The Tumor Microenvironment 1: The Oxygen Effect and Tumor Hypoxia

A. The Oxygen Effect: Historical Background

1] it has been recognized for over 100 years that *something* related to cutting off tissue blood flow during irradiation led to increased radioresistance

... it was much later however that the "something" was identified as (lack of) oxygen

MÜNCHENER
MEDIZINISCHE WOCHENSCHRIFT.
ORGAN FÜR AMTLICHE UND PRAKTISCHE ÄRZTE.

No. 24. 15. Juni 1909. Redaktion: Dr. B. Spatz, Arnulfstrasse 26. Verlag: J. F. Lehmann, Paul Heysestrasse 26. 56. Jahrgang.

Die bestrahlten Stellen durch Lapispunkte bezeichnet.
Radikm-Kapsel
ohne Druck mit Druck

Fig. 1.

Abnehmende Entzündung, Schuppung. Nichts.

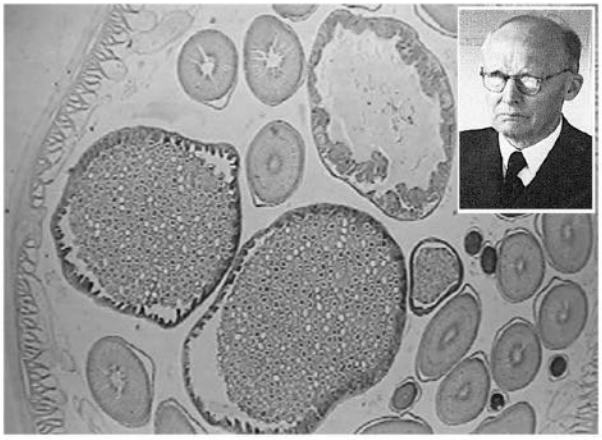
Happy-go-lucky Dr. Schwarz

In 1909, a German radiotherapist named Schwarz was studying skin reactions produced after exposure to radioactive sources...using willing volunteers, hopefully.

When the sources were merely placed on the skin surface, vigorous skin reactions appeared a few days later.

However, when the sources were bound to and compressed into the skin (thereby cutting off blood flow temporarily), skin reactions were much diminished or absent.

b. In 1921, another German, Holthusen, noted that *Ascaris* eggs were more resistant to killing by ionizing radiation if they were irradiated in the absence of oxygen



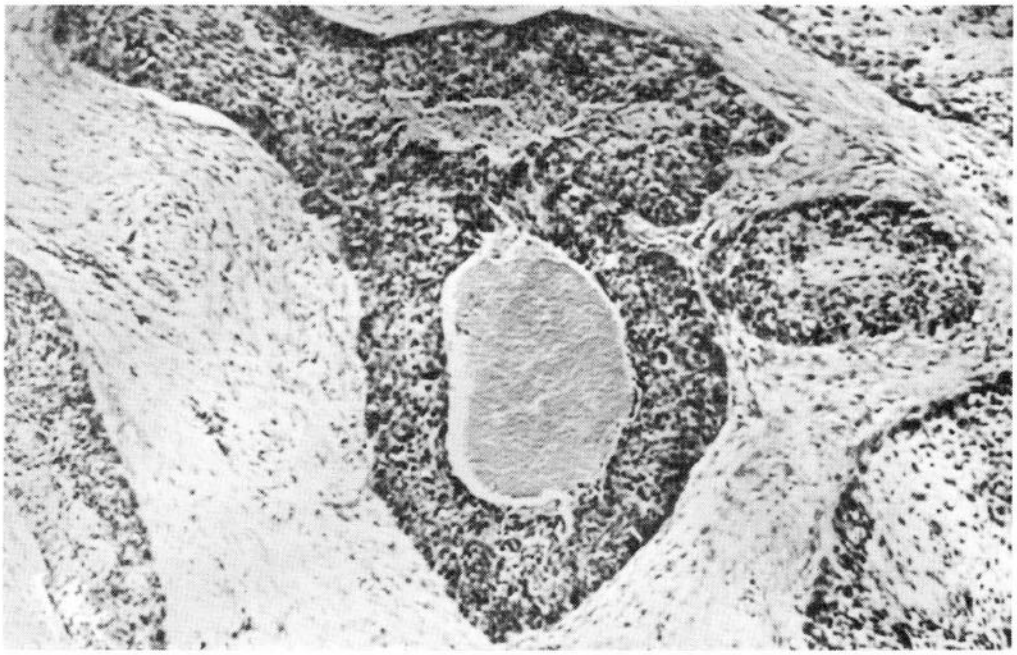
Ascaris eggs (upper) and the disgusting parasitic gut worms (lower) that arise from them.

Inset: Life-of-the-party Dr. Holthusen.

d. Thomlinson and Gray (Br. J. Cancer 9: 539-549, 1955): one of the most influential studies with respect to the oxygen effect and its importance in radiotherapy

1) this histological study of bronchial carcinomas produced compelling, albeit indirect, evidence that human tumors may contain hypoxic cells

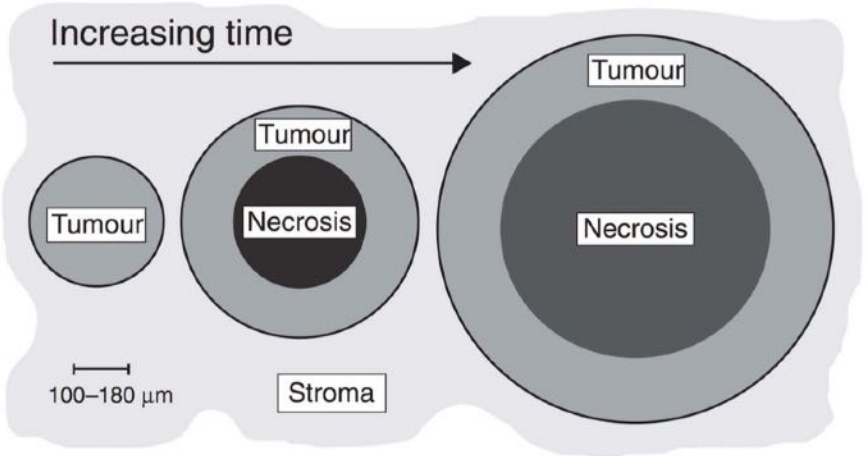
2) the structure of these tumors usually consisted of long "cords" with the host vascular supply on the periphery of the cord, and relatively few blood vessels deep within it



Transverse sections of tumor cords surrounded by stroma from human carcinoma of the bronchus.

3) Thomlinson and Gray came to the following conclusions:

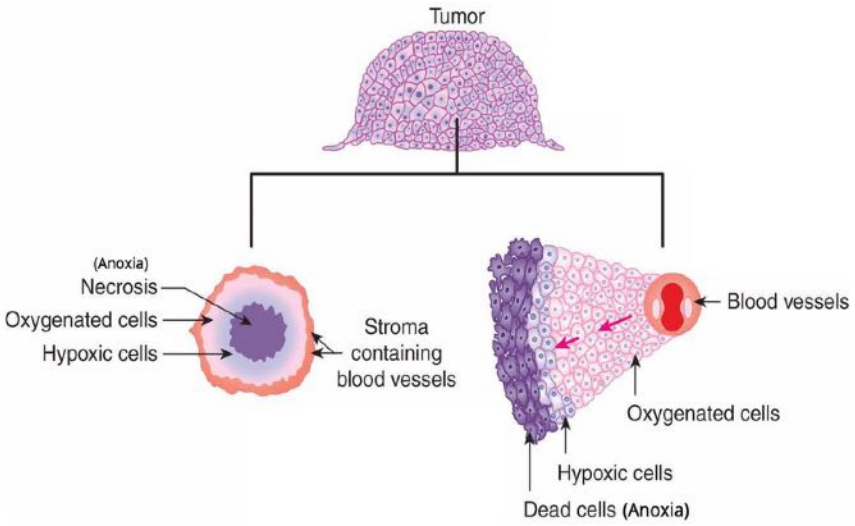
- **no tumor cord with a radius smaller than 160 μm showed evidence of necrosis**
- **no tumor cord with a radius larger than 200 μm was without a necrotic center**
- **regardless of the size of the necrotic center of the tumor cord, the thickness of the rim of seemingly viable cells was never greater than about 100-180 μm**



a. in later years, and using more realistic values for oxygen diffusion and consumption coefficients, we now estimate that the distance oxygen can diffuse in respiring tissue is about 70 μM

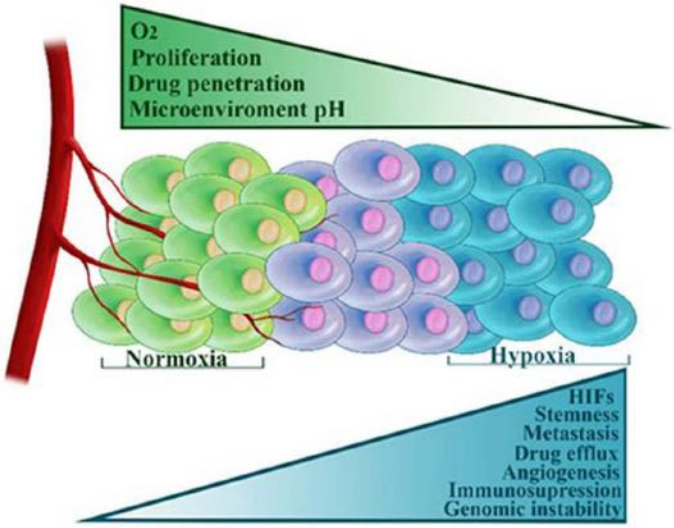
Boards

5) taking this reasoning one step further, **between the viable, fully oxygenated cells and the dead, necrotic ones, a region should exist that contains viable cells, but which are relatively low in oxygen; such cells should also be radioresistant**



What Thomlinson and Gray proposed in the mid-1950's is what we now refer to as ***chronic, diffusion-limited hypoxia***.

It occurs in tumor cells relatively distant from blood vessels, and is a consequence of high oxygen utilization in the cells closer-in to the vessel(s). They use up oxygen at an abnormally high rate, in no small part because such cells are usually actively-dividing, which takes a lot of energy to accomplish (especially repeatedly and at short time intervals).



• Oxygen and nutrient supply (and therefore, energy reserves) decrease with increasing distance from blood vessels.

• pH becomes more acidic with increasing distance from blood vessels.

6] since around 1980 however, a second type of tumor hypoxia was identified, so called **transient or intermittent hypoxia** (the phenomenon formerly known as "acute hypoxia")

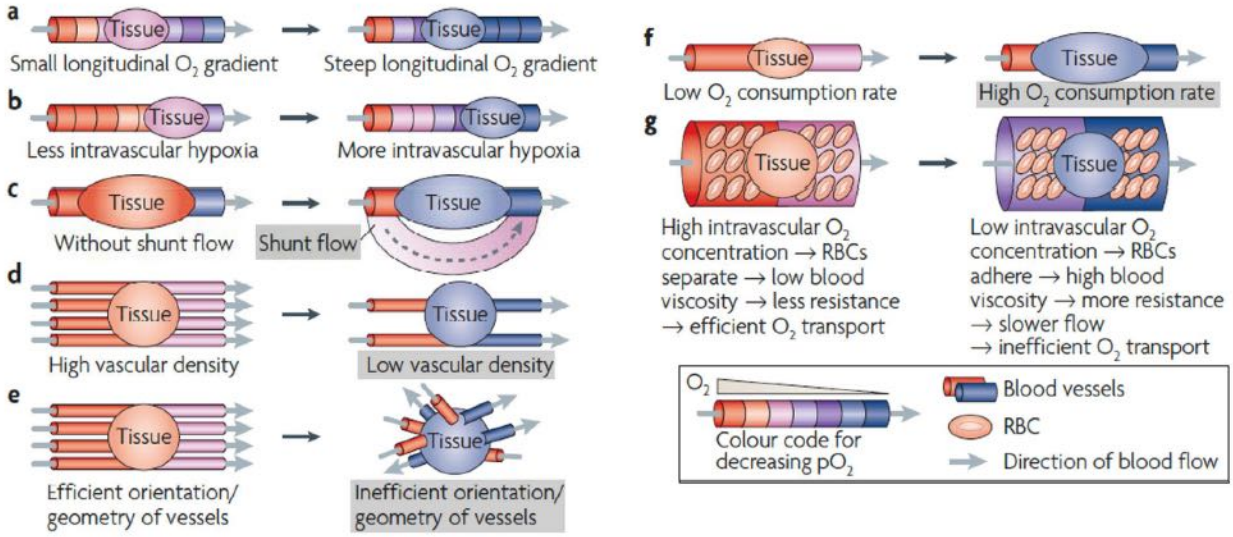
a. tumor vasculature tends to be abnormal both structurally and functionally, and this can lead to any number of physiological effects that may include...

- slowing, or total stoppage of blood flow through a vessel, i.e., "vascular stasis"
- diversion of blood flow elsewhere (vascular shunts and blind ends)
- inefficient vascular geometry
- high O2 consumption rate by tumor cells

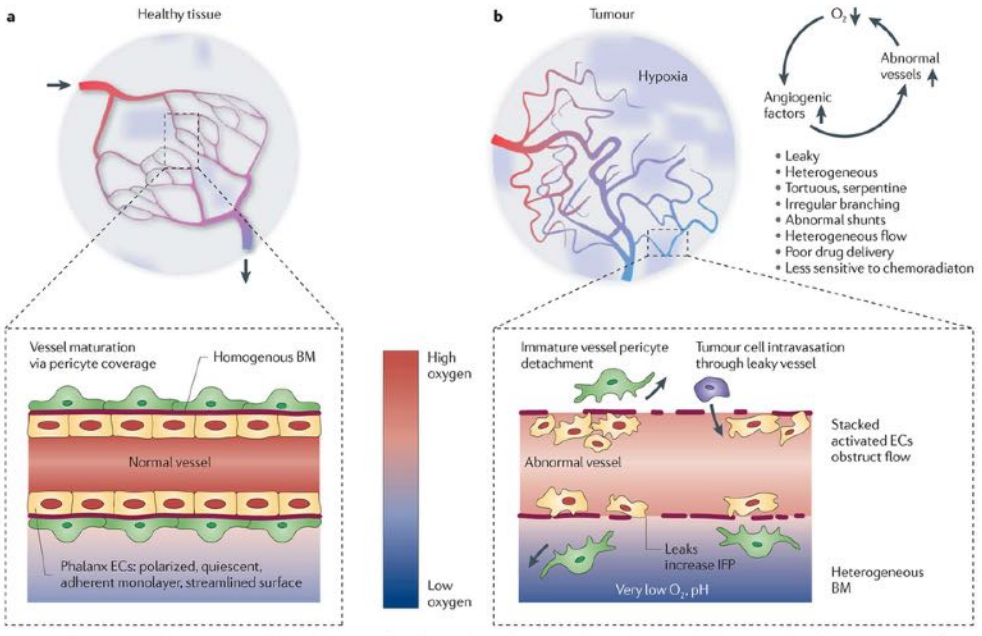
➤high interstitial fluid pressure (due to vessel leakiness), that squeezes vessels closed

...any one or more of these will have the net effect of causing transient (as in, as short as minutes) tumor hypoxia

Causes of Tumor Hypoxia



Dewhirst, MW. Nature Reviews Cancer (2008) 8, 425-437.



