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The Six Rs of Head and Neck Cancer Radiotherapy

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1. Introduction

Locally advanced head and neck cancers are usually aggressive tumours, due to the presence of hypoxia and the ability of the tumour to repopulate during treatment. The aggressive behaviour generally requires aggressive treatment, which for head and neck carcinomas consist of altered radiotherapy fractionation schedules combined with chemotherapy. Treatment fractionation, based on the 4 Rs of radiotherapy [1] is a well-accepted concept that has been re-adjusted for head and neck cancer decades ago to accommodate new radiobiological findings. The 4 Rs in terms of *repair, repopulation, reoxygenation, and redistribution* along the cell cycle have been promoted to 5 Rs with the aid of *radiosensitivity* and more recently to 6 Rs with the experimental evidence of *remote* (bystander) *cellular effects*.

The paragaphs below aim to describe the major aspects concerning the six Rs of radiotherapy applied to head and neck cancer.

2. The 5 Rs revisited

The 5 Rs of radiobiology represent a group of processes determining the response of cells and tissues to radiation, with great impact in fractionated irradiation. Balancing them one against the other has become one of the pillars of modern radiation therapy to maximise the therapeutic gain by either increasing radiation damage in the tumour cells or decreasing the damage to the normal tissues. The impact of the individual Rs varies across the different tissues, but for head and neck tumours all of them play important roles that have to be taken into account when designing successful radiotherapy schedules.

2.1. Repair

Repair is a term covering a number of processes responsible for the identification and correction of the damage to the DNA molecule induced by endogenous and exogenous factors. Sometimes the term "recovery" is used instead of "repair" because other processes are involved besides the actual repair of damage. The importance of recovery or repair in relation to irradiation has been determined in a series of experiments employing continuous irradiation at various dose-rates or split-dose experiments studying the variation of the cell survival with irradiation time or the time between individual fractions of radiation. Following these types of experiments radiation damage has been divided into two categories, non-repairable or lethal damage and repairable or sublethal damage. It should be remarked that these are operational terms that have not been correlated with any type of radiation-induced damage, with the possible exception of the irreparable clusters of damage. Sublethal damage could be removed as part of the cell recovery, unless fixated by interaction with other sublethal lesions or by being forced to be expressed by the cells. Consequently, cell survival following irradiation depends not only on the creation of irreparable lesions, but also on the competition between repair and fixation of sublethal damage that is influenced by many factors including the rate of generating new lesions and the cellular environment.

When describing the effect of radiation with the help of the well-known cell survival curves, the repairable component of damage is considered responsible for the shoulder of the curve as the accumulation of damage in the absence of the repair increases cell kill. Consequently the recovery capacity of cells could be quantified by relating the "bendiness" of the cell survival curve to its initial slope that is a measure of the irreparable or lethal damage induced by radiation. For the linear-quadratic (LQ) model [2,3] this is given by the beta/alpha ratio, which is low for acutely reacting normal tissues and most tumours including head and neck tumours and high for late reacting tissues [4]. This indicates the high potential for recovery of late reacting tissue that should be exploited in head and neck radiotherapy to limit the late complication rates.

Several models have been proposed to account for the process of recovery, including the incomplete repair model [5], the lethal-potentially-lethal model [6] or the repairable-conditionally repairable damage model [7], with the incomplete repair model, based itself on the LQ formalism, being mostly known and employed in clinical practice. According to the incomplete repair model [5], the effect of continuous irradiation delivering radiation dose D in time t is given by equation 1.

$$E = \alpha \dot{d} t + g(t)\beta(\dot{d} t)^2 \tag{1}$$

where α and β are parameters of the LQ model and g is a function describing the repair taking place in time t. If the repair is described as an exponential process, with repair constant μ, the g term is described by the expression in equation 2.

$$g(\mu t) = 2\frac{\left[\mu t - 1 + exp(-\mu t)\right]}{(\mu t)^2} \tag{2}$$

Similar expressions could be derived for the case of fractionated irradiation, when the focus is on the repair taking place between the individual fractions. Thus, the effect in fractionated radiation with time *t* between fractions is given by the expression in equation 3.

$$E = n[\alpha d + h(t)\beta d^2]$$
(3)

where *n* is the number of fractions, *d* is the dose per fraction and the repair term *h(t)* is given by the expression in equation 4.

$$h(t) = \frac{2}{n} \frac{exp(-\mu t)}{1 - exp(-\mu t)} \left[n - \frac{1 - exp(-n\mu t)}{1 - exp(-\mu t)} \right]$$
(4)

These expressions could be used together with the Biological Effective Dose (BED) formalism [8,9] to calculate the effectiveness of various treatment approaches. Thus, the expressions in equations 1 and 2 are mostly suited for brachytherapy, while expressions in equations 3 and 4 are suited for fractionated therapy when intra-fraction repair is considered negligible. Expressions have also been derived to account simultaneously for intra and inter-fraction repair [10].

