola, Palmer and Fike. 2003. “Extreme Sensitivity of Adult Neurogenesis to Low Doses of X-Irradiation.” *Cancer Research* 63(14): 4021–27.

Moeller, Chintagumpala, Philip, Grosshans, McAleer, Woo, Gidley, Vats and Mahajan. 2011. “Low Early Ototoxicity Rates for Pediatric Medulloblastoma Patients Treated with Proton Radiotherapy.” *Radiation Oncology* 6(1).

Molteni, Moulder, Cohen, Ward, Fish, Taylor, Wolfe, Brizio-Molteni and Veno. 2000. “Control of Radiation-Induced Pneumopathy and Lung Fibrosis by Angiotensin-Converting Enzyme Inhibitors and an Angiotensin II Type 1 Receptor Blocker.” *International journal of radiation biology* 76(4): 523–32.

Monje, Mizumatsu, Fike and Palmer. 2002. “Irradiation Induces Neural Precursor-Cell Dysfunction.” *Nature Medicine* 8(9): 955–62.

Monje, Vogel, Masek, Ligon, Fisher and Palmer. 2007. “Impaired Human Hippocampal Neurogenesis after Treatment for Central Nervous System Malignancies.” *Annals of Neurology* 62(5): 515–20.

Monje, Toda and Palmer. 2003. “Inflammatory Blockade Restores Adult Hippocampal Neurogenesis.” *Science* 302(5651): 1760–65.

Montay-Gruel, Meziani, Yakkala and Vozenin. 2018. “Expanding the Therapeutic Index of Radiation Therapy by Normal Tissue Protection.” *The British Journal of Radiology*: 20180008.

Montay-Gruel, Petersson, Jaccard, Boivin, Germond, Petit, Doenlen, Favaudon, Bochud, Bailat, Bourhis and Vozenin. 2017. “Irradiation in a Flash: Unique Sparing of Memory in Mice after Whole Brain Irradiation with Dose Rates above 100 Gy/S.” *Radiotherapy and Oncology* 124(3): 365–69.

Mornex, Thomas, Mohr, Hauschild, Delaunay, Lesimple, Tilgen, Nguyen, Guillot, Ulrich, Bourdin, Mousseau, Cupissol, Bonneterre, de Gislain, Bensadoun and Clavel. 2003. “Randomised Phase III Trial of Fotemustine versus Fotemustine plus Whole Brain Irradiation in Cerebral Metastases of Melanoma.” *Cancer radiotherapie : journal de la Societe francaise de radiotherapie oncologique* 7(1): 1–8.

Morshead, Reynolds, Craig, McBurney, Staines, Morassutti, Weiss and van der Kooy. 1994. “Neural Stem Cells in the Adult Mammalian Forebrain: A Relatively Quiescent Subpopulation of Subependymal Cells.” *Neuron* 13(5): 1071–82.

Moulder, Fish and Cohen. 2003. “ACE Inhibitors and AII Receptor Antagonists in the Treatment and Prevention of Bone Marrow Transplant Nephropathy.” *Current Pharmaceutical Design* 9(9): 737–49.

Mu, Björk-Eriksson, Nill, Oelfke, Johansson, Gagliardi, Johansson, Karlsson and Zackrisson. 2005. “Does

Electron and Proton Therapy Reduce the Risk of Radiation Induced Cancer after Spinal Irradiation for Childhood Medulloblastoma? A Comparative Treatment Planning Study." *Acta Oncologica* 44(6): 554–62.

Mulhern, R. K., Palmer, Reddick, Glass, Kun, Taylor, Langston and Gajjar. 2001. "Risks of Young Age for Selected Neurocognitive Deficits in Medulloblastoma Are Associated with White Matter Loss." *Journal of Clinical Oncology* 19(2): 472–79.

Mulhern, Raymond K., Merchant, Gajjar, Reddick and Kun. 2004. "Late Neurocognitive Sequelae in Survivors of Brain Tumours in Childhood." *Lancet Oncology* 5(7): 399–408.

Munoz, Rodriguez-Cruz, Greco, Nagula, Scotto and Rameshwar. 2014. "Temozolomide Induces the Production of Epidermal Growth Factor to Regulate MDR1 Expression in Glioblastoma Cells." *Molecular Cancer Therapeutics* 13(10): 2399–2411.

Munz. 2017. "Psychotherapie in Der Psychiatrie." *Nervenheilkunde* 36(10): 800–805.