Tumour vessels are structurally and functionally abnormal. **a** | In healthy tissue, a regularly patterned and functioning vasculature is formed (upper panel), with a normal vessel wall and endothelium (lower panel). **b** | In established tumours, the vasculature (upper panel), as well as the endothelium and vessel wall (lower panel) exhibit structural and functional abnormalities, leading to regions of severe hypoxia (represented by blue shading). BM, basement membrane; EC endothelial cell; IFP, interstitial fluid pressure.

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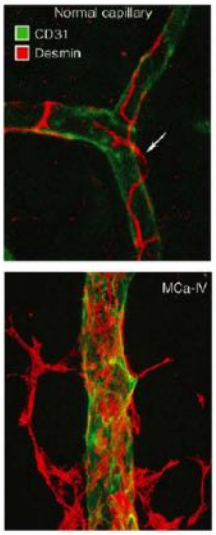


Morikawa et al., Am J Path 160: 985, 2002

Upper panel - normal microvasculature of mouse breast tissue

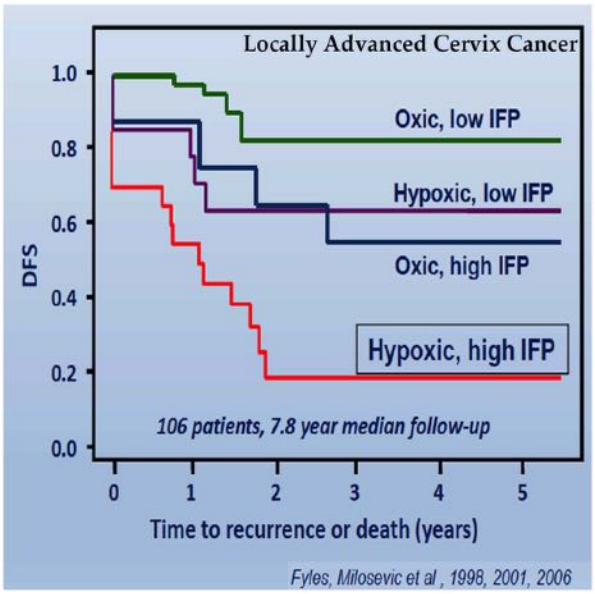
Lower panel - mammary tumor microvasculature is distended, endothelial cells disorganized, and pericytes (red) detached from the vessel

Main (?) Culprit: High Interstitial Fluid Pressure - occurs secondary to abnormal, leaky tumor blood vessels, and in the extreme is capable of collapsing vasculature, resulting in hypoxia (which could be chronic or intermittant)

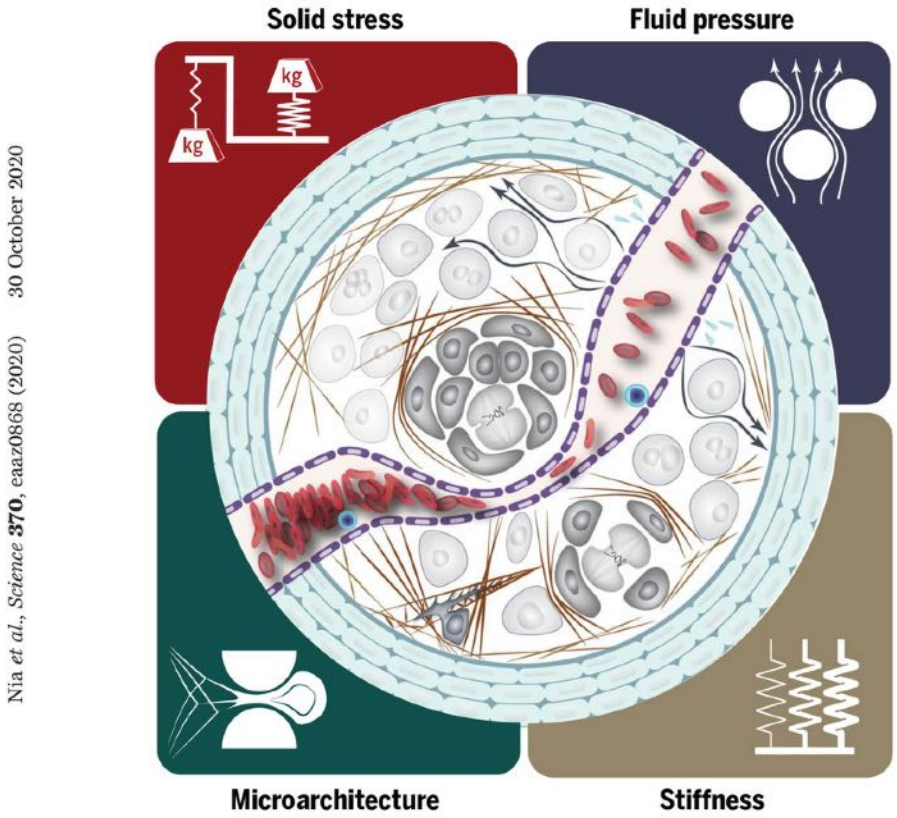
INTERSTITIAL PRESSURE IN HUMAN TUMORS

TYPE OF TISSUE	NUMBER OF PATIENTS	MEAN PRESSURE
NORMAL BREAST	8	0.0
NORMAL SKIN	5	0.4
RENAL CELL CARCINOMAS	1	38.0
CERVICAL CARCINOMAS	26	22.8
COLORECTAL LIVER METASTASES	8	21.0
HEAD AND NECK CARCINOMAS	27	19.0
BREAST CARCINOMAS	8	15.0
METASTATIC MELANOMAS	12	14.3
LUNG CARCINOMAS	26	10.0

(in mm Hg)



A New Tumor Biology Paradigm: "Physical Traits of Cancer" - a companion to the more familiar "Hallmarks of Cancer"



Nia *et al.*, *Science* **370**, eaaz0868 (2020) 30 October 2020

These newly-proposed (but not really all that new, technically) physical traits of solid tumors result from tumor cell interactions with other cells and acellular components in their surrounding microenvironment.

Clearly, these physical features can conspire to increase tumor hypoxia... not to mention influence the proliferative, angiogenic and metatstatic processes, and maybe even contribute to the tumor's ability to evade immune detection.

Physical traits of cancer. On the basis of the advancements of the past few decades, we suggest that the physical traits of cancer can be categorized into four major groups: (i) elevated solid stress, (ii) elevated interstitial pressure, (iii) increased stiffness, and (iv) altered architecture and geometry.

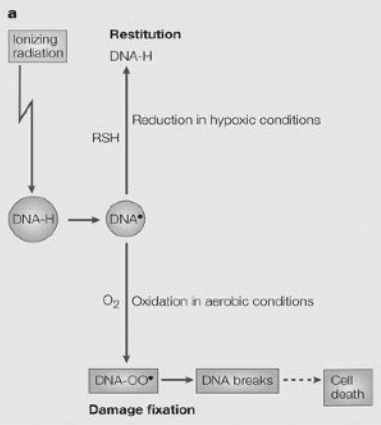
B. The Hows and Whys of the Oxygen Effect

1] ***Hypoxic cells are more resistant to (low LET) radiation than well-aerated ones because of the rapid free radical reactions that occur within a millisecond of highly-localized energy deposition events***

a. ***oxygen is able to "fix" this chemical damage to DNA and therefore render cells more radiosensitive, however in the relative absence of oxygen, this molecular damage is reversed, making cells more radioresistant***

| Radiation resistance of hypoxic cells

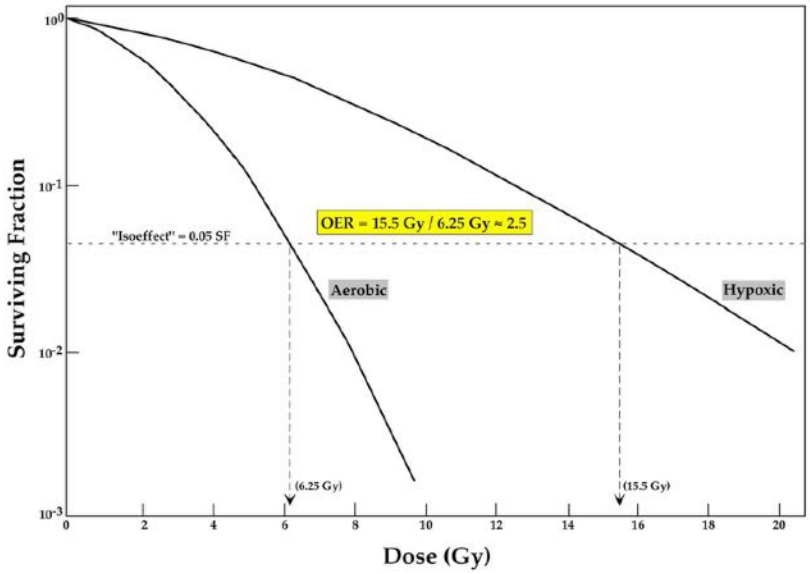
Ionizing radiation, such as that used in radiotherapy, kills cells by producing DNA damage, particularly DNA double-strand breaks. This damage results from ionizations in or very close to the DNA that produce a radical on the DNA ($DNA^{\bullet}$). This radical then enters into a competition for oxidation, primarily by oxygen (which fixes, or makes permanent, the damage), or reduction, primarily by –SH-containing compounds that can restore the DNA to its original form (see figure, part **a**). Therefore, DNA damage is less in the absence of oxygen. This effect of oxygen in sensitizing cells to radiation is illustrated in the cell-survival curve (see figure, part **b**) and is quantitated as the ratio of dose in the absence of oxygen to dose in the presence of oxygen needed to obtain the same surviving fraction of cells. For mammalian cells, this ratio is usually 2.5–3.0 (see horizontal dotted line). The oxygen partial pressure (pO_2) that produces sensitivity midway between the oxic and hypoxic responses is approximately 3 mmHg. Clinical trials, particularly with head and neck cancers for which control of the primary tumour is the main problem, have demonstrated that the more hypoxic tumours (typically those with a median pO_2 less than 10 mmHg) are more radioresistant than the less hypoxic tumours.



www.nature.com/reviews/cancer
JUNE 2004 | VOLUME 4

b. the extent or degree of radiation resistance of hypoxic cells is quantified using a radiobiological parameter called the ***Oxygen Enhancement Ratio or OER***

$$OER = \frac{\text{Dose of Radiation Under Hypoxic Conditions}}{\text{Dose of Radiation Under Aerobic Conditions}} \text{ to achieve the same biological effect}$$

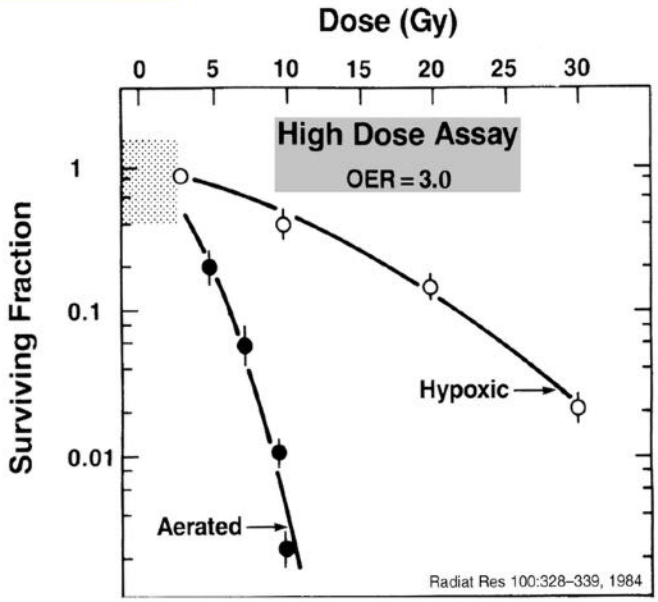


The oxygen enhancement ratio is a ratio of **doses** to yield the same effect, NOT a ratio of surviving fractions! For mammalian cells, the OER ranges between 1.0 and about 3.0 for exposure to ionizing radiation; it is a unit-less number.

2] everything you ever wanted to know about the OER...

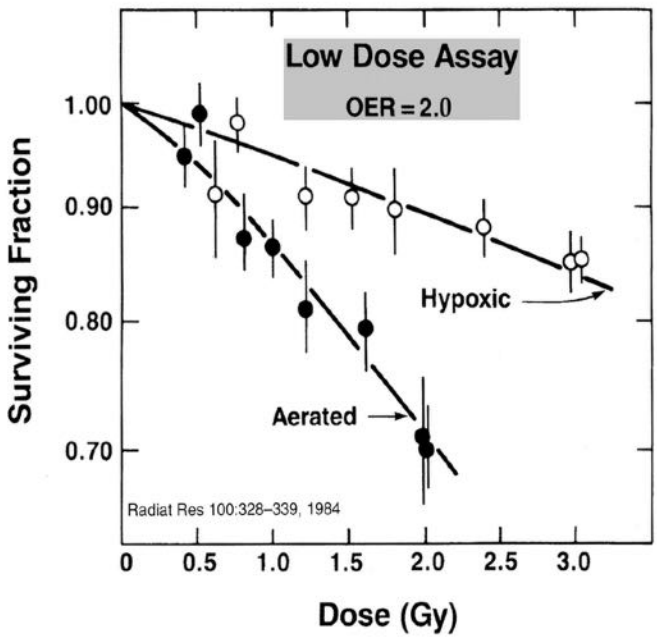
a. Is the OER a fixed value, or does it vary with the experimental/treatment conditions?

1. **when the OER is determined based on a "severe" endpoint, i.e., one that involves a great deal of cell killing such as might be achieved when large, single radiation doses are used, its value is usually in the 2.5 to 3.0 range**



The OER determined for rodent cells irradiated in culture in the presence or relative absence of oxygen, when challenged with large, single doses of X- or γ-rays that reduce cell survival to less than about 1%.

