RADIOBIOLOGY SPECIAL FEATURE: REVIEW ARTICLE

New challenges in high-energy particle radiobiology

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ABSTRACT

Densely ionizing radiation has always been a main topic in radiobiology. In fact, α-particles and neutrons are sources of radiation exposure for the general population and workers in nuclear power plants. More recently, high-energy protons and heavy ions attracted a large interest for two applications: hadrontherapy in oncology and space radiation protection in manned space missions. For many years, studies concentrated on measurements of the relative biological effectiveness (RBE) of the energetic particles for different end points, especially cell killing (for radiotherapy) and carcinogenesis (for late effects). Although more recently, it has been shown that densely ionizing radiation elicits signalling pathways quite distinct from those involved in the cell and tissue response to photons. The response of the microenvironment to charged particles is therefore under scrutiny, and both the damage in the target and non-target tissues are relevant. The role of individual susceptibility in therapy and risk is obviously a major topic in radiation research in general, and for ion radiobiology as well. Particle radiobiology is therefore now entering into a new phase, where beyond RBE, the tissue response is considered. These results may open new applications for both cancer therapy and protection in deep space.

Biological effects of densely ionizing radiation have been studied since the beginning of radiobiology. As a matter of fact, α-particles deliver the main contribution to the background radiation dose on Earth, owing to inhalation of the indoor radon.[1] Neutrons have also been extensively studied because of protection of workers in nuclear power plants; use of fast neutrons in radiotherapy; and of the exposure of the survivors of the atomic bomb in Hiroshima and Nagasaki, where a neutron component was added to γ-rays. Bacq and Alexander[2] already elegantly described the radiobiology of α-particles and neutrons in their 1955 seminal book.

More recently, radiobiology research has focused on high-energy protons and heavy ions, mostly for two reasons: charged particle therapy (CPT) in oncology and radiation protection in manned space missions. In both cases, charged particles at energies $>100 \, \text{MeV} \, \text{n}^{-1}$ are involved. The characteristic depth–dose distribution with the sharp Bragg peak at the end of the range can be exploited for killing tumours; on the other hand, densely ionizing radiation can effectively delay tissue morbidity. Notwithstanding the many differences in exposure conditions, CPT and space radiation protection share several research topics, including individual sensitivity, non-targeted effects, late stochastic

effects and so forth.[3] Research in these fields requires large high-energy accelerators and is often performed by the same research groups with a common interest in particle radiobiology. Research is rapidly moving forward owing to the diffusion of CPT centres in the USA, Europe, and Asia[4] and to the growing interest in manned space exploration, now a priority for all space agencies,[5] but with cosmic rays acknowledged as a potential showstopper.[6] We are also facing a shift in the mainstream research topics, from comparisons of cellular end points to studies of the tissues and of the microenvironment.[3,7–10]

RELATIVE BIOLOGICAL EFFECTIVENESS

For many years, particle radiobiology was focused on measurements of relative biological effectiveness (RBE). The RBE is used to scale the data from reference radiation (X- or γ-rays) to test radiation (here energetic charged particles). The radiation weighting factors w_R are among the few quantities proposed by the International Commission on Radiological Protection[11] not derived from epidemiology, but from laboratory studies in radiobiology. In space travel, radiation weighting is instead based on the radiation quality factor Q, which is a continuous function of the radiation linear energy transfer (LET), rather than on radiation quality-

specific w_R values.[12] Quality factors are derived mostly from accelerator-based experiments on relevant end points, such as carcinogenesis in animals, neoplastic transformation *in vitro*, mutations, chromosomal aberrations and so on. In CPT, the RBE-weighted absorbed dose is expressed in Gy(RBE), which replaces the former grey-equivalent (GyE) or cobalt-grey-equivalent (CGE).[13] Despite many years of studies, starting at the Lawrence Berkeley Laboratory (LBL) in the 1970s[14] and then at GSI, Darmstadt, Germany; Heavy-Ion Medical Accelerator, Chiba, Japan; Brookhaven National Laboratory, Upton, NY, and many other accelerators, uncertainty about RBE is still very high. RBE is indeed the main source of uncertainty about the estimates of cancer risk for interplanetary missions (Figure 1)[15] and in dose estimation in CPT.[4] In proton therapy, a constant value of 1.1 is used all along the spread-out-Bragg-peak (SOBP), but it is generally acknowledged that corrections should be used, at least in the distal part of the SOBP, where radiobiology shows that slow protons have an increased effectiveness.[16] The increase of RBE with depth causes a shift of the fall-off of the proton beam, *i.e.* a change of the beam range (biological effective range).[17,18] Depending on the SOBP specific characteristics, increases in depth of several millimetres are expected due to the increased RBE in the distal penumbra (Figure 2). The RBE has obviously a much greater impact when heavy ions are used, because in that case, the LET varies sharply along the SOBP. For carbon ions, currently used for therapy in Germany, Italy, Japan and China, the dose in the SOBP is modulated using appropriate models for the RBE.[19,20] Different models are used in Europe and Japan, and the calculated doses in Gy(RBE) can differ significantly.[21] Uncertainty on the RBE is often quoted as a major hindrance to a widespread use of heavy ions in radiotherapy and is a source of concern for the potential late effects. For instance, C-ions have been used very little in paediatric patients,[22] mostly for the concern about high risk of secondary cancers.

Many years of *in vitro* studies on the RBE for cell killing consistently show a bell-shaped dependence on LET (Figure 3) and

Figure 1. Estimates of uncertainties in projecting cancer risks for interplanetary space missions based on current knowledge on radiation protection on Earth. Several factors such as radiation quality of high-energy ions, space dosimetry and microgravity do not contribute on Earth and lead to large increases in risk projections. The relative biological effectiveness (RBE) is by far the main factor contributing to uncertainty. Predicting risks to individuals is difficult, as there are very few quantitative measures of individual sensitivity. Reproduced from Durante and Cucinotta[15] with permission from Nature Publishing Group.
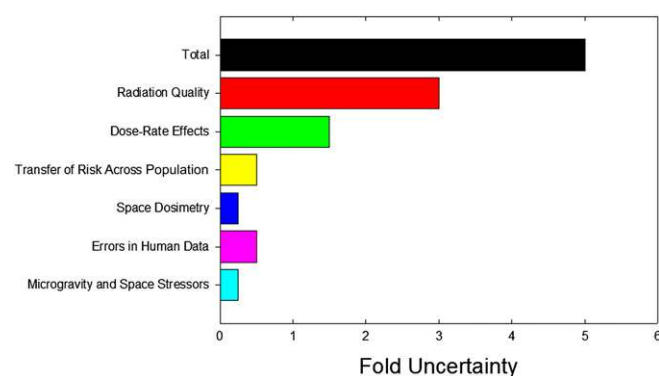