A number of studies have determined relevant repair constants from clinical and experimental data, yielding values between 0.5 and 2 h for the repair half-life, depending on the tissue and the type of experiment used to derive them [11]. More recent studies indicated that the rate of repair might depend on the dose or that second order or bi- or even multi-exponential processes might exist [12-15]. The multi-exponential processes appear to be equally divided between the fast and the slow components with median repair half-lives of 0.3 h for the fast component and about 4 h for the slow component (for a summary see [16]). In this context it has been pointed out that identification of the relevant rates might depend on the design of the experiment as it has been suggested that split-dose experiments could easily miss a fast component of repair [16].

Repair or recovery of radiation damage has been extensively exploited to spare late reacting normal tissues in head and neck radiation therapy. Thus, the low alpha/beta ratio (or high beta/alpha ratio) of these tissues in comparison to the tumours indicates their high capacity for repair in fractionated regimes if enough time is allowed between individual fractions for the recovery of sublethal damage. Consequently, decreasing the dose per fraction can protect late reacting normal tissues more than the tumour cells, this differential effect allowing an escalation of the dose to the tumours in comparison to conventional fractionation or a decrease in the expected complication rates to maximise the therapeutic gain. Indeed, several clinical studies initiated in the 1980s and 1990s have shown that increased fractionation in head and neck radiotherapy could increase the control rates for the same levels of complications or could even reduce the complication rates. Thus, Bourhis *et al* [17] performed a meta-analysis of hyperfractionated trials in head and neck cancer, and reported a significant benefit from hyperfractionation than with conventional fractionation on survival (8% at 5 years) and on locoregional control (6.4% at 5 years).

Hyperfractionated regimes employing very small fractions required that they are given twice or three times per day to keep the overall treatment times to manageable lengths that avoid problems from tumour repopulation (see section II.2). Some of these treatment schedules highlighted the clinical implications of not allowing enough time between fractions for complete recovery of sublethal damage. One of the most known examples is the Continuous Hyperfractionated Accelerated Radiotherapy (CHART) trial delivering 54 Gy in 36 fractions in 12 consecutive days [18]. While the trial demonstrated a similar level of local control with 66 Gy in 33 daily fractions for a significant reduction in late treatment-related morbidity, the reduction was much less than expected from BED calculations. Analyses of the complication rates in the conventional and hyperfractionated arms showed that the repair half-life might indeed be 4-5 h, which is quite long for the 6 h interfraction intervals in the hyperfractionated arm of CHART [19]. The high amount of residual damage after the slow component of recovery might also explain why twice daily fractionation schemes with 2 Gy per fraction sometimes led to too high rates of late effects [20]. Nevertheless, twice daily fractionation regimes employing 1.2 or even 1.6 Gy per fraction have been safely used for treatment [21-24] and illustrate how the differential recovery potential of late and acutely reacting tissue could be exploited to increase the therapeutic potential in the radiation therapy of head and neck cancers.

In fact the potential for improvement of any fractionation scheme employing n fractions of size d could be evaluated using the BED formalism. Thus, the biologically equivalent of total dose in 2 Gy fractions (EQD) could be derived for the effects in late reacting tissues using an alpha/beta ratio of 3 Gy (equation 5) and in tumours and acutely reacting tissues using an alpha/beta of 10 Gy (equation 6).

$$EQD_3 = nd \frac{1 + \frac{d}{3}}{1 + \frac{2}{3}}$$ (5)

$$EQD_{10} = nd \frac{1 + \frac{d}{10}}{1 + \frac{2}{10}}$$ (6)

where $1 + \frac{2}{3}$ is the relative effectiveness of conventional fractionation regimes in late reacting tissues and $1 + \frac{2}{10}$ is the relative effectiveness of conventional fractionation regimes in acutely reacting tissues and tumours. The expressions could be adapted to include the effect of incomplete recovery between fractions (equations 3 and 4) or for protracted irradiation (equations 1 and 2). In fact, the loss of effects due to protracted irradiation has been a cause of concern in some applications like intensity modulated radiotherapy (IMRT), although this loss might not be significant as long as the delivery time is shorter than the half-life of the quick component of recovery [16].

2.2. Repopulation

Besides repair, repopulation during treatment is another important factor that could modulate the response to fractionated regimes. Indeed, as the effects in radiotherapy are related to the inactivation of cells from tumours and normal tissues, any proliferative process taking place during treatment will increase the cell population and consequently diminish the effect of radiation therapy. The effect would therefore depend on the time available for proliferation, i.e., the overall treatment time, and will particularly be a problem for acutely reacting tissues and tumours that have significant proliferation. In late reacting tissues in contrast, proliferative activity is minimal during the few days or weeks required by most treatment schedules and the treatment duration will not influence the complication rates.

