Quantitative Analysis of Hypoxia Markers in Cervical Cancer Biopsies

By

BOJANA JANKOVIC

B.Sc. (Biophysics Honours), The University of British Columbia, 2003

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
in
THE FACULTY OF GRADUATE STUDIES
(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

June 2005
© Bojana Janković, 2005
ABSTRACT

INTRODUCTION: Although tumour hypoxia has been associated with a more aggressive phenotype and lower cure rate, there is no consensus on the method best suited for routine measurement. Recent studies have suggested that carbonic anhydrase 9 (CA9) and hypoxia-inducible factor-1α (HIF-1α) can be used as endogenous markers of hypoxia. Expression of these markers was compared to binding of the chemical hypoxia marker pimonidazole and oxygen microelectrode measurements in 78 tumor biopsies from patients with locally advanced invasive carcinoma of the cervix.

METHODS: Two or more biopsies were taken one day after i.v. infusion of pimonidazole. One biopsy was used for flow cytometry analysis of pimonidazole binding, while sequential sections from the other biopsies were used for immunohistochemical analyses of pimonidazole binding and expression of CA9 and HIF-1α. Images of stained sections were tiled and analyzed to determine the fraction of labeled tumor cells in tissue and marker colocalization. Hypoxic proportions, HP2.5 and HP5, were obtained from polarographic oxygen electrode measurements in tumours.

RESULTS AND DISCUSSION: Automated image analysis was found to be a reliable method for quantitative measurement of hypoxia marker expression or binding. The mean percentage of marker positive regions of the sections was similar for the 3 markers (between 5.5% and 7.4%). A significant but weak correlation was found between flow cytometry and image analysis methods for detecting pimonidazole ($r = 0.45$). Hypoxia
marker analysis on multiple biopsies taken from the same tumour indicated that there was substantial intra-tumour marker heterogeneity. CA9 expression correlated with pimonidazole binding ($r = 0.60$), while weaker but significant correlations were observed between both of these markers and HIF-1α ($r = 0.34$ for pimonidazole and HIF-1α, and $r = 0.42$ for CA9 and HIF-1α). Markers showed a high degree of colocalization, with 60%-80% of the regions staining with all markers if one marker were present. Nevertheless, regions of mismatch were identified and were of particular interest as they could be indicative of transient changes in tumor perfusion. No correlation was observed between oxygen electrode measurements and expression of the three hypoxia markers. Neither pimonidazole binding nor endogenous marker expression correlated with known clinical prognostic factors (Hb, nodal status, maximum tumour size, or FIGO stage). High HIF-1α expression predicted shorter progression-free survival ($p = 0.024$), while CA9 ($p = 0.333$) and pimonidazole ($p = 0.488$) had no apparent predictive value in this group of patients. Measurements of HIF-1α changes over the course of radiotherapy for 15 patients evaluated did not correlate with disease progression.

**CONCLUSIONS:** Quantitative comparisons between three hypoxia markers indicated significant but weak correlations between binding of pimonidazole and expression of two endogenous markers. Good colocalization of HIF-1α and CA9 with pimonidazole stained regions was observed confirming the ability of these proteins to mark hypoxic cells. In addition, HIF-1α emerged as a potential prognostic marker, and the predictive ability improved when two or more hypoxia markers were combined.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF EQUATIONS</td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xiv</td>
</tr>
<tr>
<td><strong>1. INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td>1.1. Overview and Goals of the Study</td>
<td>1</td>
</tr>
<tr>
<td>1.2. Structure of the Thesis</td>
<td>3</td>
</tr>
<tr>
<td>1.3. Tumour Microenvironment</td>
<td>6</td>
</tr>
<tr>
<td>1.3.1. Definitions of Hypoxia</td>
<td>6</td>
</tr>
<tr>
<td>1.3.2. Vascular Heterogeneity</td>
<td>7</td>
</tr>
<tr>
<td>1.3.3. Chronic versus Acute Hypoxia</td>
<td>9</td>
</tr>
<tr>
<td>1.4. Oxygen-response Pathways</td>
<td>16</td>
</tr>
<tr>
<td>1.4.1. Regulation of HIF-1α</td>
<td>17</td>
</tr>
<tr>
<td>1.4.2. HIF-1 Targets</td>
<td>20</td>
</tr>
<tr>
<td>1.5. Resistance of Hypoxic Cells to Therapy</td>
<td>24</td>
</tr>
<tr>
<td>1.5.1. Reduced Sensitivity to Radiation Therapy</td>
<td>25</td>
</tr>
<tr>
<td>1.5.2. Evasion of Chemotherapeutic Strategies</td>
<td>28</td>
</tr>
<tr>
<td>1.6. Malignant Progression</td>
<td>28</td>
</tr>
<tr>
<td>1.6.1. Hypoxic Subpopulations and their Prognostic Significance in Treatment Outcome</td>
<td>29</td>
</tr>
<tr>
<td>1.6.2. Other Factors That Contribute to Treatment Outcome Together With Hypoxia</td>
<td>30</td>
</tr>
<tr>
<td>1.7. Methods for Measuring Hypoxia</td>
<td>33</td>
</tr>
<tr>
<td>1.7.1. Use of Polarographic Oxygen Electrode to Measure Hypoxic Fraction</td>
<td>35</td>
</tr>
<tr>
<td>1.7.2. Use of Exogenous Markers for Measuring Hypoxic Fraction</td>
<td>37</td>
</tr>
<tr>
<td>1.7.3. Use of Endogenous Markers for Measuring Hypoxic Fraction</td>
<td>40</td>
</tr>
<tr>
<td>1.7.4. Other Techniques for Measuring Hypoxia</td>
<td>41</td>
</tr>
<tr>
<td>1.8. Strategies for Overcoming Tumour Hypoxia in the Clinic</td>
<td>43</td>
</tr>
<tr>
<td>1.8.1. Hypoxia-Modulating Therapies</td>
<td>43</td>
</tr>
<tr>
<td>1.8.2. Hypoxia-Targeting Therapies</td>
<td>46</td>
</tr>
<tr>
<td><strong>2. METHODS</strong></td>
<td>48</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>2.1. Patients</td>
<td>48</td>
</tr>
<tr>
<td>2.2. Eppendorf Electrode Measurements</td>
<td>52</td>
</tr>
<tr>
<td>2.3. Flow Cytometry Analysis</td>
<td>52</td>
</tr>
<tr>
<td>2.4. Immunohistochemical Staining of Tissue Sections for Pimonidazole, CA9, HIF-1α and CD31</td>
<td>55</td>
</tr>
<tr>
<td>2.5. Image Acquisition, Processing and Enhancement</td>
<td>60</td>
</tr>
<tr>
<td>2.6. Image Analysis: Quantifying the Fraction of Tissue Labeled for Pimonidazole, CA9, HIF-1α and CD31</td>
<td>64</td>
</tr>
<tr>
<td>2.6.1. Manual versus Automated Image Thresholding</td>
<td>64</td>
</tr>
<tr>
<td>2.6.2. Histogram Analysis as an Alternative Approach for Quantifying the Stained Tissue Fraction</td>
<td>67</td>
</tr>
<tr>
<td>2.6.3. Measurement of Hypoxic Marker Distribution in Relation to Vasculature</td>
<td>67</td>
</tr>
<tr>
<td>2.7. Image Analysis: Marker Colocalization</td>
<td>68</td>
</tr>
<tr>
<td>2.7.1. Semi-quantitative Marker Colocalization: Visual Scoring</td>
<td>68</td>
</tr>
<tr>
<td>2.7.2. Image Registration</td>
<td>70</td>
</tr>
<tr>
<td>2.7.3. Quantitative Marker Colocalization: Theoretical Calculations</td>
<td>71</td>
</tr>
<tr>
<td>2.7.4. Verification of the Use of Theoretical Calculations to Predict Marker Colocalization: Direct Measurements of Colocalized Marker Fractions by Image Manipulation</td>
<td>73</td>
</tr>
<tr>
<td>2.8. Statistical Analysis of Marker Correlations and Outcome</td>
<td>74</td>
</tr>
<tr>
<td>2.9. Use of Tumour Image Models to Examine the Effects of Noise, Contrast and Stain Distribution on Image Analysis Reproducibility</td>
<td>75</td>
</tr>
<tr>
<td>3. RESULTS</td>
<td>78</td>
</tr>
<tr>
<td>3.1. Inter- and Intra-Tumour Heterogeneity in Hypoxia Marker Labeling</td>
<td>81</td>
</tr>
<tr>
<td>3.1.1. Inter-tumour Heterogeneity in Hypoxia Marker Labeling</td>
<td>81</td>
</tr>
<tr>
<td>3.1.2. Intra-tumour Heterogeneity in Hypoxia Marker Labeling</td>
<td>85</td>
</tr>
<tr>
<td>3.1.2.1. Heterogeneity in Hypoxia Marker Labeling Among Biopsies of the Same Tumour</td>
<td>85</td>
</tr>
<tr>
<td>3.1.2.2. Reproducibility of Pimonidazole Labeling Between Sequential Sections</td>
<td>89</td>
</tr>
<tr>
<td>3.2. Numerical Correlations Between Hypoxic Fraction Determined by Pimonidazole, CA9 and HIF-1α</td>
<td>91</td>
</tr>
<tr>
<td>3.2.1 Inter-Observer Variation in Hypoxic Fraction Assessment Using Image Analysis</td>
<td>94</td>
</tr>
<tr>
<td>3.2.2. Correlation Between Hypoxia Markers in Sequential Sections</td>
<td>96</td>
</tr>
<tr>
<td>3.3. Spatial Correlations Between Pimonidazole, CA9 and HIF-1α in Sequential Tissue</td>
<td>96</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1. Antibody reference list ................................................................................................................ 59
Table 2. Image acquisition settings in Northern Eclipse ........................................................................... 61
Table 3. Semi-quantitative colocalization scoring scheme ........................................................................ 69
Table 4. Twelve colocalization parameters .............................................................................................. 69
Table 5. Statistics of clinicopathologic parameters and therapy, and hypoxic fraction in relation to these factors ........................................................................................................................................... 79
Table 6. Distribution of hypoxic fraction measured by markers and Eppendorf electrode in cervical carcinomas .............................................................................................................................................. 82
Table 7. Inter-observer correlations in measurement of marker positive fraction ........................................ 95
Table 8. Pearson’s correlation coefficients between pairs of hypoxia measurement techniques .................. 97
Table 9. Comparison between two quantitative marker colocalization techniques .................................... 103
Table 10. Correlation between colocalization (calculated semi-quantitatively) of pairs of markers and marker positive fraction .......................................................................................................................... 107
Table 11. Rates of 3-year progression-free survival for different marker combinations ............................. 118
LIST OF FIGURES

Figure 1. Vascular network in normal and tumour tissue ......................................................... 8
Figure 2. Two forms of hypoxia: chronic and acute ............................................................... 8
Figure 3. Diffusion of oxygen through the tissue in a two-dimensional model with a radial flow outward ............................................................. 12
Figure 4. Major interactions in the HIF-1 pathway under normoxic and hypoxic conditions .................................................................................. 18
Figure 5. The role of CA9 in regulating the acid-base balance intra- and extracellularly ........... 22
Figure 6. An example of permanent oxygen “fixation” by oxygen adduct formation with an unstable DNA radical, formed upon direct or indirect effect of radiation .................... 27
Figure 7. The number of tumour hypoxia-related publications, from 1965 until 2004, fitted to a polynomial function ................................................................. 34
Figure 8. Schematic diagram of the experimental procedures for quantitative hypoxia measurements used in this study ......................................................... 51
Figure 9. The output graph of flow cytometry analysis of pimonidazole-labeled tumour cells from a biopsy ................................................................. 54
Figure 10. Images resulting from the RGB Split function in ImageJ ........................................... 63
Figure 11. Tissue and stain threshold procedure demonstrated on an image of a pimonidazole-stained section .............................................................................. 65
Figure 12. Tumour image model ............................................................................................. 76
Figure 13. Histograms of hypoxic fraction distributions ........................................................ 84
Figure 14. Pimonidazole Flow Cytometry vs. Pimonidazole Immunohistochemistry ......... 86
Figure 15. Heterogeneity in hypoxia marker labeling measured in multiple biopsies from each tumour .................................................................................. 88
Figure 16. Correlation between pimonidazole positive fractions in stained sections from Set I and Set II .................................................................................. 90
Figure 17. Subcellular localizations of markers and marker staining patterns ......................... 93
Figure 18. Numerical marker correlations ............................................................................. 99
Figure 19. Histograms of colocalization frequency between pairs of markers ................. 101
Figure 20. Distributions of staining colocalization patterns with respect to pimonidazole, CA9 and HIF-α staining ................................................................. 105
Figure 21. Comparison between marker colocalization (measured quantitatively) and marker positive fraction .............................................................. 109
Figure 22. Distribution of hypoxia markers in relation to vasculature .................................. 111
Figure 23. HIF-1α change over the course of radiotherapy in 15 patients ......................... 114
Figure 24. Cumulative progression-free survival of patients with respect to individual hypoxia markers ................................................................. 117
Figure 25. Cumulative progression-free survival of patients with respect to combined hypoxia markers ................................................................. 120
Figure 26. Cumulative progression-free survival of patients with respect to pimonidazole in small (<5cm) and large (≥5cm) tumours ................................................................. 121
Figure 27. Correlation between automated and manual image analysis ................................................................. 124
Figure 28. Histogram of an 8-bit grayscale image of a tumour tissue section immunohistochemically stained for pimonidazole ................................................................. 126
Figure 29. The effect of noise on the histogram of the image (a) and image analysis (b) ................................................................. 128
Figure 30. The effect of contrast between the stained and unstained tissue on the histogram of the image (a) and image analysis (b) ................................................................. 130
Figure 31. Measured hypoxic fraction in a tumour image model vs. distribution of stain ................................................................. 132
LIST OF EQUATIONS

Equation 1. Differential equation for radial oxygen diffusion in 2D................................. 10
Equation 2. Oxygen concentration at a distance r from the centre of the blood vessel, $C(r)$ ............................................................................................................................................. 10
Equation 3. Solution for the maximum oxygen diffusion distance, b ........................................ 10
Equation 4. The role of CA9 in conversion of carbon dioxide to carbonate ion and a proton ........................................................................................................................................... 21
Equation 5. Calculation of the fraction of staining by one marker .............................................. 72
Equation 6. Calculation of the fraction of staining by two markers ............................................ 72
Equation 7. Calculation of the fraction of staining by three markers ........................................... 73
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>Avidin: Biotinylated enzyme Complex</td>
</tr>
<tr>
<td>ARCO</td>
<td>Accelerated Radiotherapy with Carbogen</td>
</tr>
<tr>
<td>ARCON</td>
<td>Accelerated Radiotherapy with Carbogen and Nicotinamide</td>
</tr>
<tr>
<td>ARN</td>
<td>Accelerated Radiotherapy with Nicotinamide</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CA9</td>
<td>Carbonic Anhydrase 9</td>
</tr>
<tr>
<td>Carbogen</td>
<td>95% $O_2 + 5% CO_2$</td>
</tr>
<tr>
<td>DAB</td>
<td>3, 3'-diaminobenzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,6-diamidino-2-phenylindole dihydrochloride hydrate</td>
</tr>
<tr>
<td>EF5</td>
<td>2-(2-nitro-1h-imidazol-1-yl)-n-(2,2,3,3,3,pentafluoropropyl)acetamide</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FC</td>
<td>Flow Cytometry</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GLUT-1</td>
<td>Glucose Transporter 1</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>HIF-1($\alpha$ or $\beta$)</td>
<td>Hypoxia Inducible Factor 1($\alpha$ or $\beta$)</td>
</tr>
<tr>
<td>HF</td>
<td>Hypoxic Fraction</td>
</tr>
<tr>
<td>HP2.5 (or HP5)</td>
<td>Hypoxic Proportion less than or equal to 2.5mmHg oxygen (or 5mmHg oxygen)</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxia Response Element</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression-Free Survival</td>
</tr>
<tr>
<td>PHD</td>
<td>Prolyl Hydroxylase</td>
</tr>
<tr>
<td>Pimonidazole/Pimo</td>
<td>1-[(2-hydroxy-3-piperidinyl) propyl]-2-nitroimidazole</td>
</tr>
<tr>
<td>pO$_2$</td>
<td>Oxygen partial pressure</td>
</tr>
<tr>
<td>PST</td>
<td>PBS containing 4% FBS and 0.1% triton X-100</td>
</tr>
<tr>
<td>PTN</td>
<td>1% BSA (w/v), 0.2% (v/v) Tween 20 in PBS</td>
</tr>
<tr>
<td>SE</td>
<td>Standard Error</td>
</tr>
<tr>
<td>TWS</td>
<td>3.5g of sodium bicarbonate and 20g of magnesium sulphate in 1L of double distilled water</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VHL</td>
<td>Von-Hippel Lindau</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to take this opportunity to thank Dr. Peggy Olive for accepting me in her research group initially as a summer student, then as an honours, and a graduate student. I am extremely grateful for her guidance, support and advice, and for always finding the time for her students! I have enjoyed our constructive and stimulating discussions and I am delighted to have had the privilege to work in such a supportive and collaborative environment! Therefore, I would like to extend my thanks to all of the members of the Olive Lab.

This work would not have been possible without Susan MacPhail, and I am grateful for her technical support, training and guidance with the immunohistochemical staining that was critical in this project. I would like to extend my gratitude to Eric Chu, who assisted me in the initial pimonidazole staining experiments. I greatly appreciate Dr. Judith Banáth’s contribution to this project in performing the flow cytometry experiments. Solmaz Sobhanifar has been extremely helpful in the exchange of hypoxia literature and discussions pertaining to this fascinating topic! I would also like to thank the BC Cancer Agency statistician, Chuck Paltiel, for generously sharing his expertise and advice on the statistical analysis methods.

I would like to thank Dr. Christina Aquino-Parsons for providing me with the cervical carcinoma biopsies and allowing me access to her Eppendorf electrode measurements and clinical parameters of patients. I am extremely grateful for Dr.
Aquino-Parsons' guidance, encouragement and constructive criticism, as a member of my supervisory committee. I would also like to thank her for her generosity with time and for her support at CARO! In addition, I would like to thank Dr. Ralph Durand for taking the time to help me with my revisions and for providing helpful comments and suggestions. I extend my gratitude to the members of my Masters thesis committee, Dr. Calum MacAulay, Dr. Paul Rennie and Dr. Vincent Duronio.

I would like to express my gratitude to Dr. Eric Stanbridge and Shu-Yuan Liao from the University of California - Irvine for performing the immunostaining for carbonic anhydrase 9 on the tumour tissue sections.

Finally, I would like to thank my mother Ljiljana Janković and my father Miroslav Janković for always being supportive and giving me encouragement and inspiration!
1. INTRODUCTION

1.1. Overview and Goals of the Study

The notion that tumour hypoxia can lead to resistance to treatment, was first suggested in the early 1950's and since then, this topic has been actively researched. In spite of our increasing knowledge concerning the role of hypoxia in tumour aggressiveness, it is not routinely measured. This is in large part a result of the technical limitations associated with the application of established methods, oxygen microelectrodes and chemical hypoxia markers. Recently, endogenous protein markers have been identified that have the potential to allow routine measurement of tumor hypoxia. However, before routine application, these methods will require careful validation against the established methods such as the oxygen microelectrode and pimonidazole binding. In addition, their predictive capacity with respect to patient outcome following therapy needs to be critically examined.

The hypotheses of this thesis were: 1) biopsy staining for two endogenous markers of hypoxia, HIF-1α and CA9, is sufficient for assessing the hypoxic status in tumours, and 2) pre-treatment hypoxia in cervical cancer, measured by these markers, is a clinically significant prognostic factor in treatment outcome. The study of endogenous markers was performed on cervical cancer because 1) hypoxia is known to develop in these tumours, 2) high pre-treatment hypoxia in cervical cancer has previously been associated with poor outcome, 3) patients with advanced cervical cancer had already been involved in a clinical study examining pimonidazole, a chemical hypoxia marker, at the
B.C. Cancer Agency, and 4) pre-treatment biopsies, as well as Eppendorf oxygen electrode measurements, were available for assessment of hypoxia. In order to critically examine the hypotheses using the available methods, the focus of this work was to answer the following questions:

1. Is intra-tumour heterogeneity less than inter-tumour heterogeneity with respect to the expression pattern and levels of hypoxia markers? Is intra-tumour heterogeneity “tolerable” when sampling issues are considered?

2. To what extent do the regions on sequential sections from cervical carcinomas, stained for pimonidazole, CA9 and HIF-1α, correlate numerically and spatially? How does hypoxia marker staining compare to hypoxic proportion extracted from Eppendorf electrode measurements of oxygen tension?

3. Do pre-treatment levels of endogenous or exogenous hypoxia markers in tumour sections have prognostic value in advanced cancer of the cervix in this patient cohort?

4. Is there evidence that levels of HIF-1α in cervical tumours fluctuate over the course of chemoradiotherapy? If so, are the fluctuations indicative of disease progression?

5. What are the main limitations of the image analysis techniques currently used for hypoxic fraction assessment? Can the accuracy of measurement of hypoxic fraction and colocalization be improved by using a combination of hypoxia markers?
When this study was initiated, it was the first one to compare HIF-1α, CA9, pimonidazole and oxygen microelectrode measurements in the same patient population, and to correlate marker expression with outcome in a patient cohort of this size. Recently, Hutchinson et al. have performed a similar study (85), however with two important differences: they used a "semi-quantitative" scoring system, and their analysis of patient survival with respect to HIF-1α expression was performed on a separate cohort of patients.

1.2. Structure of the Thesis

In the remainder of the Introduction, the circumstances and events leading to both chronic and acute hypoxia are discussed in the context of the tumour microenvironment. The heterogeneity within the tumour vascular network, vessel structure and function are described as contributing factors to tumour hypoxia. Next, the oxygen sensing mechanisms and hypoxia response pathways are reviewed, with a focus on the HIF-1 signal transduction pathway. The mechanisms by which tumour hypoxia confers tumour resistance to chemo- and radiotherapy are discussed. Following this, the association between hypoxia and malignant progression is described in the context of genes whose expression is modulated in response to changes in oxygenation. In addition, clinical, physiological and genetic parameters which influence tumour hypoxia and disease progression are considered (such as tumour size, FIGO stage, tumour cell proliferation, apoptosis and p53 status). Methods for detection of hypoxia, including the oxygen electrode and hypoxia markers, are explained in the next section. Finally,
therapeutic strategies that modulate the hypoxic fraction prior to radiation and those that target hypoxic cells are discussed.

