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Roadmap for precision preclinical x-ray radiation studies

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TOPICAL REVIEW

Roadmap for precision preclinical x-ray radiation studies

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Abstract

This Roadmap paper covers the field of precision preclinical x-ray radiation studies in animal models. It is mostly focused on models for cancer and normal tissue response to radiation, but also discusses other disease models. The recent technological evolution in imaging, irradiation, dosimetry and monitoring that have empowered these kinds of studies is discussed, and many developments in the near future are outlined. Finally, clinical translation and reverse translation are discussed.

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Contents

1. Introduction 3
2. Modern preclinical cancer radiotherapy 4
3. Preclinical models for non-malignant diseases 7
4. Precision radiation and normal tissue damage models 9
5. Imaging systems for image guidance 12
6. Irradiation systems 15
7. Treatment planning 18
8. Small animal dosimetry 21
9. Informatics systems 26
10. Monitoring systems 29
11. Clinical translation 32
12. Reversing the translational research paradigm in preclinical radiotherapy studies 34
13. Concluding remarks 37

1. Introduction

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Introduction. The first preclinical radiation biology studies, using animal models for tumors or normal tissues, are almost as old as the discovery of radiation itself. Spanning many decades, this has led to some important insights into the detailed interaction of radiation with biological structures. Unfortunately, the uncountable published radiobiology studies have not been used on a large scale to inform clinical radiotherapy trials and have in general not led to many practice-changing discoveries. Within medicine, preclinical trials are well-established and mandatory, e.g. in drug discovery. Therefore, it is surprising that in clinical radiotherapy practice it is quite common to focus on technology trials or even to adopt new irradiation or imaging technology without preclinical trials. Part of the explanation for this is that until recently preclinical irradiation technology and its integration with imaging, was not sufficiently adequate to perform precision irradiation with dose regimes mimicking clinical conditions.

It is generally accepted that this is changing rapidly now, mostly due to two evolutions which both started about 15 years ago. The first was the development of precision irradiation technology, combined with high-resolution image-guidance methods. The second was the introduction of more clinically relevant animal models for tumor development. Both progressed rapidly and probably stimulated a mutual development. Many radiobiology labs - their number still growing steadily - are now able to set up studies which can image complex and realistic tumor models with an increasing number of imaging modalities, often especially developed for small animals. These models can then be subsequently interrogated with complex radiation fields using modalities such as photons and ion beams. This required the development of much supporting technology for treatment planning, dosimetry and data management. All of these do provide now the tools to perform preclinical trials relevant for clinical practice.

From a recent conference held in this field (https://small-animal-rt-conference.com/) it is clear that this field has enormous potential to improve human radiotherapy. One only has to think of all the aspects that need to be investigated *in vivo* before e.g. Flash radiotherapy can be applied clinically. This Roadmap paper collection gathers brief assessments of the state-of-the-art in various sub-specialties (figure 1), in particular for photon irradiation studies, and reflects upon the challenges and solutions that lie in the near future in precision image-guided preclinical radiotherapy.

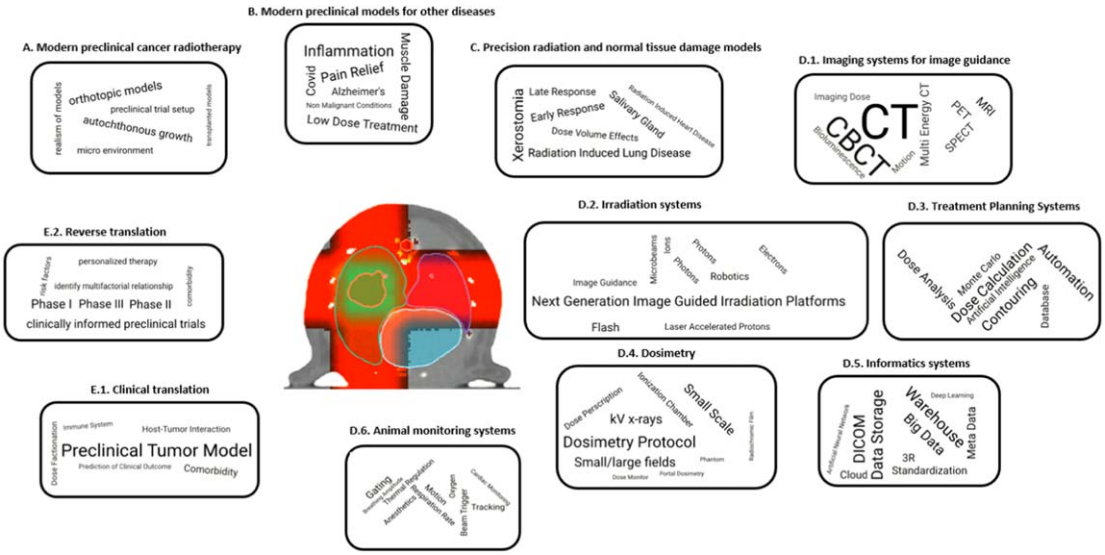

Figure 1. Contents of this Roadmap paper. Various aspects for accurate imaging and radiation targeting of certain structures in preclinical animal models are indicated. The section numbers are shown in the headers.

2. Modern preclinical cancer radiotherapy

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Status. With the increasing development of molecularly targeted and immunotherapeutic treatment approaches, it is evident that future radiotherapy-based treatment concepts will be of multi-modal nature and strongly rely on biological and preclinical research. This demands preclinical radiotherapy trials which reproduce the clinical situation as closely as possible, not only with regard to model systems and treatment regimens but also in terms of clinical standards, such as trial design, analysis endpoints, and reporting. Technologically, preclinical radiation platforms are already well-equipped to perform precise radiotherapy treatment planning and dose delivery based on on-board integrated image guidance. However, the widespread use of suboptimal cancer models in conjunction with treatment regimens of questionable clinical relevance, and poorly informative analysis endpoints so far have largely prevented effective translation of novel treatment concepts from the lab into clinical radiotherapy practice.

Current and future challenges. In order to address and overcome this gap in clinical translation, radiation biologists and preclinical radiation oncologists are currently scrutinizing and refining their experimental approaches with regard to (I) the model animals, (II) the model tumors, and (III) the concepts of preclinical trialing (figure 2) (Brix *et al* 2017).

Animal models have played a major role in shaping our current understanding of tumor biology and the development of mechanism-based cancer therapies. Several species have been employed of which the mouse has emerged as the most frequently used experimental animal for several reasons, including its relatedness to humans, accelerated lifespan, short generation time, availability of different strains and genotypes, well-characterized genome, and readily established genomic (and other) manipulation techniques (Butterworth 2019). The mouse (sub-)strain and physiological condition in terms of age, sex, hygiene, immune, metabolic, nutritional, microbiota, and behavioral status (among others) is of paramount importance for the planned experiments and accordingly needs to be carefully chosen and reported. Prospectively, large animal models, such as (mini-)pigs, and true animal cancer patients, including pet cats and dogs, should be considered in order to obtain higher-level preclinical evaluation of novel radiotherapeutic concepts that have proven promising in small animals.

Apart from the model animal, also the model tumor needs thorough attention (Gengenbacher *et al* 2017). Ideally, it replicates the underlying human genetics and genetic heterogeneity, shares common anatomy and histology to the human disease, and has a preserved microenvironment, immune cell function as well as metastasizing capacity. In terms of etiology, tumor formation can be initiated based on autochthonous growth or transplantation, respectively (figure 3). Autochthonous models (induced by random mutagenesis or targeted genetic manipulation) recapitulate the early stages of tumor initiation and progression from a genetic point of view more closely. However, they often are of multi-nodule nature, and suitable single-nodule models, which are preferred for preclinical radiotherapy trials, so far have only rarely been developed (Herter-Sprie *et al* 2014). Additionally, autochthonous models require resource-intensive monitoring in order to detect tumor onset. With transplantation models in contrast, the onset of tumor growth is well-defined, and the number and localization of tumor nodules can be distinctly controlled. Yet, they commonly fail to properly reflect the tumor microenvironment, even if genetically well-defined cell lines are transplanted orthotopically into syngeneic, immunocompetent animals. So, spontaneously growing tumors are commonly considered to be better predictors of therapeutic responses (Wisdom *et al* 2020), but the vast majority of preclinical studies still rely on tumor models with transplanted cell lines as a starting point.

The concepts of preclinical trialing represent the third crucial aspect that is currently undergoing intensive refinement. For maximal translatability, mouse experiments should come as close as possible to the clinical situation. This implies starting the treatment when tumors are fully established and—of particular importance in the field of radiation oncology—using clinically relevant beam conformality, radiation doses, dose rates, and fractionation schemes. Furthermore, the defined endpoints and (non-invasive, imaging-based) methodologies to assess these should be carefully selected. For clinical trials, response evaluation criteria, such as RECIST 1.1 (Eisenhauer *et al* 2009) or iRECIST (Seymour *et al* 2017), have been formulated in guidelines. Ideally, these should be adapted and transferred to the preclinical counterparts. Animal studies evaluating local and/or systemic control have a larger predictive power towards clinical success than tumor growth delay and tumor

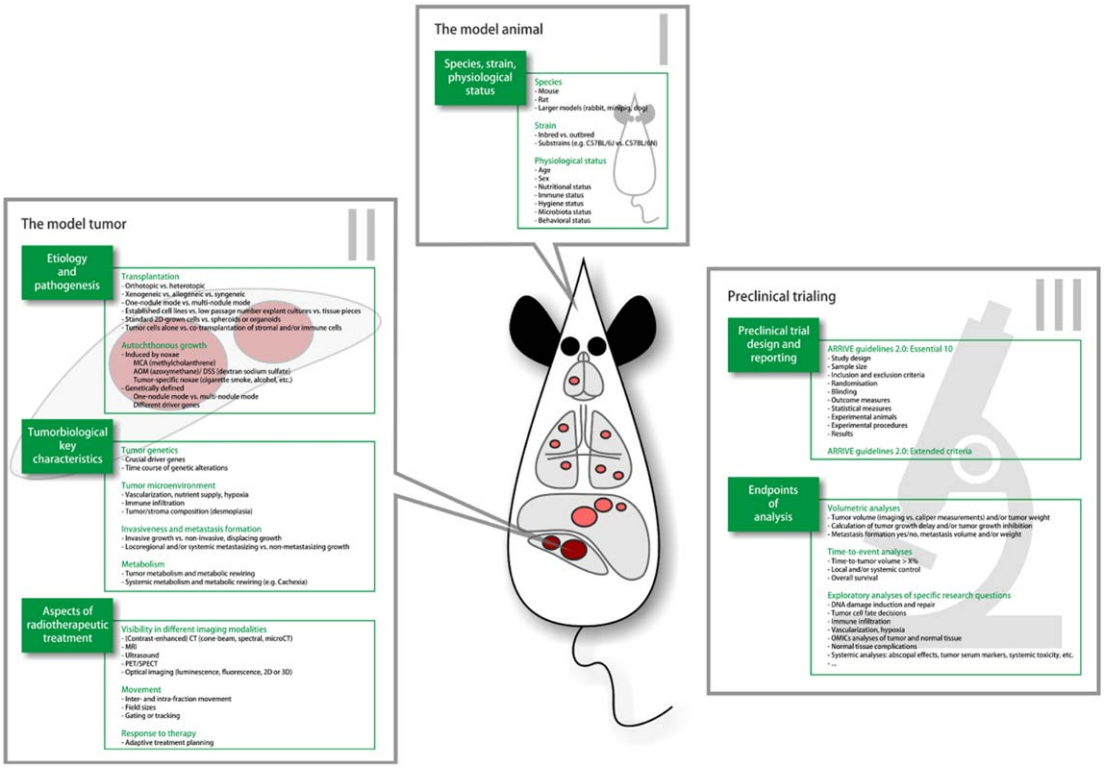

Figure 2. Aspects that need to be considered when setting up preclinical cancer radiotherapy trials.

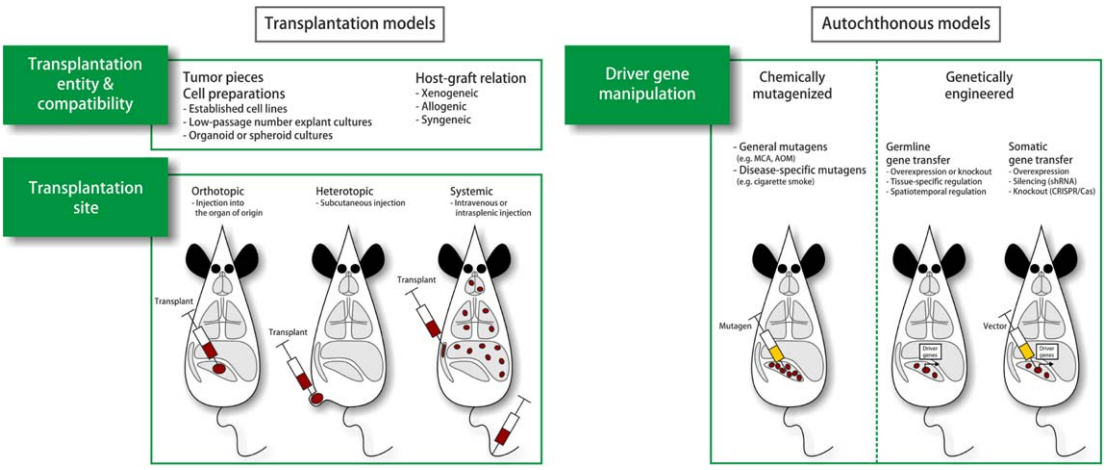

Figure 3. Transplantation tumor models and autochthonously growing tumor models used in preclinical (radiation) oncology.

growth inhibition studies, which essentially need to be classified as progressive disease (Kummer *et al* 2021). Translatability is furthermore undermined by poor reproducibility and lacking standardization of preclinical trials. In this regard, comprehensive description of preclinical trial design, experimental protocols, aspects of randomization, blinding, and endpoint assessment, including statistical sample size estimation, statistical analyses, and standardized reporting are increasingly being implemented. As such, the ARRIVE guidelines of animal experiments in general (Kilkenny *et al* 2010) and the ACROP guidelines for preclinical radiotherapy studies in particular (Verhaegen *et al* 2018) are currently being endorsed by scientific societies, journals, and funding agencies.

