PUSHING THE FRONTIERS OF RADIOBIOLOGY: A SPECIAL FEATURE IN MEMORY OF SIR OLIVER SCOTT AND PROFESSOR JACK FOWLER: REVIEW ARTICLE

Clinical trials targeting hypoxia

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ABSTRACT

The concept of tumour hypoxia as a cause of radiation resistance has been prevalent for over 100 years. During this time, our understanding of tumour hypoxia has matured with the recognition that oxygen tension within a tumour is influenced by both diffusion and perfusion mechanisms. In parallel, clinical strategies to modify tumour hypoxia with the expectation that this will improve response to radiation have been developed and tested in clinical trials. Despite many disappointments, meta-analysis of the data on hypoxia modification confirms a significant impact on both tumour control and survival. Early trials evaluated hyperbaric oxygen followed by a generation of studies testing oxygen mimetics such as misonidazole, pimonidazole and etanidazole. One highly significant result stands out from the use of nimorazole in advanced laryngeal cancer with a significant advantage seen for locoregional control using this radiosensitiser. More recent studies have evaluated carbogen and nicotinamide targeting both diffusion related and perfusion related hypoxia. A significant survival advantage is seen in muscle invasive bladder cancer and also for locoregional control in hypopharygeal cancer associated with a low haemoglobin. New developments include the recognition that mitochondrial complex inhibitors reducing tumour oxygen consumption are potential radiosensitising agents and atovaquone is currently in clinical trials. One shortcoming of past hypoxia modifying trials is the failure to identify oxygenation status and select those patient with significant hypoxia. A range of biomarkers are now available including histological necrosis, immunohistochemical intrinsic markers such as CAIX and Glut 1 and hypoxia gene signatures which have been shown to predict outcome and will inform the next generation of hypoxia modifying clinical trials.

INTRODUCTION

The first report showing the importance of oxygenation in radiotherapy was in 1909 by Schwarz who showed the radiation response of skin to be markedly reduced when the blood supply to the irradiated area was restricted by compression.[1] During the first half of the century, further experimental and clinical observations emphasised the requirement for adequate tissue oxygenation to achieve an effective radiation response, although it was not until the seminal studies of Gray and colleagues in the 1950s that the role of hypoxia was established as a major cause of radiation resistance. In their pioneering work, they demonstrated that hypoxia caused resistance to radiation in a broad spectrum of microbial, plant and mammalian cellular models using a variety of different end points.[2] The oxygen effect is mediated by the radiochemical reaction by which ionising

radiation interacts with cellular DNA. Radiation induces DNA damage via the formation of DNA free radicals. Oxygen, being highly electron-affinic, is able to "fix" the radiation-induced DNA damage by reacting rapidly with the unpaired electron of the free radical. Reactive oxygen species are generated that then undergo further interactions, ultimately leading to double-stranded DNA breaks and cell death. In the absence of oxygen, DNA free radicals are restored to their original, undamaged form by reacting with H^+ ions donated from cellular non-protein sulfhydryls, hence, the ability of ionising radiation to kill hypoxic cells is greatly reduced.

The mechanism by which hypoxia develops in tumours was first hypothesised by Thomlinson and Gray[3] based on histological studies of bronchial carcinoma. They observed

that across microscopic sections, there was a consistent distance between necrotic tissue and blood vessels. The thickness of the viable tissue in between, usually between 100 and 150 μm, was shown to be predictive of the oxygen diffusion distance, calculated using the capillary oxygen partial pressure and cellular oxygen consumption rate. It was hypothesised that as oxygen diffuses away from the vascular stroma, it gets metabolised by tumour cells. Those cells beyond the diffusion distance are unable to survive, whilst those adjacent to this necrotic tissue may be viable but hypoxic. Thus, as the tumour divides and outgrows its blood supply, areas of chronic, diffusion-limited hypoxia develop. Tumour hypoxia can also be acute in nature, owing to the transient collapse of immature blood vessels rendering sections of the cancer hypoxic for a limited period. The relative extent to which the two mechanisms occur or interact within tumours is unknown, although protection against radiation is likely to be conferred regardless of whether malignant cells are acutely or chronically hypoxic, hence both will influence the efficacy of radiotherapy.