In **Methods**, the experimental protocol, including acquisition of the biopsies, tissue processing, flow cytometry, immunohistochemistry method, acquisition and analysis of images of stained slides, oxygen electrode measurements in tumours, patient data acquisition from charts, marker correlation and colocalization and outcome analyses are described. Alternative approaches, such as automated image analysis, which was compared to manual image thresholding, and histogram analysis are introduced and explained. Lastly, generation of tumour image models, which were used for analyses of the effect of noise, contrast and distribution of stain on the accuracy of manual image analysis, is described.

In the **Results** section, the distributions of clinical parameters in the patient cohort, courtesy of Dr. Christina Aquino-Parsons, BCCA, are presented. Inter- and intra-tumour heterogeneities in hypoxia markers are presented by comparisons of marker positive fractions in the patient cohort and within the same tumour. The correlations between marker labeling on different biopsies from the same tumour are shown, and pimonidazole positive fractions, analyzed by flow cytometry and histology on two different biopsies of the same tumour, are compared. The validity of using sequential sections for comparison of labeling with hypoxia markers is confirmed by analysis of pimonidazole binding on sequential sections. Next, the inter-observer correlations in marker positive fraction measurement are demonstrated by correlations between the three
observers' measurements. The marker positive fraction correlations between pimonidazole, CA9 and HIF-1α, are presented. Colocalization scores, computed using three methods are shown: visual scoring, computational derivation of colocalization fraction, and manual image manipulation to obtain colocalized fraction of each marker combination. Assessment of prognostic abilities of individual markers, and combinations of markers are presented using Kaplan-Meier and Cox Regression analyses. The fluctuations in HIF-1α during therapy are examined and their association with patient response to therapy is discussed. Next, image analysis approaches, such as automated and manual image thresholding, and histogram analysis, are explored and compared. Lastly, the effects of noise, contrast and distribution of stain on image analysis techniques are examined.

In **Discussion**, the sufficiency of using endogenous hypoxia markers for estimating the hypoxic fraction in a tumour is argued, due to the degree of hypoxia marker correlations and colocalizations. Similarly, the reliability of colocalization calculations is examined, and possible causes for marker mismatch patterns and correlations are discussed. The associations between hypoxia markers and outcome are critically analyzed, and reasons for lack of correlation between hypoxia marker presence and patient response to therapy are discussed in the context of other predictive factors. Next, the validity of the sampling method via acquisition of a single tumour biopsy is discussed in the light of tumour heterogeneity in oxygenation. Lastly, sources of error in manual image analysis are discussed and improvements are suggested.
In Conclusions and Future Aims, an overview of the major findings is presented, with an outlook on the future of tumour hypoxia in the clinic.

1.3. Tumour Microenvironment

1.3.1. Definitions of Hypoxia

Hypoxia, a decrease in oxygen content below normal levels, is a condition caused by the imbalance between oxygen supply and its consumption in the tissue. As consumption often exceeds supply in tumour tissue, cell layers distant from vasculature may be subject to oxygen deficiency.

Oxygen tension ranges from 40mmHg in the venous blood (equivalent to approximately 6.7% oxygen; 1% oxygen = 6mmHg) and 80mmHg (range: 70mmHg-100mmHg) in the arteries (13.3% oxygen). In most normoxic normal tissues, oxygen partial pressure is typically between 20mmHg and 40mmHg. Therefore, cells are considered normoxic if their oxygen concentration exceeds 20mmHg (3.3% oxygen). Cells are termed anoxic if they have no oxygen. The border between normoxia and hypoxia is not well defined. It encompasses a range of values due to the range of oxygen partial pressures that are currently used to define hypoxia. Different categories of the hypoxic condition depend on both the degree of cell oxygenation (≤2.5mmHg to ≤10mmHg), and the mechanistic origin of hypoxia. In this study, both chronically and acutely hypoxic cells, which undergo changes in expression of genes involved in hypoxia signaling, are included.
1.3.2. Vascular Heterogeneity

Blood vessel distribution in tumours is chaotic and in sharp contrast to well-ordered vessel distribution in normal tissue, as illustrated in Figure 1. Irregular vessel configuration contributes to heterogeneity in oxygen concentration across the tumour tissue, which has been characterized by several groups (10, 49). Large intervascular distances are a common occurrence in solid tumours and are often associated with chronically hypoxic regions (156). Nevertheless, occurrence of hypoxic regions is possible in highly vascularized tumours due to the heterogeneous distribution of blood vessels, rapid oxygen consumption, and periods of non-perfusion in blood vessels (44, 157).
Figure 1. Vascular network in normal and tumour tissue. Organized vascular network in normal colon (a) and chaotic vasculature in a melanoma xenograft (b) (102).

Figure 2. Two forms of hypoxia: chronic and acute. Chronic hypoxia occurs at a distance from the blood vessel and acute hypoxia borders non-perfused vessels.
1.3.3. Chronic versus Acute Hypoxia

In tumours, oxygen and nutrient demand frequently exceeds supply. Depending on the mechanistic origin of oxygen insufficiency, hypoxia can be termed chronic or acute. Chronic hypoxia\(^1\) occurs in cells which have oxygen concentrations that are below normal due to the limited oxygen diffusion distance in metabolizing tissue (Figure 2). As oxygen diffusion distances typically range from 100\(\mu\)m to 200\(\mu\)m from the blood vessel (75, 131), it is not surprising that tumours as small as 0.5mm in diameter contain hypoxic regions (49).

Steady state oxygen levels occurring at varying oxygen diffusion rates, consumption rates and tumour geometries have been modeled previously (25). If a simple two-dimensional tumour cord geometry is considered, in which a blood vessel is in the centre of a circular cord, the oxygen diffusion is radial outward, as shown in Figure 3, described by the equations 1, 2 and 3. The steepness of the oxygen concentration gradient from the blood vessel to the tissue varies depending on the oxygen consumption rate, \(K\), and oxygen diffusion coefficient, \(D\), in tissue, both of which are considered to be constant in this case. The differential equation describing the outward flow at a distance \(r\) from the centre of the blood vessel is shown in Equation 1.
\[
\frac{1}{r} \frac{d}{dr} \left( rD \frac{dC}{dr} \right) = K
\]

**Equation 1.** Differential equation for radial oxygen diffusion in 2D.

The solution for oxygen concentration at a distance \( r \) from the centre of the capillary is represented by Equation 2.

\[
C(r) = C_0 \frac{2\ln \left( \frac{b}{r} \right)}{2\ln \left( \frac{b}{a} \right)} - 1 + \frac{r^2}{b^2} - 1 + \frac{a^2}{b^2}
\]

**Equation 2.** Oxygen concentration at a distance \( r \) from the centre of the blood vessel, \( C(r) \).

Although \( a \) and \( b \) can be arbitrarily chosen as certain distances from the capillary, in this case \( a \) is the radius of the capillary (\( a = 5 \mu m \)) and \( b \) is the maximum oxygen diffusion distance. Therefore \( C(a) = C_0 = 40 \text{ mmHg} \) and \( C(b) = 0 \). Variable \( b \) is determined by \( K \) and \( D \), and it can be obtained from the following equation:

\[
\frac{4C_0D}{Ka^2} = 1 - \frac{b^2}{a^2} + \frac{b^2}{a^2} \ln \frac{b^2}{a^2}
\]

**Equation 3.** Solution for the maximum oxygen diffusion distance, \( b \).

In this oxygen diffusion equation, factors such as the rate of oxygen consumption, \( K \), can be modified to account for the differences in metabolic rates of tumours, and

---

1 Please note that the terms chronic hypoxia and diffusion-dependent hypoxia are used interchangeably.
between cells at varying distances from the vessel. It is evident from the graph in Figure 3 that the maximum oxygen diffusion distance increases as the tumour oxygen consumption rate decreases. Therefore, the number of normoxic cells in the tumour cord of a fast oxygen-consuming tumour is smaller than that of a slow oxygen-consuming tumour. Because blood vessels surrounded by fast oxygen-consuming cells are able to sustain a smaller radius of cells, these cords are likely to contain a larger amount of hypoxic and necrotic cells. Conversely, in a slow oxygen-consuming tumour, a blood vessel in the centre of a tumour cord supplies a larger radius of cells. It is therefore tempting to speculate that a tumour composed of multiple cords of fast oxygen-consuming cells contains a larger proportion of severely hypoxic and necrotic cells compared to slow oxygen-consuming tumours. This is only true, however, if the intervascular distances of the tumours are comparable. In tumour microenvironments where intervascular distances are small enough to allow adequate oxygen supply for all of the tumour cells surrounding these vessels (i.e., the intervascular distance is less than the oxygen diffusion distance), there are no hypoxic cells provided that the blood vessels are perfused, and the rate of oxygen consumption is constant.
Figure 3. Diffusion of oxygen through the tissue in a two-dimensional model with a radial flow outward. Comparison between oxygen diffusion models in fast oxygen-consuming tumours (in red), intermediately oxygen-consuming tumours (in green) and slow oxygen-consuming tumours (in blue). The centre of the capillary is at 0μm, while its radius is approximated to be 5μm.
To overcome the inadequate oxygen supply to tumour cells, the process of angiogenesis can be initiated via the stimulation of oxygen-sensing pathways. These newly formed vessels, however, have abnormal structure and are often subject to leakage, transient blockage or collapse, causing temporary reduction in blood flow, leading to perfusion-dependent hypoxia\(^2\) (Figure 2) (39, 118). In addition, fluctuations in red blood cell flux in vessels are another possible cause of intermittent changes in tissue oxygenation (50). Similarly, high interstitial fluid pressure (IFP), caused by exceptionally permeable blood vessels and poor drainage mechanisms, may result in a temporary vessel collapse causing blood flow stasis (118). As the pressure on the blood vessel is released, the vessel regains its form allowing restoration of normal blood flow. This is one of the mechanisms by which acute hypoxia may periodically arise for varying durations of time, ranging from minutes to hours (28, 50, 158). Other mechanisms of reduced blood flow that have not been directly investigated are spontaneous arteriolar vasomotion caused by partial constriction of smooth muscle in arteriolar wall, vessel plugging by white blood cells, platelet thrombi, red blood cells or circulating tumour cells, and endothelial cell contractility or swelling resulting in vessel lumen occlusion.

While it is estimated that in some murine tumours, transient blockage or collapse of blood vessels accounts for a significant fraction of tumour hypoxia (55) and that over fifty percent of the blood vessels in SCCVII tumours transplanted in mice exhibit transient fluctuations in perfusion (57), the precise proportions of chronic and acute hypoxia in most murine and human tumours are still unknown. Although it has been

\(^2\) Please note that the terms perfusion-dependent hypoxia, transient hypoxia, intermittent hypoxia and acute
suggested that the fraction of acutely hypoxic cells exceeds the fraction of chronically hypoxic cells in most tumour types (3, 143), there is no consensus on the proportions of the two types of hypoxia, and it is likely that the proportions vary between tumour types and sizes. Direct measurement of the fraction of acutely hypoxic tissue presents a challenge, as it is not easy to differentiate between the two hypoxic subpopulations. Rofstad and Maseide have developed a method for measuring the fraction of acutely hypoxic cells by subtracting the fraction of chronically hypoxic cells (measured by the immunohistochemical detection of pimonidazole binding optimized to selectively bind chronically hypoxic cells) from the fraction of radiobiologically hypoxic cells in human melanoma xenografts (143). This method, however, is not feasible on human tumours, as the assessment of radiobiologically hypoxic cells requires excision of the tumour and pre-treatment (clamping) prior to irradiation. It also assumes that pimonidazole labels only chronically hypoxic cells without providing rigorous evidence for this. Another direct method involving two hypoxia markers utilized to measure the change in hypoxia marker labeling over a period of 2.5 to 3.0 hours demonstrated that acute hypoxia can account for at least 25% of the total hypoxic fraction in rat prostate carcinomas (89). Indirect methods, such as laser Doppler microprobes, have also been used to provide information regarding acute hypoxia by measuring fluctuations in microregional blood flow (77). These experiments have indicated that over 50% of the changes in red blood cell flux occur in less than 20 minutes in human tumours and murine xenografts (77). In human tumours, most of the changes in red blood cell flux observed were between 2-fold and 5-fold, however the changes in the flux in human tumours were slower compared to hypoxia are used interchangeably.
xenografts (77). The relationship between the proportion of non-perfused blood vessels and proportion of acute hypoxia is not invariable. The extent to which changes in red blood cell flux modulate the hypoxic fraction is highly dependent on the location of the vessels undergoing changes in blood flow, the vascular density, inter-vascular distances in the tumour microregions, vessel size and normal functionality, the magnitude of the red cell flux change, and the rate of change of blood flow. For instance, in poorly vascularized regions (i.e., regions of large inter-vessel distances), decrease in perfusion could lead to a greater increase in hypoxia, compared to well-vascularized regions (small inter-vessel distances) (44). In simulation studies of oxygen transport in well-vascularized regions, it was found that blood flow reduction of two-fold can lead to acute hypoxia in up to 30% of the neighbouring tissue (100). When two vascular markers, Hoechst 33342 and the carbocyanine dye, DiOC₇, were administered to SCCVII carcinomas in mice 20 minutes apart, approximately 8% of the vasculature was mismatched for the two markers, while additional 16% percent of the vasculature exhibited a difference in fluorescence intensity, indicating transient changes in perfusion during this period (158). The episodes of transient cessation of perfusion lasted at least 4 to 5 minutes (159). Interestingly, it was found that central tumour regions contained significantly more vessels undergoing reduction or cessation in perfusion than vessels in the tumour periphery (158). Complementary evidence for the presence of acute hypoxia, measured by hypoxia marker mismatch patterns, and the fraction of this type of hypoxia, are presented in the Results section of this work.

Questions of paramount importance in hypoxia-targeted cancer treatment are does
hypoxia modulate response to treatment and, if so, which type of hypoxia is more likely to lead to failure. Rofstad and Maseide argue that acute, rather than chronic, hypoxia promotes tumour aggressiveness (143). This can be attributed to the dynamics of the environment of acutely hypoxic cells. They profit from fluctuations in oxygen supply by undergoing normoxic periods during which they are clonogenic, and hypoxic episodes during which they are radiation resistant. Therefore, transient changes in blood flow can contribute to tumour cell resistance to radiotherapy and simultaneously allow for cell survival and proliferation, as the cells are not chronically deprived of oxygen and nutrients. Assessment of acute hypoxia may therefore be more important in predicting a patient's response to treatment. In addition, progression of tumours that are predominantly acutely hypoxic is likely to be more rapid than that of chronically hypoxic tumours. Therefore, implementation of methods with capabilities to quantify the hypoxic fraction and distinguish between different types of tumour hypoxia, would be of great benefit in clinical practice. Assessment of hypoxia at the start of treatment could aid in treatment planning by selectively targeting the resistant hypoxic populations.

1.4. Oxygen-response Pathways

Hypoxia inducible factor – 1 (HIF-1) transcription factor is a major regulator of the hypoxia-response pathways. It regulates the expression of over 60 target genes (48, 71, 148, 169), and its activity is modulated in response to oxygen and growth factors.
1.4.1. Regulation of HIF-1α

Deprivation of oxygen stimulates cellular responses which facilitate cellular management of hypoxic stress. Oxygen sensing mechanisms play an important role in initiation of such responses. Unlike HIF-1β, which is constitutively present in the nucleus, HIF-1α is stabilized or degraded depending upon oxygen conditions. Under hypoxia, unaltered HIF-1α migrates to the nucleus where it binds HIF-1β to form HIF-1. This transcription factor, in complex with p300 (a transcription co-factor), binds the hypoxia response elements (HRE's) of a number of target genes initiating their transcription (Figure 4).
Figure 4. Major interactions in the HIF-1 pathway under normoxic and hypoxic conditions. Factors contributing to HIF-1α induction and regulation are illustrated, in addition to HIF-1α binding partners and some of HIF-1 target genes.
HIF-1α protein induction and activation occur via PI3K/Akt and MAPK pathways, respectively. There is evidence for HIF-1α regulation on multiple levels of the PI3K/Akt pathway. It can occur by Akt directly, via GSK3β, which is inactivated upon Akt accumulation, or through mTOR, which has a role in stabilizing HIF-1α (Figure 4). Although the precise mechanisms have yet to be validated, current data suggest that stimulation of growth factor receptor (by EGF, IGF-1, insulin, TNF-α, etc.) results in HIF-1α induction (11).

It has been shown that inactivation of MAPK blocks the transcriptional activity of HIF-1. Phosphorylation of HIF-1α and HIF-2α by MAPK is necessary for their activation, however the exact mechanisms whereby this leads to activation of HIF-1 are not clear (11). In addition, MAPK promotes pairing of HIF-1 and p300, and modulates p300 transactivation activity (144).

In the presence of oxygen, active prolyl hydroxylase enzymes contribute to downregulation of HIF-1α. Alteration of HIF-1α by prolyl hydroxylases occurs via hydroxylation of prolyl residues in the amino- and the carboxy- terminal oxygen-dependent degradation (ODD) domains (37). More specifically, it has recently been demonstrated that inactivation of prolyl hydroxylase 2 (PHD2) enzyme prevents HIF-1α destabilization under normoxic conditions, indicating that this enzyme is essential in the HIF-1α ubiquitination process during normoxia (18).

Prolyl hydroxylation, together with acetylation of lysine in the oxygen-dependent
degradation domain (153), provides a signal for Von-Hippel Lindau (pVHL) mediated ubiquitination of HIF-1α (48, 108, 116, 147). Studies have shown that both pVHL mutations and dissociation of pVHL and HIF-1α lead to ubiquitous HIF-1α expression, while reintroduction of pVHL inhibits expression of HIF-1 target genes, confirming the critical role of pVHL in HIF-1α regulation and HIF-1 pathway (86, 116, 153, 176).

Another mechanism of oxygen sensing was suggested to occur via mitochondrial electron transport chain (ETC) complex IV (cytochrome c oxidase). Reduction of oxygen to water by complex IV is inhibited under hypoxic conditions resulting in generation of $O_2^-$ due to release of electrons upstream at complex III. This results in the production of reactive oxygen species (ROS) under hypoxia, which are, controversially, more abundant than under normoxic conditions (38, 93, 148). The reactive oxygen species play a role in the transcriptional activation of VEGF and NFκB (38, 93).

1.4.2. HIF-1 Targets

A feature that all HIF-1 target genes have in common is the hypoxia response element (HRE) located in the promoter region of the gene (Figure 4). These HRE’s are genetic enhancer sequences to which HIF-1 binds to activate their expression (3, 94, 118, 174, 175). Some of the target genes of HIF-1 are critical in malignant progression via angiogenesis and metastasis. The most studied targets of HIF-1 are carbonic anhydrase 9 (CA9), glucose transporter (GLUT-1) and vascular endothelial growth factor (VEGF). Carbonic anhydrase 9 is a membrane-bound protein that is involved in inter-cellular pH
regulation. Other carbonic anhydrases are present in the cytoplasm and are involved in intra-cellular pH modulation. Glucose transporter GLUT-1 is a transmembrane protein which facilitates glucose uptake. VEGF is a secreted protein which promotes growth of vascular endothelium from existing vessels into surrounding tissue.

While cytoplasmic CA’s catalyze the conversion of carbonate to carbon dioxide in order to facilitate its diffusion across the cell membrane, CA9 catalyzes the conversion of carbon dioxide to a carbonate ion and a proton, thereby decreasing extracellular pH, as shown in Equation 4 (138). By this mechanism, a CO₂ gradient is maintained across the cell. Carbonate is transported back to the cell, where it combines with another proton. The newly formed carbonic acid is converted to carbon dioxide and is exported from the cell, maintaining the pH-regulating cycle.

\[
CA9
\]
\[
\text{H}_2\text{O} + \text{CO}_2 \rightarrow \text{H}_2\text{CO}_3 \rightarrow \text{HCO}_3^- + \text{H}^+
\]

**Equation 4.** The role of CA9 in conversion of carbon dioxide to carbonate ion and a proton.
Figure 5. The role of CA9 in regulating the acid-base balance intra- and extracellularly. Hydrogen ions are generated as end products of anaerobic metabolism. To facilitate their diffusion across the lipid bilayer, they combine with bicarbonate to form carbon dioxide and water, in a reaction catalyzed by the intracellular CA2. Carbon dioxide then diffuses into the extracellular environment where CA9 catalyzes hydration of carbon dioxide, resulting in formation of bicarbonate and a proton (a). This bicarbonate is recycled, as it is exchanged for intracellular chloride upon re-entering the cell (b). The net outcomes of this cycle, termed Jacobs-Stewart cycle, are acidification of the extracellular environment and alkalosis of the intracellular compartment, driven by the extrusion of protons (138).
It has been shown that CA9 expression is a significant and independent prognostic factor for disease-specific and metastasis-free survival in cervical carcinomas, but not for local control following radical radiotherapy treatment (112). Although CA9 is necessary for maintaining the extracellular acidity in the tumour microenvironment, and is involved in optimization of conditions which promote tumour invasion (e.g., via the activity of metalloproteases), the molecular mechanism by which CA9 is involved in carcinogenesis is not completely clear. Expression of GLUT-1 is regulated by glucose and oxygen concentrations in the cell's environment (2). In normoxic conditions, oxidative phosphorylation is made possible by the availability of molecular oxygen as an electron acceptor. During hypoxia, the decrease in ATP synthesis leads to an increased demand for glucose metabolism via the glycolytic pathway to generate lactate. As glucose transport is the rate-limiting step for glucose utilization, overexpression of GLUT-1, a transporter which facilitates glucose intake, is one of the key events responsible for mediating cell survival in hypoxic conditions. It is therefore not surprising that increased expression of GLUT-1 positively correlates with an aggressive phenotype and recurrent disease (80). Although the fall in ATP levels is believed to be the main trigger of increase in GLUT-1 expression, this has yet to be validated, as GLUT-1 increase has been observed without a drop in the amount of ATP (15). Hypoxia can augment GLUT-1 levels by causing an increase in the production of GLUT-1 mRNA, increase in the transport of GLUT-1 to the membrane, or increase in the activity of GLUT-1 already on the cell membrane (15). Whereas GLUT-1 and CA9 are easily detectable due to their localization to the cell membrane, the study of VEGF is more complex. This growth factor is secreted by cells, forming concentration gradients from a hypoxic tumour region
to the neighbouring blood vessels, providing a critical signal for angiogenesis.