Figure 2. Biological range extension owing to a variable relative biological effectiveness (RBE) with depth in proton therapy. The orange dashed line indicates the corrected physical spread-out-Bragg-peak using the conventional RBE = 1.1. The orange line represents the correction used with the Local Effect Model version IV (LEMIV) model.[20,21] The black dashed line indicates 80% of the prescribed RBE-weighted dose. The inset shows a zoom of the distal penumbra, and the green line shows the increased range predicted by the biological model. Image and calculation courtesy of Rebecca Grün, GSI, Darmstadt, Germany. Modified with permission from the American Association of Physicists in Medicine.[18]
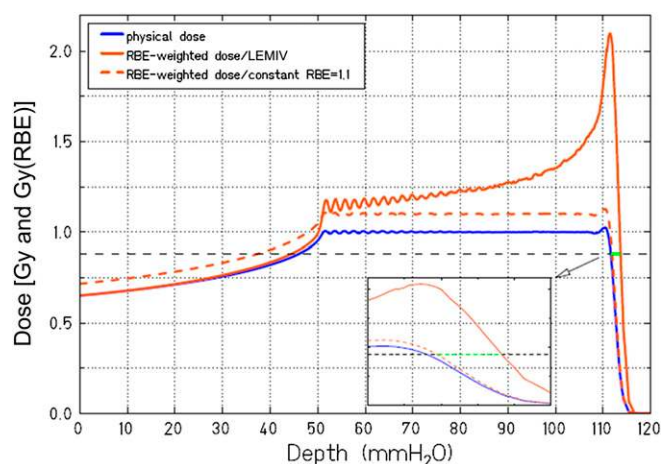
a significant dependence also on the particle charge.[23] Recent data mining of survival curves for cell cultures exposed to charged particles[24,25] confirms the spread in the RBE-LET

Figure 3. Variability of the relative biological effectiveness (RBE) (calculated at 10% survival level) *vs* linear energy transfer (LET) (dose-averaged in water, in keV μm^{-1}) *in vitro* data. Points are extracted from the large Particle Irradiation Data Ensemble (PIDE) database (https://www.gsi.de/bio-pide) developed at GSI, Darmstadt, Germany.[24] Different colours correspond to different ions as shown in the legend. The blue curves describe the approximated band of Chinese hamster V79 survival data as collected from Sørensen et al.[25] The red curves include the data on different *in vitro* cell lines collected at Lawrence Berkeley Laboratory.[14] DC, dendritic cell.
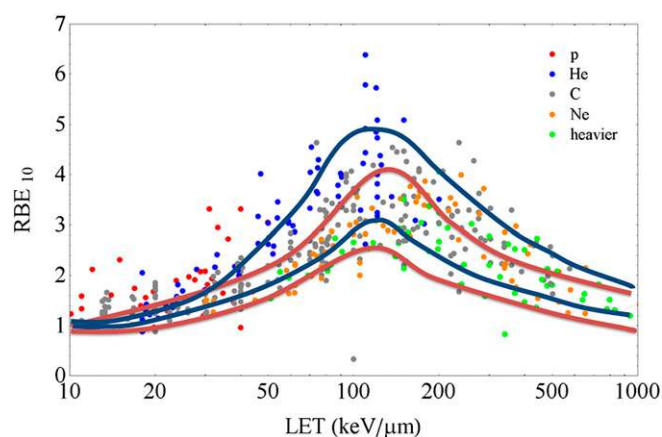
relationship originally measured at LBL.[14] RBE is in fact a function of several factors, often non-independent: biological end point, tissue type, proliferation status, dose, dose rate, oxygen concentration, culture conditions (*in vitro*) or metabolism (*in vivo*) and so forth. Replicating *in vitro* measurements on different cell lines, as done many times since the Berkeley experiments,[14] cannot reduce this intrinsic variability. Modelling will always be necessary for including RBE in the treatment planning, and here, there is room for improvements switching from phenomenological to mechanistic models.[26] It is worth noting that although the uncertainty in RBE is certainly a problem in heavy ion therapy, it should not prevent the use of this treatment modality, owing to the lack of significant side effects observed so far in Japan and Europe. Centres like the National Institute for Radiological Sciences (NIRS), Heidelberg Ion Therapy Center (HIT) or Centro Nazionale Adroterapia Oncologica (CNAO), where patients are treated with C-ions, all use Phase I/II dose escalation trials to find optimal protocols.[4] At the Institute of Modern Physics, Chinese Academy Sciences, Lanzhou, China, patients are treated with C-ions without any biological modulation, *i.e.* with an SOBP flat in dose, apparently with no major toxicity.[27]
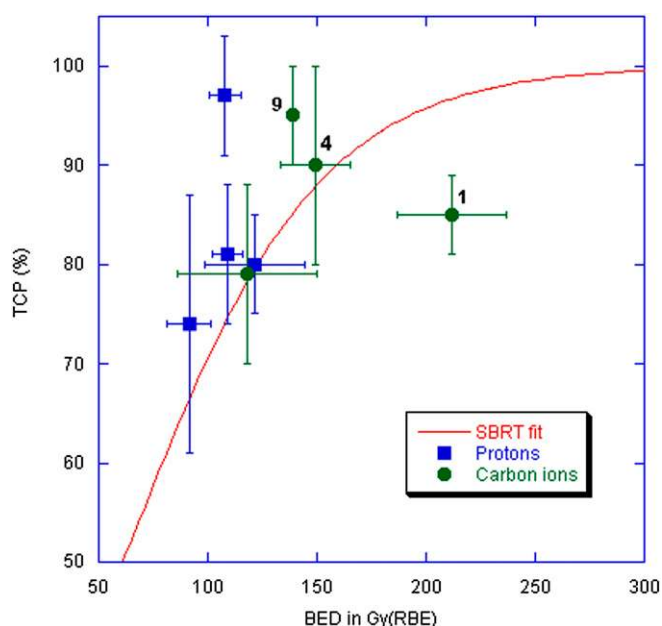
HYPOFRACTIONATION