Following the work of Gray and collaborators, an extensive number of studies detecting and measuring the extent of hypoxia in human tumours have been carried out. Three main techniques have been employed. First, directly measuring the amount of oxygen within the tumour using polarographic sensing electrodes.[4–6] Second, the labelling of metabolically active hypoxic cells via their ability to reduce extrinsic hypoxic markers that are then identified by immunohistochemical analysis or positron emission tomography.[7–10] Finally, the identification of specific gene expression and molecular activity known to be induced by hypoxia, most of which is related to the expression of proteins involved in the hypoxia inducible factor-1α (HIF-1α)[11–13] and osteopontin[14,15] pathways. In addition to these methods, non-invasive imaging modalities have also been useful in providing an indirect measure of oxygenation. Information relating to vascular density and tumour blood flow can be derived from dynamic contrast-enhanced and dynamic susceptibility MRI (DCE-MRI).[16,17] In combination with DCE-MRI, intrinsic susceptibility weighted MRI has been shown to have high sensitivity in the detection of hypoxia in prostate cancer.[18] DCE-MRI parameters alone have been shown to directly correlate with electrode-measured hypoxia levels in carcinoma of the cervix[19] and measurements from this imaging modality were independently associated with a worse outcome to radiotherapy in this tumour site.[20] From studies utilising all of the aforementioned techniques, it is now well-established that hypoxia is present to varying degrees in the majority of solid malignancies.

Hypoxia and radiotherapy outcomes

The decrease in radiation sensitivity in reduced concentrations of oxygen can be defined by the oxygen enhancement ratio; the ratio of radiation dose under hypoxia to oxic conditions required to produce equivalent cell kill. For mammalian tissues, the oxygen enhancement ratio is normally in the range of 2.5–3 and is most prominently seen after single, large doses of radiation.[21] The oxygen effect in fractionated radiotherapy is more convoluted and depends on numerous factors including patient characteristics, tumour site of origin, tumour histology, time-dose fractionation and the rate and degree of reoxygenation. However, even in the context of fractionated regimes, the oxygen concentration of hypoxic islands within the tumour is still sufficient to maintain viability of cancerous cells whilst conferring relative resistance to radiotherapy. In practical terms, the magnitude of the oxygen effect is dependent on the presence of hypoxic clonogenic stem cells within the tumour and their capacity to remain viable during prolonged exposure to hypoxia. This is likely to vary between tumour types and it has been suggested that squamous cell carcinomas with their development in a non-vascularised epithelium may be more able to maintain clonogenicity when exposed to chronic hypoxia.[22] In line with this hypothesis, a number of studies predominantly in tumour sites of squamous cell origin have shown by direct measurement of pre-treatment median tumour pO_2 values that patients with hypoxic tumours have significantly worse outcomes following radical radiotherapy.[23–31] The first of these was reported by Hockel et al who evaluated the prognostic value of low pre-treatment tumour oxygenation status in 89 cervical cancer patients undergoing radical radiotherapy or chemoradiotherapy.[28] After a median follow-up of 28 months, overall and progression-free survival rates were both significantly higher in those with a median tumour pO_2 >10 mmHg. In those with advanced disease, median pO_2 was seen to be the strongest independent prognosticator. Further studies in head and neck cancers[23–27] and carcinoma of the cervix[28–31] have consistently shown pO_2 values <10 mmHg to have an adverse effect on locoregional control, disease-free survival and overall survival in patients undergoing radiotherapy. In the vast majority, the prognostic effect of tumour oxygenation is maintained on multivariate analysis; tumour hypoxia is not dependent on tumour grade, tumour size, volume of necrosis or haemoglobin level and it is therefore a strong, standalone predictor of outcome following radiotherapy.

More recently, data have emerged to suggest that persistence of tumour hypoxia beyond the start of radiotherapy treatment may be a more significant prognostic indicator than baseline hypoxia. In an exploratory prospective cohort study of 25 patients with locally advanced head and neck cancer, fluomisonidazole-positron emission tomography imaging was used to establish tumour hypoxia levels at various time points during and before radiotherapy.[32] Fluomisonidazole imaging parameters at weeks 1 and 2 were shown to be more strongly associated with the primary end point of locoregional control than those obtained at baseline. These results were subsequently confirmed in a validation cohort[33] and demonstrate the importance of poor interfraction reoxygenation and residual tumour hypoxia as significant drivers of hypoxic radioresistance.

Strategies to overcome hypoxic radioresistance

Methods of improving radiotherapy outcomes by hypoxic modification have been the subject of experimental and clinical research since the early 1960s. The most commonly employed clinical strategies are detailed in Table 1 and can be considered in four distinct categories.

