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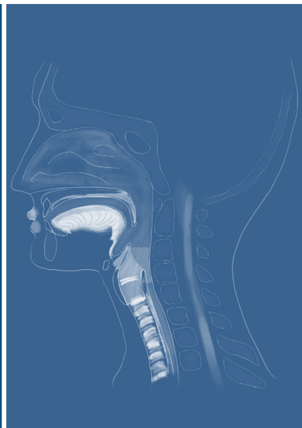

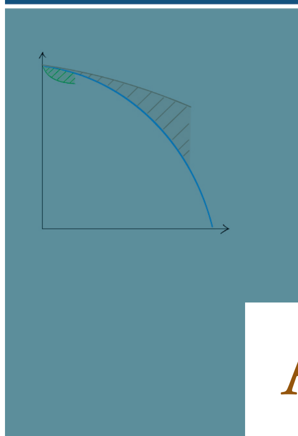
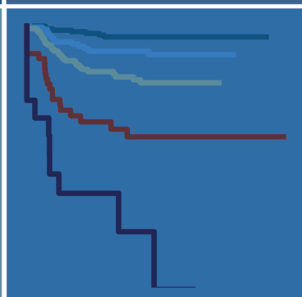
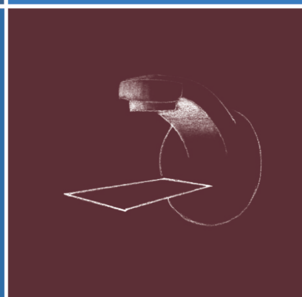
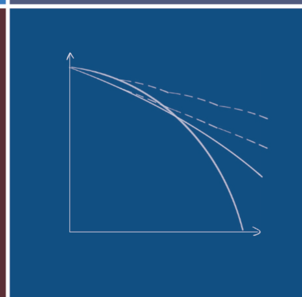

Altering Radiation Response

with Time, Volume and Fractionation

GABRIEL ADRIAN
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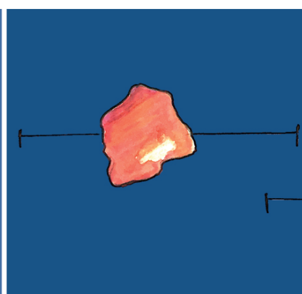
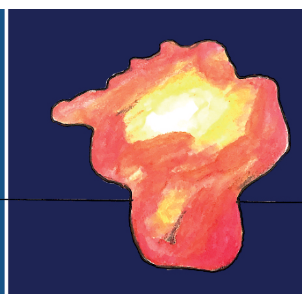
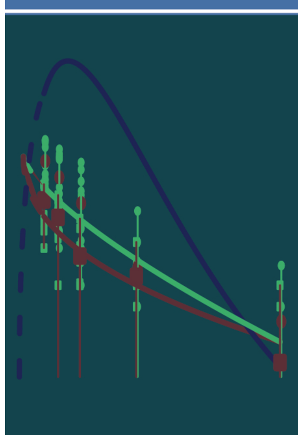
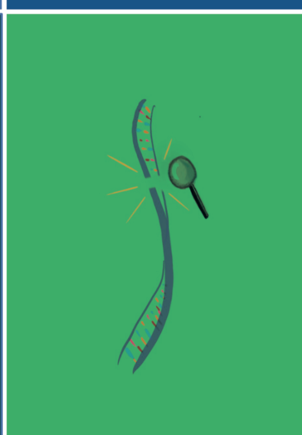

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Take Home (thesis in half a minute)

This thesis investigates different aspects of radioresistance and opportunities to overcome it. In patients with oropharyngeal cancer, we show that tumour VOLUME causes radioresistance, and altered FRACTIONATION could be a strategy to improve survival. A pre-clinical part concerns recent discoveries in radiotherapy. FLASH, the use of ultra-high dose rate where the TIME to deliver the dose is reduced to a fraction of second, has been suggested to overcome radioresistance – by inducing radioresistance in healthy tissue. We investigate a potential role for oxygen in FLASH. Lastly, cellular communications and the VOLUME of irradiated cells in vitro are shown to mediate radioresistance.

The overall conclusion is that radiation responses can be altered. There are opportunities to improve tumour cure rates using time, volume and fractionation.

GABRIEL ADRIAN is an oncologist working at Skåne University Hospital, Sweden, since 2012 and has an interest in translational aspects of radiobiology.

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Altering Radiation Response

Altering Radiation Response

with Time, Volume and Fractionation

Gabriel Adrian

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DOCTORAL DISSERTATION

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To be defended in the Belfrage Lecture Hall, D15, 3rd floor, BMC, Lund, Sweden, on Friday 14th of May, 2021 at 9.00 am (due to the pandemic, it will only be publically available via Zoom).

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Abstract
Radioresistance, the failure to achieve a desired outcome, is an obstacle in clinical radiotherapy. In this thesis we investigate factors affecting radioresistance and strategies to overcome it, both with established clinical approaches and by using novel pre-clinical discoveries.

Study I & II concern the impact of tumour volume in patients with oropharyngeal cancer. In a large, pooled cohort of 654 patients from three clinical trials, we show that tumour volume is the predominant factor for local control, progression free survival and overall survival. The negative impact of large tumour volumes could, in exploratory analyses, be mitigated by intensified radiotherapy. The studies also confirm the prognostic role of HPV/p16-associated tumours, haemoglobin level and smoking status. Based on the results, individualized treatment based on tumour volume could be suggested.

The second part of the thesis is based on pre-clinical experiments of novel discoveries. FLASH, the use of ultra-high dose rate radiotherapy where the irradiation is delivered in a fraction of a second, has been shown to spare normal tissue without hampering tumour control. Thereby, FLASH could be used to overcome radioresistance by escalating the dose to the tumour without increasing the risk of normal tissue complications. Oxygen has been proposed to play a key role in mediating the FLASH effect. We investigated the role of oxygen concentrations in a prostate cancer cell line and found that the FLASH effect appeared in hypoxic cells, but not in normoxic (study III). To further elucidate if FLASH effects are solely appearing in hypoxia, we investigated six additional cell lines under normoxic conditions and found that a FLASH effect may also appear in normoxia (study IV). We did not find any correlation between the FLASH effect and induction of DNA double strand breaks or cell cycle arrests.

In the last two decades the discovery of bystander and rescue effects has broaden the understanding of radiation responses. Not only directly hit cells are affected by the irradiation, and cellular communications contribute to part of the radiation response. We investigated if cellular communications could induce radioresistance. By varying the number of irradiated cells, adding cell conditioned medium and irradiating only half of the cells, we found that cellular communications cause a rescue effect, hence radioresistance.

In summary, the thesis underpins that radiation responses can be altered. To overcome radioresistance due to large tumour volumes, intensified radiotherapy for patients with large oropharyngeal cancers should be considered. The clinical exploitations of FLASH and bystander/rescue effects remain to be investigated.

Key words Radiotherapy, head and neck squamous cell carcinoma, tumour volume, FLASH radiotherapy, rescue effect, radioresistance, individualized radiotherapy

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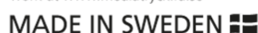

To Inger Hillerdal, my first teacher

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Adrian G, Konradsson E, Beyer S, Wittrup A, Butterworth K, McMahon S J, Ghita M, Petersson K, Ceberg C. Cancer cells can exhibit a sparing FLASH effect at low doses under normoxic *in vitro*-conditions. *Submitted.* (study IV)

Adrian G, Ceberg C, Carneiro A, Ekblad L. Rescue effect inherited in colony formation assays affects radiation response. *Radiat Res* 2018; **189**: 44-52 (study V)

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Related publications not included in the thesis:

Petersson K, **Adrian G,** Butterworth K, McMahon SJ. A Quantitative Analysis of the Role of Oxygen Tension in FLASH Radiation Therapy. *Int J Radiat Oncol*. 2020;107:539-547.

Lempart M, Blad B, **Adrian G**, Bäck S, Knöös T, Ceberg C, Petersson, K. Modifying a clinical linear accelerator for delivery of ultra-high dose rate irradiation. *Radiother Oncol*. 2019;139:40-45.

Abbreviations

BID	Bi-daily (two fractions the same day)
CT	Computed Tomography
D	Dose (in Gray)
DSB	DNA-Double Strand Break
e	The base of the natural logarithm (Euler's number ≈ 2.718)
EGFR	Epidermal Growth Factor Receptor
FDG	Fluorodeoxyglucose
Gy	Gray (unit of ionizing radiation dose)
Hb	Hemoglobin
HNSCC	Head and Neck Squamous Cell Carcinoma
HPV	Human Papillomavirus
HR	Homologous Recombination (DSB repair pathway)
LC	Local Control
LF	Local Failure
MRI	Magnetic Resonance Imaging
NHEJ	Non-Homologous End-Joining (DSB repair pathway)
OTT	Overall Treatment Time (of a radiotherapy course)
OS	Overall Survival
PET	Positron Emission Tomography
PD-L1	Programmed Death-Ligand 1
PFS	Progression Free Survival
ROC	Receiver Operating Characteristic
RT	Radiotherapy

SF	Survival Fraction
SIB	Simultaneous Integrated Boost
SSB	DNA-Single Strand Break
TCD	Tumour Controlling Dose (e.g. TCD50, the dose required to control 50% of the treated tumours)
TCP	Tumour Control Probability
TNM	Tumour, Nodal, Metastasis (classification of malignant tumours)

Abstract

Radioresistance, the failure to achieve a desired outcome, is an obstacle in clinical radiotherapy. In this thesis we investigate factors affecting radioresistance and strategies to overcome it, both with established clinical approaches and by using novel pre-clinical discoveries.

Study I & II concern the impact of tumour volume in patients with oropharyngeal cancer. In a large, pooled cohort of 654 patients from three clinical trials, we show that tumour volume is the predominant factor for local control, progression free survival and overall survival. The negative impact of large tumour volumes could, in exploratory analyses, be mitigated by intensified radiotherapy. The studies also confirm the prognostic role of HPV/p16-associated tumours, haemoglobin level and smoking status. Based on the results, individualized treatment based on tumour volume could be suggested.

The second part of the thesis concern pre-clinical experiments of novel discoveries. FLASH, the use of ultra-high dose rate radiotherapy where the irradiation is delivered in a fraction of a second, has been shown to spare normal tissue without hampering tumour control. Thereby, FLASH could be used to overcome radioresistance by escalating the dose to the tumour without increasing the risk of normal tissue complications. Oxygen has been proposed to play a key role in mediating the FLASH effect. We investigated the role of oxygen concentrations in a prostate cancer cell line and found that the FLASH effect appeared in hypoxic cells, but not in normoxic (study III). To elucidate if FLASH effects are solely appearing in hypoxia, we investigated six additional cell lines under normoxic conditions and found that a FLASH effect may also appear in normoxia (study IV). We did not find any correlation between the FLASH effect and induction of DNA double strand breaks or cell cycle arrests.

In the last two decades the discovery of bystander and rescue effects has broaden the understanding of radiation responses. Not only directly hit cells are affected by the irradiation, and cellular communications contribute to part of the radiation response. We investigated if cellular communications could induce

radioresistance. By varying the number of irradiated cells, adding cell conditioned medium and irradiating only half of the cells, we found that cellular communications cause a rescue effect, hence radioresistance.

In summary, the thesis underpins that radiation responses can be altered. To overcome radioresistance due to large tumour volumes, intensified radiotherapy for patients with large oropharyngeal cancers should be considered. The clinical exploitations of FLASH and bystander/rescue effects remain to be investigated.

Introduction

Radiotherapy. Invisible lights, energy deposited in the tumour, duration ranging from seconds to minutes, no immediate sensations and afterwards everything looks the same. But the deposited energy – the dose delivered – can cure cancer. And there is a simple relationship between dose and response. The higher the dose, the more cells are killed, and the higher the chances of a curative outcome. However, radioresistance – the failure to achieve a desired outcome – is a major obstacle for radiotherapy. The current thesis aims at investigating different aspects of radioresistance, and strategies to overcome it.

Study I & II concern clinical radiotherapy. In cohorts of patients with oropharyngeal cancer, the impact of tumour volume on radioresistance, and opportunities to improve outcome by intensified radiotherapy, are investigated. Study III-V are *in vitro*-investigations of two recent discoveries. FLASH, ultra-high dose rate radiotherapy where the irradiation time is a fraction of a second, is a promising new method to overcome radioresistance − by inducing radioresistance in healthy normal tissue. We investigated the role of oxygen for such a FLASH effect to appear, first by varying the oxygen concentrations, and then by investigating the responses for a range of cell lines in normoxia. Bystander and rescue effects have underpinned the impact of cellular communications on radiation responses. We investigated the possibility of such cellular communications to induce radioresistance.

Background

Ionizing radiation was discovered in the late 19th century and was quickly adapted and used for clinical applications; radiotherapy. Today, it is estimated that every second cancer patient will receive radiotherapy at some point during his or her illness. Radiotherapy plays an important role in the curative setting, and for some diagnoses, like head & neck cancer, it is probably the most important treatment modality. For patients with incurable cancer, radiotherapy can provide pain relief, diminish the risk of bleedings, or locally stop threatening cancer growth, such as spinal cord compression or compromised airways.

Radiotherapy is a double-edged sword in its inherent nature. Tumours and healthy normal tissues exist in close proximity to each other, and radiotherapy will inevitably affect both. Some radioresistant tumours may be hard to eradicate without inacceptable toxicity of the surrounding tissues. This therapeutic window is sometimes so narrow, or non-existent, that successful treatments are not possible. This thesis aims at investigating factors that cause radioresistance, and strategies to overcome it.

In the following sections the basis of the classical understanding of radiation mechanisms, mathematical descriptions of dose and effect, and clinical consequences and exploitations are presented. Then, some general aspects of radioresistance and a brief introduction to head & neck cancers, followed by the recent discoveries of FLASH radiotherapy, as well as bystander and rescue effects.

Classical Radiobiology

Radiation – from physics, through chemistry, to biology

Radiotherapy starts with the physical delivery of ionizing radiation in a tissue. The ionizing radiation interacts with orbital electrons causing excitations or ionizations, where secondary electrons may lead to further excitations and ionizations. The primary target in the cell is the DNA-molecule.[1] Ionizing radiation can cause direct excitation and ionizations in the DNA, but for X-ray and electron irradiations, most of the damage is induced through middle steps involving water molecules (Fig 1). In this indirect mechanism of action, water molecules become ionized and form a highly reactive ion radical, H_2O^+, which in turn reacts with another water molecule and form the highly reactive hydroxyl radical OH• (see Supplementary). Depending on oxygen concentration, subsequent radio-chemical steps yield several products, including hydrogen peroxide (H_2O_2) and superoxide (O_2^-).[2,3] Hydroxyl radicals and other reactive oxygen species can diffuse a short distance and react with DNA resulting in DNA-damage. Such DNA-damage can be single-stranded (SSB, only one of the DNA-strands affected, the other intact) or double-stranded (DSB, both DNA-strands broken). DNA-damage triggers diverse biological responses, and eventually the cell recovers with full integrity, or may be doomed to cell cycle arrests, cell death, impaired function, or carcinogenesis decades later.

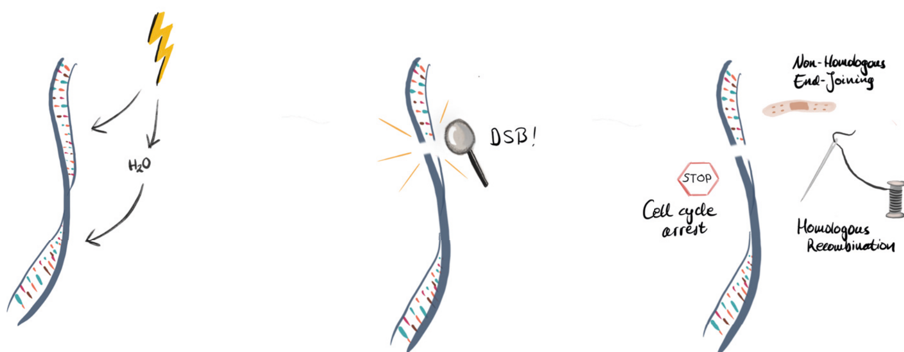

Figure 1 DNA-damage, recognition and response.