Nagai, Tsunoda, Hori and Asada. 2000. "Selective Vulnerability to Radiation in the Hippocampal Dentate Granule Cells." *Surgical Neurology* 53(5): 503–7.

Nageswara Rao, Ye, Decker, Howe and Wetmore. 2011. "Therapeutic Doses of Cranial Irradiation Induce Hippocampus-Dependent Cognitive Deficits in Young Mice." *Journal of Neuro-Oncology* 105(2): 191–98.

Nieder, C, Leicht, Motaref, Nestle, Niewald and Schnabel. 1999. "Late Radiation Toxicity after Whole Brain Radiotherapy: The Influence of Antiepileptic Drugs." *American journal of clinical oncology* 22(6): 573–79.

Nieder, Carsten, Spanne, Mehta, Grosu and Geinitz. 2011. "Presentation, Patterns of Care, and Survival in Patients with Brain Metastases: What Has Changed in the Last 20 Years?" *Cancer* 117(11): 2505–12.

Niethammer, Grabher, Look and Mitchison. 2009. "A Tissue-Scale Gradient of Hydrogen Peroxide Mediates Rapid Wound Detection in Zebrafish." *Nature* 459(7249): 996–99.

Noel, Gumin, Raju and Tofilon. 1998. "Increased Expression of Prohormone Convertase-2 in the Irradiated Rat Brain." *The FASEB Journal* 12(15): 1725–30.

Olsen, Marzulla and Raber. 2014. "Impairment in Extinction of Contextual and Cued Fear Following Post-Training Whole-Body Irradiation." *Frontiers in Behavioral Neuroscience* 8: 231.

Olsen, Weber, Akinyeke and Raber. 2017. "Enhanced Cued Fear Memory Following Post-Training Whole Body Irradiation of 3-Month-Old Mice." *Behavioural Brain Research* 319: 181–87.

Oparka, Walczak, Malinska, van Oppen, Szczepanowska, Koopman and Wieckowski. 2016. "Quantifying ROS Levels Using CM-H2DCFDA and HyPer." *Methods* 109: 3–11.

Ostrom, Gittleman, Stetson, Virk and Barnholtz-Sloan. 2015. "Epidemiology of Gliomas." In *Cancer Treatment and Research*, Springer, Cham, 1–14.

Packer, Gurney, Punyko, Donaldson, Inskip, Stovall, Yasui, Mertens, Sklar, Nicholson, Zeltzer, Neglia and Robison. 2003. “Long-Term Neurologic and Neurosensory Sequelae in Adult Survivors of a Childhood Brain Tumor: Childhood Cancer Survivor Study.” *Journal of Clinical Oncology* 21(17): 3255–61.

Paget and London. 2006. “Observations from a Ploughman.” *Nature reviews. Cancer* 573(April): 7–23.

Palmer, Shawna L., Gajjar, Reddick, Glass, Kun, Wu, Xiong and Mulhern. 2003. “Predicting Intellectual Outcome among Children Treated with 35-40 Gy Craniospinal Irradiation for Medulloblastoma.” *Neuropsychology* 17(4): 548–55.

Palmer, Theo D., Willhoite and Gage. 2000. “Vascular Niche for Adult Hippocampal Neurogenesis.” *Journal of Comparative Neurology* 425(4): 479–94.

Parihar, Allen, Tran, Macaraeg, Chu, Kwok, Chmielewski, Craver, Baulch, Acharya, Cucinotta and Limoli. 2015. “What Happens to Your Brain on the Way to Mars.” *Science Advances* 1(4).

Peißner, Kocher, Treuer and Gillardon. 1999. “Ionizing Radiation-Induced Apoptosis of Proliferating Stem Cells in the Dentate Gyrus of the Adult Rat Hippocampus.” *Molecular Brain Research* 71(1): 61–68.

Pellow, Chopin, File and Briley. 1985. “Validation of Open : Closed Arm Entries in an Elevated plus-Maze as a Measure of Anxiety in the Rat.” *Journal of Neuroscience Methods* 14(3): 149–67.

Peña, Fuks and Kolesnick. 2000. “Radiation-Induced Apoptosis of Endothelial Cells in the Murine Central Nervous System: Protection by Fibroblast Growth Factor and Sphingomyelinase Deficiency.” *Cancer Research* 60(2): 321–27.