Thanks to tremendous improvements in image-guided radiotherapy (IGRT), there is nowadays a tendency to reduce the number of fractions and increase the dose per fraction (hypofractionation).[28] The advantages for the patient and for the economy are enormous. X-ray stereotactic body radiation therapy (SBRT) and CPT are both pushing hypofractionation towards the region of 1–3 fractions (oligofractionation) with a very high dose/fraction (up to 25–30 Gy). For non–small cell lung cancer (NSCLC) and oligometastases, SBRT has proven high control rates, durable local control and little normal tissue complications.[29] At very high dose, the vascular injury, *i.e.* damage to the endothelial cells supplying the cancer tissue with oxygen and nutrients, may become a dominant pathway for tumour suppression.[30] Damage to the tumour stroma at high doses was originally demonstrated in fibrosarcoma and melanoma grown in genetically modified mice, where vascular endothelial cell apoptosis was shown >10 Gy per fraction.[31] The ceramide pathway orchestrated by acid sphingomyelinase is a major pathway for the apoptotic response. In later clinical work involving the use of single fraction high-dose spinal SBRT, investigators from the Memorial Sloan Kettering Cancer Center (MSKCC, New York, NY) recorded pronecrotic response after doses in the range of 18–24 Gy, a radiographical change consistent with a devascularizing effect.[32] Although the engagement of the vascular component in radiation response can be crucial at very high doses, it should be underlined that according to the conventional linear–quadratic model used in fractionated radiotherapy, hypofractionation leads to very high biologically effective doses (BED). This was not possible in the past because of the damage to the normal tissue, but it can now be spared, at least for parallel organs, with the modern image-guided techniques. In NSCLC, BED correlates with the tumour control probability (TCP) over a wide range of fractionated conformal radiotherapy and SBRT regimes.[33] The question remains therefore open, whether oligofractionation can be only justified by the improved physical dose distribution in IGRT and, consequently, very

high BED,[34] or it requires a different radiobiological mechanism involving vascular damage and possibly reperfusion.[35]

The high conformity granted by the Bragg peak makes CPT ideal for radiosurgery. Particle radiobiology research at high doses is needed to support and guide oligofractionation in hadrontherapy. Experiments in three-dimensional cultures of human endothelial cells[36,37] or lung cancer cells injected with basement membrane matrix into nude mice[38] showed reduced angiogenesis and vasculogenesis capability after exposure to high-energy charged particles, suggesting that the vascular damage may be particularly effective with protons or heavy ions. Hypofractionation with protons and C-ions is under way for NSCLC in several centres. Published TCP data for CPT in NSCLC[39–46] are plotted in Figure 4, along with the curve derived from X-ray SBRT trials.[33,34] CPT TCP are generally consistent with the X-ray SBRT TCP. One major deviation is observed in the single-fraction treatment at NIRS in Japan,[46] which corresponds to BED >200 Gy(RBE). RBE in the Japanese treatment plan is assumed to be independent from
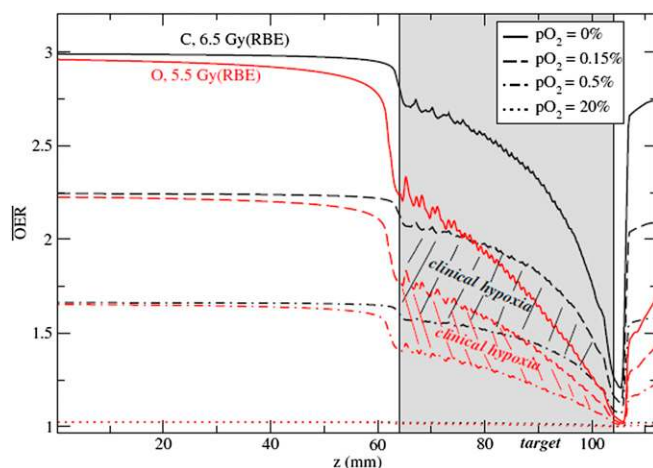
Figure 4. Tumour control probability (TCP) *vs* biological effective dose (BED) for different stereotactic body radiation therapy (SBRT) and charged particle therapy trials for non–small cell lung cancer (NSCLC). The red curve represents the fit to the X-ray SBRT data as reported in Mehta et al[33] using the common linear–quadratic formula $BED = nd[1 + d/(\alpha/\beta)]$, where *d* is the dose given in every fraction, *n* the number of fractions, and $\alpha/\beta = 8.6$ for NSCLC. Data points are calculated from published trials with protons or C-ions.[43–46] The number of fractions is shown next to the high BED C-ions data points. The single-fraction C-ion data point with the highest BED is described in Tsujii and Kamada,[46] while the highest TCP has been achieved at Tsukuba University, Ibaraki, Japan, in a trial on 58 (T1/T2, 30/28) patients treated with 66 Gy(RBE)/10 fractions for peripherally located and 72.6 Gy(RBE)/22 fractions for centrally located tumours.[42] BED for CPT was calculated using the formula above with *d* in Gy(RBE) as reported in the publications and the same α/β ratio as for X-rays.

the dose per fraction.[19] This can lead to overestimations at high dose per fraction up to a factor of 2.[21] In fact, the single-fraction trial for NSCLC at NIRS[46] gives a TCP consistent with a BED around 140 Gy X-rays (Figure 4). Moreover, the hypofractionated C-ion trial[45,46] reported no side effects, whereas a small but significant fraction of complications was reported in all other trials. On the other hand, some proton data seem to point to a higher RBE than expected, especially the trial at the University of Tsukuba.[42] This would be consistent with an RBE >1.1 for protons in NSCLC. Slow protons are more effective than X-rays in the inactivation of human tumour cell lines.[47] A recent experiment in a human NSCLC cell line reported an RBE = 1.9 for 3.9 MeV protons compared with X-rays.[48] The Tsukuba proton beam line used for the NSCLC treatment[42] has been used for *in vitro* experiments in different human cell lines.[49] Apoptosis induction was greater than two-fold the level induced by 10 MeV X-ray.[49] Other experiments have shown that the signalling cascade following exposure to protons can substantially differ from the damage response to photons.[50]

A potential advantage of CPT in hypofractionation is the reduced oxygen enhancement ratio (OER) using high-LET radiation. Hypoxia is one of the main factors reducing local controls in solid tumours,[51] and fractionation in radiotherapy has one of the main reasons in the possibility of re-oxygenation of the hypoxic areas. Re-oxygenation will be reduced in hypofractionation and, finally, lost in single-fraction/high-dose radiosurgery. Combinations with hypoxic sensitizers have been proposed for SBRT.[52] Owing to the reduction of OER using heavy ions,[14,53] CPT may be an alternative. Targeting specifically hypoxic regions can be achieved with strategies of dose-[54] or LET-painting.[55] For oligofractionation or single fraction/high dose treatments, use of ions heavier than carbon

