HYPOXIA AND ANGIOGENESIS IN RENAL CELL CARCINOMA

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Author’s Declaration

This thesis contains no material which has been accepted for any other degree in any university. To the best of my knowledge and belief, this thesis contains no material previously published or written by any other person, except where due reference is given in the text.

Signature:

........................................

Nathan Leo Lawrentschuk
Abstract

Hypoxia is one of the hallmarks of cancer. It was first postulated to occur in solid tumours by Thomlinson and Gray in 1955.\textsuperscript{1} The presence of hypoxia has been demonstrated in different types of solid tumours.\textsuperscript{2} Intratumoral hypoxia is caused by the lack of functional blood vessels in proliferating tumour tissue, resulting in low intratumoral oxygen concentrations. If hypoxia is severe or prolonged, cell death occurs.\textsuperscript{3} Malignant cells can undergo genetic and adaptive changes that allow them to escape from dying of oxygen deprivation. These changes are associated with a more aggressive malignant phenotype\textsuperscript{4,5} conferring resistance to radiation\textsuperscript{6,7} and chemotherapeutic agents.\textsuperscript{3,8,9} Hence hypoxia is known to be a key factor responsible for tumour resistance in humans.

Invasive polarographic oxygen sensor measurements have demonstrated hypoxia in solid tumours and it is generally defined to occur at an oxygen tension less than ten mmHg.\textsuperscript{10} Perhaps of more importance is that hypoxia has been demonstrated to be a prognostic indicator for local control after treatment with radiotherapy in glioma, head and neck and cervical cancers.\textsuperscript{11-13} It has also been able to predict for survival and the presence of distant metastases in soft tissue sarcomas.\textsuperscript{14} Finally, the significance of hypoxia in the activation and induction of functional molecules such as hypoxia inducible factors (HIFs) and VEGF, the modulation of gene expression (e.g. carbonic anhydrase IX), increased proto-oncogene levels, activation of nuclear factors and accumulation of other proteins (e.g. TP53) although progressing, is yet to be defined.\textsuperscript{15,16}

Thus, it is of clinical interest to understand the levels of hypoxia and numbers of hypoxic cell populations in tumours, particularly those resistant to radiation and chemotherapy. In doing so clinicians and researchers may formulate more accurate prognostic information and develop treatments targeting hypoxic cells. Renal cell carcinoma (RCC) is a tumour resistant to radiation and chemotherapy that is yet to have its oxygen status investigated.
Although the “gold standard” of oxygen tension measurement is the Polarographic Oxygen Sensor (POS or Eppendorf pO₂ histograph), non-invasive means of measuring oxygen status via imaging, immunohistochemistry or serum tumour markers are more practical. As highlighted by Menon and Fraker, it is imperative that reliable, globally usable, and technically simplistic methods be developed to yield a consistent, comprehensive, and reliable profile of tumour oxygenation. Until newer more reliable techniques are developed, existing independent techniques or appropriate combinations of techniques should be optimized and validated using known endpoints in tumour oxygenation status and/or treatment outcomes.¹⁷

Hanahan and Weinberg¹⁸ surmised that the field of cancer research has largely been guided by a reductionist focus on cancer cells and the genes within them— a focus that has produced an extraordinary body of knowledge. Looking forward in time, they believe that progress in cancer research would come from regarding tumours as complex tissues in which mutant cancer cells have conscripted and subverted normal cell types (endothelial cells, immune cells, fibroblasts) to serve as active collaborators in their neoplastic agenda. The interactions between the genetically altered malignant cells and these supporting coconspirators will prove critical to understanding cancer pathogenesis and to the development of novel, effective therapies.¹⁸

Essentially, the background outlined here not only highlights the core aim of this thesis: to better understand the oxygen status of renal cell carcinoma and the relationship of this to angiogenesis so that better targeted therapies may be pursued in the future; but it also places this research in the context of the future proposed by Hanahan and Weinberg,¹⁸ by clearly focusing on collaborators in the neoplastic agenda, rather than just tumour cells themselves, to better understand RCC.
Acknowledgements

Firstly I would like to acknowledge the tremendous help and guidance and support of my three supervisors Associate Professors Damien Bolton, Ian Davis and Andrew Scott. Firstly, Damien provided the inspiration that led to me undertaking research within the Austin Hospital at the University of Melbourne. In retrospect, although a chance meeting, it was one that has had a major impact on my life and for this I thank him. He is a true surgeon scientist and gracious mentor. Ian has been an intellectual pillar and extremely supportive of all the work in this thesis. He has been instrumental in directing and crafting the body of work presented and his clinical experience, scientific knowledge and acumen combined with an ability to see the larger picture is a talent I can only hope to master. Finally, Andrew, who allowed me complete and full access to the PET centre and the Ludwig Institute for Cancer Research, Tumour Targeting Laboratory, Austin Hospital, Heidelberg, Victoria, Australia. He quietly pushed me towards greater heights and has maintained an infectious enthusiasm to discover. Without sharing his vision, none of the thesis would be possible.

I would also like to acknowledge the spiritual guidance and friendship of Mr Ray Swann whose confidence, faith and intellect inspired me. The assistance and expertise of Dr Carmel Murone with immunohistochemistry Dr Dongmao Wang, Angela Rigopolous, Angela Mountain and Violeta Spirkoska from the Ludwig Institute for Cancer Research, Tumour Targeting Laboratory were all invaluable. To Kath Lewis, Andrew Scott’s wonder assistant and organiser, I thank you. Also, the staff in the PET Centre, Operating Suite, Austin Library and the University of Melbourne, Department of Surgery, Austin Hospital, who gave me full access and advice where necessary. The University of Melbourne, Faculty of Medicine provided exceptional support through the Melville Hughes Scholarship and also the Australasian Urological Foundation, which allowed me to pursue this body of work. The Royal Australasian College of Surgeons was also extremely supportive via a scholarship, as was the Austin Hospital Urology Unit. The urologists on the unit also ultimately let me complete the clinical work, and for this I am grateful.
Dedication

This thesis is dedicated to my family. Firstly, to my beautiful wife Janelle, who gave me the support, love and space required to complete this journey. Our wonderful children Angus and Hugo, also provided love and perspective and the necessary distractions that allowed me to refocus on what is important in life.

To my parents Leon and Judy, who have lovingly supported everything I have ever done. Leon I know understands the dedication required, as he completed his Masters at the University of Melbourne some years ago. I should never forget it is he who inspired me to ask questions and seek answers. My brother Luke and sister Sabina have also always supported my endeavours.

Finally, to Dr Peter Hewitt- a wonderful man, beyond inspirational and a true scientist. He taught me to think like a researcher and to act like a surgeon scientist- a gift I cannot thank him enough for giving. His friendship has been invaluable and I only wish he were here to read this thesis in person......
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COMMUNICATIONS PUBLISHED DURING THE RESEARCH PERIOD OF THIS
THESIS (ARISING DIRECTLY FROM THIS THESIS)


6. Lawrentschuk N, Davis ID, Scott AM, Bolton DM. Positron Emission Tomography (PET), Immuno-PET and Radioimmunotherapy in Renal Cell Cancer: an evolving


**PUBLISHED ABSTRACTS (International Presentations)**


**OTHER PRESENTED ABSTRACTS (National, State and Local Presentations)**


AWARDS ARISING FROM WORK IN THIS THESIS

2006
1. The John Blandy Prize: Award of the British Association of Urological Surgeons and the British Journal of Urology International; Awarded for the best manuscripts published in the previous year by a resident in Urology from anywhere in the world
2. Urological Society of Australasia Annual Scientific Meeting:
   a) Winner Villis Marshall Award- Prize for Best Research Paper
   b) Recipient Karl Storz Travelling Fellowship for Registrars
3. Urological Society of Australasia Australian and New Zealand Travelling Fellowship- Award for presenting papers at the American Urological Association Annual Meeting in 2006

2005
1. University of Melbourne- Melville Hughes Scholarship
2. National Health and Medical Research Council of Australia [NHMRC] Postdoctoral Research Scholarship- Awarded but funding declined due to acceptance of Melville Hughes Scholarship
3. University of Melbourne- Melbourne Research Scholarship (Awarded but declined due to Melville Hughes restrictions)
4. Royal Australasian College of Surgeons- Surgeon Scientist Scholarship (Awarded but declined due to Melville Hughes restrictions)
6. University of Melbourne, Austin Research Prize in Surgery and Anaesthesia

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1. Urological Society of Australasia Annual Scientific Meeting:
   a) Winner Villis Marshall Award- Prize for Best Research Paper
   b) Recipient Karl Storz Travelling Fellowship for Registrars
4. University of Melbourne, Austin Research Prize in Surgery and Anaesthesia
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COMMUNICATIONS PUBLISHED DURING THE RESEARCH PERIOD OF THIS THESIS (ARISING INDIRECTLY FROM THIS THESIS)

Related to Renal Cell Carcinoma, Tumour Hypoxia and Positron Emission Tomography


**Related to other areas of Urology**


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<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>avidin biotin complex</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BHD</td>
<td>Birt-Hogg-Dubé gene</td>
</tr>
<tr>
<td>CAIX</td>
<td>Carbonic anhydrase IX (refers to the protein=G250 antigen)</td>
</tr>
<tr>
<td>CA9</td>
<td>Carbonic anhydrase 9 (refers to the gene for CAIX).</td>
</tr>
<tr>
<td>c-Met</td>
<td>Proto-oncogene</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
</tr>
<tr>
<td>cG250</td>
<td>Monoclonal antibody to carbonic anhydrase IX</td>
</tr>
<tr>
<td>CD34</td>
<td>Endothelial cell surface antigen</td>
</tr>
<tr>
<td>CD31</td>
<td>Endothelial cell surface antigen</td>
</tr>
<tr>
<td>CD44</td>
<td>Endothelial cell surface antigen</td>
</tr>
<tr>
<td>DUS</td>
<td>Doppler Ultrasound;</td>
</tr>
<tr>
<td>DLS</td>
<td>Diffuse Light Spectroscopy</td>
</tr>
<tr>
<td>EAU</td>
<td>European Association of Urology</td>
</tr>
<tr>
<td>ECOG</td>
<td>European Operative Oncology Group</td>
</tr>
<tr>
<td>EF5</td>
<td>5 (2-[2-nitro-1H-imidazol-1-yl]- N-(2,2,3,3,3-pentafluoropropyl) acetamide) [a nitromidazole]</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>von Willebrand factor [endothelial cell surface antigen]</td>
</tr>
<tr>
<td>FH</td>
<td>fumarate hydratase gene</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------------------</td>
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<tr>
<td>FIH-1</td>
<td>Factor inhibiting HIF-1</td>
</tr>
<tr>
<td>$^{18}$F-FAZA</td>
<td>$^{18}$F-fluoroazomycin arabinoside</td>
</tr>
<tr>
<td>$^{18}$F-FDG</td>
<td>$^{18}$F-fluorodeoxyglucose</td>
</tr>
<tr>
<td>$^{18}$F-FMISO</td>
<td>$^{18}$F-Fluoromisonidazole</td>
</tr>
<tr>
<td>FOV</td>
<td>Field of view</td>
</tr>
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<td>Glut-1 (GLUT-1)</td>
<td>Glucose transporter 1</td>
</tr>
<tr>
<td>G250 antigen</td>
<td>Carbonic anhydrase IX</td>
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<tr>
<td>H&amp;E</td>
<td>haematoxylin and eosin stain</td>
</tr>
<tr>
<td>HER-2</td>
<td>(ErbB-2/Neu) types of tyrosine kinase receptor</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia Inducible Factor</td>
</tr>
<tr>
<td>HIF-1$\alpha$</td>
<td>Hypoxia Inducible factor-1$\alpha$</td>
</tr>
<tr>
<td>HIF-1$\beta$</td>
<td>Hypoxia Inducible factor-1$\beta$</td>
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<tr>
<td>HLRC</td>
<td>Hereditary leiomyomatosis renal cell carcinoma</td>
</tr>
<tr>
<td>HP</td>
<td>Hypoxic Fraction</td>
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<tr>
<td>HPRC</td>
<td>Hereditary papillary renal carcinoma</td>
</tr>
<tr>
<td>HREs</td>
<td>Hypoxia-responsive elements</td>
</tr>
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<td>IGF-2</td>
<td>Insulin-like Growth Factor-2</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin 2 [a cytokine]</td>
</tr>
<tr>
<td>$\beta$-D-IAZGP</td>
<td>$\beta$-D-Iodinated azomycin galactopyranoside</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Nuclear protein/antigen</td>
</tr>
<tr>
<td>LSAB</td>
<td>a streptavidin-biotin method for immunohistochemistry</td>
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<tr>
<td>LE</td>
<td>radiolabelling efficiency</td>
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5
<table>
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<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>mAb</td>
<td>Monoclonal antibody(ies)</td>
</tr>
<tr>
<td>MBq</td>
<td>megabecquerels - measure of radioactivity</td>
</tr>
<tr>
<td>mCi</td>
<td>millicurie - measure of radioactivity</td>
</tr>
<tr>
<td></td>
<td>(1 millicurie = 37 megabecquerels (MBq))</td>
</tr>
<tr>
<td>MIB-1</td>
<td>Monoclonal antibody binding ki-67</td>
</tr>
<tr>
<td>MN75</td>
<td>Antibody against CAIX</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MSKCC</td>
<td>Memorial Sloan-Kettering Cancer Centre</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>MVD</td>
<td>Microvessel density</td>
</tr>
<tr>
<td>NCCN</td>
<td>National Comprehensive Cancer Network</td>
</tr>
<tr>
<td>Oxylite Probe</td>
<td>Time-resolved luminescence-based optical sensor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer solution</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>PET-CT</td>
<td>Combined Positron Emission Tomography with Computed Tomography</td>
</tr>
<tr>
<td>pO₂</td>
<td>Oxygen partial pressure</td>
</tr>
<tr>
<td>POS</td>
<td>Polarographic Oxygen Sensor (Eppendorf pO₂ Histograph)</td>
</tr>
<tr>
<td>PNET</td>
<td>Primitive neuro-ectodermal tumours</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate-specific antigen</td>
</tr>
<tr>
<td>RECIST</td>
<td>Response Evaluation Criteria in Solid Tumours</td>
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</table>
RCC Renal cell carcinoma
mRNA messenger ribonucleic acid
ROI regions of interest
RTK Tyrosine kinase receptor
RT-PCR Reverse transcription-polymerase chain reaction
SEER Surveillance, Epidemiology and End Results (database USA)
SEM Standard error of the mean
SD Standard deviation
TCC Transitional cell carcinoma
TFE3 Transcription factor E3
TGFα Transforming growth factor alpha
TK Tyrosine kinase
TKI Tyrosine kinase inhibitors
TNF-α Tumour necrosis factor alpha
TNM Tumour, Node, Metastasis (Staging of Tumours)
TP53 Tumour protein 53
VDA Vascular disrupting agents
VEGF Vascular Endothelial Growth Factor
( also known as vascular permeability factor or VPF)
VEGFR Vascular endothelial growth factor receptor
VHL von Hippel-Lindau
VHL von Hippel-Lindau gene
pVHL von Hippel-Lindau tumour suppressor protein
WHO World Health Organisation
wt wild type
# Chapter 1

## INTRODUCTION

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1.1 Introduction
The incidence of Renal Cell Carcinoma (RCC) is increasing for all stages of the disease. Treatment options for locally advanced, recurrent and metastatic disease are few and complete responses to systemic therapy are rare. RCC is continuing to present a challenge to urologists as 20-25% of patients will have metastatic disease at diagnosis and approximately 50% will ultimately develop metastatic disease. The therapy of choice for organ-confined RCC is surgery. The 5-year survival rate is 60%; the prognosis for patients with recurrent or metastatic disease is often bleak, the 2-year survival between 0–20%. Metastatic RCC remains one of the most treatment-resistant malignancies.

A greater understanding of the basic biology of RCC will lead to new treatment options, as has occurred with the recently developed tyrosine kinase inhibitors for systemic treatment of RCC. These have spurred an interest in hypoxia and angiogenesis in RCC, the very basis of this thesis. Furthermore, more accurate and reliable methods of diagnosing, staging and monitoring for response to treatment in RCC are needed using functional and not just anatomical imaging modalities. As such, hypoxia, angiogenesis and functional imaging of RCC with PET-CT are the major themes of this thesis.

1.2 Background to the research
The concept of Molecular Target Credentialing in oncology has been advocated by the National Cancer Institute of the USA and involves cancer imaging, cancer signatures and molecular targets of prevention and treatment. Hypoxia has been identified as a model for studying the interrelationship of these three areas. This thesis is timely for almost every aspect of this credentialing has been addressed.

Hypoxia is one of the hallmarks of cancer. It was first postulated to occur in solid tumours by Thomlinson and Gray in 1955. The presence of hypoxia has been demonstrated in different types of solid tumours. Intratumoral hypoxia is caused by the lack of functional
blood vessels in proliferating tumour tissue, resulting in low intratumoral oxygen concentrations. If hypoxia is severe or prolonged, cell death occurs. Malignant cells can undergo genetic and adaptive changes that allow them to escape from dying of oxygen deprivation. These changes are associated with a more aggressive malignant phenotype conferring resistance to radiation and chemotherapeutic agents. Hence, hypoxia is known to be a key factor responsible for tumour resistance in humans.

Although the “gold standard” of oxygen tension measurement is the invasive Polarographic Oxygen Sensor (POS or Eppendorf pO2 Histograph), non-invasive means of measuring oxygen status via imaging, immunohistochemistry or serum tumour markers are more practical. As highlighted by Menon and Fraker, it is imperative that reliable, globally usable, and technically simplistic methods be developed to yield a consistent, comprehensive, and reliable profile of tumour oxygenation. Until newer more reliable techniques are developed, existing independent techniques or appropriate combinations of techniques should be optimized and validated using known endpoints in tumour oxygenation status and/or treatment outcomes.

Invasive polarographic oxygen sensor measurements have demonstrated hypoxia in solid tumours and it is generally defined to occur at an oxygen tension less than ten mmHg. Perhaps of more importance is that hypoxia has been demonstrated to be a prognostic indicator for local control after treatment with radiotherapy in glioma, head and neck and cervical cancers. Hypoxia has also been able to predict for survival and the presence of distant metastases in soft tissue sarcomas. Finally, the significance of hypoxia in the activation and induction of functional molecules such as hypoxia inducible factors (HIFs) and Vascular Endothelial Growth Factor (VEGF), the modulation of gene expression (e.g. carbonic anhydrase IX), increased proto-oncogene levels, activation of nuclear factors and accumulation of other proteins (e.g. TP53) although progressing, is yet to be defined.
Thus, it is of clinical interest to understand the levels of hypoxia and numbers of hypoxic cell populations in tumours, particularly those resistant to radiation and chemotherapy. In doing so researchers may formulate more accurate prognostic information and develop treatments targeting hypoxic cells. RCC is a tumour that is resistant to radiation and chemotherapy and is yet to have its oxygen status fully investigated.

Functional imaging, particularly with combined positron emission tomography-computed tomography (PET-CT), may improve accuracy and provide critical information that has been unavailable to date in recognising hypoxia. This is against a background where treatments for metastatic RCC using targeted therapies in the past few years have entered clinical practice, further highlighting the importance of accurate imaging for patient selection and monitoring response to treatment.

Hanahan and Weinberg\textsuperscript{18} surmised that the field of cancer research has largely been guided by a reductionist focus on cancer cells and the genes within them; a focus that has produced an extraordinary body of knowledge. Looking forward in time, they believe that progress in cancer research will come from regarding tumours as complex tissues in which mutant cancer cells have conscripted and subverted normal cell types (endothelial cells, immune cells, fibroblasts) to serve as active collaborators in their neoplastic agenda. The interactions between the genetically altered malignant cells and these supporting coconspirators will prove critical to understanding cancer pathogenesis and to the development of novel, effective therapies.\textsuperscript{18}

Essentially, the background outlined here highlights the core aim of this thesis: to better understand the oxygen status of RCC and the relationship of this to angiogenesis so that better therapies may be pursued and used clinically in the future. It also places this research in the context of the future proposed by Hanahan and Weinberg,\textsuperscript{18} by clearly focusing on collaborators in the neoplastic process, rather than just tumour cells themselves, to better understand RCC.
1.3 Research problem and hypotheses

The overall aim of this thesis is to evaluate the relationship between tumour hypoxia and angiogenesis in patients with renal cell carcinoma (RCC). Hypoxia is known to be an important stimulating factor for neovascularisation and this may correlate with defined markers of angiogenesis in some cancers. However, this relationship has not been studied in RCC. Eppendorf pO2 histography is an invasive means of measuring tissue pO2 in experimental animals and humans and will be undertaken on both to determine if renal cell tumours are relatively hypoxic. 18F-Fluoromisonidazole (18F-FMISO) PET scanning has now been characterised and validated as a marker of hypoxia. Initial studies in stroke and glioma in our institution have confirmed its ability to detect viable but hypoxic tissues. This is a non-invasive means of measuring tissue pO2 that may be compared with the invasive Eppendorf readings.

The hypotheses of this thesis are as follows:

1. RCC are relatively hypoxic, particularly in the periphery of the tumour, where viable hypoxic cells are most likely to reside.
2. In RCC a relationship exists between hypoxia and 18F-FMISO uptake. 18F-FDG uptake may be related to hypoxia in RCC because HIF-1 activates transcription of genes including glucose transporters and glycolytic enzymes, a feature of malignant cells that is the basis of 18F-FDG uptake.
3. A correlation exists between the presence of hypoxia and angiogenesis in RCC.

The aims of this thesis are therefore as follows:

1. To evaluate in vivo intratumoral hypoxia in patients with RCC who are planned for resection by means of intra-operative pO2 histography and preoperative PET studies. This will correlate with a specific pattern of oxygen readings obtained by Eppendorf pO2 histography and PET uptake of radioisotopes within the tumours.
2. To evaluate RCC resection specimens by immunohistochemical analysis of angiogenic markers and by molecular studies where appropriate and to correlate these observations with the intra-operative pO$_2$ histography and preoperative $^{18}$F-FMISO PET scans. Also, to evaluate another surrogate marker of hypoxia, serum osteopontin, as a potential indicator of tumour hypoxia by correlating it with oxygen readings by correlating levels with intra-operative pO$_2$ histography.

3. To use a human RCC tumour line with known defective VHL gene products in an animal model to undertake confirmation of the above three hypotheses using pO$_2$ histography, PET studies and immunohistochemistry.

**1.4 Justification for the research**

Significant advances have been made in the diagnosis, staging and treatment of RCC improving overall survival in select groups of patients and an overall change in the natural history of the disease. However, until the recent development of tyrosine kinase inhibitors (TKI), biologic and immune based treatments (e.g. Interleukin 2) had response rates for patients with metastatic disease around only 10-20%. Despite the development of TKI (discussed later) and related agents, a need remains for the development of therapies to extend the life by years, and not months, in patients with metastatic or high risk, locally advanced RCC. RCC continues to be a hallmark disease for the testing of such new immunologic approaches. A more comprehensive understanding of the basic biology of RCC will help more agents to be developed.

It has been stated that until newer more reliable techniques for measuring tumour hypoxia are developed, existing independent techniques or appropriate combinations of techniques should be optimised and validated using known endpoints in tumour oxygenation status and/or treatment outcomes. Thus, data on hypoxia and angiogenesis, need to be obtained from
both patients with RCC and animal models using different techniques. Only then will our understanding of the amount, extent and implications of hypoxia in RCC be elucidated.

1.5 Methodology
The data are collected from (humans and mice) in this study will fall into the groups of:

- Intra-operative measurements of oxygen tension within the tumour (RCC) and normal kidney tissue and experimental measurements in animal models
- \(^{18}\)F-FMISO PET study results will be analysed and compared to polarographic oxygen sensor measurements (Eppendorf pO\textsubscript{2} histogram)
- Immunohistochemistry of tumour tissue looking for VEGF expression, microvessel density, proliferative index (Ki-67), G250 expression and HIF-1\(\alpha\). Serum osteopontin will also be explored as a marker of tumour hypoxia.

1.6 Outline of the thesis
Chapter two is an extensive literature review covering hypoxia, angiogenesis and imaging of RCC. Chapter three covers the methodology in human and animal studies whilst chapter four is a report and analysis of human data and chapter five xenograft data. Chapter six covers conclusions and implications of the research in this thesis.

1.7 Definitions
Key words that will be used in this thesis are hypoxia and angiogenesis, both relating to tumours. They will be defined here to maintain precision throughout the thesis.
The median value for oxygen partial pressure is between 40 and 60 mmHg in normal subcutaneous tissue. A ‘typical’ tumour may have a median oxygen partial pressure of approximately 10mmHg. By definition, half the cells have oxygen levels less than 10mmHg and half have oxygen levels greater than 10mmHg. This breakpoint is significant, as cells
with oxygen levels of less than 10mmHg start to become radiation resistant. Thus “hypoxia” will be referring to tumour hypoxia and will be defined to occur at an oxygen tension less than ten mmHg, as indicated above.

Vasculogenesis is defined as a neovascularisation (creation of new blood vessels) mechanism by which endothelial progenitor cells are successively incorporated into the growing capillaries. Angiogenesis is another neovascularization mechanism which includes mitotic proliferations of endothelial cells of the pre-existing capillaries and their migration to the vascular tips forming so called "vascular sprouts" or "endothelial buds". An effective circulatory system is mandatory to the delivery of oxygen and nutrients and also aids in the excretion of metabolic waste products. Thus the development of neovascularature is a vital component of many normal physiological processes and a number of disease states. Physiological angiogenesis occurs in wound and bone fracture healing, embryogenesis, the female menstrual cycle and normal tissue that is hypoxic or nutrient-deplete. Pathologic angiogenesis occurs in a variety of disease states including cancer, arthritis, vasculopathies and retinopathies and is characterised by either excessive (e.g. cancer) or inadequate (e.g. coronary artery disease) neovascularisation. Of particular note is that angiogenic vessels in pathologic processes may be different from mature vessels and lack the organisation and contain regions of acidosis and hypoxia. This is contributed to by leaky endothelial cells where intercellular junctions are not tight and basement membranes are incomplete.