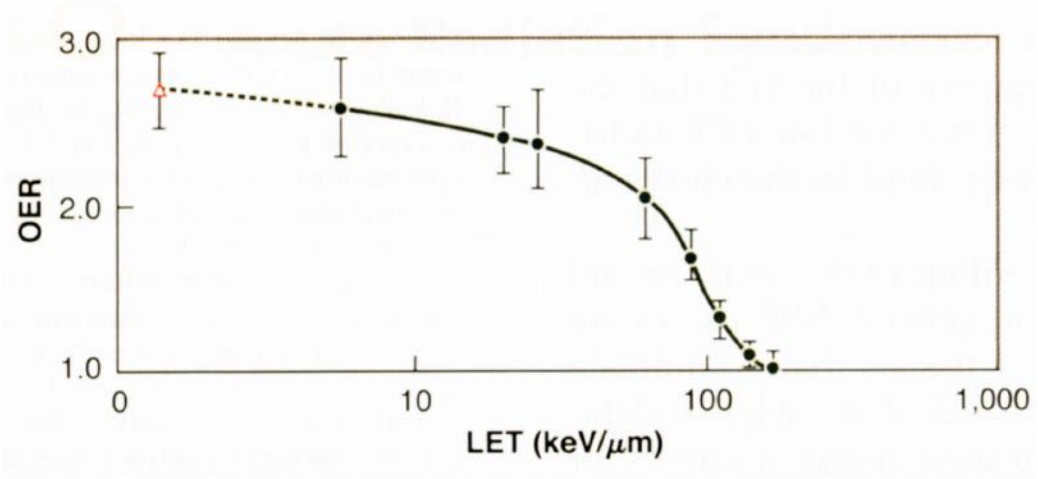
2. **however, when the OER is determined based on a "milder" endpoint (when cell survival is relatively high), such as might be achieved using small, repeated doses (think: fractionated radiotherapy), its value is lower, usually in the 1.5 to 2.0 range**



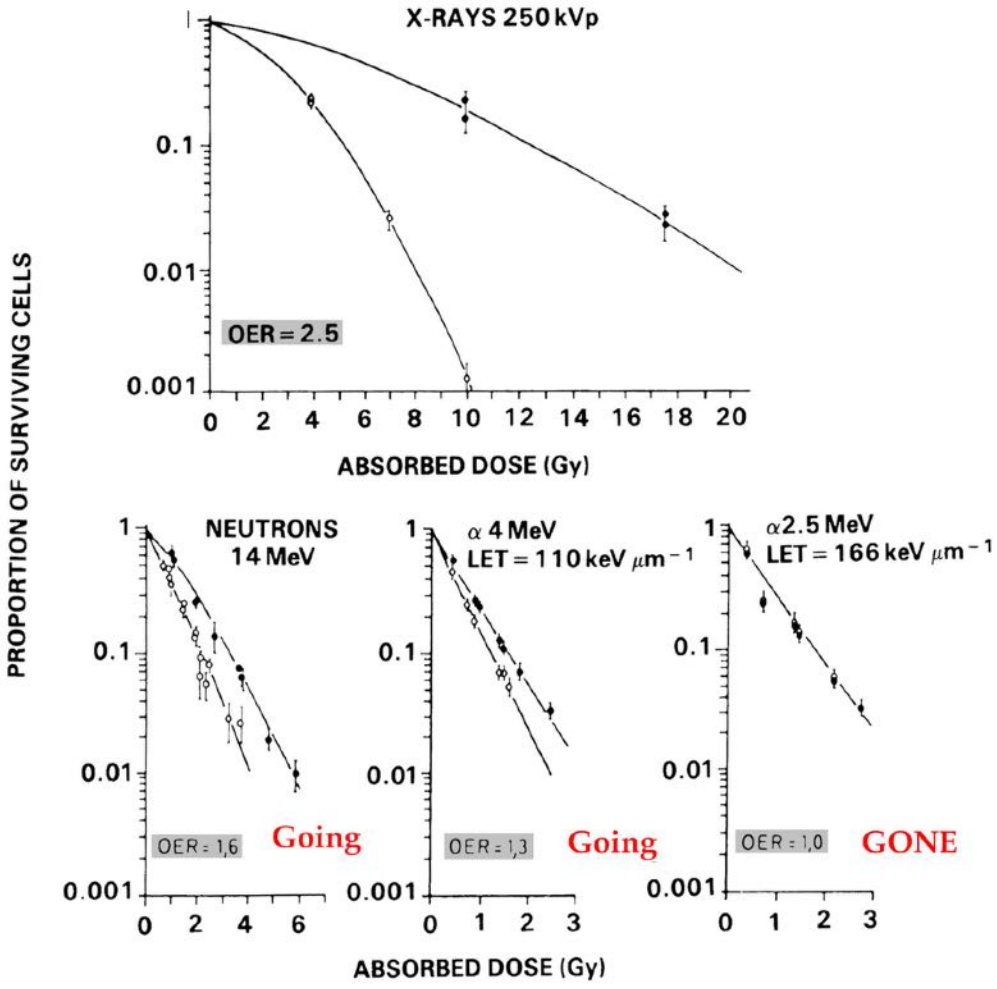
The OER determined in hypoxic and aerobic rodent cells grown in culture when challenged with small doses of X- or γ-rays that reduce cell survival to no less than about 50%.

b. How does the OER change with high versus low LET radiation?

1. **the OER decreases with increasing LET such that, at an LET of 100 keV/μm (the threshold for what is considered "high LET"), the OER approaches 1.0, that is, that there is no longer an oxygen effect, and hypoxic cells are equal in radiosensitivity to well-aerated ones**



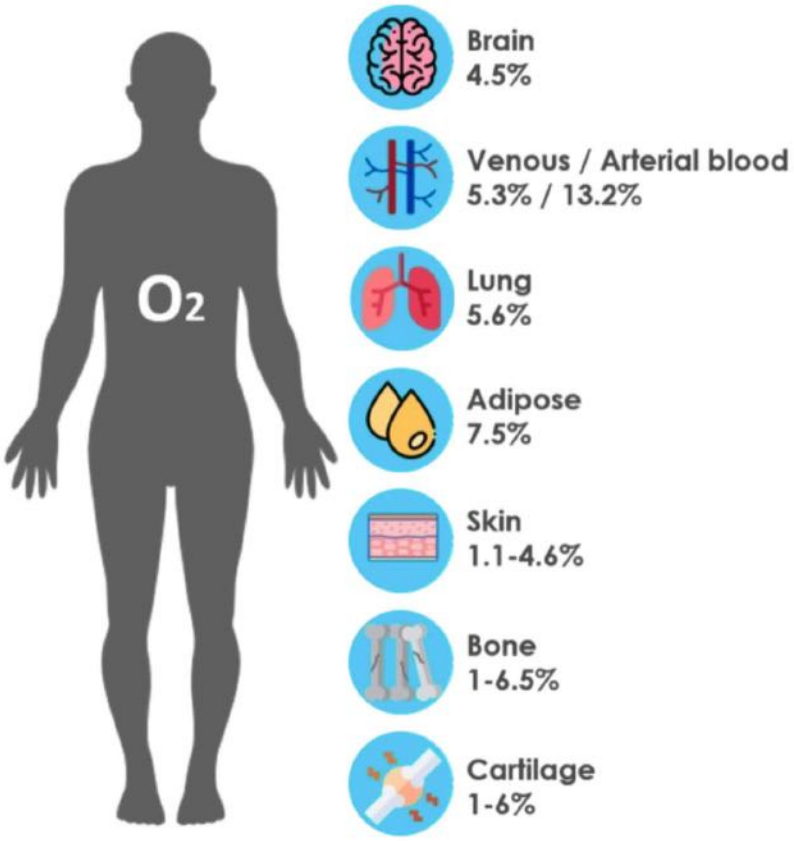
OER as a function of LET. Measurements of OER were made with cultured cells of human origin. *Closed circles* refer to monoenergetic charged particles, the *open triangle* to 250-kVp x-rays with an assumed track average LET of 1.3 keV/μ. (From Barendsen GW, Koot CJ, van Kersen GR, Bewley DK, Field SB, Parnell CJ: Int J Radiat Biol 10:317–327, 1966)



Survival curves of human kidney cells T1 irradiated under hypoxic and aerobic conditions with different qualities of radiation.

From: Barendsen, GW. Curr Top Radiat Res Q 4: 293-356, 1968

c. What, exactly, is meant by "hypoxic"?



Ranges of oxygen concentrations in normal tissues.

Compared to room air (21% oxygen), you might think these tissues are already "hypoxic"...but they aren't even close, either physiologically or radiobiologically-speaking!

Radiobiological hypoxia, i.e. when tumor cells first start to become radiation resistant, occurs at an oxygen concentration of <1%, corresponding to a partial pressure of O2 of ~10 mm Hg*. ***At 0.5% O2 (3 mm Hg), cells are at about halfway between sensitive and resistant - this is termed oxygen's k-value.*** **At 0.13% O2 (1 mm Hg), cells become fully radioresistant.**

** In clinical studies where oxygen concentrations in tumors are directly measured, the cutoff point between "aerated" and "hypoxic" is assumed to be 10 mm Hg.*

d. When does oxygen have to be present in relation to the time of irradiation to yield an aerobic (i.e., sensitive) radioresponse?

1. **for all intents and purposes, oxygen need only be present at the time of irradiation in order for the cells to respond as if they are fully aerated; this is in keeping with the fast, free radical-based mechanism of oxygen's action as a radiosensitizer** (the presence of oxygen *prior to* irradiation is also OK, provided it's still there at the time of irradiation, but adding it *after* irradiation has no effect on radiosensitivity)

C. Tumor Hypoxia *In Vivo*: Experimental and Clinical Studies

1] So it's only big honkin' tumors that contain regions of hypoxia, right?

a. Answer: That would be a **NO**. *It is a common (and persistent) misconception that only large tumors contain hypoxia, and that it tends to be centrally located in the tumor mass.*

1. the truth is that even "baby" tumors less than a millimeter in diameter are already developing microregions of hypoxia, and this very stressor serves as a major stimulus for angiogenesis; **in the absence of angiogenesis, no tumor would grow to much more than about 2 mm in diameter!**

Mammary tumor cells deliberately implanted in the flank of a host mouse begin to grow into a tumor mass, and already start to become hypoxic when they are *well* less than 0.5 mm in diameter (green staining = presence of hypoxia)! Moeller et al., CANCER CELL : MAY 2004 · VOL. 5 ·

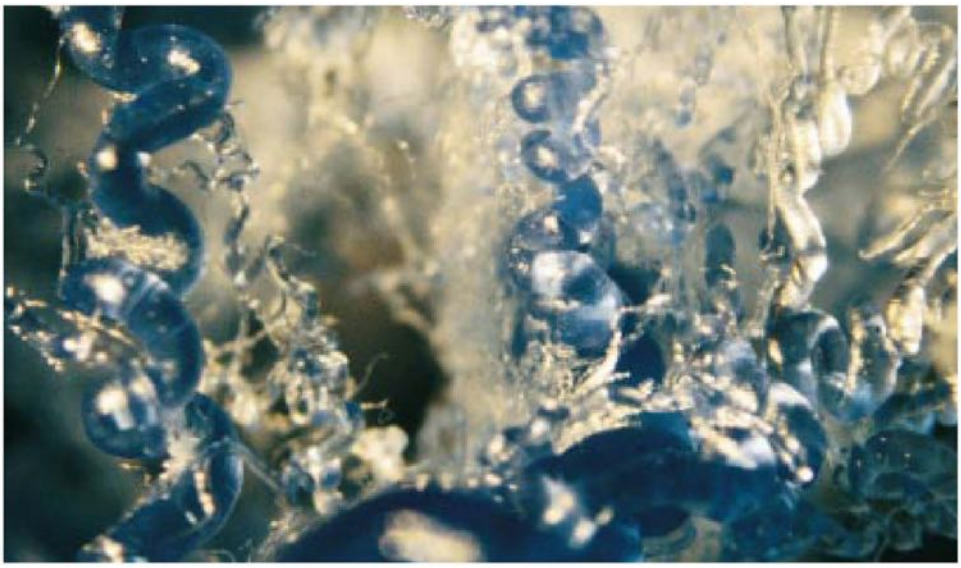
b. yet big tumors can also be hypoxic, even though they've already built an extensive vascular system...why?

Tumors develop regions of hypoxia because, unlike normal tissues, their vasculature is abnormal.

- Abnormal structurally
- Abnormal functionally
- Abnormal physiologically
- Abnormal angiogenesis

Chaotic and completely disorganized tumor vasculature. Note the dead zone in the center of the tumor mass where necrosis developed.

Polymer cast of the vasculature of a human GI tumor weighing in at about half a pound. YIKES!

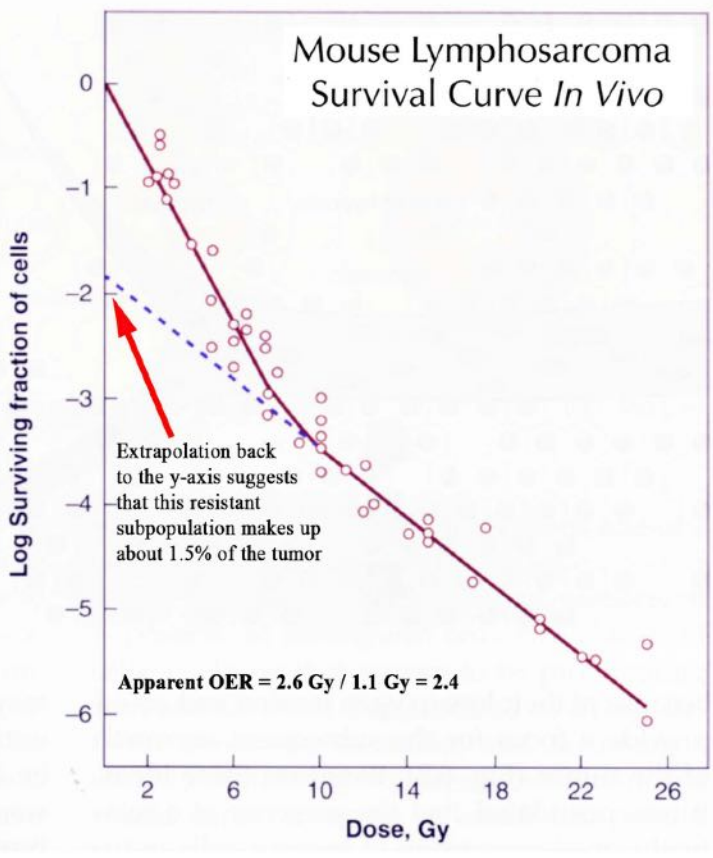


Magnified view of the same polymer cast of tumor vasculature. Needless to say, the vessels are highly abnormal, of different sizes and degrees of distension, and with corkscrews and shunts in evidence.

The vascular orientation and geometry is also a mess and most likely insufficient to reach all tumor cells, which is why regions of necrosis develop.

2] the first (direct) demonstration of the existence of tumor hypoxia in a mouse model *in vivo*, showing that such cells are both radioresistant and importantly, clonogenic

Fraction of surviving cells as a function of dose for a solid subcutaneous lymphosarcoma in the mouse irradiated *in vivo*. The first part of the curve has a slope D_0 of 1.1 Gy; the second component of the curve has a shallower slope D_0 of 2.6 Gy, indicating that these cells are hypoxic. (Adapted from Powers WE, Tolmach LJ. A multicomponent x-ray survival curve for mouse lymphosarcoma cells irradiated in vivo. *Nature*. 1963;197:710–711, with permission.)