Perkins and Liu. 2016. 93 American Family Physician *Primary Brain Tumors in Adults: Diagnosis and Treatment*. American Academy of Family Physicians.

Petersson, Jaccard, Germond, Buchillier, Bochud, Bourhis, Vozeninz and Bailat. 2016. “High Dose-per-Pulse Electron Beam Dosimetry - A Saturation Model for the Advanced Markus Ionization Chamber.” *Strahlentherapie Und Onkologie* 192(11): 848–49.

Phillips and LeDoux. 1992. “Differential Contribution of Amygdala and Hippocampus to Cued and Contextual Fear Conditioning.” *Behavioral Neuroscience* 106(2): 274–85.

Piao, Major, Auyeung, Policarpio, Menon, Droms, Gutin, Uryu, Tchieu, Soulet and Tabar. 2015. “Human Embryonic Stem Cell-Derived Oligodendrocyte Progenitors Remyelinate the Brain and Rescue Behavioral Deficits Following Radiation.” *Cell Stem Cell* 16(2): 198–210.

Prempree, Michelsen and Merz. 1969. “The Repair Time of Chromosome Breaks Induced by Pulsed X-rays of Ultra-High Dose-Rate.” *International Journal of Radiation Biology* 15(6): 571–74.

Pulsifer, Sethi, Kuhlthau, Macdonald, Tarbell and Yock. 2015. “Early Cognitive Outcomes Following Proton Radiation in Pediatric Patients with Brain and Central Nervous System Tumors Presented at the American Society of Clinical Oncology, June 6, 2010, Chicago, IL; International Society of Paediatric Oncology, London,.” *International Journal of Radiation Oncology Biology Physics* 93(2): 400–407.

Quail and Joyce. 2017. “The Microenvironmental Landscape of Brain Tumors.” *Cancer cell* 31(3): 326–41.

Quinn, Jiang, Reardon, Desjardins, Vredenburgh, Rich, Gururangan, Friedman, Signer, Sampson, McLendon, Herndon, Walker and Friedman. 2009. “Phase II Trial of Temozolomide Plus O6-Benzylguanine in Adults with Recurrent, Temozolomide-Resistant Malignant Glioma.” *Journal of Clinical Oncology* 27(8): 1262–67.

Raber, Rola, LeFevour, Morhardt, Curley, Mizumatsu, VandenBerg and Fike. 2004. “Radiation-Induced Cognitive Impairments Are Associated with Changes in Indicators of Hippocampal Neurogenesis.” *Radiation Research* 162(1): 39–47.

Raff, Miller and Noble. 1983. “A Glial Progenitor Cell That Develops in Vitro into an Astrocyte or an Oligodendrocyte Depending on Culture Medium.” *Nature* 303(5916): 390–96.

Ramanan, Kooshki, Zhao, Hsu and Robbins. 2008. “PPARα Ligands Inhibit Radiation-Induced Microglial Inflammatory Responses by Negatively Regulating NF-KB and AP-1 Pathways.” *Free Radical Biology and Medicine* 45(12): 1695–1704.

Ramanan, Kooshki, Zhao, Hsu, Riddle and Robbins. 2009. “The PPARα Agonist Fenofibrate Preserves Hippocampal Neurogenesis and Inhibits Microglial Activation After Whole-Brain Irradiation.” *International Journal of Radiation Oncology Biology Physics* 75(3): 870–77.

Ramanan, Zhao, Riddle and Robbins. 2010. “Review Article: Role of PPARs in Radiation-Induced Brain Injury.” *PPAR Research* 2010: 234975.

Read. 2011. “Drosophila Melanogaster as a Model System for Human Brain Cancers.”

Régaud. 1920. “Fondements Rationnels, Indications Techniques et Résultats Généraux de La Radiothérapie Des Cancers.” *Journal de Radiologie et d’Électrologie* 4: 433–55.

Regnard, Duc, Bräuer-Krisch, Troprès, Siegbahn, Kusak, Clair, Bernard, Dallery, Laissue and Bravin. 2008. “Irradiation of Intracerebral 9L Gliosarcoma by a Single Array of Microplanar X-Ray Beams from a Synchrotron: Balance between Curing and Sparing.” *Physics in Medicine and Biology* 53(4): 861–78.

Reinhold, Calvo, Hopewell and Van Den Berg. 1990. “Development of Blood Vessel-Related Radiation Damage in the Fimbria of the Central Nervous System.” *International Journal of Radiation Oncology, Biology, Physics* 18(1): 37–42.