Advances in science and technology to meet challenges. The multidisciplinary spirit is one of the strengths of radiation oncology. Routinely employed team-based approaches of clinicians, physicists, and biologists to define the best treatment options should be further nurtured, cultivated and expanded to other disciplines, including geneticists, veterinarians, data scientists, and others, in order to ensure early implementation of relevant

strategies into preclinical trial designs with the overarching aim to maximize the potential for clinical translation. Small animal radiotherapy platforms technologically allow the precise planning and administration of small, highly conformal beam geometries under image guidance. Recent advances include (breathing) motion management, beam gating as well as progress in on-board, non-invasive imaging, such as bioluminescence and dual-energy computed tomography (Butterworth 2019). This enables improved radiation delivery in more efficacious and less toxic treatment regimens and proper follow-up monitoring of orthotopically growing tumors. Developments in animal models do parallel this evolution, and more elaborate translational models are currently finding their way into preclinical radiation oncology. Besides advanced tumor models and refined preclinical trial designs, the application of multi-level OMICs analyses with single cell and spatial resolution will further expedite our knowledge of therapy resistance mechanisms and will support the transition from simple to multi-model approaches in order to better reflect clinical settings and to guide the development of novel multi-modality radiotherapy concepts.

Concluding remarks. The availability of precision small animal radiotherapy platforms and elaborate tumor models allows sophisticated preclinical radiotherapy research with clinically relevant treatment setups and advanced preclinical trial designs that meet clinical standards at eye level. Improving reproducibility and standardization, together with the expansion of multidisciplinarity among scientists will further support the successful translation of preclinical results into novel multi-modality radiotherapy concepts.

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3. Preclinical models for non-malignant diseases

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Status. Radiotherapy is widely used as a therapeutic treatment approach for non-malignant diseases, although there is wide geographical variation. For example, up to a third of the daily activity of radiation oncology departments in Germany involves radiotherapy for non-malignant conditions (Seegenschmiedt *et al* 2015) such as treatments of plantar fasciitis (Hasegawa *et al* 2020), Dupuytren contracture (Keilholz *et al* 1996) and to address inflammatory and degenerative disorders of the joints (Alvarez *et al* 2021), as well as painful degenerative muscoloskeletal and hyperproliferative disorders. The feasibility of radiotherapy as a modality for non-malignant disease was also successfully demonstrated in the prevention of vascular restenosis after percutaneous transluminal angioplasty in a small clinical trial using external beam irradiation (Zabakis *et al* 2005), and has also been shown to provide some clinical benefit as a treatment for non-malignant vascular anomalies in pediatric and young adult patients (Liu *et al* 2021). These studies followed work that used beta and gamma vascular brachytherapy (VBT) to reduce angiographic restenosis (Shirai *et al* 2003). In clinical situations, stereotactic radiosurgery has been widely used as a treatment for cerebral arteriovenous malformations (AVMs) (Orio *et al* 2006, Loebel *et al* 2022), although in pre-clinical models studies have been restricted to the effects of radiation arteriopathy observed in resected brain arteriovenous malformations after radiosurgery (Lawton *et al* 2008) and to prompt target activation on endothelial cells for drugs treatments against AVMs (McRobb *et al* 2019). Low-dose radiation treatments are also used for asthma, pneumonia and other lung diseases (Chew *et al* 2021) and most recently as a potential treatment for, or mitigator of, for symptoms for COVID-19 (Venkatesulu *et al* 2021), although this is associated with potential carcinogenic risks for younger COVID patients with elevated risk factors for lung cancer and heart disease (Shuryak *et al* 2021). Radiation has been investigated in the treatment of Alzheimer's disease (AD). Single dose (5 Gy, 10 Gy, 15 Gy) and fractionated (2 Gy $\times$ 5, 2 Gy $\times$ 10) cranial irradiation significantly reduced beta amyloid plaques in the brain and improve cognition in murine models of AD (Marples *et al* 2016, Wilson *et al* 2020), and supported by observations in rats (Ceyzeriat *et al* 2021) and mice (Ceyzeriat *et al* 2022). Irradiation has also been investigated as a novel treatment for other prevalent neurological diseases. X-ray synchrotron microbeams were used to demonstrated stable and long-term antiepileptic effects in different animal models of epilepsy, and showed encouraging proofs-of-concept data (Studer *et al* 2015).

Current and future challenges. The scientific rationale for many of these past studies was to exploit the anti-inflammatory properties of low doses of radiation, or/and provide pain relief, and they evolved from single cases studies or smaller clinical pilot studies without extensive pre-clinical studies. The traditional translational research path was not followed in many of the studies because preclinical animal irradiators lacked sophistication. Until recently, the majority of preclinical irradiators were not sufficiently precise to irradiate a small targeted volume, because CT image guidance and accurate beam collimation was needed for accurate targeting. A recent brain study, that investigated the role of immune cell recruitment in the irradiated brain, required precise DVH-based radiation dose planning only achievable with a sophisticated small animal irradiator. Using a murine model surgically-implanted with a cranial window over the somatosensory cortex, the brain was irradiated using CT-image-guidance with a Small Animal Radiation Research Platform (SARRP) (Whitelaw *et al* 2021). A significant change in the temporal and spatial positioning of microglial in the imaged landscape was evident after irradiation, characterized by loss of microglial cells and significant re-arrangements of microglial location within the irradiated field with time after irradiation. This small preclinical study demonstrated the feasibility of imaging microglia-neuron interactions in real-time and defined how microglia react to irradiation in the same mouse.

The second use of radiation in non-malignant setting investigated the effects of radiation treatment on prepubertal muscle stem cells to model radiation-induced muscular deficiencies (Bachman *et al* 2020). Muscle stem cells are involved in myofiber growth during prepubertal development, and to investigate how these stem cells are impacted by radiotherapy Bachman and colleagues (Bachman *et al* 2020) used a SARRP to investigate stem cell regeneration post irradiation. Using transgenic mouse models for lineage tracing of indelibly labeled stem cells, they demonstrated reductions in both stem cells number (~44% reduction in juveniles compared with ~27% loss in adults) and function after prepubertal irradiation and then derived cell fate of targeted cells. Irradiated mice exhibited deficits in myofiber size. The use of the SARRP was essential for this study because mice were irradiated at three-four weeks of age, and precise small volume irradiation was need to target the prepubertal muscle. This involved the use of a customized single-mouse SARRP bed to allow localized delivery

to the lower limb below the knee to ankle that required CT guidance scan. This study highlighted the susceptibility of prepubertal stem cells to radiation exposure.

Advances in science and technology to meet challenges. The recent advances in small animal image-guided radiation platforms have played a major role in facilitating and refining these preclinical studies. The ability to deliver a precise hemibrain irradiation in the mouse Alzheimer studies significantly reduced the number of mice required for experiments, and reduced variability in assessing immunohistochemical or immunofluorescence staining analysis as the control and irradiated brain co-exist on the same histological section. In the microglial studies, the ability to irradiate a defined area of the brain under the cranial window facilitated the precise tracking of EGFP tagged cells using 2-photon microscopy. Similarly, the prepubertal muscle stem cell studies were made possible by the ability to precisely target the radiation to the tissue of interest.

Many of these RT-based non-malignant therapeutic interventions have raised concern about the risk of low-dose radiotherapy inducing malignant transformation. Although these concerns seem largely unfounded because of the low or intermediate single and total doses that are used in the treatment of non-malignant diseases, when compared the large total doses used for oncological treatments, the ability to mimic precise localized RT in preclinical models should help to further allay those concerns.

Concluding remarks. In conclusion, the application of radiotherapy not exceeding a single dose of 5 Gy and total doses of 30 Gy [low- or intermediate-dose RT (LD-RT)] is an established and effective modality in the management of a variety of non-cancerous inflammatory, degenerative, and hyperproliferative/fibroproliferative disorders. Future studies using appropriate preclinical models and precision small animal irradiators may further expand the use of radiation in other non-malignant diseases.

4. Precision radiation and normal tissue damage models

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Status. Radiotherapy (RT) is a major anti-cancer modality with nearly half of all patients expected to receive RT at some point during their disease course. However, radiation-induced side effects are common and may severely compromise the quality of life of cancer survivors. Early side effects are mostly transient, but late side effects are in general chronic and often progressive, leading to long-term reduction in patients' quality of life and are therefore the main cause of dose limitations (Bentzen 2006). Current strategies to reduce radiation-induced side effects are focused on reduction of the physical dose using technological advances, such as Intensity-Modulated Radiotherapy (IMRT), Stereotactic Body Radiotherapy (SBRT) and hadron (mainly proton and carbon) therapy. The severity of symptoms due to normal tissue damage is both volume and dose dependent. Until recently, the majority of the animal models employed in the study of radiation-induced normal tissue injury have been limited by the fact that they involved the whole body, whole abdomen (for intestinal effects), whole head (for salivary gland effects) whole thorax (for lung effects) or whole heart RT (for heart effects), which are far removed from the clinical setting (Barazzuol *et al* 2020, Schlaak *et al* 2020). Although we will never be able to exactly copy the human clinical setting with preclinical animal models, using more precision-based radiation schemes can uncover unappreciated and unanticipated side-effects as well as pharmacologically actionable biological mechanisms underpinning late effects. This review will outline some basic findings and conclusions with respect to dose- and volume-based effects with an emphasis of the cardio-pulmonary system.

Current and future challenges. During radiotherapy for head and neck cancer, the salivary glands are unavoidably co-irradiated leading to hyposalivation and related xerostomia with devastating consequences for the quality of life of the patients. Still, many preclinical studies use whole body or whole head irradiation to investigate salivary gland effects (Barazzuol *et al* 2020) ignoring the possibilities of precision radiation in small animals. Using local proton irradiation instead, clear volume effects and interesting regional differences were observed in preclinical studies of the salivary gland, showing a critical highly radiosensitive region (figure 4) (van Luijk *et al* 2015). Interestingly, the translation of these preclinical studies in a clinical double-blind randomized trial showed promising results (Steenbakkers *et al* 2022). Similar dose-volume effects and/or regional differences have been observed in the brain, pancreas, and bladder.

For malignant tumors in the thoracic region, including breast, lung, and oesophageal cancer, which are routinely treated by RT, a significant number of patients are at an increased risk of developing radiation-induced lung disease (RILD) or radiation-induced heart disease (RIHD) due to direct or incidental dose delivery to the heart and lungs. Often, during thoracic RT part of the lung and heart are both irradiated; however, these two tissues been shown to interact biologically and damage to one enhances damage to the other (Wiedemann *et al* 2022). To study the specific effect of lung or heart using preclinical models, accurate local irradiations targeting one organ but sparing the other needs to be performed. Clinical manifestations of RIHD include cardiomyopathy, conduction disorders, myocardial fibrosis, pericarditis, acute coronary syndrome, congestive heart failure, and valvular disease. One of the best-characterized pathological consequences of incidental exposure of the heart to RT is collagen deposition and progression to fibrosis development, which can lead to acute or delayed pericarditis, cardiomyopathy, arrhythmias and in certain cases, congestive heart failure or even sudden death. The mechanisms of RIHD are multifactorial and mainly involve direct DNA damage, increased oxidative stress, vascular endothelial cell injury, continues inflammation, and fibrosis (Boerma 2012). However, until now, there is no effective treatment to ameliorate the RIHD, partially because the detailed underlying mechanisms of the RIHD remain largely unknown.

Overall, the irradiated sub-volumes and dose distribution, are critical parameters that define the clinical tolerance of the heart, lung and likely most normal tissues.

Advances in science and technology to meet challenges. Most preclinical models to study the RIHD include whole thorax, whole heart, and partial heart and lung irradiation (Schlaak *et al* 2020). Even though these models have provided valuable insights into the mechanistic understanding of normal tissue toxicity post-RT, increased morbidity, and mortality due to unavoidable lung irradiation makes the relative contribution of specific tissue

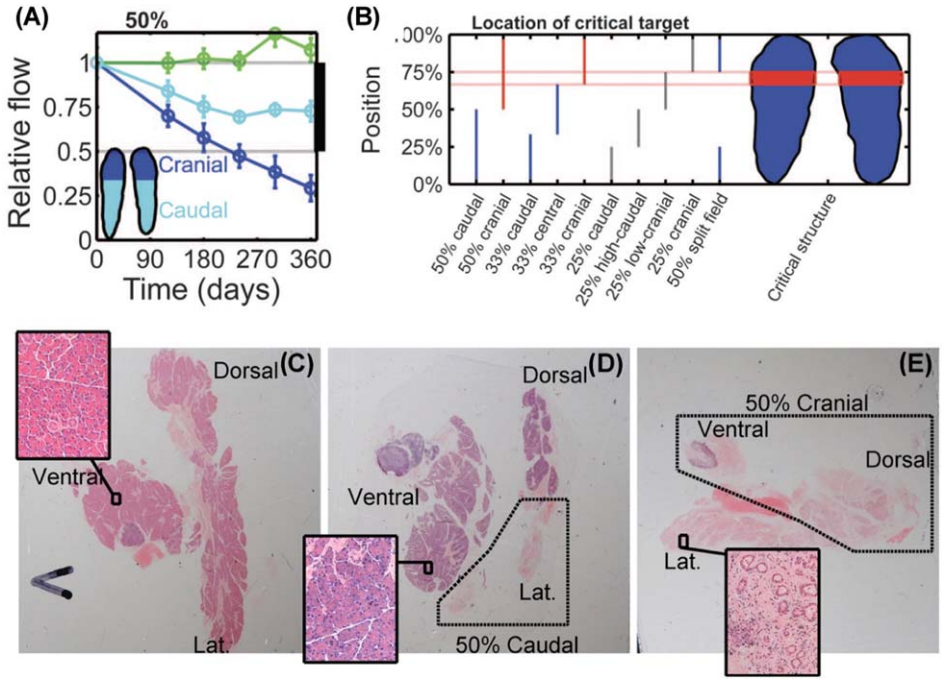

Figure 4. Local organ irradiation shows region-dependent radiosensitivity of the rat parotid gland. (A) Relative residual stimulated saliva flow rate after irradiation of various subvolumes of the rat parotid gland. Green line indicates the relative saliva flow rate of control animals. The grey line indicates the expected loss of function after 50% volume irradiation. Cranial 50% (dark blue)—caudal 50% (light blue). (B) Overview of several irradiated subvolumes. When the irradiated volume exceeded 25%, there was irreversible damage to the salivary gland [red or blue lines]. (C) Non-irradiated hematoxylin and eosin-stained rat parotid gland. (D) Irradiation of the caudal 50% region spared the critical region identified in (B); the irradiated parts of the lateral (Lat.) and ventral lobes degenerated but without visible damage to the non-irradiated sections. (E) Irradiation of the cranial 50% region, which includes the critical region containing stem/progenitor cells, led to degeneration of all lobes, including the non-irradiated sections. Black line indicates estimated position of the edge of the radiation field. Error bars indicate SEM. Adapted from (van Luijk *et al* 2015) and reprinted with permission from AAAS.