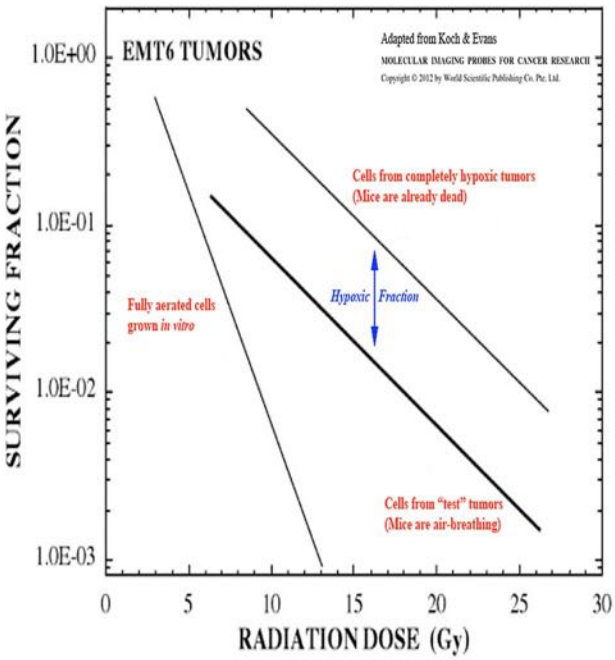


a. the biphasic shape of the survival curve for such tumors implied the presence of a resistant subpopulation of cells comprising about 1.5% of the population, and that this population was about 3 times more radioresistant (compare the slopes of the two components of the biphasic curve)

1) even so, this still doesn't unambiguously prove that the reason for the resistance was hypoxia... so how was this finally figured out? Answer: using a technique called the **"paired survival curve method"**

(a) in this method, three groups of tumor-bearing mice are irradiated:

- one group breathing air (as above),
- a second group breathing high-pressure oxygen (so their tumors would be, in theory, fully aerated), and
- a third group in which the animals were killed immediately prior (their tumors would be fully hypoxic/anoxic)



The proportion of hypoxic cells in a mouse tumor. The biphasic curve labeled *air curve* represents data for cells from tumors irradiated in mice breathing air, which are therefore a mixture of aerated and hypoxic cells. The *hypoxic curve* is for cells irradiated in mice asphyxiated by nitrogen breathing or for cells irradiated *in vitro* in nitrogen, so that they are all hypoxic. The oxic *curve* is for cells irradiated *in vitro* in air. The proportion of hypoxic cells is the ratio of the air to hypoxic curves, or the vertical separation between the curves, since surviving fraction is on a logarithmic scale.

b. the paired survival curve method became (in the 1960's) the *de facto* standard method for determining the hypoxic fraction of experimental rodent tumors; ***to date and across dozens of different types of rodent tumors (and xenografted human tumors), calculated hypoxic fractions have ranged from 0% to approximately 50%, with an average of around 15-20%***

Fraction of hypoxic cells in assorted rodent tumors (plus a couple of human ones)

Carcinomas	Per cent	Sarcomas	Per cent
Adenocarcinoma MTG-B	21	CBA sarcoma F	>50
Squamous carcinoma D	18	Gardner lymphosarcoma	1
C_3H mammary carcinoma	7	C_3H sarcoma KHT	14
Carcinoma KHJJ	19	Rhabdomyosarcoma BA 1112	15
C_3H mammary carcinoma		Osteosarcoma C22LR	14
Female	1	Fibrosarcoma RIB5	17
Male	17	Fibrosarcoma KHT	12
Squamous carcinoma G		Sarcoma EMT6	35
Intradermal	<1	CBA sarcoma F	
Subcutaneous	>46	In situ	<10
Carcinoma DC	10–30	Excised	50
C_3H mammary carcinoma		Sarcoma S	<0.01
Small	0.2	Sarcoma S (fast)	1–30
Large	>20	Sarcoma FA	30–70
WHT carcinoma MT		Sarcoma BS	5–25
In situ	>80		
Excised	5		
Carcinoma RH	2–25		
Carcinoma NT	7–18		
Human tumours			
Human tumour nodules	12–20		
Human tumour nodules	1–80		

Acta Radiologica: Oncology, 23:4, 217-225, DOI: 10.3109/02841868409136015

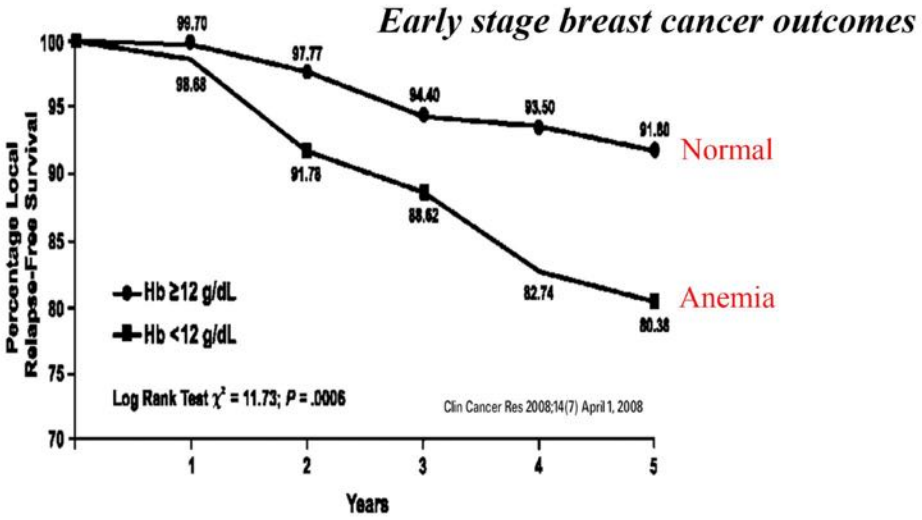
3] **Hypoxia in human tumors** - problematic to say the least, insofar as the paired survival curve method is not exactly appropriate

a. *therefore, up to about 35 years ago, there was no direct way to determine whether human tumors contained viable, radioresistant, hypoxic cells, let alone how many of them there were – only indirect or inferential methods were available*

b. **Indirect evidence for the presence of treatment-limiting hypoxia in human tumors**:

- histopathological studies akin to those of Thomlinson and Gray, where patterns of intercapillary distances and presence of necrosis in tumors were used as surrogates for (presumed) hypoxia

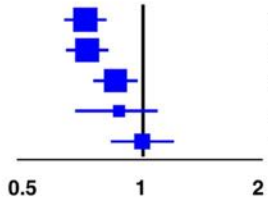
- an association between pre-treatment anemia in cancer patients and poor clinical outcome (particularly apparent in head and neck and cervical cancers) - to this day, anemia tends to be corrected by blood transfusions prior to the start of radiotherapy



- success of a few clinical trials of hypoxia-directed therapies at improving tumor control (such as, using hyperbaric oxygen breathing during irradiation, or pre-irradiation administration of select hypoxic cell sensitizers)

Head and neck cancer - meta analysis - summary

Endpoint	Events / Total		Odds ratio and 95% CI			
	Hypoxic modification	Control	Odds ratio	Risk Reduction	NNT**
Loco-regional control	1203 / 2406	1383 / 2399	0.71 (0.63-0.80)*	8% (5-10%)*	13
Disease specific survival	1175 / 2335	1347 / 2329	0.73 (0.64-0.82)	7% (5-10%)	14
Overall survival	1450 / 2312	1519 / 2305	0.87 (0.77-0.98)	3% (0-6%)	31
Distant metastasis	159 / 1427	179 / 1391	0.87 (0.69-1.09)	2% (-1-4%)	57
Radiotherapy complications	307 / 1864	297 / 1822	1.00 (0.82-1.23)	0% (-3-2%)	>>



Data from 32 randomized trials of hypoxia modification (~4,800 patients)

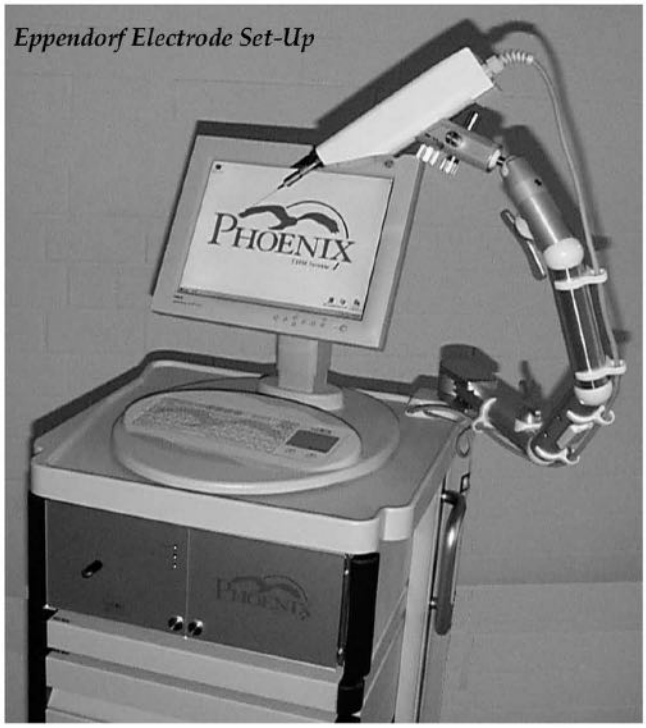
Meta Analysis - Hypoxic modification of radiotherapy in HNSCC

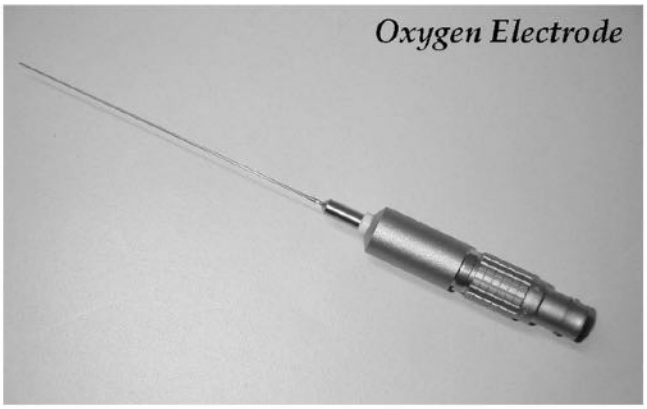
* 95% CI.
** Numbers of patients Needed to Treat to achieve benefit in one patients.

Overgaard, Radiotherapy and Oncology *100* (2011) 22–32

c. *direct* detection and quantification of hypoxia in human tumors

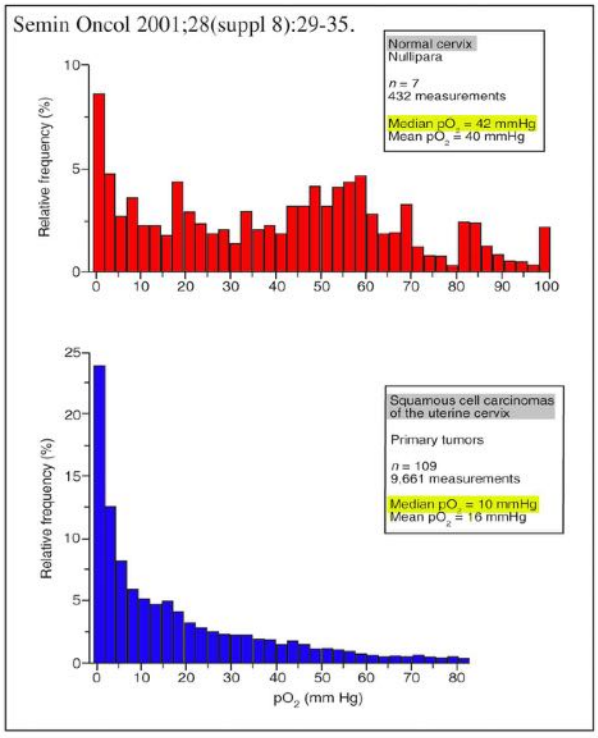
1. Gatenby and colleagues published the first paper (another rad bio classic: Int J Radiat Oncol Biol Phys *14*: 831-838, 1988) in which oxygen tension in tumors was measured using a needle-thin glass sensor called an **oxygen electrode** (akin to a pH meter, except that local oxygen tension is measured rather than acid/alkaline)



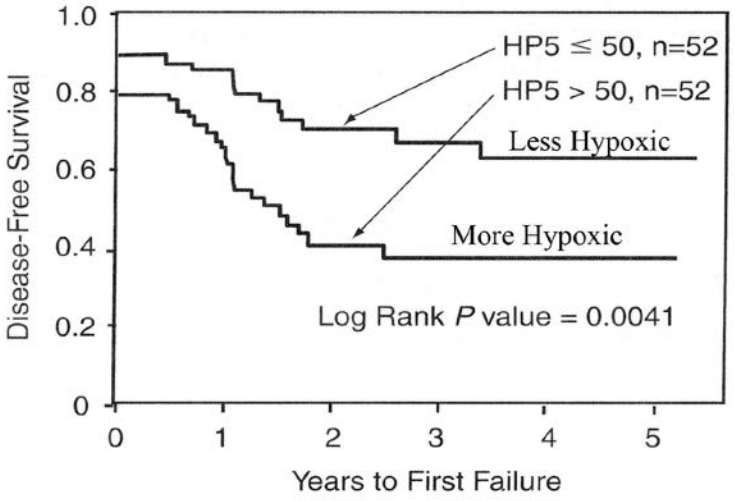


The company that produced the first commercially available oxygen electrode "workstation" was named Eppendorf.

Their company name became so well known that it practically replaced the terms "oxygen electrode".



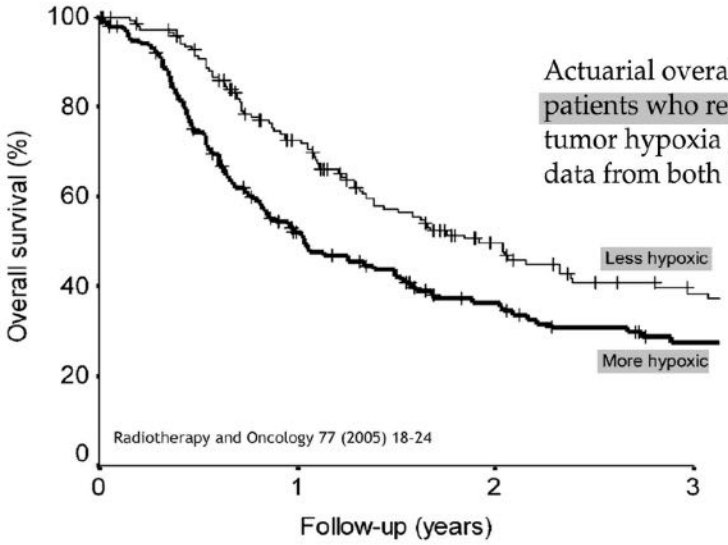
Frequency distribution of pO_2 values in normal uterine cervix and locally advanced squamous cell carcinoma of the uterine cervix (FIGO stage IB-IV).