Upon oxygen deprivation, HIF-1 activity has been shown to cause an increase in erythropoietin expression (83), which stimulates the RBC production, resulting in a greater oxygen-carrying capacity to the tissue. Therefore, administration of EPO has the potential to reduce tumour hypoxia, and thereby sensitize cells to radio- and chemotherapy (92). Nevertheless, by achieving increased oxygenation, EPO also has the potential to stimulate tumour growth. Conversely, through increases in blood viscosity, EPO may actually reduce tumour blood flow. Therefore, it is important that the appropriate dose of EPO is administered only to patients who would benefit from such therapy.

1.5. Resistance of Hypoxic Cells to Therapy

Substantial evidence from clinical studies on various tumour types indicates that there is a strong association between tumour hypoxia and reduced response to treatment, leading to poor patient prognosis (80, 163). The lack of oxygen in cells confers resistance to ionizing radiation and some forms of chemotherapy (80). In addition to conferring resistance to therapy, hypoxia promotes an aggressive tumour phenotype through selection of cells fit to survive these conditions (155). The mechanisms of hypoxia-induced aggressiveness and malignancy are discussed in Section 1.6.
1.5.1. Reduced Sensitivity to Radiation Therapy

Upon irradiation, damage to DNA can occur via a direct or indirect effect. The direct effect consists of ionization or excitation of a DNA molecule by the incident radiation. Indirect DNA damage is mediated by other molecules in a cell (largely water), which are ionized upon irradiation, and this accounts for the majority of radiation damage within a cell. Radiation causes radiolysis of water, resulting in formation of highly reactive ions and free radicals (H and OH) which most often interact with each other or neighbouring organic molecules such as DNA. Oxygen is very electron affinic, and it rapidly combines with DNA radicals. This reduces the probability that free radicals will recombine (so-called chemical repair) thus ensuring “fixation” of the damage in the DNA. Therefore binding of oxygen to a DNA radical prevents charge recombination and reversal of damage, resulting in permanent oxidized products (Figure 6). Cells having lower oxygen concentrations are therefore less susceptible to permanent DNA damage by ionizing radiation. Moreover, recent evidence suggests that changes in hypoxic cell transcriptome and proteome, including stabilization of HIF-1α, contribute to the cell’s reduced sensitivity to radiation (160). Studies of transformed mouse embryonic fibroblasts deficient for HIF-1α indicated that these cells were more susceptible to radiation than their HIF-1α-expressing counterparts under both normoxic and hypoxic conditions (160), measured by the MTT assay, which was used to assess cell growth and viability (70, 120). Unruh and colleagues speculated that this sensitivity may be a result of DNA double strand break repair enzyme deficiency in cells lacking HIF-1α, however
this hypothesis is yet to be validated (160). Clinical studies indicate that only those esophageal cancer patients lacking HIF-1α expression in their tumours exhibited a complete response to radiation therapy (151), while squamous cell head and neck cancer patients (1, 105) and cervical carcinoma patients (87) with high hypoxic fraction, measured by elevated HIF-1α expression, exhibited incomplete response to radiation and chemotherapy, marked by disease recurrence or distant metastasis.
Figure 6. An example of permanent oxygen "fixation" by oxygen adduct formation with an unstable DNA radical, formed upon direct or indirect effect of radiation.

\[ \cdot \text{DNA radical} + O_2 \rightarrow \cdot O_2' + H' \rightarrow \text{OOH} \]

DNA radical formed from direct or indirect effect of radiation
Oxygen adduct
Permanently altered ("fixed") DNA molecule
1.5.2. Evasion of Chemotherapeutic Strategies

In addition to having reduced sensitivity to ionizing radiation, hypoxic tumour cells can often evade chemotherapeutic strategies. The localization of hypoxic cells in poorly perfused areas, often distant from functional blood vessels, presents an obstacle for chemotherapeutic agents to effectively reach these cell populations (29). In addition, many chemotherapeutic drugs specifically target cycling cells and a few, like bleomycin, require oxygen for toxicity. As the fraction, and possibly the rate of cell proliferation decreases as a function of distance from the vasculature (59, 142, 154), the majority of chronically hypoxic cells are likely to be quiescent, and are therefore minimally affected by such agents (29). In addition, altered genomic and proteomic profiles, promoted by hypoxic conditions, enhance resistance to chemotherapy. Unruh and colleagues detected impaired survival of HIF-1α deficient mouse embryonic fibroblasts after treatment with carboplatin and etoposide under both hypoxic and normoxic conditions, compared to HIF-1α expressing cells, suggesting a hypoxia-independent role of HIF-1α in conferring enhanced resistance to these chemotherapeutic agents (160).

1.6. Malignant Progression

Upon oxygen deprivation, cellular adaptation to such conditions via modulation of activity of cellular pathways, can significantly improve cell survival. One of the essential events leading to adaptation to a hostile environment and, ultimately, progression of the disease, is activation of the HIF-1 pathway, which usually occurs in
response to oxygen deprivation. Expression of the key genes in this pathway has been correlated with metastatic spread and malignant progression in human tumours and xenograft models.

1.6.1. Hypoxic Subpopulations and their Prognostic Significance in Treatment Outcome

Clinical studies, as well as investigations using in vivo and in vitro tumour models, have established that hypoxic fraction is a strong, independent prognostic factor in various tumour types. Comprehensive overviews of prognostic capabilities of hypoxia markers, both exogenous and endogenous, are provided in reports by Bussnik and Evans (34, 60). The latter review underscores the importance of considering hypoxia as a continuous variable, rather than a categorical one, and stresses that the degree of hypoxia should be measured. Recently, convincing arguments have been made that moderate and not severe hypoxia has adverse effects on patient outcome (13, 46, 60, 125, 126, 173). Severely hypoxic cells do not proliferate, are likely to be nutrient and growth factor deprived, and their energy production and metabolism are compromised. If such conditions persist, these cells are destined to die (60). Moderately hypoxic cells, while expressing HIF-1α and its target genes, that enhance their survival in hypoxic conditions, are more metabolically active, and have greater levels of ATP than severely hypoxic cells (46). In addition, several groups have shown that the proportion of cells at intermediate oxygenation is dominant among hypoxic cell populations (28, 30, 59, 103, 126). Evans et
al. used the intensity of EF5 binding to distinguish between intermediately and severely hypoxic cells. They found that severely hypoxic cells (marked by high EF5 binding intensity), were rarely positive for the proliferation marker Ki-67, unlike the intermediately hypoxic cells (59). Another aggressive subpopulation of cells are acutely hypoxic cells, which can be radiation resistant during hypoxia, but regain proliferative capacity when reoxygenated. Development of a method to specifically detect these highly resistant subpopulations of hypoxic cells is a major challenge in identifying aggressive tumours in patients. Knowing the parameters such as the fraction and distribution of hypoxic cells, and the severity and duration of hypoxia will provide vital information when designing customized treatment. In addition to therapy that targets normoxic tumour cells, therapy aimed at transiently and intermediately hypoxic cells which have maintained proliferation potential may slow the progression of hypoxic tumours by aggressively targeting hypoxic cells (or sufficiently improving tumour oxygenation and increasing hypoxic cells’ sensitivity to radiation).

1.6.2. Other Factors That Contribute to Treatment Outcome Together With Hypoxia

Although the vast majority of reports indicate that HIF-1α negatively impacts patient outcome, there exists some conflicting evidence with respect to the effect of HIF-1α in promoting tumour growth, metastatic potential and response to treatment. It is important to acknowledge that tumours originating from different tissues as well as tumours at different stages of progression exhibit different genetic profiles. Hutchison and colleagues reported that increased HIF-1α expression had no prognostic value for
disease-free survival, however it predicted for poor outcome in small tumours, and favourable outcome in large tumours (85). The disparity in the literature on the HIF-1α effect on treatment outcome could indicate that its function is at least partially tumour size-dependent.

Although very few studies have quantified the extent of HIF-1α expression, the degree of its activity, and the context of its expression, these factors may be of great importance in determining the fate of the cell. Recent evidence suggests that more than 90% of cervical cancer patients are infected with the Human Papilloma Virus (HPV) (63), which inhibits the expression of the tumour suppressor p53, by stimulation of Mdm-2 mediated degradation of p53. Absence of p53 function, in combination with high HIF-1α expression, may greatly reduce the apoptotic potential of tumour cells and accelerate disease progression (22). Conversely, when p53 expression is preserved, HIF-1α plays an important role in stabilizing p53 protein upon DNA damage (36). Consequently, it has been suggested that overexpression of HIF-1α plays a role in apoptosis. Not surprisingly, in human breast cancer cell lines with functional p53, overexpression of HIF-1α and HIF-2α was detrimental to tumour growth (24). Moreover, upon DNA damage, activated p53 causes accelerated degradation of HIF-1α, leading to inhibition of CA9 expression (98). These results underscore the collaborative effect and the complexity of mutual regulation between HIF-1α and p53, leading to modulation of tumour cell’s aggressiveness.

Clinical parameters, including FIGO (International Federation of Gynecologists and Obstetricians) stage, nodal status, tumour size and hemoglobin level, have been
shown to independently predict tumour response to therapy and patient outcome in cancer of the uterine cervix (9), oropharyngeal cancer (1), and renal clear cell carcinoma (31), among others. Cellular function parameters, such as proliferative activity and apoptotic index, are also useful, and they alone or in combination with hypoxic status, have predictive capacity with respect to response to therapy (56, 78, 99).

Although radiation therapy generates less DNA single and double strand breaks in hypoxic cells than in normoxic cells, the ability of cells to repair this damage is an additional factor that must be considered when predicting a patient's response to therapy. It has been suggested that chronically hypoxic cells are repair incompetent (46), however their ability to repair varies with respect to severity and duration of hypoxia. Therefore, methods for measuring the repair capacity of hypoxic cells upon radiation, such as γ-H2AX foci quantification and comet assay, may be applied since both are feasible for clinical use. Furthermore, it is speculated that hypoxia induces genetic instability by giving rise to chromosomal rearrangements, gene amplification and polyploidy, all of which can adversely affect response to therapy and contribute to tumour progression (42, 68, 78-80). Genome instability in a cell population can accelerate its adaptation to the changing environment. With a greater rate of mutations in a population, the selection of cells with mutations which confer survival and proliferative advantage is more rapid and frequent. Combining parameters that individually predict poor response to treatment should greatly improve identification of resistant tumours at the start of therapy. However, it is first necessary to develop practical and accurate methods for measuring such parameters.
1.7. Methods for Measuring Hypoxia

Over the last two decades, a variety of methods for measuring hypoxia have been developed (14, 30, 124, 127, 166). Initially, polarographic oxygen needle electrodes were employed in a variety of tumour types. This was followed by chemical or exogenous hypoxia markers, while currently there is emphasis on the measurement of hypoxia using endogenous protein markers. The trend towards increasing number of publications on the topic of tumour hypoxia, with a specific focus on the methods for measuring hypoxia, is shown in Figure 7.
Figure 7. The number of tumour hypoxia-related publications, from 1965 until 2004, fitted to a polynomial function. Data were obtained from a PubMed keyword search on "tumor hypoxia".
1.7.1. Use of Polarographic Oxygen Electrode to Measure Hypoxic Fraction

Clinical application of polarographic oxygen electrode marketed by Eppendorf for measuring tumour oxygenation was introduced in 1990 and because of its widespread use and demonstrated predictive ability, it has been referred to as the “gold standard” for hypoxia assessment in tumours (97). Measurements of oxygen tension in a small volume at the tip of the oxygen microelectrode are captured as the needle advances in 0.4mm to 0.7mm increments into the tumour (64, 122, 123, 165). The electrode needle acquires a series of measurements along multiple tracks inside the tumour. The sampling volume consists of a hemisphere estimated to contain between 70 to 500 cells (70 cells for the electrode with an anode 12μm in diameter, and 500 cells for the electrode with an anode 17μm in diameter) around the electrode tip (46). These readings can be assembled into a histogram of frequencies of oxygen partial pressures, pO₂. The parameters extracted for hypoxia assessment in the clinic are the median partial pressure as well as two measures of the hypoxic volume: HP5 and HP2.5, which indicate the fractions of tumour with less than or equal to 5mmHg (0.83% oxygen) and 2.5mmHg (0.42% oxygen) oxygen, respectively.

In clinical practice, Eppendorf measurements with median oxygen tension values equal to or below 10mmHg, 5mmHg, or 2.5mmHg have indicated a significantly worse patient prognosis in studies of various tumour types, including cervical and head and neck carcinomas (64, 114, 122, 123). Nevertheless, the ability of oxygen electrode to accurately capture the oxygen distribution in tumour tissue has been challenged by
several groups. Oxygen electrode measurements can be affected by areas of necrosis, which may not be clinically-relevant, or by vasculature, if the tumour is highly vascularized. Similarly, the choice of sampling volume within the tumour may greatly affect the data acquired by the electrode. Recently, a study examining a series of simulations of Eppendorf electrode measurements in tumours of varying degrees of oxygenation and hypoxia distribution was performed (157). The authors of this study suggest that the reliability of electrode measurements may depend on the degree of hypoxia (157). Toma-Dasu and colleagues showed that Eppendorf electrode averaging yields accurate estimates of hypoxia distribution and mean oxygen tension only in tumours with a high hypoxic fraction. Limited reliability of Eppendorf measurements in tumours with a low hypoxic fraction is attributed to sampling in regions of small intervascular distances, where hypoxia is limited to thin layers of cells. Simulation studies have shown that the Eppendorf electrode does not permit detection of hypoxia when it is localized to thin rims of cells that are surrounded by normoxic cells (157). In contrast, transient changes in blood flow are not an obstacle in achieving accuracy by oxygen electrode measurements (157). In fact, larger regions of oxygen deficiency are more likely to be detected by the oxygen electrode, since the average oxygen tension reading does not include the contribution of the surrounding normoxic cells. Only when measurements are taken at the periphery of the hypoxic regions, is the oxygen tension overestimated due to the normoxic cells situated at the normoxia-hypoxia boundary (157). Measurements that overestimate oxygen tension are also likely to occur when the electrode needle encounters an area of a dense material such as collagen, in which case a false high oxygen pressure measurement may be obtained. On the contrary, it is possible
that oxygen tension is underestimated if the electrode needle causes sites of hypoxia to form in the cells situated along its tracks, which has been demonstrated by Olive and colleagues (130). Increased pimonidazole binding and reduced sensitivity to radiation, measured by the comet assay, were shown to be the consequences of intratumour injection of a 26 gauge needle (400\(\mu\)m in diameter) (130). Eppendorf microelectrodes may produce similar, yet reduced effects, as the probe diameter is 250\(\mu\)m and is blunt, and the damage to tissue and microvessels may be less dramatic (130). Similarly, the electrode continuously moves along the tracks, therefore the damage created by the electrode is unlikely to significantly contribute to the measurements, as long as the tumour sites already sampled are not revisited. Other major disadvantages of Eppendorf electrode use in the clinic are a high degree of discomfort and invasiveness for the patient, limited use to superficial tumours, requirement of a physician trained to operate an oxygen electrode and low spatial resolution (i.e., inability to measure the microregional variations in oxygenation).

1.7.2. Use of Exogenous Markers for Measuring Hypoxic Fraction

Development of an extrinsic hypoxia marker has been one of the main directions in research on tumour hypoxia measurement since the early nineties. Earlier studies with radiolabeled misonidazole confirmed the feasibility of using nitroimidazoles as markers for hypoxic cells in solid tumours (161). An analogue of misonidazole started receiving widespread attention after 1982, when Denekamp and colleagues found that pimonidazole was a more potent radiosensitizer of hypoxic cells than misonidazole (47).
In the early 1990's, groups led by Raleigh, Hodgkiss and Koch developed antibodies against 2-nitroimidazoles to detect hypoxic cells by immunohistochemistry (41, 81, 113). Since then, pimonidazole has been the most actively researched exogenous hypoxia marker and has been used in numerous studies of hypoxia in murine xenograft models and clinical studies (121, 162). When low doses (approximately 0.5g/m² in humans and 100 mg/kg in mice) of pimonidazole are administered for hypoxia labeling, no radiosensitization is observed and no other cytotoxic or side effects have been documented to date, hence its administration to patients appears to be safe.

Pimonidazole plasma half-life in humans was reported to be 5.6 hours (5). When activated by nitroreduction in cells that have below 0.8% oxygen, it stably binds thiol-containing molecules (5). In mouse xenografts, pimonidazole binds all hypoxic cells within 20 to 30 minutes post-injection, which is the plasma half-life of pimonidazole in mice, and is retained by them for several days (its half-life when bound is on the order of days) (141). The degree of pimonidazole binding is oxygen-dependent, and it increases with decreasing oxygen concentration (121). Pimonidazole binding is detected in chronically hypoxic regions (143), and it may not cover all regions of acute hypoxia when administered as a single injection prior to excision of biopsy. Nevertheless, it can be retained in regions of acute hypoxia, provided that the duration of hypoxia is sufficiently long during the period of drug availability (171). Occasionally, pimonidazole is detected in areas of necrosis. This is likely an indication that cells that were once hypoxic were forced into necrotic regions by proliferating cell populations.
It has been demonstrated that pimonidazole binds metabolically active, viable cells (58), indicating that it could be used as a marker of clinically-relevant, hypoxic tumour regions. Nevertheless, when administered as a single i.v. injection, it fails to label all physiologically and therapeutically relevant hypoxic regions if the time between biopsy excision and radiation or chemotherapy is several hours. This is supported by a study in which pimonidazole administered orally in mice over 6 hours, labeled twice as many regions than when administered orally over a 3-hour period (16). The additional labeling may indicate transient changes in blood flow, giving rise to new regions of hypoxia. In addition, it may signify the emergence of new chronically hypoxic cells which were forced farther from the vessels by proliferating cells near the vasculature. Under both assumptions, this finding suggests that pimonidazole may not be adequate for marking all cells which become hypoxic after it has been administered. Other potential obstacles associated with using this drug in the clinical setting are its high cost and the procedures required for routine administration.

Detection of hypoxia markers in tumour biopsies is arguably no less invasive than oxygen electrode measurements, however it is probably more convenient for the patient since the procedure for obtaining electrode measurements can be lengthy and uncomfortable. Chemical hypoxia markers, however, must be administered to the patients before tumour biopsy, and therefore at least one additional biopsy, following the biopsy for diagnostic pathological assessment, is required. Alternatively, Azuma and colleagues recommended the use of multiple biopsies from different parts of the tumour for hypoxia measurement (8), although this is not always practical or feasible.
1.7.3. Use of Endogenous Markers for Measuring Hypoxic Fraction

Since the late 1990's (36, 181), there has been growing interest in using endogenous markers to label hypoxic tissue. In this study, CA9 and HIF-α were used to detect severe to moderate hypoxia (0%-2% oxygen) (125).

HIF-1α levels increase rapidly in response to hypoxia, and are detectable by immunohistochemical staining within two to five minutes upon induction by hypoxia (93). Upon reoxygenation, HIF-1α signal is lost within four to eight minutes, as a result of rapid degradation (19, 93, 179). Because of the tight regulation of HIF-1α by PHD2 and pVHL, HIF-1α is exclusively present in cells that are currently hypoxic (in pVHL and PHD2 expressing cells). Interestingly, the duration of hypoxia determines the rate of subsequent HIF-1α degradation upon reoxygenation (18). Prolonged periods of hypoxia result in accumulation of PHD2 enzyme via HIF-1α transcriptional activity (18). Therefore, reoxygenation may result in more rapid HIF-1α loss in chronically hypoxic cells than in acutely hypoxic cells.

After initiation of the hypoxic response involving HIF-1α activation, there is a time delay before detectable levels of CA9 accumulate (due to the time required for upregulation of CA9 gene, followed by mRNA and protein synthesis and transport to the membrane). Ivanov et al. have shown that CA9 mRNA levels increase 4-fold after six hours of hypoxia, and 11-fold after twelve hours in hypoxic conditions in a glioblastoma
cell line (88), indicating that the duration of hypoxia determines the extent of CA9 expression. Because CA9 accumulation requires continuous positive regulation of its gene’s promoter, prolonged HIF-1α stability is required. If these premises are correct, all CA9 labeled cells are chronically hypoxic (174). Nevertheless, because it is so slowly lost upon reoxygenation, the possibility that acutely hypoxic cells with short periods of perfusion also express CA9, should not be excluded. Furthermore, increased CA9 expression is associated with malignant tumour phenotype (174), and is not found in normal cells, unless HIF-1α is constitutively upregulated.

Other endogenous marker candidates include GLUT-1 and VEGF, which may be used in addition to the markers investigated here. A few studies have shown a positive correlation between hypoxia measurements using oxygen electrodes and hypoxia measured using endogenous markers (2, 3, 133).

By using endogenous markers for hypoxic fraction assessment, it is possible to overcome many of the challenges associated with using oxygen electrodes and exogenous markers. Measuring levels of endogenous hypoxia markers can make use of the pre-treatment biopsy that is routinely obtained from tumours of patients for initial diagnostic pathological assessment purposes.

1.7.4. Other Techniques for Measuring Hypoxia

Functional and imaging approaches have also been considered as possible
methods for measuring hypoxia. Comet analysis is used to quantify the amount of radiation-induced DNA strand breaks produced in individual cells. As anoxic cells show only one third of DNA damage displayed by normoxic cells upon radiation, it is possible to identify the fraction of "radiobiologically" hypoxic cells in a tumour sample obtained by fine needle aspiration biopsy (129). Previous studies have reported that hypoxic fraction measured by alkaline comet assay correlates with the tumour fraction having less than 5mmHg of oxygen according to oxygen microelectrode readings (6). In the follow-up study by Olive and colleagues (127) and a study by Sauer et al. (145) the same trend was observed for microelectrode measurements and fraction of tumour having oxygen tension less than 10mmHg. Furthermore, pimonidazole binding agreed well with comet assay in identifying hypoxic regions and the two methods yielded approximately the same hypoxic fractions for a given tumour (128).

Functional imaging methods are emerging as promising, non-invasive techniques for measuring tumour hypoxia. Magnetic resonance imaging (MRI), single photon emission computed tomography (SPECT) and positron emission tomography (PET) have been used to measure tumour tissue oxygenation. PET has limited spatial resolution, however its sensitivity can be superior to other functional hypoxia imaging modalities (4). When PET is used, compounds that are selectively retained in hypoxic cells, including $^{[18}F$]-fluoromisonidazole (FMISO), Cu-60 diacetyl-bis(N$^4$-methylthiosemicarbazone) ($^{60}$Cu-ATSM), 2-(2-nitroimidazol-1[H]-yl)-N-(3-$^{18}$F]fluoropropyl)acetamide ($^{[18}F$]-EF1), [2-(2-nitro-1[H]-imidazol-1-yl)-N-(2,2,3,3,3-$^{18}$F]-pentafluoropropyl)-acetamide] ($^{[18}F$]-EF5) and $^{123}$I-Labeled iodoazomycin
arabinoside (IAZA), are administered to patients, and regions of greatest marker retention are non-invasively identified (4, 62). The most widely used PET radiotracer is FMISO, which has been shown to strongly correlate to pimonidazole and CA9 staining on tumour tissue sections (53).