(such as ^{16}O) may be beneficial, because with these ions, the OER can be further reduced in the clinically relevant hypoxia region[53] (Figure 5). Interestingly, SBRT commonly uses a non-uniform dose distribution in the tumour to achieve sharp edges on the border of the target volume.[56] The excess dose in the central region of the target may indeed be already acting, in current SBRT practice, as a dose-painting for the hypoxic fraction.[57]

Animal experiments comparing SBRT and CPT at high dose per fraction are necessary to clarify this issue and for a full exploitation of CPT in radiosurgery for both malignant and benign diseases. In fact, CPT can be potentially used for several non-cancer diseases[58] including atrial fibrillation (Figure 6), a common heart disease now treated by catheter ablation. These applications require extensive research in particle radiobiology for different tissue, such as heart, nerves, blood vessels and so on. Very little data are available for the response of these tissues to particle, whereas clinical results are used to establish X-ray tolerance doses in SBRT.[59]

Sensitivity of different tissues to high doses of charged particles is also important for protection of astronauts from intense solar particle events (SPEs), which can deliver high doses, even lethal, to unprotected crews.[6] Animal experiments in minipigs[60] suggest that the RBE for acute radiation syndrome of protons in simulated SPE is >1. Ferrets exposed to SPE-like protons had increase in fibrin clots, prothrombin time and partial thromboplastin time values post-irradiation,[61] suggesting a proton radiation-induced coagulopathy, which represents a risk for SPE in space but may find important applications in CPT.

COMBINED TREATMENTS
Although local control is generally very high with CPT, in most malignancies, radiotherapy must be combined to systemic therapies to control metastasis and increase survival. Combined radio + chemotherapy protocols are already used in many cancers, such as glioblastoma multiforme (GBM) or pancreas cancer.

Figure 5. Comparison of the computed oxygen enhancement ratio (OER) along a spread-out-Bragg-peak for carbon (black curves) and oxygen (red curves) at different pO$_2$ levels. The hatched areas represent the interesting clinical regions for hypoxia (0.15% < pO$_2$ < 0.5%). Doses indicated are prescribed relative biological effectiveness (RBE)-weighted doses in the target to achieve iso-survival. Plans for use of ^{16}O in addition to ^{12}C and ^{1}H in the clinics are currently under way at Heidelberg Ion Therapy Center (Heidelberg, Germany). Reproduced from Scifoni et al[53] with permission from IOP Publishing.

Figure 6. An *in silico* study for the application of heavy ions in the treatment of atrial fibrillation based on four-dimensional CT targets, the left pulmonary vein (LPV) and right pulmonary vein (RPV) with high-energy ^{12}C-ions. Atrial fibrillation would require a five-dimensional treatment plan for compensation of both breathing motion and heartbeat.[58] The radiobiological response of the vein and cardiac tissue are very little known and, therefore, the choice of the dose level is at present uncertain. Image and calculation courtesy of Anna Constantinescu, GSI, Darmstadt, Germany.
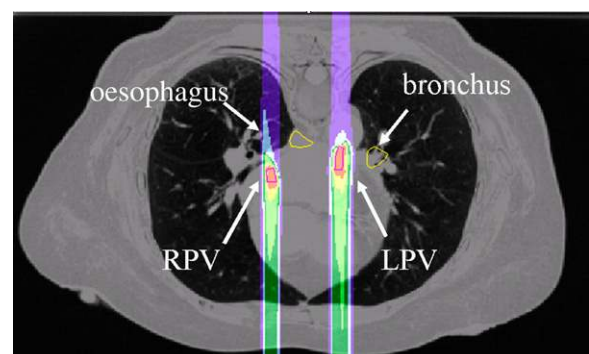
However, very few radiobiology studies specifically address the potential synergistic interaction of the drugs and ion irradiations. *In vitro* experiments on GBM provided useful indications on the combination of different drugs with C-ions.[62]

Equally important is the combination of particle therapy with immunotherapy,[63] which is now rapidly gaining practice in clinics with great expectations for cancer cure. Abscopal effects, defined as shrinkage of metastatic lesions far from the irradiation field during radiotherapy of a primary malignancy, have been reported for many years,[64] but although immune-related effects were assumed to play a role, this was hitherto not immunologically proven until a recent case of a female melanoma patient at MSKCC, receiving immune adjuvant anti-CTLA4 therapy for 1 year, resulting in disease progression, requiring focal irradiation of her spinal metastasis (28.5 Gy and 6 MV X-rays in three fractions). In the CT scan, 4 months after irradiation, vanishing of multiple metastases out of the irradiation field occurred.[65] Changes in cellular and molecular parameters indicate a comprehensive immune reaction against the tumour. More cases of abscopal effects with anti-CTLA4 treatment concurrent with radiotherapy were reported later, including one complete response (no cancer visible in PET scan).[66] This is clear clinical evidence of immune-mediated abscopal effect, formerly observed in different animal models.[67] The mechanism underlying this effect is now fairly well understood.[68] Radiation triggers cell death via DNA damage. Ceramide formation and mitochondrial damage, leading to release of mitochondrial cytochrome C and consecutive caspase activation, cause cell apoptosis. In addition to apoptosis, cells can be effectively eliminated following DNA damage by necrosis, mitotic catastrophe, autophagy and premature senescence, and these pathways are prevalent in most solid cancers.[69] Radiation damage response leads to the release of damage-associated molecular patterns, which induce dendritic cell (DC) maturation and tumour antigen uptake, resulting in priming and clonal expansion of cytotoxic lymphocytes (CTLs) in the lymph node, which harbour the T-cell receptor matching specifically to the antigen. From the lymph nodes, CTL can travel to the tumour. There, release of granzyme results in caspase activation and mitochondrial membrane permeabilization. At that point, immunity converges with the same cell death pathways as induced directly by irradiation (Figure 7). The immune-mediated cell killing is supported further by radiation through upregulation of HSP70, which aids in transferring granzyme into the cell, an increased antigen presentation via major histocompatibility complex-I and upregulation of NKG2D (natural killer group 2, member D) ligands, facilitating tumour cell killing by natural killer (NK) cells. The induction of NKG2D ligands may be the reason of the clinically[65,66] observed sensitization of unresponsive tumours to anti-CTLA4 immunotherapy[70] (Figure 8).