Rice, Spangenberg, Yamate-Morgan, Lee, Arora, Hernandez, Tenner, West and Green. 2015. “Elimination of Microglia Improves Functional Outcomes Following Extensive Neuronal Loss in the Hippocampus.” *Journal of Neuroscience* 35(27): 9977–89.

Ris, Packer, Goldwein, Jones-Wallace and Boyett. 2001. “Intellectual Outcome after Reduced-Dose Radiation Therapy plus Adjuvant Chemotherapy for Medulloblastoma: A Children’s Cancer Group Study.” *Journal of Clinical Oncology* 19(15): 3470–76.

Robbins, Payne, Tommasi, Diz, Hsu, Brown, Wheeler, Olson and Zhao. 2009. “The AT1Receptor

Antagonist, L-158,809, Prevents or Ameliorates Fractionated Whole-Brain Irradiation-Induced Cognitive Impairment." *International Journal of Radiation Oncology Biology Physics* 73(2): 499–505.

Rohde, Rea, Simon and McBride. 1979. "Effects of X-Irradiation Induced Loss of Cerebellar Granule Cells on the Synaptosomal Levels and the High Affinity Uptake of Amino Acids." *Journal of Neurochemistry* 32(5): 1431–35.

Rola, Raber, Rizk, Otsuka, Vandenberg, Morhardt and Fike. 2004. "Radiation-Induced Impairment of Hippocampal Neurogenesis Is Associated with Cognitive Deficits in Young Mice." *Experimental Neurology* 188(2): 316–30.

Rola, Sarkissian, Obenaus, Nelson, Otsuka, Limoli and Fike. 2005. "High-LET Radiation Induces Inflammation and Persistent Changes in Markers of Hippocampal Neurogenesis." *Radiation research* 164(4 Pt 2): 556–60.

Roman and Sperduto. 1995. "Neuropsychological Effects of Cranial Radiation: Current Knowledge and Future Directions." *International Journal of Radiation Oncology, Biology, Physics* 31(4): 983–98.

Röntgen. 1898. "Ueber Eine Neue Art von Strahlen." *Annalen der Physik* 300(1): 12–17.

Rosi, Andres-Mach, Fishman, Levy, Ferguson and Fike. 2008. "Cranial Irradiation Alters the Behaviorally Induced Immediate-Early Gene Arc (Activity-Regulated Cytoskeleton-Associated Protein)." *Cancer Research* 68(23): 9763–70.

Rosner, Nemoto and Lane. 1986. "Chemotherapy Induces Regression of Brain Metastases in Breast Carcinoma." *Cancer* 58(4): 832–39.

Sampson, Carter, Friedman and Seigler. 1998. "Demographics, Prognosis, and Therapy in 702 Patients with Brain Metastases from Malignant Melanoma." *Journal of Neurosurgery* 88(1): 11–20.

Sano, Sato and Tanaka. 1997. "Radiation-Induced Apoptosis and Injury of Oligodendrocytes on Neonatal Rat Brains." *Clinical Neurology and Neurosurgery* 99: S117.

Santana, Peña, Haimovitz-Friedman, Martin, Green, McLoughlin, Cordon-Cardo, Schuchman, Fuks and Kolesnick. 1996. "Acid Sphingomyelinase-Deficient Human Lymphoblasts and Mice Are Defective in Radiation-Induced Apoptosis." *Cell* 86(2): 189–99.

Sasaki, Matsumoto, Itoh, Kawabe, Ota, Yamada, Maruta, Soejima and Sugimura. 2000. "Target Cells of Apoptosis in the Adult Murine Dentate Gyrus and O4 Immunoreactivity after Ionizing Radiation." *Neuroscience Letters* 279(1): 57–60.

Sathornsumetee, Reardon, Desjardins, Quinn, Vredenburgh and Rich. 2007. "Molecularly Targeted Therapy for Malignant Glioma." *Cancer* 110(1): 12–24.

Schiff and Freund. 1898. "Beiträge Zur Radiotherapie." *Wiener Medizinische Wochenschrift* May 28: 1058–61.

Schindler, Forbes, Robbins and Riddle. 2008. "Aging-Dependent Changes in the Radiation Response of the

Adult Rat Brain." *International Journal of Radiation Oncology Biology Physics* 70(3): 826–34.