1.8. Strategies for Overcoming Tumour Hypoxia in the Clinic

Pre-treatment assessment of hypoxia may aid in tailoring cancer treatment to exploit the biological characteristics of hypoxic tumours. Treatment modification can be aimed at reducing the amount of hypoxia, or increasing the hypoxic fraction and subsequently targeting hypoxic cells specifically. The two, seemingly conflicting rationales, are explained below.

1.8.1. Hypoxia-Modulating Therapies

The goal of hypoxia-modulating therapies is to reduce hypoxia by increasing oxygen concentration in blood, or by increasing blood perfusion. Carbogen gas is composed of 95% oxygen and 5% carbon dioxide, although different proportions of gases have also been used (e.g., 99% O₂, 1% CO₂). This gas mixture is optimized so that it promotes an increase in breathing rate, acts as a vasodilator, and shifts the oxyhemoglobin dissociation curve such that hemoglobin has a decreased oxygen affinity, due to the presence of CO₂. In addition, high oxygen content in carbogen causes an
increase in the concentration of dissolved oxygen in plasma. A study by Partridge and colleagues demonstrated that administration of a gas containing 95% O2 and 5% CO2 resulted in the greatest reduction of hypoxic fraction (measured by the comet assay following radiation treatment) in human tumours compared to 97.5% O2 and 2.5% CO2 mixture and 100% oxygen (135). As a result of carbogen administration, oxygen diffusion distance increases, thereby reducing the fraction of chronically hypoxic cells, indicating that this treatment may only be beneficial to patients whose tumours are primarily chronically hypoxic (33). Carbogen may not enhance oxygen levels in cells that suffer from transient hypoxia, due to its reduced accessibility to tissue supplied by poorly perfused or collapsed blood vessels. As transiently hypoxic cells may be outside the range of oxygen diffusion distance achieved with carbogen, transient hypoxia may not be reduced by carbogen. Hence, addition of carbogen may not always improve the efficacy of therapy for all hypoxic cells in tumours. A vasoactive agent, nicotinamide, can be used in combination with carbogen to reduce acute hypoxia by increasing blood perfusion in tumours and inducing radiosensitization via other mechanisms (136).

Accelerated radiotherapy combined with carbogen (ARCO), nicotinamide (ARN) or both (ARCON) has been used in several clinical trials for treating various types of solid tumours and all treatment modalities have been shown to be both tolerable and feasible in the clinic (95). Although trends for reduction of tumour cell proliferation in human squamous cell carcinoma in vivo (33) and longer patient survival times (95) have been observed as a consequence of these therapies, the ability of these combined modalities to significantly improve response to therapy has not been rigorously validated.
The reported therapeutic gain resulting from these combined modalities varied, with 4/9 studies indicating improved local control and survival and 5/9 studies demonstrating no improvement (95). Moreover, ARN and ARCON have been associated with a higher acute toxicity rate (17, 82, 119). Ultimately, the effectiveness and feasibility of these combined modalities will depend on their ability to sensitize cells to radiation by reducing acute and chronic hypoxia, while ensuring tolerability of such therapies. Limiting toxic therapies to the subgroups of tumours likely to benefit from them should be a consideration in such trials.

Hyperbaric oxygen (2.4kPa to 4.0kPa) is an alternative approach for reducing chronic hypoxia but it is technically more difficult to administer with radiation than carbogen (67). Nevertheless, as proof of principle, it has been shown to improve the radiation response in squamous cell carcinoma of the head and neck (69) and rhabdomyosarcoma rat xenografts (72). Erythropoietin (EPO), a cytokine that stimulates production of red blood cells (RBC's), is frequently administered to patients, in order to manage anemia, a side-effect of radiotherapy (54, 61). Increase in the number of RBC’s allows for greater oxygen-carrying capacity leading to a decrease in poorly oxygenated cells. Administration of EPO should be performed with caution, however, since an increase in tumour oxygenation may lead to faster tumour growth rate resulting in low curability. Furthermore, treatment with EPO may not be effective in enhancing oxygenation (104) of tumours in which low vascular density is the primary cause of poor oxygenation.
1.8.2. Hypoxia-Targeting Therapies

The goal of hypoxia-targeting therapies is to minimize blood flow to the tumour in order to exploit hypoxia-associated properties of tumour cells. Hydralazine is a vasoactive agent that selectively decreases tumour blood flow while increasing perfusion in normal tissue. It promotes development of hypoxia in tumour tissue near vasculature, rendering the cells more susceptible to damage by tirapazamine and other bio-reductive drugs which are more cytotoxic under hypoxic conditions (162). An alternative means of reducing tumour blood flow is via application of short intense electric pulses (149). This therapy, combined with chemoradiotherapy, termed electroradiochemotherapy, and concurrent tirapazamine administration has shown therapeutic benefit via enhanced cytotoxic action of tirapazamine (115).

The majority of molecular hypoxia-targeting therapies interfere with one or more of the key components of the HIF-1 pathway. Small molecule inhibitors of HIF-1α and CA9 have been shown to reduce tumour growth rate, malignancy and invasion (71, 134, 138). Sulphonamides, CA9 inhibitors, have been found to reduce growth rates of lymphoma cell lines, renal cell carcinoma cell lines, and murine fibrosarcoma models (138). Acetazolamide has been shown to reduce the invasion rate of four renal carcinoma cell lines by inhibiting CA9 (134). Similarly, drugs that inhibit HIF-1α expression, antagonize HIF-1α interaction with CBP/p300 or block downstream function of genes such as VEGF may have important roles in tumour therapy (71). For instance, an agent
developed for circulatory disorders, YC-1 (3-(5’-hydroxymethyl-2’-furyl)-1-benzylindazole), has been shown to reduce tumour growth, vascularization and HIF-1α target gene expression in hepatoma, stomach carcinoma, renal carcinoma, cervical carcinoma and neuroblastoma \textit{in vivo} (178). Moreover, small interfering RNA therapy which targets HIF-1α, has been shown to increase hypoxia-induced tumour cell death in HeLa cells \textit{in vitro}, and to enhance the response to radiation therapy in murine HeLa and HCT116 xenografts (180). Angiogenesis inhibitors, such as inhibitors of VEGF receptor tyrosine kinases hinder growth of blood vessels in tumour tissue, causing diminished blood supply and expansion of hypoxia and necrosis (52). In addition, gene therapy approaches, which specifically target hypoxic cancer cells, have been explored in which vectors that carry pro-apoptotic or anti-proliferation genes are fused to HREs (hypoxia response elements) (43, 66, 71).
2. METHODS

2.1. Patients

At the time this project was initiated, there was an ongoing study headed by Dr. Christina Aquino-Parsons to assess the importance of tumour oxygenation status prior to treatment of patients with invasive epithelial cervical cancer at the Vancouver Cancer Centre. Ethical approval was granted by the British Columbia Cancer Agency Ethics Board and the University of British Columbia Ethics committee. Over the course of the study, more than 100 patients received pimonidazole as an intravenous infusion before the study was closed at the end of 2004. Seventy-eight patients with cervical carcinoma that participated in this project were treated at the BC Cancer Agency between September 1999 and June 2003. Charts of over one hundred cervical carcinoma patients stored in the Cancer Agency Information System (CAIS) were reviewed for treatment information, dates of treatments and biopsy excision, other clinicopathologic parameters and confirmation of the eligibility of patients for hypoxia marker analysis. In the cases of incomplete charts, Pathology Reports from Pathology Departments at the Vancouver General Hospital and Vancouver Cancer Centre, were collected and analyzed, and the information was integrated into CAIS. Subsequently, a database was created in SPSS containing all of the clinical information for the patients in the cohort.

All patients underwent initial clinical evaluation in accordance with local protocol, including a complete history and physical, CT and/or MRI of the abdomen and pelvis, chest X-ray, liver function tests, complete blood count, and serum creatinine and
electrolytes. Staging was according to FIGO guidelines. Suitable candidates for this study had a clinically visible and histologically confirmed invasive carcinoma of the cervix: squamous cell carcinoma, adenocarcinoma, adenosquamous carcinoma or carcinoma not otherwise specified (NOS), and gave informed consent. Patients were ineligible if they had liver enzyme tests greater than twice the normal laboratory values, serum creatinine $\geq$ 150 $\mu$mol/l, or a history of a peripheral neuropathy.

Seventy-four patients with cervical carcinoma (FIGO stages Ia to IVb) were treated with radical radiotherapy, forty-eight of these patients were given adjuvant chemotherapy (cisplatin), and sixty patients inhaled carbogen (95% oxygen, 5% carbon dioxide) 4 minutes before and during the daily fractions of external beam radiation therapy. Three patients underwent palliative treatment for recurrent tumours, and one patient refused treatment. All of the patients were eligible for marker correlation studies, regardless of the therapy they received. For outcome analysis, in addition to the entire patient cohort (64-74 patients for each marker alone), patients treated with radical chemoradiotherapy combined with carbogen were selected as a homogeneous subset for analysis (47-48 patients for each marker alone). This subset was selected in order to minimize the effect of variations in treatment on outcome, while the entire set was analyzed in order to maximize the number of individuals in the analysis, and thereby improve statistical significance of the results. Measurement of the fluctuations in HIF-1$\alpha$ over the course of chemoradiotherapy was performed on 15 patients. For each patient, in addition to one pre-treatment biopsy, multiple biopsies (1-4 biopsies) obtained during treatment were available for analysis. The entire study protocol is described in the
following sections, and is represented in Figure 8. Unless otherwise specified, all of the work presented here was performed by Bojana Janković.

All patients received a 20 minute i.v. infusion of 0.5g/m² of Hypoxyprobe-1 (pimonidazole hydrochloride; Natural Pharmaceuticals International Inc., Research Triangle Park, NC) dissolved in 100 ml of 0.9% sterile saline at room temperature. Two incisional biopsies (~150mg) of visible tumour were acquired from each patient approximately 24 hours later (about 4 plasma half-lives) by Dr. Christina Aquino-Parsons, a radiation oncologist at the B.C. Cancer Agency, Vancouver Clinic. One biopsy was transported immediately after excision to the laboratory and disaggregated for analysis of pimonidazole binding using flow cytometry. The other biopsy was fixed in formalin and embedded in paraffin. Sequential sections, 5μm thick, were prepared by the Pathology Department at the Vancouver Cancer Centre.
Figure 8. Schematic diagram of the experimental procedures for quantitative hypoxia measurements used in this study.
2.2. Eppendorf Electrode Measurements

Measurements of pO$_2$ were performed using an Eppendorf pO$_2$ histograph-6650 with sterile, polarographic probes 250µm in diameter (Hamburg, Germany) as previously described (64, 122, 165). In each track, the electrode was programmed to automatically advance a step length of 1mm followed by a backstep of 0.3mm to minimize tissue compression. The location of, the number and length of the tracks for each site was at the discretion of the clinician (CAP), and was dependent on the clinical size and location of the lesion, the tolerance of the patient to the procedure, and the clinical suspicion of any measurement artifacts that may have occurred. To decrease the potential variability of this invasive technique, all Eppendorf oxygen electrode measurements were performed by the same clinician, Dr. Christina Aquino-Parsons. Two parameters were extracted from Eppendorf electrode measurements, HP5 and HP2.5, the percentage of pO$_2$ values $\leq$ 5mmHg and the percentage of pO$_2$ values $\leq$2.5mmHg, respectively.

2.3. Flow Cytometry Analysis

Preparation of single cell suspensions and antibody staining for flow cytometry analysis were performed by Dr. Judith Banáth from the Medical Biophysics Department at the BC Cancer Research Centre. Tumour biopsies were minced and incubated for 30 minutes in a mixture of trypsin, collagenase and DNase, to create a single cell suspension. Cells were filtered and centrifuged through 30µm pore nylon mesh, suspended in PBS, and diluted with 95% ethanol to a 70% solution. Ethanol-fixed cells
were rinsed in PBS and resuspended in PST (PBS containing 4% FBS and 0.1% triton X-100). A fluorescein isothiocyanate (FITC)-conjugated (1:1000 dilution) anti-pimonidazole primary antibody (139) was incubated with 2x10^6 alcohol-fixed cells for 2 hours at 37°C. Samples were rinsed with PST and resuspended for DNA staining in 1ml PBS containing 1μg/ml 4,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI). Single cell suspensions were analyzed on a Coulter Epics Elite cell sorter (Coulter Corp. Hialeah, FL) for the intensity of FITC-anti-pimonidazole staining. Approximately 100,000 cells were acquired for the analysis.

Univariate histograms, plotted as cell number versus logarithm of fluorescent anti-pimonidazole antibody intensity, were analyzed by a least-squares approach for three Gaussian distributions representing aerobic, intermediately oxygenated, and hypoxic tumour cell populations. No constraints on the positions of the distribution means were imposed. Therefore, the range but not absolute fluorescence intensity determined whether a hypoxic fraction could be reliably identified within cells from different tumours. On the pimonidazole intensity histograms used to determine the hypoxic fraction, cells considered hypoxic were, on average, 10 times more fluorescent than well-oxygenated cells (Figure 9) (128).
Figure 9. The output graph of flow cytometry analysis of pimonidazole-labeled tumour cells from a biopsy. In this multimodal distribution graph, division into subpopulations of cells indicates that 22% of the cells show on average 10 times more pimonidazole adducts (area labeled in purple) than the well-oxygenated cells (area labeled in green). The remaining cells (hatched area) are at intermediate oxygenation.
2.4. Immunohistochemical Staining of Tissue Sections for Pimonidazole, CA9, HIF-1α and CD31

Immunoperoxidase method with diaminobenzidine tetrahydrochloride (DAB) substrate was performed to detect hypoxic regions indicated by the presence of pimonidazole, CA9 and HIF-1α in tumour tissue sections. In addition, a subset of tissue sections was stained for the endothelial marker CD31, using the immunoperoxidase method with Vector® NovaRED substrate, for the purpose of locating blood vessels.

The 5μm paraffin-embedded sections were placed into a pre-heated oven at a temperature of 50°C for 1 hour and 30 minutes to allow the paraffin to melt. They were subsequently immersed in xylene (2 times for 3 minutes), to complete the dewaxing process. The slides were hydrated in graded alcohols (99.5%, 95% and 75% ethanol for 6, 6 and 3 minutes, respectively), and rinsed in distilled water, then PBS, for 1 minute each. Subsequently, the sections were treated with 3% hydrogen peroxide in methanol for 10 minutes in order to eliminate the endogenous peroxidase activity and thereby prevent non-specific reactions with DAB substrate. After being rinsed in water and drained, each section was treated with 50μl of protease (1:100 v/v in PBS) for 30 minutes at 37°C. This step was performed in order to degrade the protein cross-links formed by formalin.

3 All of the steps were performed at room temperature, with the exception of the steps where the temperature is noted.

4 Upon treatment with protease, PTN, and antibodies, the slides were placed in a humidified chamber to prevent the sections from drying. The proper reagent was administered to sections, and the slides were covered with parafilm.
fixation and expose the antigenic sites. When staining for HIF-1α, the application of protease was substituted by treatment with high pH target retrieval solution, as this method was found to have improved efficacy for nuclear antigen exposure, compared to protease treatment. The slides were submerged for 30 minutes in a pre-heated solution inside a 95°C water bath. Subsequently, they were taken out of the bath and allowed to cool at room temperature for 15 minutes. They were then rinsed with PBS (3 times for 5 min).

To reduce the background staining, a blocking agent, PTN, containing 1% BSA (w/v) and 0.2% Tween 20 (v/v) in phosphate buffered saline (PBS), was applied to the sections for 20 minutes. Fifty to sixty microliters (depending on the size of the tissue section) of anti-pimonidazole, anti-HIF-α, or anti-CD31 monoclonal mouse antibody (diluted in PTN in proportions: 1:100, 1:100 and 1:20, respectively) was applied to each drained section for 2 hours (pimonidazole) or 1 hour (HIF-1α and CD31) (Table 1 contains the complete antibody reference list). After being washed three times (2 times with PBS and once with PTN) for 5 minutes, the sections were incubated in rabbit anti-mouse biotinylated secondary antibody (1:200 in PTN) for 30 minutes and subsequently washed in PBS for 5 minutes. Vectastain® Elite ABC reagent (Vector Laboratories Inc., Burlingame, CA) was mixed 30 minutes prior to use (A: 1:50 and B: 1:50 in PBS), and 50μL of this solution was applied to each section for 30 minutes. After a 5 minute PBS wash, 70μL of 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate solution was applied to sections for 10 minutes. The DAB solution was prepared by adding 32μL of DAB stock solution, 16μL of Hydrogen Peroxide solution and 8μL of Nickel solution,
respectively, and mixing after each solution is added, to 1mL of PBS. The Nickel Solution was added in order to yield a black precipitate where substrate reacted with ABC enzymes. For blood vessel staining using the CD31 marker, Vector® NovaRED substrate was mixed as per kit instructions (3 drops of Reagent 1, 2 drops of Reagent 2, 2 drops of Reagent 3 and 2 drops of Hydrogen Peroxide solution in 5mL of distilled water) and applied to yield a stain ranging from light shades of pink to moderate-intensity of purple. The slides were rinsed in distilled water for 10 minutes, and were submerged in Hematoxylin (mixed 1:3 with distilled water) for 5 seconds, to stain the nuclei blue. This step aided image analysis, as a distinct contrast between background and tissue was achieved. In addition, visualization of stained nuclei facilitated identification of individual cells. Hematoxylin was rinsed with water and the TWS buffer (3.5g sodium bicarbonate and 20g magnesium sulphate in 1L of double distilled water) for 6-10 seconds. Subsequent rinsing of slides with water removed the TWS. During the HIF-1α staining protocol, nuclei were not stained with hematoxylin, as HIF-1α is a nuclear antigen. In order to improve the visualization of cells and verify HIF-1α localization in the nucleus, the cytoplasm was stained pink with Eosin dye (1g/100mL mixed 1:1 with distilled water) for 8 seconds and rinsed with water only (no TWS). After the counterstaining procedure (hematoxylin or eosin application) was completed, the sections were dehydrated by immersion in 95% and 100% isopropanol (60 times each), respectively. Subsequently, the sections were left in 100% isopropanol for 1 minute. Following 60 dips in xylenes, the slides were immersed in xylenes for 2 minutes. Mounting medium, VectaMount (Vector Laboratories Inc., Burlingame, CA), was placed over the sections, covered by a cover-slip, after which the sections were allowed to dry.
Because the CA9 antibody was not commercially available at the time of this study, a collaboration was initiated with Dr. Eric Stanbridge at the University of California Irvine. CA9 staining was performed by Dr. Shu Liao in the laboratory of Dr. Stanbridge using the same immunohistochemical staining protocol and a CA9 monoclonal antibody was prepared as described previously (109).
Table 1. Antibody reference list. Primary (1°) and secondary (2°) antibodies are indicated.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Origin</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pimonidazole-HCl (1°)</td>
<td>Mouse anti-human</td>
<td>Natural Pharmaceuticals International Inc., Research Triangle Park, NC</td>
<td>1:100</td>
</tr>
<tr>
<td>HIF-1α (1°)</td>
<td>Mouse anti-human</td>
<td>BD Transduction Laboratories, Lexington, KY</td>
<td>1:100</td>
</tr>
<tr>
<td>CD31 (1°)</td>
<td>Mouse anti-human</td>
<td>Dakocytomation, Mississauga, ON</td>
<td>1:20</td>
</tr>
<tr>
<td>Biotinylated antibody (2°)</td>
<td>Rabbit anti-mouse IgG</td>
<td>Vector Laboratories, Inc., Burlingame, CA</td>
<td>1:200</td>
</tr>
</tbody>
</table>
2.5. Image Acquisition, Processing and Enhancement

A system consisting of a Zeiss Axioplan 2 microscope (Oberkochen, Germany) with an attached monochrome Retiga Exi cooled 12 bit CCD camera (Q Imaging, Burnaby, BC) was used for acquisition of images of stained tumour tissue sections. A 10x objective was used to view the slides and capture the images, and the light source intensity was maintained constant for all of the slides (the level of light intensity adjusted on the microscope was “5”). Images of pimonidazole- and most CA9-stained sections were focused and captured in 8-bit grayscale, while images of HIF-1α, CD31- and some CA9-stained sections were captured in 24-bit RGB using an RGB colour filter. The digitized images were visualized using Northern Eclipse 5.0 application software (Empix, Toronto, ON). The image focus and capture settings were selected in the Digital Focus function in Northern Eclipse 5.0 application software (Table 2a). Each image was manually or automatically focused prior to acquisition. Composite images of the entire tumour tissue were prepared by electronically tiling up to 300 individual frames, using the Full Control function. A computer controlled motorized scanning stage whose parameters were defined in the Full Control function (details of the settings are displayed in Table 2b), enabled capturing of sequential images. Some images was compressed by a factor of 2 to 4 prior to saving, to reduce their size.
Table 2. Image acquisition settings in Northern Eclipse. Digital Focus settings, specifying image acquisition parameters, are shown in (a), and Full Control settings, determining the steps in tiling of images, are displayed in (b).

(a) Digital Focus Settings

<table>
<thead>
<tr>
<th>Name of Function</th>
<th>Setting</th>
<th>Task Performed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Focus</td>
<td>8-bit</td>
<td>Focuses image in 8-bit grayscale</td>
</tr>
<tr>
<td>Capture</td>
<td>8-bit*</td>
<td>Captures image in 8-bit grayscale</td>
</tr>
<tr>
<td>Colour Correction†</td>
<td>White Balance</td>
<td>White balances bright field images</td>
</tr>
<tr>
<td>Exposure</td>
<td>14-30ms</td>
<td>Controls the brightness of the images</td>
</tr>
<tr>
<td>Gain</td>
<td>5.11%</td>
<td>Controls the brightness of the images</td>
</tr>
<tr>
<td>Offset</td>
<td>31.06</td>
<td>Controls the brightness of the images</td>
</tr>
</tbody>
</table>

* Images of CD31-, HIF-1α- and some CA9-stained sections were captured in 24-bit RGB.

† This function was performed only on 24-bit RGB images.