These studies beg the question of whether abscopal effects and combined immunotherapy and radiotherapy, can be enhanced by charged particles.[68] Carbon ions significantly reduce lung metastasis count in LM8 osteosarcoma mouse models and a model of squamous cell carcinoma in immune competent C3H mice.[71]

Figure 7. Pathways where radiation can synergize with immune adjuvant therapy for cancer. (1) Immunogenic cell death is promoted by ionizing radiation, through dendritic cell activation and, consequently, T-cell expansion. (2) Cytokines play a role in radiation therapy success. (3) NKG2D-ligands, sensitizing stressed cells to natural killer cells (innate immunity) are upregulated by radiation. (4) Chemokines can be induced by radiation, attracting effector T-cells to the tumour. (5) Radiation-induced interferon-gamma-dependent upregulation of cell adhesion molecule also influences antitumour immunity. (6) Heat shock proteins sensitize to cytotoxic granzymes. (7) Radiation can lead to enhanced expression of major histocompatibility complex (MCH)-I and to *de novo* expression of neoantigens. (8) Death receptors can be upregulated by irradiation. (9) CD8 T-cells are essential for the success of radiotherapy. Image courtesy of Norman Reppingen, Technical University of Darmstadt, Darmstadt, Germany. CTL, cytotoxic T cell; DC, dendritic cell; IFN, interferon; TCR, T cell receptor; TNF, tumour necrosis factor.
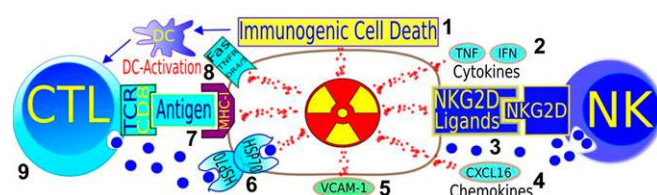

Figure 8. Simplified model of the mechanism of anti-CTLA4 treatment with ipilimumab. The clonal expansion of tumour-specific T-cells is controlled by "immune checkpoints". A strong signal via major histocompatibility complex-I leads to a more pronounced expression of the immune inhibitory molecule CTLA4. CTLA4 competes with higher affinity with the surface receptor CD28 for CD80 and CD86, which is a part of Signal 2, and necessary for the successful mounting of an immune response. Binding of CD80 and CD86 to CTLA4 is antagonizing the function of helper T-cells and cytotoxic T-cells and activating immune suppressive regulatory T-cells, conclusively leading to suppression of immune responses (upper row of cells). Therefore, blocking of CTLA4 leads to an increased expression of effector molecules (granzyme, cytokines) and an enhanced expansion of cytotoxic T-cells (lower row), increasing the "amplitude" of an immune response. These cells could be directed against the tumour, improving treatment success, but also against normal cells leading to severe treatment side effects. Radiation could assist to direct the immune processes toward treatment success,[70] as evidently seen in some animal models and preliminarily in clinical results.[65,66] Image courtesy of Norman Reppingen, Technical University of Darmstadt, Darmstadt, Germany. TCR, T cell receptor.
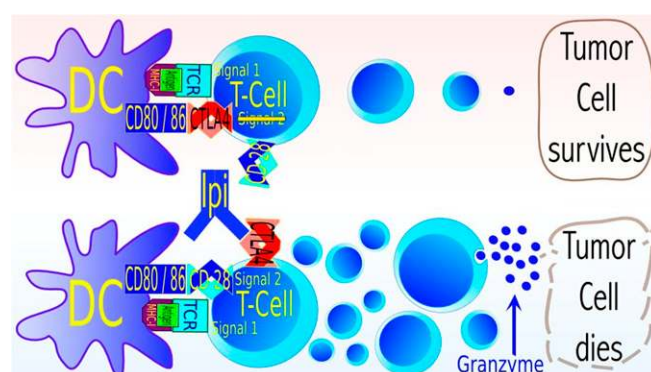
A recent study showed that heavy ion irradiation could confer tumour rechallenge resistance, which was dependent on CD8$^+$ T-cells and influenced by NK cells in this system, showing synergy with dendritic cell treatment approaches,[72] an encouraging outcome for future protocols combining immunotherapy and CPT.[73]

RADIOGENOMICS

The ultimate goal of radiogenomics is to develop a genetic risk profile individualization of radiation dose prescriptions to optimize tumour control, while minimizing normal tissue damage.[74] Genome-wide association studies have already been successful in finding novel genetic variants with high risk of developing some common diseases, for instance breast cancer.[75] Radiogenomics uses a similar approach to predict the sensitivity of the normal[76] and cancer[77] tissue to radiation.

Normal tissue

Radiogenomics is extremely attractive for the assessment of patients' normal tissue response as long as the genetic component plays a major role in determining radiosensitivity. This is clearly the case for those syndromes, such as ataxia-telangectasia (AT) or Nijmegen breakage syndrome, where germline mutations of DNA repair genes induce extreme radiosensitivity.[78] These diseases are, however, rare, confined to a limited number of families, phenotypically obvious and certainly not responsible for the observed variability in radiotherapy response. It is interesting to note that, while AT patients are homozygous for mutation in the ATM protein, a fraction about 3% of the population is heterozygous for ATM[79] and a similar fraction is hypersensitive in radiotherapy trials. ATM haploinsufficiency results in increased radiosensitivity in mice,[80,81] thus, suggesting that this subgroup may be the one more at risk of side effects in radiotherapy and radiation-induced cancer. ATM is one of the candidate genes to be screened as potential biomarkers of radiation response. Radiogenomics has used the candidate-gene approach to look for variations in many other genes involved in radiation response, e.g. those in the pathways of DNA repair (BRCA1/2), cytokine production (TGFβ), scavenging of free radicals (SOD2) and so forth.[82] Genetic variations include single nucleotid polymorphism (SNP),[83] copy number variations[84] and other epigenetic modifications. Unfortunately, although several studies have initially reported associations between radiation toxicity and SNP, a prospective study aimed to validate failed to detect any of the previously reported associations,[85] suggesting that SNP may be irrelevant for individual sensitivity.

Few studies deal with radiation quality in radiogenomics. Haploinsufficiency for ATM results in accelerated cataracto-genesis in mice exposed to both X-rays and heavy ions, and the RBE is higher for heterozygotes compared with wild-type.[86] In a recent microarray hybridization analysis of irradiated normal human bronchial epithelial cells,[87] unsupervised clustering analysis of gene expression segregated samples according to the radiation quality (Fe-ions, Si-ions or γ-rays) followed by the time after irradiation (0–24 h), whereas dose (0.5–3 Gy) was not a significant parameter for segregation. The great majority of genes with modified expression where uniquely associated to one radiation quality (Figure 9). Even if preliminary, these data suggest that gene profiling may identify pathways uniquely associated to CPT compared with X-ray therapy.