The existence of a time factor in clinical radiation therapy was recognised quite early and gave rise to the Strandquist plots [25] and the nominal standard dose (NSD) concept [26] attempting to relate the time, dose and fractionation of equivalent fractionation regimes. However, an important breakthrough linking the time factor to the proliferation of tumour cells was made following the publication by Withers and colleagues of a study analysing the total dose needed to achieve 50% control for squamous tumours of the head and neck as a function of the overall treatment time [27]. The results showed that there was an increase of the required dose at a rate of 0.5-0.6 Gy per day for treatments lasting more than about 21-28 days, which was attributed to accelerated proliferation taking place in tumours after the lag time. This report was accompanied by the development of the BED concept with proliferation [9]. Thus, the effect of proliferation with doubling time T_p for a treatment with n fractions of size d being delivered in time T could be accounted by equation 7

$$BED = nd\left[1 + \frac{d}{\alpha/\beta}\right] - \frac{\ln(2)}{\alpha T_p}\left(T - T_k\right) \tag{7}$$

where T_k is the delay in the onset of proliferation. The equation could be further adapted to include the effects of repair as described in equations 1-4.

A series of clinical studies have investigated the impact of overall treatment time and proliferation on the outcome of radiation therapy for head and neck tumours. Studies investigating only the impact of acceleration, i.e., the delivery of more than 10 Gy per week that is the norm in conventional fractionation delivered in 5 daily fractions per week, have reported better control, but also an increase in acute reactions and sometimes an increase in late reactions when the interfraction interval was too short to allow full repair of sublethal damage [20,28,29]. More successful were schedules combining hyperfractionation and acceleration by delivering two or more fractions per day [18,22,30,31]. Nevertheless, an analysis performed by Bourhis and colleagues found a small but statistically significant benefit of 2% on survival at 5 years from acceleration itself in comparison to conventional fractionation [17].

The overall treatment time analysis of head and neck tumours indicates proliferation doubling time of the order of 3 to 5 days. This corresponds to the values determined from measurements of the potential doubling time of tumours, T_{pot} [32]. Whether T_p in equation 7 is T_{pot} is still a matter of debate. Experimental studies have shown that the effective doubling time for

proliferation during radiotherapy could be either smaller or larger than T_{pot} [33]. A large multicentre analysis also failed to correlate experimental determinations of T_{pot} with treatment outcome in head and neck cancers [34]. However, a more recent analysis has shown that pre-treatment proliferation parameters are better predictors of outcome when other factors like tumour size, individual radiosensitivity and overall treatment time are taken into account [35].

The increase in acute reactions when shortening the overall treatment time indicates that compensatory proliferation is also part of the mechanisms available for the rapidly proliferating normal tissues to recover from radiation damage. However, it has been found that the kinetic parameters for acute reactions are significantly different from those from tumours [36]. Thus, acute mucosal reactions that may become a limiting factor in the radiotherapy of head and neck tumours have a T_k of 7 days and a T_p of 2.5 days. This difference has given the opportunity to search for an optimum overall treatment time that maximises tumour effect, without jeopardising the function of late and acute normal tissues [37,38].

Three mechanisms have been proposed to be behind accelerated proliferation, namely asymmetry loss and acceleration of divisions of the stem cell compartment, as well as abortive divisions of sterilised cells [39]. Recruitment of quiescent cells into the cell cycle has also been proposed. The molecular triggers for these mechanisms remain to be elucidated [40], although there are some indications that epidermal growth factor receptor (EGFR) and protein tyrosine phosphatase (PTEN) activation might be involved [41-44].

Ample modelling of the aforementioned proliferation mechanisms has been undertaken in order to quantify the extent of repopulation during treatment, to study the individual contribution of each mechanism as well as their interplay towards overall tumour repopulation [45,46]. It was shown that while cell recruitment does contribute towards repopulation to a small extent, the major mechanism responsible for accelerated proliferation of tumour cells during radiotherapy is the asymmetry loss of stem cell division.

2.3. Reoxygenation

Tumour oxygenation is known to be one of the main factors that determine the response to radiotherapy. For advanced head and neck cancer in particular, clinical trials have shown that pre-treatment polarographic measurements of tumour oxygenation indicating the presence of tumour hypoxia correlate with poor prognosis [47]. This clinical evidence of the role of tumour hypoxia in determining the outcome of the treatment has been further confirmed by several studies in which pre-treatment uptake of nitroimidazole compounds such as ^{18}F-Fluoromisonidazole (^{18}F-MISO) or Cu-diacetyl-bis(N4-methylthiosemicarbazone) (Cu-ATSM) used as Positron Emission Tomography (PET) hypoxia imaging agents was shown to predict the outcome in head and neck cancer radiotherapy [48]. Furthermore, pre-treatment tumour hypoxia does not only correlate with poor local control due to the presence of resistant cells to radiotherapy, but also to chemotherapy and poor long time prognosis because of the loco-regional spread and formation of distant metastases [49].

The mechanism of resistance of tumour hypoxic cells to radiation can be explained by the so-called oxygen effect related to the oxygen actions at the level of the free radicals formed after

the interaction of charged particles with biological material. The free radicals, which are highly reactive molecules because of their unpaired valence electron, are responsible for the break of the DNA chemical bonds which might be further made permanent by molecular oxygen. The resulting biological damage depends thus on the presence or absence of oxygen, well-oxygenated cells being more sensitive to radiation induced damage than hypoxic cells deprived of oxygen.