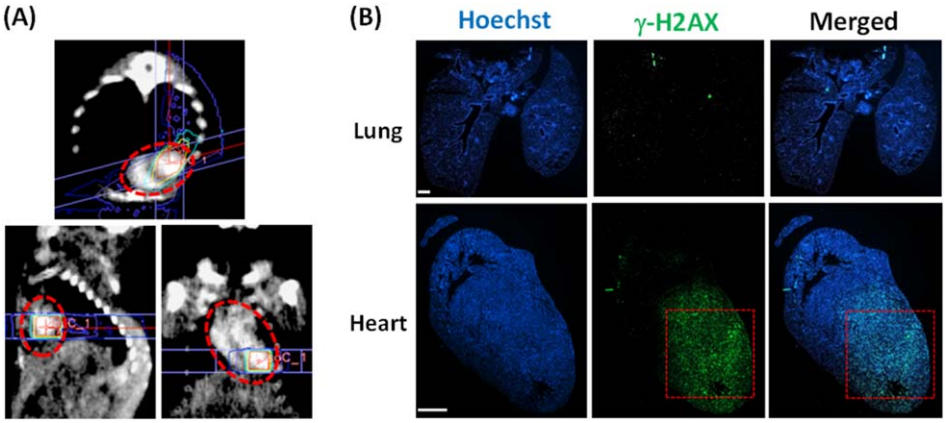

Figure 5. Dose plan in MuriPlan (Xstrahl, Suwanee, USA) and corresponding immunohistochemical g-H2AX staining of heart and lung tissue. **A,** Axial, sagittal, and frontal views of radiotherapy planning and delivery to the cardiac apex at a dose of 60 Gy with a 3 × 3 mm^2 collimator and conformal radiotherapy over a 75° arc. **B,** Positive staining in the cardiac apex and negative staining in the lungs confirms selective partial heart radiotherapy targeting. The red dotted circles outline the cardiac silhouett e. The red dotted boxes indicate the 3 × 3 mm^2 collimator. Scale bar, 1 mm. Adapted from (Ghita *et al* 2020).

damage (i.e. heart versus lung) to these endpoints quite difficult to ascertain. Recently, more physiologically relevant preclinical mouse models of RIHD have been developed, thus distinguishing them from previous models by both dose delivery and tissues involved. It has been shown that partial heart irradiation with a single dose of 12 Gy of x-rays to approximately one-third of the left ventricle led to decreased ejection fraction and increased myocardial fibrosis, a phenotype that was not observed when the whole heart was irradiated (Lee *et al* 2014). In another study using small animal image-guided RT, the group demonstrate structural and functional consequences of sub-volume targeting in the mouse heart and identified the heart base as a critical region with

increased radiosensitivity (Ghita *et al* 2020). Our group (Verginadis and Koumenis) recently developed a physiologically relevant small animal model which implements image-guided partial heart radiation with and without partial lung radiation (figure 5) (Dreyfuss *et al* 2021). We demonstrated a dose- and time-dependent cardiac dysfunction, dose- and site-dependent decrease in myocardial perfusion and significantly increased perivascular fibrosis (Dreyfuss *et al* 2021). Intriguingly, inclusion of partial lung irradiation into the treatment planning led to increased levels of cardiac toxicity compared to heart irradiation alone.

In general, clear dose-volume effects can be observed in all tissues using pre-clinical models. Moreover, regional differences in radiosensitivity can be found in most organs, due to different cellular compositions with distinct functions, density of stem/progenitor cells and intrinsic radiosensitivity. To make clinical decisions regarding the extend and type of tissue to be spared, or where to dose-escalate and by how much, a more in-depth knowledge of mechanisms behind tissue responses and the impact of dose distribution needs to be achieved. Interestingly, knowledge of such mechanisms could help to ameliorate normal tissue side effects such as for instance seen after thoracic irradiation with cardio-pulmonary side effects (Barazzuol *et al* 2020) which may be rapidly translated to the clinic.

Concluding remarks. There is an increasing need for more clinically relevant preclinical models to investigate the underlying molecular, cellular, and physiological mechanisms by which radiation causes acute and chronic normal tissue toxicities. For proper translation to the clinic the challenges are (a) developing clinically relevant pre-clinical models for sequential study of the tumor and normal tissue response in the same animal, (b) proper treatment planning and dose distribution, (c) image registration for focal irradiation studies and (d) image registration of normal and tumor response e.g. with PET using biologically relevant tracers. Finally, studies comparing ultra-high dose rate irradiation (FLASH) with conventional irradiation currently lack in-depth mechanistic background (Vozenin *et al* 2019, Diffenderfer *et al* 2020, Velalopoulou *et al* 2021), which is needed to properly compare preclinical models and suggest clinical approaches. Overcoming these challenges will help us improve the translational success and therefore, to develop new strategies that can block or even reverse the course of normal tissue effects.

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5. Imaging systems for image guidance

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Status. Within the last decade, novel technologies have been developed to improve preclinical imaging to realize real translational research. With the popularity of innovative small animal irradiation units, the importance of valid imaging has substantially increased. Optimal imaging is a significant requirement and critical feature for successful treatment planning, high-precision irradiation, and longitudinal response assessment in clinically relevant *in vivo* models. Presently available preclinical imaging modalities reflect the technologies available in the clinic adapted for preclinical radiation research (Verhaegen *et al* 2018). However, preclinical solutions have not yet reached the extent of imaging used today in human radiation oncology, where multi-modal imaging is a key factor before, during and after (image-guided) radiation therapy.

In general, the visualization of the target region and organs at risk could be improved by integrating appropriate imaging modalities, optimizing imaging protocols and parameters, or using contrast agents, specific biomarkers, or radioactive tracers. The most common 3D imaging modalities are computed tomography (CT) and magnetic resonance imaging (MRI) with superior soft-tissue contrast. For better differentiation and quantification of different tissue types, dual- and multi-energy CT were developed (McCollough *et al* 2015), complemented by novel x-ray modalities like phase contrast or dark-field imaging (Burkhardt *et al* 2021).

Nuclear medicine imaging modalities such as positron emission tomography (PET) and single-photon emission CT (SPECT) also provide tumour metabolic and functional information useful in defining molecular profiles and tumour sub-volumes with different radiobiological properties. Besides imaging, targeted internal radiopharmaceuticals with antitumor effects are promising tools for cancer treatment (James *et al* 2021).

Optical molecular imaging, including bioluminescence and fluorescence imaging (BLI), directly detects soft tissue targets via luciferase or fluorescence proteins and provides complementary molecular information (Deng *et al* 2020). Further imaging modalities are under investigation for small animal research, including photo-acoustic imaging, magnetic particle imaging, and terahertz imaging.

Over the last decade, image-guided preclinical irradiation setups were increasingly integrated into translational radiation research to improve precise guidance for therapeutic beam delivery and accuracy of target localization and reduce normal tissue toxicity. Thus, on-board imaging minimizes experimental uncertainties, which ensures the reproducibility of experimental investigations (Poirier *et al* 2020). Routinely, CT and cone beam CT are integrated into small animal irradiators. In addition, novel approaches integrated BLI or PET/SPECT image-guidance (Deroose *et al* 2007).

Current and future challenges. Besides significant technical developments, different challenges must be addressed to further advance preclinical research and bridge the translational gap between basic experimental settings and clinical radiotherapy.

Depending on the imaging modality, protocol, and intended spatial resolution, the imparted dose exposure from imaging is significant and needs to be considered, especially in longitudinal follow-up examinations. For example, a typical dose of a micro-CT examination can be 0.3 Gy or even higher (Verhaegen *et al* 2018). Consequently, there is a clear need for dose reduction or alternative approaches using non-ionizing radiation in order to avoid unnecessary radiation damage to the animals or confounding effects of the imaging dose to the overall study.

The scanning time of small animal imaging varies with the different modalities from a few minutes for standard CT up to 90 min for SPECT or PET (Vanhove *et al* 2015). Different forms of anaesthesia are therefore used for immobilization. The widely used volatile anaesthesia can easily be controlled, but also requires a dedicated anaesthesia system including vaporizer and flow metre integrated within the imaging systems. Alternatives are injected anaesthetics with a typical shorter duration of only 20 to 70 min However, anaesthesia affects cardiovascular and respiratory parameters and other physiological functions. Proper heating of the animals is urgently required to maintain a constant body temperature (Vanhove *et al* 2015). Overall, reducing the imaging time is a current challenge that needs to be addressed.

Another significant issue is the movement of the animals between initial imaging and subsequent irradiation and inter- and intrafractional organ motion, especially in abdominal and thoracic target tissues, leading to challenges in co-registration of images and uncertainties in irradiation. In human radiotherapy many

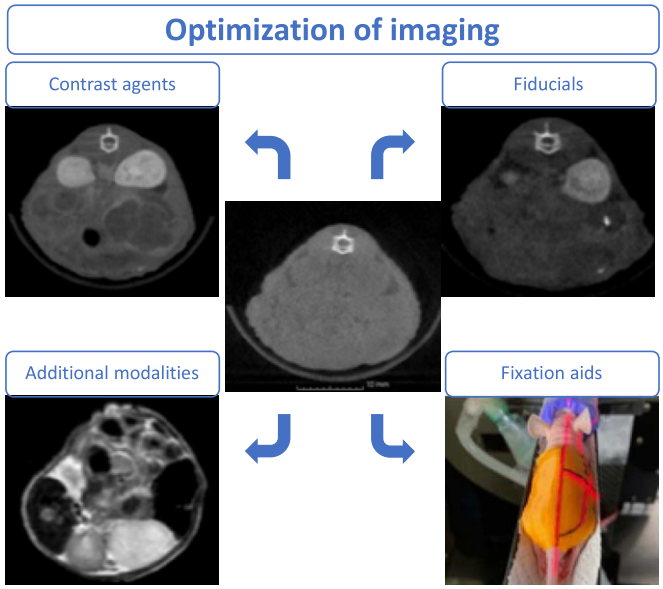

Figure 6. Example of optimized imaging for external beam radiotherapy in an orthotopic pancreatic tumor mouse model: Improved imaging by intravenous injection of iodine-containing contrast agent (upper left), by use of a liquid fiducial marker directly in the tumor tissue (upper right), by additional MRI (lower left) and by development of individual masks (lower right) to achieve stable positioning and reduced motion artefacts.

approaches deal with these issues both clinically and in research, which need to be adapted to small animal irradiation as well.

Finally, imaging for treatment planning, irradiation, and longitudinal studies generate large amounts of data. Here, significant limitations of high-throughput preclinical experiments are the lack of standardization of imaging protocols, import, and registration of data from different sources, data storage, communication and management systems, and quality assurance (QA). Efficient time-management of the whole routine workflow involving initial imaging, reference imaging, import and registration of data sets, animal handling, and transportation is still challenging (Persoon *et al* 2019).

Advances in science and technology to meet challenges. Strategies to minimize the dose caused by x-ray imaging include using non-ionizing modalities (e.g. MRI), optimizing the beam shape, appropriate tailoring of the beam energies and exposure time, and improvements in detector technology (e.g. photon counting detectors). Advanced image reconstruction protocols and methods of artificial intelligence can improve image quality and reduce artefacts and dose (Gupta *et al* 2022). Recent developments in novel imaging modalities beyond the clinical standard are very encouraging, although not necessarily dose efficient yet; in addition, not all emerging modalities can be transposed from small animals to the human scale (e.g. some optical methods are restricted by optical penetration depth in tissue), which might limit their potential for translational studies.

Various approaches to address motion management between initial imaging and fast co-localization for subsequent beam delivery can be further developed. During the (automated) registration process, supply of sufficient common features such as anatomic landmarks, contrast agents or fiducial markers can be beneficial (see figure 6). The same positioning and fixation aids such as animal holder, individual masks, or animal beds designed to provide primary immobilization solutions and stable positioning for imaging, transport, and subsequent irradiation should be generated (Verhaegen *et al* 2018). Alternatively, advanced on-board imaging modalities combined with (deformable) image registration can deal with remaining movements between initial imaging and irradiation setups. For target volumes in the thorax, 4D-CT can provide additional information over all breathing phases. Systems for surface-guided radiotherapy (SGRT) could monitor intra-fraction motion and detect anatomical variations along the treatment course, as established for human radiotherapy (Freislederer *et al* 2020). Further novel developments transposed from the clinic include preclinical MR-guidance (as in clinical MR-linacs), on-board PET-guidance as well as systems for image-guided small animal proton irradiation (Parodi *et al* 2019).

A major focus should be on optimizing and standardizing the workflow management by defining research protocols and standard operating procedures for imaging and (auto-)contouring and by using consistent nomenclature and unique IDs for animals and images. The exclusive use of DICOM ('Digital Imaging and Communications in Medicine') is recommended, as well as storing large datasets systematically in 'Picture

Archiving and Communication Systems' (PACS) for storing images. Regular QA for image quality and targeting accuracy (geometry and distortions) should be complemented by end-to-end tests comparable to the clinical setting.

Concluding remarks. Small animal imaging systems and advanced equipment for image-guidance are significant for target detection, the delivery of high-precision irradiation, and longitudinal assessment of tumour response and toxicity mimicking human treatment scenarios. Novel developments should focus on the reduction of dose and scan times, and on methods to guarantee the safe application of integrated, multi-modal imaging. To further improve the potential of translational preclinical radiation research, it is urgent to standardize and optimize the whole workflow of imaging, data transfer, processing and management, and registration of different imaging modalities. This includes the integration of the most relevant imaging modalities with the treatment device and the optimization of the animal transfer to the treatment site for remaining modalities. By adapting methods from clinical practice, sophisticated techniques can be applied to minimize motion and develop appropriate guidelines and protocols.

6. Irradiation systems

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Status. Current preclinical photon irradiation systems roughly fall into two categories: simple irradiation cabinets with limited degrees of freedom and rarely any imaging options, and more sophisticated systems with flexible treatment fields from multiple beam directions (e.g. from a rotating gantry) and image guidance. This section focusses on the latter, which have been in existence for about 15 years. There have been several in-house built systems and currently there are only two commercial vendors of image-guided platforms (Verhaegen *et al* 2011), which sell quite similar systems with a kilovolt irradiator (220–225 kV) and a cone beam CT imaging panel mounted on the same gantry. Both platforms have a treatment planning system (TPS) and a control system, along with an optional bioluminescent camera (Weersink *et al* 2014). A few users have added ultrasound imaging or PET imaging to their platform.