The first is increasing oxygenation of the tumour via the blood. The simplest approach here is hyperbaric oxygen (HBO)

Table 1.Strategies to improve radiotherapy outcomes through modification of hypoxic radioresistance

Improving intratumoral oxygenation through increased oxygen delivery by the blood
Increasing oxygen transfer from the lungs with hyperbaric oxygen
Improving intratumoral oxygen diffusion with carbogen
Increasing vascular perfusion with nicotinamide
Radiosensitising oxygen mimetics
Nitroimidazole compounds, *e.g.* misonidazole, etanidazole, nimorazole
Selective destruction of hypoxic cells
Hypoxic cytotoxins, *e.g.* mitomycin C, porfiromycin, tirapazamine
Hyperthermia
Reducing tumour cell oxygen consumption
Mitochondrial inhibitors, *e.g.* metformin, atovaquone

whereby the breathing of 2–4 atmosphere 100% oxygen during radiotherapy ensures full saturation of haemoglobin and results in extra oxygen dissolved in the blood.[34] Normobaric oxygen in association with carbogen (combined oxygen and 2–5% carbon dioxide) has also been employed on the assumption that CO_2 may both increase the intratumoral diffusion capacity of O_2 and counteract the vasoconstriction sometimes induced by pure oxygen.[35] Carbogen breathing has further been combined with nicotinamide, a vasoactive amide of vitamin B_3 known to counteract the transient fluctuations in microregional tumour blood flow causing acute hypoxia thereby synergistically targeting diffusion-limited and perfusion-limited hypoxia.[36] This underlies the ARCON strategy which sees the combination of carbogen and nicotinamide used in association with accelerated radiotherapy.[37] Here, overall treatment time is reduced and repopulation limited in addition to counteracting hypoxia.

The second category of hypoxic modifiers are the nitroimidazole oxygen mimetics, chemical agents that mimic the radiochemical effects of oxygen and preferentially sensitize the hypoxic population to radiotherapy. These compounds have a potential advantage over oxygen in that they are not metabolised by the tissues they diffuse through, thereby allowing them to penetrate deeper into the tumour and reach hypoxic regions.[38]

The third method of hypoxic modification is that of the hypoxic cytotoxins which act to preferentially destroy hypoxic cells, instead of radiosensitizing them. The foremost of these drugs is tirapazamine. In a reaction catalysed by intracellular reductase enzymes, tirapazamine is reduced to form highly reactive free radical species that induce double-stranded DNA breaks.[39] In the presence of oxygen, the additional electron is removed from the tirapazamine free radical, oxidising it back to its non-lethal state. Thus, the drug is strongly cytotoxic under conditions of hypoxia.

More recently, attention has turned to the identification of pharmacological agents that reduce the cellular oxygen consumption of tumours, thereby rendering them less hypoxic and more

sensitive to radiotherapy. Agents such as metformin and the anti-malarial drug atovaquone work in this way through the inhibition of cellular mitochondrial complexes.

Hypoxic radiosensitisation—experimental studies and clinical trials
Hyperbaric oxygen breathing
Early experimental studies in both spontaneous murine tumours and mammary carcinoma models have shown that breathing oxygen and carbogen potentiates the tumour response to irradiation.[40,41] The effect is clearly greater under hyperbaric (three atmospheres) as opposed to normobaric conditions. In the UK, the first large multicentre clinical studies evaluating the benefit of HBO inhalation were introduced early in the 1960s by the Medical Research Council.[42] Results from advanced carcinoma of the head and neck[43,44] and those of the uterine cervix[45,46] both showed significant improvements in local tumour control which translated into an overall survival benefit. However, the simultaneous delivery of radiation and HBO is technically complex and practically demanding and often resulted in poor patient compliance. Moreover, the hypofractionated schedules commonly used in the trials in conjunction with the radiosensitisation of normal tissues saw an increase in the incidence of late tissue toxicity in a number of studies. Consequently, the use of HBO was never really accepted into general clinical practice and alternative forms of improving tumour oxygenation were sought by researchers.

ARCON
ARCON describes the use of accelerated radiotherapy in conjunction with carbogen and nicotinamide. A substantial body of pre-clinical evidence exists demonstrating the beneficial effects of the three components of ARCON, both individually and in combination. Studies in the murine mammary tumour CaNT observed an enhancement ratio of 1.2 for accelerated radiotherapy over conventional fractionation.[47] The addition of carbogen further increased the ratio to 1.7 whilst the triplet combination of accelerated radiotherapy, carbogen and nicotinamide resulted in an enhancement ratio of 1.9 compared to conventional radiation alone. Thus, by utilising the complete ARCON strategy, the equivalent effect of standard radiotherapy could be achieved with almost a 50% lower radiation dose. Regarding late toxicity, the enhancement ratios of normal tissues for combined carbogen and nicotinamide have typically been much lower than for most tumours, highlighting the potential therapeutic gain with the ARCON approach.[48,49] However, experimental studies on rat spinal cord have shown carbogen and nicotinamide to reduce cord radiation tolerance by almost 20%.[50] Consequently, when ARCON is employed clinically, it is the general consensus that a lower maximum dose to the spinal cord than the conventional 46–48 Gy should be mandated.[37]

