Radiation-induced cardiopulmonary dysfunction
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SUMMARIZED DISCUSSION, FUTURE PERSPECTIVES & CONCLUSIONS
Radiotherapy is an important treatment modality for thoracic tumours. Unfortunately the radiation dose is restricted by the risk of normal-tissue toxicity of the surrounding healthy tissues such as the lung and heart. More insight into the pathology of radiation-induced cardiac and pulmonary dysfunction (RICPD) could lead to strategies to diminish/ameliorate RICPD. It may also lead to the finding of (better) predictors of the development of RICPD. This could improve radiotherapy treatment and even provide tumour dose escalation leading to a better tumour control and an increased survival of the patient.

As such, in this thesis we studied the pathology of RICPD in a rat model aiming to find (better) predictors and intervention strategies to attenuate the damage. An overview of our results is shown in Figure 1 and will be discussed here.

Classically, radiation toxicity is considered an inflammatory driven process eventually leading to fibrosis. In the lung this would lead to an early "radiation pneumonitis" and a later phase called "lung fibrosis". We studied early radiation-induced lung dysfunction (RILD) in our rat model and found an important role of pulmonary vascular damage besides the classical inflammation in the development of RILD (Chapter 2). This vascular damage in the lungs leads to an increased pulmonary arterial pressure and consequently to hypertrophy of the right ventricle (RV) of the heart. The results reported in chapter 3 showed that the lung and heart are inter-related and that respiratory dysfunction manifested through physiological changes in the whole cardio-pulmonary system rather than through changes in the lung only.

In chapter 2 we showed that the observed increased pulmonary artery pressure (PAP) after rat lung irradiation resulted into pulmonary hypertension, in an irradiated-volume dependent manner. This may originate from perivascular oedema and vascular remodelling in the whole lung, possibly initiated by loss of pulmonary endothelial cells (ECs). Loss of ECs, in both the irradiated parts of the lung as well as the shielded parts, was observed prior to breathing rate (BR) increases of the animals and morphological changes in the lung parenchyma, suggesting that the vascular response may precede parenchymal damage. The increase in pulmonary pressure correlated strongly with the increase in BRs suggesting that lung irradiation-induced respiratory dysfunction in rats may be due to the vascular remodelling and not necessarily only by lung parenchymal damage.

In chapter 3 the interaction between radiation-induced heart and lung damage was described. It was found that radiation-induced pulmonary hypertension and pulmonary perivascular oedema subsequently impaired left ventricle (LV) diastolic function by increasing the relaxation time of the LV. An explanation for this may be a decreased blood input from the pulmonary system to the heart. LV diastolic dysfunction also occurs after only heart irradiation, possibly initiated from radiation-induced cardiac (perivascular) fibrosis, as described in chapter 4. This direct effect of irradiation of the heart on diastolic function induces a secondary effect on the lung by promoting pulmonary interstitial oedema.

The findings described in chapter 2 and 3 show that heart and lung irradiation can cause independently both cardiac and pulmonary damage/dysfunction, which may subsequently affect each other through the vasculature. Moreover, co-irradiation of both organs combined these effects and enhanced respiratory dysfunction measured as a pronounced
Summarized discussion

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The findings described in chapter 2 and 3 show that heart and lung irradiation can cause independently both cardiac and pulmonary damage/dysfunction, which may subsequently affect each other through the vasculature. Moreover, co-irradiation of both organs combined these effects and enhanced respiratory dysfunction measured as a pronounced
Summarized discussion, future perspectives & conclusions