Principle illustrations of the cellular effects of radiation to the DNA. Firstly, the irradiation causes damage to the DNA, either through a direct interaction with the DNA-molecule, or via indirect action where H_2O is the predominant middle step (left panel). Through various mechanisms, the cell recognizes the damage (middle panel) and multiple cellular responses are activated. Cell cycle arrest (right panel, STOP-signal) allows the cell to repair the damage before propagation in the cell cycle. There are two key pathways for DNA-DSB-repair; the quick, but error-prone Non-Homologous End-Joining (NHEJ, illustrated as a band-aid) and the more accurate, but slower and cell cycle-dependent Homologous Recombination (HR, illustrated as a needle and thread). © Gabriel Adrian.

Cellular responses to radiation

Life on earth has arisen in the presence of ionizing radiation. Background radiation from cosmos and naturally occurring radioactive materials, have made it a pre-requisite for life to effectively handle the damage radiation induces.[4] The biological responses to ionization damages are sophisticated and involve several different approaches. The following sections outline the key responses:

Damage recognition

Every cell has a group of proteins that actively monitor the genome, looking for damages. Once DNA-damage is found, signals are triggered leading to phosphorylation of histone H2AX (γH2AX) at the site of the damage (Fig 1, middle panel).[5] The sensor proteins include the MRN-complex (MRE11, RAD50 and NBS1) that recruits the ataxia-telangiectasia mutated protein (ATM), and the Ku70/Ku80 complex that recruits the catalytic subunit of DNA-dependent protein kinases (DNA-PKcs).[6] Clinically, ATM-deficiency is known to increase the risk of cancer development,[7] as well as causing extreme radiosensitivity,[8] and low levels of DNA-PKcs also cause extreme radiosensitivity,[9] underpinning their importance in the radiation response. Following γH2AX-formation, a complex of proteins (including 53BP1 used in study III) are formed around the DNA-damage and numerous cellular responses and signalling pathways are activated.[10]

Damage repair

Single Strand Breaks are less complex than DSB and easier to repair. Excision Repair, Single Stranded Breakage Repair, Mismatch Repair, and Nucleotide Excision Repair are four of the cell's repair machineries for SSB.[1] Clinically relevant, the Base Excision Repair has gained recent focus, since PARP-inhibitors exhibit their action by blocking this process.[11] Double-Strand Breaks are the most relevant lesions for radiotherapy. Here, two main pathways for repair are available (Fig 1, right panel). Non-Homologous End-Joining (NHEJ) is independent of cell cycle phase and resolves most of the DNA-damage within few hours.[12] However, NHEJ is error-prone and the repaired DNA-chain may have deletions, insertions, or changes of base-pair.[13] Homologous Recombination (HR), on the other hand, requires the presence of a sister-chromatid, hence it is only available in late S- and G2-phase.[12] Here, the sophisticated and time-consuming HR offers a perfect repair of the DNA-damage. The BRCA2-protein is one of the proteins involved in HR, connecting the consequence of impaired HR to the higher risk of cancer development in BRCA2-mutational carriers.

Cell cycle arrest

Activation of cell cycle checkpoints is a powerful response once the cell recognizes a DNA-damage. Cell cycle checkpoints appear in the late G1-phase, S-phase, and in early- and late G2-phase.[14] Once activated, the checkpoints halt the cell cycle propagation, allowing the cells to repair the damage. After some time, depending on the damage, the arrest is released, and the cell continues its journey in the cycle. The checkpoint activation depends on different factors and their function can be impaired. Clinically relevant, the HPV-virus elicits (one of) its action by interference of the G1-checkpoint (see separate section).

Cell death

Actual cell kill after irradiation can arise in several ways. In some cases, such as for certain lymphomas, the radiation response is to commit suicide, apoptosis.[15] Thereby, such cells tend to be very sensitive to radiation. Most solid cancers, however, activate some cell cycle checkpoints and (try to) to repair the damage. As a result, cells tend to successfully complete one or two mitoses, but, due to accumulating damages, the cells fail to complete more rounds of cell division and succumb in a mitotic catastrophe.[16] Cells can also die by necrosis or complete failure of initializing cell cycle propagation, known as senescence.[6]

Clonogenic assays to determine cell death

The golden standard to determine *in vitro* responses of radiation is the clonogenic assay (also known as the colony formation assay).[17] The assay does not differentiate any cell death mechanism, instead it captures the ability of cells to undergo indefinite replication. Cells are plated as single cells in a dish or a flask, exposed to irradiation[A], and are then put in a humidified incubator and allowed to grow for 7-14 days, until colonies of at least 50 cells are formed. The assay was developed in the 50's by the seminal work of Puck and Marcus[18] and has been the backbone of many radiobiological studies since.[19] The definition of survival (clones with at least 50 cells) was found to be a reliable threshold by Puck and Marcus. Thereby, the typical initial 2-3 cell cycle rounds (divisions) of eventually dying cells, would not affect the result. However, already Puck and Marcus observed the slow-growing appearance of surviving cells after higher doses of irradiation, and the importance of slow-growing colonies and the survival definition ("50 cells") has raised concerns in the past. [16,20–24] Probably, one could argue that the clonogenic assay has inherited behaviours or limitations that affect

[A] The assay can be performed in the reverse order as well, where cells are first irradiated and then plated.

the results. Nonetheless, by capturing the capacity of irradiated cells to continue endless division, the clonogenic assay plays an important role as an *in vitro*-surrogate for complete sterilization of tumour cells.

Mathematical models describing survival

The survival fraction (SF) obtained using clonogenic assay can be visualised in a log-linear plot with dose on the x-axis and log(SF) on the y-axis (Fig 2).[25] With increasing dose, SF decreases. The survival curve typically has some kind of bendiness, hence a higher dose is more efficient in killing cells than two separate lower doses. The shape of the survival curve has been subject for many mathematical models. The single-hit multi-target model was the predominant model for many years, and still has some advantages as it reflects radiosensitivity (D_0) of a cell line. [26] With this model, the SF is described as:

$$SF(D) = 1 - \left(1 - e^{-\frac{D}{D_0}}\right)^n \quad (1)$$

where D is the delivered dose, D_0 the dose required for reducing SF to 1/e = 37%, and n the number of sensitive targets in the cell (extrapolation number where the linear part of the curve would cross the y-axis). The single-hit multi-target model generates an initial shoulder of the survival curve, described by D_q:

$$D_q = D_0 \log_e n \quad (2)$$

At higher doses ($D>Dq$) the relationship between dose and log(SF) gradually becomes linear, with the slope $-D_0^{-1}$. The D_0-value can thus be compared between cell lines and reflects the cell line specific radiosensitivity.

Nowadays, the linear-quadratic (LQ)-model is most commonly used to describe the relationship between SF and dose[26]:

$$SF(D) = e^{-(\alpha D + \beta D^2)} \quad (3)$$

where α and β are parameters describing the radiosensitivity of the cell with the unit Gy^{-1} and Gy^{-2}, respectively. The LQ-model gives a continuously bendy curve on a log-linear plot. When Chadwick and Leenhouts described the LQ-relationship in 1972, their underlying assumptions were based on DNA as the principal radiation target and that both DNA-strands were to be broken to induce cell kill (hence, DSB).[27] Such lethal breaks could be caused by one radiation event that increased linear with dose (the α-term), or by two independent events, where the probability increased with the square of the dose (the β-term). These underlying

assumptions are not necessarily true, and the LQ-relationship can also be justified as a fitting of a curve to a mathematical expression.[26,28] The ratio of the constants, i.e. the α/β-ratio (unit Gy), is most useful as it reflects the bendiness of the survival curve (Fig 2). Thereby, a single value (the α/β-ratio) can be used to compare the *fractionation* sensitivity between cell lines. It should, however, be noted that, in contrast to the D_0-value, the α/β-ratio does not reflect the radiosensitivity of a cell line.

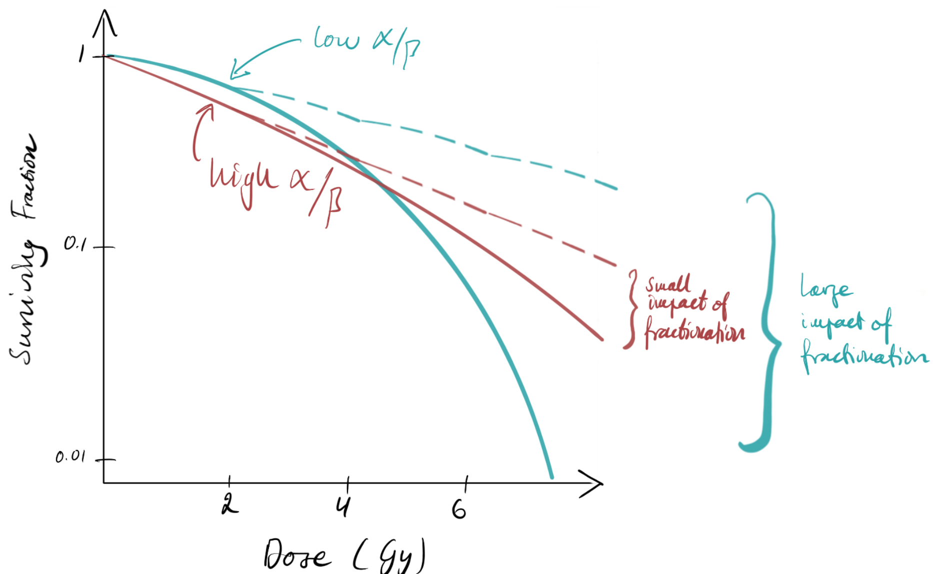

Figure 2 The bendy survival curve.
The clinical use of fractionated radiotherapy exploits the bendy survival curve. The relationship between dose (D) and surviving fraction (SF) is described by the linear quadratic model: SF = $\exp(-\alpha D - \beta D^2)$. The various bendiness for different tissues and tumours, allows a widening of the therapeutic window between normal tissue complication and tumour control. The figure illustrates the smaller impact of fractionation when the α/β-ratio is high (red curve) compared with a low α/β-ratio (turquoise curve), when the dose is delivered in four fractions (dashed lines) compared with one single dose (solid lines). For head & neck cancer with α/β ~10 Gy, the use of small fraction doses will be beneficial, since the late reacting normal tissue has an α/β ~3 Gy. © Gabriel Adrian.

The LQ-model bridges pre-clinical and clinical radiobiology, since α/β-ratios also can be determined for clinical end-points.[29] Hereby, clinically useful values of α/β-ratios for specific normal tissue end-points and tumours have been established.[30] Clinical α/β-ratios are not derived from survival curves, instead they are estimated by comparisons between (at least two) different fractionation schedules, for a given end-point (such as iso-effective local tumour control, or a

certain degree of kidney failure). In contrast to the *in vitro*-determinations, it is only the ratio between α and β, not their exact values, that can be determined with this approach. Once again, the α/β-ratio does not reflect a certain tissue or tumour's radiosensitivity, but its response to different fractionations. Interestingly, normal tissue complications tend to have a high α/β of ~10 Gy for acute reactions (such as epitelitis), but a low α/β of ~3 Gy for late complications (fibrosis, kidney failure). Tumours, on the other hand, have different α/β-ratio depending on their origin. Head and neck squamous cell carcinoma (HNSCC), lung, and cervix cancer are usually regarded as having an α/β-ratio of ~10 Gy, breast cancer ~4-5 Gy, and prostate cancer 0.5-3 Gy.[30–33] The difference in α/β-ratios between tumours and normal tissue can be exploited clinically using different fractionation schedules, as discussed below.

5 R's of radiobiology

The response to different fractionation schedules can thus be described with the LQ-model. Although some mechanistic assumptions can be included in the model[27], it does however not describe why cells, tissues or tumours have different α/β-ratios (see further discussion in the section "Radioresistance"). The clinical benefits achieved by fractionated radiotherapy can, at least to some extent, be explained by the "5 R´s of radiobiology":[34,35]

Recovery (repair of radiation induced damage),

Re-distribution (propagation in the cell cycle, from radioresistant S-phase to more radiosensitive G2-phase, as an example),

Re-population (through cell division),

Re-oxygenation (oxygenation of hypoxic or anoxic cells, leading to increased radiosensitivity),

Intrinsic Radiosensitivity.

Differential behaviours between tumour and normal tissue regarding the 5R's tend to increase the relative cell kill in tumours as the radiation is fractionated, although some factors, such as re-population in a tumour, might worsen the outcome (see section below, accelerated treatment).

Clinical exploitation through fractionation schedules

The LQ-model and the establishment of α/β-ratios for tumours and normal tissues have enabled clinical exploitations using three principally separate ways to alter fractionation.[36] A typical standard fractionation for HNSCC is 2.0 Gy per fraction, one fraction a day, five days per week, up to a total dose of 68.0-70.0 Gy in seven weeks. This can be altered:

Hyperfractionation: The administration of lower fractionation doses than 1.8-2.0 Gy, delivered twice daily, up to a higher total dose, with the same overall treatment time. Example: 1.1 Gy + 1.1 Gy (BID), five days a week, total dose 81.4 Gy.

Accelerated treatment: The administration of the same total dose, but with shorter overall treatment time. Example: 2.0 Gy / fraction, 6 days per week, total dose 68.0 Gy in six weeks.

Hypofractionation: The administration of a dose higher than 2 Gy per fraction, up to a lower total dose. Example: 2.4 Gy / fraction, 5 days per week, total dose 60.0 Gy.

The underlying hypotheses for the treatment strategies could be summarized as follows:

Hyperfractionation: exploits radiobiological differences for tumours with higher α/β-ratios compared with normal tissue[B]. Hereby, the large number of fractions, with low doses per fraction, will spare normal tissue to a higher degree than the tumour. This will allow escalating the total dose, without increases in late normal tissue toxicities, and thereby increasing the therapeutic window.[37]

Accelerated treatment: tumour cells proliferate during a radiotherapy course. There is also some evidence for an increased proliferation due to the irradiation, a phenomenon termed accelerated re-population.[38] Since proliferation of tumour cells increases the number of tumour cells the radiotherapy has to sterilize, a shorter overall treatment time should be beneficial.[39] [C]

Hypofractionation: for tumours with α/β-ratios *lower* (or comparable) to normal tisse (i.e. α/β ~3 Gy), the administration of large fraction doses will cause relatively more damage in tumour cells. Hypofractionation has proven to be clinically useful for prostate cancer and breast cancer.[31,32]

[B] It is usually the α/β-ratio of the late effects in normal tissue that can be exploited. The acute normal tissue toxicity (with higher α/β-ratio) might increase in altered fractionation schedules, but usually resolves over time (although questions have arisen around so called "consequential late effects" resulting from increased acute normal tissue toxicity).[235]

[C] A time factor can be added to the LQ-model to account for accelerated repopulation during a treatment:[236]

$$SF = e^{-n(\alpha d + \beta d^2)} e^{\gamma(T - T_k)}$$

n, number of fractions; d, dose per fraction; γ, the growth rate; T, the total treatment time; T_k, the "kick-off" time before accelerated repopulation begins. For $T<T_k$ the term $e^{\gamma(T-T_k)}$ is taken to be 1.

Combinations of the different treatment strategies are possible. A hypo-fractionated schedule has typically a short overall treatment time (hence, accelerated). Multiple daily fractions with doses <1.8 Gy can exploit hyperfractionation and acceleration.

Radioresistance

Radioresistance is a broad term that can be summarized as failure to achieve a certain outcome. That could be a tumour recurring locally after completing radiotherapy or a cell line with higher survival compared with another cell line for a certain irradiation dose.

