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Hypoxia and prognosis: the oxygen tension mounts

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1. ABSTRACT

Hypoxia has been the subject of research within the radiotherapy community for many years. Until relatively recently, reliable methods to measure hypoxia were unavailable. However since the 1990's, a series of different methodologies have been developed which have allowed large-scale studies of the prognostic significance of this tumor-associated process. Within the same time-frame biological knowledge concerning hypoxia has dramatically increased and a paradigm shift has taken place in current thinking concerning the role of hypoxia not only in treatment resistance but also as a driving force in malignant progression and continued tumor growth. This review discusses the current view of hypoxia, the methods available to measure hypoxia and the prognostic information that has been attained.

2. INTRODUCTION: A SHORT HISTORY

The concept that hypoxia influences radiation response evolved over many decades. The first study that was retrospectively attributed to be the original practical demonstration of hypoxia modification of radiation was carried out by Swartz in 1912. He noticed that skin reactions were less severe if a radiation applicator was pressed tight to the skin. The conclusion was made that blood flow interruption caused a reduction in the effectiveness of radiation because a higher dose was required to cause erythema. Later on, this would be interpreted as the first manifestation of the effect of acute hypoxia. *Ascaris* provided the model for Holthusen to discover that eggs of the nematode were relatively radioresistant in the absence of oxygen in 1921. At the time this was incorrectly ascribed to absence of cell division

under hypoxic conditions. The German connection was completed in 1923 by Petry who made the observation that radiation inhibited germination only in the presence of normal oxygen levels; this was the first direct correlation between radiosensitivity and oxygen.

During the 1930s, JC Mottram established the importance of oxygen for radiosensitivity using tumor slices irradiated in the presence or absence of oxygen. This was taken further by his colleagues who included Hal Gray. It was Gray who became the luminary in the early field of hypoxia by studying the effect of oxygen on radiation-induced growth inhibition of the broad bean *Vicia faba*. The milestone study that established the concept of hypoxia in human tumors proved to be the careful study Gray performed with Hugh Thomlinson, a pathologist at Mount Vernon Hospital, that uncovered the concept of chronic or diffusion-limited hypoxia in tumor cords found within human bronchial carcinomas (1).

In the ensuing decades hypoxia became a prominent feature of radiobiological and radio-oncological study but the ability to measure hypoxia in human tumors in a reproducible and routine way was not realized until the early 1990s with the development of the computerized oxygen electrode (2, 3). In the same period extrinsic nitroimidazole probes that depended on hypoxia-specific bioreductive metabolism were being developed, initially for autoradiographic recognition of hypoxia and subsequently immunohistochemical or positron emission tomography detection (PET) (4, 5). In more recent years the growing repertoire of proteins known to be hypoxia-regulated has led to the development of antibodies against intrinsic surrogate markers of hypoxia and the emergence and proliferation of PET scanning technology has opened up avenues for more non-invasive methods to analyze hypoxia in human tumors.

Throughout this period, and until relatively recently, hypoxia was thought of as something cancer cells needed to surmount to survive, necessitating the formation of new blood vessels to escape from areas of low oxygen. However in the last few years there has been a paradigm shift in the understanding of hypoxia suggesting that it may be a prerequisite for continued tumor growth and malignant progression (6).

3. HYPOXIA IN TUMORS

Hypoxia is a common feature of many primary tumors at the time of diagnosis and arises due to an imbalance between the cellular oxygen consumption rate and the oxygen supply to the cells. Indeed, hypoxia is thought to be a required feature of tumors vital to their development and continued growth (7). Developing microscopic tumors induce a neovasculature to sustain their potential for unlimited growth and this is achieved through angiogenesis, vasculogenesis and intussusceptions (8). These processes result in sprouting of new vessels from preexisting vessels, involvement of stem cell endothelial precursor cells and division of large "mother" vessels into smaller daughter vessels. However, the structure and function of tumor blood vessels formed by these processes in tumors is flawed (9) in that they are usually irregular and disorganized, they are leaky, hemorrhagic, tortuous, and consist of vascular plexuses that tend to erupt. Tumor blood vessels may also have an incomplete endothelial lining or incorporate tumor cells into their lining by vascular mimicry. There is often a lack of appropriate receptors leading to a deficiency in flow regulation and intermittent stasis. These inadequacies can lead to perfusion-related reductions in oxygen delivery to tumor cells resulting in acute hypoxia which is often transient. The chaotic vascular function is accompanied by an equally chaotic vascular architecture (10) where the orderly arrangement of vessels seen in normal tissues, that has developed to precisely supply the oxygen needs of the tissue, is replaced by a network that contains areas of sparse vasculature or areas where the expansion of the tumor has resulted in diffusion distances that provide inadequate oxygen and nutrient supply. This and defects due to concurrent versus countercurrent blood flow within the tumor microvessel network lead to chronic hypoxia. Hypoxia can also be exacerbated due to tumor-associated or therapy-induced anemia can and is particularly intensified in tumors or tumor areas exhibiting low perfusion rates (11).

However, the deficient tumor vasculature is not the only factor that causes hypoxia in tumors. Hypoxia may develop because of the high intrinsic oxygen consumption rate found within actively proliferating tumors (12). Indeed, in developed tumors, sub-volumes with high proliferative activity may cause locoregional hypoxia which is not directly related to the degree of vascularity, perfusion, or oxygen supply (13). In addition there are other cells present in tumors that can deplete oxygen. Infiltrating white blood cells may add to this effect by lowering pO_2 in regions of active inflammatory activity. It is well known that tumor-associated macrophages co-localize with regions of hypoxia in many cancers; an alternative view of their function is that these immune cells may actually be the source of hypoxia, providing an alternative explanation for why they are found preferentially at low pO_2 in tumors.

Whatever the evolution of hypoxia there is no doubt that it is a therapeutic problem as it renders solid tumors resistant to sparsely ionizing radiation and some forms of chemotherapy. Moreover hypoxia-driven alterations in gene expression as well as hypoxia-induced genetic instability may increase resistance to therapy and add to the development of a more aggressive tumor phenotype and further impact on patient prognosis.