A recent review showed that more than 300 studies have already been published using the image-guided type of platforms (Brown *et al* 2022). Many of the initial papers were on technical aspects such as dosimetry, calibration, system design, imaging techniques, protocols and processing, treatment planning systems, novel detectors, phantoms, and the accuracy and precision of the devices. Due to this, preclinical radiobiology studies can now be performed with a much higher level of quantification than with the previous generation of cabinets, and experiments with orthotopic tumors are now much more common. Only in the last ten years radiobiology studies were published with the advanced devices. A majority of studies were in the oncology field, either on tumor response, or about normal tissue radiation damage studies. Within the oncology studies most were about brain, lung or the gastrointestinal system. There is also a growing number of non-oncology studies where radiation is used for other purposes, such as cardiac ablation, renal damage, Alzheimer's disease and even COVID-19 (Wilson *et al* 2020, Jackson *et al* 2022).

Current commercial platforms offer many degrees of freedom such as a flexible irradiation system, variable rectangular fields and fixed collimators, and a rotating stage. However, a large majority of investigators is currently using a small number of fixed beams, often e.g. parallel-opposing beams. The potential of the research platforms has, therefore, not yet been fully reached.

One of the most important aims of preclinical studies is to identify the most promising research to transfer to clinical trials in the most efficient way, thereby also respecting the 3Rs of animal research (Replacement, Reduction and Refinement). A preclinical TPS should play a very important role in this, as it constitutes the core system where the irradiation scheme is designed. Therefore, a modern preclinical TPS should be part of the radiation platform.

Current and future challenges. The current commercial image-guided radiation research platforms present a quantum leap forward compared to the older simple cabinets, but there is still a lot of technology to develop to support radiobiology research optimally and to emulate, to some extent, the clinical workflow of image-guided radiotherapy.

Platforms should have a dose monitor. Since animals can move non-negligibly during irradiation they should also be monitored by a non-invasive camera system for motion, and for physiological parameters such as temperature, breathing rate & frequency, and possibly O_2 exchange.

As platforms advance one can expect to see more hybrid technology in the same coordinate system, such as PET/SPECT cameras, MRI equipment, but also more exciting novel equipment specifically for preclinical research such as proton and ion CT, dark field x-ray imaging, and phase contrast x-ray imaging. Bioluminescence tomographic AI-based reconstruction is only now being added to the capabilities of imaging devices in these platforms and fluorescent imaging may also not be far in the future.

Even with all this, the platforms will still lack capabilities that are quite common in radiotherapy clinics, such as robotic positioning of the beams and specimen, 6 degree of freedom couches, multileaf collimators to shape arbitrary beam shapes, and monitoring systems to enable tracking and gating of radiation beams. The standard preclinical irradiation modality is currently kV photon beams, but there are a few centers where a commercial photon platform has been docked to a clinical proton beam (Ford *et al* 2017, Kim *et al* 2019), enabling e.g. relative biological effectiveness (RBE) studies in the same coordinate system.

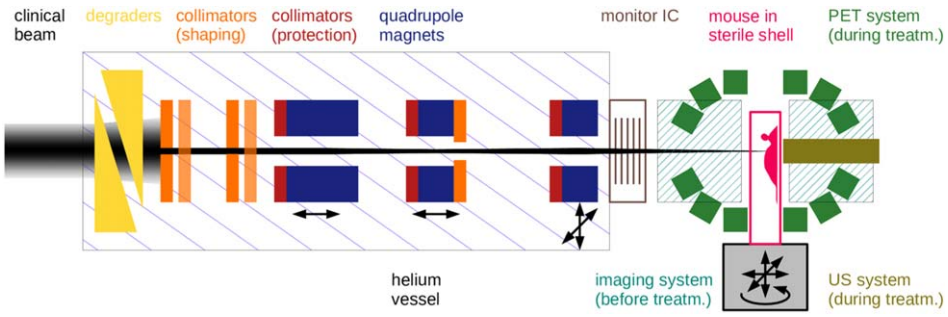

Figure 7. Schematics of the SIRMIO system, showing the beamline used to degrade, collimate and actively focus the clinical proton beam, the dedicated beam monitor and the irradiation site accommodating different modalities for image-guidance before and during treatment (Courtesy Dr Jonathan Bortfeldt).

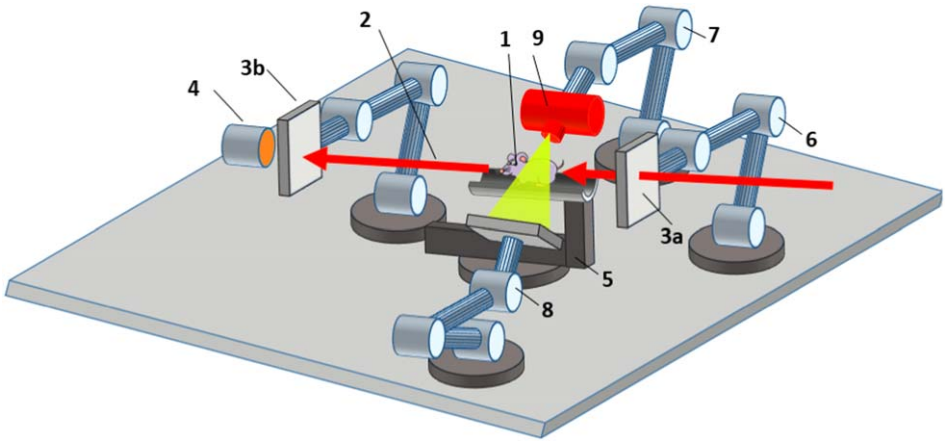

Figure 8. The IRRADION system under design. The red arrow pointing from the right is a proton/ion beam from a clinical therapy facility. The proton beam (2) traverses a mouse (1) held on a (robotic) couch (5), also hitting two TimePix sensors (3ab) and a stopping calorimeter (4). Robotic arms (6,7,8) hold the proton imaging sensors and the x-ray tube (9) which is both used for x-ray imaging with another TimePix sensor, and for photon irradiation (green beam).

The current hype about Flash radiotherapy, where very high dose rates (>40 Gy s^{-1}) are administered in less than a second, is an ideal subject to be studied with the advanced preclinical platform. However, currently, these Flash platforms are not widely available and much of the preclinical research so far has been done with modified electron beam accelerators with little or no imaging on board. Also, a preclinical Flash treatment planning system needs to be developed. Similar challenges involve research on the effects of micro/minibeam radiation therapy (MRT), a novel form of spatially fractionated radiotherapy exploiting intense micro/minibeams, which can be currently best produced at only a few dedicated beamlines for example of large-scale synchrotron radiation facilities.

The fascinating field of radiation-stimulated immunotherapy is also a likely candidate to investigate in depth on these novel devices, where response of biomarkers after irradiation should be monitorable.

Advances in science and technology to meet challenges. Occasionally, users of the image-guided preclinical platforms design some additional improvements, e.g. few-leaf variable collimators, to mimic closer the clinical irradiation devices (Woods *et al* 2019). But mostly, the innovations have come from the vendors. The commercial systems have been improving, but the scientific community now really needs a next-generation of versatile research devices. We discuss here briefly two such undertakings, which are currently at the development stage and are not yet commercially available.

Figure 7 shows the portable SIRMIO (*Small Animal Proton Irradiator for Research in Molecular Image-guided Radiation-Oncology* (Parodi *et al* 2019)) system under development at LMU in Munich (Germany) for operation at clinical proton therapy facilities. A dedicated beamline degrades, collimates and magnetically focuses the beam to sub-millimeter spot size. Solutions of pre-treatment proton imaging span from in-house detectors for tracking and residual energy measurement of single particles up to commercial sensors for position- (and single proton) resolved energy loss detection. *In vivo* monitoring of the beam range can be provided by a dedicated in-

beam PET scanner and, for intense pulsed beams, sensing of thermoacoustic emissions, ideally co-registered to (pre-treatment) ultrasonic imaging.

Figure 8 sketches the IRRADION system (Meyer *et al* 2021), under construction in Maastricht (Netherlands) and Prague (Czech Republic). It offers a flexible system with imaging and irradiation capabilities for photon/proton beams. It uses small robots to position the specimen, pick up different imaging sensors and an x-ray tube for irradiation. TimePix imaging sensor technology is used for proton and photon planar or CT imaging. A calorimeter stops the protons to determine their residual energy after traversing the mouse, enabling extraction of stopping powers from the mouse voxels.

Dedicated preclinical Flash electron irradiators are now also appearing on the market (Sordina IORT Technologies, Italy). Most likely, in the near future standard clinical proton accelerator technology beams can reach Flash dose rates for preclinical studies. Flash photon or proton research may be realized by employing x-ray tubes, laser-driven proton accelerators or by equipment like the PHASER accelerator array (Maxim *et al* 2019).

Concluding remarks. The development of versatile preclinical research platforms has just begun. By combining novel imaging sensors, adapted from different physics and engineering fields, with accurate positioning (e.g. by robots) and monitoring, high-throughput research will be facilitated. Exciting new phenomena such as Flash radiotherapy or radiation-induced immunotherapy can be unraveled with these platforms. Combined with sophisticated disease models and drugs, there is no limit to the novel therapies that these platforms may help to create. Many studies have already been initiated outside the field of oncology, which is currently the heaviest application field.

These platforms with their multitude of novel imaging methods and new ways to deliver radiation beams, can also be seen as the forerunners of clinical technology to be developed over the next decades. Experience gained with these research platforms will therefore also greatly contribute towards clinical technical innovation, which up to now has never been the case.

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7. Treatment planning

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Status. In human radiotherapy the process of treatment planning involves identifying the target volume (e.g. tumor) and the surrounding normal tissues to be spared, followed by arranging the beam directions and performing dose calculations. This was possible since the 90ies of the previous century, with the advent of various 3D imaging modalities such as x-ray computed tomography (CT) and software algorithms on powerful computers. In preclinical radiation research the development of methods for treatment planning only started about a decade ago. Before that, point dose calculations or measurements were often the state of the art.

Figure 9 shows the steps involved in modern preclinical treatment planning. Most effort so far has been done on developing dose calculation methods, from simple analytical methods, to superposition-convolution approaches and even Monte Carlo (MC) methods (van Hoof *et al* 2013, Reinhart *et al* 2017, Cho *et al* 2018). Currently, two commercial preclinical treatment planning systems (TPS) exist, based on 3D CT specimen images and 3D dose calculations. The TPS provide treatment plans, where most of the steps are performed manually, which are then sent to the research platform to irradiate the specimen with various beams. Currently, most investigations are still done with a single static beam with a fixed collimator, or at most with two-parallel opposing beams. In many cases no avoidance structures are defined. This is far removed from how patients are treated in radiotherapy. There is therefore a clear need to perform irradiation studies that mimic much more closely human radiotherapy for a variety of specializations (oncology, cardiology, neurology, urology).

The development of novel research platforms, for studies exploiting a range of radiation types (photons, ions, electrons) and for investigating modern developments such as Flash irradiation, or combined immuno-radiation therapy, demands more powerful TPS.

One of the most important aims of preclinical studies is to identify the most promising research to transfer to clinical trials in the most efficient way, and to quickly eliminate unsuccessful therapies, thereby also respecting the 3Rs of animal research. A preclinical TPS should play a very important role in this, as it constitutes the control system where the irradiation scheme is designed. Therefore, a modern preclinical TPS should be fast, accurate, easy to use for non-specialists, strongly integrated with the research platform, and also connected to a data warehouse, which currently doesn't exist for the preclinical field.

Current and future challenges. Preclinical imaging uses a wide range of imaging modalities and even some that are not common in clinical practice such as ion radiography or CT, bioluminescent imaging, photo-acoustic imaging, phase-contrast x-ray imaging, or even dark x-ray imaging (Burkhardt *et al* 2021). Most of these imaging modalities may provide useful information for treatment planning e.g. by combining two modalities where one displays the anatomy, and the other a physiological biomarker, e.g. for hypoxia. Novel contrast media are also being explored preclinically, e.g. nanoparticles for both imaging and therapy (Schuemann *et al* 2020). Before these images can be used for treatment planning, several of these modalities require much further work, e.g. to develop algorithms for true tomographic bioluminescent image reconstruction, or proton CT (Meyer *et al* 2020). Modern radiation platforms may comprise several imaging systems in the same coordinate system but for images acquired with other systems, development of registration software is required. Some imaging systems may even acquire time-dependent images, so far unused in preclinical treatment planning. Imaging may also be used to assess outcome of experiments (van der Heyden *et al* 2020), and also this information may be utilized to e.g. adapt treatment plans for different radiation fractions.

A wide range of particle types for irradiation is being studied, e.g. to assess their relative biological effectiveness compared to photons. Electron beam studies have received renewed interest with the (re)discovery of the Flash phenomenon, since photon beams with a sufficient dose rate are hard to make. A preclinical TPS must be able to model all these different beams and perform rapid dose calculation during the time span where the animal remains sedated on the platform during the whole process. Each beam modality requires specific quantitative physics information to be extracted from various imaging modalities. An example is mass density and tissue composition for MC dose calculations in photon beams. Knowledge of the latter for animal models is surprisingly poor currently. The clinically commonly used inverse planning (this process starts by specifying the required dose distribution and then deriving the beam arrangement from this) has not yet been implemented in preclinical TPS. These TPS should be able to handle complex planning with many degrees of freedom, and with inputs from various imaging modalities, without the need for the user to a treatment planning expert.

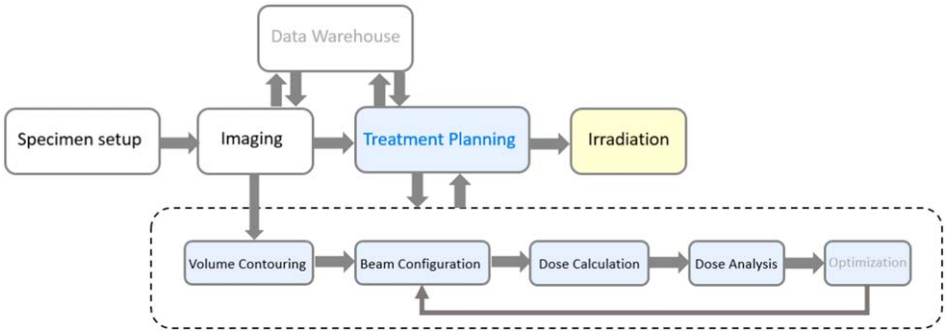

Figure 9. The current preclinical workflow. The specimen is sedated and setup for imaging, which uses nowadays mostly a form of x-ray CT imaging. The treatment planning stage (blue) involves contouring structures, setting up the radiation beams, performing dose calculations, analyzing the dose and, possibly, optimizing the process in a feedback loop. The boxes with light grey text are currently not part of the standard process.