Given the clinically proven impact of the presence of hypoxia on the treatment outcome, it is important to investigate the mechanisms of the occurrence of tumour hypoxia and its dynamics. The impaired oxygen supply to tumour cells leading to the formation of tumour hypoxia originates in the particularities of the tumour vasculature formed mainly through parasitation of the normal tissue vasculature and angiogenesis. Consequently, the major mechanisms involved in the formation of hypoxia are related to either the actual architecture of the blood vessels and the diffusion-limited delivery of oxygen, or to the functional abnormalities of tumour capillaries leading to perfusion limitations [49]. The two main forms of hypoxia associated with them are known as chronic (diffusion-limited) or acute (perfusion-limited) hypoxia. Thus, chronic hypoxia will occur when the distance from the cells to the nearer capillaries is close to exceeding the maximum oxygen diffusion distance, which under normal rates of oxygen consumption by the cells is expected to be in the order of 100-150 μm as shown by the early studies of Thomlinson and Gray [50] and confirmed later by experimental and modelling studies [51-53]. Acute hypoxia arises near the blood vessels temporary occluded, and, by its nature, has a transient character, unless the blood vessels remain blocked a long time period, depriving the cells of oxygen beyond the limit for survival.

For head and neck squamous cell carcinomas (HNSCC) in particular, which appear to be formed from nonvascularized epithelium, relatively hypoxic under normal conditions, hypoxia might pose particular reasons of concern. Thus, heterogeneous distributions of oxygen throughout the cellular microenvironment are expected in HNSCC. The impairment in the oxygen supply is not only spatially but also temporally heterogeneous.

Regardless the mechanism through which hypoxia occurs in tumours, there is a general consensus correlated to the clinical evidence that hypoxia is a negative predictive factor for the treatment and that patients might benefit from treatment strategies adapted according to the oxygenation of their individual tumours. However, it has also been suggested that chronically and acutely hypoxic cells might respond differently to radiation on the grounds of their energy supply and viability. It is well known that the combined high rate of glycolytic metabolism and poor availability of glucose result in low energy reserves for tumour cells reflected by the relative levels of ATP, ADP, AMP, Pi and PCr [54, 55]. The energy supply of chronically hypoxic cells, however, appears to decrease after a couple of hours of glucose deprivation [56] while the energy of well oxygenated cells does not decrease significantly under glucose deprivation. Therefore, one could postulate that chronically hypoxic cells are less capable to activate their DNA repair mechanisms and therefore would be more radiation sensitive compared to the acutely hypoxic cell [57] and quote in support studies on nutrient deprived cells [58,59]. Consequently, particular attention has to be paid to the cells at intermediate oxygen levels which might possess a dangerous combination of viability and

partial radioresistance that might in turn be reflected in the poor outcome to radiation treatment [60,61].

The oxygen status of the tumour cells is however not static with respect to both spatial and temporal patterns. Changes in the cellular oxygenation related to the dynamics of both chronic and acute hypoxia are generally known as tumour reoxygenation.

Reoxygenation manifests itself following two main patterns. Temporal heterogeneity in oxygenation arises in relation to acute or perfusion-limited hypoxia. Abnormal vasculature can lead to fluctuations in the blood flow due to the temporary occlusion or even backflow. These phenomena have a rather chaotic but transient character and are conventionally referred to as fast reoxygenation [62] although in some cases the change in oxygen supply might in fact be from poor to well and back to poor, thus not necessarily leading to an improvement of the oxygenation of the cells but rather to a re-hypoxiation. The temporal scale of changes in oxygenation related to acute, perfusion limited hypoxia, ranges from minutes to hours as demonstrated by several experiments using sequential injection of different fluorescent dyes for hypoxia and vascular perfusion [63,64]. Furthermore, in presence of irradiation, the dynamics of the oxygenation might be even more pronounced as shown in a study on human laryngeal squamous cell carcinoma tumour line grown as xenografts in nude mice by Bussink *et al* [51] indicating that irradiation could lead to rapid changes in oxygenation and perfusion. Chronically hypoxic cells might also change their oxygenation status during the course of fractionated therapy through the so-called slow reoxygenation. In a mixed tumour cell population with respect to oxygenation, ionizing radiation will primarily kill the sensitive well-oxygenated cells. This would result in lower oxygen consumption and hence to larger distances of oxygen diffusion which independently or in conjunction to overall tumour shrinkage might lead to the improvement of the tumour oxygenation by reoxygenation of the chronically hypoxic cells. Furthermore, during long, fractionated, radiotherapy treatments, extending over several weeks, revascularisation of the tumour through angiogenesis might also occur resulting in the reoxygenation of cells that were chronically hypoxic.