Disease-free survival probability in patients with carcinoma of the cervix treated with radiotherapy only, as a function of degree of tumor hypoxia. Tumors with a high degree of hypoxia (HP5 > 50%) had much poorer outcomes.

HP5 refers to the percentage of pO_2 measurements that were below 5 mm Hg.

Oxygen Electrode Studies in Head and Neck Cancer



Actuarial overall survival rate for 400 advanced head and neck cancer patients who received radiotherapy only as a function of degree of tumor hypoxia (measured pre-treatment). Meta-analysis of pooled data from both European and North American institutions.

2. **hypoxia markers** - a newer and different approach than oxygen electrodes for the detection and quantification of hypoxia *at the cellular level*

a) there are two types of hypoxia markers:

• *exogenously supplied biochemicals* that are uniquely metabolized only under hypoxic conditions and bind to hypoxic cells, making them "markable" by a variety of methods (radioactive label, fluorescent label, immunohistochemistry, and with MRS, PET, SPECT etc.)

• *endogenous cellular biomolecules* only expressed under hypoxic conditions (i.e., products of oxygen/hypoxia-regulated genes) that can be detected using monoclonal antibodies and then visualized

1) there is longer-term experience using the exogenous markers...however there would be a theoretical advantage to using an endogenous marker, so that the patient wouldn't need to be administerd a drug

b) Examples of Exogenous Hypoxia Markers: *pimonidazole hydrochloride* (marketed commercially as "HypoxyProbe-1") and *EF-5*

1. advantages:

• markers bind on a cell-by-cell basis, and have approximately the same oxygen concentration dependency as does radioresistance/sensitivity

• in histological sections prepared from biopsy specimens, a picture of the "geographic physiology" of tumor micro-regions can be established, and can be compared to staining patterns of other types of tumor markers and/or structural features

• dead cells do not get stained, as the drugs require metabolic activation before they bind

c) Examples of Endogenous Hypoxia Markers: ***CA9 (carbonic anhydrase)*** and ***HIF-1α*** or ***HIF-2*** (***"hypoxia inducible factors"***); ***also, Lysyl Oxidase (LOX), GLUT-1 and Osteopontin (OPN)***

1. **advantages** – similar to above, plus:

   • no drug pretreatment required, meaning that **archival tissue specimens can be used**

d) disadvantages of both types of hypoxia markers:

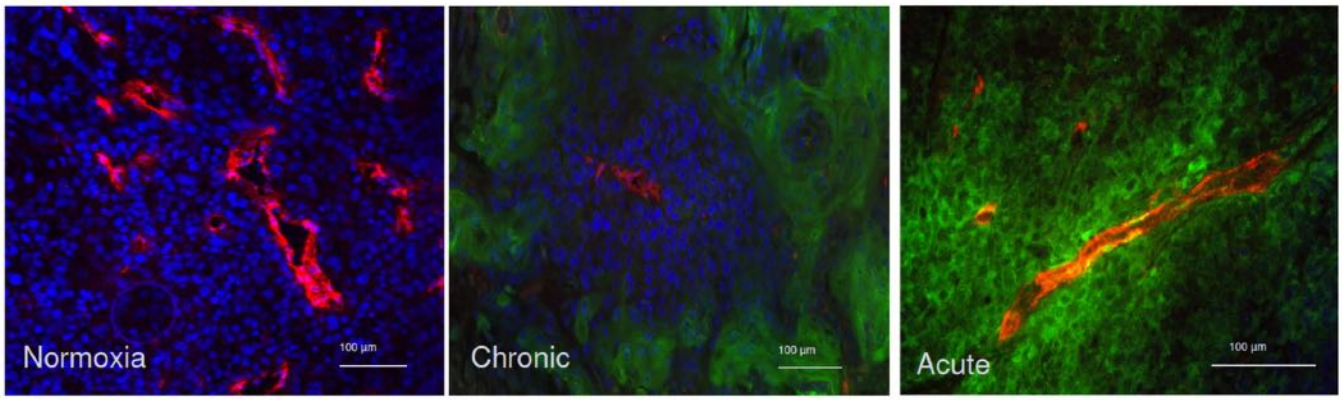
• **both require one or more invasive biopsy procedures**, as well as **labor-intensive** processing and analysis

• **not sure whether the cells being stained are *reproductively* alive or dead** (although they do have to be metabolically alive)

• **for the most part, only chronic hypoxia is detected** (especially for **the** exogenous markers), which may not turn out to be as clinically-relevant as **intermittent** hypoxia

HYPOXIA MARKER ART GALLERY!

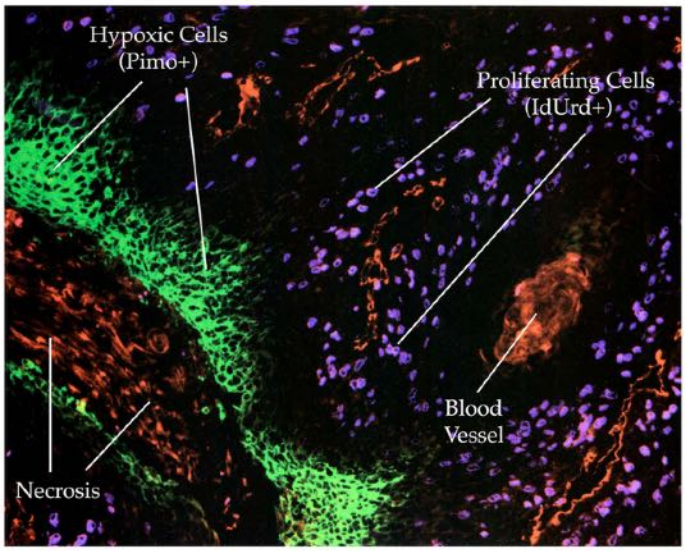
Visualizing chronic versus intermittent (versus no) hypoxia in a human tumor xenograft



Demonstration of chronic hypoxia and "geographic physiology" in a rodent tumor

Distribution of hypoxic cells (labeled with pimo, green) in a murine tumor in relation to tumor structural features such as proximity to blood vessels or necrotic zones.

Also shown is the location of cells actively going through the cell cycle (labeled with IdUrd, purple), which tend ***not*** to overlap with the hypoxic ones.



Can expression profiles for hypoxia-related genes have prognostic or predictive value?

A panel of 15 genes upregulated under hypoxic conditions does show some potential...

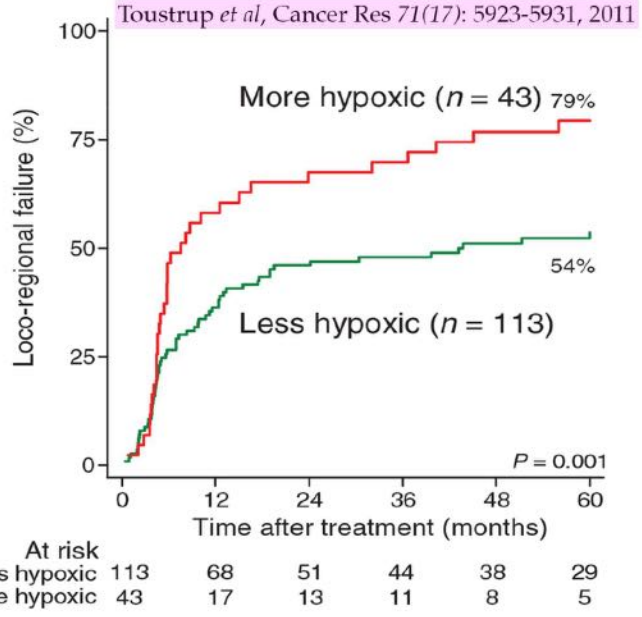
(requires some metric that includes both the number of these genes that are upregulated, and to what extent)

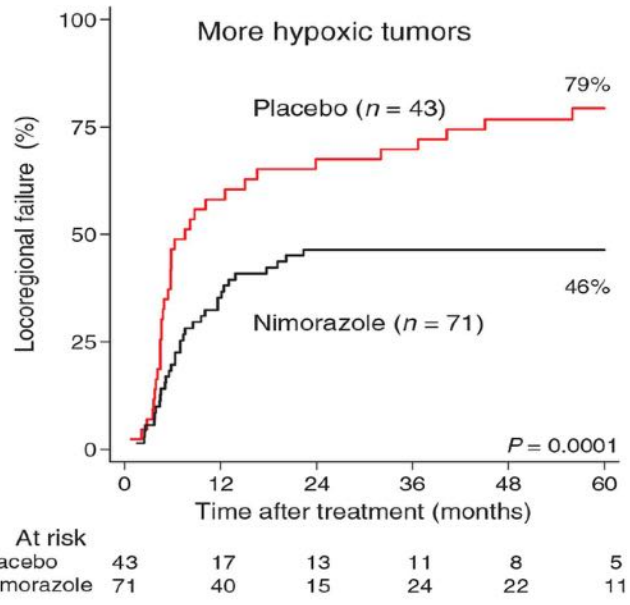
Hypoxia-responsive genes

In vitro derived genes	Included in hypoxia classifier	Function
ADM	ADM	Stress response
AK3L1		Nucleotide metabolism
ALDOA	ALDOA	Glucose metabolism
ANKRD37	ANKRD37	Protein–protein interactions
ARRDC3		Cell surface metabolism
BNIP3	BNIP3	Apoptosis
BNIP3L	BNIP3L	Apoptosis
C3orf28	C3orf28	Unknown
C18orf19		Unknown
CCNG2		Cell cycle regulation
EGLN1		Regulation of HIF-1 activity
EGLN3	EGLN3	Regulation of HIF-1 activity
ERO1L		Oxidoreductase
FOSL2		Cell proliferation
GPI		Glucose metabolism
HIG2		Stress response
IGFBP3		Cell proliferation
JMJD1A		Histone demethylase
KCTD11	KCTD11	Apoptosis
LOC401152		Unknown
LOX	LOX	Extracellular-matrix metabolism
NDRG1	NDRG1	Stress response
P4HA1	P4HA1	Extracellular-matrix metabolism
P4HA2	P4HA2	Extracellular-matrix metabolism
PDK1	PDK1	Energy metabolism
PFKFB3	PFKFB3	Glucose metabolism
RORA		Unknown
SLC2A1	SLC2A1	Glucose metabolism
SLC6A8		Glucose metabolism
CA9		pH regulation

Toustrup *et al*, Cancer Res *71(17)*: 5923-5931, 2011

Toustrup *et al*, Cancer Res *71(17)*: 5923-5931, 2011





Advanced head and neck tumors showing the highest levels of expression of these genes (indicating "more hypoxic") show higher locoregional failure rates after treatment

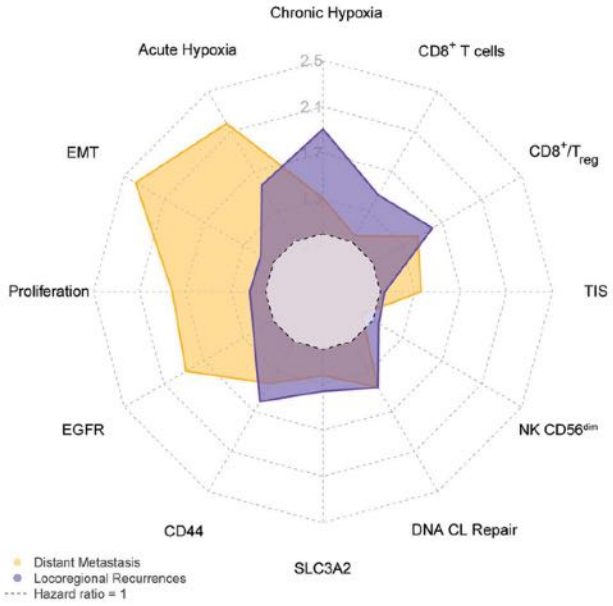
The more hypoxic tumors are also more responsive to hypoxia-directed interventions (such as treatment with the hypoxic cell radiosensitizer nimorazole).

(The less hypoxic tumors do ***not*** *benefit from the addition of nimorazole.)*

doi: 10.3389/fonc.2019.01470

Hypoxia as a biomarker (cont.)

~200 patients with advanced stage HPV-negative HNSCC



Importance of different biological factors in predicting likelihood of locoregional recurrence (purple) or distant metastasis (yellow) in advanced HPV-negative head & neck squamous cell carcinoma. (The "spider plot" shows average hazard ratios for each factor, with a higher number suggesting a greater influence.)

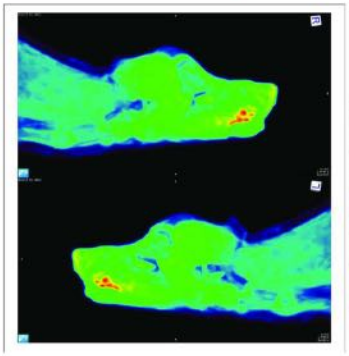
In this example, locoregional recurrence was more associated with chronic hypoxia, and the development of distant metastases was more associated with acute hypoxia.

Are we making progress in *non-invasive* (PET) methods for measuring tumor hypoxia?