Schmid, Dollinger, Hable, Greubel, Zlobinskaya, Michalski, Molls and Röper. 2010. "Relative Biological Effectiveness of Pulsed and Continuous 20 MeV Protons for Micronucleus Induction in 3D Human Reconstructed Skin Tissue." *Radiotherapy and Oncology* 95(1): 66–72.

Schmid, Dollinger, Hable, Greubel, Zlobinskaya, Michalski, Auer, Friedl, Schmid, Molls and Röper. 2011. "The Effectiveness of 20 Mev Protons at Nanosecond Pulse Lengths in Producing Chromosome Aberrations in Human-Hamster Hybrid Cells." *Radiation research* 175(6): 719–27.

Schnegg, Greene-Schloesser, Kooshki, Payne, Hsu and Robbins. 2013. "The PPARδ Agonist GW0742 Inhibits Neuroinflammation, but Does Not Restore Neurogenesis or Prevent Early Delayed Hippocampal-Dependent Cognitive Impairment after Whole-Brain Irradiation." *Free Radical Biology and Medicine* 61: 1–9.

Schüler, Trovati, King, Lartey, Rafat, Villegas, Praxel, Loo and Maxim. 2017. "Experimental Platform for Ultra-High Dose Rate FLASH Irradiation of Small Animals Using a Clinical Linear Accelerator." *International Journal of Radiation Oncology Biology Physics* 97(1): 195–203.

Schultheiss and Stephens. 1992. "Invited Review: Permanent Radiation Myelopathy." *British Journal of Radiology* 65(777): 737–53.

Schwartzbaum, Fisher, Aldape and Wrensch. 2006. "Epidemiology and Molecular Pathology of Glioma." *Nature Clinical Practice Neurology* 2(9): 494–503.

Scott, Rewcastle, Brasher, Fulton, MacKinnon, Hamilton, Cairncross and Forsyth. 1999. "Which Glioblastoma Multiforme Patient Will Become a Long-Term Survivor? A Population-Based Study." *Annals of Neurology* 46(2): 183–88.

Serduc, Van De Looij, Francony, Verdonck, Van Der Sanden, Laissue, Farion, Bräuer-Krisch, Siegbahn, Bravin, Prezado, Segebarth, Rémy and Lahrech. 2008. "Characterization and Quantification of Cerebral Edema Induced by Synchrotron X-Ray Microbeam Radiation Therapy." *Physics in Medicine and Biology* 53(5): 1153–66.

Serduc, Vérant, Vial, Farion, Rocas, Rémy, Fadlallah, Brauer, Bravin, Laissue, Blattmann and Van Der Sanden. 2006. "In Vivo Two-Photon Microscopy Study of Short-Term Effects of Microbeam Irradiation on Normal Mouse Brain Microvasculature." *International Journal of Radiation Oncology Biology Physics* 64(5): 1519–27.

Sevenich, Bowman, Mason, Quail, Rapaport, Elie, Brogi, Brastianos, Hahn, Holsinger, Massaguï¿½, Leslie and Joyce. 2014. "Analysis of Tumour- and Stroma-Supplied Proteolytic Networks Reveals a Brain-Metastasis-Promoting Role for Cathepsin S." *Nature Cell Biology* 16(9): 876–88.

Shi, Molina, Robbins, Wheeler and Brunso-Bechtold. 2008. "Hippocampal Neuron Number Is Unchanged 1 Year After Fractionated Whole-Brain Irradiation at Middle Age." *International Journal of Radiation*

Oncology Biology Physics 71(2): 526–32.

Shi, Linville, Iversen, Molina, Yester, Wheeler, Robbins and Brunso-Bechtold. 2009. "Maintenance of White Matter Integrity in a Rat Model of Radiation-Induced Cognitive Impairment." *Journal of the Neurological Sciences* 285(1–2): 178–84.

Shinohara, Gobbel, Lamborn, Tada and Fike. 1997. "Apoptosis in the Subependyma of Young Adult Rats after Single and Fractionated Doses of X-rays." *Cancer Research* 57(13): 2694–2702.

Singh, Clarke, Terasaki, Bonn, Hawkins, Squire and Dirks. 2003. "Identification of a Cancer Stem Cell in Human Brain Tumors." *Cancer research* 63(18): 5821–28.