(b) Full Control Settings

<table>
<thead>
<tr>
<th>Full Control Settings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reflector</td>
</tr>
<tr>
<td>Time Delay 1s</td>
</tr>
<tr>
<td>Snap</td>
</tr>
<tr>
<td>Resolution 50% (or 25%)</td>
</tr>
<tr>
<td>Add to Superimage</td>
</tr>
</tbody>
</table>
Image artifacts, such as bubbles created upon application of the mounting medium, dust particles and areas of obvious necrosis, were manually removed with an Eraser tool in ImageJ (http://rsb.info.nih.gov/ij) or Scion Image (http://www.scioncorp.com) application software. Pieces of tissue present in one section and absent from a sequential section (stained for a different antigen) were deleted or disregarded upon colocalization analysis. In addition, images of tissue pieces in sequential sections were rotated until they were aligned in the same manner, in order to facilitate colocalization analysis.

The 24-bit colour images were utilized for visual scoring. Prior to image analysis, each image captured in 24-bit RGB was split into three images, each one representing a colour component (Red, Green or Blue), using a function RGB Split in ImageJ. The unstained tissue was selected in the grayscale image generated from the green channel, and the stained portion of the tissue was selected in the image from the red channel. These channels were selected for the analysis, as the tissue-background contrast was the highest in the image from the green channel, while the stained-unstained tissue contrast was the greatest in the image from the red channel (Figure 10).
Figure 10. Images resulting from the RGB Split function in ImageJ. An image of HIF-1α staining acquired in 24-bit RGB is split into three images corresponding to the red, green and blue colour channels. Note that the highest stained-unstained tissue contrast occurs in the red channel, while the highest background-tissue contrast occurs in the green channel.
Digitized images of suboptimal quality (e.g. due to excessive or insufficient brightness, or noise) were enhanced using Brightness/Contrast and Color Balance functions in ImageJ to manually optimize both the stain-tissue and the tissue-background contrasts. Upon completion of the image processing steps, the images were analyzed for the fractions of tumour sections stained for pimonidazole, CA9, HIF-1α and CD31 and for hypoxia marker colocalization.

2.6. Image Analysis: Quantifying the Fraction of Tissue Labeled for Pimonidazole, CA9, HIF-1α and CD31

2.6.1. Manual versus Automated Image Thresholding

Images were analyzed independently by three observers for marker positive fractions. The Threshold function in ImageJ was used to discriminate pixels on the basis of their grayscale intensity value. All of the pixels having intensity lower than or equal to the threshold intensity, selected by an observer, were highlighted. The highlighted areas were computed by summing the highlighted pixels. Thresholding was performed twice – to differentiate tissue from background and to differentiate stained tissue from the rest of the tissue (Figure 11). The two highlighted areas were measured and the hypoxic fraction was calculated by dividing the stained tissue area by the total tissue area. The selected threshold intensities were adapted to the intensity parameters of each image analyzed in order to differentiate between the unstained and stained tissue as accurately as possible. The threshold intensities depended on the intensity of the staining, the marker being detected, the extent of the counterstain absorption, the brightness of the image and the noise in the image.
Figure 11. Tissue and stain threshold procedure demonstrated on an image of a pimonidazole-stained section. The grayscale intensities used for selecting tissue and stain were 254 and 136, respectively.
Images that exceeded the memory limitations available in ImageJ were analyzed in Scion Image using the Density Slice function, which is equivalent to the Threshold function in ImageJ. Grayscale methods for thresholding and calculating areas were equivalent in Scion Image and ImageJ (both available from NIH imaging), hence the choice of application did not affect the results.

Although care was taken to maintain consistency in concentrations and duration of treatment by antibodies, ABC and DAB reagents, between the experiments, the efficacy of these reagents, and the resulting intensity of the signal were subject to variation. For this reason, the exact relationship between the staining intensity and the amount of marker present, could not be precisely identified, or assumed consistent between experiments. All of the antibodies yielded strong signals when present, or no signal at all, and therefore only the percentage of "stained" pixels as a fraction of total tumour tissue, and not the staining intensity, was assessed.

In addition to manual image analysis, the Otsu algorithm (132) was applied as an automated image analysis technique to 10 images of pimonidazole-labeled tissue sections in order to evaluate the automated image analysis method in comparison to the manual image analysis. Otsu algorithm is an unsupervised method for threshold selection whereby the optimal threshold is derived by maximizing the measure of separability of the resultant classes in gray levels. This algorithm was installed as a plug-in in ImageJ. When Otsu Thresholding function was applied, pixels from stained tissue were automatically highlighted. With the automated thresholding method, the selection of
unstained tissue was performed manually.

2.6.2. **Histogram Analysis as an Alternative Approach for Quantifying the Stained Tissue Fraction**

The use of histogram analysis to obtain hypoxic fractions was analyzed and tested with the goal of minimizing the error in measurement of stained area. When analyzing histograms of images, intra- and inter-observer errors are minimized when the pixel subpopulations are easily distinguished. A program in Java, developed by Hart Lambur, a summer student in Dr. Peggy Olive's laboratory, and ImageJ function *Histogram List* were used to acquire data points of histograms from grayscale images. The number of pixels in an image at each intensity value were extracted, and plotted in Gnuplot, a free graphing software (www.gnuplot.info/).

2.6.3. **Measurement of Hypoxic Marker Distribution in Relation to Vasculature**

The distribution of hypoxia with respect to blood vessels was measured on co-registered images of sequential sections stained for pimonidazole and CD31. Five images of CD31-stained tumour tissue were randomly selected and areas containing blood vessels were copied and pasted to the corresponding regions in the image of pimonidazole section. The distance between the blood vessels and the nearest pimonidazole-stained region was computed in ImageJ by drawing a straight line from the
two nearest points of the two regions, recording the length of this line, and converting it to the metric system. Five such measurements were made for each of the five pairs of images with the total of 25 readings.

2.7. Image Analysis: Marker Colocalization

Semi-quantitative and quantitative colocalization analysis methods were developed with the goal of numerically assessing the proportions of all of marker co-staining combinations. The methods described below were applied to the images and compared and their strengths and weaknesses were critically analyzed and discussed.

2.7.1. Semi-quantitative Marker Colocalization: Visual Scoring

A semi-quantitative colocalization analysis was performed on all images to calculate the colocalization between the following pairs of markers: pimonidazole and CA9, pimonidazole and HIF-1α, and CA9 and HIF-1α. A discrete scoring system was used in which scores from 0 to 4 were assigned to each pair of sections (Table 3). Markers on the pairs of sections with scores greater than or equal to 2 were considered moderately to highly colocalized. The differences in the three markers with respect to the extent of stain within the same region (e.g., narrow stain vs. slightly dispersed stain covering the same region), or the variation in the staining intensity were not considered as mismatch. Such variations were expected due to different concentrations of the three markers, differences in their subcellular localization and oxygen thresholds required for marker presence.
Table 3. Semi-quantitative colocalization scoring scheme.

<table>
<thead>
<tr>
<th>Score</th>
<th>Percent Colocalization (x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>x &lt; 20%</td>
</tr>
<tr>
<td>1</td>
<td>20% ≤x &lt; 40%</td>
</tr>
<tr>
<td>2</td>
<td>40% ≤x &lt; 60%</td>
</tr>
<tr>
<td>3</td>
<td>60% ≤x &lt; 80%</td>
</tr>
<tr>
<td>4</td>
<td>x ≥80%</td>
</tr>
</tbody>
</table>

Table 4. Twelve colocalization parameters. The colocalized fraction between combinations of markers shown in the right column is expressed with respect to positive fraction of each of the markers shown in the left column.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pimo</td>
<td>Pimo only</td>
</tr>
<tr>
<td></td>
<td>Pimo &amp; CA9</td>
</tr>
<tr>
<td></td>
<td>Pimo &amp; HIF-1α</td>
</tr>
<tr>
<td></td>
<td>Pimo, CA9 &amp; HIF-1α</td>
</tr>
<tr>
<td>CA9</td>
<td>CA9 only</td>
</tr>
<tr>
<td></td>
<td>CA9 &amp; Pimo</td>
</tr>
<tr>
<td></td>
<td>CA9 &amp; HIF-1α</td>
</tr>
<tr>
<td></td>
<td>CA9, Pimo &amp; HIF-1α</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>HIF-1α only</td>
</tr>
<tr>
<td></td>
<td>HIF-1α &amp; Pimo</td>
</tr>
<tr>
<td></td>
<td>HIF-1α &amp; CA9</td>
</tr>
<tr>
<td></td>
<td>HIF-1α, Pimo &amp; CA9</td>
</tr>
</tbody>
</table>
Direct, quantitative colocalization analysis via image superposition of pimonidazole, CA9 and HIF-1α stained areas was not feasible with sequential sections because of slight tissue distortion or damage caused by sectioning. This could only be performed by comparing marker labeling on the same section.

2.7.2. Image Registration

An algorithm for registration of images of sequential sections that would enable automated pixel-by-pixel analysis of two images was developed and tested on an application created in collaboration with Craig Hennessy, a graduate student in the Department of Electrical Engineering and Computer Engineering at the University of British Columbia. The algorithm for image registration was implemented in MATLAB, and it consisted of coarse registration of two images of sequential sections, followed by fine registration. Initially, the tissue contours were aligned. The reference image, referred to as image1, was padded with zeros around its border to prevent the second image, image2, from extending outside of image1’s border. Image2 was rescaled, and the orientations of the major axes of the contours were determined. The major axis was defined as the straight line between two points inside the contour, which are the greatest distance from each other. The angle between major axes of the two contours was the coarse rotation angle. Image2 was rotated by the value determined by the major axis orientation and the centroid of the two contours was determined from the centre of mass. By overlapping the two centroids the coarse translation was determined. After coarse registration was completed, fine registration was performed. This is an iterative process.
whereby the translation values are offset by a random value of -1, 0, or 1 pixels according to a uniform distribution. If this variation resulted in an improvement, as defined by an increase in the total area of the two images which overlap, the new transformation parameters were kept and the procedure restarted. If the area of overlap decreased, the transformation changes were discarded. The registration was completed after a certain number of iterations was performed without any further improvements in the overlap between the images.

After this method was used for registration of images of sequential sections, it was concluded that this approach of automated image registration was not feasible for these sections and the nature of the stains. The differences in tissue positioning in sequential sections resulted in alignment errors during image registration, leading to overestimates of stain mismatch. The high resolution of the images also contributed to false mismatch, as the different subcellular localizations of the markers were detected as mismatched regions by this method.

2.7.3. Quantitative Marker Colocalization: Theoretical Calculations

Proportions of marker mismatch patterns were approximated using semi-quantitative colocalization scores and marker positive fractions. The calculations yielded percentages of each marker's staining occupied by that marker only, each pair of markers, and all three markers, resulting in 12 colocalization parameters (Table 4).
Two approximations were made in the marker colocalization equations:

1. The colocalization score for all three markers is the lowest of the three colocalization scores between the three pairs of markers.

2. On average, marker positive fraction is approximately the same for the three markers for a given section. As there were significant correlations between marker positive fractions, this approximation is valid in the majority of cases.

In the equations, colocalization scores were normalized with respect to the maximum colocalization score, 4, to obtain the fraction of a marker stain colocalized by another marker. The term $\text{Coloc}(\text{Marker}_1, \text{Marker}_2)$ refers to the colocalization score between markers 1 and 2, while the term $\text{Avg}(\text{Marker}_1, \text{Marker}_2)$ refers to the average marker positive fraction of markers 1 and 2. The equations used were the following:

$$\text{Marker}_1 \text{only} = \text{Marker}_1 \times \left\{ 1 - \frac{1}{4} \left[ \text{Coloc}(\text{Marker}_1, \text{Marker}_2) + \text{Coloc}(\text{Marker}_1, \text{Marker}_3) \right] \right\}$$

**Equation 5.** Calculation of the fraction of staining by one marker.

$$\text{Marker}_1 \text{and}_2 \text{only} = \frac{1}{4} \text{Avg}(\text{Marker}_1, \text{Marker}_2) \times \left[ \text{Coloc}(\text{Marker}_1, \text{Marker}_2) \right]$$

**Equation 6.** Calculation of the fraction of staining by two markers.
\[
\text{AllMarkers} = \frac{1}{4} \text{Avg}(\text{Marker1, Marker2, Marker3}) \times \text{Coloc}(\text{Marker1, Marker2, Marker3})
\]

**Equation 7.** Calculation of the fraction of staining by three markers.

**2.7.4. Verification of the Use of Theoretical Calculations to Predict Marker Colocalization: Direct Measurements of Colocalized Marker Fractions by Image Manipulation**

Colocalization was measured quantitatively in ten randomly selected (~15% of the sample) triplets (pimonidazole-, CA9- and HIF-1α-labeled) of sections in which the staining was of high quality, such that there was no ambiguity with respect to the marker positive fraction.

Two images of sequential sections labeled for different markers were analyzed simultaneously in ImageJ software. Manual thresholding was used to compute the marker positive fraction of each section. Grayscale values used for thresholding were the same as the previously recorded values used for measuring marker positive fractions for the marker correlation studies. For each pair of markers, marker1 and marker2, areas of marker1 staining not labeled by marker2 were manually deleted from the image of marker1, and vice versa. Each image was re-analyzed, using the previously recorded threshold value, for the percentage of section stained for the marker. This procedure was repeated for each marker, in combination with each of the other two markers alone, and
together.

2.8. Statistical Analysis of Marker Correlations and Outcome

Linear least squares regression analysis was used to determine the degree of correlation between the fractions of tissue sections labeled for pimonidazole, CA9 and HIF-1α. Bivariate Pearson Correlations were performed to identify pairs of clinical and hypoxia parameters which correlated significantly. To examine the differences in hypoxic fraction between populations with different values of clinical parameters, independent Student-t tests were performed. A p-value less than or equal to 0.05 was considered statistically significant.

For survival analysis, the patients were divided into two groups: those having hypoxia marker values above and equal to median, and those with hypoxia marker values below median. Two sets of patients were analyzed, those receiving identical treatment, radical radiotherapy, chemotherapy and carbogen, and the entire group of patients. The outcome analysis yielded comparable results for the two sets of patients, but only the data for the set homogenized with respect to treatment is presented. The endpoint selected for outcome analysis was progression-free survival. In addition to the possibility of comparing the relationship of hypoxia markers and outcome to other studies that have also used progression-free survival as the endpoint, there are several benefits to using this as an indicator of outcome. Since progression-free survival accounts for any sign of disease progression, including occurrences such as local failure, metastasis and death, the
probability that any one or more of these events will occur is greater than the probability of a single event. This grouping of outcome endpoints is appropriate for a modest patient cohort size, such as the one in this study, allowing for a greater probability of event occurrence. Univariate analysis of progression-free survival was performed by Kaplan-Meier analysis and differences between groups were tested by log-rank test. Multiple regression survival analysis was performed using the Cox proportional hazards model (after confirming that the hazard ratio is constant over time) on the homogenized patient subset. Both forward and backward stepwise model building methods, based on likelihood ratio tests were used in the analyses of covariates. Variables were removed from the model if their significance exceeded 0.1 and were added to the model if their significance was less than 0.05. All of the analyses were performed with the statistical application software SPSS 11.0 (Chicago, IL).

2.9. Use of Tumour Image Models to Examine the Effects of Noise, Contrast and Stain Distribution on Image Analysis Reproducibility

To examine the sources of error in manual image analysis, artificial tumour section images were generated and analyzed under varying conditions in ImageJ (Figure 12).
Figure 12. Tumour image model. This tumour model template was used for testing the effects of noise, contrast and stain distribution on image analysis. The dark rectangle in the middle of the image represents the stained tissue (precipitate formed as a result of the immunoperoxidase method with the DAB substrate), while the large gray rectangle represents the non-stained tissue, and the surrounding white background mimics the background of a tissue section image. The “stained area” comprises 5% of the total “tissue area” in this tumour image model.
Tumour image models represented tumour sections immunohistochemically stained for pimonidazole, CA9 or HIF-1α whose images were captured in grayscale. Two shades of gray assigned to the tumour model represented unstained tumour tissue (180 grayscale intensity selected according to the most dominant shade of gray of the real tumour images) and stained tumour tissue (70 grayscale intensity). The resulting histogram consisted of three peaks: at 255 grayscale (background), 180 grayscale (unstained tissue) and 70 grayscale (stained tissue). Noise was added to each of the tumour model images to mimic the real images. The addition of this parameter resulted in transformation of the three peaks in the histogram into three Gaussian distributions. Unless otherwise specified, the invariant parameters of the tumour model image were: standard deviation = 25 shades of gray, mean non-stained tissue shade of gray = 180, mean stained tissue shade of gray = 70, distribution of stain = a single stained area occupying 5% of the entire tissue. In addition, the areas of two rectangles in the image (total tissue area and the stained portion of the tissue) were not altered. Parameters varied were noise, contrast between stained and unstained tumour tissue and distribution of stain throughout the tumour model.
3. RESULTS

Distributions of clinicopathologic factors and median values and ranges of hypoxia markers and Eppendorf electrode measurements in this patient cohort are displayed in Table 5. As the clinical data were not available for all of the patients, the number of patients whose clinical parameters were available is noted (N). The most frequent causes for missing data were incomplete patient charts, procedures that were performed at other institutions, procedures that were not performed, failure to diagnose (e.g., FIGO stage, histological type or grade), or missing reports from other departments which could not be acquired (e.g., pathology reports, imaging reports). Frequencies of adenocarcinoma and squamous cell carcinoma in this study reflected the incidence of these two types of cancer in the North American population (63, 110). In agreement with previously reported observations (85, 117), hypoxic fraction measured by three hypoxia markers did not correlate with the well-established clinical prognostic factors (including FIGO stage, maximum clinical diameter, hemoglobin concentration, nodal status and tumour grade) (Pearson Correlation results are not shown).
Table 5. Statistics of clinicopathologic parameters and therapy, and hypoxic fraction in relation to these factors. For each patient subset with respect to values of the factors, the median value of the hypoxia marker positive fraction and the range of values are noted. Pimo refers to the pimonidazole positive fraction obtained from the image analysis while Pimo-FC is the flow cytometry measurement.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>N</th>
<th>%</th>
<th>Pimo</th>
<th>Pimo-FC</th>
<th>CA9</th>
<th>HIF-1α</th>
<th>HP2.5</th>
<th>HP5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histology</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamous Cell Carcinoma</td>
<td>55</td>
<td>75.3</td>
<td>5.4</td>
<td>5.4</td>
<td>4.7</td>
<td>4.0</td>
<td>39.5</td>
<td>61.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.4-27.8)</td>
<td>(0.0-28.1)</td>
<td>(0.2-28.1)</td>
<td>(0.4-21.1)</td>
<td>(1.0-89.0)</td>
<td>(2.0-100.0)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>16</td>
<td>21.9</td>
<td>1.9</td>
<td>2.9</td>
<td>7.6</td>
<td>7.0</td>
<td>35.0</td>
<td>57.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.6-8.9)</td>
<td>(0.7-22.6)</td>
<td>(1.0-23.5)</td>
<td>(1.3-30.4)</td>
<td>(0.0-80.0)</td>
<td>(0.0-81.0)</td>
</tr>
<tr>
<td>Adenosquamous</td>
<td>2</td>
<td>2.7</td>
<td>9.6</td>
<td>7.4</td>
<td>12.8</td>
<td>6.4</td>
<td>64.2</td>
<td>70.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(9.6-9.6)</td>
<td>(6.6-8.2)</td>
<td>(12.8-12.8)</td>
<td>(6.3-6.5)</td>
<td>(64.2-64.2)</td>
<td>(70.4-70.4)</td>
</tr>
<tr>
<td><strong>FIGO Stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>27</td>
<td>37.5</td>
<td>3.7</td>
<td>5.0</td>
<td>6.6</td>
<td>5.2</td>
<td>41.5</td>
<td>66.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.6-27.8)</td>
<td>(1.0-23.0)</td>
<td>(0.3-28.1)</td>
<td>(0.6-30.4)</td>
<td>(2.0-87.0)</td>
<td>(9.0-100.0)</td>
</tr>
<tr>
<td>II</td>
<td>27</td>
<td>37.5</td>
<td>4.1</td>
<td>3.0</td>
<td>4.1</td>
<td>4.3</td>
<td>39.0</td>
<td>66.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.4-11.4)</td>
<td>(0.0-20.8)</td>
<td>(0.2-23.5)</td>
<td>(0.4-21.1)</td>
<td>(10.0-81.0)</td>
<td>(32.0-96.0)</td>
</tr>
<tr>
<td>III</td>
<td>13</td>
<td>18.1</td>
<td>6.1</td>
<td>5.1</td>
<td>5.2</td>
<td>4.5</td>
<td>38.0</td>
<td>51.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1.5-11.1)</td>
<td>(0.7-28.1)</td>
<td>(1.1-14.4)</td>
<td>(2.2-17.1)</td>
<td>(1.0-83.0)</td>
<td>(0.0-92.0)</td>
</tr>
<tr>
<td>IV</td>
<td>5</td>
<td>6.9</td>
<td>6.2</td>
<td>9.2</td>
<td>10.3</td>
<td>5.4</td>
<td>33.1</td>
<td>38.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(5.6-11.0)</td>
<td>(6.3-22.6)</td>
<td>(6.4-12.8)</td>
<td>(2.9-6.5)</td>
<td>(0.0-89.0)</td>
<td>(4.0-92.0)</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well/Mod Differentiated</td>
<td>35</td>
<td>53.0</td>
<td>4.9</td>
<td>3.8</td>
<td>5.0</td>
<td>6.3</td>
<td>41.0</td>
<td>68.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.4-27.8)</td>
<td>(0.0-28.1)</td>
<td>(0.2-28.1)</td>
<td>(0.4-30.4)</td>
<td>(0.0-28.1)</td>
<td>(0.0-100.0)</td>
</tr>
<tr>
<td>Poorly Differentiated</td>
<td>31</td>
<td>47.0</td>
<td>4.6</td>
<td>5.4</td>
<td>4.5</td>
<td>3.7</td>
<td>40.3</td>
<td>57.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1.2-14.8)</td>
<td>(0.3-23.0)</td>
<td>(0.3-27.8)</td>
<td>(0.5-17.4)</td>
<td>(1.0-89.0)</td>
<td>(2.0-100.0)</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 47 (median)</td>
<td>36</td>
<td>50.0</td>
<td>3.1</td>
<td>3.2</td>
<td>4.1</td>
<td>3.4</td>
<td>40.5</td>
<td>66.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.4-11.0)</td>
<td>(0.0-20.8)</td>
<td>(0.2-23.5)</td>
<td>(0.4-30.4)</td>
<td>(1.0-80.0)</td>
<td>(0.0-90.0)</td>
</tr>
<tr>
<td>≥47 (median)</td>
<td>36</td>
<td>50.0</td>
<td>5.8</td>
<td>6.5</td>
<td>7.8</td>
<td>6.5</td>
<td>39.0</td>
<td>57.0</td>
</tr>
<tr>
<td>Parameter</td>
<td>N</td>
<td>%</td>
<td>Pimo</td>
<td>Pimo-FC</td>
<td>CA9</td>
<td>HIF-1α</td>
<td>HP2.5</td>
<td>HP5</td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>----</td>
<td>-----</td>
<td>------</td>
<td>---------</td>
<td>------</td>
<td>--------</td>
<td>-------</td>
<td>-----------</td>
</tr>
<tr>
<td>Size (largest diameter in cm)</td>
<td>69</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 5.0 (median)</td>
<td>31</td>
<td>44.9</td>
<td>5.4</td>
<td>5.0</td>
<td>6.7</td>
<td>5.6</td>
<td>42.0</td>
<td>66.0</td>
</tr>
<tr>
<td></td>
<td>(0.6-27.8)</td>
<td>(0.0-28.1)</td>
<td>(0.6-27.8)</td>
<td>(0.0-28.1)</td>
<td>(0.3-28.1)</td>
<td>(0.6-30.4)</td>
<td>(3.0-89.0)</td>
<td>(0.0-100.0)</td>
</tr>
<tr>
<td>≥ 5.0 (median)</td>
<td>38</td>
<td>55.1</td>
<td>4.7</td>
<td>5.3</td>
<td>4.3</td>
<td>3.7</td>
<td>37.0</td>
<td>54.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.4-11.1)</td>
<td>(0.2-22.6)</td>
<td>(0.2-23.5)</td>
<td>(0.4-18.0)</td>
<td>(0.0-87.0)</td>
</tr>
<tr>
<td>Pre-treatment Hemoglobin(g/L)</td>
<td>69</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 128 (median)</td>
<td>34</td>
<td>49.3</td>
<td>4.6</td>
<td>6.0</td>
<td>4.2</td>
<td>4.8</td>
<td>41.0</td>
<td>63.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1.2-27.8)</td>
<td>(0.7-23.0)</td>
<td>(1.0-28.1)</td>
<td>(0.5-18.0)</td>
<td>(1.0-87.0)</td>
</tr>
<tr>
<td>≥ 128 (median)</td>
<td>35</td>
<td>50.7</td>
<td>4.9</td>
<td>3.7</td>
<td>7.2</td>
<td>5.9</td>
<td>36.0</td>
<td>60.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.4-14.8)</td>
<td>(0.0-28.1)</td>
<td>(0.2-27.8)</td>
<td>(0.4-30.4)</td>
<td>(0.0-89.0)</td>
</tr>
<tr>
<td>Nodal Status</td>
<td>60</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>38</td>
<td>63.3</td>
<td>4.3</td>
<td>4.4</td>
<td>5.2</td>
<td>4.1</td>
<td>40.3</td>
<td>61.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.6-27.8)</td>
<td>(0.0-22.6)</td>
<td>(0.3-28.1)</td>
<td>(0.6-30.4)</td>
<td>(0.0-89.0)</td>
</tr>
<tr>
<td>Positive</td>
<td>22</td>
<td>36.7</td>
<td>6.1</td>
<td>5.7</td>
<td>4.2</td>
<td>4.8</td>
<td>38.5</td>
<td>60.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.4-20.0)</td>
<td>(0.2-23.0)</td>
<td>(0.2-27.8)</td>
<td>(0.4-18.0)</td>
<td>(2.0-87.0)</td>
</tr>
<tr>
<td>Necrosis in Biopsy</td>
<td>65</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>14</td>
<td>21.5</td>
<td>6.8</td>
<td>6.4</td>
<td>7.4</td>
<td>6.5</td>
<td>40.5</td>
<td>60.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.6-11.1)</td>
<td>(1.2-12.4)</td>
<td>(2.0-14.4)</td>
<td>(1.8-30.4)</td>
<td>(2.0-81.0)</td>
</tr>
<tr>
<td>No</td>
<td>51</td>
<td>78.5</td>
<td>4.9</td>
<td>4.7</td>
<td>5.1</td>
<td>4.8</td>
<td>39.8</td>
<td>64.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.4-27.8)</td>
<td>(0.0-28.1)</td>
<td>(0.2-28.1)</td>
<td>(0.4-21.1)</td>
<td>(0.0-89.0)</td>
</tr>
<tr>
<td>Smoking Status</td>
<td>60</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not Current Smoker</td>
<td>38</td>
<td>63.3</td>
<td>5.8</td>
<td>5.0</td>
<td>5.2</td>
<td>5.2</td>
<td>38.5</td>
<td>57.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.6-27.8)</td>
<td>(0.0-23.0)</td>
<td>(0.3-28.1)</td>
<td>(1.3-30.4)</td>
<td>(0.0-89.0)</td>
</tr>
<tr>
<td>Current Smoker</td>
<td>22</td>
<td>36.7</td>
<td>3.6</td>
<td>5.8</td>
<td>4.0</td>
<td>3.3</td>
<td>45.0</td>
<td>70.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.4-14.8)</td>
<td>(0.2-23.0)</td>
<td>(0.2-19.3)</td>
<td>(0.4-17.4)</td>
<td>(1.0-87.0)</td>
</tr>
</tbody>
</table>
3.1. Inter- and Intra-Tumour Heterogeneity in Hypoxia Marker Labeling