BR increase of the rats. Here both RV and LV of the heart were compromised and the combination of lung irradiation-induced perivascular oedema and heart irradiation-induced interstitial oedema caused excessive accumulation of pleural fluid (as described in Chapter 4), which may be the determinant of enhanced respiratory dysfunction. Cardiac diastolic dysfunction, pulmonary hypertension and the resultant pulmonary oedema and pleural effusion are all known to impair ventilation by reducing perfusion due to either backward failure of the LV or vascular damage, causing congestion of the pulmonary vasculature. Previous studies showed that inhibition of the RAS system ameliorated radiation-induced lung dysfunction. However, in most of these studies the whole thorax was irradiated including the heart and lungs. Since the heart and the lungs interact (as described in chapter 2 and 3), protection of lung damage by ACE inhibition could still be an indirect effect of direct heart protection. The exact protective mechanism was not elucidated in these studies. Therefore, in chapter 4 we studied the effect of the ACE inhibitor (ACEi) captopril on cardiopulmonary dysfunction in rats after only lung irradiation, only heart irradiation or combined lung and heart irradiation. We showed that captopril attenuated RICPD, but only if the heart was co-irradiated. Moreover, we observed that captopril attenuated early radiation-induced heart damage and consequentially prevented the influence of dose to the heart or lung toxicity. Captopril prevented LV perivascular fibrosis and the elevations in left ventricular end-diastolic pressure (LVEDP) induced by heart irradiation, thus, preventing LV diastolic dysfunction and consequent pulmonary damage. The ACEi captopril significantly improved BR and cardiopulmonary density/structure.
Consistently, radiation-induced pleural and pericardial effusion and cardiac fibrosis was reduced, resulting in an improved LVEDP only in the heart-irradiated groups. Apart from their hypotensive action, ACEI are known to have other properties such as anti-inflammatory action. Furthermore, it has been suggested that the sulfhydryl group in the molecular structure of captopril confers in a free radical scavenger activity, and this can account in part for its radioprotection. It might act as an antioxidant to reduce inflammatory reactive oxygen species and thus mitigate radiation-induced toxicity. In this study it was found that captopril reduced fibrosis in rat hearts early after irradiation, which in the field of cardiology research is a known property of captopril.

As described in chapter 2 EC loss occurred already at 2 weeks after irradiation preceding BR increases of the animals and morphological changes in the lung tissue. This early EC loss may be the initiating factor leading to perivascular oedema and vascular remodelling eventually leading to pulmonary hypertension. The importance of acute EC loss in the development of radiation-induced normal tissue damage has been established for other tissues such as intestine, spinal cord and rectum. It was also demonstrated that irradiated ECs induce migration and proliferation of vascular smooth muscle cells, one of the most prominent features of vascular remodelling in our rat model for lung toxicity. Since ECs are an important target of radiation in different organs, they may have a pivotal role in the development of early radiation-induced lung morbidity too. If so, the use of (endothelial progenitor) cell therapy may be a potential strategy to prevent further vascular damage leading to pulmonary hypertension. Therefore, in chapter 5 we studied the role and mechanism of early EC loss after irradiation and investigated the use of endothelial progenitor cells (EPCs) to ameliorate RILD. We showed that EC loss occurred very early after irradiation, already at 8 hours and 2 weeks after irradiation. The increased pressure and shear stress may induce a second phase of EC loss as observed at 2 weeks. Between these 2 phases a repair of the EC lining may have occurred e.g. by endogenous endothelial progenitor cells explaining the continuous layer between 8 hours and 2 weeks. The further loss of ECs in the irradiation field as well as in the shielded field, hypothetically due to pressure changes, blood flow and thereby shear stress may lead to vascular remodelling, increased pulmonary pressure and subsequent right ventricle hypertrophy.

Next we investigated if acute EC apoptosis was the initiating mechanism leading to EC loss. Radiation-induced pulmonary EC apoptosis was only found at a low level. As such, this could not be the only mechanism leading to EC loss, but might play an initiating role leading to a cascade of further EC loss. Hypothetically, apoptotic ECs may lead to changes in vascular integrity. As previously shown to occur hours to days after radiation in pulmonary ECs, the loss of endothelium integrity due to EC retraction results in an increased permeability to low molecular weight solutes. Early disruption of the endothelial lining with increased permeability and perivascular oedema found in our and other studies may decrease the blood flow in the irradiated vasculature. As a compensatory effect, the pressure, blood flow and thereby shear stress would increase in the vasculature damaging more ECs in both the irradiated parts of the lung as in the non-irradiated parts. Further, we performed a pilot study to investigate the effect of EPC transplantation on the development of RILD. Our preliminary results indicated that EPC transplantation at the time-points after irradiation when we observed EC loss, 8 hours and 2 weeks post-irradiation, may ameliorate respiratory dysfunction. Possibly by repair/replenishment of damaged ECs. Although further optimization of the experiment is...
needed, these preliminary findings stimulate further research to investigate the possible use of EPCs in the amelioration of RILD. Accurate prediction of the development of RILD is of great importance to optimize the treatment. So far, controversy exists about which dosimetric parameter(s) optimally predict RILD \(^20\). Moreover, only models predicting early RILD are described. Because new advances in therapies will lead to a longer life expectancy of cancer patients, the occurrence of late radiation-induced normal tissue toxicity will become more relevant. In addition, technological developments in radiation therapy result in smaller irradiated volumes of normal tissue. As described in chapter 2, radiation-induced pulmonary vascular damage plays an important role in the development of early RILD, in a volume dependent manner. The role of vascular damage and its dose-volume dependency in the development of late RILD is unknown. Therefore, changing the irradiated volume could change the dose-limiting toxicity, early or late toxicity, of a treatment. As such, in chapter 6 we studied and compared the different pathologies underlying early and late RILD, their dose-volume dependencies and investigated if decreasing irradiated volume could change the dose-limiting toxicity. By using our rat model, we found that in contrast to early RILD, late RILD predominantly depended on dose and was associated with inflammation and fibrosis, rather than irradiated volume and vascular remodelling. Consequently, the dose-limiting toxicity changed from early to late RILD when the irradiated volume was reduced. As such, new radiation techniques reducing irradiated volume, like stereotactic radiation and proton therapy, might change the dose-limiting toxicity of the radiation treatment.