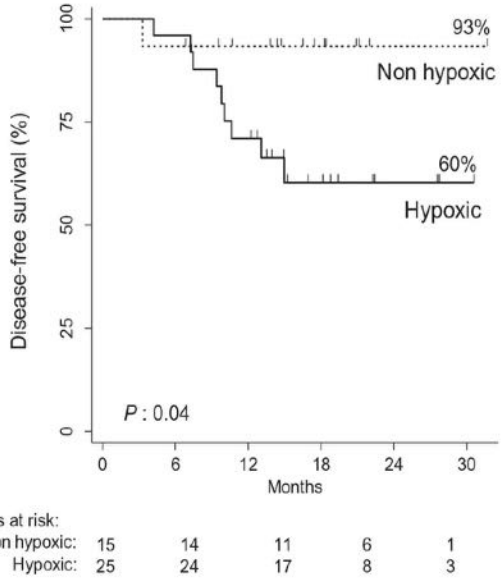
Yes, but slowly. Several markers currently being used in preclinical or early clinical studies include:

FAZA = ^{18}F-labelled fluoroazomycin arabinoside

FAZA uptake in a canine patient with a squamous cell carcinoma of the oral tongue

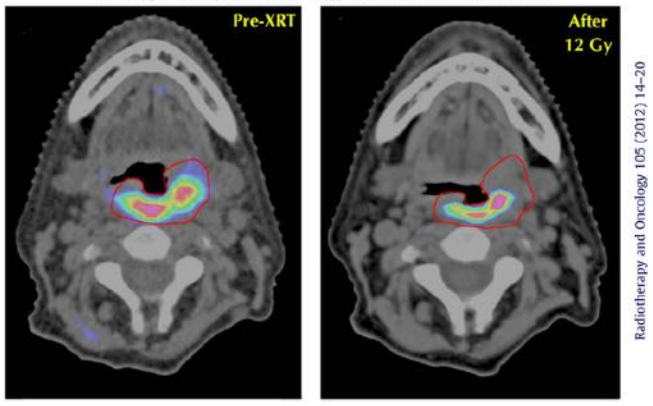


Front. Oncol., 22 November 2019
https://doi.org/10.3389/fonc.2019.01291



Radiotherapy and Oncology 105 (2012) 14–20

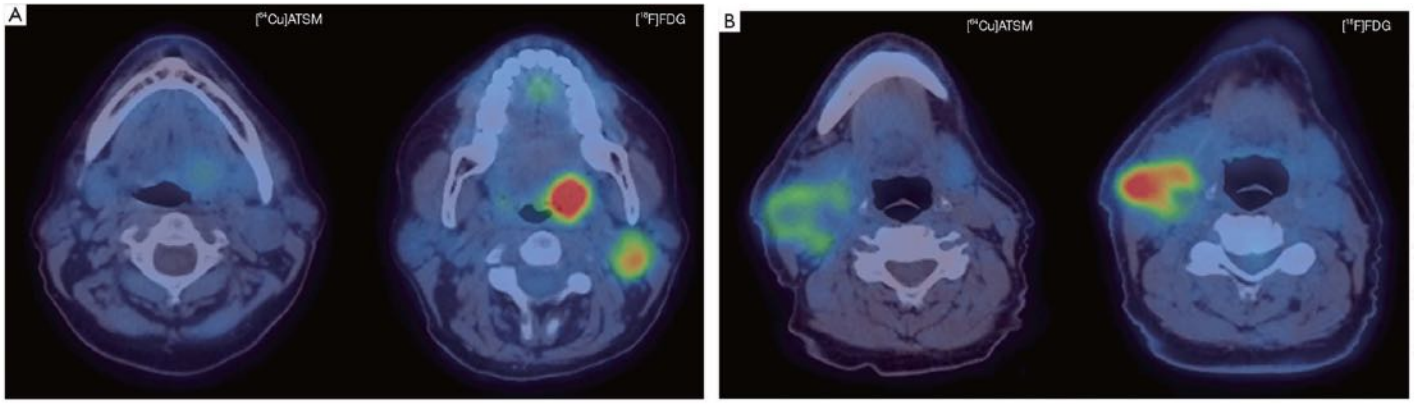
FAZA hypoxia imaging in radiotherapy treated HNSCC - DAHANCA 24



Radiotherapy and Oncology 105 (2012) 14–20

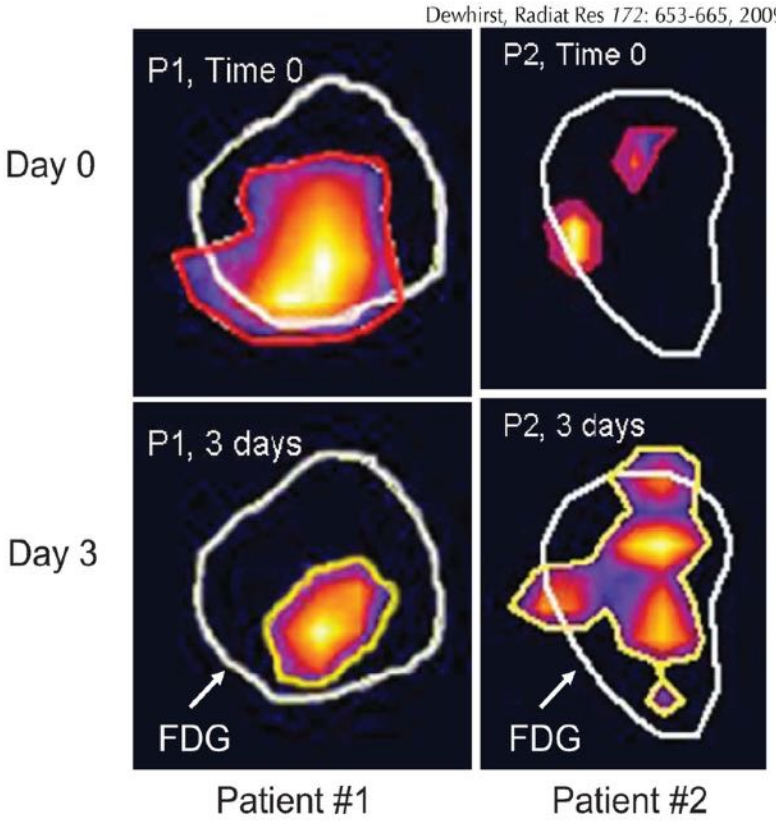
Disease free survival of patients with hypoxic versus non hypoxic tumors as assessed by FAZA PET.

Cu-ATSM = copper(II)-diacetyl-bis(N4-methylthiosemicarbazone)



[^{64}Cu]ATSM PET/CT (left) and corresponding [^{18}F]FDG PET/CT (right) of an (A) oropharyngeal primary with ipsilateral cervical lymphadenopathy showing increased FDG uptake but only mild [^{64}Cu]ATSM uptake in the primary tumour and (B) enlarged FDG-avid right cervical lymph node with increased [^{64}Cu]ATSM at the periphery.

Dewhirst, Radiat Res *172*: 653-665, 2009



Intermittent hypoxia first demonstrated for a human tumor (head and neck) using PET scanning

Differences in ^{18}F misonidazole uptake as assessed by PET scans taken 3 days apart in patients with head and neck cancer. In these scans, the ^{18}F FDG (fluorodeoxyglucose) avid area is outlined in white and the areas positive for ^{18}F misonidazole are outlined in red (time 0) and yellow (time 3 days). In patient 1, the region of hypoxia remains in the same location but shrinks between day 0 and day 3. In patient 2, the regions of hypoxia change in size and distribution between the 2 days. This is the first evidence for cycling hypoxia in human subjects.

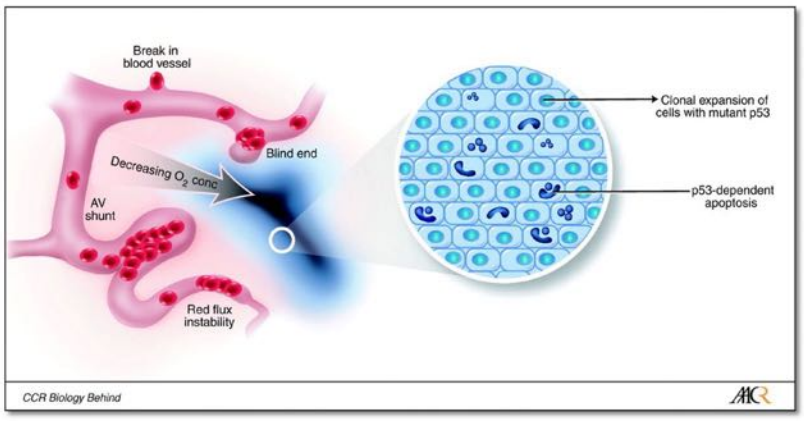
D. The Molecular Biology of Tumor Hypoxia

1] the radiation and cancer biology communities received a wake-up call during the mid-1990's when it became clear that tumor hypoxia was significantly more complex than first thought, and had implications far beyond merely being a source of resistance to radiotherapy

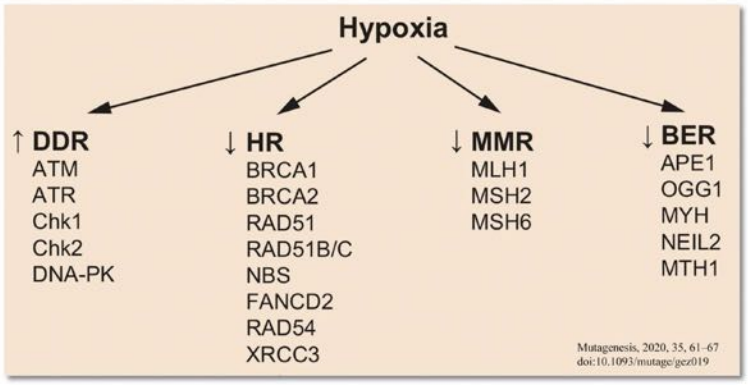
2] ***Epiphany #1: Hypoxia is a driving force for tumor progression and increased aggressiveness*** (see: Graeber et al, Nature 379: 88-91, 1996)

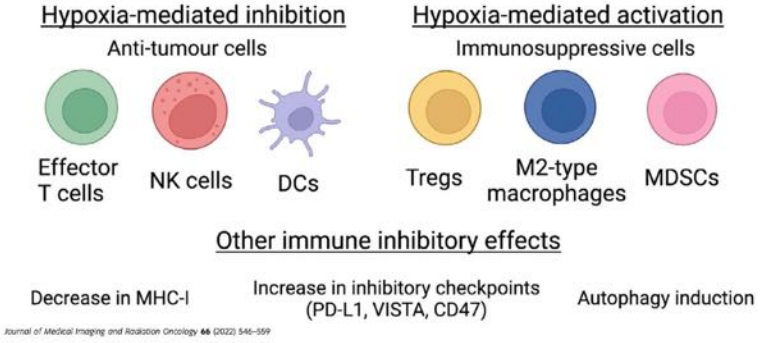
a. *normally, cells which are exposed to hypoxic conditions for extended periods of time experience growth arrest and often, apoptosis; these effects are mediated by wild-type p53*

b. *however, cells with missing or mutated versions of p53 ("loss of function", LOF) can continue to grow and don't undergo apoptosis--thus, these mutant cells eventually take over the whole tumor, thereby endowing it with more malignant characteristics than it may have had originally*



c. *hypoxia also further contributes to genomic instability by down-regulating important DNA repair pathways*





d. *to make matters worse, hypoxia also plays a significant role in creating an immunosuppressive tumor mictroenvironment; hypoxia causes:*

1. *accumulation, activation and expansion of regulatory T-cells (Tregs) that turn off immune responses*

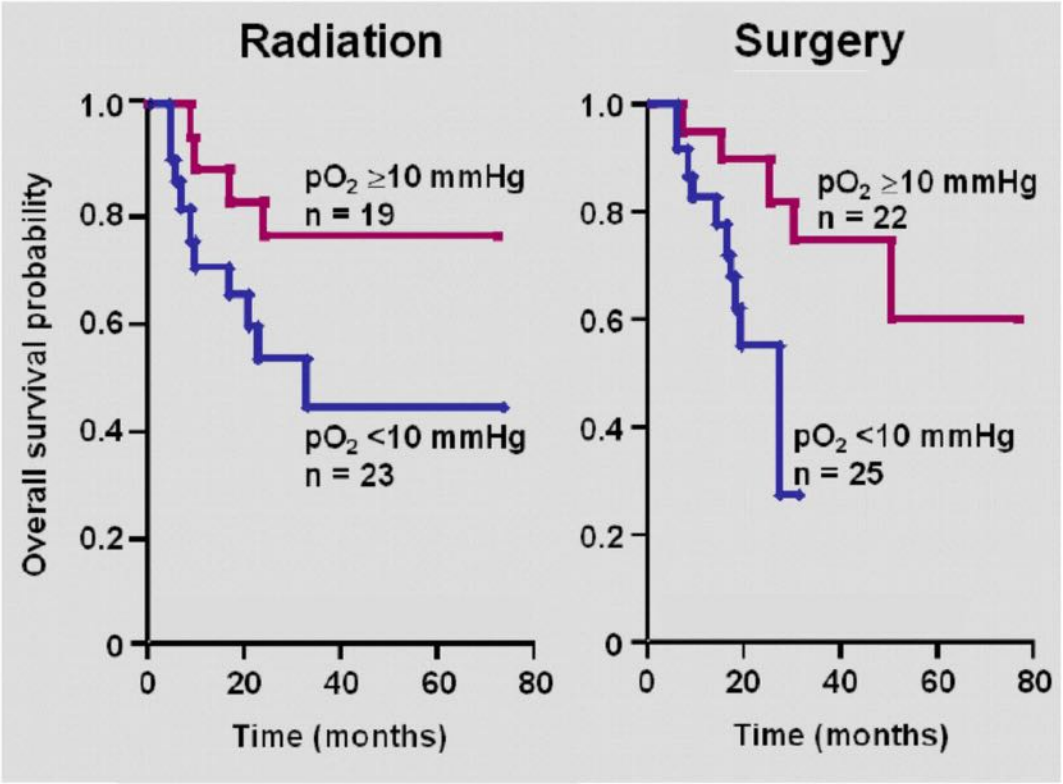
2. *recruitment and reprogramming of macrophages and MDSCs to support immunosuppression rather than activation*

3. *suppression of dendritic cell maturation, leading to impaired tumor antigen presentation, and NK cell activation*

(Important) Clinical Correlate:

c. ***patients with hypoxic tumors tend to have worse treatment outcomes even when they have not received radiation therapy, i.e., that the radiation resistance of hypoxic cells is NOT the sole cause of treatment failure***

Patients with advanced cervical carcinoma



Höckel M. et al. Cancer Res 56, 4509-4515 (1996)

3] ***Epiphany #2: Cells consider hypoxia a "stressor", and in an attempt to adapt, activate many genes to help them cope***

a. unfortunately some of the genes that become activated under hypoxic conditions are the very ones that promote increased tumor aggressiveness

Classification of the hypoxia-regulated genes

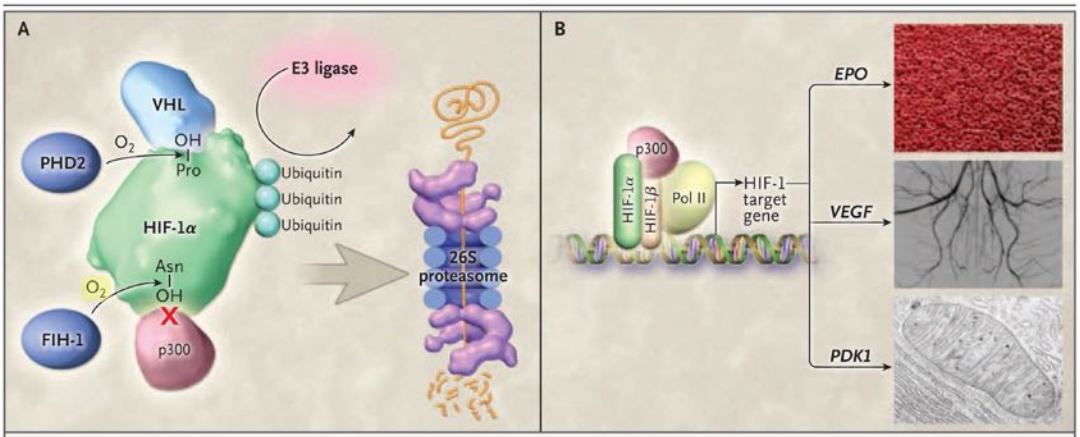
Biological function	Gene symbol/ alias
Transcription factors	Twist1, Snail, Slug, ZEB1, ZEB2, E12/E47, ID2,EWS-FLI1, β-catenin, CDX2, SMAD7
Histone modifiers	JMJD2B, JMJD2C, MLL1
Enzymes	MMP1, MMP3, MT1-MMP, LOX, ADAMTS1, ACE, ACE2, Hsulf-1, IDH2, XPA
Receptors, receptor-associated kinase	CXCR4, CX3CR1, Notch, uPAR, PAI-1, 67-kDa laminin receptor, TLR4, c-Met, RON tyrosine kinase
small GTPases, intracellular signaling molecules	Cdc42, Rac1, RhoE, IRS-2
Transporters	glut-1, MDR1, VDAC1
Membrane proteins	ANGPTL4, L1CAM, α5 integrin, CD151, CD24, CD147, Galectin-1, MUC1, Semaphorin 4D, Caveolin-1

Around a thousand genes (and counting) are upregulated under hypoxic conditions, many of which are associated with one or more of the following processes:

ANGIOGENESIS
GLYCOLYSIS ┐ "METABOLIC REPROGRAMMING"
pH REGULATION ┘
GROWTH & SURVIVAL PROMOTION
MOTILITY & INVASION
METASTASIS
DIFFERENTIATION (e.g., "STEMNESS")
IMMUNE EVASION

b) what, exactly, is turning on all of these genes? Answer: **Hypoxia-Inducible Transcription Factors**

N ENGL J MED 365;6 NEJM.ORG AUGUST 11, 2011

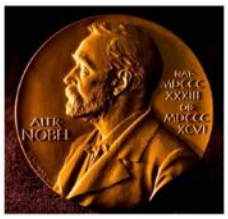


Oxygen Sensing, Gene Expression, and Adaptive Responses to Hypoxia.