Radioresistance can thus be determined in several ways. Clinically, local control rate and overall survival are central end-points to determine radiation responses, and allow comparisons between different tumours and/or treatments. There is not a clear relationship between α/β-ratios and radioresistance. As mentioned above, α/β-ratios determine the sensitivity to different fractionation schedules, and both prostate cancer with low α/β (0.5-3 Gy) and HNSCC with high α/β (~10 Gy)[30] can be cured with radiotherapy, whereas glioblastoma (α/β ~8 Gy)[40] almost inevitably recur after radiotherapy. Preclinical attempts have been made to relate survival fraction at a certain dose *in vitro* to α/β-ratios, without any consistent relationships.[41–44] Instead, by definition D_0 (equation 1) and SF_2 (survival fraction at 2 Gy) reflect radioresistance for *in vitro*-studies, and correlations between SF_2 and clinical responses have been shown.[45–47] The underlying mechanisms why different cell lines exhibit different radiosensitivity are probably many. As earlier discussed, some cell lines respond to irradiation with apoptosis and are highly radiosensitive.[15] Other explanations include vulnerabilities in the DNA to be exposed for complex DNA-damages[48], alterations in signalling pathways (such as Ras/PI3K/AKT-pathway)[49], p53 mutations[50], cell cycle distributions[51], and differences in damage tolerance[52]. The repair process is closely related to radioresistance, and phenomenological investigations have shown that cell lines have different repair capabilities using sublethal damage assays, potentially lethal damage assays[D], or low-dose rate irradiation.[53–55]

Clinical radioresistance

A radioresistant response in the clinic is influenced by several factors, which can be categorized into tumour specific and patient specific.

Firstly, tumour size affects radiation response since a large tumour consists of more cells that needs to be sterilized by the radiotherapy, and should therefore be harder to cure.[56] A large tumour may also harbour higher degree of hypoxia, causing further radioresistance.[57,58] Hypoxia is known to increase radioresistance,

[D] Sublethal damage repair describes the type of damage that can be recovered if cells are given time to recover, for instance by comparing the effect of 4 Gy with 2 Gy – 2 h recovery – 2 Gy. Potentially lethal damage repair is referred to as the increased survival obtained by halting the cell cycle propagation after irradiated, for instance by density inhibited cell cultures.

presumably through a mechanism where the presence of oxygen stabilizes the DSB and cause greater damage (the oxygen fixation hypothesis, see Supplementary).[59] Typically, a radioresistance by a factor of 3 is noted in the absence of oxygen compared with normoxic responses. In clinical cohorts with HNSCC patients, hypoxic tumours were substantially more radioresistant.[60]

Biological factors in the tumour also contribute to radioresistance. Beside hypoxia, intrinsic sensitivity (as the case for HPV-positive HNSCC)[61], differential expression of DNA-repair genes[62], differentiation grade (well differentiated being more radioresistant due to accelerated re-population)[63], stem cell richness[64], are other tumour specific factors affecting radioresistance.

Patient specific factors causing radioresistance include smoking status[65,66] and haemoglobin (Hb) level.[67,68] Both factors may be related to oxygenation levels in the tumour. In addition, performance status and age have been shown to affect the outcome.[66,69,70]

Strategies to overcome radioresistance

Radiotherapy can be altered to overcome radioresistance. Dose escalation, i.e. increasing the prescribed dose to the tumour, has historically improved outcome.[71] As discussed above, altering the fractionation schedule can be beneficial. Both dose escalation and altered fractionation schedules must be considered in relationship to normal tissue tolerance. A huge dose escalation could possibly cure a radioresistant tumour, but at the price of intolerable normal tissue complications. Hyperfractionation is capable of exploiting the radiobiological differences between tumour and normal tissue and have been shown to increase overall survival (OS) for HSNCC patients.[70] In spite of the advantages, hyperfractionation is seldom used in everyday clinic, perhaps due to the inconvenience for patients with multiple daily fractions, and the additional workload for radiotherapy departments. Modern radiation techniques enable highly conformal dose distributions, with possibilities to both spare normal tissue and escalate dose to tumours (using simultaneous integrated boost, SIB). Hereby, an improved outcome would be expected, but the clinical results are still scarce.[72,73] Carbon ion radiotherapy, although with limited access in Europe, might provide benefit, especially for hypoxic tumours.[74] The recent discovery of FLASH-radiotherapy, the administration of the irradiation in a fraction of second, offer new possibilities to escalate tumour doses without causing additional toxicity (see separate section below). The role of cellular communications to induce or protect from radiation damage have been studied in the last two decades.[75–77] At least in pre-clinical scenarios, cellular communications undoubtedly affect radiation responses.[78] Given its potential role, an increased bystander response, or an inhibition of the

counteracting rescue effect, would be useful to overcome radioresistance (see separate section below).

Besides altering the radiation itself, addition of drugs can be used to overcome radioresistance. Concurrent chemotherapy is the most used combination therapy, with a clear benefit for OS in meta-analyses of HNSCC patients.[79] Molecular targeted agents, especially epidermal growth factor receptor (EGFR)-inhibition through the antibody cetuximab, has been a clinical disappointment, at least for HPV-positive HNSCC.[73,80,81] Hypoxia modification with nimorazole has been shown to improve outcome[82], and is now tested in an on-going trial where patients are stratified based on hypoxic profiling.[E] The addition of erythropoiesis stimulating agents to increase patients' Hb-levels have been disappointing and may actually increase radioresistance.[83] It is hard to write a thesis concerning cancer in the year 2021 without mentioning immunotherapy, and that is particularly true when it comes to radiotherapy. There are data indicating a synergistic effect between radiotherapy and immunotherapy, where the combination can evoke novel immune responses resulting in durable tumour control.[84–86] Its role in the curative setting for HNSCC patients is currently investigated in several trials.[87] The Javelin Head and Neck 100 trial studied concurrent and adjuvant avelumab (programmed death-ligand 1 [PD-L1] antibody) in addition to concurrent chemotherapy. The trial was terminated prematurely and in the recently presented results a disappointing tendency towards *worse* progression free survival (PFS) for the intervention group was found.[88] The results for the similar Keynote-412 study[F] remains to be reported, and a study of adjuvant immunotherapy is still recruiting.[G] Other novel treatment options include the addition of drugs affecting apoptosis. Debio 1143 is an antagonist of inhibitor of apoptosis protein (that's a double negation, in other words, it increases the chances of apoptosis), with promising phase II results[89], and a phase III trials is recruiting.[H]

Individualized treatment

To overcome radioresistance, treatment approaches where individual tumour and patient related factors are considered, could be explored. In current practise, radiotherapy is individualized when it comes to the anatomical dose distribution. However, no individualization based on biological information is taken into account. A distinction between prognostic and predictive factors should be made,

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[E] The DAHANCA 30 trial, ClinicalTrials.gov Identifier: NCT02661152

[F] ClinicalTrials.gov Identifier: NCT03040999

[G] ClinicalTrials.gov Identifier: NCT03452137

[H] ClinicalTrials.gov Identifier: NCT04459715

where the former describes the patients overall cancer outcome, and the latter response to a specific intervention.[90] Prognostic factors can be used to risk group patients. For HNSCC patients, the subgroup of HPV-related tumours constitutes a distinct entity, where the overall good prognosis could motivate de-escalation trials, exemplifying the use of prognostic factors to individualize therapy. Large tumour volume is a prognostic factor for worse outcome (see separate section). Predictive factors are even more useful, since they inform about the response to a specific treatment intervention. For instance, predictive factors for response to altered fractionation, concurrent chemotherapy or a targeted drug, or hypoxia modification would be most useful to individualize treatment for HNSCC patients. However, there are to date few predictive biomarkers to guide treatment individualization.

The presence of EGFR-overexpression or gene amplification is a poor prognostic factor,[91] and interestingly, in post hoc-analyses EGFR-overexpression was a predictive factor for response to accelerated radiotherapy.[92,93] Hypoxia profiling using gene profiling has been shown to predict response to hypoxia modification.[94] PD-L1-status might predict response to concurrent immunotherapy.[88] Current initiatives to guide individualized treatment include RNA-sequencing to predict tumour responses and risk of normal tissue complications, histological biomarkers such as cancer stem cell markers, and functional imaging biomarkers.[71,95–98]

In the current thesis, tumour volume is investigated, both as a prognostic marker, as well as a predictive marker for response to intensified radiotherapy.

Head & Neck Cancer

Incidence, epidemiology, classifications

Cancer in the head & neck region constitute a broad entity of diseases. Globally, 700 000 new cases are diagnosed each year, accounting for around 4% of all malignancies.[99] Squamous cell carcinoma is the predominant histological subtype and arises in the mucosal surfaces inside the mouth, nose, or throat. The location of the primary tumour is sub-classified into sino-nasal, salivary duct, lip, oral, oropharyngeal, nasopharyngeal, hypopharyngeal and laryngeal cancer (Fig 3).

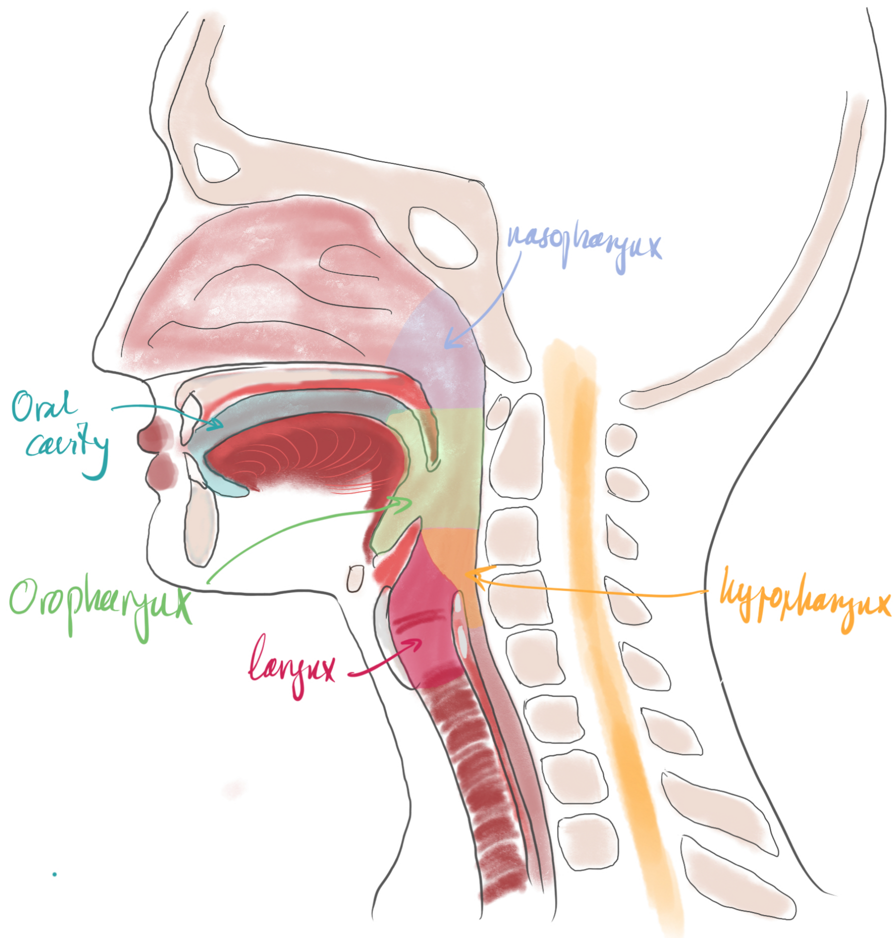

Figure 3 Illustration of the head and neck region.
Cancers in the head & neck region are sub-classified according to their primary location. © Gabriel Adrian.

The prognosis and treatment options differ between sub-sites. Generally these cancers have been attributed to exposure for tobacco and alcohol.[100] They typically occur in middle aged to elderly men, often with some degree of co-morbidity.[101] In the last decades there has been a rapid increase in oropharyngeal cancer occurring in younger patients without co-morbidities or abuse.[102] This has been attributed to an infection with the sexually transmitted human papillomavirus (HPV), mainly the high-risk type 16 and 18, see section below.

Typical presenting symptoms include sore throat, local pain, hoarseness in the voice, swallowing difficulties, a lump in the neck, or *en passant* by an observant dentist.[101] Most cancers in the head and neck region present as local or loco-regional diseases and distant metastases at time of diagnoses are rare.[103] The diagnosis is verified through a tissue biopsy, where the histological subtype, and HPV-association for oropharyngeal tumours, is determined. Imaging using computed tomography (CT) of the head & neck and thorax are usually sufficient, and could be supplemented with position emission tomography (PET) or Magnetic Resonance Imaging (MRI).[104] Based on the results of the diagnostic procedures, the cancer is staged according to Union for International Cancer Control [UICC] TNM Classification of Malignant Tumours. For oropharyngeal tumours T-classification depend on the size of the primary lesion measured in one dimension (T1: < 2 cm, T2: 2-4 cm; T3: >4 cm or has spread to the epiglottis), or invasion to adjacent tissues (T4).[105]

Human Papillomavirus

It is estimated that most people are exposed to HPV-infection, and the infection usually resolves without any consequences.[106] Unfortunately, for some individuals the infection becomes persistent and may cause cancerogenesis. The viral proteins E6 and E7 drive the process, and exhibit several actions on the infected cells; tumour suppressors p53 and retinoblastoma-associated protein (Rb) are among the targets for E6 and E7, respectively.[101] As a consequence of the Rb inactivation, the cell cycle arrest in G1 is abrogated (Fig 4). Feedback loops in the cell respond and as a result p16 is over-expressed. Pathological examination of p16-expression is therefore a surrogate marker for HPV-associated cancer, and also used in our studies.[101] The prognosis for HPV-positive oropharyngeal cancer is substantially better compared with HPV-negative,[61] and HPV-positive cells tend to be more sensitive to both chemotherapy and radiation.[107] Considering the better prognosis, de-escalation trials for patients with HPV-positive tumours are on-going.[108]

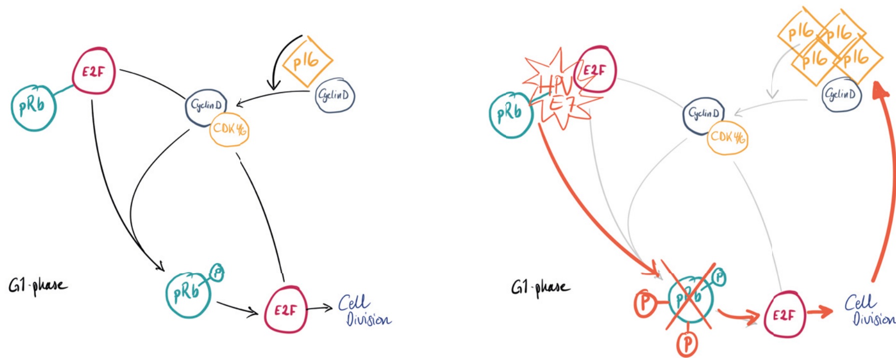

Figure 4 The HPV-protein E7 and effect on cell cycle and p16 expression.
The transition from G1 to S-phase is dependent on the E2F-transcription factor. As long as E2F is bound to Rb, the cell stays in G1. In homeostasis (left panel), the cell propagation from G1 to S is initiated by the up-regulation of Cyclin D which then binds to CDK4/6. The complex phosphorylates Rb → E2F is released → cell cycle propagation to S-phase. p16 reacts as a negative feedback loop and inhibits binding of CDK4/6 to Cyclin D. The HPV-protein E7 disturbs the machinery (right panel). Here, E7 phosphorylates Rb and E2F is released → cell cycle propagation. The cell reacts by up-regulating the negative regulator p16. However, the inhibition of CDK4/6 and Cyclin D by p16 does not affect the Rb – E2F interactions, due to the E7 protein. As a consequence, the G1/S-phase cell cycle transition is non-functional and p16 is accumulated. Illustration adopted with permission from Anna Holm, Umeå University, Sweden.

Treatment options

Treatment options for local or loco-regional HNSSC include surgery, radiotherapy, chemo-radiotherapy, alone or in combination.[104] The choice between treatment modalities depend on tumour subsite (oral tumours are predominantly treated with surgery, whereas oropharyngeal and hypopharyngeal tumours with radiotherapy or chemo-radiotherapy), how advanced the tumour is (the more advanced, the more difficult to achieve radical surgery), patient's co-morbidities, performance status and sometimes personal preference, and according to local traditions and expertise.[104] Treatment approach for each individual patient should be discussed at a multi-disciplinary conference, which was accomplished in 98% of all cases in Sweden during 2019.[109]

For oropharyngeal squamous cell carcinoma the Swedish Treatment Guidelines advocate radiotherapy or chemo-radiotherapy.[110] Surgery could be an option for cancers of the uvula or soft palatine, and the guidelines recognize that trans-oral robotic surgery could have a role, but data is limited.