In this thesis, the term angiogenesis will be used in reference to pathologic angiogenesis with the creation of new blood vessels within tumours, as it is often done in the literature. It will not be specifically referring to one mechanism or another and is not meant to incorporate lymphogenesis or arteriogenesis.
1.8 Conclusion

RCC is becoming more common yet clinicians are having minimal impact on overall survival predominantly because of a lack of understanding of the basic tumour biology. Furthermore, our ability to link and understand the functionality of the tumour with imaging hold promise for selection monitoring and treatment of patients with RCC. With information gained from this thesis researchers and clinicians may better understand RCC and impact on survival of patients.
Chapter 2
BACKGROUND

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2.7.6 Angiogenesis as a Target for Cancer treatment

2.8 Tyrosine Kinase Inhibitors and other small molecules
2.1 General principles of Cancer

Cancer cells are able to divide indefinitely and even outside the body without regard to normal control mechanisms. The challenge is to identify mechanisms to cause cancer cell death whilst maintaining a normal, healthy cellular population in any individual with cancer. This often involves exploiting the rapid division of cells and more recently switching off signals allowing growth. A greater knowledge and understanding of these principles will allow improvements in primary and adjuvant treatment, salvage therapy and palliation.

2.2 Introduction to Renal Cell Carcinoma

Primary adult renal tumours in approximately 90% of cases originate in the cells of the proximal tubule, are malignant and are known as Renal Cell Carcinoma (RCC). The remainder are benign tumours and rare malignant tumours that are currently classified as in Table 2-1 according to their origins as epithelial, mesenchymal or blastemal. Sarcomatoid variants of different subtypes have been described and generally have a worse prognosis. Oncocytic tumours are not RCC and are not discussed further.

In Australia with a population of approximately 20,000,000, malignant neoplasms were the main underlying cause of 38,000 deaths, comprising almost a third of all deaths in 2003-4 (the latest year for which there are figures). Further, malignancy was an associated cause of death in a further 10% of all deaths. RCC is estimated to account for over 100,000 deaths annually worldwide with 208,000 new cases (1.9% of the world total for cancer).

Increased use of axial imaging, ultrasound and computerised tomography (CT), has increased the incidence of all stages of RCC. In the past 15 years in the USA, the incidence of RCC has increased 24.8% from 27,200 cases in 1990 to 36,160 cases in 2005. Based on the Surveillance, Epidemiology and End Results (SEER) database in the USA, from 1988 through 2002 tumour size decreased from 66.8 to 58.6 mm, while the age adjusted incidence of RCC increased from 8.6 to 11.2 cases per 100,000 individuals. Kaplan-Meier analysis showed steadily deteriorating survival with increased cancer size above 4 cm with a median
survival of 105 months for 4 to 7 cm versus 46 months for more than 7 cm.\textsuperscript{50} So although clinicians are finding some tumours earlier, our impact on survival for larger tumours remains poor whilst incidentally detected small tumours have a better prognosis.\textsuperscript{51}

RCC cases localised to the kidney, those confined to the immediate surrounds of the kidney (regional disease) and those with metastases beyond the kidney region (distant disease) have increased annually by 3.7\%, 1.9\% and 0.68\% in recent years.\textsuperscript{52} Despite increased utilisation of imaging (CT, ultrasound) the percentage of locally advanced and metastatic cases has remained constant. A migration to lower stage disease has not occurred but a detection of smaller incidental asymptomatic tumours has happened.\textsuperscript{53} Evidence suggests that incidentally detected tumours have a better prognosis but further data are required.\textsuperscript{49,51}
<table>
<thead>
<tr>
<th>PRIMARY TUMOURS</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPITHELIAL TUMOURS</td>
</tr>
<tr>
<td>Benign Epithelial Tumours</td>
</tr>
<tr>
<td>Cortical papillary adenoma; Renal oncocytoima; Juxta-glomerular; Metanephric adenoma</td>
</tr>
<tr>
<td>Malignant Epithelial Tumours</td>
</tr>
<tr>
<td>Renal Cell Carcinoma</td>
</tr>
<tr>
<td>Clear Cell (Conventional Type) [60-80%]</td>
</tr>
<tr>
<td>Chromophobe Type</td>
</tr>
<tr>
<td>Papillary Type I and Type II (Chromophilic) [10%]</td>
</tr>
<tr>
<td>Collecting duct carcinoma with medullary carcinoma of the kidney [&lt;1%]</td>
</tr>
<tr>
<td>Renal Cell carcinoma unclassified (&lt;1%)</td>
</tr>
<tr>
<td>MESENCHYMAL TUMOURS</td>
</tr>
<tr>
<td>Benign Mesenchymal Tumours</td>
</tr>
<tr>
<td>Angiomyolipoma</td>
</tr>
<tr>
<td>Medullary fibroma; Leiomyoma; Lipoma; Haemangioma; Lymphangioma + others</td>
</tr>
<tr>
<td>Malignant Mesenchymal Tumours</td>
</tr>
<tr>
<td>Sarcoma (leiomyosarcoma, liposarcoma, malignant fibrous histiocytoma + others)</td>
</tr>
<tr>
<td>Lymphoma</td>
</tr>
<tr>
<td>BLASTEMAL TUMOURS</td>
</tr>
<tr>
<td>Mesoblastic nephroma; Adult nephroblastoma; Clear cell sarcoma</td>
</tr>
<tr>
<td>NEURO-ENDOCRINE TUMOURS</td>
</tr>
<tr>
<td>Carcinoid; Primitive neuro-ectodermal tumours (PNET)</td>
</tr>
<tr>
<td>SECONDARY TUMOURS (METASTASIS)</td>
</tr>
<tr>
<td>Common- Melanoma, Colon, Ovary Breast Lung, Oesophagus</td>
</tr>
</tbody>
</table>
2.2.1 Presentation of patients with suspected Renal Cell Carcinoma

Decades ago common presenting symptoms of RCC were haematuria, abdominal mass, pain, and weight loss. However, with frequent use of intraabdominal imaging, contemporary series have an increasing number of incidental RCC. RCC is also often locally advanced and unresectable at the time of diagnosis. Such patients are usually not curable with five-year survival rates of 25% but those with metastatic RCC fare worse with five-year survival of only 5%. Organ confined RCC has overall five-year survival of greater than 55%. Finally, paraneoplastic symptoms may be present in around 20-30% of patients. Such symptoms encompass hypertension, loss of weight, pyrexia, neuromyopathy, anaemia, polycythaemia, amyloidosis, deranged liver function or an elevated erythrocyte sedimentation rate.

2.2.2 Assessment of patients with suspected Renal Cell Carcinoma

The assessment of a patient with suspected RCC includes a clinical exam and focused history. Investigations should include serum haemoglobin, creatinine and electrolytes, calcium and liver function tests (especially Alkaline Phosphatase to delineate liver, bone metastases, and paraneoplastic syndromes). Imaging may include initial ultrasound but should have a CT (including chest) with MRI optional if the CT is not conclusive. Based on symptoms and signs and results of other investigations bone scintigraphy or brain CT may be needed.

Biopsy of renal lesions prior to definitive therapy is not routinely performed because of the risk of tract seeding and pathologic inaccuracy in differentiating oncocytoma from chromophobe RCC. Biopsy does have a role in certain situations such as prior history of non-renal malignancy, metastatic disease of unknown primary origin, previous contralateral nephrectomy for a renal cell neoplasm, a renal transplant mass, suspected renal lymphoma, history of tuberous sclerosis, and poor surgical candidacy. It is likely the indications for biopsy may expand as a renewed interest in monitoring smaller renal masses combined with improved accuracy and safer techniques become more widespread. Current Australian data supports the use of biopsy in such situations.
2.3 Epidemiology of Renal Cell Carcinoma

RCC has the highest incidence rates in North America, Australia/New Zealand and Europe (except southern areas). Incidence rates are low in Africa, Asia (except Japanese males) and the Pacific. RCC has a peak incidence in the 6th and 7th decade occurring with a predominance of men over women (1.5-3.1 to 1) and has had a constant rise in incidence over the past 50 years.\(^47\)

2.3.1 Risk Factors for Renal Cell Carcinoma

Tobacco smoking is a contributing cause to RCC development. Environmental chemical (arsenic, cadmium, asbestos and petroleum) exposure is also a factor in the development of RCC but is not as well characterised as other risk factors such tobacco and needs further evidence in larger population based analyses.\(^47\) The risk is increased by obesity\(^61\) and this was confirmed in a recent meta-analysis where a relative risk of 1.24 was demonstrated.\(^62\) There is also evidence of a genetic predisposition. Various familial syndromes have also been identified (see below).

An increased incidence of RCC in long term survivors of testicular cancer has been suggested,\(^63\) possibly due to intra-abdominal radiation.\(^64,65\) In Moller’s review of 3256 patients from 1943-1987, the relative risk of RCC was estimated at 2.3.\(^66\) An increased relative risk of 3.2 for developing RCC has also been calculated, supporting this figure.\(^67\) Current treatment protocols now aim to minimise carcinogenic exposure, with the risk of second malignancy being lower today than in past studies.\(^68\) One such case of second malignancy in the kidney following testicular cancer and relevant literature is reviewed as part of the background for this thesis (see Appendix E).\(^69\)

RCC is not a single disease; it is made up of a number of different types of cancer that occur in this organ and this is exemplified by the familial syndromes associated with it.\(^70\) The most common cause of inherited RCC is von Hippel-Lindau (VHL) disease. RCC does not have
classic precursor lesions as in other cancers.

2.3.1.1 Familial Renal Cell Carcinoma Syndromes
Besides VHL disease, a number of other familial RCC syndromes exist. These are summarised in Table 2-2. The VHL gene (VHL) is a crucial one in the development of RCC. It is involved not only in VHL disease, but also sporadic tumours. Each will be discussed.

2.3.1.2 von Hippel-Lindau (VHL) Disease
VHL disease is a familial syndrome resulting from the loss of von Hippel-Lindau protein (pVHL) function in an autosomal-dominant fashion. VHL disease results from germ-line mutations in the tumour suppressor gene (VHL) that codes for VHL and ultimately pVHL and is characterized by variable expression and the development of benign and malignant neoplasms in multiple organs: angiomas of the retina, hemangioblastomas of the central nervous system, clear-cell RCC and phaeochromocytomas. The syndrome has a high penetrance but variable expression and has an incidence around 1/50,000 live births. Approximately 40-60% of patients with VHL disease will develop RCC and of those with the disease, RCC is the leading cause of death. Additional renal disease may develop in the form of cysts that may later develop RCC.

Each patient with VHL disease inherits the germ line mutation from their affected parent but also one, normal (wild type) copy of the gene from their other parent. Knudson advanced a "two hit hypothesis" or "two-mutation theory" to explain tumourigenesis in such conditions. Each cell contains pairs of chromosomes (Figure 2-1). Both copies of the gene must be mutated or epigenetically silenced, for cancer to develop.
<table>
<thead>
<tr>
<th>Familial Syndrome</th>
<th>Mode/Gene</th>
<th>RCC Type</th>
<th>Other associations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Von Hippel-Lindau (VHL) disease\textsuperscript{71,78}</td>
<td>Autosomal dominant loss of von Hippel-Lindau protein (pVHL) function; Tumour Suppressor Gene Chromosome 3</td>
<td>Clear Cell (70% by age 60)</td>
<td>Cerebral haemangioblastomas, retinal angiomas, islet cell tumours, Cysts, Phaeochromocytomas</td>
</tr>
<tr>
<td>Hereditary papillary renal carcinoma (HPRC)\textsuperscript{70}</td>
<td>c-Met proto-oncogene tyrosine kinase; Chromosome 7</td>
<td>Papillary Type I</td>
<td>Cutaneous tumours, lung cysts, and colon polyps or cancer</td>
</tr>
<tr>
<td>Birt-Hogg-Dubè syndrome\textsuperscript{70}</td>
<td>BHD gene Tumour Suppressor Gene</td>
<td>Mixed (hybrid) chromophobe/oncocytic RCC (in 50% of patients), chromophobe RCC (in 33%), oncocytic (in 7%), and clear cell RCC (in 5%)</td>
<td>Benign fibrofolliculomas, pulmonary cysts, spontaneous pneumothorax, colon polyps and tumours, and several multifocal renal cancers</td>
</tr>
<tr>
<td>Hereditary leiomyomatosis renal cell carcinoma (HLRC)\textsuperscript{70}</td>
<td>\textit{FH} gene, which encodes the enzyme fumarate hydratase in the mitochondrial Krebs cycle</td>
<td>Papillary</td>
<td>Cutaneous leiomyomas, uterine leiomyomas (fibroids)</td>
</tr>
</tbody>
</table>
Each cell contains pairs of chromosomes. Solid circles are sites of mutations. The first mutation is usually a structural change in a specific cancer gene (single solid circle). The second mutation usually involves a much larger region of the chromosome (multiple solid circles) or epigenetic silencing rather than mutation. Both copies of the gene must be mutated or epigenetically silenced, for cancer to develop (adapted from Zbar et al. 76).
However, only a small percentage (2%) of RCC are familial with the majority occurring sporadically. Normal individuals carry two genes encoding for VHL. VHL inactivation occurs via one mechanism initially and then two other mechanisms in sporadic (non-inherited) RCC. The primary one is VHL allele deletion (loss of heterozygosity) in 84-98% of cases. Therefore, one allele is effectively not present. In the remaining allele in such patients, the second mechanisms are at work with either mutation of the gene or gene silencing via methylation. Effectively, allelic VHL inactivation occurs (“two-hits”) in the majority of sporadic clear cell RCC tumours, similar to that occurring in VHL disease (Figure 2-1). Thus VHL plays an extremely important role in the tumourigenesis of sporadic RCC in the absence of VHL disease. Non-clear RCC do not demonstrate significant VHL inactivation.

2.3.1.3 The VHL gene

VHL is located at chromosome 3p25 and this was characterized in 1993. It contains three exons and encodes a 4.5kB transcript. The VHL product is ultimately pVHL (after phosphorylation), a 213 amino acid protein, that within cells normally resides in the cytoplasm but may freely move into and out of the nucleus.

2.3.1.4 pVHL and Consequences of VHL Gene Inactivation in RCC

pVHL binds to hypoxia-inducible factor (HIF-1α), which is a transcription factor that regulates a number of proteins including vascular endothelial growth factor (VEGF), Insulin-like Growth Factor-2 (IGF-2) and the glucose transporters (e.g. glucose transporter 1 or Glut-1). Overproduction of these proteins often characterises the development of tumours and they are each discussed further below. Loss of pVHL function results in constitutive activation of the hypoxic response, is important in the development of clear cell RCC, where both copies of the gene are usually inactivated (VHL-/- RCC).
summarises the process: VHL-/− RCC cells fail to degrade HIF-1α, resulting in the constitutive activation of its target genes, a process that is required for tumourigenesis. In conditions of normoxia and normal VHL gene function, pVHL is the substrate recognition component of a ubiquitin ligase complex that targets a protein transcription factor HIF-1α, for proteolysis. Such oxygen-dependent enzymatic hydroxylations by prolyl hydroxylases occur at specific prolyl residues (residues 564 and 402 of HIF-1α). This enzymatic modification of HIF-1α al is required for the binding of the VHL tumour suppressor protein (pVHL), which is the recognition component of an E3 ubiquitin-protein ligase that targets HIF-1α for proteosomal degradation. Thus in oxygenated cells this process targets HIF-1α for rapid proteasomal destruction.

In conditions of hypoxia or defective pVHL function, the interaction between pVHL and HIF is dysfunctional; HIF-1α is not subject to proteolysis and is thus constitutively activated with overexpression. So when hypoxic conditions occur, oxygen becomes the rate-limiting step for prolyl hydroxylation, resulting in decreased ubiquitination of HIF-1α. HIF-1α translocates into the nucleus and leads to transcription of hypoxia-inducible genes. Several hypoxia inducible genes are induced by this process, including VEGF. Thus VHL-defective RCC cells fail to degrade HIF-1α regardless of oxygen tension. Examination of RCC tumours for VEGF (mRNA transcripts and/or VEGF protein) has demonstrated VEGF overexpression in the vast majority of samples (see section 2.9.3). Taken together, the above data provide compelling evidence for VHL inactivation in the majority of clear cell RCC tumours, leading to VEGF overexpression that drives tumour angiogenesis.

A further point to note is that under normoxic conditions HIF-1α transcription is oxygen regulated due to cooperative binding of pVHL and the co-repressor FIH-1, the factor inhibiting HIF-1. FIH-1 performs asparaginyl hydroxylation of HIF-1α in oxygenated cells. This further hydroxylation of HIF-1α prevents interaction with a transcriptional co-
activator, providing a second mechanism by which HIF-mediated transcription is inactivated.\textsuperscript{89} However, VHL-defective RCC cells fail to degrade HIF-1\(\alpha\) regardless of oxygen tension leading to the constitutive activation of its targets. Silencing of HIF-1\(\alpha\) by short hairpin RNA (shRNA) prevents \textit{VHL}/- RCC tumour formation in a nude mice xenograft assay showing that the constitutive activation of HIF targets is required for \textit{VHL} defective RCC tumourigenesis.\textsuperscript{81}

Although at present pVHL is recognised as a specific substrate-recognition component of the E3 ubiquitin complex, which regulates proteasomal degradation of the subunit of the hypoxia HIF, it may have other functions.\textsuperscript{71} It appears to regulate the stability of other proteins that might be involved in various steps of oncogenic processes e.g. pVHL appears to play a pivotal role in tumour suppression by participating actively as a component of p53 transactivation complex during DNA damage response.\textsuperscript{95}

2.3.1.5 Summary of pVHL Regulation in RCC

Clearly, pVHL has a significant role in the development and progression of clear cell RCC. In summary, it is a negative regulator of the cellular response to hypoxia and loss or mutation of the gene coding for its production favours the promotion of angiogenesis.\textsuperscript{96,97} Once the pVHL pathways have been understood, the next step has been to formulate mechanisms to exploit the pathways to develop therapeutic agents to combat RCC (discussed further in section 1.5.3).\textsuperscript{74}
Figure 2-2 Schematic illustration of hypoxia-inducible factor-1α (HIF-1α) and the von-Hippel-Lindau (VHL) protein function.

Under normoxic conditions HIF-1α after oxygen-dependent modification by a prolyl hydroxylase, binds to the von-Hippel-Lindau protein and is degraded via the ubiquitin pathway. In hypoxia, HIF-1α is stabilised, translocates to the nucleus forms the dimer HIF-1 with its partner HIF-1β and binds to hypoxia-responsive elements (HREs) of hypoxia-regulated genes. CAIX: carbonic anhydrase IX; VEGF: Vascular endothelial growth factor conditions (original figure).
2.4 Prognostic Factors in Renal Cell Carcinoma

The prognostic factors may be related to the pathological findings of the tumour but also tumour-related factors (e.g. chromosomal abnormalities and biochemical markers) are becoming increasingly important.

2.4.1 Pathological Findings: Stage

Staging is the oldest and most important prognostic factor in RCC. The TNM system is the most commonly is used worldwide and is constantly evolving and replaced the original Robson four stage system.\textsuperscript{19} Intuitively, those tumours confined to the kidney offer a better prognosis than those that invade perirenal fat or have spread to distant sites. Collecting system invasion by RCC is also a poor prognostic factor. In the most recent publications, the five-year survival rates for the various stages as classified by the more commonly used TNM system\textsuperscript{19} are in Table 2-3.

The 1997 TNM system was subsequently updated in 2002\textsuperscript{98} with the only change being the subdivision of T1 tumours into T1a (tumour \(\leq 4\) cm, limited to kidney) and T1b (tumour >4 cm and \(\leq 7\) cm, limited to kidney). This reclassification of stage I was done to help to optimise the selection of patients suitable for elective nephron sparing surgery, with people classified as having pT1a disease (tumour \(\leq 4\) cm) being prime candidates for this approach.\textsuperscript{99}
Table 2-3 TNM (1997) Classification of Renal Cell Carcinoma

<table>
<thead>
<tr>
<th>Tumour (T)</th>
<th>Five-year survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 Tumour 7.0cm or less in greatest dimension, limited to the kidney</td>
<td>90-100%</td>
</tr>
<tr>
<td>T2 Tumour greater than 7.0cm in greatest dimension, limited to the kidney</td>
<td>75-95%</td>
</tr>
<tr>
<td>T3 Tumour extending into major veins or invading adrenal gland or perinephric tissues, but not beyond Gerota’s Fascia</td>
<td></td>
</tr>
<tr>
<td>T3a Tumour invades adrenal gland or perinephric tissues but not beyond Gerota’s fascia</td>
<td></td>
</tr>
<tr>
<td>T3b Tumour grossly Extends into Renal Vein(s) or cava below diaphragm</td>
<td></td>
</tr>
<tr>
<td>T3c Tumour grossly extends into vena cava above diaphragm</td>
<td>60-70%</td>
</tr>
<tr>
<td>T4 Tumour invading beyond Gerota’s fascia</td>
<td>15-30%</td>
</tr>
</tbody>
</table>

Node (N)

<table>
<thead>
<tr>
<th>Node</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>N0</td>
<td>No regional lymph node metastases</td>
</tr>
<tr>
<td>N1</td>
<td>Metastasis to a single regional lymph node</td>
</tr>
<tr>
<td>N2</td>
<td>Metastasis in more than one regional lymph node</td>
</tr>
</tbody>
</table>

Metastasis (M)

<table>
<thead>
<tr>
<th>Metastasis</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>M1</td>
<td>Distant metastasis</td>
</tr>
</tbody>
</table>

Table 2-4 Fuhrman Grading of Renal Cell Carcinoma

<table>
<thead>
<tr>
<th>Grade</th>
<th>Nuclear Size</th>
<th>Nuclear Outline</th>
<th>Nucleoli</th>
<th>Five-year survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10µm</td>
<td>Round and Uniform</td>
<td>Absent or inconspicuous</td>
<td>65-76%</td>
</tr>
<tr>
<td>2</td>
<td>15µm</td>
<td>Irregular</td>
<td>Small</td>
<td>30-70%</td>
</tr>
<tr>
<td>3</td>
<td>20µm</td>
<td>Irregular</td>
<td>Prominent</td>
<td>20-50%</td>
</tr>
<tr>
<td>4</td>
<td>&gt;20µm</td>
<td>Bizarre</td>
<td>Prominent, heavy chromatin clumps</td>
<td>10-35%</td>
</tr>
</tbody>
</table>
2.4.2 Pathological Findings: Grade

The Fuhrman grading system (Table 2-4) is the most commonly used worldwide and in Australia and again offers good prognostic information. The most common drawback of such a system is that it is subjective. This is probably reflected in the wide ranges for survival when using grade alone. 19

2.4.3 Pathological Findings: Histological Type

Histological type is prognostically important. Clear cell RCC has a five-year survival of 55-60%. Papillary are usually considered to have a better prognosis than clear cell. 100 Chromophobe RCC has genetic similarities to oncocytes (a benign tumour), are of low grade and have a five-year survival approaching 100% in many series. Collecting duct carcinomas and medullary carcinomas (extremely rare) offer the worst prognosis (clinically aggressive, high grade and often disseminated at presentation). 101 Finally, sarcomatoid variants of all types of RCC exist and have their prognosis is extremely poor. 19

2.4.4 Tumour-related Factors: Immunohistochemistry

Ki-67 tumour antigen immunostaining appeared to be an additional prognostic indicator of biologic aggressiveness in RCC. 102 In multivariate analyses the estimation of growth fraction by determination of Ki-67-positive cells in tissue sections has served as an independent prognostic marker for RCC. The mean range in RCC ranged from 6-13% (total range 0-60%) in different studies whilst in benign renal cells it was generally below 1%. 103,104

CAIX and Ki67 staining have been inversely correlated (p = 0.009) in their expression in RCC. 105 However, when combined, Ki-67 significantly substratified patient subgroups defined by high or low CAIX staining. On multivariate analysis the combined parameter consisting of Ki-67 and CAIX was a significant predictor of survival (p <0.001) and it was able to displace histological grade. 105
In a small study of 44 patients a correlation was found between VEGF in the renal vein prior to nephrectomy and tumour stage and grade. Also, VEGF in the renal vein was an independent prognostic factor for predicting progression\textsuperscript{106}.

Patients at high risk of RCC progression (greater than 50% at 24 months) could be indentified by a multivariate correspondence analysis using Ki-67 labelling index, CD44, an endothelial cell surface antigen and VEGF expression, in association with tumour size, tumour nuclear grade and tumour stage\textsuperscript{107}.

2.4.5 Pathological Findings: Vascular invasion and Multifocality

The five-year survival rate for RCC when completely resecting a vena caval tumour thrombus in patients without metastases (30-69\%) is not dependent on the level of thrombus, but tumour stage.\textsuperscript{108,109} Microvascular invasion may affect tumour progression but data are lacking and it is not routinely reported.\textsuperscript{110} Tumour multifocality occurs around 5.3\% of pathological specimens and may remain undetected with preoperative imaging in up to 3.5\% of cases\textsuperscript{111} and has no apparent effect on recurrence or survival\textsuperscript{111}.

2.4.6 Tumour-related Factors: Chromosomal Abnormalities

RCC with chromosome translocations involving a breakpoint at Xp11 and resulting in gene fusions involving the TFE3 transcription factor gene have recently been recognised not only as paediatric renal tumours but as a further subtype of clear cell RCC in adults with a poorer prognosis.\textsuperscript{112} These tumours occur in younger adults and a recent series found 1.6\% of RCC to be Xp11.\textsuperscript{113} However, the most common chromosomal abnormalities involve chromosome 3; loss of 3p is consistently found in clear cell RCC.\textsuperscript{114} This will be expanded upon in coming sections.

2.4.7 Clinical Risk Factors and Renal Cell Carcinoma

Stratifying patients according to risk profile is important as it has implications for prognosis as well as treatment options, particularly with metastatic RCC. The Memorial Sloan-Kettering Cancer Centre (MSKCC) risk classification\textsuperscript{51} is based on clinically identifiable risk
factors: low serum haemoglobin levels, elevated corrected serum calcium levels, elevated serum lactate dehydrogenase levels, poor performance status and intervals less than one year between diagnosis and treatment.\textsuperscript{115,116} The factors are additive and help place patients into good (no factors), intermediate (one-two factors) and poor (three or more factors) prognostic groups. The University of California-Los Angeles Integrated Staging System\textsuperscript{117} uses similar parameters.