**Future perspectives**

"From bench to bedside"

Generally, the goal of preclinical studies as described in this thesis is to get more knowledge on how to improve medical therapies in clinic, the so-called bench-to-bedside approach. Obviously, preclinical "bench"-work cannot be translated directly to clinical "bedside"-work, but may provide important new clues on how to improve existing medical therapies or to even find new therapies. In the next sections, the translation of the present preclinical work to clinic will be discussed.

**The interaction between heart and lung damage**

*Clinical decision-making: What to spare, heart or lungs?*

In thoracic radiotherapy the dose that can be given to a tumour is limited by the risk of radiation-induced toxicity of the surrounding lung and heart tissues. Organ specific constraints are used to establish a "safe" radiation plan (e.g. MLD, MHD, Vx). However, these constraints may differ between individual patients depending for instance on age, smoking history, poor pre-treatment performance status, poor pre-treatment lung function, chronic obstructive disease, female sex \(^21-24\). Unfortunately, so far we cannot predict the exact risk of developing radiation toxicity in an individual patient.
Unfortunately, a “safe” radiotherapy plan is not always an “effective” plan with regard to tumour control. This is the case for the treatment of lung cancer. It has been predicted that a dose up to 100 Gy may be required to sterilize tumours of the size frequently treated in NSCLC. However, with the standard radiation dose of 60 Gy for lung tumours, even up to 20% of the patients develop symptoms and signs of radiation-induced lung toxicity. On the other hand, 80% of the patients do not develop (symptomatic) RILD and may receive a higher and more “effective” dose. On the other hand, the radiation susceptible 20% of the patients could be protected from the radiotherapy side effects for instance by searching for other treatment strategies than radiation. Unfortunately, until recently we are not able to make such a selection between patients.

Currently, when a radiation plan meets the given constraints for the surrounding organs, e.g. the heart and the lungs, it can be approved. However, wide variation in radiation plans is possible. When both the constraints for heart or lung are met, still changes in the radiation plan may be possible to reduce or increase the dose in either the lungs or the heart which leads to the question: “What is more important to spare, heart or lungs?”. Especially since the life expectancy of cancer patients increases due to improved treatment strategies this question becomes increasingly important. Unfortunately, these issues remain to be determined.

Now, our preclinical work shows an interaction between lung and heart damage early after irradiation. Only lung or only heart irradiation can secondarily damage the other and combined irradiation even enhances the toxicity. This heart and lung interaction has not been recognized in clinic yet and predictive modelling studies do not include heart parameters.

As such, to get an answer on this important question, clinical modelling studies predicting RICPD are needed including different dose-volume distributions of lung, heart and combined irradiation. For instance, (sub)clinical RICPD could be assessed in an objective, non-invasive manner (e.g. by performing CT, MRI (see section below), echocardiography, lung function tests, blood tests for heart damage) and related to the different dose-distributions. This may eventually lead to an answer on this important question enabling (individual) radiotherapy optimization.