Tumour volume and radioresistance in HNSCC
T-classification in the TNM-staging depends on the measured extension of the primary lesion in one dimension and should thereby reflect the tumour volume. Earlier reports have found a substantial overlap between CT-determined tumour volume and T-classification[111], and a better prognostic value of tumour volume compared with T-classification.[112–114] Several studies have addressed the connection between tumour volume and radioresistance. From a principal point of view, large tumours should contain more clonogenic cells, and therefore be harder to cure compared with smaller tumours. Baumann *et al.*, using mice models with two different HNSCC cell lines, showed that the number of clonogenic cells and the dose required to cure 50% of the mice (TCD_{50}) increased with tumour volume.[115] In a large set of clinical data from different sites, Dubben *et al.* concluded that tumour volume had large impact on outcome, and suggested that tumour volume was the most precise and relevant predictor of radiotherapy outcome.[56] Similar results are found in several studies with oropharyngeal cancer[113,116,117] and HNSCC in general.[112,114,118–120] Mathematical modelling to determine the impact of tumour volume on tumour control probability (TCP) for head and neck cancers has also found strong relationships.[121–123] However, not all studies support the relationship between tumour volume and outcome.[111,124–127] In Table 1, clinical studies addressing the connection between tumour volume and outcome after RT in retrospective HNSCC cohorts are summarized. In addition, two mathematical models could only establish a weak relationship between tumour volume and local control.[128,129] Only few publications stratify for HPV/p16-status. Thereby, the relationship between tumour volume and radioresistance in oropharyngeal cancer is an open question, requiring further analyses.

An adjacent question is how to overcome the possibly more radioresistant behaviour of large tumours. Adding concurrent chemotherapy is one option.[79] The radiotherapy itself can also be altered (as discussed above). In some phase III trials, there seem to be an increasing efficacy of altered radiotherapy schedules for higher T-classifications.[130–132] However, the MARCH meta-analysis with 11,423 HNSCC patients, did not find any interaction between tumour stage and altered fractionation for progression free survival or overall survival.[70]

To summarize, several studies suggest that large tumours are more radioresistant, but the efficacy of altered fractionation to overcome the tumour volume associated radioresistance is unclear.

Table 1 Studies on tumour volume and outcome after radiotherapy

Literature overview of publications investating the role of primary tumour volume and outcome after radiotherapy. Their main findings are listed in the last column. The statistical analyses methods (continuos data, dichotomized, or divided in four groups) are shown in the second last column.

First author (year)	Sub-site	No. of Patients	p16-str	Treatment	End-point	Analyses of tumour volume	Findings
Nathu (2000) [111]	Oroph	114	No	Radical RT, different schedules	LC	continous	Marginal impact
Hermans (2001) [124]	Oroph	112	No	Radical RT, different schedules	LC	4 groups	Marginal impact
Chao (2004) [116]	Oroph	74	No	Mixed radical and post-operative, different schedules, +/- chemo.	LRC, DMF	continous	Important factor
Studer (2007) [112]	All	172	No	Radical RT, +/- chemo	LC, DFS	4 groups	Important factor
Been (2008) [126]	Oroph	79	No	Radical RT, schedule not specified, +/- chemo	LRC	NA	Not important
Knegjens (2011) [114]	All	360	No	Radical RT, different schedules, +chemo.	LC, OS	4 groups.	Important factor
Lok (2012) [113]	Oroph	340	No	Radical RT, different schedules +chemo	LC, OS	dichotomized	Important factor
Strongin (2012) [120]	All	78	No	Radical RT, different schedules, +chemo	PFS, OS	dichotomized	Important factor
Studer (2013a) [117]	Oroph	277	No	Radical RT, schedules not specified, +/- chemo	LC	4 groups	Important factor
Studer (2013b) [118]	All (T4)	201	No	Radical RT, schedules not specified, +/- chemo	LRC, OS	4 groups	Important factor
Davis (2016) [125]	Oroph	53	Yes	Radical RT, different schedules, +chemo	DFS, OS	continous	Not important
Linge (2016) [96]	All	158	Yes	Radical RT, different schedules, + chemo	LRC, DMF, OS	Cox continous, KM-dichot	Important factor
Carpen (2018) [127]	Oroph	91	Yes	Radical RT 70Gy, +/- chemo	LRC, DFS, OS	Cox continous, KM-dichot	Not for p16+, but important for p16-negative
Schüttrumpf (2020) [68]	All	184	Yes	Radical RT, different schedules, +/- chemo	LC,LRC, OS	Cox continous, KM-dichot	Important factor

Abbreviations: p16-str, inclusion or stratification for p16/HPV-status; Oroph, oropharynx; RT, radiotherapy; LC, Local Control; LRC, Loco-Regional Control; DMF, Distant Metastases Free survival; DFS, Disease Free Survival; PFS, Progression Free Survival; OS, Overall Survival; NA, not available; Cox continous, cox regression with tumour volume as continiuos variable; KM-dichot, Kaplan-Meier estimates with logrank comparison for two volume groups.

FLASH

Curative radiotherapy is a delicate balance between tumour control and risk of normal tissue complications; the exploitation of the therapeutic window. Fractionated radiotherapy is supposed to widen that window. Dose-escalation *per se* does not widen the window. But if dose can be escalated and increase tumour control rates without increasing the risk of normal tissue complications, the therapeutic window would be widened. The novel FLASH radiotherapy, where the dose is given ultra-fast (in a fraction of a second compared with several minutes for conventional dose rate irradiation), seems to accomplish such a widening, and is thereby a most exciting new tool to overcome radioresistance.

Discovered and Rediscovered

In the late 60's researchers began to investigate the effects of ultra-high dose rate irradiation, and early on results were both promising and debated.[133] Some studies showed that radioresistance after ultra-high dose rate irradiations was induced under certain circumstances, depending on total dose as well as the initial oxygen concentration. Effects in normoxia were inconsistent. No translation to clinical practise occurred, perhaps due to technical requirements or uncertainties on tumour effects.[134] Later, Hendry *et al.* studied skin tolerance *in vivo*, and found convincing relationships between dose-rate and oxygen concentration for the risk of tail necrosis.[135] Another decade later, neither Cygler *et al*. nor Zackrisson *et al.* could detect any dose-rate dependent differences in survival fraction under normoxic or anoxic conditions *in vitro.*[136,137]

Then, in 2014, Favaudon *et al*. "rediscovered" the use of ultra-high dose rate irradiation and termed it FLASH.[138] In their study, a profound sparing of lung tissues using FLASH was shown, without affecting the ability to control tumour growth. Since then, the interest in FLASH has risen almost exponentially year-by-year, and has been proposed as the most promising new achievement for future radiotherapy.[139,140]

Main findings – normal tissue tolerance

Most experimental FLASH results concern normal tissue effects. There is now substantial evidence that, for a given dose, FLASH is less toxic compared with conventional dose rates. This has been shown various organs and animal models, including lung[138], brain[141,142], skin[135,143] and gut[144], although some opposing findings exist.[145,146] The prerequisites of the irradiation beam to trigger a FLASH sparing is being debated, and probably instantaneous dose rate, dose per pulse,

pulse repetition frequency, average dose rate, total delivery time and type of irradiation (photon, electron, proton, heavy ions) all need to be considered.[147,148]

Tumour effectiveness in vivo

For FLASH to become useful, the sparing of normal tissue must be compared to effects on the tumour level. To date, only few publications assess tumour effects (Table 2). The available results do, however, suggest that FLASH is iso-effective (*i.e.* same effectiveness) compared with conventional dose rate irradiation.

Table 2 Tumour effects of FLASH compared with conventinal dose rate irradiation
Available publications with direct comparisons between FLASH and conventional dose rate irradiation using *in vivo* models.

First authour (year)	In vivo model	Tumour type	End-point(s)	Comparison FLASH vs. conv
Favaudon (2014)[138]	Xenograft (leg)	Breast cancer (HBCc-12A), HNSCC (Hep-2)	Growth delay (mm)	Iso-effect
	Syngenic orthotopic (lung)	Lung cancer TC-1	Growth delay (biolum) Survival	Iso-effect
Bourhis (2019)[147]	Xenograft (flank)	Glioblastoma (U87)	Growth delay (mm)	Iso-effect
	Orthotopic (brain)	Glioblastoma (H454)	Growth delay (biolum)	Iso-effect
Montay-Gruel (2020)[149]	Orthotopic (brain)	Glioblastoma (H454)	Growth delay (biolum)	Iso-effect
	Xenograft (brain)	Glioblastoma (U87)	Growth delay (CT)	Iso-effect
Chabi (2020)[150]	Xenograft (leukemia)	3 different patient derived T-ALL	Cell number	Cell line specific*
Diffenderfer (2020)[151]	Allograft (flank)	Pancreas cancer (MH641905)	Growth delay (mm)	Iso-effect
Levy (2020)[144]	Syngenic ortotopic (intrapertioneal)	Ovarian cancer (ID8)	No. of solid tumours & tumour weight	Iso-effect

Abbreviations: HNSCC, head and neck squamous cell carcinoma; mm, manual measurement of tumour size using calipers; biolum, fluorosence imaging to determine tumour volume; CT, cone-beam computed tomography imaging to determine tumour volume; T-ALL, T-cell acute lymphoblastic leukemia; *, two of the cell lines were more efficiently controlled with FLASH, and the third by conventional dose rate irradiation.

In addition, very promising clinical results have been obtained with FLASH in treating cat patients with squamous cell carcinoma of the nose. Single doses of up to 41 Gy were given, with excellent tumour control, and no severe dose limiting normal tissue toxicity.[143] The first experience with a human patient was equally promising, and a single dose of 15 Gy to a cutaneous T-cell lymphoma was efficient in controlling the tumour, with very limited normal tissue toxicity.[152]

In vitro data using clonogenic assays

During the "first wave" of interest in ultra-high dose rate effects, starting in the late 60's, several investigations with clonogenic assays were conducted. As mentioned, results were inconsistent and several groups reported no survival differences depending on dose rate in normoxia[136,137,153,154], whereas other found differences at higher doses.[155,156] At lower oxygen concentration some studies did see an increased survival after ultra-high dose rate irradiation, although there were no direct comparisons to conventional dose rate irradiation.[153,154,157,158]

In the present era of rediscovered FLASH-interest, there has only been a limited number of studies using clonogenic assays to determine a FLASH response. Similar to the old data, present results are inconsistent. In normoxic conditions, no difference[159,160], a decreased survival fraction[146], and an increased survival fraction[141] after FLASH compared with conventional dose rate irradiation in normoxia have been demonstrated.

Underlying mechanisms

There is still no consensus explaining the underlying mechanism of the FLASH effect. The two prevailing hypotheses are radiation induced oxygen depletion and radical-radical interactions.

Oxygen is consumed in the radiochemical steps following irradiation.[161,162] New oxygen is continuously supplied by diffusion from nearby blood vessels, and hence a time factor between consumption (depletion) and supply should be considered. During conventional dose rate irradiations, the consumption of oxygen does not exceed the replenishment.[163] FLASH does not consume more oxygen, but it occurs during a much shorter time frame which could lead to a lowering of the oxygen concentration, hence a relatively more hypoxic tissue.[163–165] As previously discussed, hypoxia causes radioresistance, probably in a process where the DNA-damages induced by hydroxyl radicals in the presence of oxygen form an organic peroxyl radical, and thereby "fixates" the damage.[59] The differential effect between tumour and normal tissue would depend on differences in initial oxygen concentration.[148,165,166] It could be argued that the intermediate oxygen concentration, sometimes termed physoxia[167], in normal tissue is shifted towards a more hypoxic radioresistant state. But in tumours, a further decrease from an initially lower oxygen concentration does not cause the same amount of additional radioresistance.[164–166] Following the oxygen depletion hypothesis, no FLASH sparing would be found in normoxic *in vitro*-conditions at clinically relevant doses.

The radical-radical interaction hypothesis relies on the notion that approximately 2/3 of the DNA-damage induced by x-ray and electron irradiation occurs through the indirect mechanism of action (Fig 1, left panel and Supplementary). During the short time frame in which FLASH occurs, there will be a substantially higher

concentration of radicals compared with conventional dose rates.[2] This will increase the probability of radicals interacting with each other, and hence fewer radicals left to damage the DNA.[3,156] Peroxyl radical recombinations have been suggested as the main critical process, and a role for superoxide recombination has also been proposed.[2,3] These reactions may also be oxygen dependent, since the chemical reactions involve oxygen. The differential FLASH effect between tumour and normal tissue, would then be related to intrinsic ability to handle peroxyl radicals, DNA-repair capabilities, but also a dependence of oxygen concentrations in the formation and decay of radical oxygen species.[3]

As for both of the hypotheses, the underlying mechanism must both elicit a differential response depending on dose rate, and a differential response in tumours compared with normal tissue to explain the FLASH effect.

Open questions and considerations

The potential widening of the therapeutic window obtained by FLASH is promising for clinical translation. In current clinical radiotherapy, a widening of the therapeutic window is already at hand through fractionated regimens and highly conformal dose distributions. For FLASH to become truly advantageous compared with current state-of-the-art radiotherapy, the factors illustrated in Fig 5 will need to be addressed.

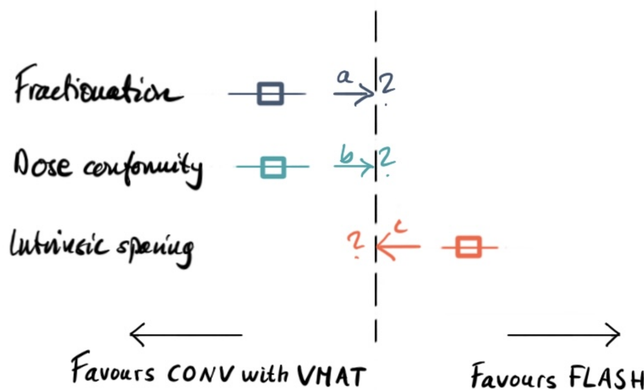

Figure 5 Widening of the therapeutic window using different radiation techniques.
Illustration how a differential tumour to normal tissue effect can be obtained, and a fictive forest plot comparison between FLASH (to the right) and state of the art radiotherapy with conventional dose rates using rotational techniques to achieve dose conformity (CONV with VMAT, to the left). The usefulness of FLASH will depend on the results of future investigations addressing fractionation effects (a), novel technical solutions to obtain dose conformity (b), and to ascertain a sparing effect for normal tissues, but not tumours (c). CONV: conventional dose rate irradiation; VMAT Volumetric Modulated Arc Therapy [rotational technique]). © Gabriel Adrian.

As discussed above, the 5 R's of radiobiology contribute to a fractionation effect in favour of the sparing of normal tissue. Most FLASH studies to date have instead used single, high doses and many models suggest that doses of 10 Gy or more must be applied to obtain a FLASH effect.[138,141,142,148,168] Contrary to this, recent work by Montay-Gruel *et al.* showed that hypo-fractionated regimens of 2 x 7 Gy preserved FLASH-sparing of cognitive functions without hampering tumour control[149], and Chabi *et al.* found significant differences for FLASH already at 4 Gy in their leukaemia mice model.[150] Thereby, benefits of FLASH may remain in fractionated therapies ('a' in Fig 5).

Modern radiotherapy with rotational techniques (such as Volumetric Modulated Arc Therapy) offers highly conformal dose distributions, with subsequent sparing of normal tissue. FLASH, as currently available, is usually applied with a single beam, with very limited possibility to modulate dose distributions. New technologies, such as the proposed PHASER linear accelerator, would overcome that dilemma ('b' in Fig 5).[169]

A general FLASH-sparing of normal tissue compared with tumours is suggested based on the current knowledge.[148] Studies to confirm its generalizability for various tissues and tumours will be important to identify the clinical situations where FLASH will be beneficial ('c' in Fig 5).

In the thesis, we investigated the FLASH-sparing effect using clonogenic assays, focusing on the role of oxygen. In study III, the impact of different oxygen concentrations was investigated for a prostate cancer cell line, and in the next study, the effect in normoxia for a range of cell lines was investigated.