4. TECHNIQUES TO ASSESS HYPOXIA IN PATIENTS

An interesting and potentially confounding aspect of the tools available to measure hypoxia in patients is the diversity of the methods used to generate the information and the nature of the information they produce. The Eppendorf microelectrode became one of the most commonly used devices for oxygen tension measurement in tissue and became regarded as the “gold standard” as the technique demonstrated the ability to identify good

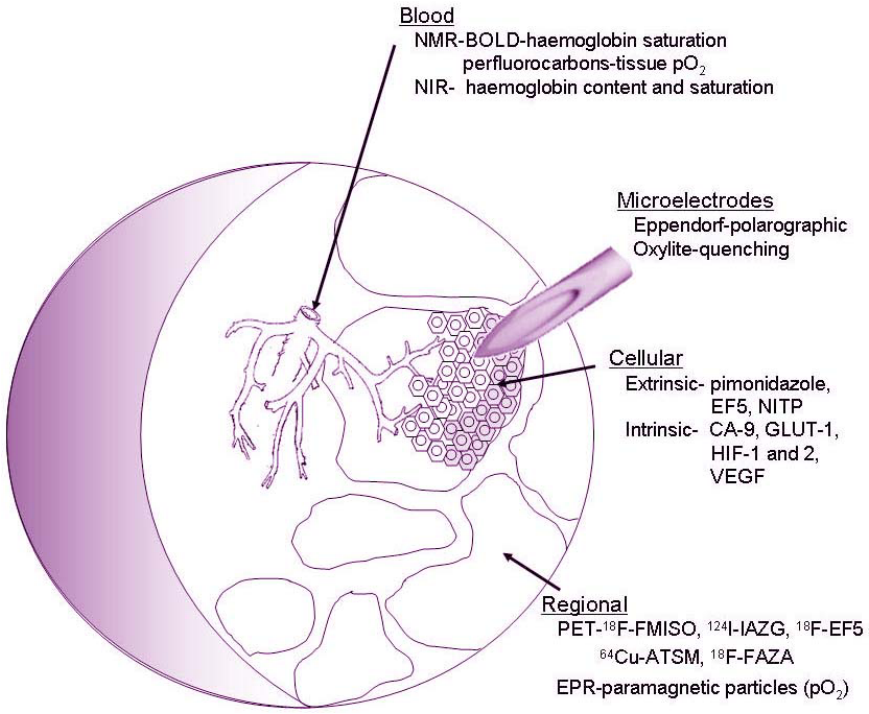

Figure 1. Schematic representation of the methods available to measure aspects of hypoxia in human tumors.

responders from poor responders in several clinical studies (14-19). However, as Olive and colleagues have pointed out (20) there may be problems in assuming that oxygen electrode measurements represent a single standard to which other methods must be compared and new methods should not be discarded on the basis that it fails to correlate with oxygen microelectrode measurements. The Eppendorf microelectrode is based on polarographic principles which mean that oxygen is consumed in the measurement process. The probe consists of a small central gold wire (the cathode) and a platinum wire (the anode) encased in a glass needle. The anode and the cathode are coupled to an electrical circuit, which transforms the signal coming from the cathode into minute changes in electrical current. The intensity of the generated electric current is directly proportional to the number of oxygen molecules that reach the electrode surface. The electrode is protected within a 300 micron metal needle which also functions as the means to penetrate tissue. The electrode measures in a volume, not at a point, and this volume is related to the size of the electrode. The early versions of Eppendorf electrodes had a 12 micron diameter central gold wire which was replaced with more robust 17 micron wire in later versions. Toma-Dasu and colleagues (21) simulated the response function of the electrode and noted that the measured value is given by the oxygen absorbed by the electrode and that this value is a weighted average of the values for oxygen tension in the 'measured volume'. Therefore, electrode measurements cannot be considered as giving a reliable distribution of intracellular oxygen concentrations in tissue. Additionally, although the 'pilgrim movement' technique was developed to minimize tissue damage, it is likely that this invasive method causes physical damage to the blood vessels and perhaps to individual cells in the tissues as the dimensions of the probe are very large compared with the cell dimensions.

The various methods to assess hypoxia in human tumors are outlined in (Figure1). Direct measurements of oxygen are difficult to make and so parameters related to oxygen delivery are often measured instead. Measurements of perfusion are an example of an indirect method that has been extensively and effectively used. NMR methods to measure pO_2 have appeal because of the widespread availability of NMR instruments and the potential for making noninvasive measurements. BOLD, or blood oxygen level dependent imaging, has been used in most NMR studies measuring changes in local oxygenation as it is sensitive to deoxyhaemoglobin. This method uses differences in the magnetic nature of oxyhaemoglobin and deoxyhaemoglobin and thus provides information related to tissue oxygen. However, the measurements only reflect changes in haemoglobin saturation and additional information is required to deduce absolute haemoglobin saturation or oxygen status. The latter has been studied by computing differences in MRI response as the patient breathes air (21% oxygen) or carbogen (95% oxygen:5% carbon dioxide) (22, 23). Although these methods do not measure pO_2 directly, they provide an indication of areas in tissue that may have compromised oxygen levels.

NMR can be used to make direct measurements of local pO_2 by measuring the oxygen-sensitive T_1 relaxation rate of perfluorocarbons (24). These compounds have to be introduced into the region of interest, either by direct injection or by vascular infusion. In the latter case, the localization of the perfluorocarbons is dependent on an,

as yet, unknown cellular uptake pathway, and deposition is limited to well perfused regions (25).

A more recent development of the perfluorocarbon technique is the use of ^{19}F echo planar magnetic resonance imaging–FREDOM (Fluorocarbon Relaxometry using Echoplanar imaging for Dynamic Oxygen Mapping), which reveals dynamic changes based on sequential maps of regional tumor pO_2 (26). This technique exploits the strong sensitivity of the spin lattice relaxation rate (R1) of hexafluorobenzene (HFB) to pO_2. Measurements in experimental tumors using the FREDOM approach have been shown to be comparable with measurements made using the Eppendorf microelectrode (27).

NIR (near infrared spectroscopy) measurements of haemoglobin can provide quantifiable data on intravascular haemoglobin content and saturation, thereby providing complementary data on the supply of oxygen to the tissue from the vascular system and the total oxygen capacity. Combining data concerning haemoglobin and pO_2 provides a complementary set of information to determine supply and demand for oxygen within a tissue (28).

EPR (electron paramagnetic resonance) oximetry is based on particulate oxygen sensitive paramagnetic materials and is capable of making repeated measurements of the pO_2 from the same site over times that can be varied from seconds to months and even years. Depending on the type of resonator that is used and the frequency that is employed, these measurements can be entirely non-invasive and can be made at depths that range from 10mm (using surface resonators and 1200MHz spectroscopy) to more than 80mm (using very low frequency spectroscopy in the range of 300–600MHz or more invasive resonators at 1200 MHz) (29).