The promising results observed in pre-clinical murine studies inspired a number of Phase I and II ARCON trials in the early 1990s, firstly at Mount Vernon Cancer Centre in the UK[51–53] and swiftly followed by other institutions across Europe.[54–57] With the presence of hypoxia in head and neck squamous cell carcinomas well-established and with an increasing recognition of the prognostic value of low pre-treatment oxygenation status in these

cancers, a number of the ARCON feasibility and toxicity studies were carried out in this tumour site.[53–58] The largest of these trials evaluated 215 patients with locally advanced carcinomas of the oral cavity, larynx, oropharynx and hypopharynx.[58] The majority of patients recruited had Stage T3 or T4 disease. Accelerated radiotherapy to a dose of 64–68 Gy in 2 Gy fractions was delivered in conjunction with carbogen and nicotinamide over a course of 36–38 days. Two fractions were given daily for the final 1.5 weeks of the regime. Local control rates were 87, 80, 60 and 29% for oropharyngeal, laryngeal, hypopharyngeal and oral cavity tumours respectively. In particular, the control rates for oropharynx and larynx were very favourable; the latter superior to any corresponding figure seen in previous reports showing great potential for organ preservation. Early mucosal and skin reactions were more severe with ARCON than those typically observed with conventional radiotherapy; confluent mucositis was seen in 91% of patients with a median duration of 6 weeks and moist skin desquamation observed in 57%. Importantly, however, there was no significant increase in severe late sequalae other than a faint suggestion of increased sensitisation of the mandible with three patients developing osteoradionecrosis.[58]

The second tumour site where ARCON has been shown to be beneficial is bladder cancer. As with laryngeal carcinoma, organ preservation in bladder cancer is a welcome alternative to surgery.[59] Although direct evidence is limited, the upregulation of HIF-1α and associated carbonic anhydrases has been observed in carcinomas of the bladder suggesting that hypoxia may be present in these tumours at a level that may contribute to their radioresistance.[60,61] In light of this, a Phase II study with ARCON was performed in 61 bladder cancer patients who had predominantly T2 and T3 stage disease.[52] Radiotherapy to a total dose of 50–55 Gy was delivered over 26 days in 20 daily fractions. In 30 patients, this was administered with carbogen whilst the remaining 31 received combination carbogen and nicotinamide. Compared with the results of previous bladder radiotherapy trials with HBO and misonidazole, significant improvements in local control, progression-free survival and overall survival were seen with no increase in either acute or late toxicity.[52]

Although Phase II ARCON studies in other tumour types such as high-grade gliomas[62] and non-small cell lung cancer[63] did not show a survival benefit, the promising results described in head and neck and bladder cancer led to further Phase III evaluation of the strategy in these tumour sites. In the first of these, the BCON study recruited 333 patients with locally advanced bladder cancer staged from T2 to T4a and randomised them to receive either radiotherapy alone or radiotherapy plus carbogen and nicotinamide.[64] The radiotherapy in both arms was delivered to a dose of either 55 Gy in 20 fractions or 64 Gy in 32 fractions. Cystoscopic examination 6 months post-treatment was used to assess the primary end point of local control. Secondary endpoints were overall survival and local disease-free survival as well as late genitourinary and gastrointestinal toxicity. Local control rates were 81 and 76% for the combination and radiotherapy alone arms respectively ($p = 0.3$) with corresponding overall survival rates of 59 *vs* 46% ($p = 0.04$); thus, a 13% absolute overall survival benefit in favour of hypoxic radiosensitisation

with carbogen and nicotinamide was observed with no reported increase in late tissue toxicity. In the second Phase III ARCON trial carried out in Netherlands in head and neck cancer,[65] 345 patients with locally advanced squamous cell carcinoma of the larynx were recruited and randomised to receive accelerated radiotherapy alone or ARCON. No benefit in the primary end point of local control was observed with hypoxic modification. 5 year regional control rates were significantly improved by ARCON (93% *(ARCON)* *vs* 86% *(accelerated alone)*, $p = 0.04$) and this effect was particularly strong in a cohort of patients with hypoxic tumours selected out by high pre-treatment pimonidazole staining [100% *(ARCON)* *vs* 55% *(accelerated alone)*, $p = 0.01$]. Toxicity rates were equivalent in both arms.