Bystander & Rescue Effects

The ability of ionizing radiation to induce damage in targeted cells is a basic assumption of radiotherapy. Cells not hit by the radiation, are not affected. However, in 1992 Nagasawa and Little found that non-irradiated cells, when being in proximity to irradiated cells, exhibited typical signs of radiation damage.[75] The effect was later termed bystander effect and was proposed as the starting point of a new paradigm in radiobiology. Bystander effects may be responsible for normal tissue complications and exhibiting effects within tumours.[76,170,171]

Bystander Effects

The first report used α-particles, and showed that although just 1% of the cells were traversed by an α-particle, 30% exhibited sister chromatid exchanges.[75] [I] Thereby some kind of communication transferring the deleterious effect of radiation between the cells must be present. This has further been studied using transfer of medium from irradiated cells to non-irradiated[172], through the use of micro-beam irradiation[173], co-culture techniques[174], or with modulated beam irradiation where only part of the cells were irradiated.[175] Typically, non-irradiated cells receiving bystander signalling showed a decreased survival fraction, and the bystander response typically saturates at low doses (<1 Gy).[171] The nature of the bystander signals is not yet fully understood, and communication through gap junctions and through soluble factors in medium have been shown. Pathways and mechanisms involved in bystander responses include cytokines, MAPK- and NF-κB-signalling, and involvement of reactive oxygen species.[76,176–178]

The other direction – Rescue Effects

Opposite to bystander effects, it has been shown that signals going the other direction, from non-irradiated to irradiated cells, can decrease the toxic effect of radiation and induce radioresistance; a rescue effect. Chen *et al.* first described rescue effects in 2011 by using the 53BP1 DSB-marker and found that irradiated cells that could communicate with non-irradiated showed significantly less DSB after 24 h.[179] Similar protective effects have been shown to be cell line specific[180] and inducible via autocrine signalling.[181] Pathways and mechanisms involved in eliciting rescue effects resemble the ones for bystander responses, and include the cytokine IL-6[182], NF-κB-activation[183], ATF-2[181] and nitric oxide[184].

[I] Sister chromatid exchanges are regarded as typical radiation induced damages.

Clinical relevance?

Bystander and rescue effects have mostly been studied *in vitro*.[185] Given their potential impact, bystander effects have by some authors been termed the 6th R in radiobiology (*r*emote effects).[186] Their relevance in the clinical setting is unknown. Concerns of bystander-induced toxicities to normal tissue have arisen, especially in the setting of highly conformal radiotherapy with sharp dose-gradients, but also to contribute to anti-tumour effects in the use of GRID[J] therapy.[187] In a theoretical dose-planning study, extrapolations from pre-clinical results to clinical scenarios suggested a considerable contribution of signalling mediated responses.[188] Some authors also suggest signalling mediated effects within the irradiated volume, affecting the radiation response.[170,189] Thereby, enhancement of bystander effects within a tumour, or inhibition of rescue effects, would be attractive to overcome radioresistance.[76,187,190]

Clonogenic assay and signalling mediated responses – open questions

A common approach to study bystander effects *in vitro* is the use of modulated beam irradiation, where part of the culture flask is shielded from the irradiation.[175,191–193] Thereby, cells in the irradiated and non-irradiated area can be analysed simultaneously. A typical result after modulated beam irradiation is illustrated in Fig 6. The non-irradiated cells, when communicating with irradiated cells, have a reduced survival fraction (a bystander response). Interestingly, in the same experiment, irradiated cells in the partly shielded flask, have an increased survival compared with whole-flask irradiation. This latter phenomenon is usually described as a lack of bystander signalling.[194,195]

Since Puck and Marcus developed the clonogenic assay in the 50's, there has been questioned raised concerning the independent radiation response of the single irradiated cell. Especially, the number of irradiated cells have been shown to both induce radioresistance as well as increasing the radiosensitivity.[196–200]

In the current thesis we studied possible contributions of bystander and rescue signalling affecting the radiation response in clonogenic assays.

[J] GRID or spatially fractionated radiotherapy is a technique where the irradiation field is not homogenous, but instead consists of several peak-and-valley regions.

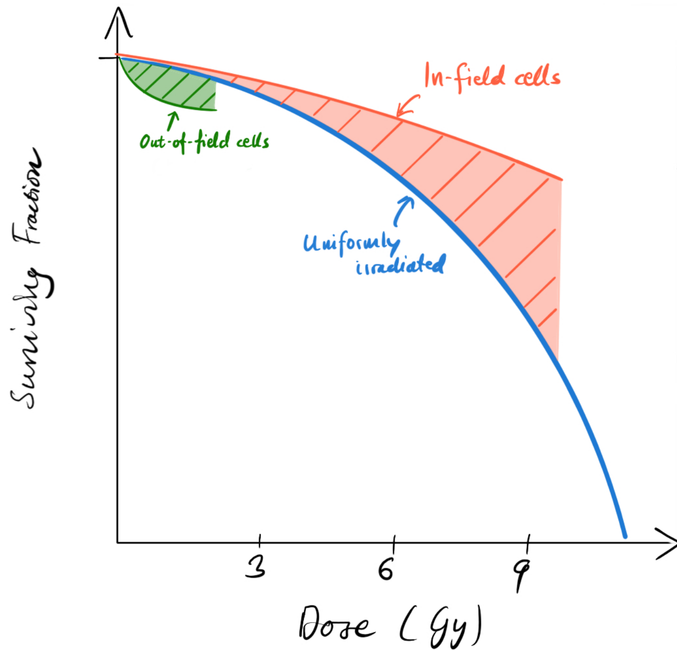

Figure 6 Cellular communications in clonogenic assays
Comparison after uniform irradiation where all cells are irradiated (blue line) with modulated beam irradiation where half of the flask is shielded from the irradiation (out-of-field cells, green line) and half the flask is irradiated (in-field cells, orange line). Although the out-of-field cells receive a very low dose, their survival decreases much more than predicted by the actual dose (differences illustrated by the striped green area), an effect that has been attributed as a bystander response. At the same time, the irradiated cells in-field survive to a much higher degree than predicted (orange striped area). In previous publications this has been assumed to be a lack of bystander signalling. In study V, we investigated if that response could instead be due to a rescue effect. The figure is an illustration inspired by previous publications. © Gabriel Adrian.

Aims

The current thesis aims at investigating radioresistance and strategies to overcome radioresistance in a clinical setting, as well as recent pre-clinical developments where radiation responses are altered.

Specific aims in the clinical part were to investigate:

- Tumour volume and radioresistance in patients with oropharyngeal tumours treated with radiotherapy (study I & II),
- How fractionation can be used to overcome radioresistance due to large tumour volumes (study I & II),
- Patient and tumour specific characteristics affecting radiation response (study II).

Specific aims in the pre-clinical part were to study

- The role of oxygen for a FLASH induced radioresistance *in vitro* (study III),
- FLASH induced radioresistance under normoxic conditions *in vitro* (study IV),
- Radioresistance due to cellular communications in clonogenic assays (study V).

Material & Methods

Clinical studies

Patients and cohorts

The clinical studies are based on results from previously reported clinical trials.[73,201,202] For the present studies, the subgroup of patients with oropharyngeal tumours in the trials was chosen since it constitutes a distinct tumour entity allowing tumour volumes to be compared. The randomized ARTSCAN-trial investigated the role of altered fractionated radiotherapy for HNSCC patients and randomized between 1.1 Gy + 2.0 Gy per day, total dose 68.0 Gy, treatment time 4.5 weeks (AF), and the control arm with 2.0 Gy per day, total dose 68.0 Gy, treatment time 7 weeks (CF).[201] For study II the cohort was expanded to include the prospective "PET-study" which studied the role of PET for evaluating the neck response after radiotherapy for HNSCC, [202] as well as the randomized ARTSCAN III-trial comparing concomitant cetuximab to cisplatin, in addition to radiotherapy for HNSCC.[73] The pooled cohort contained 654 oropharyngeal cancer patients who completed radiotherapy, were eligible for analyses of primary end-point and had available CT-scans. To our knowledge, it thereby constitutes the largest available cohort of patients with oropharyngeal cancers treated with radiotherapy. In addition, the original data was collected prospectively, in comparison to most other studies concerning tumour volume with retrospective data.

End-points

In study I the primary end-point was local control (LC), hence the ability of the treatment to completely sterilize the primary tumour. In study II, we broadened the end-points to include PFS and OS. Although PFS is an end-point often used for palliative treatments with existing tumour burden, it is an advocated end-point for head & neck cancer also in the curative setting.[203] The definition of a PFS-event was any recurrence (local, regional or distant) or death by any cause. The most definite, and possibly for patients most relevant, end-point is OS.

Tumour volumes and other characteristics

Individual CT-scans for all patients were retrieved and manually reviewed to ascertain that the contoured tumour volume (primary gross tumour volume, GTV-T) was accurately defined. The size of the GTV-T as defined by the treating physician was used as the tumour volume. As described in study I, the primary and nodal tumour volumes were not separated for 36 patients, and a separation was then performed, keeping the total volume intact.

The importance of HPV/p16 status was not well established as the ARTSCAN-trial started, and was added as an additional analysis when the mature results were presented.[201] For the PET-study and ARTSCAN III, p16-status was part of initial tumour characterization. Smoking status was recorded in all trials, although the definition varied slightly. Haemoglobin levels and performance status were part of patients' descriptions, and the Karnofsky-scale was used in ARTSCAN, whereas WHO performance status was used in the PET-study and ARTSCAN III.

In vitro-studies

Cell lines

In total, seven human cancer cell lines and one normal fibroblast cell line have been used in the pre-clinical studies (table 3). Seven cell lines were acquired from suppliers, and one of them, LU-HNSCC 4, was previously established in the lab.[204]

Table 3. Cell lines
Index of cell lines used in study I-III.

Name	Origin	Study
MM576	Melanoma	V
DU145	Prostate cancer, adenocarcinoma	III, V
HeLa	Cervix cancer, adenocarcinoma	IV
WiDr	Colon cancer, adenocarcinoma	IV
MDA-MB-231	Breast cancer, adenocarcinoma	IV
MCF7	Breast cancer, adenocarcinoma	IV
LU-HNSCC4	Floor of mouth, Squamous cell carcinoma	IV
MRC5	Normal Lung fibroblast	IV

Irradiation

Irradiations were performed using external beam irradiation. For the FLASH-studies (study III & IV), we used a modified Electra Precise linear accelerator (Elekta AB, Stockholm, Sweden), with an 10 MeV electron beam.[205] The dose rate could be shifted from 14 Gy/min (conventional dose rate) to >600 Gy/s (FLASH). In study V, a Gulmay X-ray device (Gulmay Medical D3225 unit, Byfleet, UK) delivering a 120 kV X-ray beam with a dose rate of 1.44 Gy/min was used.

Clonogenic assay

Most of our *in vitro*-results are based on the golden standard in radiobiology, the clonogenic assay. We have solely used the *pre-plating* method, i.e. counting and plating the cells in appropriate concentrations before irradiation.[17] The dilution steps are crucial, and much care was taken to assure the correct number of cells to be plated. Following irradiation, cells were incubated for 10-14 days to allow the surviving cells to grow and form colonies of at least 50 cells. Then, cells were fixated and stained with methylene-blue in ethanol. To objectively assess the number of colonies, a standardized evaluation procedure was developed. The flasks were scanned in high resolution (1,200 dpi) and the images were analysed in a self-developed code in ImageJ (NIH, Bethesda, MD).

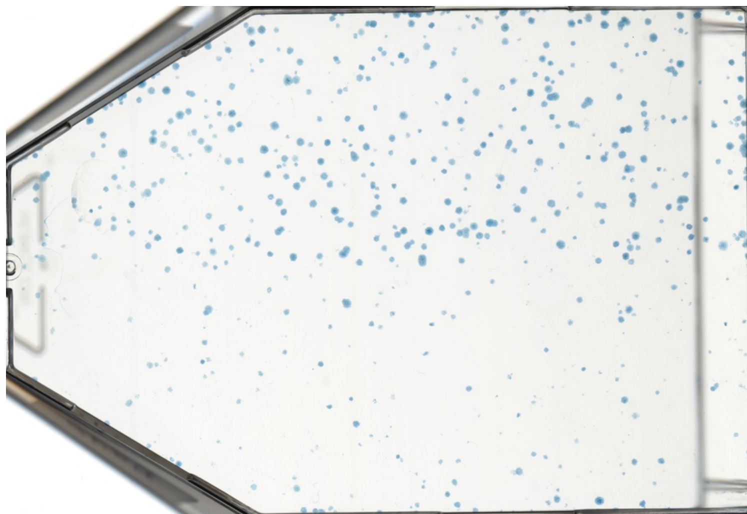

Figure 7 Modulated beam irradiations to investigate cellular communications
The clonogenic assay used with modulated beam irradiation. Representative image of a T75-flask where 1,000 melanoma cells (MM576) were seeded, and half the flask (lower part) was irradiated with 5 Gy. © Gabriel Adrian.

Modulated field

To investigate signalling mediated effects between irradiated and non-irradiated cells, we used the modulated beam approach (Fig 7). Here, part of the cell culture flask was shielded with lead during the irradiation. This assay was adopted from the Prise & Butterworth lab in Belfast, Northern Ireland.[192] After irradiation, the flasks were treated as described above.

Conditioned medium

To further investigate the role of signals secreted from cells, we used the conditioned medium approach.[181] A large number of cells were allowed to grow for 48 h, and then the growth medium was extracted, filtered through a 0.22-µm filter and centrifuged (188 × *g*) for 5 minutes to assure that no cells remained in the medium. The conditioned medium was then added to flasks with irradiated cells as described above.

Hypoxia treatment

To investigate the effect of oxygen concentration (study III), we used a hypoxia chamber (InVivo2 Hypoxia Work Station 400, Baker Ruskinn Technology Ltd, Bridgend, UK). The partial oxygen pressure (pO2) was set to resemble varying levels of hypoxia and physoxia, ranging from 1.6 to 8.8 % oxygen, compared with normoxia (20%). Cells were kept in hypoxia for 1 h prior to irradiation. The hypoxia treatment was kept as short as possible, to minimize biological adaptation to hypoxia, but still allowing the oxygen diffusion in the cell media to equilibrate. After irradiation, the cells were kept in normoxic conditions and treated as above.

Flow-cytometry

Cell cycle distributions after irradiation (study IV) were assessed with flow cytometry. Principally, a flow cytometer analyses suspended single cells as they move in a laminar flow through a laser beam. In the case of cell cycle analysis, cells were labelled with propidium iodide (PI), a fluorescent intercalating agent that stains the DNA in the cell nuclei. As the laser beam hits the PI-stained nuclei, the fluorescent PI will emit light proportionally to the amount of PI, hence the amount of DNA. Optical detectors capture the emitted light. The collected data is then analysed using dedicated software, such as ModFit LT 5.0 (BD Biosciences). After proper gating to exclude debris, the DNA-content of the analysed cell can be visualised in a histogram (Fig 8).

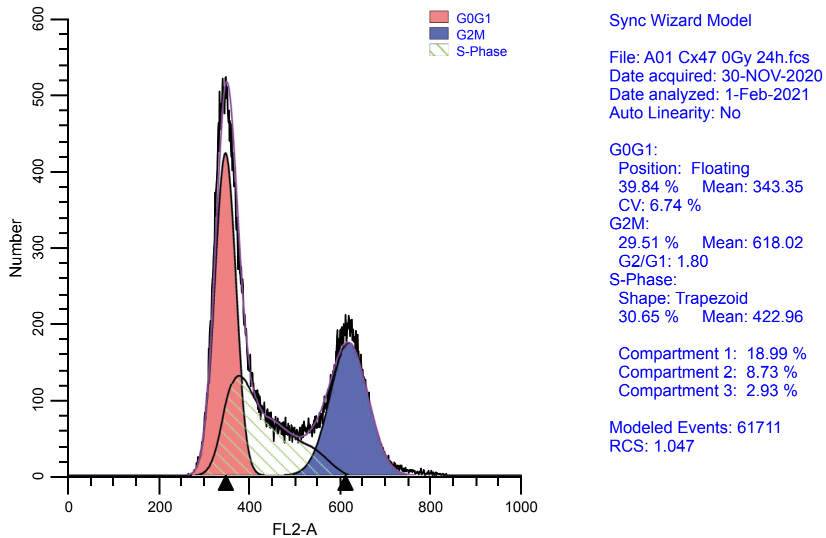

Figure 8 Cell cycle analysis using flow cytometry
Example of DNA-content histogram. In this case, controls of the LU-HNSCC4 – cell line at 24 hours after sham irradiation. 61,711 single cells were recorded. G1-cells are found to the left (red peak), S-phase (green dashed) was divided into three different compartments, and G2/M-phase to the right (blue peak). Estimated distributions per cell cycle phase are shown in the right panel. The goodness of the fit (purple line) to the data (black line) is measured by the reduced chi-squared (RCS)-value. © Gabriel Adrian.