2.5 Treatment of Renal Cell Carcinoma

In organ confined disease, surgery (either radical or partial) nephrectomy remains the standard of care but ablative therapies (cryotherapy, radiofrequency ablation and high-intensity focused ultrasound) and even monitoring of smaller masses are appropriate in certain situations, particularly where the patient is not fit for surgery. Metastatic or locally advanced disease treatment strategies may be curative or palliative and involve surgery with or without newer systemic therapies. Radiotherapy has a role in palliating metastatic bone deposits, as does surgery stabilising bones.

In a curative situation, patients are then monitored clinically, with imaging and renal function to assess for recurrence and maintain evidence that the remaining nephrons are not deteriorating.\textsuperscript{20}

2.5.1 Surgery

Radical nephrectomy (excising the kidney intact including Gerota’s fascia) remains the “gold-standard” for treating primary tumours in those with localised disease. However, for smaller RCC, generally 4cm or less, partial nephrectomy has been of equal oncological value in selected patients or when nephron sparing is paramount.\textsuperscript{118,119} Both procedures may be done by conventional open surgery or laparoscopic techniques.\textsuperscript{120,121} A formal lymph node dissection is a staging tool but therapeutic efficacy is unproven.\textsuperscript{122} The chances of cure by surgery most strongly depend on the stage and grade of the disease.\textsuperscript{123}
Cytoreductive nephrectomy may also be performed in locally advanced disease or metastatic disease where a patient is a candidate for adjuvant therapy (e.g. immunotherapy). Prospective randomized trials have shown a significant survival advantage and longer time to disease progression in such patients. Upfront cytoreductive nephrectomy also potentially offers palliation from local tumour symptoms (e.g. persistent haematuria, pain or a paraneoplastic syndrome). Cytoreductive surgery prior to systemic therapy with newer targeted agents such as tyrosine kinase inhibitors has not been established but is being investigated.

At present, surgery remains the mainstay of curative treatment for kidney cancer. Overall, the 5-year survival rate is 60%; for patients with metastatic disease, the 2-year survival is 0–20%. It has been recognized that since RCC are widely chemo and radio-resistant, new treatment options for non organ-confined disease or local recurrence after surgery are urgently needed.

### 2.5.2 Tyrosine kinase inhibitors and other small molecules

A later section is devoted to these important molecules (section 2.8).

### 2.5.3 Chemotherapy and Immunotherapy

Thus far RCC has been found to be insensitive to current chemotherapeutic regimens. One explanation is the overexpression of the drug transporter P-glycoprotein (Pgp, P-170) which may contribute to chemoresistance and has been observed in 70% of tumours.

RCC has been reported to regress spontaneously, including where metastatic disease is present, suggesting immune system involvement in surveillance and potentially destruction of RCC. Immunotherapy with the cytokines interleukin-2 (IL-2) and interferon-α have since been demonstrated to have modest responses in RCC with only around 12% of patients having a response that in many cases is not sustained. High-dose IL-2 may lead to long-term disease free survival in a small percentage of patients but results in significant
haemodynamic and other toxicities requiring intensive support. Combining immunotherapy with cytoreductive nephrectomy has improved results but only modestly. Current immunotherapy strategies have not improved response rates against metastatic RCC and side effects remain significant, imposing the search for new therapeutic targets. Therapeutic cancer vaccines have little proven effect in the area of RCC.

2.5.4 Monoclonal Antibodies

The ability to target human tumours with mAb has only arisen in conjunction with the recognition that many human neoplasms have antigens that may be recognised by the human immune system spontaneously. Anti-tumour antibodies and cell-mediated immunity have both been observed.

Monoclonal antibodies have an anti-tumour effect by either immune effector function initiated when the antibody binds to a receptor on the tumour and initiates antibody- or by dependent cellular cytotoxicity and complement dependent cytotoxicity; some even have direct effects on the neoplastic cells. Ultimately, the effects may lead to reduced angiogenesis and altered tumour oxygenation, but this has not been previously studied in RCC.

Another targeting role for mAb is in radioimmuotherapy where in the strictest sense the mAb has the ability to destroy cancer cells due to immune function but more importantly, by radiation from an attached isotope once bound to a target antigen- preferably only expressed on tumour cells. This is not part of this thesis but a variation, using radioisotopes for imaging, radioimmunoscintigraphy or immune-PET has been investigated (see section 2.11.12).

2.5.4.1 Monoclonal antibody types

In tumour research and treatment, there are two broad groups of monoclonal antibody that may be raised against antigens: murine and humanised. An example to illustrate the
differences may be explained with the antibody G250, specific for CAIX. Mouse anti-human (murine) were first engineered. However, in the clinical setting when used for their immune function against RCC, immune system responses to the mouse portion of the antibody precluded multiple dose schedules for the murine antibody.\textsuperscript{143-145}

The generation of a chimeric form of G250 (Mouse Fv and human IgG\textsubscript{1} Fc) refers to a mAb cG250 engineered from the original murine mAb G250.\textsuperscript{146,147} This resulted in reduced immunogenicity of the murine G250 whilst retaining the antigen specificity for CAIX of the parent murine antibody that has been demonstrated with in vitro competitive binding studies with targeting RCC.\textsuperscript{143,146}

Monoclonal antibodies will be discussed in greater detail in section 2.11.12.

**2.5.5 Radiotherapy**

Radiotherapy has been used chiefly as a palliative tool but may also be used to irradiate the symptomatic kidney if surgery is not an option. Radiotherapy still maintains an important role in treating bony metastatic disease.\textsuperscript{148}

**2.5.6 Arterial Embolisation**

Embolisation of larger tumours by placing coils in the renal artery larger tumours has been utilised prior to surgery or rarely to palliate symptomatic RCC. It is normally reserved for treating angiomyolipomas and trauma.\textsuperscript{149-151}

**2.6 Hypoxia in Tumours**

Hypoxia will now be outlined covering the current definitions and the implications of hypoxia in tumours.
2.6.1 Definitions of Hypoxia

Tissue hypoxia arises when an inadequate supply of oxygen compromises biologic functions of that tissue.\textsuperscript{152} In normal tissues there are finely tuned regulatory processes leading to increases in blood flow and therefore hypoxia does not generally develop because increases in tissue pO\textsubscript{2} consumption are generally matched by increases in blood flow. Hypoxia is occurs in physiological systems in general because regulation of blood flow fails to meet the increased pO\textsubscript{2} demand.\textsuperscript{153} There are five basic causative mechanisms of tissue hypoxia\textsuperscript{153} summarised in Table 2-5.
<table>
<thead>
<tr>
<th>Type of Hypoxia</th>
<th>Mechanism</th>
<th>Aetiology (examples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxaemic hypoxia</td>
<td>Low pO\textsubscript{2} in arterial blood</td>
<td>Pulmonary disease, high altitude</td>
</tr>
<tr>
<td>Anaemic hypoxia</td>
<td>Reduced ability of the blood to carry O\textsubscript{2}</td>
<td>Anaemia methaemoglobin formation carbon monoxide poisoning</td>
</tr>
<tr>
<td>Circulatory or ischaemic hypoxia</td>
<td>Reduced tissue perfusion, local or generalised</td>
<td>Vasospasm, reduced circulating volume</td>
</tr>
<tr>
<td>Diffusional hypoxia</td>
<td>Deterioration of the diffusion geometry</td>
<td>Altered diffusion distances, concurrent versus countercurrent blood flow within microvessels</td>
</tr>
<tr>
<td>Histotoxic or cytotoxic hypoxia</td>
<td>Inability of cells to use O\textsubscript{2} because of intoxication</td>
<td>Cyanide poisoning</td>
</tr>
</tbody>
</table>

Table 2-5 Hypoxia- causative mechanisms
Biochemists often define hypoxia as oxygen-limited electron transport.\textsuperscript{154} Physiologists and some clinicians when referring to hypoxia are identifying a state of reduced oxygen availability or decreased oxygen partial pressures below critical pressures resulting in restriction or abolishment of organ, tissue or cellular function.\textsuperscript{155} A final manner in which to define hypoxia generally at a tissue or cellular level is when the oxygen partial pressure falls below a critical value causing the oxygen consumption rate or ATP production rate to progressively decrease.\textsuperscript{153}

Anoxia is the state where no oxygen is detected in the tissue (oxygen partial pressure=0mmHg)\textsuperscript{153} In solid tumours delivery of oxygen to respiring neoplastic and stromal cells is frequently reduced or even abolished by a deteriorating diffusion geometry, severe structural abnormalities of tumour microvessels, and disturbed microcirculation.\textsuperscript{153,156}

Additional factors contributing to the hypoxia are anaemia and the formation of methaemoglobin or carboxyhaemoglobin that serve to reduce the capacity of the blood to transport oxygen. Subsequently acute or chronic areas with extremely low (down to and sometimes at zero) oxygen partial pressures exist within solid tumours. Such microregions of extremely low oxygen partial pressures are heterogenously distributed within the tumour mass and may even be located adjacent to regions with normal oxygen partial pressures. In contrast to normal tissue, neoplastic tissue can no longer fulfil physiologic functions. Therefore tumour hypoxia cannot be defined by functional deficits. However, areas of necrosis are indicative of the loss of vital cellular function and these regions are sometimes only detectable on careful histological assessment.\textsuperscript{153} Oxygen consumption will continue to a cellular oxygen partial pressure of 0-10mmHg.\textsuperscript{157,158} This is an important point to consider when examining tumour hypoxia.
2.6.2 Introduction to Tumour Hypoxia

Tumour oxygenation status is an independent prognostic indicator in cancer because it influences tumour progression and treatment outcome. Furthermore, due to a specific histologic structure, avascular, well-differentiated hypoxic regions in tumours exhibit low drug uptake and may play a part in drug resistance.\textsuperscript{159} Antihypoxia treatment strategies are available in the clinic including hypoxic cell radiosensitisation and hypoxic cytotoxic therapy. A variety of new treatments are under development, including hypoxia-induced gene therapy, HIF-target therapy and anti-angiogenic and anti-vascular therapies.\textsuperscript{24} As described in the introductory chapter, a pO\textsubscript{2} of less than 10mmHg is generally regarded as hypoxic tissue in oncological literature.\textsuperscript{10}

2.6.3 Hypoxia in Tumours - an overview

The low levels of oxygenation observed in solid tumours are the result of two processes, both of which limit the amount of oxygen available to cells. The first of these is chronic hypoxia and the second of these is acute hypoxia\textsuperscript{32}. Thomlinson and Gray first demonstrated hypoxia in the 1950s whilst studying human lung tumours\textsuperscript{160}. They observed a relatively constant distance (around 100-150\textmu m) between blood vessels and the edge of necrosis in tumours and calculated that this distance corresponded to the oxygen diffusion distance through tissue. These findings helped support the hypothesis that cells were becoming necrotic due to a lack of oxygen diffusing to the tissue. Thomlinson and Gray also proposed that one or two layers of cells would still remain viable, but be functioning with much lower levels of oxygenation.\textsuperscript{6}

The second factor, acute hypoxia was proposed by Brown in the late 1970s.\textsuperscript{161} He believed that this acute form of hypoxia was due to fluctuating blood flow which was subsequently supported by studies of tumour blood flow using fluorescent dyes.\textsuperscript{162-164} The fluctuation in tumour blood flow that was demonstrated suggested that tumour blood vessels are often temporarily occluded, resulting in acute hypoxia. However, Brown also stressed that it is
important to remember that acute and chronic hypoxia in solid tumours are just two ends of a continuous spectrum with fluctuations in blood flow without total occlusion producing a dynamic situation with fluctuating oxygen diffusion distances in many parts of tumours\textsuperscript{32}.

### 2.6.4 Biological Consequences of Tumour Hypoxia

Growing evidence from experimental and clinical studies points to the fundamental, pathophysiologic role of hypoxia in solid tumours. Intratumoral hypoxia is a consequence of a structurally and functionally disturbed microcirculation, with deterioration of the diffusion geometry and of tumour-associated anaemia. Hypoxia-induced changes of the in the neoplastic and stroma cells may lead to neoplastic growth impairment through molecular mechanisms, resulting in cellular quiescence, differentiation, and apoptosis. Alternatively, hypoxia-induced changes activate non-specific stress response, anaerobic metabolism, angiogenesis, tissue remodelling, and change of cell contacts may promote tumour propagation by enabling neoplastic cells to overcome the nutritive deprivation through adaptation or escape from the “hostile” environment.

Whether the phenotypic result of hypoxia-induced change is impairment, stasis, or promotion of neoplastic growth is thought to be determined by the genome of the tumour cells and additional microenvironmental factors. Tumour cells with genomic alterations (such as loss of apoptotic potential) allowing their survival under hypoxia will aggravate tumour hypoxia. Sustained hypoxia increases genomic instability, genomic heterogeneity, and the selection pressure of the microenvironment. New variants even better adapted to survive and proliferate under reduced pO\textsubscript{2} will be selected through clonal expansion. These variants will further drive the vicious circle of malignant progression which is clinically characterized by an increasing probability of local, perifocal, regional, and distant spread.\textsuperscript{165}

### 2.6.5 Hypoxia and Angiogenesis- the paradox

Paradoxically, even in the presence of a blood supply a tumour can remain hypoxic and this may be explained in a number of ways. Firstly, Thomlinson and Gray demonstrated that the
distance of a tumour from its blood supply is important. Necrosis occurs 100-150um from a blood vessel and tumour cells adjacent to these necrotic areas may be viable but hypoxic, forming a peri-necrotic area of hypoxic cells.\(^1\) Secondly, tumour vasculature demonstrates greater permeability than normal tissues, which leads to an increased amount of leakage into the interstitial space, thereby limiting oxygen diffusion. This is predominantly due to large leaky blood vessels and capillaries, impaired blood flow patterns, neovascularisation, high interstitial fluid pressures and aberrant lymphoid vessels.\(^{166}\) These phenomena are also inconsistent with time, so different parts of the tumour will have varying levels of molecules depending on access by diffusion driven molecules that will vary accordingly.\(^{167}\) Finally, poorly developed blood vessels within a tumour that open and close intermittently will deliver oxygen sporadically, also contributing to tumour hypoxia.\(^{168,169}\)

**2.6.6 Radioresistance of Hypoxic cells**

The radioresistance of hypoxic cells is due to the role of oxygen in radiation damage. Ionising radiation causes cell death due to its ability to form free radicals in DNA. Once the free radicals are formed, oxygen rapidly reacts with the free electron, this reaction “fixes” (makes permanent) the radiation damage which ultimately is DNA cross-linking. Cells with low levels of oxygen are more likely to have the radical neutralised by donation of a hydrogen molecule from sulfhydryl-containing compounds before the damage is “fixed”, thus avoiding cell death.\(^32\) In general, compared to aerobic cells, it has been calculated that a 2-to-3-fold higher radiation dose is required to kill hypoxic mammalian cells.\(^{170}\)

**2.6.7 Chemoresistance of Hypoxic cells**

Studies performed in animal models and in human xenografts have demonstrated the chemoresistance of hypoxic cells. The reasons for this chemoresistance are two-fold:

1) Hypoxic cells are located further from functioning blood vessels than aerobic cells, and thus less active drug reaches them.

2) Hypoxic cells divide less rapidly than aerobic cells and most anticancer drugs target proliferating cells.\(^{32,171}\)
Given the importance of tumour oxygenation status in therapy and in predicting disease progression, it is imperative that reliable, available, affordable and technically simplistic methods be developed to yield a consistent, comprehensive, and reliable profile of tumour oxygenation.\textsuperscript{17}

2.6.7.1 Exploiting hypoxia with chemotherapy
An example of a chemotherapeutic drug exploiting hypoxia is tirapazamine and it belongs to a group of cytotoxic compounds known as “hypoxic cytotoxins”. Tiripazamine was discovered whilst researching hypoxic radiosensitisers that had differential activity against hypoxic cells. \textit{In vitro}, the dose required to kill hypoxic cells was less than 1\% of that required to kill well perfused (aerobic) cells and was confirmed in animal models.\textsuperscript{32,172} Most cancer chemotherapeutic drugs have decreasing activity with increasing distance from the vasculature, as the drug is less able to diffuse to these distant cells. Tirapazamine displays the opposite profile.\textsuperscript{32,172} It enters cells and is reduced by intracellular reductases, by the addition of an electron, to form a highly reactive radical. In the presence of oxygen, the molecule is converted back to its stable non-toxic parent molecule, and a superoxide radical is formed.\textsuperscript{173} Unfortunately, no role for this agent in RCC has yet been demonstrated but related agents are used to image hypoxia (section 1.11.11).

2.6.8 Quantification of Hypoxia
Quantification of hypoxia is determined by a number of parameters such as tumour microvascular density, blood flow, blood volume, blood oxygen saturation, tumour tissue \textit{pO}_2, and resistance to oxygen diffusion within the tumour. The measurement and interpretation of data obtained with currently available methods is complicated by the heterogeneity in tumour oxygenation.\textsuperscript{17} Currently available techniques can be broadly classified into direct invasive methods, direct non-invasive methods, and measurement of surrogate endogenous markers of tumour oxygenation.
Invasive Measurements of Tissue Oxygen Tension

Direct measurements of tissue oxygenation developed in the late 1960’s, when the Clark oxygen electrode became available.\textsuperscript{174} Of all methods available to measure tissue or tumour oxygenation, although not perfect,\textsuperscript{175} the direct invasive method using a polarographic oxygen sensor probe (POS) is considered the 'gold standard'.\textsuperscript{8,17} The POS probe is also known as the Eppendorf pO\textsubscript{2} histograph, after its original manufacturer (Eppendorf Hamburg, Germany) and the graph created by its measurements. An electrode is mounted on the tip of a needle and advanced through the tissue of interest. A resulting histogram reflects the oxygen status of the tissue being examined and median levels of oxygen partial pressure are calculated.\textsuperscript{32}

Another probe that has been developed and that is considered equivalent in terms of its ability to measure oxygen status in tissues is the time-resolved luminescence-based optical probe (Oxylite probe, Oxford Optronics, UK hereafter referred to as oxylite probe).\textsuperscript{176} This system is a probe using fibre-optic sensors that use dyes whose luminescence is quenched by oxygen.\textsuperscript{177} Essentially these sensors rely on the measurement of the oxygen-quenched lifetime of a luminescent molecule immobilized at the tip of an optical fibre (the probe). The optical fibre is used to guide the excitation and emission light transients to the measurement system.\textsuperscript{8} This is discussed in section 3.11 and is only licensed for use in animal studies.

Although the integrated approach to delineating tumour oxygen status can be a powerful tool in tumour therapy, it has its limitations. Whereas it has the potential to provide useful information in early stage disease in many types of cancers, such as the identification of the unique pathophysiological parameter that is most relevant for the behavior of malignancies, it may become impossible to carry out when there is extensive metastatic spread. It also appears that tumours that are very large or very small may not be ideal for such a study because both Doppler ultrasound (DUS) and diffuse light spectroscopy (DLS) are non-invasive methods, and the depth of penetration they give is limited. Furthermore, data from
oxygen profiles of several different tumour types need to be analyzed to determine whether it is possible to combine the individual data to arrive at a unique value that represents the oxygen status of a tumour. Such values could then be compared against thresholds that would have to be reached for optimal response to the different tumour treatment modalities. DUS and DLS in the present study were used to give relative blood volume and blood flow. These techniques could be further refined and standardized to yield absolute values of blood volume and blood flow in tumours. Nevertheless, given the central role that tumour oxygenation plays in the pathophysiology of tumours and therefore in prognosis and in the prediction of the tumour’s response to treatment, it is relevant and imperative to resolve the shortcomings of such an integrated approach, given the heterogeneity and complexity in tumour oxygenation.178

**Summary of Direct Invasive Measurements in Human Tumours**

A complete summary of relevant human clinical studies is included in Appendix E. Other tumours studied more than once fall into five broad categories (Table 2-6): squamous cell carcinoma of head and neck and cervix; soft tissue sarcomas, gliomas of the brain and prostate adenocarcinoma.
Table 2-6 Summary of studies in human tumours using invasive oxygen sensor probes

<table>
<thead>
<tr>
<th>Tumour Type</th>
<th>Number of studies and patients</th>
<th>Tumour pO$_2$ (range and median; mmHg)</th>
<th>Control Tissue pO$_2$ (range and median; mmHg)</th>
<th>Tumour Hypoxic Fraction (range and median; HP$_5$)</th>
<th>Tumour Hypoxic Fraction (range and median; HP$_{10}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervix</td>
<td>14 studies, 11,179-191 805 patients</td>
<td>4-12.5 (10)</td>
<td>10-19 (14.5)</td>
<td>48-65 (52)</td>
<td>48-61 (51)</td>
</tr>
<tr>
<td>Head and Neck</td>
<td>15 studies, 13,192-205 815 patients</td>
<td>7-44 (12.5)</td>
<td>36-67 (44)</td>
<td>30-74 (44)</td>
<td>0-64 (41)</td>
</tr>
<tr>
<td>Prostate</td>
<td>6 studies, 206-212 313 patients</td>
<td>2.4-10.8 (4.5)</td>
<td>27-30 (29)</td>
<td>14-60 (33)</td>
<td>21</td>
</tr>
<tr>
<td>Brain</td>
<td>5 studies, 8,213-217 96 patients</td>
<td>5-53 (12)</td>
<td>18</td>
<td>16-45 (37)</td>
<td>0-68 (37)</td>
</tr>
<tr>
<td>Soft tissue</td>
<td>5 studies, 12,14,218-220 67 patients</td>
<td>14-21 (21.5)</td>
<td>49</td>
<td>17-29 (23)</td>
<td>20-44 (34.5)</td>
</tr>
</tbody>
</table>

HP$_5$= percentage of readings ≤5mmHg; HP$_{10}$= percentage of readings ≤10mmHg;
Other studies with only one report in the literature include cancer of the vulva and breast and melanoma and pancreas. There is intra and inter-tumour heterogeneity reported for all tumours studied.

A brief summary of what is known about human tumours other than RCC is as follows:

- Human tumours have significant regions of hypoxia - this has been demonstrated in a variety of tumours such as cervical, breast, brain and prostate.

- The oxygen levels throughout tumours are heterogeneous and thus median or mean measures are better for quantifying tumours.

- Hypoxia is a dynamic state that may alter acutely or chronically within tumours.

- Hypoxic tumours in patients undergoing radiotherapy in some instances have a worse prognosis than those where the tumour oxygen status is more representative of normal tissue.

- The level of oxygen that defines a truly hypoxic tumour although considered to be 10mmHg is really a continuum and may differ for different tumours.

- Only tumours easily accessible from the skin have been accessed with direct invasive oxygen measurements with the one exception of a small study of pancreatic cancer (which demonstrated hypoxia) - little is known of solid abdominal organs such as the bowel, liver, spleen and kidneys.

*Summary of Direct Invasive Measurements in Xenograft Tumours*

A complete summary of relevant xenograft studies is included in Appendix C.
Other tumours studied in multiple studies fall into five broad categories: squamous cell carcinoma; soft tissue sarcomas, breast adenocarcinoma, gliomas of the brain and prostate adenocarcinoma with a smattering of other tumour types. Of the forty studies, the majority reported a $pO_2$ under 5mmHg in tumours but there was a wide range (1.8-37.8mmHg). The $HP_5$ and $HP_{10}$ was not routinely reported but ranged from 61-96% and 6-89% respectively; most measurements for both were over 70% when reported.

Here is a summary of current knowledge regarding xenografted tumours including RCC and what questions remain:

- Studies of xenografted tumours have been conducted using murine tumour cells and human tumour cells
- In general the tumour study demonstrate hypoxia and often at levels around 5mmHg or lower
- Tumour heterogeneity is apparent and as for human tumours global assessment rather than point measurements are required to assess any given tumour oxygen status
- Only one study exists with RCC and this is in a murine model using five mice using xenografted murine (not human) tumour cells
- No studies of RCC using human tumour cells xenografted into a murine model exist
2.6.8.1 Direct Invasive Measurements of Normal Renal Tissue Oxygen Tension

No studies have been conducted in humans apart from in a solitary transplant kidney with a $pO_2$ of 30mmHg. Studies of six dog kidneys have been performed that demonstrated heterogeneity with a mean $pO_2$ of 38mmHg.

2.6.8.2 Non-Invasive Measurements of Tissue Oxygen Tension

**Immunohistochemistry- quantifying hypoxia directly**

Immunohistochemical hypoxia marker techniques using markers avid for hypoxic cells have been investigated since 1981 when $^{14}$C-misonidazole, a radiolabelled analogue of nitromidazole, was used *in vitro* to demonstrate binding to hypoxic cells. Both pimonidazole and EF5 (analogues of nitromidazole) are now commonly used and provide a spatial map of the distributions of hypoxia in tissue. With this technique, biopsies of tumours are usually minced and incubated to provide a single cell suspension. Cells are then filtered, centrifuged and resuspended with pimonidazole binding solution. Visual scoring and flow cytometry to analyse for binding is undertaken. Pimonidazole studies may also be done using adduct immunohistochemistry.

An advantage of such hypoxia markers in comparison with oxygen electrode measurements is that markers can provide an indication of hypoxia at a single cell level. Problems of necrosis and the impact of normal tissue can therefore be minimised. The other theoretical advantage over electrode measurements is that markers are only selectively reduced in visible hypoxic tissues. The disadvantages are the requirement of an infusion 24-48 hours prior to surgery and there is no elimination of potential sampling errors. Furthermore, immunohistochemical markers will only give an impression of hypoxia in regions displaying chronic hypoxia whereas the polarographic oxygen electrodes may give information on acute and chronic hypoxia due to real time *in vivo* measuring.
Some data has been gathered to suggest that 2-nitromidazoles (either pimonidazole or EF5) are not totally reliable as extrinsic markers of hypoxia. This is because of the presence of necrotic areas that may preferentially bind the extrinsic markers or even keratinising areas where non-specified binding may occur.

Imaging of hypoxia
This will be discussed in section 2.11 where imaging of RCC and hypoxia are outlined in detail.