Taking advantage of the changes in tumour oxygenation and expecting that they will result in an improvement of radiosensitivity by fractionating the dose and thus increasing the treatment duration is one of the first approaches clinically used for overcoming tumour hypoxia. However, the search for the optimal dose per fraction and number of fractions in which the treatment has to be delivered is far from being over considering that one has to find the right balance between the fractional dose that might overcome hypoxia and the number of fractions that will ensure proper reoxygenation accounting at the same time for the normal tissue and organs at risk.

There are several clinical studies indicating that the oxygenation of head and neck tumours is indeed dynamic. The early studies of changes in tumour oxygenation in advanced head and neck carcinoma using polarographic electrodes were inconclusive in proving that reoxygenation positively correlates to increased local control most likely due to the inherent limitations of the technique [65-67]. More recent studies, however, using PET imaging, started to shed more light on the clinical evidence of oxygenation changes and reoxygenation in head and neck tumours [68-70]. The general consensus is that hypoxic subvolumes identified with the

use of PET imaging in head and neck cancer are inversely correlated with the response to radiotherapy and generally with the treatment outcome. The improvement in the tumour oxygenation together with the observed geometrical stability of the persistent hypoxic regions during the course of radiotherapy suggest that head and neck tumours are strong candidates for treatment strategies accounting for tumour hypoxia at the time of treatment planning and/or treatment adaptation based on hypoxia PET imaging.

Clinical implementation of hypoxia-driven radiotherapy is, however, still in its infancy. Several strategies for dose-painting approaches based on hypoxia in head and neck tumours have been proposed and they are under various stages of validation. Among them one could mention the planning study by Thorwarth *et al* [71] on dose escalation to head and neck hypoxic subvolumes based on PET imaging which was followed by a still ongoing clinical trial and the dose prescription and treatment planning method based on hypoxia PET imaging proposed by Toma-Dasu *et al* [72] which is currently under clinical validation.

In addition to dose escalation, treatment strategies focusing on overcoming hypoxia could include radiosensitizers or hypoxic cytotoxins. Lin and Hahn [48] presented a conceptual multimodal adaptive clinical trial approach focusing on radiation dose escalation to hypoxic regions highlighting the importance of pretreatment hypoxia imaging in order to properly select the patients that would be expected to benefit from hypoxia targeted treatments. They envisaged that serial imaging should be performed during therapy to evaluate treatment response and to select in a step-wise manner the highest-risk areas warranting treatment modifications, such as radiation dose escalation and to select the candidates for radiosensitizers.

A special class of strategy in the management of advanced head and neck is represented by the anti-angiogenic treatment which addresses the vascular endothelial growth factors and their respective receptors on endothelial cells as well as their role in role in promoting the growth and progression of carcinoma of the head and neck. Several anti-angiogenic treatments have shown promising results in the clinical setting such as those using tyrosine kinase inhibitors or bevacizumab [73]. Nevertheless, the current results suggest that multimodal therapies combining anti-angiogenic agents with chemo/radiotherapy have the potential to further increase the overall clinical benefit.

2.4. Redistribution

Similar to other tumour cell populations, squamous cell carcinomas of the head and neck proliferate in asynchronous growth. Therefore cells will be distributed unevenly through the cell cycle phases. Yet, the most probable distribution is the exponential one, with the largest population in G1 and smallest in mitosis. Partial synchronisation can occur as a result of cell arrest in one or more cycle phases due to the effect of radiotherapy or chemotherapy.

Cellular redistribution or reassortment along the cell cycle plays an important role in the success of fractionated radiotherapy, given that cells present various radiosensitivities along the four phases of the cell cycle. Given the relatively short average cell cycle time of squamous cell carcinomas (around 33 hours) [62], cells that survive a first dose of radiation will tend to

be in a resistant phase however, within a few hours they may progress into a more sensitive phase where they can be hit by radiation and killed.

Cells situated in the S phase are known to be about three times more radioresistant than cells undergoing mitosis. Since the duration of the S phase is about one third of the cell cycle length, there are large numbers of cells escaping the effect of radiation during a single hit. Fractionated radiotherapy assists in overcoming this challenge due to cellular redistribution between two consecutive doses.

Head and neck tumour cells have a high cell turnover, thus a relatively short cell cycle time. Cellular redistribution along the cell cycle for rapidly proliferating tumours consents to a more uniform cell kill than in slowly growing tumours. This rationale justifies the implementation of hyperfractionated radiotherapy schedules in head and neck cancers, which also hinders tumour repopulation during treatment.

Cells in the quiescent phase also play an important role during treatment as they can be triggered back into the cycle by cell loss due to radio- or chemotherapy. Quiescent cells are usually more resistant to radiation than cycling cells, fact that makes cellular recruitment (i.e. the process whereby quiescent cells re-enter the cell cycle) a double-edged sword: once they reach mitosis, newly cycling cells can increase the pool of tumour cells via cell division and, at the same time, cell killing can be more effective among cycling cells (as compared to quiescent cells) due to an overall higher radiosensitivity.