Another development in the direct measurement of pO_2 has been the development of an oxygen sensing technology based on fluorescence. The commercial system is called the OxyLite and involves the insertion of a fiber-optic probe directly into the tumor or tissue. The technique uses a ruthenium complex-based fluorophore compound embedded in a rubber matrix at the tip of a 220 micron diameter fiber-optic probe. The fluorophore is excited by photodiodes emitting at 460nm and fluorescence lifetime signals are generated which are inversely proportional to the oxygen tension at the probe tip. The method is temperature-sensitive, thus quantification requires temperature correction, which is accomplished by thermocouples attached to the fiber-optic probes. The calibrations are stable and the sensitivity is highest at lower pO_2 values. The fiber optic oxygen sensor can be used to continuously monitor changes in pO_2 at a single location (30, 31).

Perhaps, the most exciting area of development for probes to measure hypoxia is in the field of PET. The attraction of PET is its non-invasive nature, the short half-life of the radionuclides use to label the probes enabling repeated measurements and the possibility to combine it with other imaging modalities such as CT to obtain more precise information. A number of PET tracers have been developed including the 2-nitroimidazole compounds fluoromisonidazole (^{18}F-MISO), ^{18}F-EF5 and fluoroazomycin arabinoside (^{18}F-FAZA) as well as the compound ^{64} or ^{60}Cu diacetyl-bis (N4-methythiosemicarbazone) (Cu ATSM). The current drawback of PET-based approaches to assess hypoxia is the limited resolution of 5 to 8 mm which probably undermines its current ability to provide reliable and informative prognostic information but doesn't inhibit its ability to assess changes in hypoxia markers during treatment (32, 33).

Most of the current studies assessing the impact of hypoxia on prognosis are carried out using biopsy techniques with either using two 2-nitroimidazole binding drugs that require injection or intrinsically-expressed hypoxia-regulated protein expression. At the present time, there are two injectable agents being studied pimonidazole (1-(2-nitro-1-imidazolyl)-3-Npiperidino-2-propanol)) (34, 35) and EF5 (36, 37) (nitroimidazole [2-(2-nitro-1H-imidazol-1-yl)-N- (2,2,3,3,3-pentafluoropropyl) acetamide). These two agents are injected intravenously up to 48 h preceding surgical biopsy or excision and activated by the same bioreductive mechanism. However, there are substantial differences in the chemical side chain which confers differences in their in vivo stability, pharmacokinetics, biochemistry and biodistribution.

Cells undergo a variety of biological responses in response to hypoxic conditions and changes in the expression of many genes occur depending on the severity and duration of exposure. Hypoxia-inducible genes regulate several biological processes, including glucose metabolism, cell proliferation, angiogenesis, metabolism, apoptosis, immortalization and migration. The expression of many of these genes is orchestrated by hypoxia-inducible transcription factor 1 (HIF-1). HIF-1 is a heterodimer that consists of the hypoxic response factor HIF-1alpha and the constitutively expressed aryl hydrocarbon receptor nuclear translocator (ARNT or HIF-1beta). In the absence of oxygen, HIF-1 binds to hypoxia-response elements (HREs), which activate the expression of many hypoxia-response genes (38). Hypoxia-specific protein expression has become an attractive surrogate for the assessment of tumor oxygenation status in clinical specimens as it requires no injection and can be assessed using simple immunohistochemical staining. However, there are many caveats to the interpretation of the staining and the reliability of the markers to reflect hypoxia. HIF-1, which activates many of the genes studied in this context, is not exclusively regulated by hypoxia. Growth factors (IGF-1 and 2), cytokines (TNF- alpha, IL-1beta), nitric oxide and genetic alterations leading to gene overexpression (v-src) or gene inactivation (PTEN, p53, pVHL) have all been shown to influence the regulation of HIF-1 (39-41). The oxygen concentration dependence and the temporal pattern of activation of gene expression will also be a complicating and largely unknown variable in human cancers. Nevertheless, HIF-1alpha, GLUT-1 and CA-IX have been

Table 1. Methods to measure hypoxia in human tumors

Method	Advantages	Disadvantages
Probe-based methods Eppendorf OxyLite™	measures pO_2 directly multiple measurements measures pO_2 directly, makes multiple measurements, doesn't consume O_2, can be combined with laser Doppler blood flow, sensitive at low pO_2 values	Invasive, restricted to accessible cancers, consumes O_2, inability to distinguish necrosis from hypoxia, unable to distinguish patterns of hypoxia invasive, restricted to accessible cancers, inability to distinguish necrosis from hypoxia, unable to distinguish patterns of hypoxia, tends to smooth out heterogeneity due to larger measuring volume, clinical probe problems
Nitroimidazoles Pimonidazole EF5	similar O_2 dependence to radiobiological hypoxia, measures on a cell-by-cell basis, stable and soluble, can be combined with other biomarkers to give information on patterns of hypoxia inverse proportional binding to O_2 over entire physiological and pathological range, measures on a cell-by-cell basis, quantitative methodology to measure maximal binding, can be combined with other biomarkers to give information on patterns of hypoxia	indirect, requires injection and biopsy depends on nitroreductases in tissue, may not detect perfusion-limited hypoxia due to half-life and interval between injection and biopsy, perfusion may limit access to hypoxic cells indirect, requires injection and biopsy, long half-life and may not detect perfusion-limited hypoxia due to half-life and interval between injection and biopsy, perfusion may limit access to hypoxic cells
Hypoxia-regulated proteins HIF-1 CA-9 and GLUT1	intrinsic, stabilization coincides with radiobiological hypoxia, can be studied retrospectively, measures on a cell-by-cell basis, can be combined with other biomarkers to give information on patterns of hypoxia intrinsic, depend of HIF-1 activation by hypoxia, , can be studied retrospectively, measures on a cell-by-cell basis, can be combined with other biomarkers to give information on patterns of hypoxia	Can be activated in the presence of oxygen by growth factors, cytokines, oncogenes leading to, biopsies may not be representative Temporal pattern of activation, unlikely to reflect perfusion-limited hypoxia, hypoxia-independent upregulation of HIF-1, biopsies may not be representative
Imaging methods BOLD-MRI ^{19}F NMR EPR PET	non-invasive, high spatial resolution, and real-time detection of fluctuations in oxygenation/perfusion NMR spin lattice relaxation rate of the PFC is enhanced in direct proportion to the dissolved O_2, using FREDOM, repeated quantitative maps of regional pO_2 can be monitored measures absolute pO_2 with minimal invasiveness, sensitive (variations of less then 1mmHg can be measured), make s measures in a few seconds, measurement at the same site over long periods of time non-invasive, combine with CT, repeated measurements, combine with other probes	indirect measurement of O_2 through deoxyhaemaglobin saturation, unable to distinguish patterns of hypoxia indirect measurement of O_2 through PFC relaxation, PFC spin–lattice relaxation rates may also depend on other physiological or histological parameters eg temperature, unable to distinguish patterns of hypoxia instrumentation costs, probes for human use unable to distinguish patterns of hypoxia low spatial resolution, nitroimidazole probes may be limited by perfusion

put forward as strong candidates as surrogate hypoxic markers (42).