2.6.8.3 Surrogate endogenous markers of tumour oxygenation

Immunohistochemistry- quantifying hypoxia indirectly
Immunohistochemistry has become an important development in the detection of hypoxia in tumours. This involves the immunohistochemical detection of proteins whose expression is increased in hypoxic cells. Hypoxia stimulates the transcription of many genes including erythropoeitin, VEGF, enzymes involved in the glycolytic pathway, the glucose transport proteins (e.g. Glut-1) and genes that promote cell survival such as insulin-like growth factor. Immunohistochemistry gives the advantage of being able to not only detect tumour hypoxia without exogenous markers of hypoxia and also may enable the correlation of the expression of such hypoxia-induced proteins and clinical outcome. For example, the expression of HIF-1α has been correlated with clinical outcome in both head and neck cancers and cervical cancer. However, there has been conflicting evidence as to the reliability of HIF-1α as a surrogate intrinsic marker of oxygen status in cervical carcinomas that are readily accessible by oxygen probes. In one study, there was a lack of correlation between expression of HIF-1α and oxygenation status in identical tissue areas. Against this, in a small study, HIF-1α expression, CAIX, and glucose transporter 1 (Glut-1) were correlated with direct Eppendorf electrode measurements of tumour oxygenation in carcinoma of the
A further study has demonstrated a weak correlation with high HIF-1α expression and low pO2 within tumours.\textsuperscript{240}

Furthermore, CAIX (and for example one of its antibodies cG250) has emerged as a promising endogenous marker of hypoxia as it is strongly expressed in chronically hypoxic cells that are considered an adverse prognostic indicator in non-small cell lung cancer and head and neck cancer.\textsuperscript{241,242} Other markers such as VEGFs, and certain proliferation markers (e.g. Ki-67), play a role in tumour proliferation, tumour growth, angiogenesis, and loss of cell adhesion.\textsuperscript{243}

\textit{Serum Markers of Hypoxia- Osteopontin}

Very few serum markers of hypoxia exist except for osteopontin. This will be discussed at in the section 2.10.

\textbf{2.6.9 Hypoxia in Renal Cell Carcinoma}

Although RCC has been treated systemically with TKI exploiting pathways relating to hypoxia no invasive studies exploring measurements of tissue oxygen tension exist. Indirect measurements via imaging are discussed later.
2.7 Angiogenesis in Tumours

2.7.1 Molecular mechanisms underlying Renal Cell Carcinoma pathogenesis

Multiple intracellular signal transduction pathways operate to transmit and regulate extracellular messages that control cell proliferation, differentiation, and survival in normal cells. In RCC, as in other cancers, deregulation of one or more of these pathways occurs leading to tumour initiation and progression, inhibition of apoptosis, and tumour angiogenesis.244

These molecular pathways will now be explored in the context of angiogenesis. A further section detailing related molecular mechanisms will then be outlined.

2.7.2 Angiogenesis

Both tumours and normal tissues need a blood supply for oxygen, nutrients, and waste removal. However, whereas normal vasculature is hierarchically assembled into efficient networks of arteries, capillaries, and veins, the blood vessels of tumours are a mess being chaotic, leaky, inefficient, and barely functioning.245 Hence angiogenesis in tumours has been the focus of considerable interest both in understanding the basic biology of tumours, its role in tumour progression and prognosis and is a target for tumour therapy. It is not clear why there should be such a difference between vessel creation in normal and tumour tissues. Some evidence suggests that some tumour vessels have a defective cellular lining composed of disorganized, loosely connected, branched, overlapping or sprouting endothelial cells. Openings between these cells contribute to tumour vessel leakiness.38 Certainly, tumour vessels may lack the signals to mature but they could also have their maturation actively suppressed. 245 These hypotheses are still being investigated.
2.7.3 Regulation of Angiogenesis

Normal adult vasculature is generally quiescent in nature, with endothelial cells dividing approximately every ten years. In contrast, the growth of tumours requires constant vascular growth and remodeling in order for solid tumours to grow beyond 1-2 mm$^3$ in size.\textsuperscript{246} Thus neovascularisation (via angiogenesis) is critical for the growth of malignant tumours and for the development and survival of metastases.\textsuperscript{247} This was known from our earlier understanding of tumour biology.\textsuperscript{1} However, it was not until 1971 that Folkman first proposed that angiogenic substances may be responsible for angiogenesis by inducing the replication of endothelial cells.\textsuperscript{248} Since then, there have been landmark studies demonstrating the crucial role of angiogenesis in cancer biology\textsuperscript{245,249} that have helped to advance our understanding of tumour physiology and the tumour microenvironment.\textsuperscript{39}

The tumour microenvironment refers to the complex milieu of cellular and non-cellular components comprising fibroblasts, endothelial cells, immune cells and metabolites of cellular respiration in which tumour cells exist.\textsuperscript{250} Tumour microenvironment may be influenced by oxygen status, in particular, hypoxia as well as a multitude of other factors from pH to distance of cells from blood vessels.\textsuperscript{251} An elaborate interplay between these components and tumour cells exists with implications for immunological recognition of tumour cells.\textsuperscript{250} Hence the tumour microenvironment may act as a powerful selective force.\textsuperscript{39} For example, tumours have been shown to alter their antigen and cytokine profiles, desensitise and impair immune defences, signal fibroblasts to facilitate metastasis, and take advantage of acidic and hypoxic conditions that impede normal cells.\textsuperscript{250}

Spontaneously arising tumour cells are not usually angiogenic at first.\textsuperscript{252} Although angiogenesis occurs in a series of complex and interrelated steps,\textsuperscript{37} it is generally accepted that for a tumour to form its own blood supply, it undergoes two broad phases. The first is the pre-vascular phase, also known as the “angiogenic switch”.\textsuperscript{253,254} This is when a tumour acquires an angiogenic phenotype. After this switching phase has occurred, the tumour is
able to enter the second phase and induce angiogenesis within itself via the production of VEGF and other growth factors. Once new vasculature is formed in and around the tumour, it may grow rapidly and acquire metastatic potential.

The “angiogenic switch” involves more than simple upregulation of angiogenic activity and is thought to be the result of a net balance of positive and negative regulators. Tumour growth is although to require disruption of this balance and hence this switch must turned on for cancer progression.

Like other homeostatic processes within the human body, angiogenesis is regulated by a balance between endogenous pro-angiogenic and anti-angiogenic factors. Endothelial cells, the crosstalk between angiogenic factors and their receptors and the interaction between vasculogenesis and lymphangiogenesis are all factors that may contribute to the switch. When the “angiogenic switch” is turned on to facilitate tumour growth, the equilibrium between pro-angiogenic and anti-angiogenic factors has been disrupted, resulting in increased secretion of the former and decreased of the latter. Pro-angiogenic growth factors bind to endothelial cells causing basement membrane degeneration, endothelial cell proliferation and migration and ultimately the formation of new blood vessels by tubule formation and extra-cellular matrix remodeling.

At present the focus for investigation and therapy regarding angiogenesis is concentrated on discovering pro-angiogenic agents and blocking them. Combining agents acting on different targets in the angiogenic process for the treatment of RCC is now being investigated as a treatment modality.
2.7.4 Angiogenesis and Hypoxia

Tumours like all living tissues require oxygen. Although a tumour may obtain oxygen by direct diffusion from its environment its major supply is via a blood supply derived from its host. When a tumour reaches a particular size its oxygen consumption will outstrip oxygen diffusion through tissue at which point the tumour will die. In the absence of a blood supply a tumour will not enlarge beyond two to four millimetres. Thus, a tumour must maintain a vascular supply to sustain itself.

Paradoxically, tumour progression is associated with both increased microvessel density and intratumoral hypoxia. Thus, even in the presence of a blood supply a tumour can remain hypoxic. The apparent paradox may be explained in a number of ways. Firstly, the distance of a tumour from its blood supply is important. Thomlinson and Gray demonstrated that necrosis occurred 100-150µm from a blood vessel and that tumour cells adjacent to these necrotic areas were viable but hypoxic, forming a peri-necrotic area of hypoxic cells.

Secondly, tumour vasculature is usually more permeable than normal, which leads to an increased amount of leakage into the interstitial space, thereby limiting oxygen diffusion. Inratumoural pressure may also be raised in conjunction with this phenomenon further inhibiting diffusion. Also, poorly developed blood vessels within a tumour that open and close intermittently will deliver oxygen sporadically, also contributing to tumour hypoxia. Finally, the tumour vessels created have been described as highly irregular, tortuous having arterio-venous shunting, blind ends and increased intercapillary distances.

Clearly, the microenvironmental physiology of tumours is quite different from that of normal tissues. These structural and physiological abnormalities result in low oxygen perfusion in some areas of tumour tissue thereby becoming hypoxic. Eventually, these processes may lead to necrosis in some instances. Furthermore, treatment with antiangiogenic agents may lead to a change in tumour vascularity and oxygenation without a change in tumour size.
2.7.5 Angiogenesis and Autocrine loops in Cancer progression

Many of the tumour markers identified and associated with angiogenesis are involved in autocrine loops (e.g. VEGF). Such loops are mediated by growth factors or mitogens, and are usually secreted by a few specialized cells to induce cell proliferation in paracrine, autocrine, or even endocrine manner. If a cell that usually does not produce growth factors suddenly starts to do so (because it developed an oncogene or had a suppressor gene such as VHL blocked), it will thereby induce its own uncontrolled proliferation (autocrine loop), as well as the proliferation of neighboring cells (paracrine stimulation).

2.7.6 Angiogenesis as a Target for Cancer treatment

Several strategies for the development of anti-angiogenic therapeutic modalities have been employed, including agents that (1) decrease the activity of specific angiogenic factors, (2) decrease the activity of endothelial survival factors, (3) increase the activity of naturally occurring anti-angiogenic agents, or (4) indirectly downregulate angiogenic and survival factor activity.

2.7.6.1 Antiangiogenic and vascular disrupting therapies

The term "antiangiogenesis" was introduced to describe treatments designed to prevent the induction of new blood vessels and perhaps reduce the number of those already present. These are distinct from vascular disrupting agents (VDA) that are a new class of potential anticancer drugs that selectively destroy tumour vasculature and shut down blood supply to solid tumours, causing extensive tumour cell necrosis. VDA are rapidly being developed and have moved from animal models to clinical trials of human malignancies and may offer an alternative or complimentary modality to antiangiogenic agents in the near future.

Several approaches inhibit tumour angiogenesis and multiple antiangiogenic compounds have been clinically evaluated. Some consider anti-angiogenic therapy as the seventh
therapeutic modality in the treatment of cancers in addition to surgery, chemotherapy, radiotherapy, hormonal manipulation, vaccines and immunotherapy. The excitement and renewed interest in RCC and its biology combined with anti-angiogenic agents for treatment has lead to the development of specific international conferences, such as the First International Conference on Innovations and Challenges in Renal Cancer, held in 2004 and again in 2006.

The most effective anti-angiogenic agents have promiscuous activity blocking multiple targets (e.g. VEGF and PDGF). However, mutant tumour cells may over time and produce redundant angiogenic factors. Therefore, for long-term use in cancer, combinations of angiogenesis inhibitors or broad spectrum angiogenesis inhibitors will be needed.

2.7.6.2 Concept of vessel “normalization” with antiangiogenic agents
There is now a body of literature arguing that that antiangiogenic agents can decrease tumour vessel permeability and interstitial fluid pressure in a process of vessel “normalization.” The resulting normalized vasculature has more efficient perfusion, but little is known about how tumour interstitial fluid pressure and interstitial fluid velocity are affected by changes in transport properties of the vessels and interstitium that are associated with antiangiogenic therapy. So, in essence the agents are not antiangiogenic by starving tumours of supply but by remodelling and perhaps restoring some degree of “normality”. This in itself may open a window of opportunity for other agents including chemotherapeutics to act more cohesively by being delivered more efficiently to the tumour than when the vessels were leaky and disorganised. There is evidence for this approach in colorectal cancer but it has not been attempted in RCC as yet.
2.8 Tyrosine Kinase Inhibitors and other small molecules

Until recently, an incomplete understanding of the molecular mechanisms underlying RCC has limited the development of successful non-surgical therapies. However, a better understanding of cancer genetics helped identify protein targets within certain patient populations in which well-defined and specific therapy has demonstrated a robust clinical response. To date, the most commonly defined protein targets have been growth factor signaling pathways that involve receptor tyrosine kinases (RTKs) on the surface of epithelial or stromal cells and their cognate ligands (Figure 2-3). Such receptors and their downstream targets, namely, signaling through vascular endothelial growth factor (VEGF) in endothelial cells, platelet-derived growth factor (PDGF) in endothelial cells and pericytes, and the epidermal growth factor receptor (EGFR) pathway or other ligand/receptor pathways in tumour cells are all reasonable and rational therapeutic targets.

Using this information, the management of metastatic RCC has undergone a “revolution” in recent years due to targeted therapies with molecules named tyrosine kinase inhibitors (TKI), because they exploit the RTK pathway. Currently, there are several approaches to the inhibition of RTK (Figure 2-4): small-molecule inhibitors of the intracellular tyrosine kinase, and monoclonal antibodies that target the extracellular domain. Anti-ligand antibodies such as bevacizumab have also been developed to bind to and inhibit VEGF. Finally, fusion proteins such as aflibercept (VEGF Trap) also act as decoys to the receptor and interfere with binding of VEGF

Measuring response to chemotherapy and immunotherapeutic agents is normally conducted using imaging. The most commonly used classification to monitor the effect of systemic therapies on the growth of solid tumours is the Response Evaluation Criteria in Solid Tumours (RECIST) criteria. TKI have used this classification. This classification system gives rise to terms such as complete or partial response.
Tyrosine kinase receptor and where small molecules may impact upon them: 
A) Anti-angiogenic factors acting at a ligand level; 
B) Anti-ligand or anti-receptor antibodies and 
C) The Tyrosine Kinase Inhibitors
More recently, the term “biological response modifier” has entered medical parlance and reflects the mechanism and effects of the treatment options outlined. Biological response modifiers can act passively by enhancing the immunologic response to tumour cells or actively by altering the differentiation/growth of tumour cells. Thus with such a mechanism of action whereby the tumour is starved of nutrients and oxygen, anti-angiogenic agents may be tumouristatic rather than tumouricidal which is not ideal considering cancer is a chronic disease requiring ongoing treatment unless complete cure is achieved.

2.8.1 Receptor Tyrosine Kinases as Targets for therapy

Tyrosine kinases play a central role in the activation of signal transduction pathways and cellular responses that are critical in oncogenesis.

The TKI are low-molecular-weight, ATP-mimetic proteins that bind to the ATP-binding catalytic site of the tyrosine kinase domain of receptors, resulting in blockade of intracellular signaling. TKI may target a specific receptor or have targets on multiple receptors. The RTK need to break into sub-units for activation of the internal TK domain, which triggers a cascade of phosphorylation and activation of signal transduction pathways. The best characterized intracellular pathways activated by EGFR are the Ras-Raf-mitogen-activated phosphokinase pathway that leads to cell division, and the PI3K/Akt pathway that results in stimulation of angiogenesis, survival and promotion of invasion. Their high expression in tumours including RCC makes them appealing anti-tumour targets.

The number of RTK identified is well over 100 with mutations and overexpression of TK regularly found in human malignancies. However, agents that specifically inhibit the activity of these molecules have been clinically developed for only a few tyrosine kinases. Some examples of clinically targeted RTKs include the EGFR, the closely related HER-2 (ErbB-2/Neu), platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR), and c-kit/stem cell factor receptor. These new targeted
therapies are designed to take advantage of the molecular differences specific to tumour cells compared with healthy tissues. The goal is to achieve better tumour responses with improved safety profiles than those associated with cytotoxic chemotherapies. As outlined in Figure 2-4, there are several points where tyrosine kinases may act. Specifically, TKI act on the receptor themselves interfering with binding and signaling whilst antibodies and anti-angiogenic factors act prior to binding of the ligand to the receptor.

2.8.2 Advantages and side effects of Tyrosine Kinase Inhibitors

Approaching tumours by targeting the tyrosine kinase receptor (RTK) has potential advantages. Firstly, the agents provide a novel mechanism of action that is different from other immune modulating mechanisms that have proven to have minor, if any benefits to date. Secondly, such agents tend not to be genotoxic and target endothelial cells, which are more stable than malignantly transformed cancer cells. Therefore the risk of developing secondary mutations as a mechanism of resistance to TKI is theoretically lower than for standard chemotherapeutics but this has not been borne out when such agents are used in other malignancies (e.g. gastrointestinal stromal tumours).²⁹³

The question of cross resistance among the TKIs has not been resolved in a recent study, twenty-nine patients received sorafenib followed by sunitinib (group A) and 20 patients received sunitinib followed by sorafenib (Group B). The median duration of stable disease for Groups A and B was 20 and 9.5 weeks, respectively. Median time from starting first TKI to disease progression after second TKI (time to progression) in Groups A and B was 78 and 37 weeks, respectively. Multivariate analysis revealed that Group B had a shorter time to progression than Group A (risk ratio [RR] 3.0; P=.016). Median overall survival was 102 and 45 weeks in Groups A and B, respectively (P=.061). They concluded that the longer duration of disease control in patients who received sorafenib followed by sunitinib warrants further investigation.²⁹⁴ Conversely in a smaller but similar trial the efficacy data of second-line sunitinib were close to published results of first-line treatment, suggesting limited clinically relevant cross-resistance.²⁹⁵
The experienced side effects of such agents are unique and relatively non-overlapping with standard cytotoxic chemotherapeutic agents. This opens the window in the future for combination treatments either with multiple tyrosine kinase inhibitors and similar agents and/or with standard chemotherapeutic agents.\textsuperscript{286} TKI are relatively well tolerated with common side effects being fatigue, diarrhea, mild neutropaenia, elevation of serum lipase, and anemia. Hand-foot syndrome is also common but variable in extent. Hypertension and cardiac ischemia are relatively infrequent and deaths are extremely rare secondary to the TKI.\textsuperscript{296,297}

### 2.8.3 Tyrosine Kinase Inhibitors in Renal Cell Carcinoma

As RTK (e.g. EGFR) are expressed in up to 90% of clear cell RCCs makes appealing the theory that this receptor might act as a potential antitumour target.\textsuperscript{290} The first specific selective tyrosine kinase inhibitor for clinical investigation was gefitinib (Iressa, AstraZeneca, Macclesfield, United Kingdom). It is orally active, acts on the EGFR and the antitumour activity of gefitinib has been assessed in human RCC models.\textsuperscript{298,299} In an early trial evaluating 18 patients with RCC they reported no complete or partial responses, whilst 13 had progression of disease within 4 months of the commencement of therapy.\textsuperscript{300} Antibodies directed against the EGFR, such as cetuximab, have shown very limited antitumour effect in RCC. The TKI field has since developed further and rather than inhibiting just one RTK they have action at multiple RTK.\textsuperscript{301}

In Figure 2-4, the major TKI agents and where they exploit the pVHL pathway as targets for cancer therapy are indicated. This is a key diagram as it links two major processes: the
Figure 2-4 Sites of action of Tyrosine Kinase Inhibitors and other small molecules

![Diagram showing sites of action of Tyrosine Kinase Inhibitors](image)

Table 2-7 Targeted agents in RCC demonstrating clinical efficacy in phase III human trials

<table>
<thead>
<tr>
<th>Targeted Agents</th>
<th>Line of treatment</th>
<th>Overall Survival (months)</th>
<th>Progression-Free Survival (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorafenib vs. placebo</td>
<td>2nd</td>
<td>17.8 vs. 14.3</td>
<td>5.5 vs. 2.8*</td>
</tr>
<tr>
<td>Sunitinib vs. Interferon (IFN)</td>
<td>1st</td>
<td>NA</td>
<td>11 vs. 5.1*</td>
</tr>
<tr>
<td>Temsirolimus vs. IFN</td>
<td>1st</td>
<td>10.9 vs. 7.3*</td>
<td>5.5 vs. 3.1*</td>
</tr>
<tr>
<td>Bevacizumab + IFN vs. placebo + IFN</td>
<td>1st</td>
<td>NA vs. 19.8</td>
<td>10.2 vs. 5.4*</td>
</tr>
<tr>
<td>Bevacizumab + IFN vs. IFN</td>
<td>1st</td>
<td>NA</td>
<td>8.5 vs. 5.2*</td>
</tr>
<tr>
<td>Everolimus (RAD001) vs. placebo</td>
<td>2nd</td>
<td>NA vs. 8.8</td>
<td>4 vs. 19</td>
</tr>
</tbody>
</table>

* Statistically significant, NA = not available
pVHL/HIF and hypoxia axis with the downstream stimulation RTK and their activities which include the promotion of angiogenesis (e.g. through VEGF, PDGF and other molecules).

The most widely clinically investigated and current therapeutic TKI and other small molecules will now be addressed in the setting of clear cell RCC.

There are now several published studies of five targeted agents that have shown evidence of clinical efficacy in RCC in phase III studies (Table 2-7). Each agent will be discussed in further detail.

2.8.3.1 Sunitinib in Renal cell Carcinoma
Sunitinib, is a first-line oral agent for use in advanced or metastatic RCC and is a multi-targeted tyrosine kinase inhibitor of RTK implicated in tumour angiogenesis, tumour growth and metastatic progression. \(^\text{307,308}\) received sunitinib. In the most recent study results, large phase 3 study of sunitinib versus interferon-\(\alpha\) until disease progression as first-line systemic therapy in metastatic RCC. The duration of progression free survival in the sunitinib versus the interferon-\(\alpha\) group was better at 11 months versus 4 months respectively. Partial responders in the sunitinib group were higher (43% versus 9%) but complete responders were similar (0.5% versus 1.6%). Overall survival data was not a part of the study. Sunitinib did confer the greatest advantage in the groups with fewer MSKCC risk factors having progression free survival at 14 months versus 8 months compared to similar patients in the interferon-\(\alpha\) group. \(^\text{302}\) Results have supported the efficacy and manageable adverse-event profile of sunitinib as a single agent in second-line therapy for patients with cytokine-refractory metastatic clear-cell RCC. \(^\text{296}\)

2.8.3.2 Sorafenib in Renal Cell Carcinoma
Sorafenib is a multikinase inhibitor of tumour-cell proliferation and angiogenesis with its effects mediated by inhibiting targets such as VEGFR and PDGFR as well as Raf Kinase. In
a phase 3, randomized, double-blind, placebo-controlled trial of sorafenib, in 903 patients with advanced clear-cell renal-cell carcinoma that was resistant to standard immunotherapy received either continuous treatment with oral sorafenib or placebo- the primary end point was overall survival. The median progression-free survival was 5.5 months in the sorafenib group and 2.8 months in the placebo group. Partial responses were reported as the best response in 10% of patients receiving sorafenib and in 2% of those receiving placebo. It is considered a second-line agent when other treatments (e.g. sunitinib or immunotherapy) have failed although more data is required.

2.8.4 Other small molecules in Renal Cell Carcinoma

2.8.4.1 Bevacizumab

Bevacizumab is a monoclonal antibody developed to bind to and inhibit VEGF (VEGF-A) thus interfering with blood vessel networks that supply tumour cells as the lack of a constant source of blood may slow tumour growth. Bevacizumab (Avastin™) was the first U.S. Food and Drug Administration (FDA) approved biological therapy designed to inhibit the formation of new blood vessels to tumours.

A randomised trial of 649 patients of good performance status with metastatic RCC treated only by nephrectomy or partial nephrectomy received bevacizumab combined with interferon-α or interferon-α with placebo. The duration of progression free survival in the bevacizumab combined with interferon-α group was better at 10.2 months versus 5.4 months respectively. Overall survival data was not part of the first interim analysis. Partial responses in the bevacizumab group were higher (32% versus 11%) but complete responders were similar (1% versus 2%).

In a previous phase II trial, bevacizumab monotherapy was demonstrated to improve progression free survival in patients previously treated with immunotherapy to 4.8 months compared to placebo at 2.5 months. Bevacizumab has also been combined with erlotinib, an epidermal growth factor receptor TKI with a median survival of 18 months in a trial of 63
patients but more data are required.\textsuperscript{310}

2.8.4.2 Aflibercept (VEGF TRAP)

Aflibercept is a protein comprised of segments of the extracellular domains of human VEGF receptors VEGF-R1 and R2 fused to the constant region (Fc) of human IgG1 with potential antiangiogenic activity.\textsuperscript{311} Aflibercept, functioning as a soluble decoy receptor, binds to proangiogenic VEGFs, thereby preventing them from binding to their cell receptors. Disruption of the binding of VEGFs to their cell receptors may result in the inhibition of tumour angiogenesis, metastasis, and ultimately tumour regression.\textsuperscript{283,312} A further interesting point is that unlike antibody complexes that are usually rapidly cleared, the Aflibercept forms inert complexes with tissue- and tumour-derived VEGF that remain stably in the systemic circulation, where they are readily assayable, providing unprecedented capability to accurately measure VEGF production.\textsuperscript{313}

2.8.4.3 Temsirolimus in Renal Cell Carcinoma

Temsirolimus is a specific inhibitor of mTOR (mammalian target of rapamycin) kinase, a regulator of HIF-1\textsubscript{\alpha} (causing G1 cell-cycle arrest).\textsuperscript{314-316} As such, the mTOR is a key signalling node coordinating cell cycle progression and cell growth in response to genetic, epigenetic, and environmental conditions. Pathways involved in mTOR signalling are dysregulated in precancerous human tissues. Cell arrest may be dysregulated and conversely cell growth are mediated.\textsuperscript{317}

The history of the mTOR is interesting because it began with discovery in the 1960s by a medical expedition to \textit{Rapa Nui} (Easter Island) leading to the discovery of rapamycin in soil samples.\textsuperscript{318} A decade later characterization of rapamycin’s antifungal properties was realised but it was not until the 1980’s that rapamycin’s anti-tumour effects first documented.\textsuperscript{319} By 1991 TOR was first identified in yeast\textsuperscript{320} and then the elucidated mechanism of action of rapamycin was proposed\textsuperscript{321} followed by the mTOR identification in 1995.\textsuperscript{322} In 1999 the
USFDA approved the first mTOR inhibitor, sirolimus, as an immunosuppressant. Temsirolimus followed in 2007 with approval but as the first anti-neoplastic agent.