Figure 9. Differentially expressed genes in human bronchial epithelial cells exposed to γ-rays or heavy ions. Venn diagram shows the numbers of genes, either specific to or overlapping with different radiation types. Courtesy of Michael D. Story (UT Southwestern, Dallas, TX). Reproduced from Ding et al[87] with permission from BioMed Central.
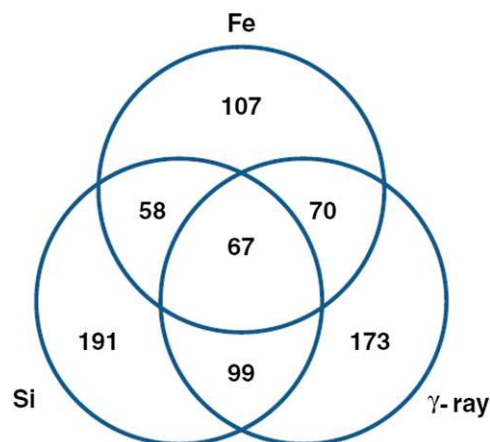
Tumour tissue

The integration of molecular data with local control data can allow the generation of biomarker signatures that predict tumour response to therapy.[77] The method has been applied to *in vitro* survival curves of different human tumour cell lines. Microarray profiling identified 22 genes differentially expressed in radiosensitive cancer cells and 18 genes associated with resistant cell lines.[88] In a molecular regulatory network model based on mRNA expression profiles, it has been possible to identify 10 hubs of interactions associated to radiosensitivity.[89] It is not clear whether these patterns can be used for all cancers, or they are tissue-specific. Individual NSCLC patients' responses to chemotherapy are highly variable,[90] and similar spread is measured irradiating NSCLC cell lines.[91] Molecular markers, including epidermal growth factor receptor, K-ras, vascular endothelial growth factor, mammalian target of rapamycin and anaplastic lymphoma kinase have been proposed as potential biomarkers of response of NSCLC to ionizing radiation.[92] For head-and neck-squamous cell carcinoma, a recent study shows that Ku80 overexpression was an independent predictor for both local recurrence and mortality following radiotherapy.[93] The results are easy to interpret, Ku80 being a key molecule for DNA double-strand break repair.[94]

The variance in radiosensitivity associated to the genetic background is reduced when cells are exposed to densely ionizing radiation.[95] The interindividual variability in radiosensitivity can be assessed with organotypic tissue slices from the human tumours.[96] A recent study showed interindividual variability for GBM slices treated with temozolomide and a smaller variability after C-ions.[97] The RBE decreases for cells with high α/β ratios, *i.e.* by decreasing intrinsic radioresistance and, eventually, reaches unity for very radiosensitive cell lines.[98] For this reason, high-LET heavy ions are preferentially used for radioresistant tumours. This is also a very attractive feature for targeting specific resistant sub-volumes in the tumour, such as the cancer stem cell niche.[99] Expression of molecular biomarkers of radioresistance should therefore represent an indication for CPT.

Radiogenomics in tumour tissues can be used for synthetic lethal genetic screen. Two genes are synthetic lethal if mutation of either one is compatible with viability but mutation of both leads to death.[100] So, targeting a gene that is synthetic lethal to a cancer-relevant mutation should kill only cancer cells and spare normal cells.[101] Synthetic lethality, therefore, provides a conceptual framework for the development of cancer-specific cytotoxic agents[102] and can be extended in combined therapies, targeting combination of genes conferring radioresistance to cancers.[103]

Carcinogenesis

If gene expression has a key role in determining sensitivity of the normal and tumour tissue, it can also be argued that it affects the risk of radiation-induced cancer. The question is whether radiation carcinogenesis is a purely stochastic process or if there exists a sub-population of sensitive individuals.[104] Should this be the case, radioprotection would be useless for many normal individuals and insufficient for the (few) sensitive subjects. In the analysis of cancer risk in children from 160 families irradiated in Israel after World War II for an epidemic of *tinea capitis*, it was found that the increased meningioma incidence in irradiated children was determined by a subgroup of only 17 families (11%), where 4 of 5 children developed meningioma.[105] In addition, the large European cohort study GENE-RAD-RISK[106] has recently concluded that in carriers of BRCA1/2 mutations, any exposure to diagnostic radiation before the age of 30 years is associated with an increased risk of breast cancer. The relative risk in the mutant carrier was 1.9 and increased with dose.[106]

Genetic predisposition may also drive the risk from exposure to charged particles. This is a critical issue for paediatric patients, a group that is often referred to proton therapy because much normal tissue can be spared compared with X-rays and, therefore, toxicity is reduced.[107] For these patients, the risk of second cancer is, however, substantial and the genetic predisposition to cancer very likely because the tumour was expressed at a young age.[108] This problem will be discussed in the next section.

LATE EFFECTS

Risk of late morbidity is a major issue for charged particle radiobiology. For space travel, cancer and non-cancer late effects represent the major showstopper for long-term interplanetary missions beyond low-Earth-orbit (LEO).[15] In CPT, as noted above, the problem is mostly related to second cancers in paediatric patients.[108]

Carcinogenesis

Epidemiology in subjects exposed to energetic charged particles is very limited. No increased cancer incidence is observed in the astronauts' cohort, consistent with the low-dose exposure in LEO experienced so far by crews on the International Space Station and other space missions.[15] For hadrontherapy, comparison of chromosomal aberrations in peripheral blood lymphocytes of patients treated either with C-ions or X-rays for similar tumours generally shows a higher damage after conventional radiotherapy compared with heavy ion treatment, both for passive modulation[109] and active raster scanning.[110] These results, apparently in contradiction with the increased effectiveness of heavy ions in the induction of chromosomal aberrations,[111] are caused by the

different treatment planning. With heavy ions, the integral dose is reduced because, thanks to the Bragg peak, only a few fields (1–3) at different angles are used compared with conformal and intensity-modulated therapy with X-rays (up to 9–10 fields). Therefore, the volume of normal tissue exposed is strongly reduced, and therefore less chromosomal aberrations observed, and a reduced late morbidity expected. Preliminary results from a cohort study of 558 patients treated with proton radiation from 1973 to 2001 at the Harvard Cyclotron Laboratory, Cambridge, MA, showed a trend towards a reduced risk of second malignancies, consistent with the reduced integral dose to the normal tissue.[112] Voxel-phantom models able to predict the second cancer risk in different organs (Figure 10) with different treatment modalities are now available[113,114] and could guide the choice of the oncologists in defining the best treatment options for paediatric patients. The epidemiological data used in these models are, however, insufficient and the uncertainty is consequently very high.[108] *In vitro* and *in vivo* experiments remain mandatory for understanding the mechanisms and reducing uncertainties.