Oxygen mimetics

Since the recognition in the early 1960s that the extent of radiosensitisation directly relates to the electron-affinity of the sensitising agent,[66] the nitroaromatics have been extensively studied as potential hypoxic modifiers. A number of drugs including metronidazole, misonidazole, nimorazole and pimonidazole have been tested and shown to be highly effective in the preferential radiosensitisation of hypoxic cells *in vitro*.[67–71] This success drove a wave of clinical trials in the late 1970s evaluating the effectiveness of misonidazole as a hypoxic modifier. However, the majority of these trials failed to show any significant benefit of misonidazole with severe peripheral neuropathy seen to be a major limiting toxicity.[72] Whilst in murine models the rapid pharmacokinetics and efficient clearance of nitroimidazole compounds generates a relatively high therapeutic ratio, the high volume of distribution and longer half-life of the drugs in humans results in considerably greater toxicity and limits the administration of the drugs at the higher doses required to mediate effectual radiosensitisation. Thus, the clinical evaluation of misonidazole was compromised by the resultant structure of the clinical trials whereby, the drug could only be given at low doses and with a paucity of radiation treatments.[73] These limitations stimulated the development of a second generation of nitroaromatic compounds with superior pharmacokinetics and less toxicity. Pimonidazole, etanidazole and nimorazole have all been evaluated in randomised controlled trials.[74–77] The results of piminidazole and etanidazole in carcinoma of the uterine cervix[74] and head and neck[75,76] respectively were disappointing with the concurrent use of these drugs not shown to afford any additional benefit over conventional radiotherapy alone. Contrariwise, in the Danish Head and Neck Cancer 5 (DAHANCA 5) study, where the radiosensitising effect of nimorazole was evaluated in 422 patients with pharyngeal and supraglottic carcinomas, a highly significant benefit was seen both in terms of 5 year local control rates [33% *(conventional)* *vs* 49% *(nimorazole)*] and 5 year disease-free survival [(41% *(conventional)* *vs* 52% *(nimorazole)*] rates.[77] No increase in late radiation toxicity was observed. Unfortunately, this high quality study has somewhat lost relevance amidst the raft of negative nitroaromatic trials, many of which were carried out with older generation compounds and lacked statistical power. The routine use of nimorazole as a radiosensitiser is standard practice in Denmark alone and general interest in nitromidazoles as hypoxic sensitisers is waning amongst radiation oncologists. However, given the considerable gain observed in DAHANCA

5, another large randomised phase III trial evaluating the benefit of synchronous nimorazole as an adjunct to intensity-modulated radiotherapy in locally advanced head and neck cancer is now recruiting in the UK (NIMRAD).[78] This trial will also look to validate a hypoxic gene marker predictive of a better response to hypoxic modification and its results are therefore eagerly awaited.

Hypoxic cytotoxins
Agents that are preferentially toxic against hypoxic cells are desirable in principal. Mitomycin C has been clinically evaluated as a potential radiosensitiser, predominantly in patients with head and neck and cervical cancer. Dobrowsky et al randomised 123 patients with mainly Stage T3 and T4 disease to receive continuous hyperfractionated accelerated radiotherapy (CHART) to a total dose of 55.3 Gy in 33 fractions over 17 consecutive days with or without concurrent mitomycin C.[79] At 4 years, the addition of mitomycin C was shown to significantly improve both actuarial survival [51% *(mitomycin C) vs* 31% *(CHART alone)*, $p < 0.05$] and locoregional control rates [57% *(mitomycin C) vs* 32% *(CHART alone)*, $p < 0.05$]. In a multicentre Phase III randomised controlled trial of 160 patients with FIGO stage IB2-IVA cervical cancer,[80] concurrent mitomycin C significantly improved 4 year actuarial disease-free survival rates [71% *(mitomycin C) vs* 44% *(conventional)*, $p = 0.01$] with the greatest benefit seen in patients with Stage III-IV disease [75% *(mitomycin C) vs* 35% *(conventional)*, $p = 0.03$]. However, no significant benefit in overall survival or local recurrence rates was observed.[80]