One of the known risk factors in head and neck cancer is the infection with HPV (human papillomavirus). A large number of studies have proven that head and neck cancers that are positive for HPV have higher cellular radiosensitivity than their non-HPV counterparts [74,75]. One of the explanations for this behaviour is the impaired DNA repair ability found among HPV-positive tumours and a considerable G_2 arrest. These experimental studies have shown that irradiated HPV-positive cells progress faster through the S phase and then accrue in G_2/M [75]. This unusual behaviour alters the expected cellular distribution along the cell cycle, accumulating the HPV cells in the more radiosensitive phases. Therefore, patients that tested positive for HPV respond better to the effect of radiotherapy and have a more favourable prognosis than non-HPV patients [74].

2.5. Radiosensitivity

Radiosensitivity is the tumour feature which aims to account for the fact that tumours respond differently to radiation therapy in a manner correlated with the intrinsic radiosensitivity of the cells derived as derived from *in vitro* experiments.

The intrinsic radiosensitivity influences the overall response of tumours to (chemo-) radiotherapy. The origins of the intrinsic radiosensitivity are related to the genetic instability of individual tumours leading to variations in response even among tumours of the same histological type [76]. Therefore, identifying *a priori* the alterations in the intracellular pathways involved in the DNA response, regulation of cell cycle and cell proliferation or responsible for activating the apoptotic signal, might offer the possibility of identifying the patients

expected to respond poorly to radiation therapy due to intrinsic radioresistance and customising the treatment based on individual radiobiological and genetic features of the tumours.

The most often mentioned pathways that were identified as clinically relevant in relation to the intrinsic radiosensitivity are the activation of Epidermal Growth Factor Receptor (EGFR), p53 and Ki-67 proteins signalling cascades.

For head and neck in particular, the activation of the phosphatidylinositol-3-kinase (PI3-K)/ protein kinase B (AKT) pathway has been shown to be associated not only with intrinsic radioresistance but also with other well-known tumour features responsible to poor outcome, cell proliferation and tumour hypoxia. This is because the PI3-K/AKT is a key element for the regulation of several cellular processes like apoptosis, invasion and proliferation. Consequently, it has been proposed that the manipulation of this signal-transduction pathway could be used in the management of head and neck cancers. Given the activation of this pathway by the stimulation of receptor tyrosine kinases like the EGFR, it has been suggested that markers for PI3-K/AKT activation should be related to predictors of EGFR sensitivity. Furthermore, inhibiting the PI3-K/AKT pathway will antagonise radiation-induced cellular defence mechanisms that in turn will result in enhancing the effectiveness of radiation therapy [77].

More recently, a biomarker that encodes the p53 protein, TP53, has been identified as the most commonly altered gene in squamous cell carcinomas of the head and neck leading to radioresistance [78].

As already mentioned above, the presence of Human Papilloma Virus (HPV) influences the response of the response to radiotherapy for head and neck squamous cell carcinoma [79]. Thus, the HPV status of the tumour could be regarded as a strong and independent prognostic factor for the success of the treatment, both in terms of local regional control and overall survival. This is due the increased cellular radiosensitivity caused by compromised DNA repair capacity in HPV-positive cells [74]. This might indicate that radiosensitivity and repair in cells should be correlated. Two main mechanisms have been identified for the repair of the double strand breaks of the DNA, homologous recombination (HR) and nonhomologous end-joining (NHEJ). Nevertheless, it has been shown that mutations in genes that impair HR often cause only modest or no radiation hypersensitivity. In contrast, mutations in NHEJ genes appear to lead to greater radiation hypersensitivity [80]. These complex relationships may in fact be the reason for difficulties in finding a correlation between repair and radiosensitivity [81] and why these are considered as two independent Rs in radiation biology.

3. The 6th R: Remote bystander effects

Remote cellular effects or bystander effects occur when non-irradiated cells that are located nearby irradiated cells undergo cellular damage similar to the irradiated cells. This experimental observation contradicts the formerly accepted theory of radiation-induced targeted cell kill [82]. While targeted cells can be killed by radiation, according to the bystander theory, non-targeted cells can also present signs of radiation damage that eventually kills the cell. This

happens as a consequence of cellular communication when radiation-hit cells direct damage signals through gap junctions to the neighbouring non-targeted cells, which then act as being hit by radiation.

Bystander effects have been evidenced in both tumour and normal cells, which implies that such remote effects could have clinical implications. The finding that gamma-ray-induced bystander effects have influence on epithelial cells and not fibroblasts, suggested that tissue architecture and also cell communication play a significant role in this process [83]. Since squamous cell carcinomas originate from epithelial cells, the bystander effect becomes an important consideration in the treatment of head and neck tumours (table 1).

It is known that in normal tissues, gap junctions physiologically connect one cell to the adjacent one to enable the transmission of genetic signals between cells. Both metabolic cooperation between cells and the regulation of normal tissue homeostasis requires the involvement of gap junctions. This normal phenotype is usually lost during head and neck carcinogenesis. Although the complete function of gap junctions in head and neck neoplasms is not fully clarified, experimental studies demonstrate that gap-junctional intercellular communication (GJIC) could mediate apoptotic cell death in non-targeted squamous cell carcinoma adjacent to individually targeted squamous cell carcinomas of the head and neck [84].