Confocal microscopy and DSB detection

DSB-foci were identified with a 53BP1-marker detected with confocal microscopy. In brief, the procedure was as follows: at specific time points after irradiation, cells were fixated with paraformaldehyde and permeabilised with Triton-X. Following blocking steps, the primary antibody (53BP1) was added, and after washing steps, the fluorescent secondary antibody was added, followed by DAPI to stain the nuclei. The fluorescent signals were detected using confocal microscopy, which enables high-resolution images in thin planes (z-axis). To further enhance the images, the z-stack projections were deconvoluted before merging (Fig 9). The final images were analysed in Image J using a self-developed code to automatize the procedure.

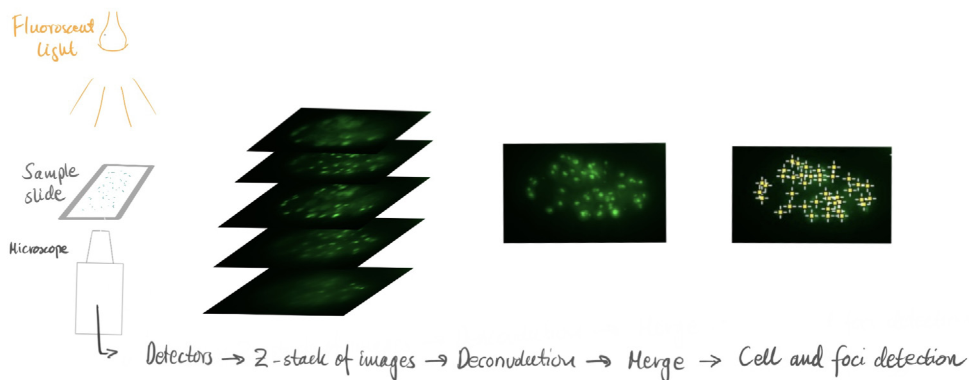

Figure 9 Identification of DNA double strand breaks
Sample slides stained with the DNA-double strand break-marker 53BP1 were placed in the microscope (to the left) and Z-stack images were obtained (second image from the left, here illustrated with five z-projections of a single cell). To enhance resolution a deconvolution step was ran before merging (second from the right). The final images were then analysed in ImageJ to identify cells and DSB-foci (to the right, each cross represents a double strand break-foci). © Gabriel Adrian.

Statistical Methods

The Kaplan-Meier method was used to illustrate time-to-event, with log-rank test for comparisons between groups (study I & II). Uni- and multivariable cox-regression models were used to provide effect sizes, and the proportional hazard assumption tested with Schönfeld residuals test (study I & II). Logistic regression was used to estimate dose-response curves (study I). Cumulative incidence was used to analyse local failure rates, with regional-, distant recurrences and death as competing risks (study II). Group comparisons and regression analyses in the presence of competing risks were investigated with the Gray's test and the Fine-Gray model, respectively (study II). Interactions were investigated with the likelihood ratio test (study I & II). Median follow-up time was determined with the inverse Kaplan-Meier method (study II). The student T-test was used for comparisons of normally distributed data between two groups, and one-way ANOVA for multiple groups comparisons (study I, II & V). Nonparametric data was compared with Wilcoxon rank sum test (two group comparisons) and with Kruskal-Wallis test for multiple groups (study I, II, III & IV). Receiver operator characteristics (ROC) analyses were used to define cut-off values and to investigate area under the curve for predictors (study II). Bootstrapping was used to estimate statistical dispersion when appropriate (study IV & V). The F-test was used to investigate if two different sets of parameters were superior compared with a common fit of the survival curves (study IV).

Methodological Considerations

Clinical studies

The clinical studies in the thesis were all *post hoc* analyses of clinical trials. Results and conclusions based on post hoc analyses are less reliable than from prospective trials where analyses are pre-defined. The drawback of post hoc analysis lies within the fact they are specified *after* data was seen. Thereby, problems with multiple testing and risks of finding significances by chance increase. As such, post hoc analyses should be regarded as hypothesis generating, and validation should be confirmed in other cohorts, or ideally in new, prospective trials.

In study II we included three different cohorts and performed pooled analyses. It was still a post hoc analysis, but regarding the relatively large number of patients (654 patients), the credibility of the results was strengthened.

In comparisons to retrospective studies, the current analyses relied on prospective controlled trials, with continuous data monitoring as the trials were conducted, which maximized the reliability of the data integrity.

In the multivariable cox-regression analyses, 205 out of the 654 patients were excluded due to missing data. This was mostly due to a lack of p16-information or Hb in the ARTSCAN cohort (189 out of the 205 patients with missing data were in the ARTSCAN cohort). Within the ARTSCAN-cohort, there were no differences comparing patients with or without missing data. When stratifying for p16-status, no significant difference in outcome between the cohorts was seen (Suppl. Fig in study II).

The size of the GTV-T-volume as defined by the treating physician was used as the primary tumour volume in the analyses. Imaging material was usually non-contrast enhanced computed tomography (CT)-images, and for some ARTSCAN III patients a complementary FDG-PET was available. In addition to the imaging material, the delineation by the treating physician could be influenced by physical examination as well as the patient's record with detailed tumour descriptions. Such information could not be retrieved retrospectively for the current study, and we therefore decided not to re-delineate the GTV-T volumes. Since modern treatment of head & neck cancers often involves PET/CT-images and/or MRI-images, the absolute volumes as delineated by non-enhanced CT, should be handled cautiously. However, the relative impact of increasing tumour volume should be valid. The findings were only based on the subgroup of oropharyngeal tumours, and the validity of the findings for other HNSCC sub-sites must be studied separately.

In vitro studies

One could argue that the translational usefulness of studies on irradiated cells *in vitro* is limited. This was elegantly articulated by F.G. Spear in the early days of radiobiology[19]:

> "An isolated cell *in vitro* does not necessarily behave as it would have done if left *in vivo* in normal association with cells of other types. Its reactions to various stimuli, including radiations, however interesting and important in themselves, may indeed be no more typical of its behaviour in the parent tissue than Robinson Crusoe on his desert island was representative of social life in York in the mid-seventeenth century".

Despite the fears, pre-clinical radiobiology, including *in vitro*-models with single cells, has extended and deepened the understanding of radiation responses. The prevailing LQ-model to describe fractionation effects, has its roots in *in vitro* survival curves, and is now used in every-day clinical radiotherapy. As new treatment modalities are developed, as in the case of FLASH-radiotherapy, it is of crucial importance – not the least from an ethical perspective – to perform accurate and detailed *in vitro*-studied, in order to optimize its transition to *in vivo*-models and, in the end, patients. The recent failure of cetuximab in the treatment of head & neck cancer, exemplifies that initially promising *in vitro*-results might translate into clinical flops.[73,80,81] Lessons learned from cetuximab include the need of a variety of pre-clinical models, and to publish negative results to avoid a selection-publication bias.[206]

Strengths of the current *in vitro*-studies were that they rely on the clonogenic assay, the golden standard in radiobiology. As presented in the following chapters, the assay inherits challenges. Nevertheless, it forms the ground on which many radiobiological discoveries have been made. Our current clonogenic assays were evaluated using a standardized method, aiming at increasing reproducibility.

For the clonogenic assays in study III-IV we were limited to small culture flasks (T12.5) due to the size of the FLASH beam. Larger flasks would have allowed seeding more cells and reaching even lower SF-numbers. Another limitation in study III was that the findings are only based on one cell line (DU145). In study IV, a total of six cell lines were investigated, however only one of them was non-cancerous (MRC-5). Additional studies with even more cell lines to validate the findings could be considered.

The use of flow cytometry also offers challenges; preparation of cells must be accurate, cells should not attach in the suspension, the cells should flow as laminar as possible. The analyses must be performed methodically, for instance, a small change in the ratio of the G2-peak and G1-peak can have major impact on the

results. To avoid such interference, the G2/G1-ratio was firstly defined using controls cells, and then kept constant throughout the analyses.

High-resolution imaging has opened new ways of studying radiation responses and enables characterisation of individual DSB-foci. For the current analyses, we examined the γH2AX and the 53BP1-markers, and found the latter one to be more reproducible between repetitions and across cell lines. As it comes to image analyses, different settings, such as thresholds for colour level, brightness, and object identification, affect the analyses. To minimize such parameters, a standardized evaluation method using the raw image data was used in the self-developed Image J-code.

Statistical Considerations

For a given data set, there might sometimes be several statistical tests or ways of presenting the data. The use of local control with the Kaplan-Meier approach or cumulative incidence of local failure with competing risk assessment is one such example. Study I was analysed according to the former, and study II to the latter, with regional or distant failure or death as competing risks. Local failure is thus different from 1-Kaplan-Meier$_{\text{Local Control}}$. The inclusion of competing risks enables patients that experience a different failure (regional, distant or death) to be excluded from the analyses and do no longer contribute to the curve. This can be useful, since a patient that is dead will never experience a later local failure.[207] In the Kaplan-Meier approach with local control, patients are censored at the time of other failures/death, and the sizes of sequential "steps" on the curve are 1/(number of remaining patients). Depending on the incidence of competing risks, the two methods may provide different estimates. Generally, Kaplan-Meier-based estimates tend to over-estimate effects when competing risks are present. For the cohort in study II, local control at 5 years was 86.6%, and the cumulative incidence of local failure was 12.5%, illustrating the (small) difference between the two methods for the current data set. Comparisons between groups when competing risks are present should preferably be investigated with the Gray's test instead of the log-rank test. Depending on the incidence of the competing risks, the test may provide different results.[207] Similarly, regression analysis when competing risks are present should be considered for analysis with subdistributional hazard regression such as the Fine-Gray model, instead of Cox proportional hazard regression model.[207] However, the coefficients from a Fine-Gray model may be challenging to interpret, since they denote the rate of occurrence of an event for those who have not yet experienced *that* event.[208] It is advocated that the magnitude of the hazard ratios obtained using the Fine-Gray model should be interpreted cautiously.[208] As stated above, several statistical methods may be appropriate for a given data set, and in survival analyses, the two

methods may provide answers to slightly different questions. Reassuringly, in study II the results were similar for the two approaches.

Dichotomization of post hoc-retrieved data is debatable, especially if the cut-off for dichotomization is chosen *a posteriori*. There is a risk of “data-fishing” where a specific cut-off maximizes differences and artificially inflates an effect, rather than reflecting a true interaction. In study I, we first analysed the continuous data (in a Cox-regression model) and found a significant interaction, and then used dichotomization to visualize the findings. Thereby, findings mainly rely on continuous (non-dichotomized) analyses. Also, in study II, most of the analyses treat tumour volume as a continuous variable. The exploratory effect of intensified radiotherapy were conducted in the same manner as in study I; first we investigated a possible interaction using the continuous tumour volume, and then dichotomized the tumour volume to visualize the results in a Kaplan-Meier plot. The reason for choosing the specific cut-off-value was illustrated in a supplementary figure.

Comparisons of SF-data in the *in vitro*-studies have generally used the Wilcoxon rank-sum test (also known as Mann-Whitney U-test), which doesn’t require the data to be normally distributed. In study IV we also used the F-test to allow comparisons of the whole SF-curve, instead of comparing discrete dose-levels. In this case, the extra sum of squares F-test compared if separate fitting to the FLASH and conventionally irradiated data points was superior to fitting all data to one curve. Bootstrapping was used to estimate statistical dispersion (study IV and V). Bootstrapping is a way of resampling the material by randomly “picking” data from the whole data set, and then performing the desired calculations in the picked subset of data-points. This can be repeated for instance 10,000 times, and the results of all calculations can then be used to estimate a statistical dispersion. The method thus provides an estimate based on the original data, and depending on the variance in the data, a confidence or interquartile range can be determined. However, the estimate still only reflects the variance in the original data.

Statistical calculations in study I-IV were made in script-based RStudio (RStudio Team (2015). RStudio, Inc., Boston, MA, URL http:// www.rstudio.com). The use of scripts is advantageous since it allows the analysis steps to be saved and can easily be reproduced.

Ethical considerations

The clinical data used in study I & II derive from three trials, conducted with proper approval from ethical committees.[73,201,202] In the current studies we re-analysed the anonymous original data, in a manner somewhat similar to a meta-analyses with individual patient data. As such, new ethical approvals were not required. However, one could still argue that the ethical questions may arise when handling individual patient data. We did not perform any additional analyses, nor did we identify any new patient specific characteristics that could potentially intrude patient's integrity. The large size of the cohort, 654 patients, and the relatively common cancer type also diminish the risk of indirect identification of individual patients, which may otherwise be a challenge for rare diseases. The analyses and presentation of the patients' outcomes were anonymous.

When doing *in vitro*-experiments with established cell lines the ethical concerns are considerably less. However, one could discuss the ethical issues connected to the establishment of cell lines. This was illustrated by the sad history of Henrietta Lacks, whose cervix cancer became the immortalized HeLa-cell line.[209] The HeLa cells were used by Puck & Marcus[18], and in additionally 113,000 publication on PubMed[K]. Henrietta Lacks never gave her consent to growing her cells *in vitro*. Her name, medical record, and genome have been published. Her family has suffered.[210] The handling of Henrietta Lacks' cells have caused much discussion and raised the awareness of ethical concerns in cell studies, and stressed the importance of informed consent.[211]

[K] PubMed-search with the term ”HeLa” generated 113,375 hits on the 29th of March 2021.

Results & Discussion

Study I & II

Tumour volume and radioresistance in patients with oropharyngeal tumours treated with radiotherapy.

The analyses of tumour volume and outcome after radiotherapy in study I-II strongly propose that tumour volume is a major determinant for radioresistance in oropharyngeal HNSCC. In total, 654 patients with oropharyngeal tumours treated within prospective clinical trials were evaluated. In cox-regressions, tumour volume was significant for local failure (LF), PFS and OS, even within each separate T-classification. When adjusting for Hb-level, smoking-, performance-, and p16-status, the importance of tumour volume remained. The analyses constitute, to our knowledge, the largest cohort of oropharyngeal tumours treated with radiotherapy. The studies add substantial evidence to the previous inconsistent findings between tumour volume and outcome (Table 1). The studies underline the importance of stratifying HNSCC patients based on p16/HPV-status. As expected, p16-positive tumours were significantly more radiosensitive, and in addition, significantly smaller compared with p16-negative tumours.

Tumour volume seems to be a very robust prognostic factor. The addition of p16-status is essential, not the least considering the significant interaction between p16-status and tumour volume for LF, PFS and OS. The current results are in line with other recent findings for HNSCC patients treated with radiotherapy.[68,96] Noteworthy, tumour volume remained a predominant factor even when contemporary state-of-the-art techniques for prognostications were used. Linge *et al.* investigated the usefulness of hypoxia gene expression analyses, but tumour volume was more informative and remained the most influential factor in multivariate analyses.[96] An additional study also investigated the role of gene expression analyses to determine outcome after chemo-radiotherapy for 197 HPV-negative HNSCC patients.[58] Interestingly, CT-determined tumour volume was included as a parameter and was found to be the most important marker for OS, thereby outperforming the different gene expression profiles for hypoxia, stem cell richness, proliferation, immune infiltration and repair genes. For loco-regional

control, chronic hypoxia was the most important factor, and a positive correlation between chronic hypoxia and tumour volume was found.

How fractionation can be used to overcome radioresistance due to large tumour volumes

In study I, we showed that the negative impact of a large tumour volume was less pronounced for patients treated with the AF schedule compared with the CF-schedule. For the cohort as a whole, the AF schedule was not superior to CF. A significant interaction between treatment schedule and tumour volume for LC was found. Thereby, the efficacy of AF was not universal for all patients. Instead, AF was more efficient with increasing tumour volumes. Surprisingly, a tendency of worse outcome after AF compared with CF for small tumours was found. This result should be handled cautiously, as post hoc analyses with dichotomized data may inflate results.