At an experimental level, tirapazamine has been shown to have heightened cytotoxicity against hypoxic cells compared to mitomycin C.[81,82] Further pre-clinical studies have shown the drug to enhance cell death induced by both fractionated radiation[83] and by cisplatin chemotherapy[84]; the synergistic effect with the latter seen to be particularly potent. Evaluating clonogenic survival in transplanted murine RIF-1 tumours, Dorie and Brown showed tirapazamine and cisplatin monotherapy to produce 0.5 logs and 2 logs of cell kill respectively. The simultaneous administration of the two drugs resulted in around 2.5–3 logs of cell kill. However, when tirapazamine was given before cisplatin, a much more substantial cell kill of 6–7 logs was observed with no concurrent increase in systemic toxicity.[84] The synergistic effect is thought to be due to a delay in the repair of cisplatin-mediated DNA cross-links in cells pre-exposed to tirapazamine under conditions of hypoxia, although the exact mechanism underlying this delay is yet to be elucidated. In light of this, clinical trials evaluating the benefit of tirapazamine as an adjunct to radiotherapy have often included concurrent cisplatin and have predominantly taken place in head and neck cancer where platinum-based chemoradiation is standard of care. Unfortunately however, the experience thus far has been disappointing. In a Phase II study, 59 patients with Stage IV head and neck cancer underwent cisplatin-based chemoradiotherapy with and without tirapazamine.[85] No significant difference in relapse rates or overall survival was seen between the two groups with a suggestion of poorer tolerance of treatment in those receiving tirapazamine. A separate but similarly structured Phase II trial in head and neck cancer showed non-significant trends favouring the tirapazamine arm in terms of local control and progression-free survival[86] and based on these results, a large Phase III trial of 861 patients was

carried out. Patients with Stage III/IV squamous cell carcinoma of the oropharynx, larynx, hypopharynx or oral cavity were randomised to receive definitive radiotherapy to a total dose of 70 Gy over 7 weeks concurrently with either cisplatin alone or cisplatin plus tirapazamine.[87] The addition of tirapazamine to chemoradiation was not shown to improve overall survival, progression-free survival or quality of life.

Mitochondrial inhibitors
Reducing the cellular oxygen consumption rate (OCR) is an attractive alternative strategy to overcome hypoxic radioresistance. Experimental studies have shown the anti-hyperglycaemic agent metformin to reduce the OCR and tumour hypoxia through inhibition of mitochondrial complex I[88,89] [88,89]. However, the observed reduction in the OCR was only around 10–20% prompting a search for agents with a similar mechanism of action that may have a more profound effect. In a high-throughput OCR screen of over 1500 pharmacological agents, the anti-malarial drug atovaquone was seen to reduce cellular oxygen consumption by more than 80% in a variety of cancer cell lines, an effect mediated by inhibition of mitochondrial complex III.[90] The investigators went on to demonstrate that atovaquone practically abolished tumour hypoxia in mice bearing FaDU (hypopharyngeal carcinoma) and HCT116 (colorectal carcinoma) xenografts and brought about a significant delay in FaDU xenograft tumour growth when delivered in combination with radiotherapy. In light of these encouraging pre-clinical results, an early phase I trial in the UK is now underway using functional imaging and circulating hypoxia markers to assess whether atovaquone reduces tumour hypoxia in patients with non-small cell lung cancer (ATOM).[91] If successful, larger clinical trials will be undertaken to investigate whether this inexpensive drug with an excellent toxicity profile improves the effectiveness of radiotherapy and results in a long-term clinical benefit.

Meta-analysis of randomised trials evaluating hypoxic radiosensitisation

It is well-established from experimental studies that solid tumours contain hypoxic regions and that cells within these islands are more resistant to radiation. Over the last 50 years, a number of clinical trials have evaluated the benefit of different methods of hypoxic radiosensitisation, although many have proved inconclusive, in part due to poor trial structure, practical difficulties and relatively small patient numbers. To address this, a recently updated meta-analysis has been performed analysing the results of 10,108 patients undergoing primary radiotherapy recruited to 86 different randomised studies evaluating various hypoxia-modifying therapies in a range of tumour types, shown in Table 2.[22]

The findings were analysed with respect to overall survival, local control, distant progression and treatment-associated complications. Locoregional control and overall survival were significantly improved by hypoxia modification; odds ratios being 0.77 [95% CI (0.71–0.86)] and 0.87 [95% CI (0.80–0.95)] respectively. No difference was seen with respect to the risk of distant progression or complications related to radiotherapy. Subanalysis of the local control data by tumour site showed the same positive

Table 2.Breakdown of studies and results from a meta-analysis of trials evaluating hypoxic modification of radiotherapy[22]

Descriptor	Number of trials	Outcome	
End point analysis		**Overall outcome**	
Locoregional control	70	OR 0.77 (0.71–0.84) in favour of hypoxic modification	
Overall survival	84	OR 0.87 (0.80–0.95) in favour of hypoxic modification	
Distant metastases	28	OR 0.93 (0.81–1.07) NS	
Radiotherapy-associated complications	21	OR 1.17 (1.00–1.38) NS	
Tumour site		**Locoregional control**	**Overall survival**
Head and neck	31	OR 0.73 (0.64–0.82) in favour of hypoxic modification	OR 0.86 (0.76–0.98) in favour of hypoxic modification
Uterine cervix	19	OR 0.80 (0.69–0.94) in favour of hypoxic modification	OR 0.91 (0.78–1.05) NS
Bladder	11	OR 0.82 (0.62–1.08) NS	OR 0.88 (0.65–1.18) NS
CNS	10	–	OR 0.86 (0.60–1.21) NS
Lung	10	OR 0.84 (0.61–1.17) NS	OR 0.83 (0.52–1.33) NS
Mixed (pancreas, oesophagus, other)	5	OR 0.71 (0.42–1.20) NS	OR 0.55 (0.24–1.24) NS
Method of hypoxic modification		**Locoregional control**	
HBO	26	OR 0.67 (0.57–0.80) in favour of hypoxic modification	
Normobaric oxygen and carbogen	5	OR 0.79 (0.58–1.09) NS	
Hypoxic radiosensitisers	54	OR 0.80 (0.72–0.89) in favour of hypoxic modification	