Temsirolimus is administered intravenously and has been studied in recommended in the National Comprehensive Cancer Network (NCCN) and European Association of Urology (EAU) guidelines for poor risk patients (Table 2-8). In the largest study, a phase 3 study of 626 patients with poor prognosis metastatic RCC, patients were randomised to temsirolimus or interferon-α or both. At interim analysis the median progression-free survival was 3.9 versus 1.9 and 3.7 months respectively. Everolimus is another mTOR inhibitor being investigated and has just had phase three trial results published as outlined above in Table 2-7.

2.8.5 Second generation Tyrosine Kinase Inhibitors
The first of the “second generation” TKI inhibitors is pazopanib and this is currently being investigated in clinical trials for patients with metastatic RCC. It is another multi-targeted TKI against VEGF-R-1/2/3, platelet-derived growth factor receptor (PDGFR)-alpha, PDGFR-beta and c-kit. There are far too many other agents under development to detail but many will enter phase II and III trials shortly.

2.8.6 Guidelines for the use of Tyrosine Kinase Inhibitors and small molecules
Reflecting the number of trials published, current EAU guidelines for management of RCC have incorporated TKI into treatment recommendations aided by the MSKCC risk classification system. The 2008 NCCN guidelines have also included TKI and small molecules (Table 2-8).
Table 2-8 Tyrosine kinase inhibitors in Renal Cell Carcinoma—guideline recommendations

<table>
<thead>
<tr>
<th>Metastatic RCC Setting</th>
<th>Sunitinib</th>
<th>Sorafenib</th>
<th>Temsirolimus</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FIRST LINE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Risk Profile (MSKCC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good (no factors)</td>
<td>EAU Indicated</td>
<td></td>
<td>EAU Indicated</td>
</tr>
<tr>
<td>Intermediate (1-2 factors)</td>
<td>EAU Indicated</td>
<td></td>
<td>EAU Indicated</td>
</tr>
<tr>
<td>Poor (3 or more)</td>
<td>EAU Indicated</td>
<td></td>
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<tr>
<td><strong>SECOND LINE</strong></td>
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<td>NCCN Indicated#</td>
<td>EAU Indicated</td>
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<tr>
<td></td>
<td></td>
<td>NCCN Indicated#</td>
<td></td>
</tr>
</tbody>
</table>

*MSKCC risk factors: low serum haemoglobin levels, elevated corrected serum calcium levels, elevated serum lactate dehydrogenase levels, poor performance status and intervals <1 year between diagnosis and treatment.

# Only category I NCCN indications included in this table
In summary, despite recent advances, there is a need for improved strategies to integrate anti-EGFR agents with conventional therapies and to explore combinations with other molecular targeted approaches including other antireceptor therapies, receptor-downstream signaling transduction inhibitors, and targeted approaches interfering with other essential drivers of cancer, such as angiogenesis.\textsuperscript{329}

By no means has the use of TKI abrogated our need to understand the biology behind TKI. In fact it has stimulated further questions and a need to go behind the clinical trials and marketing.\textsuperscript{330} Concerns about the cost and side effect of combination therapy have lead to studies investigating sequencing of agents such that full doses of agents may be given whilst avoiding the toxicity and side effects.\textsuperscript{294,331,332} The landscape is changing rapidly and already new proposals above those in guidelines are being outlined (Table 2-9 adapted from Bellmunt\textsuperscript{333})

It is important to acknowledge that patients enrolled into phase III trials may have different characteristics to those of the clinical population i.e. patients in the clinic will have heterogenous characteristics unlikely to match any phase III population.\textsuperscript{333} No single TKI or small molecule will benefit all patients. Evidence shows that as little as 24\% of treatment decisions are based on level 1 evidence\textsuperscript{334}. Physicians should consider data beyond those from phase III trials when making treatment decisions.\textsuperscript{333}

\textbf{2.8.7 Tyrosine Kinase Inhibitors in Non-clear cell Renal Cell Carcinoma}

Non-clear cell RCC accounts for approximately 25\% of all patients with metastatic RCC. It is refractory to standard immuno(chemo)therapy and, to date, no specific trials have been reported to evaluate the efficacy of novel targeted drugs in the different subtypes of metastatic nccRCC\textsuperscript{335} Despite this, the 2008 NCCN guidelines have also included TKI and small molecules as options for non-clear cell RCC.\textsuperscript{328}
<table>
<thead>
<tr>
<th>Clinical Setting of metastatic RCC patient</th>
<th>Patient Group</th>
<th>Therapy (Level 1 evidence)</th>
<th>Other therapy options (≥ level 2 evidence)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>Good or intermediate risk</td>
<td>Sunitinib Bevacizumab ± Interferon alpha</td>
<td>High dose interleukin-2 Sorafenib Clinical Trial Observation</td>
</tr>
<tr>
<td></td>
<td>Poor risk</td>
<td>Temsirolimus</td>
<td>Sunitinib Clinical Trial</td>
</tr>
<tr>
<td>Refractory</td>
<td>Cytokine</td>
<td>Sorafenib</td>
<td>Sunitinib±Bevacizumab</td>
</tr>
<tr>
<td></td>
<td>VEGF; mTOR</td>
<td>Everolimus Clinical Trial</td>
<td>All options above</td>
</tr>
</tbody>
</table>
2.8.7.1 **Summary and future of TKI in RCC**

In clear cell RCC complete responses are rare but in pivotal or supportive studies but partial responses of between 25-38% have been reported in patients having radical nephrectomy and being refractory to cytokine treatment.\(^{336}\) Measuring response to these new therapies is normally conducted using imaging and the most commonly used system today is the RECIST.\(^{284}\) Hence disease stabilisation\(^ {337}\) has been used in the context of TKI rather than complete response or cure.

However, the RECIST criteria may not be an ideal way of assessing response to TKI and small molecules, particularly in RCC. Molecular and functional imaging (such as PET-CT) has added a new dimension to cancer research. Biological information is needed to incorporate in future RECIST criteria, thereby providing a realistic evaluation method for treatment response.\(^ {338}\) The classic example for TKI in RCC being that loss of function of tumour perhaps detected on functional imaging may provide better prognostic information than shrinkage; or the ability to distinguish scar tissue from functional tumour may alter management decisions such as when to perform surgery rather than considering all tissue residual tumour. Future studies should incorporate functional imaging so such questions may be answered.

### 2.9 Molecular mechanisms underlying Renal Cell Carcinoma

Multiple intracellular signal transduction pathways operate to transmit and regulate extracellular messages that control cell proliferation, differentiation, and survival in normal cells. In RCC, as in other cancers, deregulation of one or more of these pathways occurs leading to tumour initiation and progression, inhibition of apoptosis, and tumour angiogenesis.\(^ {244}\)

These molecular pathways in relation to RCC will now be explored.
2.9.1 Hypoxia Inducible Factor-1 (HIF-1)

HIF-1 is a heterodimer composed of two subunits, HIF-1α and HIF-1β, which are basic helix-loop-helix-PAS domain proteins. HIF-1β is constitutively expressed whereas the expression of HIF-1α is maintained at low levels in most cells under normoxic conditions (Figure 2-2).^39^

2.9.2 HIF-1 regulation

HIF-1 is a transcriptional complex that mediates a broad range of cellular and systemic responses to hypoxia. HIF-1 activity depends mainly on the intracellular level of HIF-1α protein, which is regulated to be in inverse relation to the oxygen concentration by an oxygen-dependent enzyme, prolyl hydroxylase 2 (PHD2). Thus, cells respond to tissue hypoxia by sensing the oxygen concentration as the enzyme activity of PHD2, regulating the HIF-1 activity and consequently changing the expression of various hypoxia-responsive molecules (Figure 2-2).^39,339^
Table 2-10 Mechanisms responsible for increased HIF-1α expression in cancer cells

<table>
<thead>
<tr>
<th>Alteration in tumour</th>
<th>Molecular mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxia</td>
<td>Decreased ubiquitination</td>
</tr>
<tr>
<td>pVHL loss-of-function</td>
<td>Decreased ubiquitination</td>
</tr>
<tr>
<td>p53 loss-of-function</td>
<td>Decreased ubiquitination</td>
</tr>
<tr>
<td>PTEN loss-of-function</td>
<td>Increased synthesis</td>
</tr>
<tr>
<td>P13K/AKT/FRAP signalling</td>
<td>Increased synthesis</td>
</tr>
<tr>
<td>SRC gain-of-function</td>
<td>Increased synthesis</td>
</tr>
<tr>
<td>P14ARF loss-of-function</td>
<td>Decreased nucleolar sequestration</td>
</tr>
</tbody>
</table>

p53= tumour suppressor protein  
PTEN= phosphate and tensin homolog deleted on chromosome 10  
Pl3K-phosphatidylinositol-3-kinase  
AKT= protein kinase B  
FRAP= FKBP-rapamycin-associated protein
In summary, HIF is a heterodimeric transcriptional activator consisting of HIF-1α and HIF-1β subunits. In response to hypoxia, stimulation of growth factors, and activation of oncogenes as well as carcinogens, HIF-1α is overexpressed and/or activated and targets those genes which are required for angiogenesis, metabolic adaptation to low oxygen and promotes survival. HIF-1 is critical for both physiological and pathological processes. Cellular response controlled by hypoxia-HIF-1 cascade is involved in pathological situations such as solid tumour growth, diabetic retinopathy and rheumatoid arthritis. Under these pathological situations, the activation of hypoxia-HIF-1 cascade often leads to the acceleration of disease progression. The cascade induces the activation of key regulations systems through more than 40 proteins, including members of the VEGF, glucose transporter (e.g. Glut-1) and carbonic anhydrase (CA) family in the respective tumour cells. Related to this is cG250 that forms the antibody against CAIX.

2.9.2.1 HIF-1α and Tumour progression
Semenza has outlined four lines of evidence indicate that HIF-1 contributes to tumour progression:
1) HIF-1 controls the expression of gene products that stimulate angiogenesis, such as vascular endothelial growth factor, and promote metabolic adaptation to hypoxia, such as glucose transporters and glycolytic enzymes, thus providing a molecular basis for involvement of HIF-1 in tumour growth and angiogenesis
2) In mouse xenograft models, tumour growth and angiogenesis are inhibited by loss of HIF-1 activity and stimulated by HIF-1alpha overexpression
3) Immunohistochemical analyses of human tumour biopsies indicate that HIF-1alpha is overexpressed in common cancers and that the level of expression is correlated with tumour grade, angiogenesis, and mortality
4) In addition to intratumoral hypoxia, genetic alterations in tumour suppressor genes and oncogenes induce HIF-1 activity.
On the last point 4), the HIF-1α subunit of the HIF-1 transcription factor is particularly attractive as an agent to investigate and understand because the mechanisms of protein accumulation under hypoxic conditions are well characterized at the molecular level.\textsuperscript{234,342}

2.9.2.2 HIF-1α expression in human cancers

Many common tumours display dramatic overexpression of HIF-1α when analysed immunohistochemically.\textsuperscript{343} This is a consequence of both intratumoral hypoxia and genetic alterations. Hypoxia appears to be a universal stimulus for HIF-1α expression whilst the effect of genetic alterations (other than the VHL loss-of-function) appear to be dependent on complex signal transduction pathways that are active in any particular tumour cell (Table 2-11).\textsuperscript{85}

It is likely that HIF-1α overexpression contributes to the “angiogenic switch” in tumours. This is supported but not confirmed by increased vascular density and HIF-1α overexpression in brain, head and neck and ovarian carcinoma.\textsuperscript{344-346} HIF-1α overexpression is also associated with an increased risk of mortality and treatment failure (Table 2-11) although there is no certainty of the exact underlying mechanism. Finally, it is possible that more than one mechanism (Table 2-10) will be active in any particular tumour cell population. For example, in oesophageal cancer it was the HIF-1α overexpression when combined with BCL2 overexpression that was associated with treatment failure.\textsuperscript{347}

To further add to matters, some squamous cell cancers have demonstrated better prognosis with HIF-1α overexpression in early stages.\textsuperscript{237,341} The conclusion is that these tumours are not hypoxic and that the expression of HIF-1α in oral SCC is rather oncogene/tumour suppressor gene related rather than hypoxia-driven due to a lack of downstream expression of targets of HIF-1α expression e.g. CAIX.\textsuperscript{341}
### Table 2-11 Association of HIF-1α overexpression with adverse clinical outcome

<table>
<thead>
<tr>
<th>Tumour Type</th>
<th>Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast cancer, lymph node negative</td>
<td>Mortality</td>
</tr>
<tr>
<td>Cervical cancer, early stage</td>
<td>Mortality</td>
</tr>
<tr>
<td>Oligodendroglioma</td>
<td>Mortality</td>
</tr>
<tr>
<td>Oropharyngeal squamous cell carcinoma</td>
<td>Treatment failure, Mortality</td>
</tr>
<tr>
<td>Oesophageal cancer, early stage, BCL2-positive</td>
<td>Treatment failure</td>
</tr>
<tr>
<td>Ovarian cancer, p53 mutant</td>
<td>Mortality</td>
</tr>
</tbody>
</table>

BCL2= B-cell leukemia/lymphoma gene (encodes anti-apoptotic protein)  
p53= tumour suppressor protein
HIF-1α has also been found to have no prognostic significance in locally advanced carcinoma of the cervix.\textsuperscript{240} An hypothesis would be that a possible switch in large tumours for an association between high HIF-1α expression and good outcome relates to tumour size-related changes in the balance of genes up-regulated by HIF-1α. Whereas angiogenesis-promoting genes might be preferentially up-regulated in small tumours, proapoptotic genes might be induced in large tumours. This hypothesis needs testing in future work.\textsuperscript{240}

Recent research into breast cancer has proposed that different regulation pathways of HIF-1α overexpression may exist.\textsuperscript{3} Firstly, hypoxia induced, perinecrotic HIF-1α overexpression with strong expression of hypoxia associated genes (CAIX and Glut-1), which is associated with a poor prognosis. Secondly, diffuse HIF-1α overexpression lacking major hypoxia associated downstream effects, resulting in a more favorable prognosis. This may help to explain differential expression in some tumour types and the effects on prognosis and why correlation between the level of HIF-1α expression in some tumours and patient outcome, using disease-free survival as the end point may not always be easily linked.\textsuperscript{348}

2.9.2.3 HIF-1α expression in Renal Cell Carcinoma

HIF-1α protein expression was found in 40% of clear cell RCC with 80% of them having VHL mutations.\textsuperscript{349} In RCC harboring VHL mutations/hypermethylation, over-expression of HIF-1α is reported.\textsuperscript{350} HIF-1α expression has further been correlated directly with higher levels of VEGF production.\textsuperscript{349} These data indicate that HIF-1α is involved in tumourigenesis and progression of RCC.

When examined closely, one would therefore expect HIF-1α to correlate with stage and prognosis. This is not the case. HIF-1α expression in RCC has been analysed by Western blot in tissue where there was no association between HIF-1α and gender, stage, grade, tumour size, or vein invasion. Conventional clear cell RCCs had significantly higher HIF-1α expression compared with papillary and chromophobe types. In clear cell RCC, HIF-1α was
a contributing prognostic factor for favorable prognosis. However, concerning angiogenesis, no single angiogenic factor has been shown as an independent prognostic marker in RCC.

2.9.2.4 HIF-2α and Renal Cell Carcinoma

Much is known of HIF-1α, not of HIF-2α. Recent reports have led to the suggestion that HIF-2α may be the oncogenic form of HIF, at least in human RCC. Other evidence is emerging between the role of HIF-2α and its ability to promote tumourigenesis of VHL-/- RCC cells by constitutively activating the TGF-α/EGFR oncogenic pathway. It appears that transient and stable silencing of EGFR is sufficient to prevent HIF-2α-dependent tumourigenesis in multiple VHL-/- RCC cell lines. To support this association, it is likely that accumulation of endogenous HIF-2α, likely the result of saturation of the h-domain of VHL by overproduced wild-type HIF-1α activation and proliferation. HIF-2α potentially promotes autonomous growth of RCC cells by specifically activating the TGF-α/EGFR pathway. In summary, EGFR is a central downstream target of HIF-dependent tumourigenesis.

2.9.2.5 Inhibitors of hypoxia-inducible factor-1α (HIF-1α)

Only one inhibitor of HIF-1α, PX-478, has been tested in human tumour xenografts, with good effect.

2.9.3 Vascular Endothelial Growth Factor

Of the many pro-angiogenic factors that have been studied, the one most characterised is vascular endothelial growth factor (VEGF). Vascular endothelial growth factor (VEGF) was originally known as vascular permeability factor (VPF) is a potent inducer of microvessel hyperpermeability. VEGF is 50,000 times more potent on a molar basis compared to histamine with resect to effects on microvessel permeability. Due to their intimate
involvement with hypoxia, both VEGF and EGF have been referred to as hypoxia-regulated genes and are both important for tumour progression in RCC.\textsuperscript{360}

Currently, there are six subtypes of VEGF recognised: VEGF-A (VEGF or VPF), placental growth factor and VEGF-B to E.\textsuperscript{361} VEGF-A is a dimeric glycoprotein with a molecular weight of approximately 45 kDa.\textsuperscript{253} From hereon VEGF will represent VEGF-A in this thesis. It is a member of the platelet-derived growth factor (PDGF) superfamily of growth factors that mediates its effect on cells via one of three receptors: VEGFR-1, VEGFR-2 and VEGFR-3.\textsuperscript{83} Of these, it is widely recognised that VEGFR-1 and VEGFR-2 are the principal receptors mediating effects of VEGF-A on the vascular endothelial cells. Both of these receptors are tyrosine kinases expressed on vascular endothelium.\textsuperscript{37,361} VEGFR-3 (Flt-4), expressed on lymphatic and vascular endothelium,\textsuperscript{83} Upon binding of VEGF-A to the extracellular domain of its receptor, dimerisation and autophosphorylation of the intracellular receptor tyrosine kinases occurs and a cascade of downstream proteins is activated. VEGFR-2 appears to be the main receptor responsible for mediating the proangiogenic effects of VEGF-A\textsuperscript{83}

2.9.3.1 VEGF and Angiogenesis

VEGF has been found to have critical importance in both normal and tumour-associated angiogenesis through increased microvascular permeability to plasma proteins, induction of endothelial cell division and migration,\textsuperscript{362} promotion of endothelial cell survival through protection from apoptosis,\textsuperscript{363} and reversal of endothelial cell senescence.\textsuperscript{83,364}

As discussed, VEGF expression results from inactivation of pVHL tumour suppressor gene, which is observed in the majority of RCC cases.\textsuperscript{83} Further, the contribution of the pVHL pathway in early carcinogenesis has been partly revealed with regard to angiogenesis and cell cycle control.\textsuperscript{365}
The expression of VEGF is upregulated in a large proportion of human malignancies, and may be associated with markers of hypoxia. However, VEGF expression can be induced in the absence of hypoxia and hypoxia does not always provoke VEGF upregulation in tumours.\textsuperscript{366}

Tumour expression of angiogenic factors is another measure of angiogenic activity.\textsuperscript{367} In fact, some studies have demonstrated that tumour expression of VEGF was not correlated with tumour microvessel density, and the former but not the latter was prognostic of outcome in cancer patients.\textsuperscript{368,369} The relationship between circulating VEGF level and tumour angiogenesis may be more complicated than a simple linear correlation. Of course, it is possible that circulating VEGF may have other unknown biologic significance that explains its prognostic value.\textsuperscript{367}

VEGF contributes to the induction of stroma in at least 2 ways- as an endothelial, growth factor and by increasing microvascular permeability to plasma proteins, leading to alterations of the extracellular matrix including fibrin deposition.\textsuperscript{356} When plasma proteins including fibrinogen are extravasated into the extravascular space, fibrinogen clots to form fibrin, and other proteins such as fibronectin may also be incorporated into the fibrin clot.\textsuperscript{370}

Interestingly, some studies have demonstrated a correlation between serum VEGF and tumour expression of VEGF\textsuperscript{371,372} whereas other did not show a significant correlation\textsuperscript{373,374} Further, some studies have demonstrated that tumour expression of VEGF was not correlated with tumour MVD, and the former but not the latter was prognostic of outcome in cancer patients.\textsuperscript{368,369}

\textbf{2.9.3.2 VEGF and Hypoxia}

VEGF has been demonstrated to be unregulated by hypoxia measured by direct oxygen probe in human tumours.\textsuperscript{178,375} Therefore, circulating VEGF may be a reliable surrogate marker of
hypoxia. This has not been explored in RCC.

2.9.3.3 VEGF and Renal Cell Carcinoma

VEGF was demonstrated to have increased expression in RCC in the 1990s. VEGF is also expressed in normal kidney but in much smaller quantities. RCCs with VHL gene mutations or of advanced grade produced significantly higher concentrations of VEGF. In a study by Nicol et al of 27 clear cell RCC, analysis demonstrated 3-37 fold increases in VEGF expression when compared to normal parenchyma. VEGFR-1, but not VEGFR-2, are expressed in epithelial and stromal cells of clear cell RCC.

In clear cell RCC, VEGF expression has been positively correlated with both nuclear grade and size of the tumour. Furthermore, a significant correlation was observed between VEGF expression and microvessel density. Finally, cumulative survival rate was significantly lower in the group of patients with clear cell RCCs expressing VEGF. This study suggests that VEGF is involved in angiogenesis in conventional RCCs and appears to be a potential prognostic factor in these tumours.

In non-clear cell RCC, different expression patterns of VEGF and receptor mRNA levels leading to the supposition that different pathways might be involved in regulating angiogenesis in the specific RCC types.

In a study of the prognostic significance of several circulating angiogenic factors in patients with untreated RCC, plasma VEGF level was significantly higher in patients with lymph node or distant metastasis compared with those with organ-confined cancer. In support of this premise, two further studies of patients with RCC treated by nephrectomy also revealed a significant association between elevated serum VEGF level and tumour stage, histopathologic grade, and survival. However, a smaller study did not find a significant relationship between serum VEGF and tumour grade, stage, or patient survival in RCC.
Some studies demonstrated a correlation between serum VEGF and tumour expression of VEGF\textsuperscript{371,372} whereas other did not show a significant correlation\textsuperscript{373,374}.

Other studies have demonstrated that tumour expression of VEGF was not correlated with tumour MVD, and the former but not the latter was prognostic of outcome in cancer patients.\textsuperscript{368,369}

### 2.9.3.4 VEGF and anti-angiogenic therapies

VEGF is an appealing target for anticancer therapeutics. In addition, VEGF is easy to access as it circulates in the blood and acts directly on endothelial cells. VEGF-mediated angiogenesis is rare in adult humans (except wound healing and female reproductive cycling), and so targeting the molecule should not affect other physiological processes.\textsuperscript{362} Tumour blood vessels, formed under the influence of VEGF, are disorganized, tortuous and leaky with high interstitial pressure, reducing access for chemotherapies. Inhibiting VEGF would reduce the vessel abnormality and increase the permeability of the tumour to chemotherapies.

Several approaches to targeting VEGF have been investigated. The most common strategies have been receptor-targeted molecules and VEGF-targeting molecules. The disadvantage of receptor-targeted approaches is that the VEGF receptors also bind different members of the VEGF super-family and affect systems other than angiogenesis.

Antiangiogenic therapies for tumours that express high levels of angiogenic factors such as VEGF may vary in their efficacy, with potentially lowered effectiveness in specific sites (e.g. such as the brain) highlighting the complexity of molecular interactions.\textsuperscript{386} The best-studied and most advanced approach to VEGF inhibition is the humanized monoclonal antibody bevacizumab (Avastin), which is the only anti-angiogenic agent approved for treatment of cancer.\textsuperscript{362}
Tyrosine kinase inhibitors target the vascular endothelial growth factor (VEGF) receptor \(^{387}\). Bevacizumab is a monoclonal antibody developed to bind to and inhibit VEGF and has been discussed in section 2.8.4.

2.9.4 Carbonic Anhydrase (CAIX)
Carbonic anhydrases are zinc-containing anhydrases being metalloenzymes with the majority being transmembrane enzymes that catalyse the conversion of carbon dioxide to carbonic acid. \(^{388}\) Thus, their primary role is to regulate the acid-base balance and respiration but they are also involved in a variety of biological functions including gluconeogenesis, bone resorption, gastric acid production, ureagenesis, epithelial cell interactions and cell proliferation. \(^{389}\) Fourteen isoforms have been identified thus far and although their exact role in carcinogenesis remains unclear, they may protect against hypoxic damage in tumours. \(^{390}\)

Carbonic anhydrase IX (CAIX) is an outer cell membrane protein that is normally present on the mucosa of the alimentary tract, mainly in the stomach. \(^{221,391}\) It is also known as G250 tumour antigen and has had a monoclonal antibody raised against it (see section 1.11.12). Ectopically, it is expressed in various human tumours, mostly carcinomas (cervical, renal, colorectal, lung, mammary and others). \(^{189,392}\) The protein is composed of 459 amino acids and may serve as a receptor or ligand in the regulation of complex intracellular communications, cellular proliferation and importantly, oncogenesis. Importantly, it has been suggested that it may serve as a biomarker in early stages of tumourigenesis. \(^{391,393}\)

A recent study has shown that CAIX, apart from its classical enzyme activity of reversibly hydrating carbon dioxide extracellularly to facilitate the net extrusion of protons from inside to outside the cell, can also be a key player in the modulation of cell adhesion processes and participate in the regulation of cell proliferation in response to hypoxic environment to ultimately contribute to tumour progression. \(^{394}\) It has been demonstrated that the sole tyrosine moiety of CAIX present in its intracellular domain can be phosphorylated in an
epidermal growth factor dependent manner, suggesting that it can feed into the growth factor receptor dependent signalling pathways. This has implications as the tyrosine phosphorylated CAIX can interact with the regulatory subunit of PI-3-Kinase, contributing to Akt activation. This could create a positive feed back loop that can form the basis of a vicious cycle that could contribute to the progression of clear cell RCC. Such pathways confirm that CAIX signalling may be a part of both the hypoxia driven and hypoxia independent pathways that occur in the cancer cell.

2.9.4.1 CAIX expression
CAIX expression is primarily regulated by the HIF-1 complex, and therefore has increased expression in hypoxic environments. However, HIF-1 itself is modulated by a variety of mechanisms including cytokines, growth factors and other mediators - not just hypoxia. Thus CAIX expression will also be influenced by factors other than oxygen level. For example, low-normal glucose and bicarbonate levels when included as microenvironmental stress factors, were additive to hypoxia in encouraging CAIX expression.