**The role of pulmonary hypertension in the development of RICPD**

In our rat model we found the important role of vascular remodelling leading to pulmonary hypertension in the development of early RICPD. To translate these findings to routine clinical practice, clinical studies that confirm the findings regarding the pathogenesis of RICPD as obtained in our preclinical studies are required. Obviously, it will not be possible to study radiation-induced vascular remodelling in patients histologically. However, non-invasively method to assess pulmonary hypertension in patients is possible by using e.g. MRI. The pulmonary artery pressure (PAP) as well as the size and thickness of the heart’s ventricles can be measured with cardiac MRI, which is already in use as a tool for diagnosing heart disease and for assessing the extent of damage from progressive heart disease. In addition, blood flow can be measured, enabling the assessment of velocities, after which pressures can be derived. Using these measurements it is possible to estimate the mean PAP. Currently, a pilot study has started in our institution to test the hypothesis that radiotherapy for lung cancer induces an increased PAP. Non-small lung cancer patients, referred for curative high dose (60 Gy) chemo-radiotherapy undergo
cardiac MRI before treatment, and 6 and 12 weeks after treatment. When this study shows an increased PAP after lung irradiation, radiation-induced vascular damage may play an important role in the development of RILD in patients too. This would be an important new finding in the pathophysiology of radiation-induced RICPD in patients, which may open possibilities for treatment optimization. For instance, including the PAP as a predictor may develop new/better prediction models. Interestingly, it has been observed that patients already suffering from pre-existing pulmonary vascular disease, manifesting as subclinical increases in PAP, have an increased risk for RILD \(^{32}\). As such, the individual risk of developing RICPD could be predicted before treatment by PAP measurements enabling a more personalized treatment. Finally, the findings could indicate an important role of radiation-induced vascular damage in patients as well which may be the basis for new intervention strategies targeting vascular damage. All these strategies could eventually lead to an optimization and broadening of the therapeutic window of radiation treatment.

**Prediction of RICPD**

An accurate prediction of the development of RICPD based on dose metrics is of major importance for treatment optimization. So far, the interaction of lung and heart damage as observed in our rat model has not been recognized in patients yet. Interestingly however, a recent modelling study by Cella et al. \(^{33}\) showed that the heart dose plays an important role in the development of radiation-induced lung fibrosis in Hodgkin lymphoma patients treated with chemo-radiotherapy. Another clinical study showed enhancement of the risk of RILD when pre-existing cardiac disease exists \(^{34}\). As such, these studies are suggesting an interaction between heart and lung damage in patients who undergo thoracic radiotherapy. Until recently, only prediction models have been established predicting RILD. However, controversy exists about which dosimetric parameter(s) optimally predict RILD \(^{20}\). Heart dose-volume parameters are not incorporated in most of these prediction models. As such; incorporating cardiac dose metrics in existing predictive models may offer new opportunities to improve its accuracy. Of course, this remains to be determined in well-designed and adequately powered prospective observational studies.

Moreover, only models predicting early RILD are currently available. However, development of models predicting early as well as late RILD, or actually RICPD as discussion above, may be of importance since new advances in therapies like stereotactic radiation technique \(^{35,36}\), irradiation with protons \(^{37}\) or specific molecular targeted therapies \(^{38}\) lead to a longer life expectancy of cancer patients. Moreover, new radiation techniques provide higher conformity and thus to reduced irradiated normal tissue volumes. Changing volume could change the dose-limiting toxicity, early or late RILD, of a treatment. In chapter 6 we found that early RILD was associated with vascular remodelling in an irradiated-volume dependent manner while late RILD was associated with inflammation and fibrosis in a dose-dependent manner. Consequently, the dose-limiting toxicity changed from early to late dysfunction when the irradiated volume was reduced. In patients, early and late RILD are also due to different pathologies. As such, reducing irradiation volume by new radiation techniques might change the dose-limiting toxicity in patients too.

In our rat study in chapter 6 we only studied early and late RILD and its underlying pathology and dose-volume dependency. Given the important influence of heart co-irradiation on the development of RICPD, different dose distribution including the heart should be included in the next study. It would be interesting to study the dose-volume
dependencies and pathologies underlying early and late RICPD and compare this to only lung irradiation. This could provide important information about the influence of heart co-irradiation on the development of late RICPD, which is becoming increasingly important as the prognosis of cancer patients improves. As such, to optimize radiation therapy treatment, models predicting both early and late toxicity may have to be used and heart dose-volume parameters should be incorporated. To establish these models clinical modelling studies should be performed.