5. WHAT IS THE BEST MEASURE OF HYPOXIA?

In the preceding section the techniques that are available to measure aspects of hypoxia were briefly outlined but the question that arises is -which is the best one to consider? Each of them has their advantages and disadvantages as outlined in (Table 1). A key feature in deciding which should be the method of choice is deciding what aspect of hypoxia is clinically relevant. The field of hypoxia research has not been without controversy. Many years of attempts to overcome hypoxia within tumors using oxygen modification or radiosensitizing drugs was summarized by Overgaard and Horsman (43) in 1996 whose overview analysis showed that hypoxia modification significantly, although modestly, improved the locoregional tumor control after radiotherapy with an odds ratio of 1.21 after considering 30 years of clinical trials in 10,000 patients. However, head and neck cancer (H&N) and, to a lesser extent bladder cancer, were the only two tumor sites where benefit was significant. The lack of success of drug-based sensitizers could partly be attributed to the administration of inadequate drug due to patient toxicity, drug hydrophilicity, perfusion-limited access to hypoxic areas and scavenging by endogenous thiols. However, the same is not true for the application of hyperbaric oxygen therapy and although there was an overall improvement in locoregional control with the combined modality (62%, versus 53%, $p<0.0001$) in 19 trials encompassing 2488 patients with tumors at various sites, this was mainly evident in H&N cancer and also associated with unconventional fractionation schemes (44). Some of the technical limitations and toxicity of applying hyperbaric oxygen in conjunction with radiation have been circumvented by the more recent use of carbogen-breathing in the ARCON schedules (45). However, as with all the other studies of oxygen modification, only H&N and bladder cancer (46) demonstrate a potential benefit from overcoming hypoxia.

The issue of hypoxia is not straightforward. Different forms of hypoxia exist in tumors and different oxygen levels exist in tumor cells. Figure 2 presents a view that dissimilar compartments within the hypoxic component of tumors may have different clinical implication. Hypoxia, indicated by pimonidazole binding, has been observed to be associated with keratinized areas in well differentiated squamous cell cancer of the head and neck (47). Moreover, it was shown that this was not a consequence of locally high reductase levels suggesting that this staining pattern was consistent with a chronic-type of hypoxia caused by differentiating cell migration from the vascular supply by the formation of new cells in the dividing layers close to the vessels. In (Figure 2) this type of hypoxia is considered to be of little clinical threat as differentiating squamous cells are already on a pathway to maturation and death. However, this form of hypoxia would not be discriminated by oxygen probe-based methods or any of the imaging modalities. The limited data set of Janssen and colleagues demonstrated that areas of keratinization were present in 10 of the 20 (50%) tumors and, of a total of 64 areas scored for pimonidazole, 36 (56%) were positive.

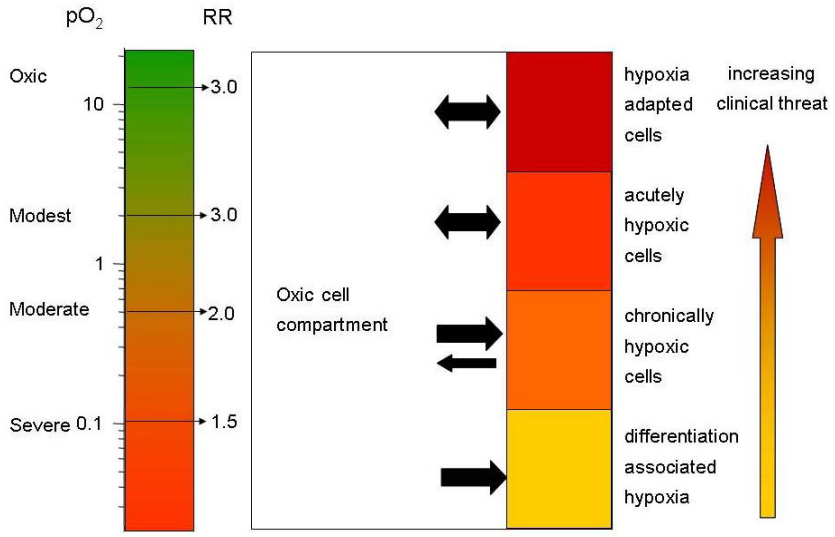

Figure 2. Different hypoxic compartments within tumors and the relationship between hypoxia and relative radiosensitivity (RR).

The concepts of perfusion-limited (acute or transient) and diffusion-limited (chronic) hypoxia has been the subject of much debate (48) and their implications for treatment outcome have yet to be fully elucidated. Chronic hypoxia occurs towards the end of the nutrient gradient around each blood vessel and develops slowly and progressively worsens, leading eventually to starvation-induced cell death. Acute hypoxia is more rapidly fluctuating and caused by the closure of individual capillaries or by blood flow ceasing or reducing significantly for some minutes or hours. Do these two forms of hypoxia have the same effect on the cellular response? The distinction between chronic and acute is important because cells exposed to these different types of hypoxia will differ in energy supply, gene expression, and DNA repair capacity and thus sensitivity to drugs or radiation. Experiments with both hypoxia and nutrient deprivation, or just prolonged hypoxia extending into the post-irradiation period, have shown that the shoulder on the single-dose survival curve is diminished or lost, and the ability to repair between fractions is also lost (49, 50). In 4 xenografted human tumors, chronic hypoxia was associated with increased radiosensitivity (51) attributed to a general breakdown of cellular metabolism. However, in human tumors this is only likely to be true of those chronically hypoxic cells that have endured significant oxygen and nutrient deprivation for a prolonged period. Many of the cells that are affected by diffusion-related issues will be at intermediate oxygen tensions. Similarly, it is likely that much of the hypoxia associated with perfusion issues will also tend to be in the intermediate oxygen range due to the transient nature of microvessel fluctuations in blood flow (52).

Modeling has suggested that the tumor response to radiation is highly dependent upon the cells at oxygen levels intermediate between fully oxygenated and hypoxic within the range of 0.5-20 mm Hg (0.06-2.6% O_2) (53). This study also suggested that, for diffusion-limited hypoxia, the impact of full reoxygenation between fractions is much smaller than previously realized. Taken together, it would seem that acute hypoxia may present a greater clinical threat than chronic hypoxia (Figure 2).