Novel therapeutic methods like gene therapy are widely used to investigate bystander effects in cancers including head and neck. A number of viral vectors have been developed that are able to transfer genes to therapy of tumours known as gene transduction. The occurrence of a bystander effect after wild-type p53 gene transduction has been investigated for human squamous cell carcinomas of the head and neck [85]. Wild-type p53 gene transduction for apoptosis-inducing molecular therapy has been shown capable of producing a bystander effect in squamous cells *in vitro.* Additionally, it was demonstrated that this phenomenon requires intercellular contact between wild-type p53 transduced and bystander, non-transduced cell populations. The study concluded that other therapies associated with apoptosis (such as radiotherapy or chemotherapy) might also demonstrate bystander effects.

Enhancing gap-junctional intercellular communication in squamous cell carcinomas of the head and neck and understanding the other mechanisms behind cancer cell communication may lead to increased therapeutic efficacy.

Bystander effect	Mode of cellular communication	Reference
Growth inhibition	Intercellular contact between wild-type p53 transduced and non-transduced cell population	Frank *et al* 1998 [85]
Apoptotic cell death		Frank *et al* 2005 [84]
Genomic instability	Cellular interaction influenced by stromal fibroblasts	Kamochi *et al* 2008 [86]
Cell dormancy and proliferation arrest	Gap-junctional intercellular communication	Kucerova *et al* 2013 [87]

Table 1. Bystander effects in squamous cell carcinoma of the head and neck

An interesting observation that could have implications in the development of new thera-peutical agents for cancer was reported by Cogan *et al* [88]. The group has shown that bystander signals from cancer cells after exposure to chromium VI results in DNA damage in neigh-bouring cells that is strongly dependent on telomerase (complex enzyme that maintains the telomere length). Low dose exposure to Cr(VI) was able to induce cancer cells to continuously secrete bystander signals that caused DNA damage in the neighbouring cells. However, these bystander signals were telomerase-dependent, meaning that the status of the telomere (negative or positive) has dictated the affinity of cancer cells for bystander signals.

It is well known that telomere activation has a therapeutic potential for cancer, given that with each cell division, the length of telomeres shortens, fact that triggers cell senescence. In order to survive, cancer cells are required to employ a mechanism to stabilise this process of telomere reduction. There are several reports in the literature showing that telomerase promoter mutations (TERT) are more prevalent in aggressive cancers and they are a major indicator of poor prognosis among head and neck cancer patients [89,90]. The effect of chromium VI on telomerase offers, therefore, a potential anticancer avenue that needs to be further explored.

Bystander effects largely relate to nontargeted effects after exposure to low dose radiation. While tumours are targeted with high doses, the surrounding normal tissue receives much lower doses of radiation and organs that are out-of-field even lower doses. Exposure of nontargeted tissue raises several concerns regarding the risk of second primary cancers. However, it was shown that low dose exposure of healthy cells (2 - 50 mGy) stimulates intercellular induction of apoptosis in the precancerous cell population, via cytokine and reactive oxygen species signalling [91]. This selective elimination of precancerous cells could reduce the incidence of second cancers following radiotherapy.

There is a limited number of studies on the risk of second cancers after primary head and neck carcinoma treatment and they reveal the observation that among these patients second cancers occur, most often, due to smoking [92] and drinking habits [93]. Second cancers are common among long-term survivals, however they are not necessarily second primaries. Recurrences occur very often and they arise mostly in the treatment field [94]. Common sites for second primary cancers are the lung and the esophagus. These occurrences are often linked to the same risk factors as for the primary head and neck cancer, i.e. tobacco smoking and alcohol consumption. The radiation-induced incidence of second primary cancers after head and neck radiotherapy was analysed in a study by Yamamoto *et al* [95]. The group has concluded that radiotherapy of the primary tumour is not thought to be associated with an increased risk of second tumours. Furthermore, it was underlined that a clonal relationship exists between the primary head and neck cancer and second primaries, suggesting that the latter are a result of micrometastatic foci migration from the site of origin.

Beside bystander effects, there is another phenomenon that is linked to low dose cellular exposure, namely the adaptive response. This effect of low dose radiation on cells was first demonstrated by Olivieri *et al* [96] after observing that human lymphocytes growing in radioactive thymidine solution were more resistant to the effect of subsequent high doses of radiation than the control group grown in non-radioactive culture. Cellular radioresistance to subsequent doses was materialised through a reduction of chromosomal aberrations.

In vitro experimental studies have shown that the two low-dose phenomena: the bystander effect and the adaptive response basically coexist. However, their effect on cells works in opposition, as bystander effects result in excess cell kill, while the adaptive response confers resistance to subsequent doses of radiation.

According to the adaptive response model, the isoeffect per dose fraction is not a valid theory anymore, as the first dose of radiation should kill a higher percent of the tumour cell population than the subsequent doses. Furthermore, for those patients that undergo pre-treatment diagnostic examinations that employ low doses, the adaptive response might work even for the first treatment dose.