### Intervention strategies

**ACE-inhibition**

In our preclinical study we found the ameliorating effect of the ACE inhibitor (ACEi) captopril directly on heart toxicity and indirectly on lung toxicity. Although many differences exist between our animal studies and radiotherapy in clinical practice, such as single dose versus fractionation, the presence of patient related co-morbidities, and the increasing use of concomitant systemic agents, there are indications that the same mechanisms play a role in humans as described below.

A few clinical retrospective studies have been published investigating the effect of ACEi on the development of RILD. Kharofa et al. 39 found a difference in occurrence of radiation pneumonitis (RP) in patients treated for stage I-III NSCLC/SCLC. RP occurred in 2% of the patients using ACEi versus 11% in non-users (p=0.03). Another study by Wang et al. 40 found a difference in occurrence of RP in treated stage I-III NSCLC patients of 34% in the patients using ACEi versus 46% of the patients not using ACEi (p=0.06). The difference was not found to be statistically significant, however when the patient group was divided in patients receiving ≤ 20 Gy and > 20 Gy mean lung dose (MLD) and male and female a significant decrease was found in the occurrence of RP for ACEi users receiving ≤ 20 Gy MLD (p < 0.01) and male patients (p=0.04). Strikingly, also in these studies heart dose-volume parameters were not included. Since we found a direct protecting effect of ACEi on the heart and indirectly on lung toxicity in our rat model (chapter 4), including heart parameters in these retrospective studies may have given even greater differences in the occurrence of RP favouring ACEi use. A recently published retrospective clinical study suggested that ACEi protect the heart directly rather than the lungs 41. Symptomatic RP was scored in lung cancer patients receiving stereotactic radiotherapy and found a difference of 6.1% versus 16% in favour of patients using ACEi. Interestingly, in the univariate analysis they performed, the risk of RP was dependent on the localization of the tumour; patients with centrally located tumours had a higher risk of developing RP than patients with peripheral tumours. Although they did not include heart dose in their analysis, this could be due to an interaction between radiation-induced heart and lung damage as seen in our preclinical studies. As such, a new retrospective clinical study could be performed to assess the effect of ACEi use on the development of RICPD, this time including heart dose-volume parameters.

To further translate our findings to clinical practice, clinical studies should be performed to investigate whether ACEi exerts the same protective effect on acute radiation-induced heart damage and consequently on RILD as shown in our preclinical study. Treatment with ACE inhibitors should start directly after irradiation in order to protect against acute heart
damage that potentially bears consequences during the later stages of irradiation damage. Importantly, ACEi should not exert a protective effect on the tumour for instance inducing radioresistancy. Fortunately, ACEi have been found to not change the radiosensitivity of lung tumour cell lines \(^\text{42}\) and even reduce the risk of colorectal cancer \(^\text{43}\). As such, ACE inhibition with the clinically approved agent captopril may be a promising strategy to reduce early RICPD induced by thoracic radiotherapy, especially in patients receiving a considerable dose to the heart.

**Cell based therapy**

In chapter 5 we found that pulmonary EC loss may be the initiating factor in the development of vascular remodelling. Therefore, we hypothesized that cell therapy to replenish/repair lost ECs might prevent the further development of vascular remodelling leading to early RILD. Since endothelial progenitor cells (EPCs) derived from the bone marrow had shown to be able to repair and regenerate damaged blood vessels \(^\text{44,45}\), these cells were transplanted early after irradiation. Our pilot study showed engraftment of EPCs in the pulmonary vasculature of irradiated rats and a possible reduction of RILD. However, further optimization of the transplantation is needed. For example, a different amount of transplanted cells, different time points of transplantation or a more selected group of cells could lead to a better response. Further, two distinct subsets of EPCs could be transplanted: the early outgrowth EPCs and the late outgrowth EPCs, of which the former mostly promotes angiogenesis through paracrine effects and the latter forms vessels \(^\text{46,47}\).