CNS, central nervous system; HBO, hyperbaric oxygen; OR, odds ratio.

overall trend across all sites, but a statistically significant benefit only in carcinoma of the head and neck and uterine cervix. Overall survival results were similar with favourable outcomes in all studies except CNS, but a significant benefit described only in head and neck. These observations support the hypothesis that squamous cell carcinomas may benefit the most from hypoxic modification of radiotherapy, hence, these tumours should be the main target group for further investigation.

Patient selection
Despite the negative findings of a number of individual clinical trials, the conclusions from the meta-analysis demonstrate an improved outcome when hypoxic radiosensitisation is utilised. The varied results between trials draws attention to the significant phenotypic heterogeneity amongst tumours of the same histology and site of origin. The recruitment of unselected patient populations to these studies is, therefore, likely to be a major contributor to the inconsistent results.

In order to maximise the future clinical efficacy of hypoxic radiosensitisation, there is a clear need for the development of biomarkers capable of selecting patients likely to benefit from treatment based on an accurate assessment of the oxygenation status of their individual tumour. Previous approaches to assessing tumour hypoxia include direct electrode measurements,[23–31] although this technique is limited by tumour heterogeneity and its invasive nature. Nitromidazole compounds have also been used successfully in clinical studies as exogenous chemical hypoxia probes. In a translational

substudy in the Phase III trial evaluating ARCON in laryngeal cancer,[65] tumour biopsies following injection of pimonidazole were obtained from 76 participating patients and analysed for the presence of hypoxia using immunohistochemistry. Using a cut-off value of 2.6% to dichotomize tumours into hypoxic and well-oxygenated categories, regional control was significantly improved by ARCON compared to accelerated radiotherapy in those patients with hypoxic tumours [100% *(ARCON)* vs 55% *(accelerated alone)*, *p* = 0.01]. This effect was lost in those with well-oxygenated tumours [96% *(ARCON)* vs 92% *(accelerated alone)*, *p* = 0.7], highlighting the importance of appropriate patient selection based on tumour biology in exploiting the ARCON approach. Despite the success of the pimonidazole assay as a predictive tool, there are concerns that this technique may not be sufficiently sensitive to detect more moderate hypoxic tumour phenotypes. Stabilisation of HIF-1α is thought to be mediated via the inhibition of proline hydroxylases that occurs upon removal of O_2. This has been shown to occur at higher oxygen tensions (0.4–1.6%) than those at which hypoxia-reduced nitromidazole stabilises via adduction to –SH-containing compounds.[92] More recently, therefore, attention has turned to analysis of tumours at a genomic level in the search for biomarkers predictive of the benefit of hypoxia-modifying treatment.

Hypoxic gene expression signatures
Hypoxia induces changes in gene expression and those up- and downregulated in response can be used as surrogate markers of tumour hypoxia. Gene expression can be quantified at either

the protein or mRNA level using immunohistochemistry or gene expression microarrays respectively. Those found to be substantially upregulated from their baseline normoxic levels are classically grouped together and referred to as a "hypoxia gene expression signature.[93] Various hypoxia signatures have been shown to have strong, independent prognostic value across a number of different tumour sites. Sorensen et al identified a group of genes upregulated by hypoxia in four separate tumour cell lines (cervical, hypopharygeal and three oral carcinomas) *in vitro*.[94] This was used to develop a 15 gene hypoxia expression classifier in head and neck carcinoma based on direct comparison of tumour hypoxia levels measured with oxygen electrodes.[95] The resulting 15 gene signature was subsequently validated and shown to be prognostic in a subset of 323 head and neck cancer patients in the DAHANCA 5 study.[95] Hypoxic signatures are prognostic across other tumour sites. Gene expression microarray data in 59 head and neck cancer clinical specimens was used to develop a hypoxic metagene comprising 99 genes upregulated in response to hypoxia.[96] This was shown to be prognostic in 295 breast cancer and 60 head and neck cancer patients. The metagene was subsequently reduced and evaluated in 4 independent data sets consisting of 80 head and neck, 216 lung and 295 breast patients. The resultant smaller common meta-signature had significant prognostic worth across all three tumour sites.[97]