3.1.1. Inter-tumour Heterogeneity in Hypoxia Marker Labeling

While similar ranges, means and medians were observed for pimonidazole, CA9 and HIF-1α staining, these measurements differed significantly from Eppendorf oxygen electrode measurements (Table 6).
Table 6. Distribution of hypoxic fraction measured by markers and Eppendorf electrode in cervical carcinomas.

<table>
<thead>
<tr>
<th></th>
<th>Pimo</th>
<th>Pimo-FC</th>
<th>CA9</th>
<th>HIF-1α</th>
<th>HP2.5</th>
<th>HP5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Samples</td>
<td>69</td>
<td>78</td>
<td>68</td>
<td>67</td>
<td>59</td>
<td>59</td>
</tr>
<tr>
<td>Mean HF (%)</td>
<td>5.5</td>
<td>6.9</td>
<td>7.4</td>
<td>6.4</td>
<td>42.3</td>
<td>58.6</td>
</tr>
<tr>
<td>Median HF (%)</td>
<td>4.9</td>
<td>5.0</td>
<td>5.1</td>
<td>4.8</td>
<td>39.0</td>
<td>63.0</td>
</tr>
<tr>
<td>Standard Deviation of HF (%)</td>
<td>4.5</td>
<td>6.5</td>
<td>6.5</td>
<td>5.3</td>
<td>24.7</td>
<td>24.0</td>
</tr>
<tr>
<td>Range of HF (%)</td>
<td>0.4-27.8</td>
<td>0.0-28.1</td>
<td>0.2-28.1</td>
<td>0.4-30.4</td>
<td>0.0-89.0</td>
<td>0.0-100.0</td>
</tr>
</tbody>
</table>
Distributions of marker positive fraction for all three markers are shown in Figure 13, where the hypoxic fraction was divided into 5% intervals, and the frequency of each range of positive staining was plotted for each marker. The frequencies of all three markers exhibited negative exponential relationships with respect to the hypoxic fraction interval while the histograms resulting from oxygen electrode measurements resembled Gaussian distributions. As electrode results are averages of the measurements taken in different parts of the tumour, it is not surprising that the measurements in the patient cohort follow a Gaussian distribution.

According to the hypoxia marker results, approximately 38% to 47% of the tumours have a hypoxic fraction between 0% and 5%, while 22% to 32% of the tumours have a hypoxic fraction between 5% and 10%, indicating that the majority of tumours studied have a low hypoxic fraction. Similar trends were observed by Ljungkvist et al. (111) for head and neck squamous cell carcinomas, Nordsmark et al. (121) and Vaupel et al. (164) for cervical carcinomas. The former group measured hypoxic fraction with pimonidazole, while the two latter groups measured pO₂ using the oxygen electrode.
Figure 13. Histograms of hypoxic fraction distributions. Measurements of hypoxic fraction are performed with (a) Pimonidazole - Image Analysis (N = 69), (b) Pimonidazole - Flow Cytometry (N = 78), (c) CA9 (N = 68), (d) HIF-1α (N = 67) and Eppendorf Electrode: (e) HP2.5 (N = 59) and (f) HP5 (N = 59).
3.1.2. Intra-tumour Heterogeneity in Hypoxia Marker Labeling

3.1.2.1. Heterogeneity in Hypoxia Marker Labeling Among Biopsies of the Same Tumour

There is considerable evidence that tumour hypoxia can be highly variable throughout the tumour tissue (7, 27, 33, 45, 51). In the first part of this study, the intra-tumour heterogeneity in hypoxia was examined by analyzing sections from biopsies taken at the same time from different locations in the tumour. The pimonidazole positive fractions in two different biopsies, prepared and measured using two distinct methods, flow cytometry and image analysis, correlated only moderately (Pearson's correlation coefficient = 0.45) (Figure 14).
Figure 14. Pimonidazole Flow Cytometry vs. Pimonidazole Immunohistochemistry. The line of best fit is \( \text{Pimo-FC} = 0.68 \text{Pimo} + 3.46 \).
Hypoxic fraction measurements from two to three different biopsies from the same tumour, obtained by image analysis, are plotted in Figure 15. Sections of multiple biopsies from each patient were stained for pimonidazole (6 patients), CA9 (6 patients) and HIF-1α (9 patients). There was heterogeneity in the distribution of all three markers within the tumours. The variation in the stained fraction and the slopes of the graphs in Figure 15 indicate that the heterogeneity in HIF-1α appears to be the lowest. In addition, the ratios of the lowest and the highest fractions of marker staining within the biopsies from the same tumour are, on average, 0.54, 0.56, and 0.60 for Pimonidazole, CA9, and HIF-1α, respectively. These ratios confirm that two-fold variations in the hypoxic fraction throughout the tumour tissue are not uncommon.
Figure 15. Heterogeneity in hypoxia marker labeling measured in multiple biopsies from each tumour. Pimonidazole (a), CA9 (b) and HIF-1α (c). The graphs in the top row indicate the hypoxic fraction measurements for each patient, while the graphs in the bottom row indicate the highest hypoxic fraction measurement vs. the lowest hypoxic fraction measurement among the biopsies of the same tumour.
3.1.2.2. Reproducibility of Pimonidazole Labeling Between Sequential Sections

Before comparing the staining of different markers on sequential sections, it was important to establish whether immunohistochemical staining of sequential sections was reproducible and whether hypoxic fraction was consistent between sequential sections. Therefore, fractions of the tumour tissue stained for pimonidazole in two sequential sections were compared. Two sets (Set I and Set II) of sixteen sections labeled for pimonidazole were available for comparative analysis. It is possible that occasionally, several consecutive sections were removed between the two “sequential” sections in Set I and Set II. This could have occurred during the sectioning procedure, prior to acquiring a Set II section. If so, this would result in the morphologic differences and variation in tissue distribution observed in several pairs of sections from the same biopsy. Similarly, the presence of functional vasculature outside of the plane of the section may cause differential hypoxia marker expression (or binding) in sections of the same biopsy at a sufficient distance from one another. Nevertheless, the correlation between the hypoxic fractions labeled by pimonidazole in Sets I and II was strong and significant, with a correlation coefficient of 0.99 (p = 0.036) (Figure 16).

The hypoxic fraction as labeled by pimonidazole, was homogeneous between sequential or proximal sections of the same biopsy. The strong correlation also confirmed the reproducibility of the immunohistochemical staining protocol used in this study. This was taken as evidence supporting the assumption that comparisons of marker labeling between sequential sections is a reliable method for analysis of hypoxia in tumour biopsies.
Figure 16. Correlation between pimonidazole positive fractions in stained sections from Set I and Set II.
3.2. Numerical Correlations Between Hypoxic Fraction Determined by Pimonidazole, CA9 and HIF-1α

The dark precipitate, resulting from the DAB substrate and peroxidase enzyme reaction in areas where the marker was present varied in subcellular localization (Figure 17a), intensity and distribution (Figure 17b-d), for pimonidazole, CA9 and HIF-1α. Pimonidazole produced a strong signal in the cytoplasm (Figure 17a), labeling the entire cell, with a narrow range of intensities in the vicinity of 0 (grayscale black). The distinct staining patterns and strong intensity facilitated the differentiation between stained and unstained tissue. Furthermore, pimonidazole staining was characterized by sharp borders and a consistent intensity within the stained area, while the staining patterns were patchy or ribbon-like. In contrast, CA9 staining was restricted to the cell membrane (Figure 17a). The staining ranged from dark grey to black in the grayscale images, and it was dark brown in colour images. Patterns of CA9 positive regions were similar to those of pimonidazole, although the borders between stained and unstained tissue were somewhat less distinct. Staining of HIF-1α was the least distinguishable, as the dynamic range of the staining intensity was prevalent in the lighter shades of gray, compared to pimonidazole and CA9. Moreover, HIF-1α was localized to the nucleus (Figure 17a), as expected, which may have contributed to underestimating the fraction of cells labeled by HIF-1α. The pattern of expression of HIF-1α in tumour sections ranged from focal and strictly localized, to weak and more diffuse throughout the tissue section. The two different patterns may indicate different genetic profiles that can influence HIF-1α.
stabilization, such as pVHL or PHD2 mutation. Because HIF-1α is rapidly stabilized under hypoxia, there is a possibility that its expression increased between the times of biopsy and fixation. To lessen this effect, biopsies were placed in a formalin-containing dish immediately (on the order of seconds) after the biopsy was acquired.
Figure 17. Subcellular localizations of markers and marker staining patterns. Different subcellular localizations of pimonidazole, CA9 and HIF-1α are shown in (a). Staining patterns of the three markers are shown in the images of sequential tumour tissue sections (b-d).
3.2.1 *Inter-Observer Variation in Hypoxic Fraction Assessment Using Image Analysis*

Three observers (PLO, SM, and BJ) independently measured the fractions of tumour sections stained for pimonidazole, CA9 and HIF-1α. To minimize the subjectivity of image analysis, threshold selection criteria were established by the observers. The tissue selected as "stained" was required to be visibly darker from the unstained tissue, especially for pimonidazole and CA9 stained sections. For HIF-1α stained sections, lighter shades of gray (i.e., higher threshold values) were usually included when measuring the stained fraction, since the staining intensity was lower. In addition, all observers analyzed a subset of images together prior to commencing manual image analysis independently. As a result, the scoring scheme used by the three individuals was assumed to be consistent and representative of hypoxic fraction. The correlations between inter-observer measurements indicate that there is significant agreement between the observers (Table 7). In the cases where the observers’ values showed large discrepancies (there were less than 10 such cases per marker), the images were re-analyzed by all observers together, and a consensus for the marker positive fraction value was reached.
**Table 7.** Inter-observer correlations in measurement of marker positive fraction. The correlations are represented as $r^2$ values of linear regression analysis.

<table>
<thead>
<tr>
<th>Marker</th>
<th>PLO/SM</th>
<th>PLO/BJ</th>
<th>SM/BJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pimonidazole</td>
<td>0.78</td>
<td>0.77</td>
<td>0.79</td>
</tr>
<tr>
<td>CA9</td>
<td>0.79</td>
<td>0.76</td>
<td>0.80</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>0.78</td>
<td>0.70</td>
<td>0.60</td>
</tr>
</tbody>
</table>
3.2.2. Correlation Between Hypoxia Markers in Sequential Sections

Numerical correlations between different methods for measuring hypoxia are shown in Table 8. Three hypoxia markers detected by immunohistochemistry, pimonidazole detected using flow cytometry, and two parameters from oxygen electrode measurements, HP5 and HP2.5, are shown. Numerically, pimonidazole correlated with CA9 ($r = 0.60; p < 0.01$) and HIF-1α staining ($r = 0.34; p < 0.01$), and CA9 correlated with HIF-1α ($r = 0.42; p < 0.01$). Interestingly, flow cytometry measurements yielded hypoxic fractions that correlated with CA9 but not HIF-1α positive fractions, which may be due to a combination of tumour heterogeneity, insufficient sample size for a significant correlation or the different hypoxia marking properties of the two markers and methods. Eppendorf measurements did not correlate with any other method for measuring hypoxia, but as expected, HP2.5 and HP5 strongly correlated.
Table 8. Pearson's correlation coefficients between pairs of hypoxia measurement techniques. The bolded letters with the symbol ** indicate that the correlation is significant at the 0.01 level (2-tailed).

<table>
<thead>
<tr>
<th>Marker Statistics</th>
<th>Pimo</th>
<th>Pimo-FC</th>
<th>CA9</th>
<th>HIF-1α</th>
<th>HP2.5</th>
<th>HP5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>0</td>
<td>0.45**</td>
<td>0.00</td>
<td>0.60**</td>
<td>0.00</td>
</tr>
<tr>
<td>Pimo-FC</td>
<td></td>
<td>1</td>
<td>0.36**</td>
<td>0.00</td>
<td>0.13</td>
<td>0.31</td>
</tr>
<tr>
<td>CA9</td>
<td></td>
<td></td>
<td>1</td>
<td>0</td>
<td>0.42**</td>
<td>0.00</td>
</tr>
<tr>
<td>HIF-1α</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>0</td>
<td>-0.02</td>
</tr>
<tr>
<td>HP2.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>HP5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>
The linear correlations and the equations of lines of best fit between hypoxic fractions determined by the bioreductive drug and endogenous markers are depicted in Figure 18 (a, b and c). These results point in favour of using CA9 and HIF-1α as markers of hypoxia in human cervical carcinomas, because they are comparable to pimonidazole. Their ability to selectively mark the same tumour regions as pimonidazole signifies that they are both sensitive and selective markers of hypoxic cells. The non-zero y-intercepts of all the marker correlation graphs may indicate the presence of background levels of HIF-1α and CA9 in normoxic cells. Alternatively, this may merely reflect the higher oxygen concentration threshold for endogenous marker expression, compared to pimonidazole. As previously shown by Pearson’s correlation analysis, HP2.5 value from Eppendorf measurements did not correlate with any of the hypoxia markers (Figure 18d).
Figure 18. Numerical marker correlations. CA9 positive fraction plotted against pimonidazole positive fraction is shown in (a). The line of best fit is: CA9 = 0.9 Pimo + 2.5. HIF-1α positive fraction vs. pimonidazole positive fraction is shown in (b). The line of best fit is: HIF-1α = 0.4Pimo + 4.2. HIF-1α positive fraction plotted against CA9 positive fraction is shown in (c). The line of best fit is: HIF-1α = 0.4CA9 + 3.9. Box and whisker plot of fractions labeled by pimonidazole, CA9 and HIF-1α in sections of tumours that have below median HP2.5 (below 39.0%) vs. those that have above median HP2.5 is shown in (d). Inter-quartile ranges, median values and outliers are marked. There were no significant differences in the marker positive fractions between the groups below and above median HP2.5.
3.3. Spatial Correlations Between Pimonidazole, CA9 and HIF-1α in Sequential Tissue Sections

3.3.1. Semi-Quantitative Colocalization Analysis

In 72%-80% of the tumour sections, more than half of pimonidazole, CA9 and HIF-1α staining colocalized with at least one other marker (colocalization scores: 2-4). Nevertheless, the extent of marker colocalization was highly heterogeneous (Figure 19). The colocalization patterns observed provide support for the existence of chronic hypoxia, labeled by all markers, and acute hypoxia, labeled by one or two markers only.
Figure 19. Histograms of colocalization frequency between pairs of markers. Frequencies of colocalization scores between pimonidazole and CA9 (a), pimonidazole and HIF-1α (b) and CA9 and HIF-1α (c) are plotted.
3.3.2. Quantitative Colocalization Analyses

Colocalization calculations indicated that in the majority of labeled areas, all markers are present. As shown in Table 9 and Figure 19a, approximately 60%-70% of the stained areas were labeled by all markers, while the remainder was mostly labeled by a combination of any two markers. The smallest portion of the labeled area (approximately 5%-7%) contained one marker only.
Table 9. Comparison between two quantitative marker colocalization techniques. Calculated colocalization, and the verification method, measured colocalization are displayed, in addition to the possible explanations for the patterns of hypoxia markers observed.
Out of ten sections on which colocalization was measured, six exhibited high levels of at least two markers (marker positive fraction above median is considered high). Four out of these six sections had high levels of all markers, while two had low pimonidazole, but high CA9 and HIF-1α positive fractions. The remaining four sections had low levels of all markers. These measurements, similarly to the calculations, indicated that 65%-77% of the stained areas were labeled by all markers (Table 9). The colocalization fractions including pimonidazole, however, differed between the two methods. The global trends were similar in that both methods showed that the majority of the labeled tissue was labeled by all markers, while the rest was labeled by combinations of two markers, with a very small proportion labeled by one marker alone (Figure 20).
Figure 20. Distributions of staining colocalization patterns with respect to pimonidazole, CA9 and HIF-α staining. Calculated (a) and measured (b) colocalizations are shown.
3.3.3. The Relationship Between Colocalization and Fraction of Marker Positive Staining

For some of the colocalization parameters, there was discrepancy between the colocalizations derived from the theoretical calculations and direct measurement (Table 9). The difference in the average marker positive fraction in the measured subset and the entire group was investigated as a possible cause of colocalization variation. There was a positive correlation between the extent of pimonidazole and CA9 colocalization (visually scored) and positive fraction of both markers (Table 10). A similar trend was observed between the CA9 positive fraction and its colocalization with HIF-1α. In contrast, the fraction of HIF-1α positive staining did not correlate to its colocalization with other markers.
Table 10. Correlation between colocalization (calculated semi-quantitatively) of pairs of markers and marker positive fraction. Note that bolded letters with a symbol ** indicate a significant correlation (p-value is less than 0.05).