More studies are needed to explain these two effects in head and neck cancer cell lines and to determine their magnitude for the *in vivo* state.

4. Conclusions

The management of locally advanced head and neck cancers is demanding. Tumour hypoxia, accelerated repopulation during treatment and inherent radioresistance are the main culprits for the suboptimal tumour control. The Rs of radiotherapy, as described above, play an important role in treatment design, particularly when it comes to dose fractionation in radiotherapy. Questions then arise as to how fractionated the treatment should be and what other parameters should be taken into consideration in order to achieve a high therapeutic gain.

As demonstrated by clinical trials, conventionally fractionated treatments are not efficient for head and neck cancer patients. Instead, altered fractionation schedules should be employed to overcome the radiobiological challenges. Accelerated fractionation is a rather aggressive protocol that, however, is needed in response to an aggressive tumour. Treatment breaks are often scheduled in these situations to allow time for normal tissue repair. Hyperfractionated radiotherapy is a perfect way to apply the Rs of radiotherapy for rapidly proliferating tumours such as head and neck carcinomas. By giving more than one dose fraction a day, tumour repopulation during treatment is minimised and tumour reoxygenation is stimulated. Hyperfractionated schedules were shown to provide the greatest benefit among these patients.

While hypoxia and repopulation are usual characteristics of head and neck cancers, the extent of hypoxia and the degree of tumour proliferation differ from patient to patient. These pre-treatment disparities lead to different post-treatment tumour responses. Decades ago, the idea of predictive assays for tumour oxygenation, proliferation and radioresistance has been embraced with high optimism. However, due to several technical and clinical challenges, the routine implementation of such predictive assays has been hindered and other methods to characterise the metabolic properties of tumours have been designed. Advanced imaging techniques such as BOLD-MRI (Blood Oxygen Level Dependent – Magnetic Resonance Imaging) or PET (Positron Emission Tomography) can give valuable indication regarding oxygenation and proliferation. The goal to gather such information is to design individualised

treatments, based on patient-specific parameters. Personalised treatment is therefore the key solution for the management of advanced head and neck cancers. This will be achieved with (figure 1):

• routine clinical implementation of *predictive assays*, which in the current era are often *PET*-based,

• *protection* of normal tissue to diminish adverse effects,

• improved *perfusion* of oxygen inside the tumour to allow for reoxygenation and better chemotherapy delivery.

These P's of head and neck cancer treatment can lead to an enhanced therapeutic ratio by increasing tumour control and decreasing normal tissue toxicity.
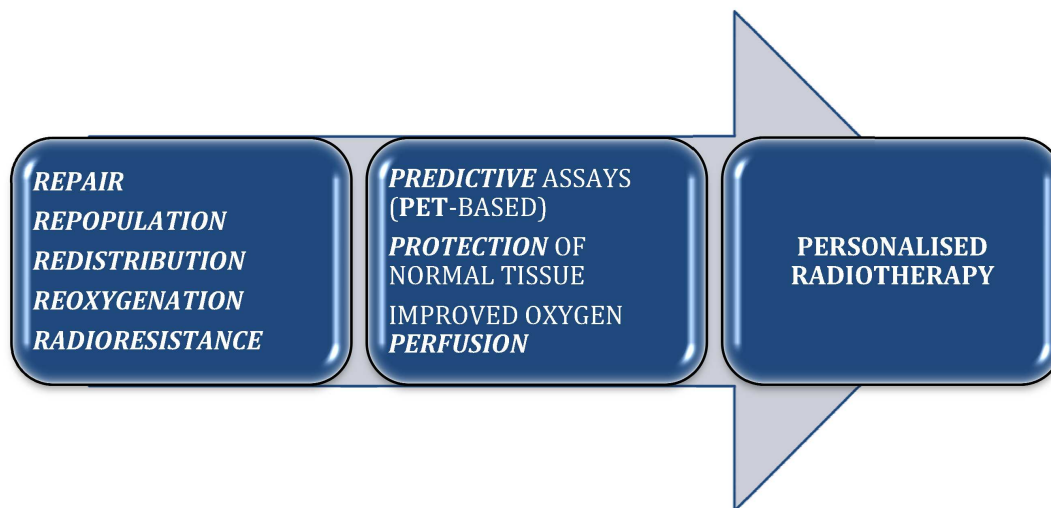

REPAIR
REPOPULATION
REDISTRIBUTION
REOXYGENATION
RADIORESISTANCE

PREDICTIVE ASSAYS (**PET**-BASED)
PROTECTION OF NORMAL TISSUE
IMPROVED OXYGEN *PERFUSION*

PERSONALISED RADIOTHERAPY

Figure 1. The Ps and Rs of head and neck cancer management towards Personalised Radiotherapy.

A more accurate patient selection for the administration of chemotherapeutical agents or altered fractionation schedules could lead to a better management of head and neck cancer with personalised treatment planning and delivery.

Acknowledgements

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