In study II, additional patients from the ARTSCAN III trial provided further possibilities to investigate the role of fractionation to overcome radioresistance. The control arms in the trials were treated to the same radiation dose (68.0 Gy in 34 fractions), whereas the experimental arms differed, but both provided intensification of the radiotherapy. The pooled analyses suggest a role for intensified radiotherapy to overcome radioresistance in large tumours. As illustrated in a supplementary figure, the efficacy of intensified radiotherapy increased with increasing tumour volume. To our knowledge, this is the first time an interaction between tumour volume and intensified radiotherapy for HNSCC has been shown. Congruent to these results, similar findings have been made for lung cancer in two different cohorts.[212,213] The interaction suggests that radiobiological behaviour differs with tumour volume. This hypothesis is supported by the findings by Linge *et al.* where a significant interaction between hypoxic gene expression profiles and tumour volume was found.[96] Also, van der Heijden *et al.* found significant correlation between tumour volume and hypoxic gene expression profiling[58], and Liotta *et al.* found a correlation between tumour volume and stem cell frequency.[214] An interpretation could be that the underlying mechanism of radioresistance differs depending on tumour volume. For large tumours, the number of clonogenic cells, or radiobiological relevant factors such as hypoxia or stem cell frequency could be more predominant, causing radioresistance that can be mitigated by intensified radiotherapy. In large tumours, these factors contribute to the "bulk" of all radioresistant phenotypes. In smaller tumours, however, the radiotherapy usually does a good job of eliminating all clonogenic cells. However, when harbouring for instance specific gene expressions, it may reflect an underlying biology resulting in another type of

radioresistance. In large tumours, corresponding subtypes may also exist, but they cannot be "resolved" due to the generally more radioresistant phenotype.

The discrepancies between analyses based on T-classifications compared with tumour volume warrant some comments. Even though both classifications relate to the physical extension of a tumour, T-classifications are limited to four ordinal steps. Tumour volume is based on the radiographic appearance of tumours, and allows evaluation as a continuous variable. In our analyses, overlap between T-classification and tumour volume was seen, and the methods classify different subsets of patients.

Patients and tumours' specific characteristics affecting radiation response (study II)

A significant raise in the prevalence of HPV-associated oropharyngeal cancers was found comparing the sequentially recruited cohorts (during two decades, 1998-2018). Our results confirm the good prognosis for these patients, and the adjusted hazard ratio for OS (HR 0.3) is comparable to previous reports.[61,68,102] The study also confirms the independent impact of smoking status (LF and PFS) and Hb-level (PFS and OS), with a threshold of 130 g/L. The current analyses can, however, not assess if erythrocyte transfusions for patients with lower Hb-level affect the outcome. In univariable analyses performance status was significant for all end-points, congruent to previous results[66,215], however no significant effect remained in the multivariable model where tumour volume, p16-status, Hb-level, smoking status and age were included. Age was for understandable reasons related to OS, but not to LF in multi-variable regressions, providing additional knowledge to the treatment of elderly patients.[216]

Study III & IV

The role of oxygen for a FLASH-sparing radioresistance in vitro

In study III we developed an *in vitro*-model where we could vary the oxygen concentration to resemble physiologically relevant oxygen levels to study the effect of irradiation with electron beams with FLASH (>600 Gy/s) compared with conventional dose rate (14 Gy/min). For the prostate cancer cell line DU145 the survival fraction after FLASH was not significantly different compared with conventional dose rate in normoxia (20% oxygen). However, at 18 Gy in hypoxic conditions (1.6% oxygen), a significant sparing was seen for FLASH. We then further investigated different oxygen concentrations and could derive a relationship between oxygen concentration and FLASH induced sparing. The data was fit to the linear quadratic model (equation 3), where we assumed a dose rate dependence for the β-term. It should be noted that the model is based on the assumption of iso-effectiveness in normoxia and anoxia (0% oxygen). With our set-up, we were not able to study anoxia, and relied on previous reports.[136,137] Following our publication, Khan *et al.* showed congruent results using traditional clonogenic assays (normoxic) and 3D-spheroids (hypoxic core), and found no differences in normoxia, but a clear FLASH sparing in the 3D-spheroid.[160]

Study III suggests that oxygen concentration plays a role for FLASH to induce radioresistance. The results do, however, not explain any underlying mechanism, and cannot differentiate a mechanism based on oxygen depletion or radical-radical interactions for instance.

FLASH induced radioresistance under normoxic conditions in vitro

Although several data sets, including our own (study III), implied relationships between oxygen and FLASH effect[153,154,157,158], results were inconsistent[141,146,159] and required further investigations. We therefore studied a possible FLASH induced sparing under normoxic conditions for six human cell lines and found a general trend of increased survival fraction after FLASH compared with conventional dose rates. The survival curves were used to estimate a dose modifying factor of 1.1-1.3 for the different cell lines. The differences between cell lines suggest inherent biological susceptibility for FLASH, which was further supported by the findings of Chabi *et al.*[150] Three cell lines were investigated for radiation induced DSB and cell cycle arrests, but the results were comparable for FLASH and conventional dose rates. The lack of difference in cell cycle arrest contrast the findings by Auer *et al.,* who found a lower G2-fraction for HeLa cells at 10 h post proton FLASH irradiation compared with conventional dose rate proton irradiation, but no survival differences.[217] Differences in beam parameters and quality (electron vs. proton), or the method used to determine cell cycle

distributions may contribute to the discrepancies.[148] Considering the higher survival fraction after FLASH compared with conventional dose rate irradiation, one would have suspected less DSB for the FLASH irradiated samples, but we found no such differences. When Fouillade *et al.* did similar experiments, lung fibroblasts, but not lung cancer cells, showed less DSB after FLASH.[168] The discrepancies warrant further investigations, including addressing DSB-complexity and repair kinetics, and comparisons between 53BP1 and γH2AX to identify FLASH induced DSB.[10] Together, the results by us, Chabi *et al.* and Fouillade *et al.* suggest that the magnitude of the FLASH effect may be cell line specific.[150,168]

General discussion

The finding of a dose modifying factor of >1 for all six cell lines *in vitro* (study IV) raises concerns about FLASH being iso-effective to control cancer growth. One could discuss the requirements to state that such an iso-effect is present *in vivo*. In the published *in vivo*-experiments no significant differences in tumour growth after FLASH compared with conventional dose rates were seen, for the typically 4-15 irradiated mice per data point (Table 2). A lack of statistic difference does not necessarily imply that two treatments are equally effective. In clinical science, non-inferiority trials are conducted to determine if a new treatment is as effective as the standard regimen. In such trials, an acceptable worse outcome (where the acceptable difference is denoted Δ) is pre-specified, and the smaller Δ the larger the sample size.[218] A similar approach for *in vivo* FLASH-studies would be costly and require a large number of animals, which would not be in line with the intention of Replacement, Reduction, Refinement for *in vivo*-studies.[219] The most acknowledged *in vivo* model to compare radiotherapy treatments with curative potential is the local tumour control assay (TCD_{50}-assay).[220,221] In contrast to growth delay-assays, the TCD_{50}-assay reflects the sterilization of all clonogenic cells. The tumour growth delay assay captures the killing of cells in general, which does not necessarily correspond to sterilization of clonogenic cells.[6] In this context, it is noted that the available FLASH results for tumour effectiveness primarily evaluated tumour growth delay (Table 2). However, a translation of FLASH to the clinic does not require iso-effective tumour control. Instead, a differential tumour to normal tissue response (increased therapeutic window) is the most relevant parameter. This will allow radioresistant tumours to receive higher total doses, increasing cure rates, without increasing normal tissue toxicity.

The normal tissue tolerance to FLASH has been studied by several groups, and with few exceptions[146], a substantial decreased toxicity has been shown. Brain, gut, skin, lung, constitute separate tissues with different biology and micro-

environments. Similarly do tumours differ in origin, biological behaviours and have different micro-environments. It would therefore not be surprising if some tumours are spared by FLASH, and the differential tumour to normal tissue effect being less pronounced. Future work to understand the fundamental underlying mechanisms will hopefully generate predictive markers for when FLASH provides benefit. Despite these concerns, the available FLASH results are most encouraging, provide substantial evidence for normal tissue sparing, and motivate for translation to clinical trials.

Based on our results, some of the open questions mentioned earlier could be elaborated on. If the findings of a FLASH effect at low doses also correspond to a widening of the therapeutic window, it would further encourage fractionated FLASH therapy in the clinic, and have implications for the (disadvantageous) dose distribution. Compared with single dose irradiation using one beam, a more conformal dose distribution could be obtained by using several beam entries. Perhaps a hypo-fractionated regimen of 3 x 20 Gy, where the 20 Gy were to be delivered with 4-5 beam entries of 5-4 Gy each, separated in time would still provide a beneficial FLASH sparing?

As already mentioned, it has been hard – both in the past and in the revived interest in FLASH – to reach a decisive answer to the importance of oxygen in the FLASH effect. Like the inconclusive findings in the past, the results in the current thesis are also inconclusive. It could, however, be stated that FLASH effects *in vitro* are not solely dependent on low oxygen concentrations and high doses of radiation. One possibility could be that the FLASH effect relies on a yet undefined mechanism X. Mechanism X could then be affected by oxygen concentrations as well as be dependent on inherent biological features, varying from tumour to tumour, and to normal tissue.

Study V

Radioresistance due to cellular communications in clonogenic assays

In study V we showed that clonogenic assays comprise cellular communications affecting the radiation response. For the two studied cell lines, an increased survival fraction, hence radioresistance, was found as the number of irradiated cells increased. Radioresistance could also be mediated through transfer of conditioned medium. For irradiated cells communicating with non-irradiated cells after modulated beam irradiations, similar increases in survival fractions were seen. Together, the results suggest that cells in clonogenic assays do not behave as independent elements.

The increased survival of the in-field irradiated cells are in line with previous reports where modulated beam irradiation have been used.[175,192,193,222–225] Although these studies mainly have been focused on the bystander effects in the non-irradiated cells, they also reported the increased survival of the irradiated cells. Following our study, another group confirmed the protective nature of signals for the in-field cells, and reported interesting findings on repair dynamics and cell-cycle arrests.[226] The responses of irradiated cells in-field is clearly different from uniformly irradiated flasks.[191,225,226] Therefore, modulated beam irradiations may be a useful approach to investigate other aspects of the radiation response and how it is affected by cellular communications. The importance of cell densities in clonogenic assays was also later independently confirmed.[227]

The clonogenic assay is widely used in radiobiological research and its robustness has been addressed numerous times. The cut-off value to define survival (50 cells), the importance of slow-growing colonies, and the number of irradiated cells, are some of the concerns that have been addressed since the assay was introduced in the 50's.[16,20–22,196–200,228,229] [L]

The cell density relationship found in study V affects the shape of the survival curve (Fig 10). Considering the range of survival curves that can be obtained from the 3D-surface in Fig 10, one could speculate if there is a need to standardize the assay. At a poster at the European Radiation Research Society's Annual Meeting in 2017, we proposed such a method and standardized the outcome to a "50-colony-clonogenic assay":

[L] As a curiosity it could be mentioned that several of the authors who studied the clonogenic assay in the 60'-70's were also involved in ultra-high dose rate studies, illustrating cyclic events in radiobiological research.

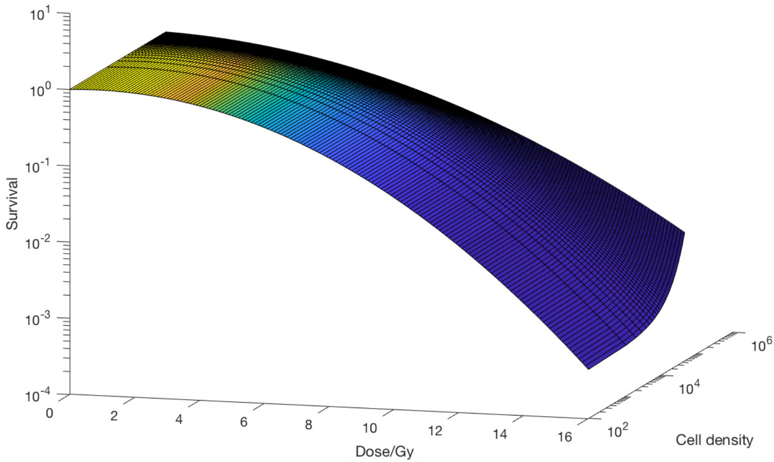

Figure 10 The survival curve and cell density
Cellular communications affect the outcome after irradiation in clonogenic assay. The obtained survival fraction (y-axis) after a given dose (x-axis) is dependent on the cell density (z-axis). If single survival curves were to be drawn from the 3D-surface, several different shapes with different α/β-ratios or D_0-values can be obtained. The illustration is based on the survival of DU145 in study V. © Gabriel Adrian.

Study V showed a linear relationship in the range studied for a given dose (Fig 5 in study V). For N = number of surviving colonies (arbitrary, for example 50 colonies), PE = Plating Efficiency, N_0 = number of seeded cells, SF = surviving fraction, k and m constants (from linear regression) for a given dose,

$$SF = k * N_0 + m \tag{4}$$

By definition,

$$SF = \frac{N}{PE*N_0} \tag{5}$$

Hence,

$$k * N_0 + m = \frac{N}{PE * N_0} \rightarrow N_0 = -\frac{m}{2k} \overset{+}{(-)} \sqrt{\left(\frac{m}{2k}\right)^2 + \frac{N}{PE*k}}$$

Thereby,

$$SF = \frac{N}{PE * \left(-\frac{m}{2k} + \sqrt{\left(\frac{m}{2k}\right)^2 + \frac{N}{PE*k}}\right)} \quad (6)$$

Using this method, Fig 10 can instead be presented as a single curve (Fig 11).

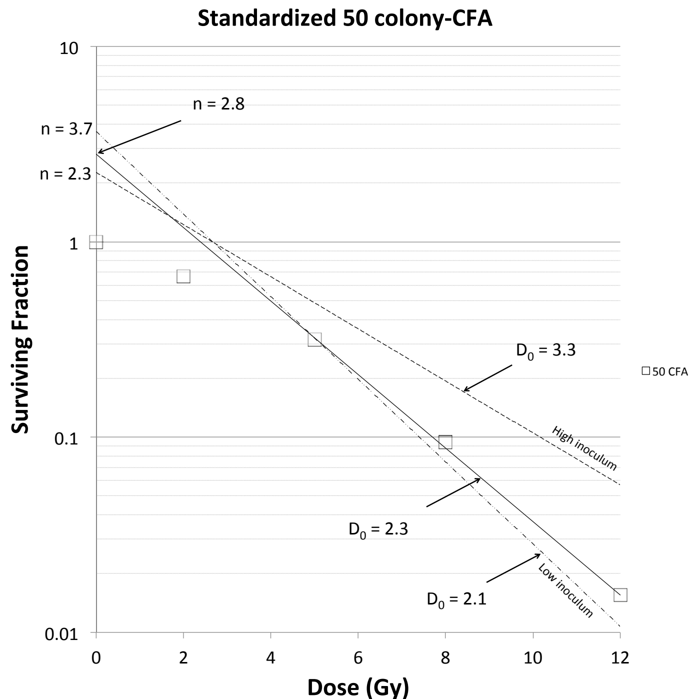

Figure 11 Adjusted survival curve
Using the information in the 3D-survival curve (Figure 10), the survival curve can be collapsed into a single curve (squares denote the calculated survival curve for a fixed survival of 50 cells per irradiation dose). Corresponding D_0 and n-values for low inoculum, high inoculum and the standardized 50-cells clonogenic assay are shown. © Gabriel Adrian.

Such an approach could be useful when the absolute D_0-value or α/β-ratio is important to compare between two treatments. For the clonogenic assays in study III-IV, it is the relative change between FLASH and conventional dose rate irradiations that was compared. In such situations, no corrections need to be made. However it is important to keep the growth medium volume and the number of irradiated cells at a given dose constant to allow a comparison.

Conclusions & Future Perspectives

1st Main Conclusion

Radiation responses can be altered, and there are opportunities to improve tumour cure rates using time (ultra-fast deliveries with FLASH allows dose-escalation), volume (exploitations of the bystander effects), or fractionation (intensified radiotherapy for patients with large tumour volumes).