Before addressing the issue of hypoxia adaptation, the levels of hypoxia found within human tumors will be addressed. On the left side of Figure 2 is a hypothetical nomogram relating oxygen concentrations to relative radiosensitivity (RR) and a classification of the severity of hypoxia taken from Evans and Koch (54). Taking H&N cancer as an example, the hypoxic fraction measured as the percentage of cells binding pimonidazole had a mean value of 6% in a small series of 20 patients prospectively studied (55). Importantly, all 20 tumors showed some evidence of hypoxia as the range varied from 0.3 to 17.2%. In a recent analysis of multicenter data on 397 H&N patients assessed by the Eppendorf microelectrode, the median pO_2 value was 9mm Hg (1.2% O_2) with 38% of values below 5mm Hg (0.66% O_2) and 19% below 2.5mm Hg (0.33% O_2). Therefore it is likely that the majority of hypoxic cells reside in the moderate to modest classification. Modeling has suggested that the hemispheric detection volume of a 17mm Eppendorf microelectrode samples in the region of 500 cells and suggests that the electrode can never determine the absence of oxygen in a single anoxic cell layer or even two adjacent cell layers because of contamination by better-oxygenated layers that are within its detection volume (56). This led to the conclusion that, when very low pO_2 values are detected by the probe, it is likely to be measuring areas in which blood flow has temporarily ceased, i.e. areas of acute hypoxia.

The final compartment of the hypoxic fraction is hypoxia-adapted cells. This concept has been brought to the forefront by Peter Vaupel and his colleagues (57-60). Again, the influence of hypoxia on tumor growth is not straightforward. On the one hand hypoxia-induced

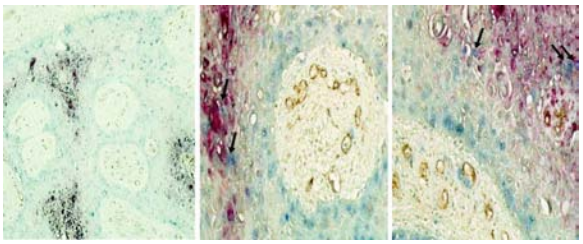

Figure 3. Triple immunohistochemical staining of a recurrent squamous cell carcinoma of the bronchus for pimonidazole (purple), Ki-67 (blue) and CD31 (brown). On the left is a x100 image, the middle image was captured at x200 and the right hand image was taken at x 400. The arrows indicate the presence of proliferating cells within hypoxic regions.

proteome changes in tumor and stromal cells may lead to the arrest or impairment of neoplastic growth through molecular mechanisms resulting in cellular quiescence, differentiation, apoptosis, and necrosis. Whilst on the other hand, hypoxia-induced proteome changes in tumor and/or stromal cells may promote tumor propagation by enabling the cells to adapt to nutritional deprivation or to escape their hostile environment. Genomic changes that reduce the potential for cell cycle arrest, differentiation, and apoptosis may favor hypoxia-associated mechanisms that promote tumor growth and dissemination, such as stress adaptation, anaerobic metabolism, angiogenesis, tumor cell detachment and subsequent adhesion, tissue remodeling, and migration (60). The scenario that is now envisaged by many researchers is that hypoxia is involved in a vicious circle which is a fundamental biological mechanism for the progression of malignant disease through clonal selection of neoplastic cells with persistent genomic changes leading to apoptotic insensitivity and increased angiogenic potential which stabilizes and further aggravates tumor hypoxia.

The questions that arise from this concept are:

- What proportion of cells can be considered to be hypoxia-adapted in tumors?
- Do these cells exist in both the hypoxic and oxic compartments?

The biology predicts, in some tumors, this population will be a self-perpetuating, highly resistant sub-population that will be found within the oxic and hypoxic compartment depending on the physiological remodeling of the tumor as it progresses. A hallmark of this phenotype is that cells may continue to proliferate in spite of hypoxia. Figure 3 shows an example of this phenotype (unpublished observations). These images were obtained using a novel triple immunohistochemical staining protocol in a biopsy from a patient with recurrent squamous cell carcinoma of the bronchus who received an injection of pimonidazole, 16 hours prior to the surgical procedure. The specimen was stained for pimonidazole to identify hypoxic cells (purple), Ki-67 to identify proliferating cells (blue) and CD31 to identify blood vessels (brown). The low power image (x100) shows a pattern that would be consistent with diffusion-limited hypoxia. The blood vessels are associated with the stroma surrounding, and within, the tumor islands. The blue proliferating cells are found in the peripheral layers of the tumor islands close to the blood vessels. Hypoxia develops at a distance from the blood supply. In the higher power images, the existence of proliferating cells (blue) within the areas of hypoxia are indicated by the arrows. Of course, this is anecdotal but in a prospective study of 31 bladder cancer patients whom were also injected with pimonidazole and dual stained for hypoxia and proliferation (Ki67), we were able demonstrate significant proliferation close to and within hypoxic regions (61). More recently the co-localization of CA-IX with an injected marker of DNA synthesis, iododeoxyuridine (IdUrd), has been studied (62). There was a high degree of overlap between IdUrd and CA-IX which was highest at intermediate distances from the blood vessels (100-150 micron) and tumors with the highest fractions of co-localization had the worst disease-free survival rates. These observations dispute the view that hypoxia and proliferation are "mutually exclusive" in human tumors and suggests the presence of a population of tumor cells with proliferative capacity can certainly exist under intermediate hypoxic conditions and may be associated with tumor aggressiveness and the worst disease-free survival rates.

So what matters most; overall or regional hypoxia or insidious subpopulations of hypoxia-adapted cells? This is a fundamental question that can only be answered using multiple different techniques. So far, there has been no correlation between hypoxic fractions measured by oxygen electrodes and that obtained with pimonidazole in patients (63, 64). A multitude of studies have reported correlations or lack of correlations between different intrinsic proteins (mainly HIF-1alpha, GLUT-1 and CA-IX) and various proven assays of hypoxia (mainly oxygen electrodes and pimonidazole) (20, 42, 61, 63, 65-68). Whilst, it is biologically and clinically important to explore these correlations, the fact that correlations do not exist or are weak may not be important as the different methods are relaying different information about hypoxia (Figure1) and may have different prognostic significance. For instance when CA-IX expressing cells were isolated by flow cytometric cell sorting form SiHa human tumor xenografts, they were found to be clonogenic, radioresistant and preferentially able to bind pimonidazole (69). More recently, activation of CA-IX has been shown to take place in a HIF-1 independent manner under conditions of high cell density but low oxygen concentration (70). These observations might suggest that CA-IX might identify cells that are associated with hypoxia adaptation and important for local control and survival. Irrespective of which is the most important aspect of hypoxia to measure, there are now a battery of techniques that can provide salient information on each of the different facets of hypoxia.