Slade and Stanic. 2016. "The Impact of RTOG 0614 and RTOG 0933 Trials in Routine Clinical Practice: The US Survey of Utilization of Memantine and IMRT Planning for Hippocampus Sparing in Patients Receiving Whole Brain Radiotherapy for Brain Metastases." *Contemporary Clinical Trials* 47: 74–77.

Smoll and Drummond. 2012. "The Incidence of Medulloblastomas and Primitive Neurectodermal Tumours in Adults and Children." *Journal of Clinical Neuroscience* 19(11): 1541–44.

Snyder, Hong, McDonald and Wojtowicz. 2005. "A Role for Adult Neurogenesis in Spatial Long-Term Memory." *Neuroscience* 130(4): 843–52.

Sofroniew. 2009. "Molecular Dissection of Reactive Astrogliosis and Glial Scar Formation." *Trends in Neurosciences* 32(12): 638–47.

Song, Stevens and Gage. 2002. "Astroglia Induce Neurogenesis from Adult Neural Stem Cells." *Nature* 417(6884): 39–44.

Spalding, Bergmann, Alkass, Bernard, Salehpour, Huttner, Boström, Westerlund, Vial, Buchholz, Possnert, Mash, Druid and Frisén. 2013. "XDynamics of Hippocampal Neurogenesis in Adult Humans." *Cell* 153(6): 1219–27.

Stafford, Hirai, Deng, Chernikova, Urata, West and Brown. 2016. "Colony Stimulating Factor 1 Receptor Inhibition Delays Recurrence of Glioblastoma after Radiation by Altering Myeloid Cell Recruitment and Polarization." *Neuro-Oncology* 18(6): 797–806.

Stahel, Smith, Bruchis and Rabb. 2008. "Peroxisome Proliferator-Activated Receptors: 'Key' Regulators of Neuroinflammation after Traumatic Brain Injury." *PPAR Research* 2008: 538141.

Stollg and Jander. 1999. "The Role of Microglia and Macrophages in the Pathophysiology of the CNS." *Progress in Neurobiology* 58(3): 233–47.

Streit, Mrak and Griffin. 2004. "Microglia and Neuroinflammation: A Pathological Perspective." *Journal of Neuroinflammation* 1(1): 14.

Stupp, R., Brada, van den Bent, Tonn and Pentheroudakis. 2014. "High-Grade Glioma: ESMO Clinical Practice Guidelines for Diagnosis, Treatment and Follow-Up." *Annals of Oncology* 25(suppl 3): 93–101.

Stupp, Roger … Mirimanoff. 2005. “Radiotherapy plus Concomitant\nand Adjuvant Temozolomide for Glioblastoma.” *The New England Journal of Medicine* 352(10): 987–96.

Stupp, Roger … Mirimanoff. 2009. “Effects of Radiotherapy with Concomitant and Adjuvant Temozolomide versus Radiotherapy Alone on Survival in Glioblastoma in a Randomised Phase III Study: 5-Year Analysis of the EORTC-NCIC Trial.” *The Lancet Oncology* 10(5): 459–66.

Su, Zhang, Zhao, Aschner, Chen and Luo. 2014. “The Interaction between Microglia and Neural Stem/Precursor Cells.” *Brain Research Bulletin* 109: 32–38.

Tada, Yang, Gobbel, Lamborn and Fike. 1999. “Long-Term Impairment of Subependymal Repopulation Following Damage by Ionizing Irradiation.” *Experimental Neurology* 160(1): 66–77.

Taylor, Northcott, Korshunov, Remke, Cho, Clifford, Eberhart, Parsons, Rutkowski, Gajjar, Ellison, Lichter, Gilbertson, Pomeroy, Kool and Pfister. 2012. “Molecular Subgroups of Medulloblastoma: The Current Consensus.” *Acta Neuropathologica* 123(4): 465–72.

Thippu Jayaprakash, Wildschut and Jena. 2017. “Feasibility of Hippocampal Avoidance Radiotherapy for Glioblastoma.” *Clinical Oncology* 29(11): 748–52.

Tofilon, Fike, Tofilona and Fikeb. 2016. “The Radioresponse of the Central Nervous System : A Dynamic Process The Radioresponse of the Central Nervous System : A Dynamic Pr.” *http://dx.doi.org/10.1667/0033-7587(2000)153[0357:TROTCN]2.0.CO;2* 153(4): 357–70.