2nd Main Conclusion

But, altered radiation responses should be handled cautiously, and there are risks of diminished tumour cure rates, using time (ultra-fast delivery with FLASH may spare tumour cells), volume (rescue effect may protect against the radiation), or fractionation (altered fractionation may impair outcome for some patients).

Study I & II

The studies demonstrate:

- A large tumour volume causes radioresistance in patients with oropharyngeal HNSCC.
- Tumour volume adds additional prognostication to T-classification.
- Individualized radiotherapy, where patients with a large tumour volume are prescribed intensified radiation treatment, may increase cure rates and overall survival.
- There is an interaction between tumour volume and intensified radiation treatment for cure rates and overall survival.

The current findings have some future implications:

Tumour volume should be included in prognostication of HNSCC patients.

The interaction between intensified radiotherapy and tumour volume warrants further investigation. A hypothesis is that radiobiological behaviour differs with tumour volume (Fig 12). Individualized treatment options for HNSCC patients based on tumour volume should be investigated. Intensified radiotherapy schedules could be offered for patients with large tumours. However, the current analyses do not support a role for intensified radiotherapy in patients with small tumours. Instead, additional markers, for instance identification of radioresistant small tumours with gene expression profiles, can select patients for other strategies to overcome radioresistance. The on-going phase III trials with the Debio 1143 agent and the DAHANCA 30 trial will provide interesting results for these patients. At the same time, the current analyses also recognize the excellent prognosis for small HPV/p16-positive tumours, where de-escalated treatment could be investigated.

Future work also includes further confirmations of the interaction between size of the primary tumour and altered fractionation. Even though tumour volume might be superior to T-classification, the latter still reflects the size of the primary tumour, and in older studies, tumour volume will not be available. A meta-analysis of response to altered fractionated radiotherapy in relationship to T-classification would further test the hypothesis that radiobiological behaviour differs with tumour volume. As different alterations in the fractionation schedules aim at overcoming different mechanisms of radioresistance, such a meta-analysis should be carefully designed. For instance, it is noted that well- and moderately differentiated tumours respond better to accelerated radiotherapy[63] and tend to be

smaller compared with poorly differentiated tumours.[230 M] On the other hand, one could argue that hypoxic tumours respond better to multiple daily fractions[231,232], and large tumours tend to be more hypoxic.[58] Thereby, different radiotherapy schedules might provide differential benefit depending on tumour volume. If this holds true, it would explain the lack of an interaction between tumour stage and altered fractionation in the large MARCH-metaanalysis.[70]

In a future patient trial, personalized radiotherapy schedules, with novel fractionation schedules aiming at overcoming radioresistance due to tumour volume, should be tested. To optimize the schedules, thorough TCP-models incorporating tumour volume and HPV/p16-status should be developed.

The importance of tumour volume for HNSCC sub-sites other than oropharynx should be investigated.

The mechanism underlying the proposed tumour volume hypothesis (Fig 12) is worth exploring.

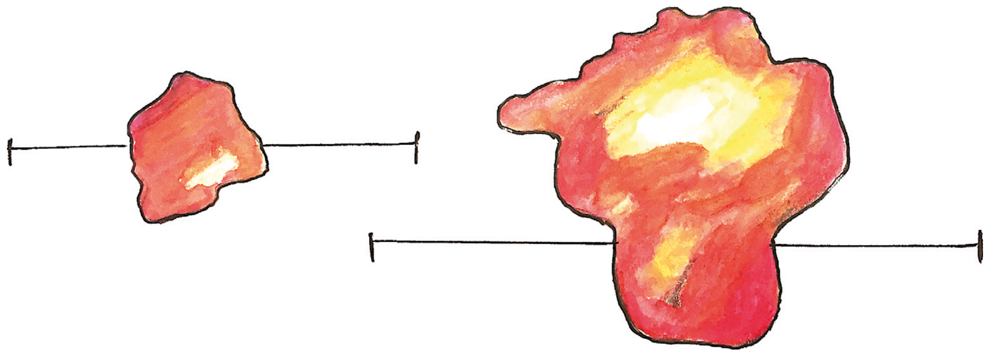

Figure 12 The tumour volume hypothesis
Future work will address the hypothesis that radiobiological behaviour differs with tumour volume. The colours illustrate a hypothesised difference in the proportion of radiobiological relevant factors, such as hypoxic cells or stem cell frequency. © Gabriel Adrian

[M] The reference concerns oral tumours. Unpublished data for oropharyngeal tumours in the PET-study suggests a similar trend, with median tumour volume of 11 cm^3 for well- to moderately differentiated tumours, compared with 19 cm^3 for poorly differentiated.

Study III & IV

The studies demonstrate:

- The FLASH effect depends on the oxygen concentration.
- The FLASH effect can also occur in normoxic conditions and is probably dependent on additional factors, other than oxygen.

For good reasons, there is a huge interest in FLASH within the radiotherapy society[N], and the number of reviews, commentaries, or modelling papers almost outnumbers publications with actual experimental data. The proposed widening of the therapeutic window is most encouraging, with astonishing clinical results in clinical cat patients.[143]

The findings in study III-IV provide some additional experimental data to the field. The results are phenomenological observations of FLASH and do not provide any mechanistic explanations. The finding of oxygen dependence is congruent to other results.[153,154,157,158,160] In study IV, a general FLASH sparing for six cell lines in normoxia was seen, with different magnitude in between cell lines. These results could indicate inherent biological sensitivity to FLASH, which is further supported by other recent results[150] and warrant confirmatory investigations. Together, study III & IV show that a FLASH effect can be found *in vitro*. The results suggest that the FLASH effect depend on, but is not restricted to, low oxygen levels, and inherent biological susceptibilities.

For the FLASH field as a whole, there are several interrelated questions that need to be clarified (Figure 13). A simple *in vitro*-model does not necessarily reflect the differential FLASH effect seen *in vivo*. On the other hand, *in vitro*-models will allow explorations of beam parameters, more detailed investigations of oxygen dependence and further studies in possible inherent biological differences affecting FLASH responses. It will then be indicated to bridge the gap to *in vivo*-models and study a cell line exhibiting a certain FLASH effect *in vitro* in mice models. Characterization of the therapeutic window requires *in vivo*-models and clinical trials.

A wish list to decipher the FLASH box in the coming years:

- TCD_{50}-experiments with single doses comparing FLASH to conventional dose rate irradiation, with simultaneous normal tissue complications assessment (addressing "therapeutic window"),

[N] In the meeting survey of the 2019 Annual Meeting by the American Society for Radiation Oncology (ASTRO) FLASH was the highest ranked discovery that "needs to be translated into the clinic".

- Fractionation studies, with relevant *in vitro*-models and TCD_{50} –assays *in vivo* (addressing “therapeutic window”),
- Experiments with a wide range of tumour types, to distinguish if there are predictive markers for FLASH susceptibility (addressing “inherent biology”),
- Detailed investigations in beam parameters and possible threshold effects, in large scale *in vitro* clonogenic assay experiment (addressing “beam parameters”),
- Further *in vitro* and *in vivo*-models with different oxygenation status and interference in redox-biology, with detailed analyses of DNA-damage response (addressing “oxygen” and “redox”)
- Carefully designed clinical trials for patients with indications for soft X-ray or electron beam irradiation to superficial targets.

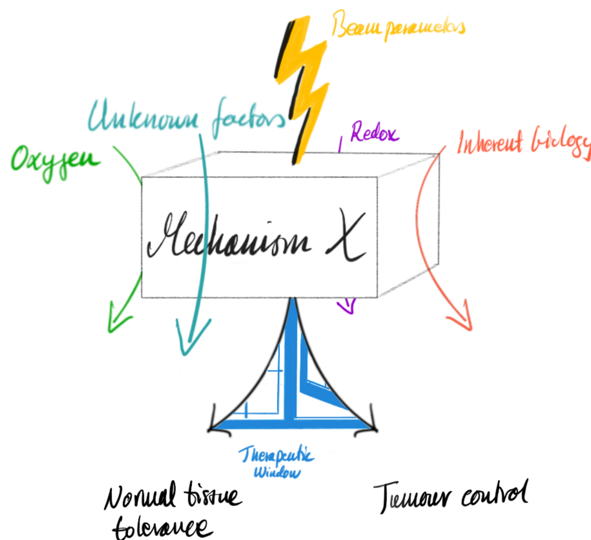

Figure 13 The FLASH box.
The underlying mechanism(s) for FLASH is (are) yet to be defined and is here simply denoted Mechanism X. Mechanism X is responsible for the differential response for FLASH compared with conventional dose rate. Based on the results in the present thesis, and work by many others, it seems as both oxygen (in green) and inherent biology (in red) affect Mechanism X. Currently unknown factors (in turquois) and redox biology (purple) may also affect Mechanism X. Beam parameters (in yellow) are crucial to evoke Mechanism X. Once Mechanism X is triggered, the most important differential effect is elicited; the widening of the therapeutic window (in blue), where normal tissue is spared more than tumours. Future experimental work is essential to increase the knowledge in all the interrelated areas surrounding the FLASH box. In several aspects, both *in vitro* and *in vivo* models will be useful, whereas the important therapeutic window will require tumour models allowing simultaneous evaluation of normal tissue tolerance and tumour control. The opening of the box will reveal the true name of Mechanism X. Is it radical-radical interactions? Oxygen depletion? Or a little bit of both and/or something else? © Gabriel Adrian

Study V

The study demonstrates:

- The response of irradiated cells *in vitro* depends on interactions between cells, both irradiated and non-irradiated.
- Cells may become radioresistant due to these interactions.
- The golden standard in radiobiology, the clonogenic assays, has limitations.

Bystander and rescue effects have broadened the understanding of radiation responses. It is now clear that the behaviour of irradiated cells is dependent on their surroundings, and the irradiated cells may in turn affect the surroundings. The results in study V, together with other publications, suggest that signalling mediated effects appear in clonogenic assays, the golden standard in radiobiology. Thereby, the clonogenic assay has inherent limitations that may affect the outcome. To allow direct comparison between two treatments, it is essential to keep the number of cells and the medium volume constant for a given dose.

These inherent features could also be exploited to investigate fundamental radiobiological phenomenon. Modulated beam irradiations have provided interesting results, not the least the increased in-field survival. Split-dose recovery, the DNA-repair dynamics and the effect on cell cycle arrests found in-filed, should be further investigated.[225,226] It seems as cellular communications alter fundamental radiobiological behaviours, and further characterizations could generate novel insights in cellular responses to radiation.

Populärvetenskaplig sammanfattning

Strålbehandling är en hörnsten för att behandla många typer av cancer och utgör den viktigaste behandlingsmodaliteten för patienter med cancer i oropharynx (mellersta delen av svalget innefattande halsmandlar och tungbasen). Ofta är strålbehandlingen framgångsrik och ger bot utan att orsaka alltför svåra biverkningar. Men i vissa fall är tumörer strålresistenta och behandlingen misslyckas.

Den här avhandlingen belyser aspekter av strålresistens och utforskar nya behandlingsstrategier mot strålresistens. Två kliniska delarbeten undersöker hur tumörvolym för patienter med orofarynxcancer påverkar behandlingsutfallet. I en patientkohort om totalt 654 patienter visar vi att tumörvolym är en dominerande prognostisk markör för utfallet efter strålbehandling, både för att uppnå lokal tumörkontroll och för överlevnad (studie II). Den negativa effekten av stor tumörvolym kan delvis motverkas genom att intensifiera strålbehandlingen, antingen med att ge två strålfraktioner om dagen eller att komma upp i en högre total stråldos (studie I & II). Studierna bekräftar också tidigare kända prognostiska markörer, däribland att det går bättre för patienter som har HPV/p16-associerad cancer, högt hemoglobinvärde eller är icke-rökare.

Avhandlingens andra del utgörs av pre-kliniska studier av nya upptäckter som kan användas för att motverka strålresistens. FLASH innebär att ge strålbehandlingen med ultrahög dosrat, där hela stråldosen levereras på bråkdelen av en sekund istället för under flera minuter. Denna nya teknik har visat på sparande effekter i normalvävnad utan att tappa i tumöreffekt. Därigenom skulle FLASH kunna användas för strålresistenta tumörer, genom att tillåta en högre total stråldos utan att orsaka mer skador i normalvävnad. Det har tidigare spekulerats kring att syrekoncentrationen är avgörande för den sparande FLASH-effekten. I studie III visar vi att FLASH-sparande effekter uppstår när prostatacancerceller är hypoxiska (låg syrekoncentration), och att det inte finns någon signifikant FLASH-effekt vid normal syrekoncentration. För att undersöka huruvida det kan uppstå en FLASH-effekt också vid normal syrekoncentration, studerade vi ytterligare sex cellinjer och kunde då påvisa en viss FLASH-sparande effekt (studie IV). FLASH-effekten kunde inte korreleras med induktion av dubbelsträngsbrott på DNA eller aktivering av cellcykelarrest.

De senaste två decennierna har upptäckten av bystander och rescue effekter belyst att strålrespons är ett samspel mellan bestrålade och icke-strålade celler. Vi undersökte om kommunikation mellan celler kan påverka strålresistens (studie V). Genom att variera antalet celler, överföra cellmedium eller bestråla endast hälften av cellerna, visar vi att strålresponsen för de bestrålade cellerna är beroende på kommunikation mellan celler. Ju fler celler i närheten av den bestrålade cellen, desto mer strålresistent blir cellen. Studien belyser att strålrespons är ett komplext system där den enskilda cellen är beroende av den biologiska miljö den vistas i. Att cellulär kommunikation påverkar strålresistens kan medföra interventions-möjligheter för att öka strålkänsligheten.

Sammantaget belyser resultaten i avhandlingen att strålrespons kan förändras. Strålbehandling är inte one-size-fits-all, istället finns utrymme att individualisera behandlingar. Närmast förestående är sannolikt att intensifiera strålbehandling för orofarynxcancerpatienter med stor tumörvolym. Framtida studier krävs för att undersöka vilka patienter som kan gagnas av FLASH. Bystander och rescue effekter är viktiga att beakta vid pre-kliniska försök, och deras inverkan i den kliniska vardagen är ännu okänd.

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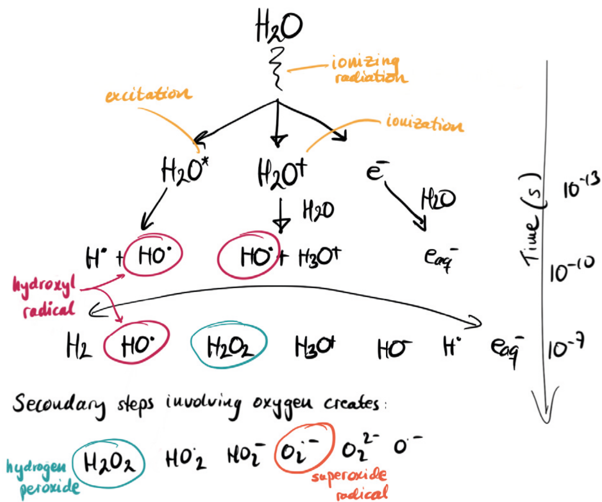
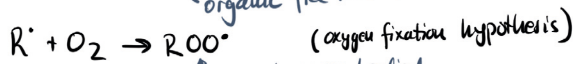
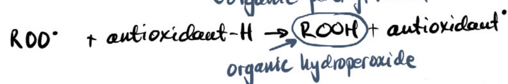
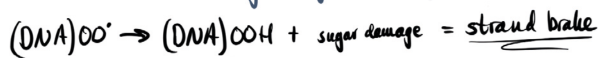
Supplementary

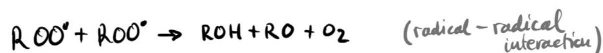


Reactions with organic molecules, RH (such as DNA)









Radiolysis of water, central reactive oxygen species and reactions with organic molecules
Schematic illustration of the initial radiochemical steps following irradiation of water, subsequent steps involving oxygen, followed by an example of reactions with organic molecules. Hydroxyl radical (pink), hydrogen peroxide (turqouis), superoxide radicals (orange), and organic hydroperoxide (blue) are high-lighted. For detailed descriptions, see ref [3,164,233,234]