6. HIGHLIGHTS OF HYPOXIA AND PROGNOSIS

Rather than give a comprehensive and repetitive review of studies where a hypoxia-based assay has been correlated with prognosis, this section will discuss some notable recent studies.

The work of Nordsmark and colleagues has been influential in proving the utility of the Eppendorf oxygen electrode measurements in H&N cancer. Her early study (71) established that high levels of hypoxia were associated with decreased locoregional control (33% versus 77% at 2 years). The prognostic significance of this observation was validated and consolidated in an independent confirmatory study (18) where, using the same cut-offs, 2-year locoregional control was 90% in patients with "well oxygenated" tumors compared to 45% in hypoxic tumors. The significance of pO_2 measurements in H&N cancer can now be considered unequivocal with the publication of the multicenter analysis of nearly 400 patients (72). This analysis demonstrated that the pre-treatment tumor oxygenation status, expressed as the proportion of values less than 2.5 mmHg ($HP_{2.5}$), was the most significant predictor of survival in univariate and multivariate analysis for this group of patients treated by radiotherapy alone or in combination with surgery, chemotherapy or a radiation sensitizer. Furthermore, the analysis, using $HP_{2.5}$ as a covariate, supported a monotonic relationship between oxygenation and prognosis, rather than a threshold effect, suggesting that prognosis in very hypoxic tumors is extremely poor. However, the heterogeneity introduced by combining data from different centers did reduce the prognostic separation between oxic and hypoxic tumors with only a ten percentage point difference (38% versus 28% respectively) at 3 years. In addition, the analysis left tantalizing unanswered questions as well as important considerations for other types of study. First, even though loco-regional tumor control and death from disease are the most relevant endpoints among H&N cancer patients, these data were not available for the entire population and overall survival time was used as the treatment outcome measure instead. Second, only one of the studies employed the use of a radiosensitizer (nimorazole; 67 patients) but data was not presented on the differential outcome of hypoxic versus oxic tumors treated with or without a sensitizer. Third, only a weak and non-linear correlation was found between tumor hypoxia and haemoglobin concentration and the latter parameter did not prove significant in multivariate analysis after adjusting for $HP_{2.5}$; this may have implications for some of the NMR methods that rely on haemoglobin parameters.

The discriminatory power of hypoxia measurements to identify patients who would benefit from oxygen modification has now been demonstrated by Kaanders and colleagues (73) but should be treated with some caution as only 38 patients were available for analysis and these were distributed among 4 groups of interest. However in this small and heterogeneous data set, the group of patients who had the worst outcome were those with hypoxic fractions greater than the median and who did not receive ARCON. This study also provided two other interesting observations. Microvessel density provided similar prognostic information to pimonidazole despite a lack of mutual correlation whilst CA-IX demonstrated no prognostic power in spite of a correlation with pimonidazole.

CA-IX has become the most popular and studied potential surrogates for hypoxia. Carbonic anhydrases are wide-spread enzymes, present in mammals in at least 14 different isoforms. Some of the isozymes show cytosolic localization (CA-I, CA-II, CA-III, CA-VII, CA-XIII) whereas others, including CA-IX, are membrane-bound (CA-IV, CA-IX, CA- XII and CA-XIV) (74) Carbonic anhydrases are zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide ($H_2O + CO_2 = H^+ + HCO_3^-$and are vital to many biological and physical functions. CAIX is one of over 50 genes that are inducible by hypoxia through HIF-1alpha. Recently, it was shown that hypoxia regulates both expression and activity of CA-IX in order to enhance extracellular acidification (pHe) which is likely to be vital in tumor survival under conditions of low pH and hypoxia (75). Experimental evidence has shown that CA-IX protein levels steeply increase from 4 to 24 hour under hypoxia (76) and that CA-IX induction occurs at a threshold of pO_2 of 20 mmHg (2.6% O_2) and downward (77). Several threads of evidence suggest that CA-IX expression is linked to chronic hypoxia. It was shown that that glioblastoma cells, exposed to 1.5% O_2 had much more elevated levels of both CA-IX message and protein at 24 hours compared to earlier times (78). The strongest staining for CA-IX correlates with strong pimonidazole binding in areas of human H&N tumors that are consistent with chronic hypoxia (73). However, weaker binding of CA-IX has been noted closer to blood vessels and may be caused by modest levels of hypoxia or through the HIF-1 independent pathway involving phosphtidylinositol-3-kinase signaling under conditions of high cell density but low oxygen concentration as previously discussed (70). Also, as mentioned previously (62), significant overlap between CA-IX and proliferating cells has been noted in H&N cancer cells likely to be at intermediate oxygen tensions.

In a recent review of intrinsic markers of hypoxia (42), 5 studies involving CA-IX and prognosis were discussed. Three of the studies, in cervix (68), H&N (79) and non-small cell lung cancer (80), showed a significant association of high CA-IX with worse prognosis whilst two studies both from the H&N region (73, 81) were not significant. Since that review, two further studies have emphasized the prognostic significance of CA-IX in H&N cancer (82, 83). In a series of 67 patients treated by radiotherapy with and without chemotherapy, the combined assessment of patients with both CA-IX and GLUT-1,expressed above the median, showed an independent correlation with local control ($p = 0.02$) and disease-free survival ($p = 0.04$) with a trend for regional control ($p = 0.06$) (83). In a larger study of 198 patients treated in the randomized trial of CHART versus conventional radiotherapy, overexpression of both CA-IX and HIF-2alpha showed independent association with locoregional control ($p = 0.0002$ and $p < 0.0001$, respectively) and poor survival ($P = 0.002$ and 0.0004, respectively). Both markers maintained their independent prognostic significance in multivariate analysis. CA-IX and HIF-2alpha represent proteins from two different hypoxia response pathways and their co-expression had an additive effect, supporting their independent role (82). The utility of CA-IX in H&N cancer needs to be underlined by a definitive study to show that modification of hypoxia

benefits patients with high levels of this hypoxia surrogate marker. As yet, there has been not been a substantial study in a retrospective series of patients treated with sensitizers, hyperbaric oxygen tirapazamine or ARCON.

Several studies have now demonstrated the prognostic significance of CA-IX overexpression in non-small cell lung cancer (NSCLC) (84-88); these studies were carried out in surgical resections. The largest of these studies (84) investigated the interaction of EGFR with CA-IX and MMP-9 and developed a model in which the study population was divided into three groups. They found that the group that had co-expression of EGFR with CA-IX or MMP-9 or both (n=70) had the worse prognosis than those groups which were composed of tumors which either had expression of EGFR without co-expression of MMP-9 or CA-IX (n=21) or had no expression of EGFR (n=75). In this study, the investigators drew a distinction between different patterns of CA-IX staining which they classified as perinuclear , membranous and cytoplasmic. The perinuclear staining pattern had the strongest association with EGFR and was used in the survival analysis. In Cox regression analysis, tumors which had co-expression of EGFR with CA-IX or MMP-9 or both had a hazard ratio of 3.25 (p=0.0004).