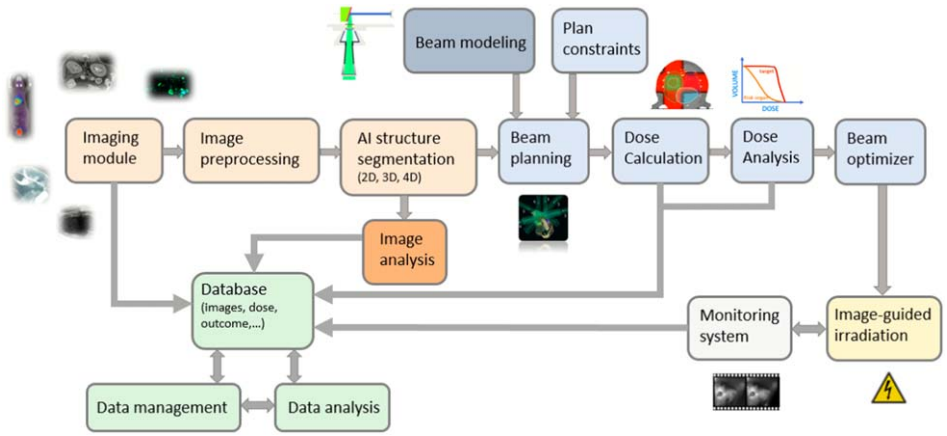

Figure 10. The preclinical study workflow of the future, where the connections between strict treatment planning actions and other tasks become more complex, allowing for more powerful and efficient studies.

The development of TPS should keep up with technological advances in research platforms (e.g. robotic systems, complex collimators, dynamic collimators, ion beams, inverse Compton beams). On the low-end side of simple irradiation platforms that do not use image guidance, currently no commercial dose calculation software exists. Essential is also the efficiency of the TPS so that it doesn't become a bottleneck in the irradiation process.

Expensive clinical photon TPS usually handle only megavoltage beams, whereas preclinical TPS must be able to handle kilovolt beams, with their issues of higher biological damage per unit dose, higher dose heterogeneity, the higher required accuracy on atomic composition and the clinical non-equivalence of different photon energies. Preclinical irradiation margin recipes are virtually non-existent currently and should be developed (Vaniqui *et al* 2019).

Advances in science and technology to meet challenges. The treatment planning technology currently used clinically is far ahead of the preclinical TPSs, so we must look towards the former for solutions, but several issues are unique to animal research. Figure 10 sketches a possible future preclinical TPS, including multi-modality imaging, a database, the planning and execution phase, and a monitoring system. Figure 9 identifies two major bottlenecks preclinically: the speed of dose calculation and structure contouring. Most preclinical research is presently done with kilovolt photon beams, requiring MC simulation to fully model the photon interactions in heterogeneous anatomies. Speedup methods may be borrowed from MC kilovolt imaging dose calculations on Graphical Processor Units (Jia *et al* 2012), but also artificial intelligence (AI) may be employed. Specialized forms of imaging such as dual-energy CT or spectral CT are now underdevelopment which will enable extracting the required medium characteristics for dose calculations.

AI has the potential to circumvent the very slow manual structure contouring process, reducing the time of up to one hour to mere seconds (Schoppe *et al* 2020, Lappas *et al* 2022). Very rapid progress is expected in this field, where until recently the slower atlas-based contouring (van der Heyden *et al* 2018) received much

attention. Most efforts went into autocontouring for avoidance structures in oncology studies, but work is also needed for autodelineating tumors or other structures to be targeted with radiation in non-oncology studies (e.g. epilepsy). AI may also play a role in optimizing the dose, inverse planning the dose, denoising the inherent statistically noisy MC dose, choosing the most suitable plan for a certain fraction from a precalculated library, and even in fully automatic dose calculation starting from a possible range of beam conditions.

The flexibility of modern MC codes enables simulation of the wide range of particle beams and irradiation conditions, including dynamic beams, encountered preclinically. It may even be used to examine the intricate dose rate distributions needed for Flash therapy. Development of preclinical data warehouses, possibly with links to clinical databases will empower large-data studies (Persoon *et al* 2019).

Concluding remarks. Currently available preclinical TPS systems allow much more accurate and versatile image-guided preclinical radiation research, compared to previous generations of radiobiology studies. However, TPS need much more development to mimic human radiotherapy technology, to optimally exploit information extracted from various types of imaging, to streamline and automate their use for non-specialists, and to allow studies of new treatment modalities e.g. Flash. Bottlenecks in the workflow, such as dose calculation speed and automatic structure contouring will soon be solved, saving tremendous amounts of time. Preclinical database systems, powerful (preferably automated) analysis methods for a wide range of data sources, and monitoring systems have yet to be developed. The availability of such advanced TPS will stimulate more advanced studies. The tremendous rise of AI, now still in its infancy, should be harnessed to empower preclinical TPS.

8. Small animal dosimetry

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Status. As eluded to in earlier sections, commercial conformal small animal micro-irradiation (micro-IR) devices are scaled down versions of their clinical counterparts. Micro-IR vendors offer collimated beams down to 0.5 mm diameter; this is an order of magnitude lower compared to the smallest clinical beam field size and represents a significant challenge for the accurate delivery of a prescribed dose to a target. The result of scaling down radiation technology to accommodate small animals has meant re-examining external beam protocols intended to measure radiation dose. Fortunately, micro-IR devices do also come equipped with fields large enough that the established guidelines for kV dosimetry remain broadly valid (e.g. AAPM TG61, IAEA TRS-398).

Despite existing standard-of-practice dosimetry protocols, dose reporting in biological studies is far from adequate. Incomplete reporting can be largely attributed to insufficient physics support or lack of training to users who are mostly biologist. A 2011 Workshop for Radiobiology Dosimetry Standardization initiated by National Institute of Standards and Technology resulted in publication of required dosimetric reporting data (Desrosiers *et al* 2013). A subsequent large-scale literature review of the mandatory requirements revealed that the majority of published studies provided about half of the required information (Draeger *et al* 2020). The lack of adequate reporting does not invalidate the published results but weakens the relevance of reported dose-toxicity and dose-efficacy relationships. The appropriate reporting of dosimetry studies is essential for inter-study comparisons and further meta-analysis.

Micro-IR small animal studies are also susceptible to the specific experimental conditions that are often challenging to control, therefore where possible, effort should be spent on best practices to reduce as much as possible the experimental variability. Tumour response and normal tissue toxicity models are characterized by steep profiles particularly sensitive to possible dosimetry inaccuracies that can lead to unreliable tumour or normal tissue dose-response. Conversely, more accurate dosimetric data can help better determine alpha/beta values for specific endpoints with improved accuracy in the determination of biological effective doses, for instance, when dealing with innovative radiation schemes (e.g. FLASH). In this direction, full 3D preclinical *in vivo* dosimetry represent a promising tool, however, dedicated and validated vendor's solutions are currently not available (Granton *et al* 2012, Verhaegen and Georg 2017, Anvari *et al* 2020).

Current and future challenges. Established orthovoltage reference dosimetry protocols using traceable calibration factors (e.g. AAPM TG61) are applicable to micro-IR devices with minor accommodations due to the limited measurement space and beam quality determination (i.e. first and second half-value-layer). Figure 11 illustrates a traceable dosimetry chain for kilovoltage dosimetry from the primary standard measurements to treatment delivery and absorbed dose assessment/verification. For collimated beams with diameters smaller than the sensitive length of stipulated ionization chambers (IC) described in the aforementioned dosimetry protocols (e.g. for PTW 30012 being ~25 mm), dose determination requires a suitable secondary dosimeter. Table 1 lists a number of dose measuring devices, their spatial resolution and energy response.

Dose determination for smaller fields is subject to additional uncertainties due to the reliance of a secondary dosimetry method (not to be confused with secondary-standard dosimeter). Radiochromic film is an example of a reliable secondary dosimetry method, which allows to measure depth-dose profiles with a reasonable accuracy by using films sandwiched in solid water (figure 11(E)).

For simple irradiation geometries (e.g. parallel opposed beams), point-dose along the central axis prescriptions can be achieved with reasonable accuracy (~6%) using tabulated depth-dose data (Subiel *et al* 2020), good laboratory practices and following published protocols traceable to primary and/or secondary standard dosimetry laboratories. However, a limitation of generalized (forward) dose prescription methods rise from the neglected scatter radiation component that contributes to the target tissue absorbed dose; this condition can deviate significantly from standard reference conditions.

The influence of object size was examined using Monte Carlo methods (BEAMnrc/ EGSnrc) (Kawrakow *et al* 2000). For 250 kVp spectrum and a field size of 25 mm and 30 cm SSD, the dosimetric impact at surface and at a depth of 2 cm is less than 2% when the object/specimen being irradiated is larger than the field size. The deviation is larger near the end of a depth dose profile when the object depth is smaller than the reference depth (see figure 12). Furthermore, the dissimilarity in scatter conditions at surface and a depth of 2 cm for varying

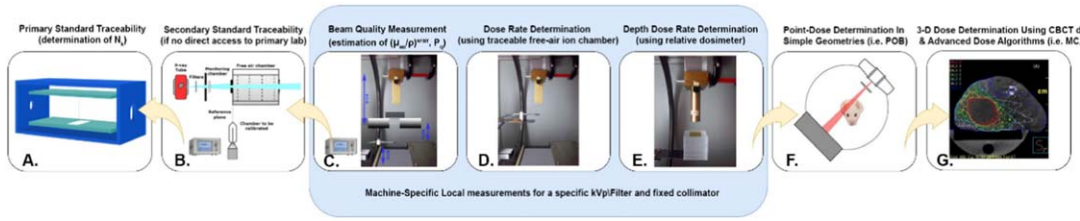


Figure 11. (A)–(G) The discrete steps in a traceable small animal dosimetry chain. (A)–(B) The ionization calibration factor N_k preferably directly disseminated at a primary standard laboratory and directly calibrated together with the institute's electrometer at the desired beam quality i.e. energy/filtration. (C)–(E) Local measurements of the beam quality, evaluation of the mass energy absorption ratios $(\mu_{en}/\rho)^{w/air}$ and chamber correction factor $P_{q\ cham}$, dose rate, and depth-dose measurements in full scatter conditions using an appropriate dosimeter such as radiochromic film. (F)–(G) Point-dose determination in simple geometries such as parallel-opposed beams to 3D dose determinations using acquired Cone Beam Computed Tomography images, tissue assignment, and application of dose algorithms utilizing analytical or Monte Carlo methods. 3D dose algorithms can account for lack of backscatter conditions, but may still rely on corrections derived from relative measurements for beam sizes less than 5 mm due to the challenge of actually modelling the focal spot and beam alignment.

beam sizes compared to the reference beam size requires the use of secondary dosimeters mentioned above or model-based dosimetric methods like superposition/convolution.

Advances in science and technology to meet challenges

Improvement in dose accuracy. A future improvement in dose accuracy within small animal RT can be achieved through the use of primary standards in orthovoltage energy range calibrated directly to absorbed dose to water in place of air kerma. This would lead to simplification of dosimetry formalism and reduction of uncertainties. Several National Metrology Institutes have worked toward achieving that goal and developed new national standards of absorbed dose to water for orthovoltage x-ray beams (Krauss *et al* 2012, Pinto *et al* 2016). The possibility to calibrate the IC directly in terms of absorbed dose to water is not universally available, however, Laboratoire National Henri Becquerel (France) offers such services on request (Perichon *et al* 2013). Small-field dosimetry using micro IC is currently limited. However, recent work indicates that small-volume IC could be used for dosimetry of micro-IR devices (Silvestre Patallo *et al* 2021).

Pre-treatment verification using heterogenous media. In orthovoltage protocols, the complex anatomy and tissue heterogeneity characteristic of small animal models, causes possible non-negligible deviation of the delivered on-axis absorbed dose when compared to the planned dose prescription. Pre-treatment plan verifications in anatomically correct tissue equivalent zoomorphic phantoms can provide more accurate estimates of the absorbed dose in target tissues. Soultanidis *et al* fabricated a mouse phantom made of tissue equivalent materials utilizing conventional milling, moulding as well as 3D-printing techniques (Soultanidis *et al* 2019). Subsequently, (Silvestre Patallo *et al* 2020), after careful detector characterization, employed that phantom with traceably calibrated alanine dosimeters at pre-selected locations to carry out end-to-end dosimetry test in micro-IR devices. These phantoms are useful for dose planning and verification in non-standard tissues such as in bones (Bazalova and Graves 2011).

In vivo *dosimetry.* Furthermore, accurate *in vivo* dosimetry tools can provide plan verification capturing potential errors due to equipment failure or incorrect specimen positioning for example. *In vivo* dosimetry can be as simple as point-based measurements such as that provided by optical scintillators (Le Deroff *et al* 2020) or more advanced 3D techniques utilizing on-board electronic portal imaging device as a dosimeter and reconstruct the dose in the acquired CT image (Granton *et al* 2012, Anvari *et al* 2020). EPID-based techniques require accurate characterization of the detector and the processing of large amounts of data, which currently is not offered on the commercial systems. Accurate EPID-based *in vivo* dosimetry would solve a number of dose verification and reporting issues with minimal experimental interference.

Concluding remarks. Despite well-established dosimetry in clinical radiotherapy, dose measurements in external beam pre-clinical and radiobiology studies are frequently inadequate or not sufficiently characterized, thus undermining the reliability and reproducibility of the published findings. The capability of micro-IR devices to deliver highly focal beams to multiple animal model systems provides new research opportunities to bridge laboratory research and clinical translation, however this adds additional complexity to dose determination due to application of very small fields. Lack of standardization and inaccurate dosimetry assessment in preclinical research can hamper translational opportunities for new radiation therapy

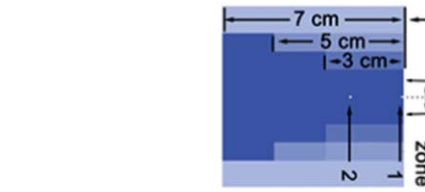
Table 1. Available detector systems for kV x-ray dosimetry. Note, that all of the detectors listed below rely on cross-calibration against an ionization chamber calibrated at a primary or secondary standard dosimetry laboratory (PSDL, SSDL).