In well-oxygenated cells (Panel A), prolyl hydroxylase domain 2 (PHD2) uses oxygen to hydroxylate hypoxia-inducible factor 1 (HIF-1α) on a proline residue (Pro–OH). The von Hippel–Lindau (VHL) protein binds to HIF-1α containing Pro–OH and recruits a ubiquitin E3 ligase. The polyubiquitination of HIF-1α flags the protein for degradation by the 26S proteasome. Factor inhibiting HIF-1 (FIH-1) also uses oxygen to hydroxylate HIF-1α on an asparagine residue (Asn–OH). HIF-1α containing Asn–OH cannot be bound by the coactivator protein p300, thereby preventing HIF-1α from activating gene transcription. Under hypoxic conditions (Panel B), the Pro and Asn hydroxylation reactions are inhibited, and HIF-α (i.e., either HIF-1α or HIF-2α) rapidly accumulates, dimerizes with HIF-1β, recruits p300, binds to hypoxia response elements, and activates the transcription by RNA polymerase II (Pol II) of hundreds of target genes, such as the following: *EPO*, encoding erythropoietin, which is the hormone that stimulates red-cell production (photomicrograph at top); *VEGF*, encoding vascular endothelial growth factor, which is the angiogenic factor that stimulates blood-vessel formation (angiogram in middle); and *PDK1*, encoding pyruvate dehydrogenase kinase 1, which inhibits the conversion of pyruvate to acetyl coenzyme A for oxidation in the mitochondrion (electron micrograph at bottom).

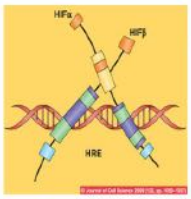
The researchers (Drs. Gregg Semenza, Peter Ratcliffe and Willian Kaelin) who figured out how HIF-1 works won the 2016 Lasker Prize for medicine, considered the "American Nobel".

And then in 2019, they upped the ante by winning the *real* Nobel Prize!



(However it was radiobiologists studying tumor hypoxia who did much of the ground work.)

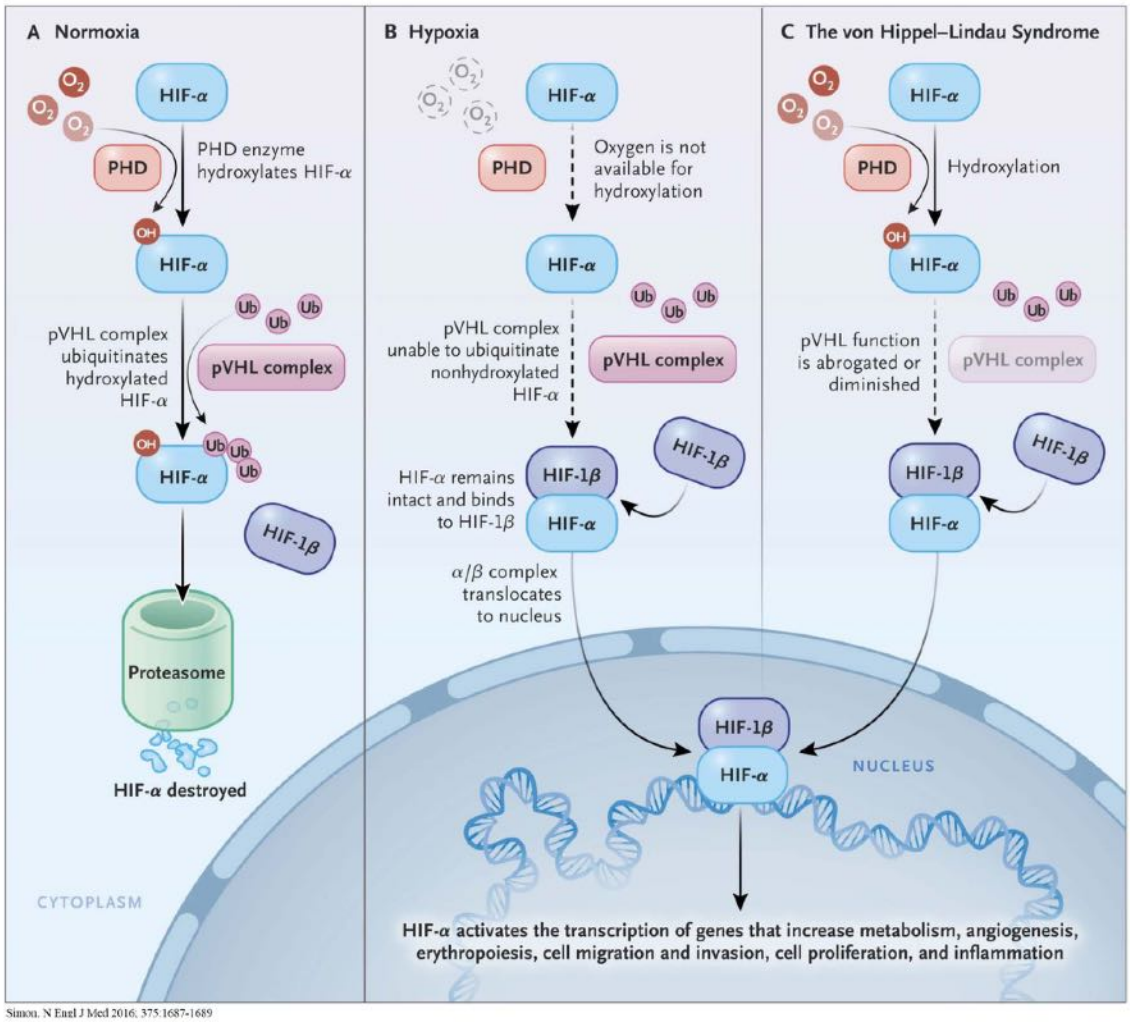
1. the principal transcription factor is called HIF-1, which has both an "α" and "β" variant; these bind to promoter regions (called "hypoxia responsive elements" or HRE's) of genes that are responsive to **oxygen** concentration, or lack thereof



2. **HIF-1β, also called "ARNT" (aryl hydrocarbon receptor nuclear translocator), is constitutively active, and serves as HIF-1α's binding partner; only when both HIFs are bound to a gene's HRE is the gene activated and transcription able to occur**

(a) when oxygen is present, any HIF-1α (normally located in the cytoplasm of the cell) is degraded, however as the oxygen concentration drops, HIF-1α is stabilized, transported into the nucleus, and allowed to bind to the HRE's, activating transcription of the target genes

Note that the stabilization of HIF-1α starts at an O_2 concentration of ~1%, higher than for "radiobiological hypoxia"



Simon. N Engl J Med 2016; 375:1687-1689

Also of interest is that in patients with von Hippel-Lindau Syndrome (another cancer predisposition syndrome), HIF-1α is active even in the presence of oxygen because pVHL is lacking, so it can't be ubiquitinated and targeted for proteasomal degradation.

As such, all of the hypoxia-activated genes get turned on...even in the absence of hypoxia. (Definitely not a good thing cancer-wise.)

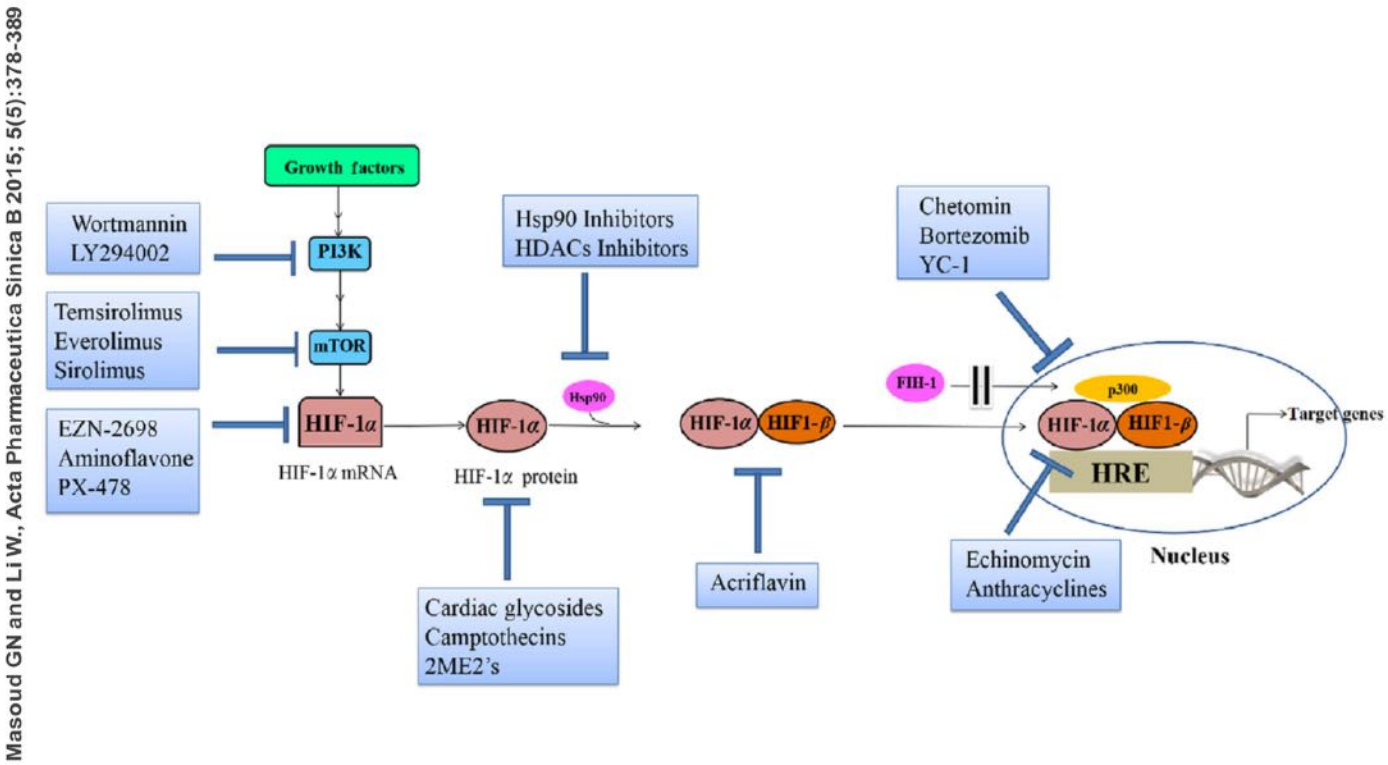
Emerging Science!

Leveraging HIF-1 gene activation for clinical advantage?

c) One area of research interest has been to use a hypoxia-responsive promoter (HRE) and link it to a toxin gene for the purposes of killing hypoxic cells, selectively via gene therapy

d) What about trying to inhibit HIF-1α as a clinical strategy

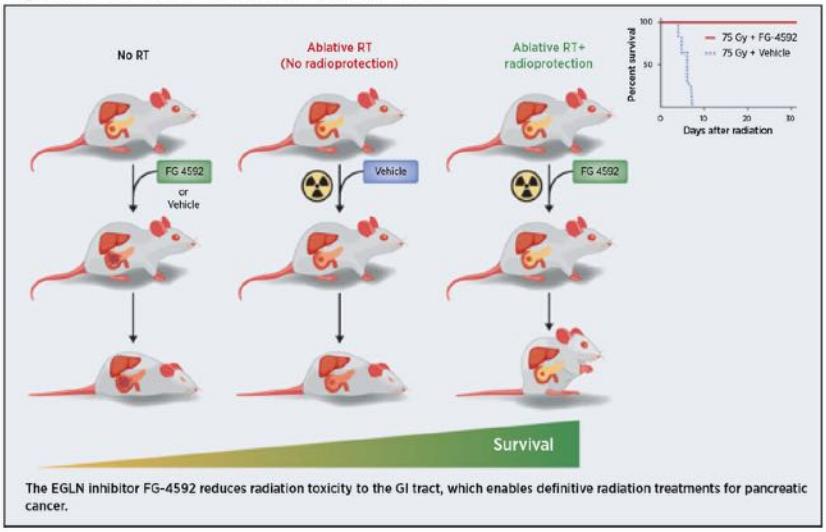
There are lots of drugs already out there that inhibit HIF-1α to varying extents (although this is not what they were developed for), and many more are in the pipeline that are more specific



e) What about trying to radioprotect normal tissues via activation of HIF-1 target genes?

Drugs called EGLN inhibitors inactivate the prolyl hydroxylases that target HIF-1α for degradation under well-aerated conditions, meaning it would become stabilized despite the presence of oxygen; this would have the effect of turning on all the hypoxia-activated genes (i.e., similar to what happens when the VHL protein is mutated or absent).

Fujimoto *et al.* Cancer Res 2019;*79 (9)*:2327–2338



The EGLN inhibitor FG-4592 reduces radiation toxicity to the GI tract, which enables definitive radiation treatments for pancreatic cancer.

In the long term, this would be considered a bad idea, but in the short term, if some of the genes associated with radioprotection of vasculature and promotion of angiogenesis (and maybe some others?) were activated, this could have the effect of protecting critical normal tissues from radiation injury.