<table>
<thead>
<tr>
<th>Marker Positive Fraction</th>
<th>Colocalization Patterns</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pimo-CA9</td>
<td>Pimo-HIF-1α</td>
<td>CA9-HIF-1α</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pearson</td>
<td>p</td>
<td>Pearson</td>
<td>p</td>
</tr>
<tr>
<td>Pimo</td>
<td>0.35**</td>
<td>0.004</td>
<td>0.23</td>
<td>0.069</td>
</tr>
<tr>
<td>Pimo-FC</td>
<td>0.36**</td>
<td>0.004</td>
<td>0.20</td>
<td>0.109</td>
</tr>
<tr>
<td>CA9</td>
<td>0.28**</td>
<td>0.019</td>
<td>0.01</td>
<td>0.920</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>0.09</td>
<td>0.478</td>
<td>0.13</td>
<td>0.317</td>
</tr>
</tbody>
</table>
Generally, sections that had more staining exhibited greater marker colocalization (Figure 21a) and less staining by one marker alone (Figure 21b). This may be purely due to reasons of probabilistic nature: as the fractions of staining by two markers increase, so does the probability of their overlap.
Figure 21. Comparison between marker colocalization (measured quantitatively) and marker positive fraction. Fractions labeled by all markers, with respect to pimonidazole (P, C & H on P), CA9 (P, C & H on C) and HIF-1α (P, C & H on H) (a) and fractions of marker positive area labeled by one marker only (b).
3.4. Tumour Vasculature Labeled by CD31 in Relation to Hypoxia Markers

Previously, vascular density has been found to correlate inversely with hypoxia marker staining (90, 106). Similarly, in this study, vascular marker staining was inversely related to hypoxia markers evidenced by hypoxia marker localization predominantly at a distance from the vessels (Figure 22). However, despite the visual observations of an inverse relationship, a significant inverse association was not found between the fraction of the tumour section occupied by the vasculature and fraction positive for any of the hypoxia markers. This may be due to the presence of acute hypoxia, illustrated in Figure 2, and the heterogeneity in tumour cell oxygen consumption, illustrated in Figure 3. Analysis of CD31 and pimonidazole staining distribution in images of sequential sections indicated that in the majority of tumour tissue sections, the minimum distance of pimonidazole binding was 100μm to 200μm from the nearest blood vessel. The average distance between blood vessels and the nearest pimonidazole-stained region was approximately 130μm, confirming that pimonidazole was mostly associated with chronically hypoxic regions. Pimonidazole was rarely detected near vasculature, indicating that the fraction of acute hypoxia is very small, or that pimonidazole fails to detect all hypoxic regions.
Figure 22. Distribution of hypoxia markers in relation to vasculature. Images of sequential sections labeled by CD31 (a), CA9 (b) and HIF-1α (c) are shown. Note that CD31 is expressed in the centre of these nodules, but CA9 and HIF-1α staining are confined to the periphery.
3.5. Changes in HIF-1α Expression in Response to Radiotherapy in Recurring and Non-Recurring Patients

Due to the oxygen effect, radiation treatment of tumors should preferentially kill well-oxygenated cells. When this occurs, more oxygen is available to previously hypoxic cells. It has been shown that increased pre-treatment apoptosis in carcinoma of the cervix is related to larger increases in post-radiotherapy tumour oxygenation (150). One of the possible mechanisms for this, suggested by Sheridan and colleagues, is migration of surviving cells toward the blood vessel, whereby previously hypoxic cells replace the normoxic cell populations that are dying as a consequence of radiation-induced damage. It is therefore possible that the reduction in the HIF-1α expression upon irradiation may be indicative of the rates of cell death, reoxygenation and repopulation by previously hypoxic cells. Alternatively, an increase in HIF-1α may indicate that the radiotherapy resulted in damage to the blood vessels causing an increase in hypoxia. Consequently, the change in HIF-1α over the course of therapy may provide a useful measure of tumour response to radiotherapy, and hence additional information for predicting patient outcome following radiotherapy.

HIF-1α assessment over the course of therapy was performed on 1-4 biopsies of fifteen patients, who had not received pimonidazole, acquired from 2 days until 13 to 178 days after initiating therapy. The median time of last biopsy was 25.5 days after commencing radiation treatment (the mean was 39.3 days). Although no consistent pattern was observed in HIF-1α fluctuations among the patients (Figure 23a), in 11/15
patients, HIF-1α decreased within two weeks after the first radiotherapy treatment. In 6 out of these 11 patients, HIF-1α decreased by more than 2-fold. All of the patients in this cohort who progressed (5/15) experienced an initial decrease in HIF-1α levels during the first two weeks of treatment, followed by a consistent decline with respect to pre-treatment levels (Figure 23b).
Figure 23. HIF-1α change over the course of radiotherapy in 15 patients. Fractions of HIF-1α staining in biopsies obtained during therapy is shown in (a) and change with respect to pre-treatment HIF-1α levels for each patient is shown in (b). The patients who progressed are highlighted in pink (a) and in red (b).
3.6. The Prognostic Value of Tumour Hypoxia and Clinical Parameters

Previously, it has been shown that high hypoxic fraction, measured with hypoxia markers or Eppendorf electrodes, is strongly linked to poor treatment response. In this study, the prognostic significance of pimonidazole, CA9 and HIF-1α was investigated using Kaplan-Meier survival analysis. In both cohorts (entire set and homogenized subset of patients), correlations were observed between progression free-survival and marker positive fractions. The results are shown for the "homogenized patient cohort" only (Figures 24, 25, 26).

The groups of patients were stratified according to the level of hypoxia marker whose prognostic capacity was being evaluated. For each analysis, the patients were divided into those having marker levels above or equal to median value of the marker for the entire patient cohort, and those having marker levels below median value. Because a "biologically relevant" hypoxic fraction threshold value is not yet established, and hypoxia marker predictive capacities have been assessed in this manner previously (117), the median values were selected to be the subgroup inclusion criteria. For Eppendorf measurements, stratification was performed according to median values of HP5 (and HP25), as well as HP5 (or HP2.5) > 50%, which has been suggested to provide a useful prognostic threshold (64, 137). In the analysis of predictive capacity of combined markers, the cohort was again stratified into two groups: the patients having both markers equal to or above median value and the patients with all other combinations of marker values (with the exception of CA9, where below median levels of CA9 were combined.
with above or equal to median levels of HIF-1α and pimonidazole). Dichotomization was performed instead of stratification into multiple groups having different combinations of marker values for several reasons: the number of patients in the study was not sufficient to stratify the patients into more than two categories for combined marker studies, as this would reduce the statistical power of the analysis. Secondly, the patients having high levels of both pimonidazole and HIF-1α are more likely to contain aggressive hypoxic subpopulations, confirmed by the presence of both markers in a large fraction of the tissue. These patients are expected to respond to treatment to a lesser degree than those patients with a high fraction of staining by one of these two markers alone, or no markers.

Tumours with high levels of pimonidazole adducts exhibited a weak, non-significant, association with poor outcome (Figure 24a). Interestingly, high CA9 expression was associated (but insignificantly) with a high probability of progression free survival (Figure 24b). HIF-1α is the only marker, which, alone, inversely and significantly (p=0.024) correlated with progression-free survival (Figure 24c). The rates of 3-year progression-free survival with respect to different levels of hypoxia markers that predict patient outcome are shown in Table 11. Oxygen electrode measurements failed to exhibit prognostic capacity. When patients were divided into groups according to their HP5 and HP2.5 values (above or equal to median, or below median), the log-rank test indicated no correlation with outcome (p = 0.923 for HP5 and p = 0.619 for HP2.5). Similar results were obtained when stratification was performed according to the 50% threshold of HP5 and HP2.5 mentioned previously.
Figure 24. Cumulative progression-free survival of patients with respect to individual hypoxia markers. Marker positive fraction below median is represented by solid lines, and fraction equal to or above median is represented by dashed lines. The fraction of recurring patients divided by the number of patients in the subgroup is also noted (n). The markers are as follows: a. Pimonidazole (median = 4.9%), b. CA9 (median = 5.1%), c. HIF-1α (median = 4.8%).
Table 11. Rates of 3-year progression-free survival for different marker combinations. The patients were divided into groups with respect to marker values indicated below (4.8% for HIF-1α, 4.9% for pimonidazole and 5.1% for CA9). Mean progression-free survival and hazard ratios are also noted.

<table>
<thead>
<tr>
<th>Hypoxia Marker(s)</th>
<th>Rate of 3-Year PFS ± SE</th>
<th>Mean PFS (in days)</th>
<th>Hazard Ratio (row1:row2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients</td>
<td>73% ± 6%</td>
<td>1379</td>
<td></td>
</tr>
<tr>
<td><strong>HIF-1α</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥4.8%</td>
<td>57% ± 11%</td>
<td>1173</td>
<td>4.38</td>
</tr>
<tr>
<td>All other patients</td>
<td>88% ± 6%</td>
<td>1341</td>
<td></td>
</tr>
<tr>
<td><strong>Pimo and HIF-1α</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥4.9% and ≥4.8%</td>
<td>42% ± 17%</td>
<td>886</td>
<td>3.83</td>
</tr>
<tr>
<td>All other patients</td>
<td>79% ± 6%</td>
<td>1458</td>
<td></td>
</tr>
<tr>
<td><strong>CA9 and HIF-1α</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 5.1% and ≥4.8%</td>
<td>45% ± 15%</td>
<td>942</td>
<td>3.43</td>
</tr>
<tr>
<td>All other patients</td>
<td>80% ± 7%</td>
<td>1467</td>
<td></td>
</tr>
<tr>
<td><strong>Pimo, CA9 and HIF-1α</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥4.9%, &lt; 5.1% and ≥4.8%</td>
<td>33% ± 19%</td>
<td>536</td>
<td>5.00</td>
</tr>
<tr>
<td>All other patients</td>
<td>78% ± 7%</td>
<td>1446</td>
<td></td>
</tr>
</tbody>
</table>
When high levels of HIF-1α were combined with low CA9 or high pimonidazole, or both, there was a significant increase in the predictive capacity with respect to poor treatment outcome (Figure 25a-c).

In the analysis of tumours with a maximum diameter less than 5cm, pimonidazole reached borderline significance (p=0.088; Figure 26a) with respect to outcome. In the cohort of patients with large tumours (with maximum diameter equal to or greater than 5cm), however, pimonidazole was not predictive of outcome (Figure 26b). Similar trend of greater marker prognostic significance (computed using Kaplan-Meier analysis) in small tumours than in large ones was observed for HIF-1α. For this set of analyses, in which patients were stratified according to tumour size, the entire patient set was included in order to improve the statistical power of the analysis.
Figure 25. Cumulative progression-free survival of patients with respect to combined hypoxia markers. a. Pimonidazole (flow cytometry) and HIF-1α (solid line: Pimo < 5.0% or HIF-1α < 4.8%; dashed line: Pimo ≥ 5.0% and HIF-1α ≥ 4.8%), b. CA9 and HIF-1α (solid line: CA9 ≥ 5.1% or HIF-1α < 4.8%; dashed line: CA9 < 5.1% and HIF-1α ≥ 4.8%), c. Pimonidazole (flow cytometry), CA9 and HIF-1α (solid line: CA9 ≥ 5.1% or Pimo < 5.0% or HIF-1α < 4.8%; dashed line: CA9 < 5.1%, Pimo ≥ 5.0% and HIF-1α ≥ 4.8%).
Figure 26. Cumulative progression-free survival of patients with respect to pimonidazole in small (<5cm) and large (≥5cm) tumours. a. Pimonidazole in tumours whose maximum diameter is less than 5cm (median = 4.9%) and b. Pimonidazole in tumours whose maximum diameter is greater than or equal to 5cm (median = 4.9%). Pimonidazole positive fraction below median is represented by solid lines, and fraction equal to or above median is represented by dashed lines.
Although hypoxic fraction conveys important information about tumour biology and aggressiveness of tumour cells, it does not render a complete account of the tumour microenvironment (e.g., acute vs. chronic hypoxia) and characteristics of the tumour (e.g., mutations in key genes which may enhance or impede tumour progression in response to hypoxia). Clinical parameters were also assessed using log-rank statistical analysis on their ability to significantly predict outcome for this patient cohort. Maximum tumour diameter equal to or exceeding 5cm correlated with shorter progression-free survival (p = 0.027), as did positive nodal status (p = 0.048), while hemoglobin levels below 115g/L (p = 0.056) and increasing FIGO stages (p = 0.073) exhibited borderline significance in predicting shorter progression-free survival. Tumour grade failed to correlate with progression-free survival (p = 0.442).

To account for some of the known clinical parameters and combine this data with hypoxic fraction, four covariates: pimonidazole, HIF-1α, maximum tumour diameter and presenting hemoglobin were included in the Cox Regression analysis. The number of covariates was limited to four due to the size of the patient cohort, as advised by BC Cancer Agency statistician, Chuck Paltiel. These parameters were categorized (a value of 0 was given when the values of the parameter were less than median, and 1 was given when the values of the parameter were greater than or equal to median, with the exception of hemoglobin where 115g/L was used instead of median). Cox Regression was performed twice, using forward and backward likelihood ratio test based methods, which yielded the same results. HIF-1α and presenting hemoglobin were indicated as significant prognostic factors for progression-free survival (p = 0.021 and p = 0.023,
respectively).

Combining known clinical parameters with hypoxic status, could enhance the accuracy of predicting patient prognosis and, more importantly, contribute to the selection of optimal treatment for individual patients with respect to these variables.

3.7. Image Analysis Methods and Factors That Affect Their Accuracy

3.7.1. Automated vs. Manual Thresholding

Manual image analysis (by thresholding or visual scoring) has been utilized in the majority of the studies on hypoxia marker correlations on stained tissue sections. In addition to possible errors in the hypoxic fraction measurement due to the quality of staining, inaccuracies due to observer subjectivity are inevitable when this method is used. Therefore, there is a need to explore a more consistent approach, such as automated image analysis, as a potential future method. Automated thresholding based on Otsu algorithm and manual thresholding were applied to 10 images of pimonidazole stained tumour tissue sections. The correlation between these thresholding modalities was strong and significant, both in the number of highlighted pixels ($r^2 = 0.97$), and in the grayscale intensity threshold values selected by the observer and by Otsu algorithm ($r^2 = 0.46$) (Figure 27).
Figure 27. Correlation between automated and manual image analysis. Areas of stained sections obtained by Otsu algorithm and by manual thresholding (a). Threshold values (in grayscale intensity) selected by Otsu algorithm and by manual thresholding (b).
3.7.2. Histogram Analysis

The Gaussian distribution in the 8-bit grayscale interval between approximately 200 and 255 represented the distribution of the pixel intensity in the background of the image (i.e., the non-tissue portion of the image). The smaller normal distribution in the intensity range between 100 and 200 represented the shades of gray of the unstained tissue. Lastly, the pixels with grayscale values in the range of 0 to approximately 100, represented the stained portion of the tissue. A combination of factors, including the noise in the images, insufficient contrast between the stained and the unstained tissue and a small subpopulation of pixels representing the stained tissue, resulted in limited reliability of the stained tissue threshold values extracted from histograms (Figure 28). For this reason, the histogram analysis was not always feasible for analyzing the information from the images acquired in this study. Moreover, histogram analysis alone results in loss of information on hypoxia distribution. It follows that the use of histogram analysis alone to measure the hypoxic fraction is not sufficiently informative and is not always practical. Nonetheless, histogram analysis may be of benefit when performed in parallel with analysis of images that have acceptable contrast, reasonably low noise, and a sufficient hypoxic fraction, so that the hypoxic subpopulation can be distinguished in the histogram of the image.
Figure 28. Histogram of an 8-bit grayscale image of a tumour tissue section immunohistochemically stained for pimonidazole. Note that the stained tissue subpopulation (typically ranging from 0 to 100 in grayscale intensity) is indistinguishable from the unstained tissue subpopulation (here reaching approximately 230 in grayscale intensity).
3.7.3. The Effects of Noise, Contrast and Stain Distribution on Image Analysis

Reproducibility

3.7.3.1. The Effect of Noise on Image Analysis

From the histograms of the stained tumour images, it was observed that the range of grayscale intensities of tumour sections stained with eosin or hematoxylin is normally distributed over the 8-bit grayscale interval [0:255], but is typically between 100 and 200 intensity values (hematoxylin was generally represented by the darker intensity values than eosin). Possible reasons for differential absorption of hematoxylin or eosin stain by the tissue may be slight variations in the conditions during the preparation of the slides from day to day, the solution used to fix the tumour tissue, variation in the ionic strength of the staining solution from daily use and different strengths of solvents utilized after the staining. To test the effects of different levels of noise typically observed in images of real tumours, the standard deviation applied to the original tumour model image was varied from 0 to 45 in grayscale (in intervals of 10-15 shades of gray). Averages of three to six measurements of the hypoxic fraction (at each value of noise) were plotted against the corresponding values of noise. With increasing noise in the image, the three subpopulations in the resulting histograms progressively merged into one (Figure 29a) and the overestimate of the hypoxic fraction rapidly increased (Figure 29b).
Figure 29. The effect of noise on the histogram of the image (a) and image analysis (b). Measured hypoxic fraction in a tumour image model vs. standard deviation (noise in image) (b). The true hypoxic fraction is 5%. The errorbars represent standard error.
3.7.3.2. The Effect of Contrast on Image Analysis

The contrasts between the background, non-stained tissue, and stained tissue can considerably affect the accuracy of image analysis. In the majority of real images of tumour tissue sections, the boundary between tissue and background is distinct. On the contrary, there is large variation in the contrast between the non-stained and stained tissue. In order to determine the minimum tolerable contrast for reasonably accurate image analysis, the contrast between the stained and non-stained tissue was varied from 80 to 160 shades of gray (in intervals of 10-15 shades of gray), as shown in Figure 30. The averages of three to six hypoxic fraction measurements (at each value of contrast) were plotted against contrast. With decreasing contrast, the stained tissue subpopulation, visible in the histogram representing the contrast of 100 shades of gray disappeared, as shown in the histogram of contrast of 20 shades of gray (Figure 30a). In addition, there was a correlation between decrease in the contrast and decline in the accuracy of the hypoxic fraction measurement (Figure 30b). With decreasing contrast, the overestimate of hypoxic fraction increased, while at a contrasts of 120 and above, the hypoxic fraction measurement was close to the true hypoxic fraction.
Figure 30. The effect of contrast between the stained and unstained tissue on the histogram of the image (a) and image analysis (b). Measured hypoxic fraction in a tumour image model vs. standard deviation (b). The true hypoxic fraction is 5%. The errorbars represent standard error.
3.7.3.3. The Effect of Distribution of Stain on Image Analysis

The "distribution of stain" parameter was varied in tumour models by using the following configurations:

1) One stained area: 5% of the tumour area
2) Two stained areas: 4% and 1% of the tumour area
3) Three stained areas: 2 x 2% and 1% of the tumour area
4) Five stained areas: 5 x 1% of the tumour area

Varying the distribution of the staining on the tumour model did not significantly affect the hypoxic fraction measurement (Figure 31), indicating that the stain distribution does not significantly contribute to error in image analysis of a stained tumour section. Nevertheless, there were differences in the hypoxic fraction measurement between 1) and 4), indicating that a large number of small "stains" is more likely to lead to overestimating the overall stained fraction than one large stained area.
Figure 31. Measured hypoxic fraction in a tumour image model vs. distribution of stain. The true hypoxic fraction is 5%. The errorbars represent standard error.
4. DISCUSSION

4.1. How Can Numerical Correlations and Colocalization Patterns of Pimonidazole, CA9 and HIF-1α be Applied in the Clinical Setting?

The search for a practical and robust method for measuring tumour hypoxia in the clinic has been a major catalyst in the development of exogenous and endogenous hypoxia markers. Previous studies have shown correlations between various markers and other techniques used for measuring hypoxia. Nevertheless, some studies have elucidated the shortcomings and the differences between the methods. Possible causes for marker mismatch patterns may be the differences in marker sensitivity with respect to degree and duration of hypoxia and temporal changes in oxygenation (96, 125, 143, 175). Although critical analysis of marker mismatch patterns is essential because colocalization reflects the nature of hypoxia, to our knowledge, no previous study has quantified spatial correlations between the staining of three hypoxia markers.

4.1.1. Numerical Correlations Between Markers in Relation to Different Marker Kinetics

The pimonidazole positive fractions, obtained by either flow cytometry and image analysis, showed similar mean and median values and were significantly correlated. However, the correlation was not as strong as might be expected. The shortcomings of flow cytometry analysis are the possibility of cell loss during sample processing resulting
in inaccurate hypoxic fraction measurement, and lack of spatial information of hypoxia marker distribution. Among the techniques for hypoxic fraction assessment used in this study, the strongest correlation existed between pimonidazole and CA9, both in the fraction of hypoxia, and their spatial distribution (Figures 18 and 19). In agreement with the results presented here, other groups have reported a correlation between pimonidazole and CA9 in tumour sections of cervical and other carcinomas (3, 85, 96). Results from this study indicate that pimonidazole and CA9 both correlated numerically and spatially with HIF-1α (Figures 18b, 18c, 19b, 19c). In contrast, in a study of ten head and neck tumours, no numerical nor spatial correlation was found between HIF-1α and pimonidazole (90). The lack of correlation may be due to small patient cohort or to pixel-by-pixel matching of HIF-1α and pimonidazole stains on consecutive sections, leading to overestimated mismatched fraction, as was encountered in this study upon use of an automated image registration method. In a novel approach for quantifying tumour hypoxia using HIF-1α, introduced by Vordermark and Brown, flow cytometry analysis of HIF-1α expression correlated well with pimonidazole flow cytometry readings (167). Similarly, stable binding of a bio-reductive drug EF5, was also found to correlate with HIF-1α spatially and temporally (168).

The graph of CA9 versus pimonidazole positive fractions indicates a linear relationship with a slope of 0.9 (Figure 18a), which differs from the results obtained by Olive and colleagues, where the slope of this graph is 2. This was hypothesized to reflect the higher threshold for CA9 induction (2% oxygen) compared to pimonidazole binding (1% oxygen) (125). Extrapolation from the CA9 vs. pimonidazole graph indicated that
when there was no pimonidazole binding, CA9 was present in approximately 2.5% of the cells (Figure 18a). Although it is possible that this reflects different oxygen thresholds required for detection of the two markers, or the presence of background levels of CA9 in normoxic cells, this offset should be taken with caution, given the limited precision of our measurements. Notably, the CA9 staining performed in the Stanbridge laboratory, was of considerably higher intensity in the first series of slides, which facilitated the distinction between stained and unstained tissue in the first series compared to the second series.

While pimonidazole and HIF-1α labeling can be found in chronically and acutely hypoxic cells, CA9 expression may be associated mainly with chronically hypoxic cells and is indicative of the cell’s hypoxic history. It is possible that CA9 may be detected in cells that have undergone repeated exposures to transient hypoxia, during which CA9 accumulates, however this has not been experimentally validated. It has also been shown that pimonidazole weakly correlates with the oxygen diffusion-limited fraction (defined as the proportion of tumour tissue farther than 120μm from the nearest blood vessel) (90), providing evidence that pimonidazole is associated with chronic hypoxia. However, its ability to detect acute hypoxia should not be underestimated. The time-frame between pimonidazole administration and excision of the biopsy allowed for the formation of stable pimonidazole adducts in any cell that was hypoxic over the course of pimonidazole availability. Possible limiting factors to the specificity of pimonidazole-adduct binding to hypoxic cells is its association with areas of necrosis, differentiation- and keratinization, the latter caused by increased levels of nitroreductases in these cells (90, 91, 140).