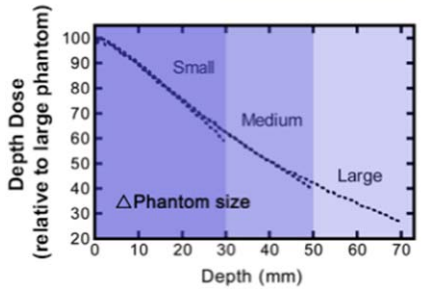
Detector	Uncertainty ($k = 2$)	Common use	Cross section of the sensitive volume/resolution	Limitations
Ionisation chambers[a]	<4% (<1% possible)	Reference dosimetry	(⌀ ~2 to 5 mm) depending on the size of sensitive volume. Typically used for field sizes above 1 cm × 1 cm	Calibration required by PSDL or SSDL
		Commissioning Dose calibration QA Dose range: mGy −>1 kGy		Size limitations (cannot be used for the very small fields)
Radiochomic films	<5% (2%–3% possible)	Planar dose distributions	Possible sub-mm resolution (if microscopy is used for scanning the film)	Processing required 24h post-irradiation (due to self-development)
		Imaging dose range: 0.1–200 Gy		Inter-batch variations
Alanine	<3% (1.5% possible)	*In vivo* dosimetry	Resolution down to 0.3 mm for the smallest pellet size (standard pellet size is 5 mm)	Processing required at an electron paramagnetic resonance EPR spectroscopy facility (often at PSDL)
		Reference dosimetry Audit purposes Dose range: 10 Gy–150 kGy		For small (2 mm ϕ) pellets delivery of >100 Gy is required Temperature dependence (0.2% C^{-1} correction)
TLDs	<5% (1.5% possible)	*In vivo* dosimetry	Typically 2–5 mm resolution (depending on the detector size), but micro-TLDs ($1 \times 1 \times 1$ mm^3) also available	Post-irradiation processing
		Audit purposes dose range:<200 Gy		Large energy dependence sensitivity to light complicated calibration procedure
OSLDs	<4% (1.5% possible)	*In vivo* dosimetry Audit purposes dose range: <10 Gy	Typically 2–5 mm resolution (depending on the detector size)	Post-irradiation processing Large energy dependence sensitivity to light Lack of dose calibration protocols Temperature dependence
Silicon diodes	<5% (3% possible)	*In vivo* dosimetry Detector arrays Relative and small field dosimetry	~1 mm^2 active area (500 μm resolution possible)	Temperature dependence (0.5% C^{-1}) Dose rate dependence Energy dependence Sensitivity changes with accumulated dose
		Dose range: <10 Gy		
MOSFETs	<5% (3% possible)	*In vivo* dosimetry Small field dosimetry	Extremely small effective volume (approx. 100 μm × 100 μm)	Finite life (~100 Gy) Energy dependence

Table 1. (Continued.)

Detector	Uncertainty ($k = 2$)	Common use	Cross section of the sensitive volume/resolution	Limitations
				Temperature dependence Sensitivity changes with accumulated dose
		Detector arrays Dose range: <10 Gy		
Diamond detectors	<3% (1.5% possible)	*In vivo* and small field	Typically ~ 5 mm resolution	Require pre-irradiation Significant variability among available detectors
		Dosimetry Dose range: <10 Gy		
Gel dosimeters	5%–10%	3D dosimetry	Limited by scan resolution/noise (can be as low as was 0.2 mm resolution, however with increased noise)	Time consuming and complex preparations
		Audit purposes Dose range: <10 Gy		Limited reproducibility Post-irradiation polymerization and ion diffusion Possible oxygen contamination hampering the readout

[a] For orthovoltage beams, typically the additional uncertainty of 6% ($k = 2$) in D_w determination is associated with current IC calibration procedures, which require conversion of air kerma measurement to absorbed dose to water.





Relative Dose Percentage at surface and at 2 cm depth

Phantom	R, D (cm)	Field size (mm) 25	10	5	2	1	
Large	3.5, 7	100.0	95.7	94.2	93.0	91.2	At surface (dose zone 1)
Medium	2.5, 5	99.6	95.6	94.2	93.1	91.2	
Small	1.5, 3	98.9	95.6	94.1	93.1	91.2	
Large	3.5, 7	100.0	89.6	85.1	82.1	80.8	At 2 cm (dose zone 2)
Medium	2.5, 5	100.4	89.8	85.1	82.1	80.8	
Small	1.5, 3	98.4	89.1	84.9	82.1	80.8	

Figure 12. Depicts the influence of scatter on object and beam size in differing cylindrical geometries having an equal width/depth of 3, 5, or 7 cm and an SSD of 30 cm irradiated with the EGSnrc 250 kVp spectrum. The resulting percent depth dose (relative to the large phantom) shows that the percent depth dose is hardly affected when the phantom size changes. The percent depth dose also illustrates that at the exit of the beam, depth dose values deviate from a (larger) object reference. The embedded table provides relative dose percentages for phantoms and field sizes relative to large phantom with 25 mm field at surface and at 2 cm depth (both shaded in grey) for the two dose zones. The table illustrates that depth doses at orthovoltage energies are more sensitive to changes in field size than changes in object size. These values are only indicative and should be evaluated for other geometries and energies.

applications. Also, lack of coherent reporting in the literature prevents from obtaining reproducible results by other research centres. Guidelines aimed at both biologists and physicists are required to achieve sufficient standardization. Therefore, innovations in pre-clinical dosimetry and comprehensive reporting are essential to be implemented and routinely used across all radiation research centres to assure the highest level of accuracy and reproducibility essential for the comparability of published pre-clinical data (Verhaegen *et al* 2018).

9. Informatics systems

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Status. Within the field of small animal/pre-clinical research, most information technology development efforts have been invested in optimizing image acquisition equipment, image reconstruction methods, and data captures (ACROP *et al* 2018). As such, the large variety of equipment and investigational options available nowadays is mostly powered by hardware miniaturization, which consequently has led to an exponential increase of data captures of many different types and with extreme high resolutions, and different dimensionality (2–3–4D). Besides this, the higher install base and availability of pre-clinical research equipment facilitate even more complex and combined captures of data. This 'big data' gathering increases the need that the data is acquired and stored within its context to ensure data quality and efficient research output (Zullino *et al* 2022). Instrumental for this need is the field of pre-clinical research, where data is nowadays stored at many different stations within the organisation and parts of data-sets are often duplicated and sometimes processed differently, which results in the fact that keeping track of the status, origin, and context of data-sets is difficult, let alone making inter-institutional collaboration possible. Tracking the status, origin and context of the data used becomes even more difficult when multiple institutes or data-providers are involved in a research project. Furthermore, computing power demands have grown drastically in order to analyse the acquired data and generate results due to the increased amount, size, and dimensionality. As a result, informatics systems are gaining more and more importance and interest within the field of pre-clinical research to further innovate and guarantee quality of executed research projects (Kain *et al* 2020). Note, for example, that the DICOM standard has been updated recently in such a way to include pre-clinical use cases and specialized data-definitions to support, for instance, Flash such that the dose profile can be used in hindsight for dose computations (DICOM *et al* 2021). To optimize pre-clinical and small animal research and improve reproducibility of studies, share data, and move towards the next step in large-scale/big data analytics, efficient and consistent/normalized execution of experiments is mandatory which can be facilitated by informatics systems (SABER *et al* 2019).

Current and future challenges. Currently, one of the biggest challenges in the field of pre-clinical research lies in the reproducibility of research (Zullino *et al* 2022). At present, spreadsheets, simple databases, and/or notebooks are still used predominantly to keep track of and register pre-clinical studies, making it hard to access and, therefore, reuse the data. This residing way of storing of data is mainly due to the fact that accessing, querying, and storing the data with contextual information is currently one of the biggest impediments, with standardization being one of the challenges to properly unlock the full potential of large data acquisition. Additionally, by adding well-described meta-data, data analysis itself could also be drastically simplified. Such an improved structured and protocolized way of data captures is, for instance, useful in the development of personalized medicine, which has inaugurated co-clinical studies where clinical and pre-clinical studies are more closely associated. This has led to the need of more consistently monitored and stored preclinical data and the need for 'small-animal' hospitals (DICOM *et al* 2021). These 'small-animal' hospitals improve the experimental statistics and increase the number of animals included a study. Storing the large amounts of data in context and making them searchable is one of the major challenges for the midterm to prevent redundant experiments, but also to increase the amount of data for proving hypotheses by sharing data. This cannot only enhance the power of pre-clinical research, but also prevent unnecessary and unethical spill of resources and small animals (3R principle) (3RANIMAL). Note that to be able to interpret pre-clinical data many metrics and factors need to be taken into account and registered to improve experimental statistics such as anaesthesia, mouse model (genetically engineered, mouse strain), cancer model (cell or fragment, orthotopic, xenograft), animal demographics, date and site of cell/fragment implant, date of fragment excision, and facility-vivarium information (DICOM *et al* 2021). For the future, therefore, an interlinked data-mesh connected to high-performance computing (HPC) power is necessary to search data across institutes, calculate and analyse metrics from the research projects, and apply data-science/machine learning approaches to capture new knowledge from previous experiments or execute in silico research.

Advances in science and technology to meet challenges. The last couple of years a lot of effort has been put into the development of the definition of data exchange standards. The first steps have been set by describing interoperability standards to create queryable data with more context that is relevant for analysis, while utilizing HPC more often. For instance, the DICOM community and workgroups created specific pre-clinical research additions to the standard such as (small) animal imaging additions to the DICOM part 3 document, see also the visualization in figure 13. Furthermore, the number of advanced image processing and analysis methods have

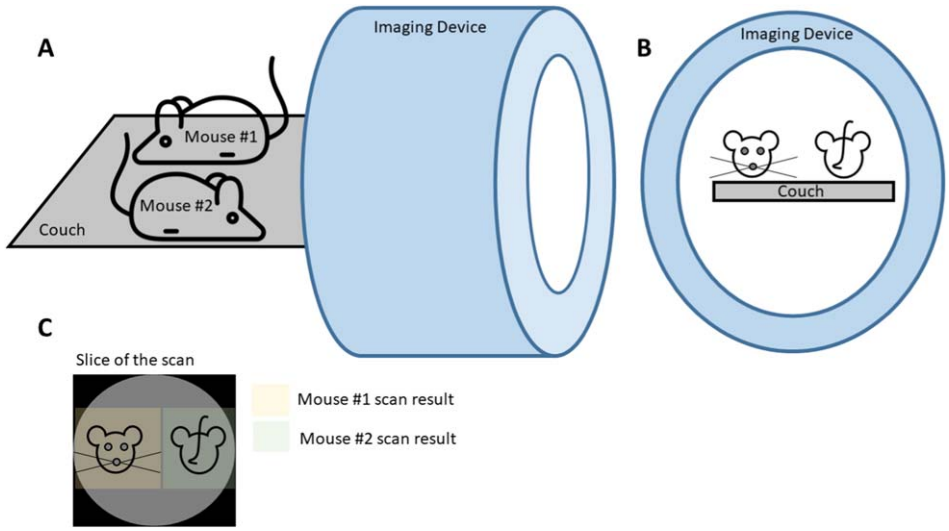

Figure 13. An example of information which has been added into the DICOM standard and meta-data/context which is added to describe scanning conditions and make specimens query able while being scanned in one single scan. (A) and (B) show the positioning of the mice into the scanner while (C) shows the areas of interest how the mice should be divided into independent DICOM scans.

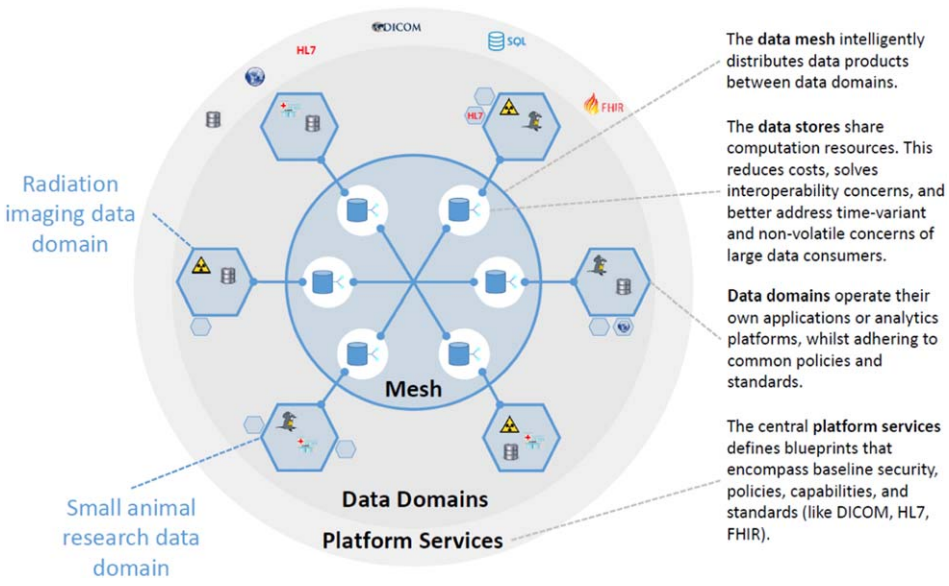

Figure 14. Explanation of a data-mesh, data domains of biological, imaging, treatment data are interconnected by a mesh and knowledge can be transferred from one domain by the other by interconnected platform services.

grown drastically by utilizing advanced hardware like GPU-computing and other affordable HPC architectures. The increase in computing power has enabled deep-learning, federated learning (facilitated by edge-computing), artificial neural networks and multi-layer perceptron, evolutionary algorithms to be applied at a larger scale in less computing time, and, on bigger datasets, improving the performance of algorithms (AIRO *et al* 2018, AIPRE *et al* 2021, AIPRE2 *et al* 2021). These technologies facilitate the discovery of new insights in complex biological processes and mechanism by processing enormous amounts of diverse data (AIPRE2 *et al* 2021). Another trend in recent years has been the introduction of cloud-based technology and services of big companies such as Google, Amazon, and Microsoft, which also focus on the life-sciences and healthcare market. Besides 'the bigger fish', also services provided by smaller providers (local or by universities at an enterprise level) have led to improved data storage, security and governance, resolving issues such as data-duplication and making data centrally accessible instead of residing at many different systems within the enterprise. Cloud technology and the new data-mesh paradigms (DATA-MESH and Dehghani 2020, Kain *et al* 2020) as visualized in figure 14, which creates domain based dataspaces/containers, combine expert knowledge, data persistency,

and semantic description and interoperability by adding domain and data specific meta-data. This is required to execute big-data studies, validate study results, or include more data in studies.