Cell density is another factor thought to contribute to CAIX expression- occurring because of pericellular hypoxia due to the increased density of cells competing for oxygen. Further to this a cell density-dependent activation of the phosphatidylinositol-3-kinase/AKT pathway also seems to have an effect on CAIX expression which requires only minimal HIF-1 activation.

2.9.4.2 CAIX as a surrogate intrinsic marker of hypoxia
CAIX, which is up-regulated by HIF-1, is an attractive alternative marker of hypoxia compared to HIF-1α because the protein is highly expressed on the cell surface and has a long half life in hypoxic tissue (unlike HIF-1α). Furthermore, specific, high-affinity monoclonal antibodies suitable for immunohistochemistry have been developed (e.g. the chimeric monoclonal antibody cG250 that binds to CAIX). In studies, CAIX immunohistochemical staining is accepted as a reliable marker for chronic hypoxia.
To date, there have been conflicting reports as to whether or not CAIX expression correlates with oxygen status in tumours. In particular, whether greater CAIX expression equates to the presence of hypoxia. The only study of direct oxygen measurements via electrode in human tumours supporting a direct correlation between hypoxia and CAIX was in carcinoma of the cervix. In a further study of non-small cell lung cancer (NSCLC), CAIX expression appeared to correlate with the degree of hypoxia in tumours. CAIX expression was present in 82% of specimens. However, direct oxygen probing was not used and the implication of hypoxia was only confirmed by a further experiment where a cell-line of NSCLC was grown under hypoxic and normoxic conditions, with those in hypoxic conditions expressing more CAIX. HIF-1α and CAIX expression have also been co-localised in NSCLC suggesting their relationship with hypoxia.

2.9.4.3 CAIX expression and tumour progression
CAIX is expressed in many human tumours. For example, in cervical cancer it is present in over 80% of specimens and it is present in almost all RCC, particularly clear cell (discussed below). CAIX expression is more prevalent in perivascular regions. Co-localisation of CAIX with Ki-67 expression, indicates that proliferating cells are expressing CAIX.

The data relating to CAIX expression to tumour progression are again conflicting. CAIX expression has demonstrated no correlation with clinical tumour size, stage, histology, histologic grading, patient age, menopausal status, parity, or pretherapeutic haemoglobin level in cervical adenocarcinoma. Against this, other studies have found that increased CAIX expression correlated with worse survival in cervical carcinoma, lung cancer, and breast cancer.
In complete contrast to this low CAIX expression and poor prognosis has been shown in studies with cervical carcinoma\(^{402}\), colorectal carcinoma\(^{393}\), and oesophageal cancer.\(^{403}\) RCC is discussed below but it in general has supported lower expression with worse prognosis.

2.9.4.4 CAIX expression in RCC

In RCC synthesis of CAIX is switched on by the loss of the tumour suppressor gene VHL.\(^{392}\) CAIX expression has been studied immunohistochemically and with RT-PCR analyses and is present in 87-100% of clear cell RCC.\(^{404-406}\) CAIX is also expressed in the majority of non-clear cell RCC but not in those consisting of chromophobe histology or in benign renal lesions, including oncocytopas.\(^{406,407}\)

The expression of CAIX and its association with grade and stage has been analysed. Tumours of low clinical stage showed a striking increase in CAIX expression, and high CAIX expression.\(^{406}\) Another study supported the association of high-grade and stage tumours with significantly lower expression of CAIX than low grade and stage tumours.\(^{404}\) Alternatively, when patients with localized RCC were analysed altogether, CAIX staining initially did not appear to stratify survival comparing low CAIX expression (≤85%) and high expression (>85%), there appeared to be no relationship.\(^{408}\) Low expression was constant across stages and grade was not predictive of progression. A trend at higher stage and grade for less CAIX expression existed but was not significant.\(^{408}\) This was repeated in a smaller study where stage was not able to be correlated with CAIX expression unless grouped. In 28 tumours of low stage (I and II) CAIX expression was significantly higher than 9 higher stage tumours (II, IV).\(^{406}\)

Patient outcome and CAIX expression in RCC has been analysed. In a study by Bui et al of a cutoff of 85% CAIX staining provided the most accurate prediction of survival.\(^{408}\) Low CAIX (≤85%) staining was an independent poor prognostic factor for survival for patients with metastatic RCC. In patients with non-metastatic RCC low CAIX predicted a worse outcome similar to patients with metastatic disease and overall CAIX expression decreased
with development of metastasis. A further analysis demonstrated increased CAIX expression to be associated with a good patient outcome.\textsuperscript{406} In patients with metastatic RCC receiving immunotherapy (IL-2), groups with high CAIX expression exhibited a longer median survival.\textsuperscript{409}

In summary, patients with non-metastatic RCC, CAIX expression is inversely proportional to tumour progression over time.\textsuperscript{410} Further, that CAIX expression is inversely proportional to stage in RCC.\textsuperscript{406} On all these points, it must be remembered that studies often conflict regarding CAIX expression and stage or prognosis in various tumours, making any meaningful conclusions without further data premature.

The majority of studies appear to support the hypothesis that in RCC decreased CAIX expression occurs in tumours with the highest malignant potential. For example, high Ki-67 staining and low CAIX staining has correlated significantly with poorer median survival.\textsuperscript{105} This is unlikely to be explained by the loss of differentiation because there has been no correlation with Fuhrman grade.\textsuperscript{408} Furthermore, the overall expression of CAIX appears to decrease with development of metastases; the level of CAIX is less in the metastatic lesion than in the parental primary tumour. This suggests that CAIX may play a functional role in tumour progression.\textsuperscript{410} Therefore, it is hypothesised that in the earlier stages of tumour progression, noxious conditions such as hypoxia or ischemia induce CAIX expression as an adaptation to confer proliferation advantage for tumour growth and spread; however, when this malignant potential is attained in the later stages of tumour growth, continued CAIX expression is no longer a requirement.\textsuperscript{408} An alternative hypothesis that the cumulative effects of genetic lesions involved in cancer progression could alter the pathways of hypoxia response \textsuperscript{411} and therefore affect CAIX expression. This may explain why cervical cancer accumulates the ability to express CAIX and retains it whilst in RCC it appears to longer need the ability to express CAIX to progress. Additional studies will be needed to determine whether genetic changes underlie differences in CAIX expression in the primary tumours and in metastatic lesions.\textsuperscript{408}
2.9.5 Epidermal Growth Factor Receptor (EGFR)

The Erb family of tyrosine kinase receptors includes: ErbB1 or epidermal growth factor receptor receptor receptor (EGFR), ErbB2, ErbB3 and ErbB4. They bind extracellular ligands to promote cell growth, proliferation and malignant progression. The natural ligand to EGFR is the epidermal growth factor (EGF), a small 53-amino acid protein with a molecular weight of 6 kDa. Transforming growth factor alpha (TGF-α) is another EGFR ligand. These are important ligands in tumourigenesis and there is in vitro evidence in pancreatic cancer suggesting that tumour necrosis factor alpha (TNF-α) increased the expression of the EGFR ligand, transforming growth factor (TGF-α), at the mRNA and protein level in all cell lines. This may be part of an autocrine loop that becomes active in tumours promoting growth.

2.9.5.1 EGFR and tumour progression

EGFR is a receptor tyrosine kinase that is frequently over-expressed in human cancers and is associated with tumourigenesis, and increased tumour proliferation and progression. Expression is generally an indicator of poor prognosis and has been associated with resistance hormone therapy and cytotoxic drugs.

Activation of EGFR leads to recruitment and phosphorylation of several downstream intracellular substrates, leading to mitogenic signaling and other tumour-promoting cellular activities. In human tumours, receptor overexpression correlates with a more aggressive clinical course. Taken together, these observations indicate that the EGFR is a promising target for cancer therapy. As an example, EGFR appears to regulate VEGF and it has been demonstrated that blockade of the EGFR also results in an antiangiogenic effect. EGFR is also linked to HIF and VHL gene expression.

2.9.5.2 EGFR and RCC

EGFR is a transmembrane glycoprotein detected on many cells and tissues including neoplastic and normal kidney. The overexpression of EGFR is seen in 47-90% of all cases.
Further, mRNA expression of EGFR has been demonstrated to be about 19 times higher in RCC than in adjacent normal parenchyma.\textsuperscript{422} Strong reactivity for EGFR was identified as predicting the patients' survival both during uni- and multivariate analysis.\textsuperscript{421}

The fact that EGFR is expressed in up to 90\% of RCCs makes appealing the theory that this receptor might act as a potential antitumour target.\textsuperscript{290} This growth factor is part of a family of closely related cell-surface receptors with intrinsic tyrosine kinase (TK) activity that include EGFR (HER1 or erbB1), erbB2 (HER2/neu), erbB3 (HER3), and erbB4 (HER4). The RTK need to dimerise for activation of the internal TK domain, which triggers a cascade of phosphorylation and activation of signal transduction pathways.\textsuperscript{281} The best characterized intracellular pathways activated by EGFR are the Ras-Raf-mitogen-activated phosphokinase pathway that leads to cell division, and the PI3K/Akt pathway that results in stimulation of angiogenesis, survival and promotion of invasion.\textsuperscript{281}

HIF activates the TGF-\(\alpha/\)EGFR pathway in VHL-defective RCC cells.\textsuperscript{22,423} Thus at a molecular level, the links between RCC and EGFR are beginning to be understood. For example, recently VHL loss has been shown to activate nuclear factor kappa B (NF-kappaB), a family of transcription factors that promotes tumour growth.\textsuperscript{424} The inability to produce pVHL drives NF-kappaB activation by HIF-1\(\alpha\) accumulation, which induces expression of TGF-\(\alpha\), with consequent activation of EGFR and other signaling cascades (phosphatidylinositol-3-OH kinase/protein kinase B (AKT)/IkappaB-kinase alpha/NF-kappaB).\textsuperscript{424} In simpler terms, this is a TGF-\(\alpha\) autocrine loop that is a consequence of VHL inactivation in renal proximal tubule epithelial cells. It may provide the uncontrolled growth stimulus necessary for the initiation of tumourigenesis in RCC.\textsuperscript{414}

Much of the evidence thus suggests that HIF-mediated constitutive EGFR activation provides permanent self-sufficiency in growth signaling that drives the growth autonomy of VHL-defective RCC cells, a hallmark of cancer.\textsuperscript{18,423}
2.9.5.3 **EGFR and Hypoxia**

One possible role of EGFR in the hypoxia driven angiogenesis cycle lies with the downstream targets of HIF that promote tumourigenesis. However, to our knowledge there are no direct studies of oxygenation levels and EGFR expression in human tumours. In animal models, some indirect evidence has emerged linking EGFR with hypoxia in human EGFR-expressing A431 squamous cell carcinoma xenografts by using hypoxia imaging and external markers of hypoxia.\(^{299}\)

2.9.5.4 **EGFR blocking therapies**

Thus inhibition of the EGFR is a rational strategy for the development of new cancer therapeutics.\(^{425-427}\) Potential therapeutics include anti-EGFR antibodies\(^{428}\) and small molecular weight tyrosine kinase inhibitors of the EGFR.\(^{429,430}\) However none to date have any role in the treatment of RCC.

2.9.6 **Glucose Transporter (Glut-1)**

Facilitative glucose transport is mediated by members of the Glut protein family that belong to a much larger superfamily of 12 transmembrane segment transporters. Six members of the Glut family have been described. These proteins are expressed in a tissue- and cell-specific manner and exhibit distinct kinetic and regulatory properties that reflect their specific functional roles.

Glut-1 is a widely expressed isoform that provides many cells with their basal glucose requirement. It also plays a special role in transporting glucose across epithelial and endothelial barrier tissues, particularly in growing and dividing cells.\(^{182,431}\) Glut-1 expression is dually controlled via HIF-1 and in response to reduced oxidative phosphorylation.\(^{432,433}\) Immunohistochemistry is the typical method for determining Glut-1 expression. The intensity of staining is generally described as negative (0-20%), weak (21-50%) and positive (> 51%) staining in the lesion.\(^{434}\)
2.9.6.1 Glut-1 and Hypoxia

Glucose transport is acutely stimulated by hypoxic conditions, and the response is mediated by enhanced function of the facilitative glucose transporters Glut-1, Glut-3, and Glut-4. The expression and activity of the Glut-1 mediated transport is coupled to the energetic status of the cell, such that the inhibition of oxidative phosphorylation resulting from exposure to hypoxia leads to a stimulation of glucose transport.\textsuperscript{433}

Glut-1 expression correlates with tumour hypoxia and predicts metastasis-free survival in advanced carcinoma of the cervix.\textsuperscript{182} This was supported in a further study.\textsuperscript{239} Based on these studies one might consider that Glut-1 expression could be a surrogate marker of tumour hypoxia. In contrast, another study has found a lack of correlation between Glut-1 expression and oxygenation status in locally advanced cervical cancers.\textsuperscript{435}

Prospective potential surrogate intrinsic hypoxia markers such as Glut-1 are only validated further if the prognostic significance of tumour hypoxia can be extrapolated to its expression.\textsuperscript{182} The absence of Glut-1 is significantly prognostic for metastasis-free survival.\textsuperscript{182} This is consistent with observations that hypoxia is associated with the formation of metastases in human tumours.\textsuperscript{14} Previous studies have demonstrated that Glut-1 expression is an indicator of poor prognosis in non-small cell lung\textsuperscript{436} and colorectal cancers.\textsuperscript{437} Death in such cases can be attributed to metastatic spread and are attributable to hypoxia-induced malignant changes as well as the possible effects of glucose starvation on metastatic potential and invasive capacity.\textsuperscript{182,438}

2.9.6.2 Glut-1 and Renal Cell Carcinoma

Although RCC consists of glycogen-rich cells, the carcinoma itself has a low potential for metabolising glucose\textsuperscript{434}. This was based on a study of 19 patients with only 11 tumours positive for Glut-1 immunostaining with larger tumours more likely to stain positive and there was no correlation between clinical stage or grading and Glut-1 expression.
2.9.7 Nuclear Antigen Ki-67 and Proliferation index

Although the role of Ki-67 has not been completely delineated, it is recognised as a nuclear protein from a family of MPM-2 antigens with a role in cell division whose expression is limited to the mitotic phase of the cell cycle. Specifically, the phosphorylation of the Ki-67 protein during mitosis is associated with the condensation of the chromosomes and the separation of sister chromatids. There may also be a role in the control of higher order chromatin structure.\textsuperscript{439,440}

The growth fraction of the tumour, i.e., the percentage of cells entering the mitotic cycle, should be theoretically related to the likelihood and rapidity of recurrence\textsuperscript{440} Thus quantification of tumour cell proliferation may be useful for outcome prediction. The Ki-67 nuclear antigen is present throughout the cell cycle, immunostaining with monoclonal antibody Ki-67 provides a reliable means of rapidly evaluating the growth fraction of normal and neoplastic human cell populations.\textsuperscript{441}

Ki-67 antigen expression is normally assessed by immunohistochemical analysis using the monoclonal antibody MIB-1. The actual measurement of the amount of Ki-67 antigen present is referred to as the labeling index\textsuperscript{441} or proliferation index\textsuperscript{441}. Typically, the Ki-67 antigen labeling index is determined by counting a total of at least 1000 neoplastic nuclei. Those cells with nuclei that are dividing, or staining positive, are divided over those that are negative, or quiescent\textsuperscript{440} There may also be a relationship between Ki-67 expression and metastatic potential of tumours. In pancreatic islet cell tumours it has been demonstrated that those with a low proliferation index were unlikely to metastasize.\textsuperscript{442}

2.9.7.1 Ki-67 expression and tumour progression

Ki-67 expression has been characterized in many tumours. For example, Ki-67 over-expression is of prognostic value in transitional cell carcinoma (TCC) of the upper urinary tract,\textsuperscript{443} and brain tumours.\textsuperscript{444} Also, in early breast cancer, increased Ki-67 expression is a sign of poor prognosis that interestingly is associated with a good chance of clinical response.
to chemotherapy. This is because chemotherapy targets dividing cells which is the same group of cells that Ki-67 is identifying.

2.9.7.2 Ki-67 nuclear antigen and Renal Cell Carcinoma
Ki-67 antigen expression was not a prognostic factor in patients with tumour venous thrombus and RCC. A further study also reported that Ki-67 expression did not add prognostic value. Further studies have demonstrated that both Ki-67 and p53 expression correlates with proliferation.

Ki-67 immunostaining appeared to be an additional prognostic indicator of biologic aggressiveness in renal cell carcinoma. Increased Ki-67 expression in clear cell RCC predicts poor cancer-specific survival.

2.9.7.3 Ki-67 Therapy
Ki-67 directed antisense oligonucleotides are potent inhibitors. Antisense oligonucleotides are designed to specifically hybridize with complimentary target mRNA and inhibit protein expression. Oligonucleotides accumulate in tumour tissues and in the kidney making RCC an ideal target. Phosphothioate-modified antisense oligonucleotides against the Ki-67 antigen have been shown to be potent antitumoural agents in bladder and prostate cancer-derived cells in animal models. They induce apoptosis in vitro and effectively inhibit tumour growth 'k' of target protein expression and proliferation of tumour cells in vitro. Also, they inhibited tumour growth and lung metastases formation in murine RCC. Interestingly, tumour vascularisation was not significantly affected. Although models have been developed no human studies exist in RCC.
2.9.8 Microvessel Density (MVD)

Tumour microvessel density (MVD) has been demonstrated in many cancers and thus has been widely used as an index of tumour angiogenesis. In essence, MVD is measuring the number of vessels in a defined area as a marker of angiogenesis which is expected to be increased in tumours. MVD is one but not the only one indicator of tumour angiogenic activity as outlined above. Measuring MVD is normally done by immunohistochemistry studies. The basis of most studies is the staining of endothelial cells with an antibody to cell surface antigens such as CD31, CD34, factor VIII related antigen (von Willebrand Factor) or CD105.

The widespread clinical use of microvessel density has so far been hindered by different measurement techniques. Typically, counting of vessels may be performed in over a number of fields and averaged or over certain “hot spots” where the maximal number of small vessels within the tumour are identified under low power (x100) before counting under high power (x 400 or even x200). Such “hot spots” may be a solitary field or multiple fields (usually three). The number of vessels in a single field or average number of vessels over fields are then calculated.

2.9.8.1 MVD and Tumour Progression

Many studies have demonstrated that the microvessel count assessed by immunostaining correlated with the risk of metastasis, recurrence and the prediction of patient survival. MVD was shown to be highest with histopathologically aggressive ductal carcinoma-in-situ lesions of breast cancer and is associated with increased VEGF expression. Further, high MVD in invasive disease has been correlated with a greater likelihood of metastatic disease and a shorter relapse-free and overall survival in patients with node-negative breast cancer. In summary, the bulk of accumulating data indicates that microvessel density in the area of most intense neovascularization in invasive breast carcinoma is an independent, significant and accurate prognostic indicator in predicting poorer survival.
2.9.8.2 MVD and Renal Cell Carcinoma
Monitoring of MVD in experimental models of RCC also gives an indication of the role of MVD in tumour behaviour. Immunohistochemical assays demonstrated that ZD1839 treatment resulted in a significant decrease in CD34 positive neovessels compared with controls in SKRC-49 xenografts.298

Expression of HIF-alpha proteins was associated significantly with up-regulation of VEGF mRNA and protein and increased MVD. Up-regulation of HIF-alpha in clear cell RCC was found to involve increased mRNA as well as protein expression, suggesting that both VHL-dependent and VHL-independent mechanisms are involved.290 MVD has been associated with aggressiveness in RCC being increased with sarcomatoid variants and with collecting duct tumours.291

His study suggest that demonstration of intense angiogenesis and micro-vascular invasion may be a predictor of a more aggressive tumour mandating closer follow up and consideration of adjuvant therapy.292 MVD counts alone did not add prognostic value for RCC patients.293

2.9.8.3 MVD and Hypoxia
Attempts have been made to link MVD with hypoxia, due to the angiogenesis and hypoxia pathways regulated by HIF-1. The assumption being underlying this approach is that MVD will correlate with the degree of hypoxia.221 Expression of HIF-alpha proteins was associated significantly with up-regulation of VEGF mRNA and protein and increased microvessel density. Up-regulation of HIF-alpha in CC-RCC was found to involve increased mRNA as well as protein expression, suggesting that both VHL-dependent and VHL-independent mechanisms are involved.290

Failure of the Krebs cycle in HLRCC tumours causes inappropriate signalling that the cell is in a hypoxic state, leading to angiogenesis and perhaps directly to clonal expansion and tumour growth through some uncharacterized, cell-autonomous effect.291
As outlined, MVD is a prognostic indicator for many cancers. Therefore, the assessment of MVD in biopsy samples taken over the course of treatment would seem to be a straightforward strategy to monitor the efficacy of antiangiogenic therapy. However, this approach is not only invasive but also potentially unreliable because the greatest density of new vessels (i.e., angiogenic “hot spots”) is typically found in the tumour periphery and, as the tumour grows, the central portion of the tumour may become hypovascular and even necrotic. Therefore, it is difficult to obtain comparable biopsy samples to quantify tissue response accurately. The number of microvessels within the invasive component of a primary tumour reflects the degree of tumour angiogenesis. At present the most widely used method to assess neovascularization is the quantitation of intratumoral microvessel density by immunohistochemical methods in which specific markers for endothelial cells are employed.

### 2.9.9 High Cell density and Hypoxia

It was suggested over thirty years ago by Werrlein and Glinos that oxygen availability and microenvironmental pO	extsubscript{2} gradients were directly related to cell density and the whole cell population respiratory activity in culture. Other studies later demonstrated that confluent cells being cultured are exposed to pericellular hypoxia under normoxic conditions due to diffusion-limited oxygen delivery.

In vitro experiments high cell density-mediated pericellular hypoxia is a crucial factor inducing expression of the intrinsic hypoxia marker CAIX in cervical cancer cells. Even in normoxic conditions cell density appeared to influence the gene expression and composition of the microenvironment of the tumour cells. In human studies, brain tumour and breast cancer response to chemotherapy correlated positively with cell density.

Cell density has not been investigated in clinical studies of RCC.
2.10 Osteopontin

Osteopontin is a single-chain polypeptide with a molecular weight of approximately 32,600 and a molecular mass of 44kDa.\textsuperscript{475,476} It is a secreted and calcium-binding phosphorylated glycoprotein, which is able to bind to cell surface integrins. Its name arose because it was identified as a key noncollagenous bone matrix protein.\textsuperscript{477} It is expressed in a limited number of normal tissues such as kidney, it is present in body fluids and is abundant in bone and other mineralised tissues.\textsuperscript{478} Due to its presence in other organs, it has been referred to by other names such as bone sialoprotein, urinary stone protein, uropontin and nephropontin to name but a few.\textsuperscript{476} Despite the diversity of tissue expression, the function of osteopontin is not fully understood.\textsuperscript{478}

2.10.1 Osteopontin in Tumourigenesis

The proposed mechanisms by which osteopontin may enhance malignancy fall into four broad categories.\textsuperscript{477} Firstly, there is evidence that osteopontin enhances the growth of transformed cells in suspension, including experiments with inducible osteopontin\textsuperscript{479}. Secondly, the ability of cells to migrate is linked inextricably with tumourigenicity. Osteopontin has been clearly demonstrated to participate in pathways that regulate the migration in diverse cell populations including fibroblast, macrophages, osteoclasts and tumour cells.\textsuperscript{480} Thirdly, another key element in tumourigenesis, is the ability to invade, requiring the induction of proteinases to dissolve the extracellular matrix.\textsuperscript{481,482} Finally, osteopontin is believed to act in concert with several growth factors including hepatocyte growth factor (HGF)\textsuperscript{483} and Epidermal growth factor (EGF)\textsuperscript{484} to induce malignancy. With the diversity of functions suggesting osteopontin function in any given situation may be determined by its microenvironment.\textsuperscript{477}
2.10.2 Osteopontin Function

Osteopontin expression is enhanced in a number of physiological and pathological events, including maintenance or reconfiguration of tissue integrity during inflammatory or remodelling processes. In particular, its expression has been implicated in vascular remodelling, renal diseases, lactation, calcification and remodelling of mineralised tissues. Osteopontin is secreted into various bodily fluids including blood, urine, milk and cochlear fluid. Due to its strong affinity for hydroxyapatite, it accumulates in bone and other sites of mineralisation.

2.10.3 Osteopontin in Human Tumours

Osteopontin is a secreted plasma marker that has recently been found to be overexpressed in a variety of human tumours. In patients with head and neck squamous cell cancers it inversely correlated with tumour pO$_2$. Those with high serum osteopontin levels had a worse prognosis (statistically significant) that also correlated with polarographic oxygen electrode measurements of hypoxia in the tumours. Furthermore, osteopontin gene expression inversely correlated with that of $VHL$ expression in their study. e.g. osteopontin levels in tumour tissue have also been associated with tumour progression in other studies breast, lung, prostate and colon cancer.

Osteopontin is linked to hypoxia and angiogenesis and the relationship is complex. The links between the two have been proposed to be mediated through Akt activation and the induction of HIF-1$\alpha$ expression. The phenomenon of osteopontin overexpression is not universal in all tumours. For example, there has been differential expression between different tumour types in the same organ. Also, in xenografted models, variable expression of osteopontin has also been noted in response to altered tumour architecture such as after radiotherapy. Certainly, our understanding of angiogenesis and osteopontin is in its infancy.
2.10.4 Osteopontin in Renal Cell Carcinoma

Osteopontin may be involved in the progression of RCC through angiogenesis promotion.\(^{497}\)

Osteopontin may have other associations with tumourigenesis such as in bone where it has a role in adhesion, migration and cell survival.\(^{498}\) In immunohistochemical studies, RCC has demonstrated expression of osteopontin in 72\% of cases.\(^{499}\) Further, osteopontin has also been found to reflect cancer stage in RCC, with high expression associated with a higher stage.\(^{499}\)

Plasma osteopontin has been studied in one retrospective series where high serum osteopontin levels were associated with distant metastases and poor survival in RCC patients. They concluded that osteopontin may be a tumour marker to monitor new treatment strategies in patients with advanced RCC but needs evaluation in prospective studies.\(^{500}\)

No studies of serum osteopontin or immunohistochemical expression of osteopontin in RCC patients have been published.