Neoplastic transformation *in vitro* by heavy ions has been studied in immortalized mouse C3H 10T1/2 fibroblasts,[115] primary Syrian hamster embryo fibroblasts,[116] and in the HeLa X human skin fibroblast hybrid cell line CGL1.[117] All these systems are very artificial and great caution should be exercised in extrapolating data to the *in vivo* situation, especially since each cancer may display a different behaviour

Figure 10. Dose and risk distribution for second cancer. Images of a 9-year old girl who received craniospinal irradiation for medulloblastoma using passively scattered proton beams at MD Anderson Cancer Center. The colour scale illustrates the difference for absorbed dose, incidence and mortality cancer risk in different organs. Radiation absorbed dose depends strongly on patient anatomy and treatment factors. Risk of second cancer incidence and mortality varies strongly with radiation dose, but, importantly, it also varies strongly between organs, with the patient's age at exposure and attained age, sex, genetic profile, and other factors. Reproduced from Newhauser and Durante[108] with permission from Nature Publishing Group.
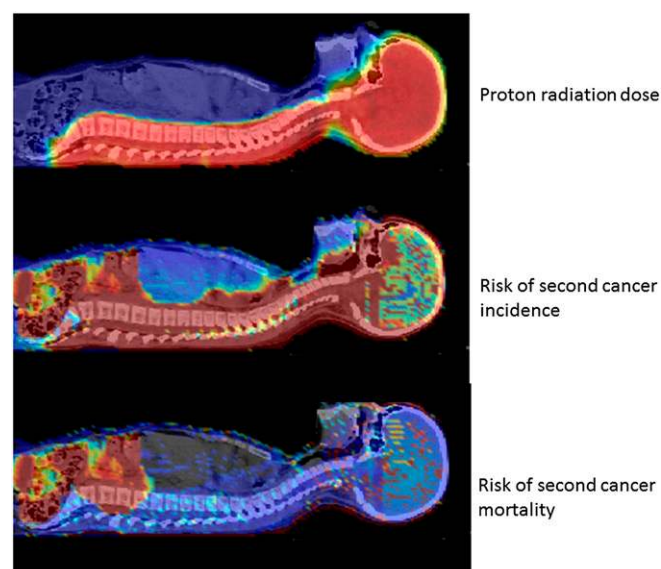
Figure 11. Relative biological effectiveness (RBE) for *in vitro* neoplastic transformation plotted *vs* the linear energy transfer (LET) of different charged particles. Data on C3H 10T1/2 mouse fibroblasts are from Yang et al;[115] Syrian hamster embryo (SHE) fibroblasts were used in Han et al;[116] and HeLaxskin fibroblasts hybrid cells (CGL1) were studied in Elmore et al.[117]
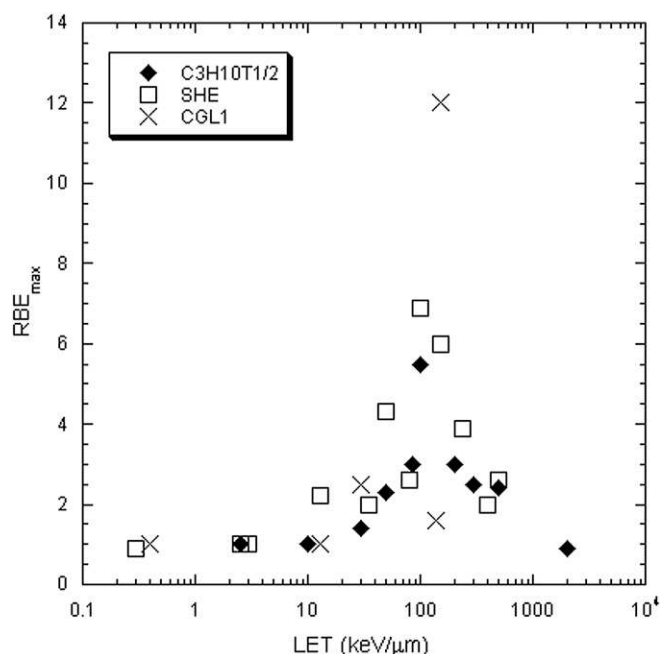
in vivo. Nevertheless, the general trend confirms the RBE-LET relationship observed for cell killing, mutations and chromosomal aberrations, *i.e.* a bell-shaped curve with a peak around 100–200 keV μm^{-1} (Figure 11).

Animal studies report the induction of different tumours in rodents and have been recently reviewed.[118] In many cases, the trend for solid tumour induction is not in contradiction with the *in vitro* data in Figure 11, although generally RBE values are lower than those observed with fission neutrons. However, *in vivo* data point to quantitative and qualitative differences in the RBE for different types of cancers. For instance, recent work at the Brookhaven National Laboratory accelerator has shown that the RBE of 1 GeV n^{-1} Fe-ions for cancer induction in mice is about 1 for leukaemia and about 20 for hepatocellular carcinoma.[119] How can the RBE be so different? The reason is most likely because of the different nature of liquid and solid cancers. Although leukaemia is strongly related to specific chromosomal aberrations (in mouse, deletion of PU.1 gene in chromosome 2), solid cancers are associated to genomic instability, *i.e.* to late tissue effects. Radiation can act both as an initiator of the carcinogenic process (*e.g.* by inducing mutations or chromosome aberrations) or in promotion (related to tissue inflammation). For liquid cancers, radiation could act as an initiator, whereas for solid cancer, as a promoter. Although heavy ions are more effective than X-rays in the induction of chromosomal rearrangements, most of the aberrations are lethal, and the RBE drops to about one in the surviving population.[120] On the other hand, heavy ions are very effective in the induction of inflammation,[121] and this process is a driver for promotion of carcinogenesis.[122,123]

Non-cancer effects

Recent epidemiological studies suggest that non-cancer late effects, particularly, cardiovascular diseases, may contribute to health risk after moderate to low doses of ionizing radiation.[124] The radiobiology of charged particles for these end points and tissues is scarcely known, as already noted for potential applications of CPT in radiosurgery. The major degenerative late effects that could result from exposure to high-energy charged particles are:

– acute and late damage to the central nervous system (CNS)
– cataract formation
– cardiovascular diseases
– other diseases related to accelerated senescence, including digestive and respiratory diseases, endocrine and immune system dysfunction.