EC loss may be an initiating step in RILD, but also other cell types are involved like pulmonary epithelial cells. As such, repair of vascular damage may not be sufficient to prevent the development of RILD. Interestingly, in a model of bleomycin induced lung fibrosis, it was found that bone marrow derived Prominin-1+ CD133 cells are involved in lung regeneration \(^\text{48}\). These pulmonary epithelial progenitor cells (PEPCs) were expanded from adult mouse lungs after digestion and culture of distal airways. After injection of these cells intratracheally, PEPCs engrafted into the lungs and differentiated into type II pneumocytes. Furthermore, PEPCs suppressed pro-inflammatory and pro-fibrotic gene expression, prevented the recruitment of inflammatory cells, and protected bleomycin-exposed mice from pulmonary fibrosis. Furthermore, in the lungs, it is possible to isolate a distinct stem-cell population from normal adult human lungs that is capable of regeneration of virtually all element of an injured mouse lung \(^\text{49}\). Transplantation of these cells resulted into development of human bronchioles, alveoli, and pulmonary vessels within 14 days after transplantation. Human lung stem cells obtained from regenerated lung tissue were able to self-renew and create lung parenchyma in vivo in another mouse with lung damage. So, human lung stem cells may have a crucial role in lung homeostasis and tissue regeneration and may therefore be suitable to repair RILD.

Besides pulmonary vascular remodelling, inflammation plays an important role in the development of RILD. Inflammatory cells migrate to damaged tissue \(^\text{50}\), as such; repair of the pulmonary vasculature and the parenchyma may inhibit migration and accumulation of inflammatory cells.

As we described in chapter 2 and 3 heart and lung irradiation independently lead to secondary changes across each other. Hypothetically, prevention of RILD by transplantation of EPCs and PEPCs may also prevent the indirect effect on the heart. Vice versa, prevention
of radiation-induced heart damage (RIHD) may also prevent indirect lung damage. The vasculature is also an important component of the heart, so EPC transplantation may have a protective effect on the heart as well.\(^5\) This could be studied in our rat model. Unfortunately, it is not yet possible to repair cardiomyocyte damage. Although cardiac stem/progenitor cells and cardiomyocytes can be cultured in vitro, transplantation in the heart does not yet seem to lead to sufficient functionally integrated and long-term survival of cardiac stem cells.\(^5\)

In summary, cell replacement strategies can potentially be developed to prevent RICPD. When successful in preclinical studies, it may open the way for the development of clinical protocols for the use of these strategies in radiotherapy. Successful amelioration of radiation-induced side effects may allow dose escalation and improve tumour control, and will improve the post-treatment quality of life of the patients.

**Conclusions**

In this thesis we tried to get more insight into the pathophysiology of RICPD to find new/better predictors of the development of RICPD and possible attenuating intervention strategies.

By using our rat model, we found that pulmonary vascular remodelling plays an important role in the development of RILD possibly initiated by acute endothelial cell (EC) apoptosis. This knowledge provided a new possible target to diminish RILD; transplanting endothelial progenitor cells (EPCs) shortly after irradiation to repair/replenish endothelial cells. Our preliminary results indicated that EPC transplantation may ameliorate RILD.

Another finding in this thesis is the interaction between heart and lung damage in the development of RICPD. Only lung or only heart irradiation can secondarily damage the other and combined irradiation even enhances the toxicity. This heart and lung interaction has not been recognized in clinic yet and predictive modelling studies do not include heart parameters. As such, to optimize radiation therapy treatment, heart dose-volume parameters may be incorporated in prediction models.

Next, we found an ameliorating effect of the ACE inhibitor (ACEi) captopril directly on early heart toxicity and indirectly on early lung toxicity. As such, ACE inhibition may be a promising strategy to reduce early RICPD induced by thoracic radiotherapy, especially in patients receiving a considerable dose to the heart.

Finally, we found that in contrast to early RILD, late RILD predominantly depended on dose and was associated with inflammation and fibrosis, rather than irradiated volume and vascular remodelling. Consequently, the dose-limiting toxicity changed from early to late RILD when the irradiated volume was reduced. Currently, only models predicting early RILD are available. This finding suggests that for treatment optimization models predicting both early and late toxicity may have to be used.

To conclude, in this thesis we presented new insights into the pathophysiology of RICPD possibly leading to the development of better/new prediction models. Besides, intervention strategies to ameliorate RICPD have been demonstrated. Translation of these preclinical findings to clinic may improve radiotherapy treatment and even provide tumour dose escalation leading to a better tumour control and an increased survival of the patient.
References