Although the characterisation of prognosis using hypoxic gene signatures is useful, it is the predictive ability of the assay that is perhaps more important; the ultimate goal being a biomarker able to predict the benefit of hypoxic modification of radiotherapy for individual patients such that the appropriate population can be selected for. Two of the aforementioned prognostic gene signatures have been evaluated for their predictive value. Toustrup and colleagues analysed the outcomes of the 323 head and neck patients in the DAHANCA 5 study in whom their 15 gene hypoxia classifier was validated.[95] Patients were randomised to receive nimorazole with radiotherapy or radiotherapy alone. Those whose tumours were retrospectively selected out by the classifier as being "more hypoxic had significantly improved locoregional control with the addition of nimorazole whilst patients with "less hypoxic" tumours did not benefit from hypoxic modification. Buffa and colleagues evaluated their reduced hypoxia metagene in the two described Phase III randomised trials of hypoxic modification with carbogen and nicotinamide; the Dutch ARCON trial in laryngeal cancer[65] and the bladder cancer carbogen and nicotinamide trial (BCON) in the UK.[64] Laryngeal tumours classified by the signature as having a high hypoxia score derived significantly greater benefit from ARCON compared to those with a low score; 5 year regional control rates for hypoxic tumours being 100% *(ARCON) vs* 81% *(accelerated alone)* (*p* = 0.009) with corresponding rates for less hypoxic tumours being 90 *vs* 91% (*p* = 0.9).[98] The signature was not able to predict benefit from hypoxic modification in bladder cancer. This has since been investigated by Yang et al who derived a new 24-gene hypoxic signature through the analysis of transcriptomic data in bladder cancer available through public databases.[99] The classifier was tested

using tumour specimens from 76 patients in the UK BCON trial and was shown to predict benefit from adding carbogen and nicotinamide to radiotherapy, accounting for other clinical and histological factors. More recently, the same investigators used four independent cell lines to derive a gene expression signature reflecting hypoxia in prostate cancer.[100] The resultant 28-gene classifier was validated retrospectively in seven separate cohorts of patients with localised prostate disease and was shown to have strong prognostic value, the significance of which remained in multivariate analysis after accounting for various presenting tumour parameters. Interestingly, this signature was also evaluated using samples from the BCON trial and was shown to predict benefit from hypoxic radiosensitisation with carbogen and nicotinamide in bladder cancer patients.[100] This demonstrates the exciting prospect of common meta-signatures that have the potential to predict the benefit of hypoxic modification across various tumour sites and can therefore be used broadly to stratify patients and optimise therapy.

To date, studies have analysed the predictive strength of the various hypoxic gene signatures retrospectively. Moving forward, such classifiers now require prospective validation as biomarkers. This can be achieved in clinical trials through the upfront allocation of patients to hypoxia-modifying treatment based on their gene expression score. The common hypoxia metagene developed by Buffa and colleagues[97] is now undergoing prospective qualification in a randomised Phase III trial in head and neck cancer investigating the hypoxic modification of intensity-modulated radiotherapy with nimorazole.[78]

CONCLUSION

Over 100 years have passed since the initial report by Schwarz describing the importance of oxygenation in the response to radiotherapy. A substantial body of experimental and clinical evidence has emerged demonstrating that hypoxia is a major mediator of radioresistance and a meta-analysis of over 10,000 patients showed a significant local control and survival benefit with hypoxic modification of radiotherapy in some tumour types. The strong evidence in head and neck cancer in particular suggests that hypoxic radiosensitisation should be used far more frequently in this tumour site than is currently the case. Various factors may underlie the lack of uptake of hypoxic modification in routine clinical practice. It is unfortunate that the lack of potential financial gain with the use of relatively cheap and simple drugs does not attract significant commercial or pharmaceutical interest. However, inconsistent results from individual clinical trials have also not helped the cause. Whilst small patient numbers and underpowered studies are partly responsible, the inability to appropriately select patients likely to benefit has been a major obstacle. Traditional measures of hypoxia such as microelectrodes and chemical probes are unrealistic when considering implementation on a large scale. Recent results of hypoxic gene expression signatures suggest that this approach could enable accurate prediction of benefit from hypoxic radiosensitisation and suitable patient stratification. Prospective validation of these candidate

signatures is eagerly awaited and if successful may pave the way for a welcome renaissance of hypoxia-modifying therapies in modern radiation oncology.

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