Tome, Gokhan, Brodin, Gulinello, Heard, Mehler and Guha. 2015. “A Mouse Model Replicating Hippocampal Sparing Cranial Irradiation in Humans: A Tool for Identifying New Strategies to Limit Neurocognitive Decline.” *Scientific Reports* 5: 14384.

Tomé, Gökhan, Gulinello, Brodin, Heard, Mehler and Guha. 2016. “Hippocampal-Dependent Neurocognitive Impairment Following Cranial Irradiation Observed in Pre-Clinical Models: Current Knowledge and Possible Future Directions.” *The British Journal of Radiology* 89(1057): 20150762.

Town. 1967. “Effect of High Dose Rates on Survival of Mammalian Cells.” *Nature* 215(5103): 847–48.

Tsao, Rades, Wirth, Lo, Danielson, Gaspar, Sperduto, Vogelbaum, Radawski, Wang, Gillin, Mohideen, Hahn and Chang. 2012. “Radiotherapeutic and Surgical Management for Newly Diagnosed Brain Metastasis(Es): An American Society for Radiation Oncology Evidence-Based Guideline.” *Practical Radiation Oncology* 2(3): 210–25.

Tsao, Li, Lu, Xu and Wong. 1999. “Upregulation of Vascular Endothelial Growth Factor Is Associated with Radiation-Induced Blood-Spinal Cord Barrier Breakdown.” *Journal of Neuropathology and Experimental Neurology* 58(10): 1051–60.

Twijnstra, Boon, Lormans and Ten Velde. 1987. “Neurotoxicity of Prophylactic Cranial Irradiation in Patients with Small Cell Carcinoma of the Lung.” *European Journal of Cancer and Clinical Oncology* 23(7): 983–86.

Vigliani, Duyckaerts, Hauw, Poisson, Magdelenat and Delattre. 1999. “Dementia Following Treatment of Brain Tumors with Radiotherapy Administered Alone or in Combination with Nitrosourea-Based Chemotherapy: A Clinical and Pathological Study.” *Journal of Neuro-Oncology* 41(2): 137–49.

Villano, Seery and Bressler. 2009. “Temozolomide in Malignant Gliomas: Current Use and Future Targets.” *Cancer Chemotherapy and Pharmacology* 64(4): 647–55.

Villasana, Rosenberg and Raber. 2010. “Sex-Dependent Effects Of56Fe Irradiation on Contextual Fear Conditioning in C57BL/6J Mice.” *Hippocampus* 20(1): 19–23.

Vittori, Motaln and Turnšek. 2015. “The Study of Glioma by Xenotransplantation in Zebrafish Early Life Stages.” *Journal of Histochemistry and Cytochemistry* 63(10): 749–61.

Warrington, Csiszar, Johnson, Herman, Ahmad, Lee and Sonntag. 2011. “Cerebral Microvascular Rarefaction Induced by Whole Brain Radiation Is Reversible by Systemic Hypoxia in Mice.” *American Journal of Physiology-Heart and Circulatory Physiology* 300(3): H736-44.

Warrington, Csiszar, Mitschelen, Lee and Sonntag. 2012. “Whole Brain Radiation-Induced Impairments in Learning and Memory Are Time-Sensitive and Reversible by Systemic Hypoxia” ed. Ken Arai. *PLoS ONE* 7(1): e30444.

Weiss, Epp, Heslin, Ling and Santomasso. 1974. “Oxygen Depletion in Cells Irradiated at Ultra-High Dose-Rates and at Conventional Dose-Rates.” *International Journal of Radiation Biology* 26(1): 17–29.

Wen, Schiff, Kesari, Drappatz, Gigas and Doherty. 2006. “Medical Management of Patients with Brain Tumors.” *Journal of Neuro-Oncology* 80(3): 313–32.

Wen and Kesari. 2008. “Malignant Gliomas In .” *The New England journal of medicine* 359(5): 492–507.

Wheeler, Payne, D’Agostino, Walb, Munley, Metheny-Barlow and Robbins. 2014. “Impact of Breathing 100% Oxygen on Radiation-Induced Cognitive Impairment.” *Radiation Research* 182(5): 580–85.

Wilson, Karajannis and Harter. 2014. “Glioblastoma Multiforme: State of the Art and Future Therapeutics.” *Surgical Neurology International* 5(1): 64.

Winocur, Wojtowicz, Sekeres, Snyder and Wang. 2006. “Inhibition of Neurogenesis Interferes with Hippocampus-Dependent Memory Function.” *Hippocampus* 16(3): 296–304.