Concluding remarks. The first steps within pre-clinical research to optimize studies have been taken, however there is still a large gap to fully realize a 'small-animal' hospital. The next steps will be to optimize small-animal studies by transparent data-access and apply novel data strategies like data-meshing to build a data environment based on domain knowledge and data-context, while further developing technologies such as FHIR and DICOM (Web). Pre-clinical research is still in the early stages of the development, however some steps such as a definition of standards is shaping up. The stage where pre-clinical systems are at is unique, as novel and future-proof technology (such as data-meshing) can be used to revolutionize pre-clinical research. The informatics system as proposed will eventually create a (deep) learning and knowledge discovery environment where results can be obtained more easily and are translated into a clinical environment more easily to improve patient treatments.

10. Monitoring systems

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Status. 3D imaging of animals (e.g. CT, MRI) typically requires them to be anaesthetised to avoid movement artefacts. Anaesthetisation is also beneficial when partially irradiating animals to ensure that the targeted volume is accurately irradiated. If the level of anaesthesia is too light the animal may become distressed and feel pain, while if too deep can cause death. Therefore, it is essential to maintain respiratory function, circulatory function and body temperature, and to monitor the depth of anaesthesia (Wolfensohn and Lloyd 2013).

The depth of anaesthesia is generally assessed by monitoring the respiration rate and amplitude. This is most commonly achieved via detection of movement resulting from inflation and deflation of the lungs. For example, changes in air pressure in a small reservoir bag (balloon) positioned between the animal and the cradle can be used, but may impact on the reproducibility in positioning of the animals. A range of other techniques have also been used, such as piezoelectric pressure sensors, diffuse optical reflectance, pressure difference produced in an enclosed box or face mask, and qualitative monitoring by an operator using live video imaging of chest movement (Grimaud and Murthy 2018).

Anaesthesia affects thermoregulation, resulting in a fall in body temperature unless measures are taken to prevent this (Wolfensohn and Lloyd 2013). Body temperature is commonly monitored using rectal or skin surface probes and is important as hypothermia is one of the commonest causes of mortality in small rodents. These can also be used to provide feedback for any heating system used to maintain the temperature of the animal. In addition, respiratory depression can also result in a reduction in oxygen saturation, as a result oxygen supplementation is often required to prevent hypoxia developing, which not only can be detrimental to the animal but potentially impact on the biological response to radiation treatment (Sorensen and Horsman 2020).

Assessment of cardiovascular signs, such as using an electrocardiogram (ECG) to determine cardiac function, can be useful in not only monitoring welfare of the animal, but also provide important information in assessing response and reproducibility between experiments. Additionally, they can provide gating information while imaging to avoid cardiac movement artefacts.

Current and future challenges. An ideal monitoring system should be able to monitor breathing rate and amplitude, heart rate, temperature, oxygen and pH levels. It should be possible to use the respiratory and cardiac monitoring systems to provide gating or beam tracking for imaging and targeted beam delivery from image-guided pre-clinical irradiators. It would also be useful if the temperature, oxygen level and potentially respiratory rate monitoring could provide feedback to help maintain required levels. In addition, it should ideally be non-invasive, contactless, cost-effective and easy to implement, use and maintain. The design of the system should also minimise set up time for individual animals in order to maximise animal throughput.

It is also important that materials used and the position of the monitoring systems, do not negatively impact on the quality of images generated or beam delivery. For example, high Z-materials such as metals and glass can result in CT artefacts and unwanted attenuation of x-ray treatment beams. Ferromagnetic materials must be avoided if used with MR imaging. It would be beneficial to minimise or avoid any distortion of the animal, such as is often observed with reservoir bags.

With regards to animal imaging, while anaesthesia helps reduce degradation of movement artefacts, there is still some significant underlying movement associated with respiratory function and to a lesser extent from cardiac function. Respiratory motion can also be problematic when targeting tumours and organs within the thorax and abdominal regions, especially when located close to the diaphragm. Movement of the target in and out of the treatment field will result in increased heterogeneity in dose across the tumour and reduction in the average dose, with increased dose to the surrounding normal tissue (van der Heyden *et al* 2017). While this can be compensated for by increasing the field size to include the motion, this would increase the volume of normal tissue irradiated. Ideally treatment should be delivered using respiratory gated beam delivery or target tracking.

Advances in science and technology to meet challenges. Figure 15 provides a sketch of an ideal monitoring system. Improved imaging quality can be achieved by triggering or gating image capture at particular points through the respiratory and if required the cardiac cycle. This can be used to generate a single 3D image of a particular stage through the cycles, or a 4D video image showing the variation in structure as a function of time through the

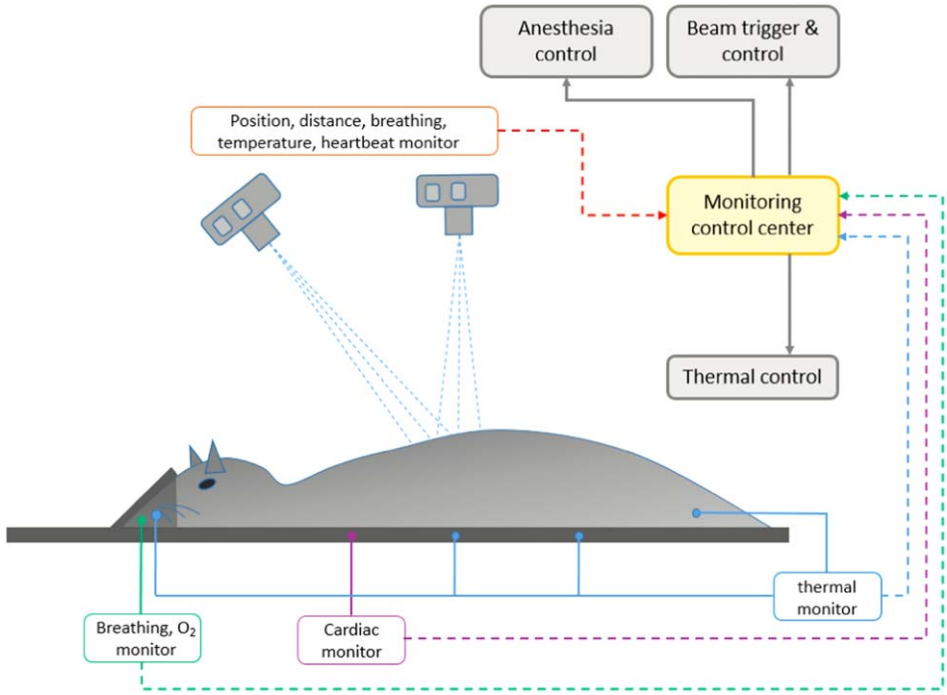

Figure 15. Ideal monitoring system. It consists of several thermal monitors in direct contact with the specimen, embedded in the stage and the gas anaesthesia nose cone. The latter is for monitoring the breathing frequency and pattern, and oxygen level. This pattern is also monitored by one or more cameras with capabilities for colour, depth and heat vision, or other types of motion sensors. These cameras also monitor the positioning of the specimen, its temperature, respiratory motion and heartbeat signals from superficial blood vessels. Together with signals from a cardiac monitor, these signals are led to a Monitoring control centre, from which the beam can be controlled and triggered and which is also linked to the anaesthesia and thermal control systems. With recent advances in affordable camera and sensing technology, image processing and artificial intelligence, it may be possible to emulate developments in the clinic to develop systems capable of tumour tracking based on breathing systems or body surface motion to enable radiation beam tracking.

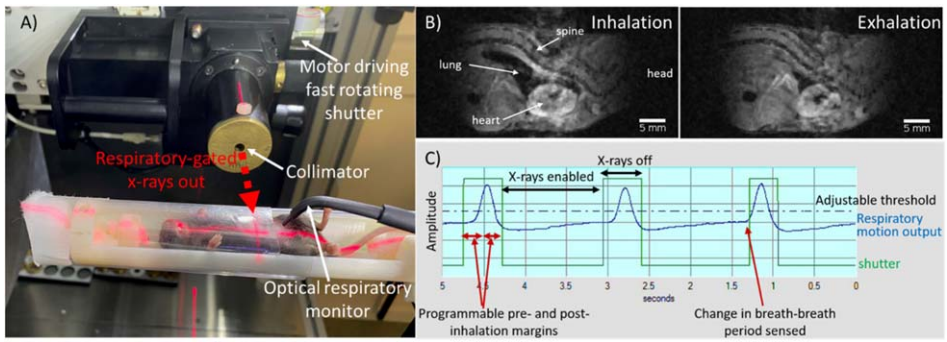

Figure 16. Respiratory-gated beam delivery. The incorporation of a fast shutter in conjunction with optical respiratory monitoring enables beam to be delivered only during the resting phase of the breathing cycle. The use of adaptive gating control can be used to account for variations in the breathing period and minimises impact of irregularities in the pattern (Hill *et al* 2017). (A) Mouse is anesthetised in a cradle. A rectal probe is used to monitor temperature and control cradle temperature. An optical probe is used to monitor respiration rate through movement of the surface of the mouse, as well as triggering respirator gated beam delivery during the resting phase following exhalation. (B) demonstrates internal movement of internal anatomy with T2 MR images of a mouse during a period of maximum inhalation and the resting phase after exhalation (reproduced from (Hill *et al* 2017) with permission © 2020 Radiation Research Society). C) Shows a typical breathing (blue line) and x-ray gating (green line) traces. Adaptive gating predicts the timing of the next breath based on breathing rate, but will detect changes in breathing period, closing the fast shutter early if inhalation is detected and staying closed until exhalation is detected.

cycles. Respiratory-gated beam delivery (figure 16) can also be used to achieve high precision in dose delivery to the target/tumour while minimising dose to surrounding tissues. This will also facilitate complex dose delivery such as 'dose-painting'.

There is an increasing need to perform multimodality imaging on animals combined with an image-guided preclinical irradiator. This would require the animal to be transported between imaging equipment and irradiator in the same cradle while still anesthetized to minimised movement of the animal and their internal

organs between machines, which will also make registration of images easier and more reliable (Kersemans *et al* 2017). This procedure will therefore benefit from having common monitor equipment on all devices.

One aspect of monitoring that is not routinely performed is oxygen saturation measurements which can be performed using a fibre-optic probe, although there have been recent studies trying to use skin colour variations to measure oxygen saturation (Kim *et al* 2021). It not only plays an important role in the physiological state of an organ/tissue, low oxygen concentrations can also result in a significant increase in resistance to radiation (Sorensen and Horsman 2020). This is currently of particular interest as the oxygen status of normal tissue has been proposed as a key parameter in normal tissue sparing associated with ultra-high dose rate associated with FLASH radiotherapy. In addition to baseline oxygen measurements prior to irradiation, the ability to measure the temporal variation of oxygen within the FLASH irradiated volume could provide useful mechanistic data; with oxygen reacting with radiation induced macromolecule radicals on the millisecond time scale which corresponds to a diffusion distance of approximately two micrometres (Wardman 2020). pH probes are now also available that could be integrated in the platforms (Hao *et al* 2018, Garcia-Guzman *et al* 2021).

Concluding remarks. Animal monitoring is probably one of the areas where image-guided precision small animal irradiation will progress the most in the near future. Considering that this is hardly ever done currently, but that the technology seems available to do it, one can expect rapid evolution in this field. Affordable camera technology, rapid sensors, real-time data processing, automated databases, and artificial intelligence can all contribute to rapid progress for monitoring animals under treatment in much more detail than is currently done.

When this wealth of data becomes available, much more detailed and reproducible experiments can be performed. Tracking and gating irradiations will also be enabled, which has so far only been done in a few pioneering efforts. In combination with advanced multi-modality imaging methods, the small animal research systems will then evolve into much more advanced study platforms.

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11. Clinical translation

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Current status. The development, adoption and ongoing refinement of precision small animal radiation platforms has had a substantial positive impact on the quality and clinical relevance of preclinical radiation research. In parallel with these technological advancements, an increasing library of preclinical tumour models is becoming available, including patient-derived xenografts (PDX), syngeneic models and genetically engineered models (GEM) (Hackam and Redelmeier 2006). After decades of reliance on subcutaneous models, there is now increasing use of orthotopic models, in which tumours are either implanted in the relevant host organ or genetically induced in a tissue specific manner (van der Worp *et al* 2010). As well as providing greater clinical relevance than subcutaneous tumours, orthotopic models are of particular value in radiation studies because they (i) better reproduce the cancer microenvironment, which involves complex host-tumour interactions, and (ii) enable simultaneous analysis of the effects of radiation on tumours and the relevant normal tissues.

Despite this progress, many challenges remain. In many disease contexts it is clear that preclinical studies fail to accurately predict or reproduce clinical outcomes. And while new technologies enable accurate imaging and delivery of preclinical RT to many anatomical sites, delineation and targeting of tumours in the abdomen and pelvis remains difficult. Finally, although some useful normal tissue models have been developed and validated, considerable further work is needed in this area. An overview of the current challenges and limitations of preclinical models used in precision radiation studies and the advances in preclinical modelling to meet these challenges is represented in figure 17.

Current and future challenges. As mentioned above, imaging the abdomen and pelvis is very challenging in small animals and accurate delineation of tumours and normal tissues is not usually possible using existing CT imaging techniques. Fusing MRI and/or PET images with CT datasets is beneficial but also expensive and time-consuming. Various contrast agents with differing routes of administration are available and should be investigated further.

It is also increasingly apparent that clinically relevant comorbidities are not usually represented in animal models; indeed most studies are conducted in young, healthy mice. As an example, many cancer patients have chronic obstructive pulmonary disease (COPD), caused by smoking, air pollution, professional exposure or ageing. COPD affects multiple organs including the cardiovascular system and also causes sarcopenia and cachexia. Animal models that recapitulate this and other human diseases may come closer to representing the majority of cancer patients (Gosker *et al* 2009).