CNS complications, including necrosis and leukoencephalotopy, have been reported in patients treated for brain tumours both by chemotherapy, X-rays[125] or protons.[126] However, exposure to heavy ions seems to produce a distinct damage in the CNS also at low doses, perhaps by single particle traversals. First evidence of these effects came from reports of visual phosphenes, described as sudden "light flashes", first reported by Buzz Aldrin after the first Moon landing and then by almost all the astronauts in the Apollo programme.[127] Light flashes are believed to be produced by a direct interaction of an energetic charged particle with the retina or the eye, as confirmed by studies in space[128] and in dedicated experiments in cancer patients treated with C-ions using spot-scanning.[129] The observation of light flashes brought attention to the possible effects of charged particles on the brain function. Calculations suggest that for a 3-year mission to Mars at solar minimum, 2–13% of the "critical sites" of cells in the CNS would be directly hit at least once by iron ions, and roughly 20 million of 43 million hippocampal cells and 230 000 of 1.8 million thalamus cell nuclei would be directly hit by one or more particles with $Z > 15$ on such a mission,[130] without considering the additional contribution caused by δ-rays. Evidence of behavioural effects induced by doses as low as 0.2 Gy of Fe-ions was produced in a series of experiments in Sprague-Dawley rats.[131] A review of behavioural end points in rats exposed to different ions conclude that low doses of heavy ions can indeed produce behavioural alterations in rats, including disruption in motor behaviour, taste aversion learning, spatial learning and conditioned place preference.[132] The large uncertainty on these behavioural end points and the interindividual variability in the animal experiments make, however, very difficult any reliable risk estimates. There could be a dose threshold, and it seems to be charge- and energy-dependent, and defining an RBE is even more difficult, considering that most of these end points are not observed at all with X-rays.[131] These observations have therefore triggered a number of cellular and molecular studies on neural cells and tissue, to gain understanding of the mechanisms of heavy ion-induced damage in the brain. These studies are ongoing, and despite some evidence of the involvement of reactive oxygen species (ROS) and neuro-inflammation,[133] they are still preliminary and do not presently allow drawing a firm conclusion.

On the contrary, we have now clear evidence that space radiation induces ocular cataract in astronauts,[134] and indeed lens opacification is the only proven space-radiation effects actually observed in crews of space missions. Recent epidemiological evidence does not support the existence of dose threshold for radiation-induced lens opacification, although the shape of the dose–response curve is still unclear.[135] However, animal studies show that the RBE of heavy ions for cataract induction is as high as 50 at doses <100 mGy,[136] thus explaining the high effectiveness for accelerated caratactogenesis in space.

Apart from cataractogenesis, the uncertainty on other, more harmful, non-cancer late effects, especially cardiovascular diseases, is much higher. There is now clear epidemiological evidence that high radiation doses induce late cardiovascular diseases. A clear correlation between radiation dose to the heart in breast cancer patients and late ischaemic heart diseases has been recently shown in a cohort of Swedish and Danish females treated by radiotherapy for breast cancer.[137] A-bomb survivors' data also support these results.[138] It is not clear, however, whether this cardiovascular risk has a threshold at low doses. This is very important for both radiotherapy and space exploration. Proton therapy can efficiently spare the heart during the treatment of left-side breast cancer,[139] whereas with X-rays, a large fraction of the heart is generally exposed. Breast cancer is therefore becoming a major application of protontherapy.[140–142] Breast cancer is indeed the most frequent cancer in females, and if proven, superior CPT could have a much larger use than in the past. The advantage here is not in the TCP, but in the reduced normal tissue toxicity, and this advantage, already considered the main justification for paediatric use of protons,[107] is likely to be decisive in the future of CPT. In space, a mission to Mars would bring the total dose around 1 Sv,[143] a region where epidemiological data suggest an increase of cardiovascular diseases.[138] An examination of possible biological mechanisms indicates that the most likely target of radiation-induced cardiovascular damages are endothelial cells and subsequent induction of an inflammatory response.[144] Again, inflammation and, in general, non-targeted effects mediated by the microenvironment are the major pathways in radiation response of the organisms,[122] driving both cancer and non-cancer late effects, as well as for radiation therapy.[145]

Generally speaking, the epidemiological evidence of non-cancer late effects in exposed individuals is consistent with radiation-induced acceleration of the ageing process. As a matter of fact, it has been shown that radiation accelerates age-related diseases such as endocrine dysfunctions,[146] digestive and respiratory diseases,[138] immune system function impairment.[147] What can be the mechanism relating radiation exposure to ageing? Premature cellular senescence can be induced by radiation and other genotoxic agents, and this process is driven by telomere shortening.[148] Even single nuclear traversals of energetic charged particles can induce accelerated senescence of cultured human fibroblasts.[149] The link between radiation exposure and accelerated ageing may be mediated by a persistent oxidative stress.[150] Indirect evidence of ROS involvement is provided by experiments on protection from late non-cancer effects using antioxidants.[151] For example, accelerated radiation-induced formation of aortic lesions occurred in mice that were on a high-fat diet, whereas smaller lesions were

observed in their irradiated transgenic littermates that over-expressed CuZn-superoxide dismutase, which is expected to decrease chronic oxidative stress.[152]

CONCLUSIONS

Radiobiology research, in general, is underfunded both in the USA and Europe. However, it is clear that charged-particle radiobiology studies are needed both for cancer therapy and radiation protection in space. In CPT, medical physics can improve the precision and quality of the treatments, but IGRT is rapidly progressing in X-ray therapy as well. Only radiobiology can identify areas of potential breakthrough in radiotherapy, *i.e.* specific tumours, fractionation schedules and combined treatments where the benefit of CPT will be decisive for the patient. In space travel, cosmic radiation is recognized as a potential show-stopper. Passive shielding is not a solution to the problem, and active (magnetic) shielding is still not technologically feasible. Again, radiobiology studies are mandatory for reducing uncertainties in late radiogenic risk and developing biomedical countermeasures. These ambitious goals should be pursued with innovative research going beyond the traditional RBE measurements. In this review, we have identified several new key topics in particle radiobiology, such as effects of high doses (hypofractionation), radiogenomics, combined treatments (with chemo- and immunotherapies), and studies on late effects focused on the response of the microenvironment. This research needs funding and accelerator facilities willing to invest in research. There are already several accelerator facilities involved in biomedical research for clinical[153] and space[154] radiobiology, and others have been proposed.[155] The exploitation of these facilities is, however, dependent on the funding available for users interested in high-energy particle radiobiology. In the USA, both the US National Cancer Institute (NCI) and the US Department of Energy (DOE) are considering further investments in CPT, including radiobiology.[156] In Europe, Horizon 2020 should be the platform to continue the funding gathered in past years through the European Network for LIGht ion Hadron Therapy (ENLIGHT) platform[157] and related European Union-funded projects.[158] The potential gain of this research is likely to be very high.

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