Wong, Hess, Gleason, Jaeckle, Kyritsis, Prados, Levin and Alfred Yung. 1999. “Outcomes and Prognostic Factors in Recurrent Glioma Patients Enrolled onto Phase II Clinical Trials.” *Journal of Clinical Oncology* 17(8): 2572–78.

Wrensch, Minn, Chew, Bondy and Berger. 2002. “Epidemiology of Primary Brain Tumors: Current Concepts and Review of the Literature.” *Neuro-Oncology* 4(4): 278–99.

Wu, Coultrap, Pinnix, Davies, Tailor, Ang, Browning and Grosshans. 2012. “Radiation Induces Acute Alterations in Neuronal Function” ed. Philip J. Tofilon. *PLoS ONE* 7(5): e37677.

Yan, Kowal, Akkari, Schuhmacher, Huse, West and Joyce. 2017. “Inhibition of Colony Stimulating Factor-1

Receptor Abrogates Microenvironment-Mediated Therapeutic Resistance in Gliomas." *Oncogene* 36(43): 6049–58.

Yang, Yang, Li, Li, Wu, Cheng and Tang. 2017. "Pathophysiological Responses in Rat and Mouse Models of Radiation-Induced Brain Injury." *Molecular Neurobiology* 54(2): 1022–32.

Yarnold and Haviland. 2010. "Hypofractionated Adjuvant Whole Breast Radiotherapy: Progress and Prospects." *Acta Oncologica* 49(8): 1288–92.

Yazlovitskaya, Edwards, Thotala, Fu, Osusky, Whetsell, Boone, Shinohara and Hallahan. 2006. "Lithium Treatment Prevents Neurocognitive Deficit Resulting from Cranial Irradiation." *Cancer Research* 66(23): 11179–86.

Yock, Yeap, Ebb, Weyman, Eaton, Sherry, Jones, MacDonald, Pulsifer, Lavally, Abrams, Huang, Marcus and Tarbell. 2016. "Long-Term Toxic Effects of Proton Radiotherapy for Paediatric Medulloblastoma: A Phase 2 Single-Arm Study." *The Lancet Oncology* 17(3): 287–98.

Yoon, Cramer, Miles, Reinsvold, Joo, Kirsch and Oldham. 2017. "A Precision 3D Conformal Treatment Technique in Rats: Application to Whole-Brain Radiotherapy with Hippocampal Avoidance: Application." *Medical Physics* 44(11): 6008–17.

Yuan, Gaber, McColgan, Naimark, Kiani and Merchant. 2003. "Radiation-Induced Permeability and Leukocyte Adhesion in the Rat Blood-Brain Barrier: Modulation with Anti-ICAM-1 Antibodies." *Brain Research* 969(1–2): 59–69.

Yuan, Gaber, Boyd, Wilson, Kiani and Merchant. 2006. "Effects of Fractionated Radiation on the Brain Vasculature in a Murine Model: Blood-Brain Barrier Permeability, Astrocyte Proliferation, and Ultrastructural Changes." *International Journal of Radiation Oncology Biology Physics* 66(3): 860–66.

Zhang, Jihong, F.G. Stevens and D. Bradshaw. 2012. "Temozolomide: Mechanisms of Action, Repair and Resistance." *Current Molecular Pharmacology* 5(1): 102–14.

Zhang, Rui, Howell, Taddei, Giebeler, Mahajan and Newhauser. 2014. "A Comparative Study on the Risks of Radiogenic Second Cancers and Cardiac Mortality in a Set of Pediatric Medulloblastoma Patients Treated with Photon or Proton Craniospinal Irradiation." *Radiotherapy and Oncology* 113(1): 84–88.

Zhao, Payne, Tommasi, Diz, Hsu and Robbins. 2007. "Administration of the Peroxisomal Proliferator-Activated Receptor γ Agonist Pioglitazone during Fractionated Brain Irradiation Prevents Radiation-Induced Cognitive Impairment." *International Journal of Radiation Oncology Biology Physics* 67(1): 6–9.

Zlobinskaya, Girst, Greubel, Hable, Siebenwirth, Walsh, Multhoff, Wilkens, Schmid and Dollinger. 2013. "Reduced Side Effects by Proton Microchannel Radiotherapy: Study in a Human Skin Model." *Radiation and Environmental Biophysics* 52(1): 123–33.