Another crucial topic relates to dose, fractionation and scheduling which are crucial components of clinical radiotherapy regimes but difficult to reproduce in the preclinical setting. Implanted tumours typically have volumes of around 100 mm^3 before irradiation (Lindenberger *et al* 1986, Cosper *et al* 2020) compared to 100 cm^3 for clinical T3 lung tumours. If directly proportionate to the number of clonogenic tumour cells, this 1000-fold difference in volume would equate to 20 Gy less being required to control implanted versus clinical tumours, assuming 50% of cells survive each 2 Gy fraction (Steel 2002). While the true dose difference is likely to be less because larger tumours have greater necrotic fractions (Khalil *et al* 1995), our current understanding of this important issue is very limited, and most preclinical doses are selected empirically.

The emergence of immune modulating strategies as potent cancer therapies has underscored the crucial role of immunity in many therapeutic and mechanistic areas including radiation oncology and biology. Preserving the immune system in preclinical models is likely to be beneficial for all preclinical studies in these areas and is clearly of critical importance when investigating immunological therapies. However syngeneic or genetically engineered tumour models may be less clinically relevant than tumours of human origin.

In general terms, the accuracy with which preclinical models predict treatment outcomes in humans remains unknown in most cases. More robust methods for evaluating the clinical relevance of these models are needed; these methods must also be capable of demonstrating where models are not representative.

Advances in science and technology to meet these challenges. Recent advances in animal modelling are beginning to deliver more sophisticated systems that recapitulate clinically relevant environments. The best examples of these are mice with humanised immune systems (Wang *et al* 2021), lung disease or cardiovascular disease. One possible approach to the important question of how can we either validate our models or show that they are not helpful is to investigate differences in gene expression profiles between rodent and human disease and only use

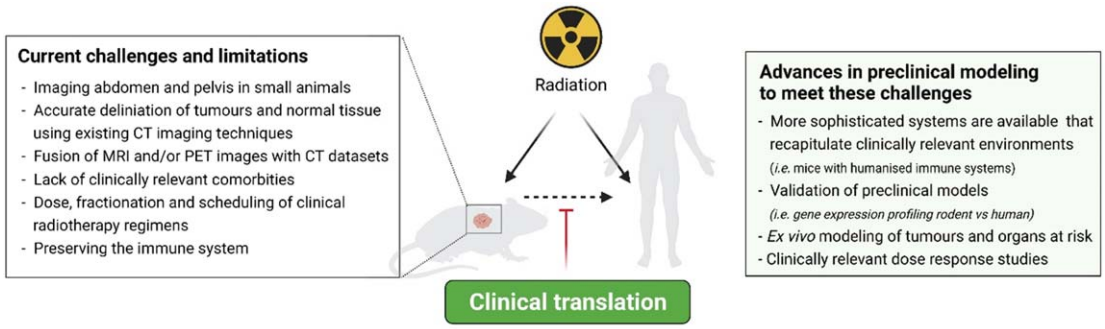

Figure 17. Precision preclinical radiation studies—From preclinical studies to clinical translation. An overview of the current challenges and limitations of preclinical models used in precision radiation studies and the advances in preclinical modelling to meet these challenges is represented. Figure created with BioRender.com.

models with demonstrable similarities (Seok *et al* 2013). Where *in vivo* modelling of radiation responses is particularly challenging, *ex vivo* models of both tumours and organs at risk are showing promise, as exemplified by the growing use of patient-derived organoids and tissue slice cultures for mechanistic studies (Suckert *et al* 2020).

The radiation dose issue requires specific investigation. Dose response studies can provide useful information but it is important to account for variations in fractionation, differences in biology between tumour types, and differences between the growth-delay and tumour control endpoints often determined pre-clinically versus clinically meaningful endpoints such as disease-free or overall survival.

Concluding remarks. Technological advances in preclinical radiotherapy systems and biological advances in preclinical models of cancer and relevant normal tissues provide unprecedented opportunities to undertake high quality preclincial radiation studies. However, sustained efforts are required to increase the clinical relevance of these studies, with particular emphasis on tumour-host interactions and dose/fractionation issues.

12. Reversing the translational research paradigm in preclinical radiotherapy studies

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Status. Improvements in cancer care are driven by the translation of basic research into the clinic through the largely unidirectional process of translational research. The translational research paradigm was first formalised in 2009 by the Translational Research Working Group (TRWG) of the National Cancer Institute (NCI) who defined the process of translational research as that which 'transforms scientific discoveries arising from laboratory, clinical, or population studies into clinical applications to reduce cancer incidence, morbidity, and mortality' (Report of the Translational Research Working Group of the National Cancer Advisory Board 2007). Based on this definition, translational research constitutes a unidirectional continuum consisting of multiple stages involving (T1) translation to humans including observational studies and Phase I and II clinical trials; (T2) translation to patients including Phase III clinical trials and the development of guidelines, and (T3) translation to clinical practice including Phase IV clinical trials and dissemination research (Westfall *et al* 2007).

Similar to other fields of cancer research, translational research continues to play a critical role in driving innovations in radiotherapy (RT) based on preclinical research into the clinic. Examples are drawn from physics such as imaging and radiation delivery technologies that have improved target volume delineation and conformality and also radiobiology, through the optimisation of fractionation schedules and combinations of RT with chemotherapy and molecular targeted therapies. Whilst it remains difficult to accurately measure the success and efficiency of translation across the spectrum of RT research, it is clear that preclinical studies play an important role at the early stages of the T1 process by providing low-level evidence to support first in human studies.

Over the past decade, the translational relevance of preclinical RT studies has significantly improved due to advances in tumour modelling and the implementation of precision small animal irradiators that have allowed previously unimaginable approaches to be explored in the laboratory (Butterworth 2019). The combined use of contemporary mouse models with small animal irradiators represents a major step forward for the radiobiology field in being able to more accurately replicate clinical exposure scenarios. However, these approaches remain challenging due to limitations in being able to fully recapitulate clinical phenotypes, the delivery of clinically relevant treatment protocols and ensuring high levels of reproducibility. Despite these challenges, preclinical models of tumour and normal tissue response critically support the T1 process prior to larger practice changing studies.

Current and future challenges. The translational research paradigm has driven innovations from the laboratory to the clinic yet challenges often remain with respect to impact beyond early phase trials. A recent modelling study estimated the probability of success for oncology drug development programs to be 3.4% (Wong *et al* 2019). An alternative approach is to challenge the classical linear research paradigm by taking knowledge gained from clinical trials and real-world patient data back into the laboratory to directly inform new discoveries. This process of reverse translation in RT research is illustrated in figure 18.

The process of reverse translation uses patient data from clinical trials or the real-world during standard-of-care clinical practice including treatment planning and imaging data, tumour or normal tissue biomarkers, genome sequencing, and clinical endpoints. Irrespective of origin, large volumes of data can be evaluated and used to formulate new hypotheses to explain observed outcomes. These ideas can be taken back into the laboratory to design preclinical RT experiments to test novel hypotheses pertaining to mechanism and potential interventions that can initiate a new iteration of the translational process. Within this framework, clinical knowledge directly informs the design of innovative preclinical RT experiments to bridge the scientific gaps that could lead to new investigational therapies and drug-RT combinations.

A major challenge lies in developing improved, patient-informed disease models that accurately capture the clinical phenotypes and RT responses of real world patients. This requires the molecular characterisation of tumours and normal tissues using different omics-based approaches (for example, next-generation sequencing, metabolomics, radiomics and microbiota assessment). Another important consideration is that the complexity of cancer patients can be confounded by comorbidities with one-third of patients having at least one pre-existing chronic disease (Ritchie *et al* 2017). The degree to which comorbidities may contribute to cancer burden and RT outcomes remains unclear yet the co-existence of one or more chronic health conditions is more common in older patient populations (Roughead *et al* 2011). In lung cancer, for example, hypertension, smoking, diabetes, obesity, and pre-existing lung and cardiovascular diseases are known comorbidities, yet there is limited

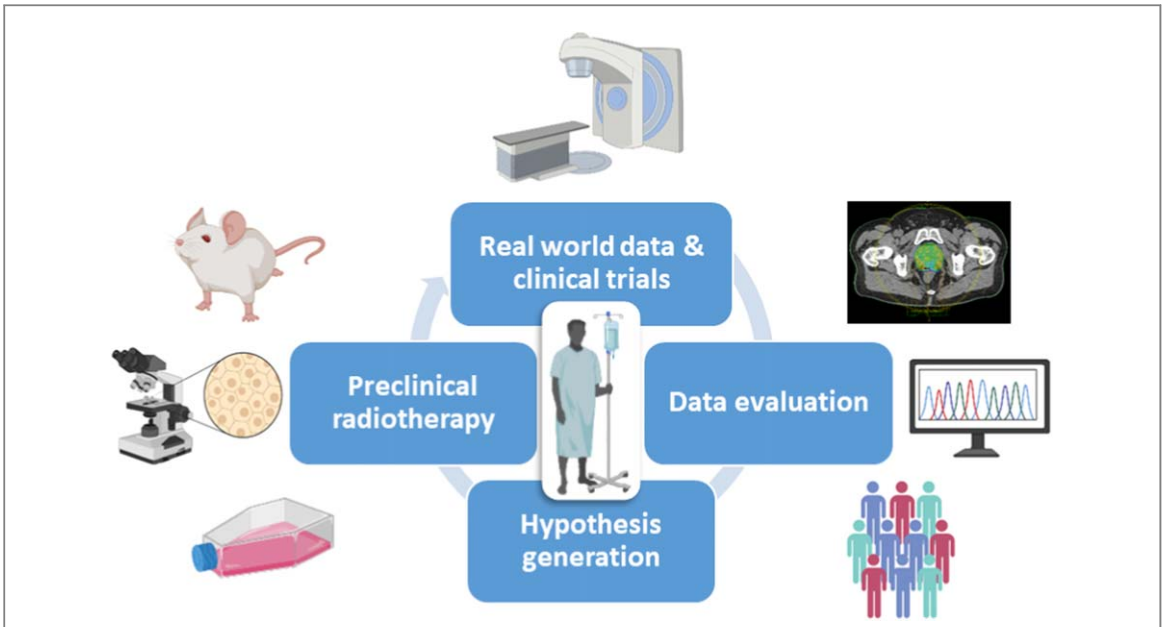

Figure 18. An overview of the process of reverse translation in radiotherapy (RT) research.

mechanistic evidence from preclinical studies and there are currently no standards for integrating these factors into decision making in the clinic. Also, comorbid patients are likely to be prescribed multiple drugs to manage chronic diseases and as part of their cancer treatments. The use of multiple drugs, known as polypharmacy, may impact treatment responses due to drug-drug or drug-RT interactions that may have important consequences on outcomes. A further challenge lies in the analysis of the large volumes of real-world and clinical trial data. It is clear that the rate at which data is generated from these sources vastly outpaces our understanding of the clinical and biological factors driving patient outcomes and relationships to treatment response.

Advances in science and technology to meet challenges. We have recently demonstrated the power of reverse translation in being able to accurately recapitulate clinical observations of regional radiosensitivity in the heart. In a retrospective analysis of patients treated with curative-intent RT for lung cancer, McWilliam and colleagues identified a highly significant region located in the base of the heart where higher doses of RT were associated with worse patient survival (McWilliam *et al* 2017). Also, data from 803 patients who had received stereotactic ablative radiotherapy (SBRT) showed dose to the upper region of the heart was significantly associated with non-cancer death (Stam *et al* 2017).

Based on these data, we developed a novel preclinical mouse model using a precision small animal irradiator to target subvolumes of the heart with a 3 × 9 mm collimator. Our data demonstrated the base of the heart as a differentially radiosensitive region based on structural and functional parameters that did not correlate with clinical mean heart dose (MHD) or the volume of the heart receiving 5 Gy (V5) (Ghita *et al* 2020). This model accurately recapitulated the clinical phenotype from real-world data and opens up new opportunities to explore the mechanisms of radiation response in the heart towards identifying actionable targets for protection or mitigation and to optimize RT dose constraints. This important study clearly demonstrates the validity of reverse translating clinical data, however, several key challenges need to be addressed to maximise the full potential of this approach.

To fully deliver personalised RT treatments in the clinic, precision animal models that reflect the variability observed across patient populations should be used to identify the multifactorial relationships between genotype, phenotype and patient-specific risk factors on cancer burden and treatment response that cannot be achieved in human studies. Also, the generation of large multidimensional datasets requires improved analytical methods that may be achieved using artificial intelligence to explore, for example, correlations between patient-specific factors, biomarkers, imaging features and spatial dose correlations at the individual voxel level (Appelt *et al* 2022). Again, these data can be taken back to the laboratory to develop an improved mechanistic understanding of outcomes and to identify novel targets and opportunities for further treatment optimisation.

Concluding remarks. The majority of RT research follows a classical, linear research paradigm from bench to bedside. Considerable opportunities exist to reverse translate real world data back into the laboratory. This approach can be enabled using precision animal models to gain *de novo* insight into the underlying basis of

treatment response in specific patient subgroups. By adopting a multidisciplinary team approach across biology, physics and clinical oncology, reverse translation may maximise real world knowledge and inform the next evolution of radiation-based cancer therapies.

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13. Concluding remarks

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This Roadmap on research in Precision Image-Guided Preclinical Radiotherapy provides a broad non-exhaustive overview in short vignettes of the most relevant aspects where rapid progress may be seen in the near future. Experts, many of them pioneers in the field, gave their impression of the state-of-the-art and their opinion on where the research can possibly lead. This is always a precarious exercise, and will most likely need frequent updates, as so often in research. A recent example is the re-discovery of the Flash effect, which now is leading to a race to develop empowering technology and to identify the most relevant biological experiments, enabling swift clinical translation. This Roadmap is aimed both at the novice, who will find a wealth of information succinctly described which can be used as a starting point, as well as at the expert, who may find the brainstorms and the compilation of recent references, many of them review papers, useful.

Three major themes are addressed in this Roadmap: (1) development of novel preclinical technology which is rapidly enabling much more powerful, accurate and efficient radiobiological experiments, and may also be used as a basis to develop derivative clinical technology (e.g. spectral CT, dark field x-ray imaging), (2) development of novel biological models with a wide variety of modern techniques, allowing for much more realistic experiments, and (3) clinical translation. Especially the latter still requires much thought about what is needed to increase the success rate of translation of preclinical into clinical trials. Note that one section deals with non-cancer diseases, for which there is tremendous potential using the current and future platforms to discover new mechanisms and cures.

If this Roadmap can help to inspire researchers and companies to focus and augment their efforts in this field, the will feel that our exercise was successful.

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