2.11 Imaging of Renal Cell Carcinoma and Hypoxia in Tumours

Evaluation of a renal mass should accomplish two objectives:
1) differentiation of benign from malignant lesions and
2) accurate staging of malignancy to allow precise planning for surgery or other therapeutic modalities.\(^{501}\)

In this context, urological imaging available includes computerised tomography, ultrasonography whilst studies using angiography, fluoroscopy, MRI and PET-CT are considered second line.\(^{502}\) In clinical practice, despite imaging advances, many lesions are unable to be labelled definitely as benign or malignant.\(^{503}\) This is also true when staging and re-staging patients where scar tissue or reactive lymph nodes may be mistaken for disease; this is where functional imaging with PET-CT is developing a role. Furthermore, PET-CT
with new radiotracers, including radiolabelled monoclonal antibodies, the imaging of tumour hypoxia and cellular proliferation are developing and may impact on the diagnosis, staging and future treatment of RCC. Clinicians need to be aware of the role of relevant modalities in RCC, and these will now be discussed.

**2.11.1 Computed Tomography in Urological Imaging**

A high-quality CT scan dedicated to the kidneys includes images obtained before and after intravenous contrast administration. Standard slices of 5mm are taken, but these may be reduced to 2-3 mm. The timing of image acquisition is critical and CT scans typically have 3 phases:

1) **Non-contrast phase**: Critical for delineating mass composition (e.g. fluid versus solid, fat versus other tissue) and identifying calculi.

2) **Nephrogenic phase**: approximately 90 seconds after contrast administration when contrast is being filtered into the nephrons. This delay is essential in ascertaining whether a mass “enhances” with contrast, this indicating if it has an active blood supply, making a tumour a more likely diagnosis. Hounsfield units (measure of density) from areas of interest, such as within the mass, are then compared to non-contrast scans to determine the degree of enhancement.

3) **Pyelogram phase**: contrast is accumulating in the collecting system of the kidney and ureter. Masses within the collecting system are often delineated. This is particularly important for transitional cell carcinomas rather than RCC.

**2.11.2 Multidetector Computed Tomography**

The first major advance in CT technology since its inception was the development of spiral or helical CT, enabling large data collection in a single breath because the gantry now move continuously around the patient. The second advance was the concept of simultaneous multiple acquisitions per gantry rotation with dual-slice scanner technology in 1992. Multi-slice technology was further advanced with the introduction of four-slice multidetector row scanners in 1998. Since then evolution has occurred with 32-, 40 and now 64-slice technology.
Multidetector CT allows faster data acquisition compared with single detector CT, without any loss of image quality because of short gantry rotation intervals combined with multiple detectors at each level, providing increased coverage\textsuperscript{504}. This, along with short interscan delays, allows image acquisitions in multiple phases of renal parenchymal enhancement and contrast material excretion in the collecting system after administration of a single bolus of intravenous contrast.\textsuperscript{506} As such the CT images obtained provide improved spatial resolution providing high quality three-dimensional data sets of the renal vessels comparable with angiography and conventional urography.\textsuperscript{507}

Currently the accuracy for CT staging at 91% staging for detecting local invasion, lymph node and IVC involvement.\textsuperscript{508} The role of multidetector CT, particularly in staging of renal vein extension, has not been explored but has now been done so as part of this thesis.\textsuperscript{509} It was compared to MRI in a group of patients with IVC extension and found to be just as accurate.

\textbf{2.11.3 Magnetic Resonance Imaging in Urological Imaging}

MRI when introduced, the main advantages were that it permitted multiplane imaging, it did not involve the use of ionizing radiation and there was no evidence of cellular toxicity.\textsuperscript{502} Apart from the first point which has been overcome by multidetector CT scanners, the two final points still make MRI advantageous (e.g. imaging young children or pregnant women).\textsuperscript{502,503} Disadvantages include cost, accessibility, specialised radiologists and metal implants or foreign bodies being contraindications.

In RCC, MRI has proved particularly useful in staging by determining the degree of spread, particularly along the renal vein and into the inferior vena cava (IVC), as the involvement (or lack thereof) will affect the surgical approach used.\textsuperscript{502,510} However, even this role has been challenged by multidetector CT (see above).
2.11.4 Ultrasonography of Renal Masses
Investigation of general abdominal conditions often results in renal masses being discovered incidentally on ultrasound or CT. However, as a primary investigative tool for delineating urinary tract related symptoms such as haematuria, ultrasound is often the investigation of choice because it is non-invasive and relatively inexpensive. Ultrasound has even been used for limited screening in an asymptomatic targeted population for renal masses. In some cases ultrasound and CT are complimentary, such as investigating the nature of a hyperdense lesion on CT that is a clearly defined cyst on ultrasound. Overall, accuracy for staging of RCC alone is reported at 50-78%. This may be improved in the future with the development of ultrasound contrast agents, particularly using microbubbles (air or contrast), that are able to better assess the blood flow and hence the degree of tumour vascularity and hence better direct smaller masses for monitoring or surgery.

2.11.5 Transoesophageal Echocardiography of Renal Masses
Transoesophageal echocardiography has a limited but important role in RCC- to document the level of IVC involvement by tumour thrombus intraoperatively and to ensure complete thrombus removal.

2.11.6 Positron Emission Tomography
Positron emission tomography (PET) is functional imaging using radioisotopes that undergo positron emission decay (table 1). Positrons are emitted from the unstable radionuclide and interact with an electron in the surrounding tissues, resulting in an annihilation reaction with subsequent release of two, 511-keV photons in opposite directions. A ring detector surrounding the patient then detects the coincident photons and registers the interactions in the image.

PET studies supply unique information about tissues by tagging radioisotopes to metabolically activity substances in the body. The radioisotope Fluorine-18 combined with the D-glucose analogue: 2 fluoro-2-deoxy-D-glucose (18F-FDG) is the most commonly used radionuclide, but other radioisotopes are used (Table 2-12)
The success of positron emission tomography (PET) in oncological imaging is based on the knowledge that malignancy leads to an alteration in cellular biochemical reactions. Synthesis of positron emitting analogues from molecules of organic matter can demonstrate these biochemical processes, thus highlighting the presence of malignancy. Hence tumour imaging with $^{18}$F-FDG is based on the fact that tumours are more metabolically active than their normal surrounding tissues and thus will metabolise more glucose. Tumour cells actively take up and shuttle $^{18}$F-FDG into glycolysis. Once cycling has commenced, glucose is phosphorylated to $^{18}$F-FDG-6-phosphate. This metabolite becomes trapped and cannot proceed along the normal pathway of glucose metabolism. Eventually, increased amounts accumulate within malignant cells. This abnormal concentration of $^{18}$F-FDG in tumour cells produces a detectable signal greater than the background, allowing isolation of tumour deposits. The signal detected by the PET camera arises from the emission of radiation in the form of $\gamma$-photons when a positron collides with an electron.

Quantitative analysis of accumulated radioisotope within different tissues forms the basis of reporting PET studies. A standardized uptake value (SUV) for normal tissue is established based on radioisotope uptake and areas of interest within the same tissue are compared to this index for reduced or increased uptake. Tumours typically have an increased SUV compared to normal indicating radioisotope accumulation. Such SUV measurements may be likened to Hounsfield Units for measuring contrast uptake on CT.
Table 2-12 Commonly used isotopes in PET studies and their half-life

<table>
<thead>
<tr>
<th>Radioisotope</th>
<th>Half-life</th>
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<tbody>
<tr>
<td>Oxygen-15</td>
<td>2 mins</td>
</tr>
<tr>
<td>Nitrogen-13</td>
<td>10 mins</td>
</tr>
<tr>
<td>Carbon-11</td>
<td>20 mins</td>
</tr>
<tr>
<td><strong>Fluorine-18</strong></td>
<td><strong>110 mins</strong></td>
</tr>
<tr>
<td>Bromine-75</td>
<td>98 mins</td>
</tr>
<tr>
<td>Copper-64</td>
<td>12.7 hours</td>
</tr>
<tr>
<td>Yttrium-86</td>
<td>15 hours</td>
</tr>
<tr>
<td>Zirconium-89</td>
<td>78 hours</td>
</tr>
<tr>
<td>Iodine-124</td>
<td>100 hours</td>
</tr>
</tbody>
</table>

Figure 2-5 Schematic illustration of Positron Emission Tomography
2.11.6.1 Limitations of $^{18}$F-Fluorodeoxyglucose ($^{18}$F-FDG)

PET offers an advantage over other modalities as no allergy to $^{18}$F-FDG is known and implants are not contraindicated. Diabetics provide some concern regarding fasting status and timing of radioisotope injection. Also, in the past there may have been limitations with oncologic imaging including a reduced ability to visualise very small tumours (less than 0.5cm) and difficulty in differentiating between malignancy and chronic inflammation. 524 Fortunately, many of these perceived limitations have been addressed and resolved with the development of combined PET and computed tomography (CT) discussed below.

2.11.7 Combined Positron Emission-Computed Tomography

PET is a stronger as a functional rather than anatomical modality. As such, in the past, it relied on software-based image fusion for the alignment of functional and anatomical images. Hence data from a CT or MRI scan taken on a different machine non-contemporaneously to the acquisition of the PET data was used to identify sites of abnormal $^{18}$F-FDG uptake. Image registration could not account for differences in patient positioning, scanner bed profiles and involuntary movement of internal organs. An alternative approach is a scanner acquiring both function and anatomy during a single session: a fusion of technologies rather than a fusion of the images post hoc. 525 Commercially available scanners with clinical CT and PET scanner mounted together in a single gantry known as PET/CT have overcome image registration problems. At this stage non-contrast CT images are obtained using helical, multi-slice protocols, but contrast studies may be used in the future.
2.11.8 Positron Emission Tomography in Urologic Cancers

Before considering PET or PET-CT and its role in imaging RCC researchers must consider how it is currently being utilised in genitourinary imaging of tumours. Several factors make interpretation of radiotracer activity in the genitourinary tract (particularly $^{18}$F-FDG) with PET and PET-CT challenging. Firstly, most radiotracers are excreted through glomeruli but are not reabsorbed by tubules and so accumulate in the renal collecting system. This may make identification of collecting system, ureteric, bladder, prostate and small parenchymal lesions difficult, even with hydration and diuretics. Furthermore, previous studies of $^{18}$F-FDG at our institution indicate that radiotracer uptake is only moderate in most primary renal tumours, but may vary. It should be noted that uptake in metastatic disease can be higher than in the primary.\textsuperscript{526}

As alluded to, PET-CT with better anatomical localisation will further enhance genitourinary imaging and most studies have thus far been published in the pre PET-CT era. The use of PET in urologic malignancies other than RCC are summarised in Appendix E.

2.11.9 Positron Emission Tomography ($^{18}$F-FDG) in Renal Cell Carcinoma

Previous studies of $^{18}$F-FDG at our institution indicate that radiotracer uptake is only moderate in most primary renal tumours, but may vary. It should be noted that uptake in metastatic disease can be higher than in the primary.\textsuperscript{526} Although $^{18}$F-FDG is the most commonly used radioisotope in oncologic imaging, new radioisotopes are being developed to overcome the difficulties of diagnosing renal masses (discussed below). Despite limitations, fourteen studies have been published using $^{18}$F-FDG to demonstrate RCC (Table 2-13).
Table 2-13 A summary of the literature of $^{18}$F-FDG PET in Renal Cell Carcinoma

<table>
<thead>
<tr>
<th>STUDY Year</th>
<th>First Author</th>
<th>PATIENTS</th>
<th>DIAGNOSIS Patients (N)</th>
<th>STAGING Patients (N)</th>
<th>RE-STAGING Patients (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sensitivity (%)</td>
<td>False negatives (FN)</td>
<td>False negatives (FN)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FN=17; 60; FN=6</td>
<td>N=54; 75; FN=32</td>
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<tr>
<td>2004 Kang</td>
<td></td>
<td>66</td>
<td></td>
<td></td>
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<tr>
<td>2003 Majhail</td>
<td></td>
<td>24</td>
<td>---</td>
<td>N=24; 64; FN=9</td>
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<td>2003 Jadvar</td>
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<td>25</td>
<td>---</td>
<td>N=25; 71; FN=6</td>
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<td>2003 Chang</td>
<td></td>
<td>15</td>
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<td>---</td>
<td>N=15; 90; FN=1</td>
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<tr>
<td>2003 Aide</td>
<td></td>
<td>53</td>
<td>---</td>
<td>N=53; 47; FN=25</td>
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<tr>
<td>2002 Miyakita</td>
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<td>19</td>
<td>N=19; 32; FN=13</td>
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<tr>
<td>2002 Brouwers</td>
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<td>20</td>
<td>---</td>
<td>---</td>
<td>N=20; 69; FN---</td>
</tr>
<tr>
<td>2002 Safaei</td>
<td></td>
<td>36</td>
<td>---</td>
<td>---</td>
<td>N=36; 87; FN=4</td>
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<tr>
<td>2000 Montravers</td>
<td></td>
<td>20</td>
<td>N=13; 85; FN=1</td>
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<td>N=7; 100; FN---</td>
</tr>
<tr>
<td>1998 Ramdave</td>
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<td>22</td>
<td>N=17; 94; FN=1</td>
<td>N=8; 100; FN=0</td>
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<td></td>
<td>10</td>
<td>N=9; 90; FN=1</td>
<td>N=2; 100; FN=0</td>
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<tr>
<td>1996 Hoh</td>
<td></td>
<td>21</td>
<td>---</td>
<td>---</td>
<td>N=21; 100; FN=0</td>
</tr>
<tr>
<td>1996 Bachor</td>
<td></td>
<td>26</td>
<td>N=26; 77; FN=6</td>
<td>N=3; 100; FN=0</td>
<td>---</td>
</tr>
<tr>
<td>1995 Bachor</td>
<td></td>
<td>9</td>
<td>N=9; 89; FN=1</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>1994 Kocher</td>
<td>Abstract</td>
<td>10</td>
<td>N=10; 40; FN=6</td>
<td>N=10; 100; ---</td>
<td>---</td>
</tr>
<tr>
<td>1991 Wahl</td>
<td></td>
<td>4</td>
<td>N=4; 100; FN=4</td>
<td>N=4; 100; FN=4</td>
<td>---</td>
</tr>
</tbody>
</table>
2.11.9.1 Diagnosis of RCC
The major modality for diagnosis of RCC is CT or ultrasound, with MRI having a specific role in evaluating tumour thrombus extension. The major difficulty with diagnosis of lesions with $^{18}$F-FDG-PET is false negative results due to difficulties with urinary excretion leading to variable detection of primary tumours between 60-90%. One must emphasise that no imaging modality has ideal specificity and around 10% of lesions thought to be RCC are benign (e.g. oncocytomas). The role of PET in diagnosing primary renal tumours is likely to remain limited in the near future. However, this may change when PET/CT is standard and cumbersome systems presented in studies such as using dictated PET reports and then analysing imaging studies (e.g. CT) alongside to establish accuracy of PET are abandoned. New radiotracers may change the ability of PET to diagnose lesions.

2.11.9.2 Staging of RCC
The identification of lymph node metastases is still problematic for conventional imaging with CT since the limiting size is 4 mm and results in a false negative rate of 10%, especially in the presence of micrometastases. A higher false positive rate of 3-43% is mainly due to reactive hyperplasia. Against this, PET staging of RCC has been promising sensitivities between 64-100% with the majority approaching 100% for metastatic disease (Table 2-13). Of course micrometastatic disease cannot be accounted for that may have been missed but the difference in sensitivity highlights the difficulties in the urinary tract for primary evaluation where urinary excretion and renal accumulation are problematic in areas of interest. Clinically with staging the most important role of imaging is detecting occult lymph node visceral metastases, but bony disease is also important. Traditionally the realm of nuclear medicine bone scintigraphy, PET is able to accurately bony metastatic deposits, and may in the future replace traditional bone scintigraphy but further studies are required.
2.11.9.3 Re-staging of Renal Cell Carcinoma

PET has been shown to be accurate in the detection of recurrent RCC with sensitivities between 87-100% in the published studies of almost one hundred patients.

Thus, $^{18}$F-FDG-PET is highly sensitive and accurate at detecting local disease spread and metastatic disease in patients with RCC. Importantly, in one study it was highlighted that the PET study altered patient management in 40% of patients. $^{526}$ Ultimately, that is the real benefit to patients, especially in cases where other forms of anatomical imaging such as CT are inconclusive because they lack the ability to delineate functional capacity of tissues in most instances.

2.11.10 Positron Emission-Computed Tomography using Hypoxic tracers

No techniques to evaluate the intratumoral hypoxia have entered widespread clinical use and hypoxia cannot be predicted by tumour size, grade or histology. The critical tumour oxygen partial pressure is proposed to be 8-10mmHg. $^{544}$ Below this level ATP depletion, intracellular acidosis and decreased energy supply occurs. Normal tissues typically have median oxygen concentrations ranging 40-60mmHg whilst half of solid tumours studied have median values below 10mmHg. Tumour-to-tumour variability in the oxygenation pattern is more pronounced than the intratumour heterogenicity. $^{545}$ As well as imaging with $^{18}$F-FDG other specific radiotracers have been developed to improve imaging of tumour hypoxia non-invasively.

2.11.10.1 $^{18}$F-Fluorodeoxyglucose ($^{18}$F-FDG)

Experimental data using human cancer cell lines suggests that hypoxic conditions lead to increased FDG uptake. $^{546}$ $^{547}$ Further support has come from studies investigating the uptake pattern of FDG as well as the expression of glucose transport proteins and hexokinase and finding a relationship with the hypoxia marker HIF-1α. $^{548}$ Taking it a step further and comparing $^{18}$F-FDG uptake to direct oxygen measurements POS the data is
less convincing. For example, no correlations could be shown between $^{18}$F-FDG PET parameters and polarographically determined tumour oxygenation status.$^{203}$

Finally, data comparing the uptake of $^{18}$F-FDG with the hypoxic volume as assessed by an hypoxic PET radiotracer $^{18}$F-FMISO (discussed later) a correlation coefficient of 0.63 has been reported. However, a considerable discrepancy between $^{18}$F-FDG and $^{18}$F-FMISO uptake has been reported in head and neck, breast cancer, soft tissue sarcoma and glioblastoma.$^{218,549,550}$

2.11.10.2 $^{18}$F-Fluoromisonidazole ($^{18}$F-FMISO)

Recently, radiolabelled nitroimidazoles (e.g. $^{18}$F-Fluoromisonidazole or $^{18}$F-FMISO) have emerged as non-invasive techniques for the assessment of tumour hypoxia. Nitroimidazoles (e.g. metronidazole) are effective against organisms thriving in hypoxic environments. Nitroimidazoles are metabolised by intracellular nitroreductases, and at low oxygen levels serve as competing electron acceptors. They are reduced and form covalent bonds to macromolecules, thus becoming biochemically trapped within these hypoxic yet metabolically active cells.$^{551}$ Such drugs bind to cells at a rate that is maximal under conditions of severe hypoxia and is inhibited at increasing oxygen concentrations. Variation in radiotracer uptake between individuals is to be expected in tissues. In particular, the sensitivity of nitroimidazole (e.g. $^{18}$F-FMISO) imaging for the detection of hypoxic tissue will be determined by several factors including delivery, retention and metabolism of the nitroreductase.$^{552}$

The advantages of $^{18}$F-FMISO are that it is highly stable after injection. Up to two hours after administration of $^{18}$F-FMISO, no metabolites of it were found in the blood of humans or mice. Uptake of $^{18}$F into bone would indicate free fluorine and this has never been observed.$^{553-555}$
Table 2-14 Comparison of clinical studies using $^{18}$F-FMISO PET to detect hypoxia

<table>
<thead>
<tr>
<th>Study</th>
<th>Tumour</th>
<th>Patients</th>
<th>Modality of treatment</th>
<th>Results-any hypoxia</th>
<th>Scan following treatment</th>
<th>Region of Interest Ratio for positive study†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rischin</td>
<td>Head and Neck</td>
<td>32</td>
<td>Chemo-radiotherapy +</td>
<td>32/45</td>
<td>6/29 hypoxia post</td>
<td>Positive; focal uptake greater than background</td>
</tr>
<tr>
<td>Rajendran</td>
<td>Head and Neck</td>
<td>73</td>
<td>Pretherapy</td>
<td>58/73</td>
<td>Nil</td>
<td>≥ 1.2 Tumour: Blood ratio</td>
</tr>
<tr>
<td>Cherk</td>
<td>Lung</td>
<td>17</td>
<td>Nil</td>
<td>17/17 had T:N &gt;1</td>
<td>Nil</td>
<td>NA</td>
</tr>
<tr>
<td>Zimny</td>
<td>Head and Neck</td>
<td>22</td>
<td>Nil</td>
<td>11/22 overall (50%)</td>
<td>Nil</td>
<td>↑ uptake in tumour tissue cw normal</td>
</tr>
<tr>
<td>Bentzen</td>
<td>Soft Tissue Tumours</td>
<td>13</td>
<td>Surgery</td>
<td>2/7 malignant (29%); 0/6 benign (0%)*</td>
<td>Nil</td>
<td>↑ uptake in tumour tissue cw normal</td>
</tr>
<tr>
<td>Rajendran</td>
<td>Soft Tissue Sarcomas</td>
<td>19</td>
<td>Chemotherapy</td>
<td>14/19 (76%)</td>
<td>10 : 5/6 positive prior reduced, 2 ↑, 2 same</td>
<td>≥ 1.2 Tumour: Blood ratio</td>
</tr>
<tr>
<td>Rischin</td>
<td>Head and Neck</td>
<td>15</td>
<td>Chemotherapy &amp; Radiotherapy</td>
<td>14/15 (93%)</td>
<td>13/14 uptake reduced or absent</td>
<td>↑ uptake in tumour tissue cw normal</td>
</tr>
<tr>
<td>Rasey</td>
<td>21NSCLC; 7 Head and Neck; 4 Prostate; 5 other (1 metastatic RCC)</td>
<td>37*</td>
<td>Radiotherapy</td>
<td>36/37 (97%)</td>
<td>Nil</td>
<td>≥1.4 Tumour: Plasma ratio</td>
</tr>
<tr>
<td>Yeh</td>
<td>Naso-pharyngeal carcinoma</td>
<td>24</td>
<td>Nil</td>
<td>24/24 (100%)</td>
<td>Nil</td>
<td>≥1.24 Tumour: Normal tissue ratio</td>
</tr>
<tr>
<td>Koh</td>
<td>NSCLC</td>
<td>7</td>
<td>Radiotherapy</td>
<td>5/7 uptake reduced</td>
<td></td>
<td>≥1.4 Tumour: Plasma</td>
</tr>
<tr>
<td>Koh</td>
<td>Head &amp; Neck (5); Renal RCC with metastases (1); NSCLC (1); Adenoid cystic (1)</td>
<td>8</td>
<td>Radiotherapy</td>
<td>6/8 overall (75%); H&amp;N 5/5 (100%); RCC negative</td>
<td>Uptake reduced in 6/8</td>
<td>≥1.4 Tumour: Plasma</td>
</tr>
<tr>
<td>Valk</td>
<td>Glioma</td>
<td>3</td>
<td>Nil</td>
<td>2/3 (66%)</td>
<td>Nil</td>
<td>≥1.1 Tumour: Plasma</td>
</tr>
<tr>
<td>Gagel</td>
<td>Head &amp; Neck</td>
<td>16</td>
<td>Radiotherapy</td>
<td>High correlation (r &gt; 0.7) between FMISO and hypoxia</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

* Region of Interest Ratio Considered Positive- The ratio of Hypoxic Tissue to Normal Tissue considered as significant increased uptake of and therefore positive, in that study

† In the study, 6/7 malignant and 3/6 benign tumours were positive for uptake but the amounts did not remain higher than normal tissue over time; NSCLC- Non-small cell lung cancer
2.11.10.3 Studies comparing $^{18}$F-FMISO uptake to oxygen tension in tumours

Many of the studies validating $^{18}$F-FMISO as an hypoxic tracer are based on the distinction between normoxic and hypoxic tissue when animals with xenografts are made to breathe carbogen gas (95% oxygen, 5% CO$_2$) or other variants against normal air or better oxygenated mixtures.$^{565-568}$ $^{18}$F-FMISO has is now accepted as a validated hypoxia marker as already discussed and has taken on a prognostic$^{556}$ and treatment planning role in some tumours. Complimenting this literature there are many examples of POS being use to predict oxygen status and even prognosis (Appendix E). However, studies comparing $^{18}$F-FMISO uptake to “gold standard” POS (or oxylite probe) measurements have not routinely been conducted. The bodies of literature have almost developed in parallel. There are some exceptions. One study in metastatic head and neck cancer patients found that POS measurements correlated with the $^{18}$F-FMISO tumour: muscle ratio but only with the frequency of HP5 (pO$_2$ readings 5 ≤mmHg).$^{569}$ Yet this was not reproduced in a similar study where only a light trend to increased uptake with pO$_2$ and $^{18}$F-FMISO uptake was demonstrated.$^{205}$

In tumour xenografts, more comparative studies have also been conducted. In one such study, tissue pO$_2$ was found to be at best “broadly consistent” with $^{18}$F-FMISO uptake.$^{570}$ This study did not correlate individual measurements with SUV and was only conducted in three tumours. Bentzen and colleagues$^{571}$ compared $^{18}$F-FMISO) PET with POS in breast carcinomas ranging 150-1,500 mm$^3$. PET studies significantly discriminated between tumours of carbogen and air-breathing mice and the POS determined a significantly lower percentage of measurements below 2.5 mmHg for carbogen-treated mice compared with air-breathing mice. However, no direct correlation between the two methods was seen. Another study using POS and $^{18}$F-FMISO PET did not correspond well when comparing data from the C3H mammary carcinoma and squamous-cell carcinoma. The former tumour had moderate POS-estimated hypoxia and marked FMISO uptake whilst the latter had a more severe POS hypoxia, but relatively minor FMISO uptake.$^{572}$
In a further study of murine xenograft models of squamous-cell carcinoma and mammary carcinoma, actual correlations could not be made between pO₂ and 18F-FMISO uptake. It was concluded the difference was likely caused by scattered foci versus confluent areas of viable hypoxic tissue in the tumours.\textsuperscript{572} Other investigators\textsuperscript{571,573} have had similar issues with discordance and asserted that tumour necrosis is at least partially responsible.