Two studies of CA-IX in bladder cancer have both shown an association between CA-IX overexpression and shorter overall survival (89, 90). The study of Hoskin and colleagues was carried out in a series of 64 patients previously treated for invasive bladder cancer using ARCON; GLUT-1 was also studied. In univariate and multivariate analyses both CA-IX GLUT-1 and were independent predictors for overall and cause specific survival. but not for local control or metastases-free survival. Unfortunately, all patients in this study received ARCON so no discrimination based on treatment was possible.

CA-IX has been studied a variety of other cancers including renal (91-93), breast (94, 95), soft tissue sarcoma (96), astrocytic tumors (97) and gastric and oesophageal cancer (98) where overexpression has been universally associated with poor prognosis and survival. Interestingly, a sizeable study (n=110) of CA-IX in locally advanced cervical carcinoma treated with radiotherapy or chemoradiotherapy (66) did not confirm the prognostic significance seen in an earlier study of cervix cancer (68) in spite of pO_2 measurements being strongly correlated with outcome.

Before discussing the merits of GLUT-1 and HIF-1alpha an interesting series of papers has been published by Mayer and colleagues which have assessed the direct correlation between CA-IX (99), GLUT-1 (100) and HIF-1alpha (101) with oxygen electrode measurements by performing staining on biopsy specimens taken from the tumor pO_2 measurement tracks obtained directly after pO_2 measurement in locally advanced cervix cancer. Each paper had the same message, none of the hypoxia-regulated proteins correlated with any of the parameters generated from the pO_2 data. The authors interpret the data as stating that none of the markers should be considered as reliable substitutes for hypoxia measurements. However, this is may be somewhat unfair interpretation. The pO_2 measurements were made by taking 36 readings in each of two tracks, starting at a tissue depth of 5 mm, with a 0.7 mm separation between readings. This resulted in an overall measurement track length of ~25 mm. The needle core biopsies were ~2 mm in diameter and 20 mm long and were taken immediately following pO_2 measurements. In each of the papers, the figures, with examples of the staining patterns, clearly show the expected unpredictable microregional expression patterns of the markers. It is certainly feasible that the needle track could progress down a "vein" of hypoxia or an uninterrupted region of oxia. Furthermore, the hypoxic markers were expressed in a semi-quantitative manner and intensity was not taken into account whilst the electrode measurements were quantitative. These studies highlight the sampling problems that can exist and emphasize that the two different approaches might provide different but complementary information.

GLUT-1 belongs to an expanding family of known mammalian glucose transporters. Two different families have been identified, sodium glucose cotransporters (SGLTs) and facilitative GLUTs (102). Thirteen members of the family of facilitative GLUTs (GLUT-1–GLUT-13) are known and these various transporters consist of 12 transmembrane domains and are only capable of carrying glucose and other sugars down a gradient. They exhibit different substrate specificities, kinetic properties, and expression in tissue depending on the cellular demand and regulation. GLUT-1, which is almost ubiquitously expressed in all cell types, mediates glucose transport into erythrocytes and through the blood–brain barrier and its expression is also upregulated in many tumors (103). Since the review of Vordermark and Brown, GLUT-1 has been studied in a variety of different tumor types. As was the case with CA-IX, overexpression of GLUT-1 has been associated with worse prognosis in H&N cancer (83, 104, 105), breast cancer (106, 107), NSCLC (108, 109), bladder cancer (90, 110, 111), gastric (112) and oesophageal cancer (113, 114). In addition, similar findings have been published in colorectal (115-117), ovarian (118), thyroid (119) and gallbladder cancer (120). Furthermore, even though GLUT-1 showed no significant correlation with pO_2 measurements in locally advanced cervix cancer, overall survival ($p = 0.004$) and recurrence-free survival ($p = 0.007$) were significantly shorter for patients with expression of GLUT-1 (100).

The HIF system and its role in angiogenesis, glucose and energy metabolism, tumor development, and ischaemic disease has bee the subject of much interest (121). HIF is composed of an alpha/beta heterodimer of two proteins both of which contain basic-helix–loop–helix PAS domains, that bind to a core DNA sequence (G/ACGTG) in hypoxia response elements (HREs) coupled to target genes. Transcriptional regulation by oxygen is mediated by the alpha subunits of which three forms (HIF-1alpha, HIF-2alpha, and HIF-3alpha) have been defined in humans, each encoded by a distinct gene locus. HIF-1 is a

key regulator of the cellular response to hypoxia and HIF-1alpha, and to a lesser extent HIF-2alpha, has attracted much attention as a potential surrogate to identify hypoxic cells in tumors (42).

In H&N cancer, there were contrasting observations made concerning HIF-1alpha overexpression and clinical outcome in the recent review (42). Two studies involving radical radiotherapy-treated patients both showed a negative impact of overexpression on several clinical endpoints whilst a surgical series suggested that overexpression was favorable for outcome. More recently, this same observation has been made on early stage tumors of the oral floor also surgically resected (122). The authors suggest that HIF-1alpha expression in these tumors may not be hypoxia-driven but due to alterations in oncogene or tumor suppressor genes. However, why this scenario should be associated with better prognosis is still unclear. Another recent study on surgical specimens failed to demonstrate any prognostic information concerned with HIF-1alpha (123).

There have been no further studies of HIF-1alpha in H&N cancer patients treated by radiotherapy although HIF-2alpha overexpression was strongly correlated with worse local and survival in specimens from the CHART trial (82). In cervix cancer, the opposite of H&N cancer was reported. One study of mainly surgically resected tumors (although some also received radiotherapy) showed worse disease-free survival when HIF-1alpha was overexpressed (124) whilst a radiotherapy treated cohort showed no influence (125). Four more recent publications have added to the confusion (67, 126-128). In one study of advanced stage disease, where all patients were treated with radiotherapy, there was a significant positive correlation between high HIF-1alpha expression and the recurrence-free survival rate ($p = 0.04$) but not with local control rate whereas the HIF-1alpha status predicted distant metastasis with strong significance ($p = 0.03$) (126). In a second study of radiation-treated patients, a similar result was found; HIF-1alpha overexpressing cancers showed a significantly shorter local progression-free survival ($p = 0.04$) and overall survival ($p = 0.01$) (127). A third study also showed that strong/moderate expression of HIF-1alpha was an independent prognostic factor for shorter progression-free survival and cervical cancer-specific survival (128). Interestingly, the authors also found no association between HIF-1alpha expression and infection with different HPV types. Conversely, another recent study concluded that HIF-1alpha had no prognostic significance in locally advanced cervix cancer treated by radiotherapy (67).