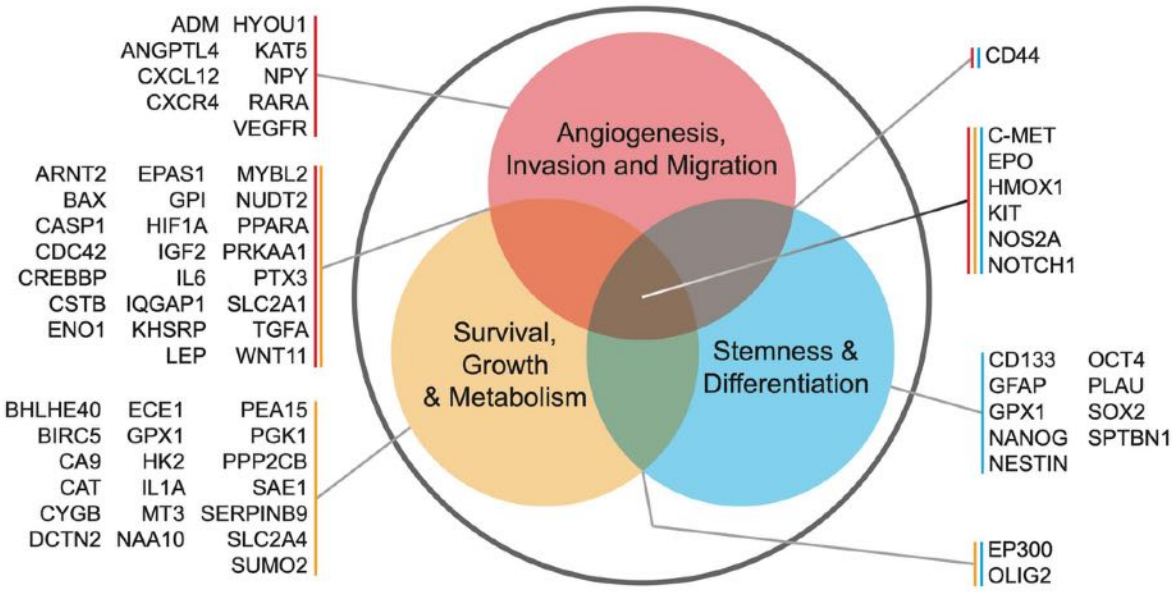
In a mouse model of pancreatic cancer, when animals were treated with the EGLN inhibitor FG-4592, significantly higher radiation doses could be tolerated without causing as much potentially fatal GI bleeding. This resulted in much improved local tumor control for a type of tumor that is notoriously difficult to eradicate locally.

APPENDIX MATERIALS

Methods for the detection of hypoxia in tumors

Invasive methods	
Polarographic oxygen electrode	Direct method
Luminescence-based optical sensor	Direct method
Pimonidazole	Immunohistochemistry
HIF-1α	Immunohistochemistry
Carbonic anhydrase IX	Immunohistochemistry
18F-2-nitroimidazolpentafluoropropylacetamiede (EF5)	Immunohistochemistry
Non-invasive methods	
18F-fluoromisonidazole (18F-FMISO)	PET
2-deoxy-2-(18F)fluoro-D-glucose (18F-FDG)	PET
18F-fluoro-erythronitroimidazole (18F-FETNIM)	PET
18F-fluoro-azomycin-arabinoside (18F-FAZA)	PET
18F-2-nitroimidazolpentafluoropropylacetamiede (EF5)	PET
18F-EF3	PET
18F-FRP170	PET
18F-flortanidazole (HX4)	PET
[(68) Ga]-HP-DO3A-nitroimidazole	PET
Cu-ATSM	PET
^{131}I-IAZGP	PET
^{89}Zr-labeled cG250-F(ab')$_2$	PET
^{123}I-Iodoazomycin arabinoside	SPECT
^{99m}Tc-cyclam-2-nitroimidazole	Planar scintigraphy
^{68}Ga-metronidazole	MRI
Oxygen-enhanced MRI	MRI
Dynamic contrast-enhanced MRI	MRI
Blood oxygen level-dependent MRI "BOLD"	MRI
Tissue oxygen level dependent MRI "TOLD"	MRI

Interrelationships Between Select Hypoxia-Responsive Genes



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Why should you assess hypoxia pre-treatment and use it to help stratify patients into different treatment groups? ANSWER: Because the impact of any hypoxia-directed therapies become much more apparent!

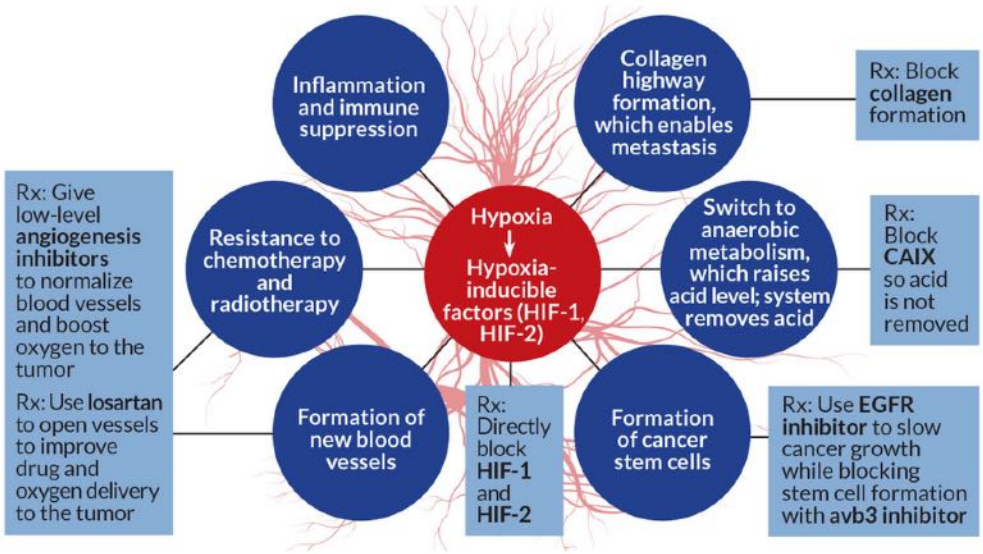
Select clinical trials where pretreatment assessment of tumor hypoxia helped demonstrate significant improvements in outcomes:

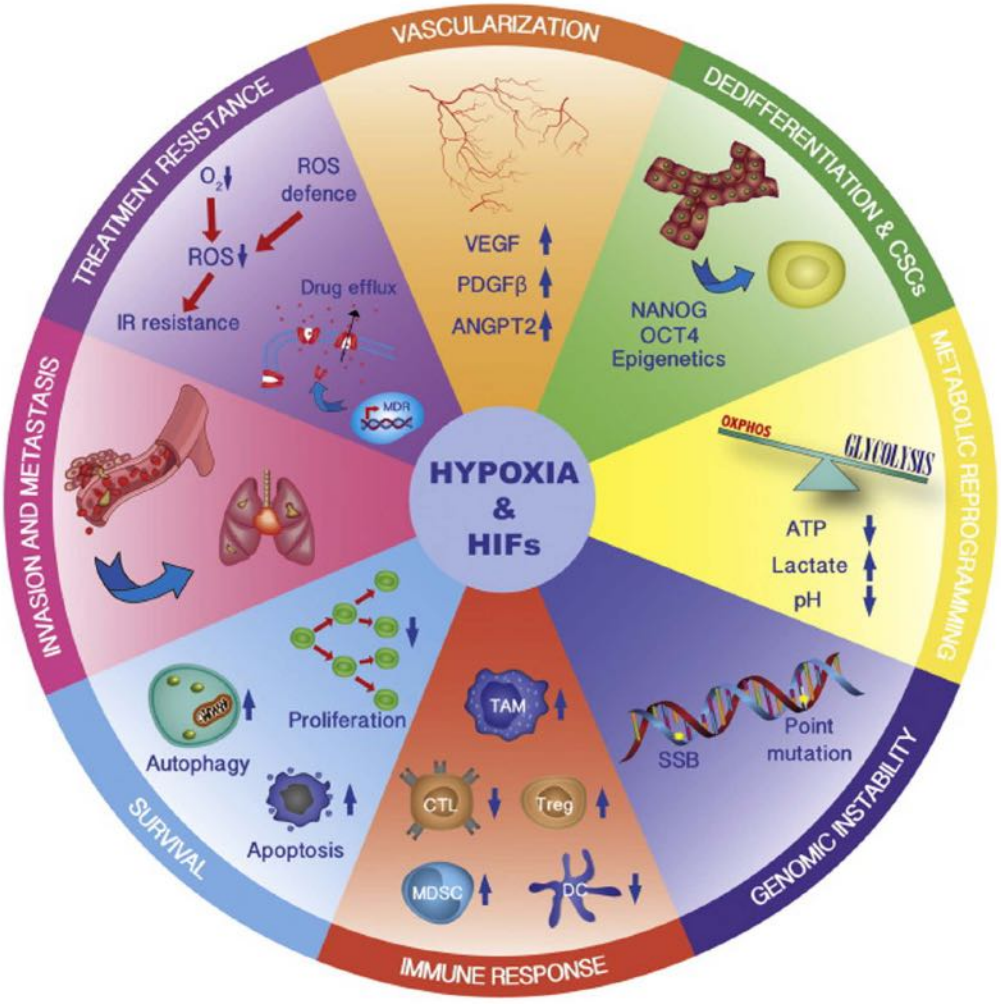
Method	Cancer	Treatment	*n*	*p*	Reference
Pimonidazole	HNC	AR ± CON	38	0.010	Kaanders et al.
^{18}F-FMISO PET	HNC	CRT ± tirapazamine	45	0.006	Rischin et al.
Osteopontin	HNC	RT ± nimorazole	320	0.006	Overgaard et al.
CA9	HNC	RT ± nimorazole	320	0.800	Eriksen et al.
Pimonidazole	HNC	AR ± CON	79	0.010	Janssens et al.
15-gene	HNC	RT ± nimorazole	323	0.003	Toustrup et al.
Osteopontin	HNC	CRT ± tirapazamine	578	0.870	Lim et al.
26-gene	HNC	AR ± CON	157	0.009	Eustace et al.
26-gene	Bladder	RT ± CON	185	0.400	Eustace et al.
Necrosis	Bladder	RT ± CON	231	0.001	Eustace et al.
CA9	Bladder	RT ± CON	189	0.030	Eustace et al.
EGFR low	HNC	AR ± CON	272	0.009	Nijkamp et al.
^{18}F-FAZA PET	HNC	CRT ± tirapazamine	24	0.004	Graves et al.
mir-210	Bladder	RT ± CON	183	0.070	Irlam-Jones et al.
24-gene	Bladder	RT ± CON	151	0.015	Yang et al.

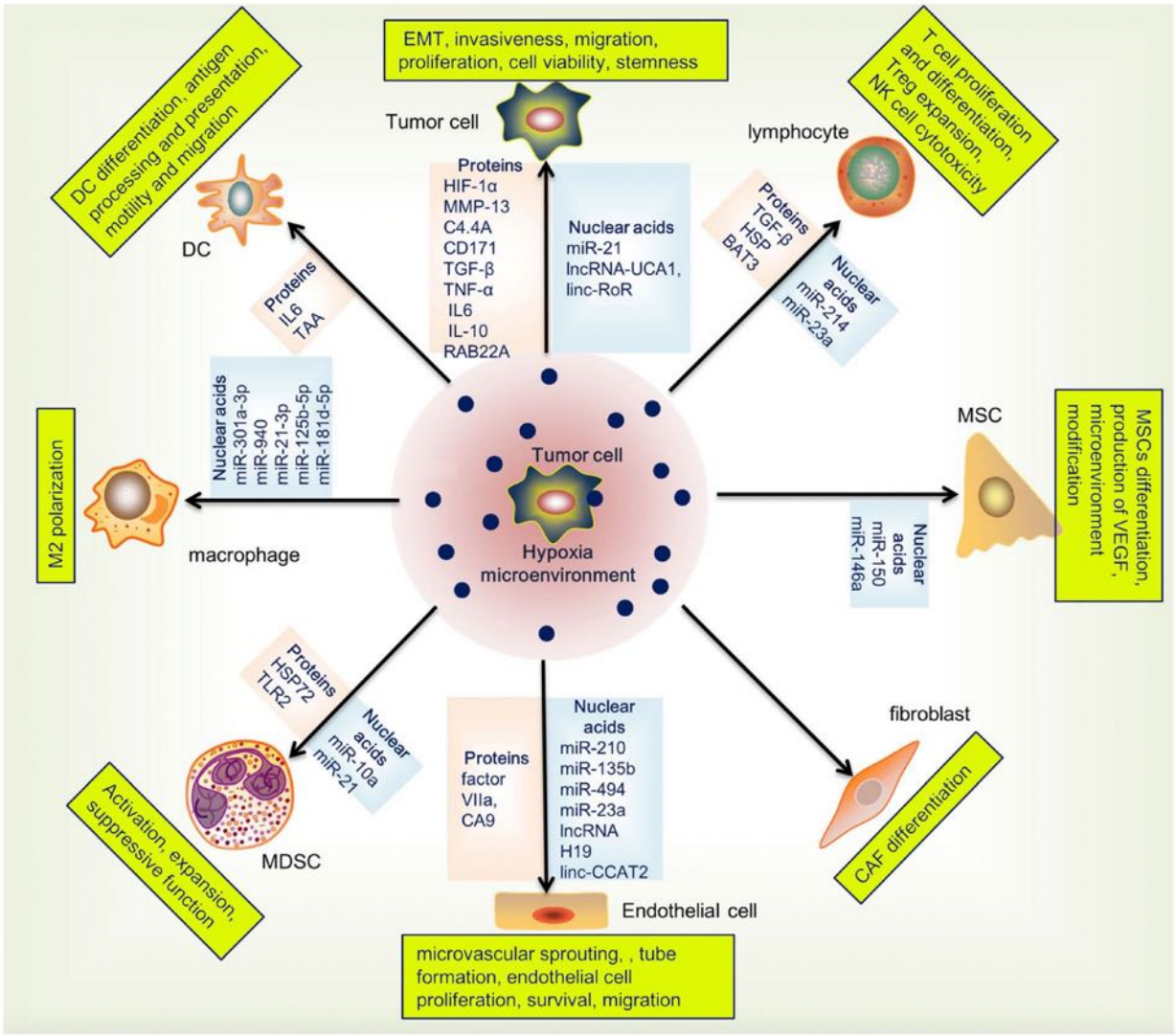
Abbreviations: HNC, head and neck cancer; AR, accelerated radiotherapy; CON, carbogen and nicotinamide; CRT, chemo-radiotherapy; RT, radiotherapy.

The HIF-1 Wheels of (Mis)Fortune

Schematic representation of the role of hypoxia-induced accumulation of HIF-1α in human cancers. *HIF-1α-regulated gene products may play pivotal roles in tumor progression, aggressiveness, and metabolic adaptation, and may contribute to increased resistance of hypoxic tumors to radiotherapy and chemotherapy.*







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