HIF-1α stabilization can occur in response to both acute and chronic hypoxia and
its presence is an indicator of hypoxia immediately before biopsy. Although CA9 protein production ceases after normal oxygen levels are re-introduced to the cell, previously formed protein can remain for days. The extent of a cell’s response to acute hypoxia depends on the frequency and the magnitude of changes in oxygenation. Fluctuations can occur on the order of hours, in which case it is likely that a response similar to that of chronic hypoxia is sustained. In contrast, changes in oxygenation can occur every 4 to 5 minutes in SCCVII tumours (158) to 15 to 30 minutes (57, 101) in acutely hypoxic tissue of some murine tumours. For prolonged durations of acute hypoxia, it is questionable whether a molecular response to acute or chronic hypoxia is triggered. If the above assumptions about oxygenation changes and marker kinetics are taken into consideration, different patterns of hypoxia marker expression can provide valuable information about the temporal variations in oxygenation.

Pimonidazole, CA9, and HIF-1α positive fractions did not correlate with oxygen electrode readings (Figure 18d), although it has been observed previously that tumours with high oxygen partial pressure (pO₂) show a trend for decreased pimonidazole binding (121) and lower HIF-1α expression (73). The relationship between marker expression and oxygen electrode measurements is a complex one, as they do not always sample the same tumour microenvironment, and while the oxygen electrode measures the average oxygen partial pressure of up to several hundred cells, the marker staining provides a hypoxic profile on a single-cell level. The reports of a lack of correlation between HIF-1α and Eppendorf electrode measurements (117) are therefore not surprising.

In addition, the differences in distributions of hypoxia markers and oxygen
electrode measurements (exponential and normal distributions, respectively; Figure 15) may exist because the two measurements sample different parts of the tumour, with the markers providing information on a single-cell level, and the oxygen electrode measuring the average oxygen concentration throughout tumour tissue. Since the electrode measures oxygen partial pressure, values of a continuous variable, pO2, collected throughout the tumour are used to estimate the fraction of the tumour containing less than or equal to 2.5mmHg (or 5mmHg) of oxygen. Therefore, the oxygen electrode measurements provide information about the average oxygen partial pressure in different parts of the tumour, while the hypoxia markers detect the fraction of microenvironments contained within a small sample of a tumour having an oxygen concentration below a threshold value. In addition, electrode measurements may overestimate the hypoxic fraction by including areas of necrosis. Similarly, the hypoxic fraction of a necrotic tumour may be overestimated by increased sampling of necrotic tissue. While the electrode may have a sampling advantage in obtaining oxygenation status in different parts of the tumour, hypoxia markers are more sensitive indicators of hypoxia in tumour microenvironments, and are less likely to produce false positive results than the oxygen electrode measurements. It should be pointed out that none of these methods provides an indication of the proportion of viable hypoxic cells, although the dynamic nature of HIF-1α expression makes this marker a better indicator of this property.
4.1.2. Deciphering the Quantified Hypoxia Marker Staining Patterns

As expected, in the majority of tumour sections, pimonidazole, CA9, and HIF-1α localized to the same regions, nevertheless, areas of mismatch of the three stains were not infrequent (Figures 19 and 20; Table 9). Evidence from qualitative analysis supports the spatial correlation previously reported between CA9 and pimonidazole (175).

Regions marked by all three markers (64.7%-77.1%) are likely to be chronically hypoxic, as the duration of hypoxia was sufficient for CA9 expression, continuing HIF-1α stabilization, and pimonidazole accumulation. These data indicate that approximately 65%-77% of the tumour hypoxia is likely to be chronic, while the remaining 23%-35% might be considered transient (Table 9). These results are in agreement with the reported fraction of functional, well-perfused blood vessels in tumours (60%-70%) measured using Hoechst 33342 as a perfusion marker and CD31 antibody staining as a vascular marker (34). However, as the relationship between hypoxia marker binding and vasculature distribution and functionality was not specifically assessed on all tumour tissue sections, this result needs further support.

There are a number of possible explanations for variation in the marker co-staining patterns (summarized in Table 9):

1. Transient changes in oxygen concentrations in cells during the 18-20 hours prior to the excision of biopsy (55, 157, 158).
2. Different threshold oxygen concentration required for exogenous marker binding compared to HIF-1α or CA9 expression. Supporting evidence is provided by Vukovic and colleagues who showed that in cervical cancer xenografts, the HIF-1α signal peaks closer to the nearest blood vessel than the exogenous hypoxia marker EF5 (HIF-1α: 138μm vs. EF5: 204μm from the nearest vessel) (168). Olive et al. made a similar observation on the CA9 and pimonidazole-stained patient cervical tumor sections (125).

3. Upregulation of the HIF-1 pathway causing a constitutive expression of HIF-1α and CA9, regardless of oxygen concentration (175, 176).

4. Lack of pimonidazole drug in hypoxic cell layers farther from the vasculature due to its retention by other hypoxic cells closer to the blood vessels (not shown to present a problem in several studies) (5, 90, 124).

5. Increased pimonidazole levels in necrotic tissue, which has been observed previously (124). This may be due to shifting of cells, previously labeled with pimonidazole, into necrotic regions, which is driven by proliferation of normoxic cells closer to the vessel.

6. Decreased transcription of HIF-1α at large distances from the blood vessel due to a combination of oxygen, nutrient and growth factor deprivation. Pimonidazole, on the other hand, may still bind to cells in these regions because cells at very low glucose levels can still metabolize and bind pimonidazole (59, 172).

7. Differences in the kinetics of endogenous marker up-regulation, stabilization and loss, and binding and retention of the exogenous marker, driven by the changes in oxygen levels, as discussed above.
The staining technique used and comparison of sequential sections, rather than multiple labeling on a single section, may be additional contributing factors to greater marker mismatch. Although their effect has not been thoroughly studied, both sources of error are most likely insignificant when examining global patterns of marker staining. Of course, for accurate single cell colocalization studies, marker expression or binding must be examined on the same section. This method, however, is not practical on paraffin-embedded sections where different antigen retrieval methods often need to be optimized for each antigen. Similarly, the use peroxidase enzyme and substrate reaction for development of a stain does not allow the use of DAB, as this results in black precipitate, which would render the study of colocalization of two or more markers impossible due to the inability to assess colocalization of black stain with stain of any other colour.

4.1.3. Accuracy of Colocalization Calculations

Although most calculated colocalization estimates agreed well with the directly measured colocalized fractions of marker staining, occasionally, the calculations under- or overestimated the measured colocalized fractions (Table 9). To reduce the errors in colocalization equations, the colocalizations with respect to pimonidazole should be assigned a higher score, since both CA9 and HIF-1α usually cover all regions labeled by pimonidazole. For calculations of colocalized fractions with respect to the two endogenous markers, marker positive fraction should be higher, as the average tumour fractions labeled by CA9 or HIF-1α are typically slightly higher than that of pimonidazole.
4.2. Hypoxic Status in Biopsies Measured Using Pimonidazole, CA9 and HIF-1α: Potential for Identifying Patients That May Benefit from Hypoxia-targeting Therapies

When considering hypoxia markers as potential predictive tools of patient outcome following therapy, it is important to view hypoxia in the context of other factors known to impact patient response. Studying hypoxia markers in the context of key regulators of apoptosis and proliferation, as well as other clinical factors, is a more promising approach in yielding meaningful prognostic information. Other factors such as aneuploidy or S-phase fraction, which have also been shown to correlate with poor prognosis in cervical carcinomas (56, 170, 177), may be of great importance in patient outcome predictions.

4.2.1. HIF-1α Expression in Relation to Patient Outcome

Of the three hypoxia markers, only HIF-1α was found to be an independent prognostic factor of progression-free survival (Figure 24c). Its elevated expression may not only signify hypoxia, but also act an indicator of “aggressiveness” of cells, as it is known to be a potent factor in cell survival under harsh conditions. Other studies of outcome in relation to HIF-1α expression in cervical carcinomas support the results presented here (23, 32, 87). Furthermore, similar results have been reported for a number of cancer types. In breast cancer, increased expression of HIF-1α correlated with shorter
progression-free survival and overall survival, regardless of the nodal status (26, 32, 84,
146). Birner and colleagues showed that HIF-1α expression negatively correlated with
survival in oligodendrogliomas (20) and oligodendroglial neoplasms with chromosome
1p deficient tumours (21). In nasopharyngeal carcinoma (84) and squamous cell cancer of
the oropharynx (1), HIF-1α expression was inversely proportional to overall survival and
time to local failure, and disease-free survival times, respectively.

Conflicting evidence for the role of HIF-1α in cancer progression has been
presented over the years. A study on squamous cell carcinomas of the head and neck
reported a positive correlation between elevated HIF-1α expression and survival (12), yet
several reports have suggested that HIF-1α does not have prognostic ability in cancer of
the cervix (73, 117) and non-small cell lung cancer (65, 107). While it is important to
take into consideration all of the available outcome reports, it is necessary to examine the
possible reasons for lack of correlation, such as the level and the context of HIF-1α
expression observed, methods of scoring HIF-1α, administration of different adjuvant
therapies to patients that may influence the efficacy of the treatment, small patient cohort
in most studies and short follow-up times (some studies’ median follow-up times are less
than two years).

Fluctuations in HIF-1α in response to therapy were not found to be indicative of
patient outcome, although in all of the recurring patients, there was a decrease in HIF-1α
during the first two weeks of treatment consistent with reoxygenation. A study in a larger
cohort of patients, in which biopsies are regularly assessed for changes in HIF-1α
expression with respect to treatment, may elucidate more significant correlations. However, since pre-treatment HIF-1α status has prognostic value, the additional therapeutic benefit of measuring HIF-1α changes in response to therapy should be carefully validated.

4.2.2. CA9 Expression in Relation to Patient Outcome

CA9 expression was not a significant predictor of progression-free survival, although there is a trend for a positive correlation between increased CA9 expression and favourable outcome (Figure 24b). There is supporting evidence for this in the literature, although the precise role of CA9 in disease progression has yet to be clarified (31, 112). There is also considerable evidence that overexpression of CA9 predicts for poor outcome. Increased CA9 expression correlated with shorter relapse-free survival time in breast carcinomas (40) and was found to correlate with increased metastatic potential in cervix carcinomas (112), while it was associated with poor prognosis in non-small-cell lung cancer (152). To explain these findings, several roles of CA9 have been suggested, such as its function in loss of contact inhibition and anchorage independence, an important factor in tumour metastasis (134). Furthermore, the established role of CA9 in regulating the extracellular pH may contribute to optimization of growth conditions in the tumour microenvironment (134).

Some studies indicate that CA9 by itself fails to predict for overall and
progression free-survival, however in combination with HIF-1α, its predictive ability is enhanced (74, 84). The lack of correlation may be due to sampling error, the possibility of non-hypoxia specific CA9 expression, induced by other factors, or the inadequacy of CA9 in marking aggressive hypoxic subpopulations.

4.2.3. Pimonidazole Binding in Relation to Patient Outcome

In this study, pimonidazole binding failed to predict progression-free survival (Figure 24a). Although pimonidazole is expected to bind both chronically and acutely hypoxic cells, there is evidence that it is predominantly found in chronically hypoxic cells (35, 76). It has been argued, however that acute hypoxia is mainly responsible for enhancing tumour aggressiveness (35, 76). Therefore, the possibility that pimonidazole staining provides limited insight into acute hypoxia, which is a critical microenvironmental factor in tumour progression, could explain the fact that its link to outcome is weak.

Tumor size is known to be an important predictive factor in cancer of the cervix. The value of pimonidazole as a prognostic marker varied in tumours of different dimensions. In small tumours (< 5cm in maximum diameter), a large fraction of pimonidazole labeling predicted poor outcome with borderline significance, while in large tumours (≥5cm), no trend was observed (Figures 26a and 26b). It can be argued that in small tumours, there is considerable uncertainty with respect to the ability of cells
to adapt to the harsh microenvironment, and contribute to further tumour growth and metastasis, if no other parameters are known for these tumours. The more cells are hypoxic and viable, and hence labeled for pimonidazole, the more potential there may be for metastatic spread. Since tumour size is known to be a general predictor of progression-free survival in most tumour types, and large tumours exhibit a weaker response to therapy than small tumours, the hypoxic fraction in large tumours may no longer be a significant predictor of progression and treatment outcome. Furthermore, large tumours have already, in some capacity, demonstrated their ability to cope with nutrient and oxygen deprivation, and hence the level of pimonidazole binding in these tumours may be somewhat less relevant with regard to tumour progression.

4.2.4. Combination of Hypoxia Markers and Patient Outcome

When a combination of hypoxia markers was taken into account, the differences in the treatment outcome between the subgroups of patients were more profound. Remarkably shorter progression-free survival times were observed in patients that had equal to or above median levels of both HIF-1α and pimonidazole, compared to the rest of the patients (Figure 25a). An even more dramatic trend was observed when patients having pimonidazole and HIF-1α levels above or equal to median and CA9 below median, were compared to the rest of the patients (Figure 25c). These results provide evidence that tumors with more transient hypoxia, marked by the presence of pimonidazole and HIF-1α, and absence of CA9, are more aggressive.
All of the patients selected for outcome analysis were administered carbogen gas prior to radiotherapy, which has been shown to reduce the hypoxic fraction in tumours (135). It is possible that carbogen administration improved patient outcome and made hypoxia a less important predictive factor. Pre-treatment hypoxia measurements do not indicate which tumors will respond to carbogen breathing. However, as carbogen is thought to primarily reduce chronic hypoxia, it is expected that its administration may only alter the prognostic ability of CA9 and pimonidazole, but not HIF-1α. This could explain why only HIF-1α levels were predictive in this cohort.

Although Kaplan-Meier survival analyses revealed associations between hypoxia markers and progression-free survival, the statistical analyses should be interpreted with caution as it is unlikely that a single factor, such as hypoxia, determines the patient’s response to therapy. Even though this method shows statistically different probabilities of progression-free survival for two subgroups of patients segregated based on their hypoxia marker levels, it is still not possible to predict with confidence the prognosis of an individual patient and the response of a single tumour to therapy.

In this study, as well as in other similarly designed studies (85, 117), hypoxic fraction measured by three hypoxia markers did not correlate with known clinical prognostic factors (Table 5; Pearson correlations not shown). These factors, including (FIGO) stage, maximum clinical diameter, hemoglobin concentration and nodal status, correlated with survival independently of oxygenation status. Complementing the knowledge of clinical parameters with hypoxic status, may improve the accuracy of
predicting individual patient’s response to therapy and outcome.

4.3. Sampling Method Evaluation: Is a Section from a Biopsy an Adequate Representation of the Tumour with Respect to Hypoxic Status?

Variability in vascular architecture, density and perfusion throughout the tumour is the underlying cause of oxygen gradients and its heterogeneous distribution. Indeed, it was found that hypoxia, measured by pimonidazole, CA9 and HIF-1α, varied considerably within tumours (Figure 15). Similarly, Janssen and Nordsmark observed intra-tumour variation in pimonidazole and HIF-1α labeling (90), and pimonidazole and oxygen electrode (121), respectively. In contrast, Mayer et al. reported agreement between HIF-1α staining in different biopsies of the same tumour (117). To reduce sampling errors in hypoxic fraction assessment, some research groups advocate the use of multiple biopsies from different regions of the tumour (2, 3, 33). However, it has been shown that there is less intra-tumour than inter-tumour variability in oxygenation (27, 164). Furthermore, there is consistency in selecting the location of the tumour from which the biopsies are acquired as they all originate from the most accessible parts of tumours (they are all near the surface). As a result, comparison between hypoxic fractions in analogous pieces of tumours from different patients is a valid approach for comparing staining patterns of different markers. In order to improve the reliability of the sampling, larger biopsies can be acquired from the tumours, from which several sections on different parts of the biopsy are analyzed for hypoxic fraction and blood vessel distribution. Alternatively, as Airley and colleagues suggested (2), obtaining multiple
biopsies from different parts of the tumour may also provide more insight into the different tumour microenvironments and may encompass more variation in vessel distribution and function. This is obviously much more important when staining patterns are used to predict response to treatment.

4.4. Image Analysis Technique Improvements

4.4.1. Alternative Image Analysis Methods: Otsu Automated Thresholding and Histogram Analysis

Automated image thresholding using Otsu algorithm strongly correlated to manual image analysis for identifying pimonidazole staining (Figure 27). This is not surprising as pimonidazole staining was distinct from non-stained tissue, facilitating quantification of the labeled region. Quantitative histogram analysis was not feasible on the images acquired in this study due to the considerable noise, poor contrast and a low hypoxic ("stained") subpopulation of pixels, which did not allow for distinguishing subpopulations on the histograms (Figure 28). To improve the accuracy of image analysis, quality of images needs to be maximized by optimization of the staining protocol and contrast enhancement, and standardized, clearly defined scoring system must be implemented to reduce the variability in judgment when thresholding.

4.4.2. The Optimal Colocalization Analysis Method

The visual scoring method, used in combination with colocalization calculations
to yield proportions of co-staining patterns with respect to each marker, is a feasible and accurate method. It was verified by comparison to results of manual manipulation of images to yield marker colocalization fractions. The verification method, however, is impractical for routine use due to the labour-intensive nature of the technique, memory requirements in ImageJ and the time required for the analysis. Performing visual scoring with respect to each hypoxia marker, and using these scores as input to colocalization equations is an efficient, practical and reliable method for colocalization analysis that can be used on large sets of data. In addition, automated image registration was tested by registering images of sections stained for two different hypoxia markers. The accuracy of this method was compromised since the markers were localized to different subcellular compartments and the staining for different markers was performed on consecutive pieces of tissue, which were not identical.
5. CONCLUSIONS AND FUTURE AIMS

Staining of tumour tissue sections for pimonidazole, CA9 and HIF-1α indicated that these three hypoxia markers provide similar average hypoxic fractions and ranges in hypoxic fractions and have comparable staining patterns. In particular, pimonidazole and CA9 exhibited the highest correlation, confirming previously reported results. Areas stained by any one marker were very likely to be co-stained by both of the other markers, and only 1%-10% of the regions stained by one marker failed to be stained by the others. On average, pimonidazole staining alone occurred in only 0.9% of the regions stained by any one or a combination of the three markers, suggesting that the use of CA9 and HIF-1α can be considered a robust method for detecting regions of hypoxia. CA9 is a more stable marker than HIF-1α due to its longer half-life, and it can be considered more robust as it colocalizes with pimonidazole more frequently than HIF-1α. Nevertheless, the increase in CA9 in response to hypoxia is slow, and hence the use of this marker alone is not appropriate for measuring transient changes in tumour hypoxia. One conclusion from these results is that there is justification for using both HIF-1α and CA9 because they provide different and potentially complementary information on tumour hypoxia.

Despite the fact that in most pimonidazole positive regions, HIF-1α and CA9 were also expressed, independent correlations of hypoxic fractions with outcome suggested that these markers are not interchangeable. Based on their different oxygen dependencies, this is not surprising. High HIF-1α expression in pre-treatment biopsies
significantly correlated with shorter progression-free survival, while CA9 and pimonidazole had no predictive capacity. It should be taken into consideration, however, that the effect of hypoxia on outcome may have been underestimated in this study, as the administration of carbogen, together with chemoradiotherapy, likely contributed to the reduction of hypoxia and a high level of local control, respectively, achieved in patients. In addition to pre-treatment marker assessments, monitoring the changes in hypoxia and proliferation marker expression over the course of therapy, can be useful in quantifying the radiation response, growth and repopulation in a tumour. The significance of hypoxia marker fluctuations in response to therapy must be investigated further in the context of tumour biology and kinetics.

Sampling of tumour tissue must be performed in such a way to ensure that the measurements obtained from the sample are representative of the entire tumour. One way of achieving this may be acquisition of larger biopsies, and sampling from more than one region of the biopsy. Alternatively, obtaining several biopsies from different parts of the tumour may result in a more representative hypoxic fraction measurement. Although the issue of heterogeneity has been explored extensively in the past, no consensus has been reached yet on the optimal sampling method.

Manual image analysis for assessing hypoxic fraction and visual scoring for assessing colocalization are robust methods that are feasible for routine use in the clinic. Since the validity of the analysis depends on the quality of the images, it is necessary that the immunohistochemical staining produces a distinct signal that is easily distinguishable
from non-stained tissue. Moreover, the quality of images should be improved by optimizing the contrasts (tissue-background and stained tissue-unstained tissue contrasts) and brightness of the tissue in the image in an image processing software such as ImageJ. To ensure the reproducibility of the method, clearly defined, standardized method for image analysis should be implemented.

Measurement of the pre-treatment hypoxic status of a tumour may be used to guide planning of therapy which will target hypoxic cells. Complementing hypoxic status information with other parameters, such as the ability of tumour cells to repair DNA damage or their proliferative capacity should also improve prognostic capabilities of the pre-treatment assessment and may aid the selection of optimal therapy for the patient. In addition, these results suggested that the significance of hypoxic fraction may vary with respect to other clinicopathologic factors. In large tumours, pimonidazole positive fraction did not predict for progression-free survival, however in small tumors it was a significant predictor. The context-dependent relevance of hypoxia should also be considered when selecting the patients who may benefit from tumour hypoxia assessment.

In the future, hypoxic status information, integrated with other clinical parameters and gene expression results, may be used in an algorithm for decision making with respect to therapy planning and modification, and patient prognosis. Before this is done, optimization of sampling methods, scoring systems for assessing hypoxia, and image analysis procedures must be achieved, so that standard protocols can be followed in the
clinical setting. Kinetics of hypoxia markers must also be further characterized to allow inference of temporal variations and degree of hypoxia on the basis of marker mismatch patterns.
REFERENCES


54. Dunst, J. The Use of Epoetin Alfa to Increase and Maintain Hemoglobin Levels


85. Hutchison GJ, V. H., Loncaster JA, Davidson SE, Hunter RD, Roberts SA, Harris AL, Stratford IJ, Price PM, West CML Hypoxia-Inducible Factor 1alpha Expression as an Intrinsic Marker of Hypoxia: Correlation with Tumor Oxygen, Pimonidazole Measurements, and Outcome in Locally Advanced Carcinoma of...


98. Kaluzova, M., Kaluz, S., Lerman, M. I., and Stanbridge, E. J. DNA Damage Is a


122. Nordsmark M, O. M., Overgaard J Pre-Treatment Oxygenation Status Predicts Radiation Response in Advanced Squamous Cell Carcinoma of the Head and


153. Tanimoto K, M. Y., Pereira T, Poellinger L Mechanism of Regulation of the