HIF-1alpha continues to provide prognostic information in breast cancer. It was previously demonstrated to have be an independent factor for unfavorable prognosis in patients with lymph node-positive breast cancer (129). More recently, high levels of HIF-1alpha had an association of borderline significance with decreased overall survival ($p = 0.059$) and disease-free survival ($p = 0.110$) in a study of 150 early breast cancer patients (130). Sub group analysis revealed that women with lymph node negative tumors were most at risk if HIF-1alpha was overexpressed where the overall survival was 96% in the low HIF-1alpha group and only 77% ($p=0.008$) in those tumors with high levels; a similar result was found with disease-free survival. In contrast a study of 77 patients with node positive disease also showed a significant reduction in survival if HIF-1alpha was overexpressed (131). In the largest study of any hypoxic marker to date, 745 unselected patients with invasive breast cancer have been studied (132) Univariate analysis showed that high levels of HIF-1alpha expression (cutoff = 10%) significantly correlated with poor overall survival ($p = 0.019$) and with high metastasis risk among the whole group of patients ($p = 0.008$). Multivariate analysis showed that the HIF-1alpha predictive value was independent of other current prognostic indicators. In addition, HIF-1alpha was significant for metastasis risk ($p = 0.03$) and relapse ($p = 0.035$). In a study of 200 patients with invasive breast cancer HIF-1alpha had a significant negative relationship with between disease-free survival ($p = 0.01$) with a relative 2.2 fold increased risk for recurrences compared with the HIF-1alpha negative group (106). The authors classified the staining pattern as diffuse or perinecrotic and noted that the diffusely overexpressed HIF-1alpha was not associated with either CA-IX or GLUT-1 expression and that these patients had a significantly better prognosis than patients with perinecrotically overexpressed HIF-1alpha.

In bladder cancer there has been mixed results with HIF-1alpha. In a series of 93 primary transitional cell carcinomas, patients characterized by HIF-1alpha overexpression had significantly worse overall ($p=0.009$) and disease-free survival ($p=0.03$) (133). In a study of superficial and invasive human bladder cancer, there was a significant association of HIF-1alpha expression with recurrence and survival in superficial disease but not invasive (110). Patients with invasive bladder cancer all received radical radiotherapy and it was GLUT-1 that showed s a significant association with survival. In 140 patients with superficial primary urothelial bladder HIF-1alpha overexpression had only a marginal adverse influence on progression-free survival ($p = 0.058$), but when combined with p53 overexpression, the unfavorable impact was statistically important ($p = 0.028$) (134).
High levels of HIF-1alpha have been significantly associated with worse clinical outcome in a variety of other solid tumors including oesophagus (135, 136), glioblastoma (137), pancreas (138), colorectal (139, 140) and renal cancer (141). However, in gastric (142), mesothelioma (143) nodular melanoma (144) and NSCLC (88, 145), HIF-1alpha has shown little or none prognostic significance.

The previous sections underscore that intrinsic surrogate markers of hypoxia can certainly provide prognostic information but a feature of many of the studies is that they have been performed outside of the radiotherapy setting and very few have studied the consequences of oxygen modification. Perhaps one of the most interesting and promising studies that has been published recently is the work of Rischin and colleagues using ^{18}F-MISO PET scanning in the TROG98.02 trial which compared conventional radiotherapy combined with either cisplatin and 5-FU or cisplatin and tirapazamine (32).

Although the number of patients entered into the imaging sub-study was small (45 patients), the results hold promise for the future. Overall, hypoxia was detected in 32 of 45 patients (71%); this was defined with reference to ^{18}FDG uptake. The ^{18}F-MISO scan was interpreted to be positive if there was greater activity within the sites of tumoral uptake of ^{18}FDG than the activity present in adjacent or mirrored soft tissue sites. In patients treated with the chemotherapy/radiation schedule, one of 14 (7%) patients without hypoxia experienced local failure compared with 6 of 9 (67%) patients with a hypoxic primary tumor (p=0.015). In the tirapazamine arm, none of the 8 patients with hypoxic tumors before treatment failed locally. This was significantly different to patients with hypoxic tumors treated in the chemotherapy arm (p=0.011). Only 3 patients had non-hypoxic tumors treated in tirapazamine arm so no robust conclusions could be drawn. This study clearly shows the feasibility of PET as a non-invasive method to study total tumor hypoxia and provide prognostic and potentially predictive information that could be useful for optimal treatment selection. The development of more sophisticated analyses (33) and better probes such as ^{18}F-labeled fluoroazomycin arabinoside (^{18}F-FAZA) which has shown superior biokinetics to ^{18}F-MISO in experimental studies (146) will further improve this methodology.

7. PERSPECTIVE

No matter how you measure it, the presence of hypoxia is a consistent adverse prognostic parameter in many tumors irrespective of the treatment they receive. It is clear that hypoxia can interact with cytotoxic treatment but the development of hypoxia-driven tumor progression may already predispose the patient to failure in spite of oxygen modifying treatment. Each of the methods mentioned in this review has its advantages and disadvantages, its limitations and potential. Taking H&N cancer as an example, the oxygen electrode, extrinsic markers (pimonidazole), intrinsic markers (HIF-1alpha, HIF-2alpha, CA-IX, GLUT-1) and PET (^{18}F-MISO) have all shown prognostic significance in this disease. Each of the techniques has shown that hypoxia is not only present but prevalent and extensive. This tumor site also represents the cancer where oxygen modification has shown most benefit and where current modifiers, nimorazole, ARCON, tirapazamine are in clinical use. The popularity of oxygen modifying treatment has waned in recent years due to the relative failure of radiosensitizers and the popularity of altered fractionation schedules and concurrent chemoradiation. It seems that the pendulum may swing back in favor of oxygen modification as the fractionation bubble has somewhat burst and the now unequivocal demonstration that hypoxia is prognostic. It would appear that we now have tools to select patients for oxygen modifying treatments but now we need new and better treatments or consider combining treatments such as ARCON and tirapazamine to combat the different forms of hypoxia that exist in tumors. Identification of hypoxia-driven gene expression may also identify new targets to combat aggressive tumors. Discriminating whether hypoxia generates an aggressive tumor phenotype or whether an aggressive tumor phenotype generates hypoxia will also be a key issue in the development of new treatment strategies. It would seem that the next few years may be a fruitful period in hypoxia research and lead to new avenues to combat this clinical problem.

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