Thus various explanations for the ability of 18F-FMISO uptake to correlate with hypoxia but not the exact range of POS measurements have emerged. These range from heterogeneity of 18F-FMISO uptake both as an intrinsic phenomenon and also due to the impact of pO₂ measurements obtained in necrotic tissue.\textsuperscript{565-567} Another explanation put forward is the architecture of the tumour and in particular how the angiogenesis impacts on structure with different sized vessels, and different structure of these tumour vessels.\textsuperscript{572} Sorensen et al also suggested that the uptake FMISO might reflect factors other than just low PO₂, such as microregional differences in metabolic activity and differences in activity of various enzymes.\textsuperscript{572}

Given this information it is not surprising that conflicting literature using other models exists regarding correlation between the 18F-FMISO uptake and pO₂ levels and such studies may help explain the differences. For example, in soft tissue sarcomas a correlation between POS and 18F-FMISO was not found.\textsuperscript{218} The reasons put forward include tumour heterogeneity and the fact that PET is gaining a more global assessment over POS. Certainly, these situations are slightly different whereas they did not find hypoxia whereas I did. But there is no reason why the same issues make correlating the exact pO₂ with the SUV\textsubscript{max} in a tumour with uptake are not occurring. In fact, it is likely that with such great uptake distinguishing actual pO₂ level becomes more difficult because the majority of the tumour and not small portions have increased uptake contributing to a higher overall global count.

Other more subtle differences may also account for conflicting results. Almost all use around two hours\textsuperscript{574} to allow biodistribution of 18F-FMISO prior to PET scanning whilst some with
positive results are beginning to use up to four and a half hours for distribution.\textsuperscript{557} The impact on \(^{18}\)F-FMISO uptake has not been studied in many patients or xenograft models and may be of interest in further studies.

In summary, although \(^{18}\)F-FMISO uptake correlates with hypoxia, there are differences between this method and POS oxygen measurements. There is some evidence that the hypoxic fraction correlates but sparse evidence that the SUV recorded on PET may correlate with a specific pO\(_2\). In simple terms, \(^{18}\)F-FMISO-PET provides information on the tumour oxygenation status on a global level- that is why it has been effective for example when facilitating dose painting in radiation treatment planning.\textsuperscript{575} POS on the other hand provide targeted measures of hypoxia that must have the pooled data analysed and the results are influenced by placement of the probe and the heterogenous nature of hypoxia within tumours.

2.11.10.4 Comparison of \(^{18}\)F-FMISO and \(^{18}\)F-FDG uptake in the same tumours

Despite \(^{18}\)F-FMISO and \(^{18}\)F-FDG-PET studying different tumour parameters some comparisons have been made in the literature to delineate if indeed there is some similarity. If there was this could potentially link the two radiotracers and the hypoxic pathway with the concept that glucose utilisation is related to hypoxia.\textsuperscript{569}

In head and neck cancer some studies have taken place. At our institution, such as study found poor correlation between the two different radioisotopes.\textsuperscript{558} This has been confirmed in a further study\textsuperscript{576} but in contrast another study found a moderate correlation of \(^{18}\)F-FMISO and \(^{18}\)F-FDG uptake. A small study of lung cancer patients\textsuperscript{205} and one of sarcoma patients also failed to demonstrate a correlation.\textsuperscript{549}

Thus the majority of studies have found no correlation between \(^{18}\)F-FMISO and \(^{18}\)F-FDG uptake. Interestingly some investigators have used the addition of the uptake by both radioisotopes as a means of prognosis, recognising they measure different parameters within tumours.\textsuperscript{557}
2.11.10.5 Other hypoxic radiotracers used in PET

$^{64}$Cu-diacetyl-bis(N4-methylthiosemicarbazone ($^{64}$Cu-ATSM) is similar to $^{18}$F-FMISO and is an intracellular agent trapped in viable cells under low cellular partial pressure of oxygen has been shown to be bioreduced$^{577,578}$. Although numerous preclinical studies exist,$^{579}$ few clinical studies in humans have been published. Limited experience with this radiotracer in uterine malignancies$^{580}$ non-small-cell lung cancer$^{581,582}$ and colon cancer$^{583}$ exist but more data is but more data is required. In particular, data that compares radiotracers to POS measurements. Another similar radiopharmaceutical with oxygenation-dependent retention is $^{18}$F-fluoroazomycinarabinofuranoside ($^{18}$F-FAZA) Again a promising but not well-characterised hypoxia-specific tracer.$^{584,585}$ Other tracers such as $^{123}$I-IAZA (similar to ($^{18}$F-FAZA $^{18}$F-FAZA )deiodinates in vivo with substantial uptake in the thyroid making it technically challenging$^{586,587}$. Other potential radioisotopes that require much more development but show promise are $^{18}$F-fluoroerythronitroimidazole which is easier to prepare, less costly, and more hydrophilic than $^{18}$F-FMISO$^{588}$ and $^{18}$F-EF5$^{589}$ which is analogous to piminidazole which is used for immunohistochemical demonstration of hypoxia in sections of tissue.
2.11.11 Molecular Imaging of Renal Cell Carcinoma with PET

*Radioisotopes based on Acetate*
Acetate is converted to acetyl-coenzyme A in the mitochondria, followed by rapid clearance as carbon dioxide through the citric acid cycle. $^{11}$C-Acetate can demonstrate areas of high metabolism and blood flow such as are found in normal renal parenchyma. Increased uptake in renal malignancies without significant excretion into the urinary tract has been demonstrated but further studies are needed to delineate its role in RCC.  

*Radioisotopes based on Choline*
Choline is a precursor for phospholipid synthesis and has been investigated as a tracer in several tumours. $^{524}$ Choline is necessary for phospholipid synthesis in cell membranes, cholinergic neurotransmission, methyl metabolism, transmembrane signalling as well as lipid cholesterol transport and metabolism. $^{524}$ Phosphorylcholine has been found at high levels in most cancers and at low or undetectable levels in normal tissues, using MRI studies. $^{501}$ In tumour cells, choline metabolism is directed toward cell membrane synthesis, and the de novo synthesis of choline is negligible in tumour cells. $^{524}$

Choline may be combined with carbon ($^{11}$C-Choline) or fluorine ($^{18}$F-Fluoroethylcholine and $^{18}$F-Fluoromethylcholine). One disadvantage of fluorine–based radioisotopes is their prompt urinary excretion leading to rapid masking of background uptake. Alternatively, $^{11}$C-Choline has reduced urinary excretion, resulting in a better target to background ratio. It has been used for cancer detection in a variety of tumours with prostate cancer also visualised. $^{592}$ Studies of RCC are lacking and normal uptake in renal tissue will need to be overcome but would be aided by reduced renal excretion.
Other Radioisotopes

Tumour tissues that have high proliferation rates require high rates of DNA synthesis and tracers such as those based on the nucleic acid thymidine have been developed. The advantages of a proliferative marker over $^{18}$F-FDG is that a proliferation marker (e.g. $^{18}$F-Fluorothymidine or $^{18}$F-FLT) may give better specificity in the assessment of tumours or better accuracy in the evaluation of early response. This is because proliferation is more sensitive than glucose utilisation.$^{593}$

A radiotracer that reflects DNA synthesis is radiolabelled thymidine (TdR) and has been used to track cell proliferation both in vitro and in vivo. Rapid metabolism by thymidine phosphorylase makes TdR a cumbersome molecule to analyse as large numbers of molecules result from breakdown requiring analysis but current PET software is able to perform the task.$^{594}$ No studies using radiolabelled fluorothymidine (FLT) for RCC have been trialled in humans so patients at our institution are currently being investigated with this radioisotope.

Radiotracers based on methionine provide a similar measure of proliferation to those based on glucose, but instead reflect amino acid metabolism. No studies with renal cell carcinoma have been published.

2.11.12 Radioimmunoscintigraphy (Immuno-PET) and Renal Cell Carcinoma

Radioimmunoscintigraphy using positron emitters (immuno-PET) for tumour visualisation has developed because antibodies may now be linked with positron emitters. Monoclonal antibodies (mAb) have been labelled with $^{111}$In or $^{99m}$Tc as radioisotopic imaging agents in prostate, colorectal and ovarian carcinoma.$^{595}$ Immuno-PET involves radiolabelled mAb bound to an antigen on tumour cells and the radionuclide then emits radiation ($\gamma$-photons) that is detected by a camera mounted within a PET scanner (Figure 6). The half-life of common PET tracers ($^{11}$C, $^{18}$F) is too short for use with antibodies in vivo. Positron emitters
The optimal uptake of such antibody conjugates in tumours is normally several days for intact immunoglobulins.

One antibody of interest in RCC is G250. The G250 antigen (CAIX) is a membrane-associated carbonic anhydrase thought to play a role in the regulation of cell proliferation in response to hypoxic conditions, and may be involved in oncogenesis and tumour progression. cG250 refers to a monoclonal antibody that was raised by immunization of mice with human RCC homogenates. Previous immunobiochemical studies revealed that cG250 is highly expressed in RCC with selective uptake of mAb G250 in antigen-positive cells versus antigen-negative cells. G250 has been labeled with $^{89}$Zr and $^{124}$I for immuno-PET with promising results. Targeting of RCC by cG250 has been amongst the highest demonstrated in solid tumours. Recently, Divgi et al using $^{124}$I-cG250 appeared to identify accurately clear cell RCC in a study of 26 patients with a sensitivity of 94% which is promising but needs further data.
Specific monoclonal antibodies radiolabelled with a particular radioactive isotope bind to a site on the tumour. Where the radiation interferes with cellular turnover and kills cells, this is radioimmunotherapy. Where the radiation is recorded by a PET detector to determine location and uptake of antibodies is straightforward immuno-PET. Both concepts may be used individually or together depending on the radioisotope properties.
2.11.13 Radioimmunotherapy and Renal Cell Carcinoma

Radioimmunotherapy is an extension of radioimmunoscintigraphy. Broadly, it involves delivery of radiation to the target cells by linking the radionuclide (e.g. $^{131}$I) to a mAb that targets a specific antigen expressed by tumour cells. However, it is different to immuno-PET as a different radiation (generally $\beta$-particles) is responsible for the destruction of cellular DNA rather than the images obtained in PET that detect $\gamma$-photons. Thus, it is possible to have radioisotopes that only emit $\beta$-particles, such as $^{90}$Y that are not suitable for imaging with PET. However, a radioisotope such as $^{131}$I emits both $\gamma$-photons and $\beta$-particles, making it suitable for imaging and treatment. The mAb itself may also act as an immune modulator helping to destroy cells through ADCC; or the mAb may mediate direct effects on the cell depending on the nature of the target for which it is specifically directed.

Therapeutic studies have focused on use of radiolabelled monoclonal antibodies with radionuclides including $^{131}$I, $^{111}$In or $^{99m}$Tc. Early therapeutic studies were in patients with lymphoma expressing multiple tumour antigens and more specialised agents such as those targeting with anti-CD80 monoclonal antibody (galiximab) are now available.

Several studies of patients with metastatic RCC have been undertaken with radiolabelled $^{131}$I-G250 with only modest results as already discussed, but with some complete responders. More studies are needed.

2.11.14 Summary of PET-CT and Renal Cell Carcinoma

The status of PET is in evolution. Certainly, $^{18}$F-FDG-PET has a role in staging and re-staging disease whilst other radiotracers need further clinical data. The use of PET-CT may alter the ability to detect pelvic and other small metastatic deposits improving sensitivity of staging and re-staging. Immuno-PET is advancing rapidly and an array of monoclonal antibodies against a variety of tumour antigens will lead to further imaging capabilities. It
will also have an increasing role in the monitoring of radioimmunotherapy for dosage and response to treatment. Finally, radioimmunotherapy is being studied and once toxicity and doses are adjusted correctly, this may be the key to treating RCC in the future.

2.12 Summary of Angiogenesis, Hypoxia and Imaging in Renal Cell Carcinoma

Clearly, RCC is a tumour reliant on angiogenesis and the mechanisms behind this are slowly being unravelled, particularly with the appreciation of the pVHL pathway in clear cell RCC and its relationship to hypoxia. This has spurned the new TKI that have had some success in treating metastatic RCC.

However, some areas of basic understanding have not have not been investigated or require greater understanding in RCC:

This is despite many papers linking hypoxia to RCC. As such, no direct oxygen studies demonstrating tissue oxygenation or hypoxia within normal or neoplastic renal tissue have been published.

1. Although oxygen status is important to the pVHL pathway, and hypoxia is constantly referred to in relation to RCC, no one has directly
   a. measured RCC or normal renal tissue oxygen levels
   b. studied oxygen levels in human RCC xenografts

2. No studies have compared tissue oxygen levels with indirect immunohistochemical measurements of hypoxia and angiogenesis

3. Researchers have no non-invasive methods of targeting tumour hypoxia in RCC. In particular, there are no studies examining $^{18}$F-FMISO, the most studied hypoxic PET radiotracer, in RCC. Further, no studies of FLT in RCC xenografts or humans with RCC.
4. No extensive studies of serum osteopontin, an surrogate marker of tumour hypoxia have been conducted in primary RCC or in conjunction with oxygen measurements

5. There are no studies of $^{124}$I-cG250 in RCC tumour xenograft models that have examined tumour oxygen levels

6. The effect of systemic therapies on oxygen status within tumours has not been investigated.

7. No therapeutic studies in xenografts of systemic treatments in conjunction with oxygen measurements within tumours

8. Orthotopic models for developing xenografts in kidney cancer have not been explored using modern surgical equipment

After the completion of this thesis, many of these questions will have been explored and our understanding has been advanced so that research into RCC may evolve further.
Chapter 3

MATERIALS AND METHODS

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3.1 MATERIALS AND METHODS: HUMAN STUDIES

The materials and methods section is divided into those methods used in the human studies of this thesis and those used in the xenograft studies related to this thesis. The broad categories covered each section for each method in each are:

1) Direct measurements of oxygenation in tumours and normal tissue
2) Imaging of tumours
3) Measurement of surrogate endogenous markers of tumour oxygenation via immunohistochemistry
4) Xenograft tumour transplantation using new methodology

As many of the methods are routine procedures in our laboratory detailed methodological evaluation will not be given here. However, where required, methods will be described in detail.

3.2 Study Patients

Patients with suspected RCC were recruited from urology outpatient clinics at the Austin Hospital, Heidelberg, Victoria, Australia from end of 2001 until 2004. Patients were enrolled after being provided with patient information sheets and consented for the study that had been endorsed by the Human Research Ethics Committee at the Austin Hospital. Patients were all consented prior to participation.

It was aimed to get approximately fifty patients enrolled. The study essentially had four components it involved the use of up to four different methods in investigating hypoxia and angiogenesis in RCC.
1) Surgery with tissue for histology and immunohistochemistry on tumour specimens, including staining for evidence of angiogenesis and cellular proliferation, as well as other marker of hypoxia

2) Polarographic oxygen electrode studies for direct measurement of tumour oxygenation with a probe at the time of nephrectomy of tumour and normal renal tissue

3) Pre-nephrectomy (pre-operative) assessment of tumour hypoxia non-invasively with positron emission tomography (PET) studies using $^{18}$F-Fluoromisonidazole [$^{18}$F-FMISO], a radiotracer specific for hypoxia in other tumours pre-nephrectomy to ascertain if the measurements of hypoxia correlate with tumour uptake.

4) Pre-nephrectomy measurement of serum osteopontin, a tumour marker correlating with hypoxia in head and neck cancers. (added to the protocol part way through the study)

These four methods are essentially in themselves four different experiments in helping to characterise oxygenation of RCC and the methods will be outlined that were utilised in each of them. As an inclusion criterion, all 52 patients had at least method one (nephrectomy, histology and immunohistochemistry) completed as part of the study.

### 3.3 Measuring oxygenation in tumours and normal tissue

Hypoxia may be detected in normal tissues and tumours by invasive techniques such as oxygen probes, non-invasive imaging techniques and molecular markers studied via immunohistochemistry. Each technique has advantages and disadvantages and must be tailored to the individual tissues or tumours being studies.\(^{24}\) Not every tissue or tumour has had their oxygen level accurately recorded and more investigation needs to be given to accurate methods of measuring which human tumours might have significant numbers of hypoxic viable cells.\(^{604}\)
There are two broad groups of techniques to study tissue oxygenation:

A) Direct or Invasive oxygen measurements
   1) Polarographic Oxygen Sensor (POS)
   2) Oxylite Probe

B) Indirect or Non-invasive techniques for assaying the presence of hypoxia
   1) Non-invasive Imaging Techniques using Positron Emission Tomography with radiotracers avid for hypoxic environments e.g. $^{18}$F-FMISO
   2) Immunohistochemistry:
       Surrogate Markers of hypoxia e.g. CAIX expression
   3) Serum Markers of Hypoxia in Tumours:
       Osteopontin is a secreted plasma marker that has recently been found to be overexpressed in a variety of human tumours.\(^{478}\)

In this thesis, both direct and indirect measurements of oxygenation are utilised. I chose methods that have been used in similar experiments in the past or that would be readily applied to RCC. I avoided experimental techniques that have limited data. As no one had invasively measured tissue oxygenation in the kidney, the choice was for a polarographic probe. For non-invasive investigation of tissue oxygenation I focused on PET and combined PET-CT as I am in a leading centre for this imaging having the first experience with this modality in Australia and having an onsite cyclotron to create radiotracers. Finally, I utilised immunohistochemistry and immunoassays that our laboratory had previous experience with to ensure high quality results.
3.4 Direct measurements of Oxygenation in tumours and normal tissue: 

Human studies

Direct measurements of tissue oxygenation developed in the late 1960’s, when the Clark oxygen electrode became available. Of all methods available to measure tissue or tumour oxygenation, the direct invasive method using a polarographic oxygen sensor probe (POS) is considered the ‘gold standard’. The POS probe is also known as the Eppendorf pO2 histograph, after its original manufacturer (Eppendorf Hamburg, Germany) and the graph created by its measurements. Another probe that has been developed and that is considered equivalent in terms of its ability to measure oxygen status in tissues is the time-resolved luminescence-based optical probe (Oxylite probe). This is discussed in section x below and was used only in animal studies as it is not licensed for use in human studies.

3.4.1 The Polarographic Oxygen Sensor Probe

Measurements of tissue oxygenation were obtained using a single computer-controlled polarographic oxygen sensor probe (Phoenix Probe, Helzel Medical Systems, Germany).

Polarographic Oxygen Sensor Probe Mechanics

The oxygen measurements were performed in awake, room air–breathing patients with a computerized polarographic electrode. Briefly, the tissue oxygen partial tension was measured with a 12- or 17-mm, glass-insulated gold microcathode, which is covered with a Teflon membrane and recessed in a jacket tube of steel. The electrode has a diameter of 0.3 mm (25 g) and was inserted subcutaneously through a 22-g intravenous catheter. The gold cathode is biased with 2700 mV toward a silver/silver chloride anode (electrocardiogram pad placed elsewhere on the body), with a resulting current of 0.01 to 3.0 nanoamperes. This current is proportional to the oxygen partial tension in the connecting electrolyte (represented by test tissue). All measurements were immediately preceded and followed by calibration and recalibration, respectively, in room air (20.9% oxygen) and pure nitrogen (0.0% oxygen).
Thus, in a polarographic sensor molecular oxygen is reduced at the surface of a cathode by applying a negative voltage between cathode and anode. In neutral or alkaline electrolytes, the following overall reaction occurs:\textsuperscript{606}

At the cathode \( O_2 = 4e^- + 2H_2O = 4OH^- \)
At the anode \( 4 Ag + 4 Cl^- = 4 AgCl + 4e^- \)

Therefore, the reduction current \( e^- \) is proportional to the number of oxygen molecules being reduced. The polarographic reaction can be tested by measuring the so-called polarogram.\textsuperscript{607}

First, by increasing the negative voltage an increase of the reduction current is produced, but then in spite of a further voltage increase the reduction current remains constant and a plateau is formed.\textsuperscript{606}

\subsection*{3.4.2 Polarographic Oxygen Sensor Probing in Humans}

All patients had measurements of normal renal tissue and tumour tissue in the same kidney whilst being monitored under general anaesthesia. All patients were warmed externally with warming blankets and had oxygen status maintained with oxygen saturations of 99-100\% throughout the measurements. General anaesthesia using these techniques has been shown to have no significant effect on polarographic electrode measurements in humans and the probe automatically adjusted for tissue temperature.\textsuperscript{189,608} Where appropriate, the measurements were taken when the situation was stable but when unstable, it was not considered to be safe and measurements were delayed until the patient was stable or abandoned in favour of progressing with the surgery.

\textit{Determining track length and number of tracks}

Oxygen tension measurements are typically made along a number of tracks within the tumour.\textsuperscript{221} Often the track length, number and location are determined arbitrarily by the investigator.\textsuperscript{221} Typically the oxygenation status of individual tumours is estimated from
multiple POS probe measurements (often between fifty and one hundred) obtained along two or three different electrode tracts. The track length used in our studies was based on preoperative CT imaging aiming to measure from the outside of the tumour and into the centre based on precise CT measurements. Necrotic tissue was avoided so as not to adversely affect the pO₂ measurements (necrotic tissue is not hypoxic and has a pO₂ of 0mmHg).

Advancing the probe within tissue and recording data
As described previously, each probe was calibrated prior to use in normal saline to ensure accuracy. The procedure for using the POS was consistent and as follows: an Ag/AgCl reference electrode was placed on the subject’s skin. A small 1mm deep stab wound incision (2 mm wide) was made with a knife (#11 blade) over the tumour because the electrode was not able to penetrate through the renal capsule. A calibrated polarographic needle electrode (0.3 mm in diameter) that contained a 12-µm insulated gold electrode was inserted into the tumour at a pre-determined superficial location (away from necrotic areas noted on preoperative CT scan). The electrode was oriented such that as it advanced, it transgressed a large portion of the tumour diameter and avoided structures such as the renal hilum to prevent excessive haemorrhage or readings within collecting system (Figure 3-1).
Figure 3-1 The polarographic oxygen sensor entering a tumour in a patient from this thesis

These figures demonstrate Left: The probe itself, Centre: The breakdown of the probe in diagram form provided by the manufacturer Helzel Medical Systems and Right: The probe entering into the tumour of a patient from the study at open surgery prior to nephrectomy
The probe was driven forward by a computer-controlled micromotor for approximately 1.0 mm and then it retracted by approximately 0.3 mm before making a measurement of oxygen pressure. The backward probe movement minimizes artifacts caused by pressure on the sensing probe membrane. The potential for tissue damage by the probe needle itself has been identified but is relatively small and almost eliminated by the backward movement. Thus the net forward motion for each measurement was 0.7 mm, and an oxygen tension reading was taken at each of those steps. Each reading takes approximately 1 sec to complete. The current between the insulated gold cathode and the reference electrode was proportional to the tissue oxygen tension at the cathode tip. The spatial resolution of the reading was approximately 100µm. The relative frequencies of the individual pO₂ values were then displayed in a histogram to give the oxygen profile for that needle track in the tissue studied.

It has been estimated that somewhere between 20 and 50 measurements per tumour were required to accurately assess the oxygen levels of the tissue. Because each measurement is taken at 0.7-mm stepwise intervals, a 1-cm probe tract was approximately 12 individual measurements and so on. Usually two to three passes were made first into normal tissue in the same kidney, and then into the tumour, each track being in a different orientation.

The same protocol was used in all patients. This allowed measurement of the partial oxygen pressure with a high spatial sampling frequency within a few minutes. The pO₂ needle probes samples the entire tumour environment including malignant cells, normal tissue components, microvessel and necrosis. Necrotic areas noted by CT imaging were avoided.

Recording and displaying of data with the POS followed all similar Eppendorf type probes, where a histogram system is programmed into the software of the system to advance the probe continuously in small steps, withdrawing partially to relieve tissue compression before each reading. Further, it is programmed to calculate tissue pO₂ values based on an external
Figure 3-2 The Eppendorf Probe being used within a patient having a nephrectomy

A histogram is demonstrated on the computer monitor and the probe with sterile cover is placed within normal tissue and tumour at nephrectomy
calibration and also to summarize the measurements into a frequency histogram with 2.5 mmHg bins.\textsuperscript{178}

It is important to stress that due to technical difficulties with POS data in the past, it is imperative that measurements are not contaminated by measurements in stroma or necrosis.\textsuperscript{572} This was made possible by avoiding necrotic areas of tumour based on the CT scan and when the tumour was on view. It should also be noted that POS measurements have no effects on the animals or xenograft tissues for histology or immunohistochemistry.\textsuperscript{186}

**Reporting of probe data**

There is no universal means of recording or reporting data that is obtained from POS probes. Furthermore, several studies using different methods of pO\textsubscript{2} measurement have shown that in many tumour entities tissue oxygenation is quite heterogeneous and thus cannot be sufficiently described by a single value.\textsuperscript{611} Individual data points although recorded at the time, are rarely reported but rather summaries of the overall oxygen status within a tumour are conducted. This is because of heterogeneity of oxygen status within tumours. This allows for a global assessment of tumour oxygen status rather than focusing on readings outside the range that could potentially bias results.\textsuperscript{204,214}

The common factor in any recording and then reporting of oxygen measurements is that a steady state is reached in the tissue prior to any recording with the oxygen probe.\textsuperscript{605} This takes up to several minutes but is an important factor in gaining reproducible and reliable measurements.\textsuperscript{204,214} That is what was done in all of the human experiments I conducted.

Regarding overall reporting, various investigative studies have used determinants such as:

1) The median or mean of oxygen measurements in a tumour is usually reported.\textsuperscript{214,221} In reporting results in human studies, the median for each patient is calculated based on the readings of one or multiple tracks through the tissue. Then the median of the median values for all patients or grand median is then reported.\textsuperscript{211}
2) The mean of the means of each track through a tissue obtained and the mean of all of the individual measurements (to ensure the absence of any bias introduced by a larger track through one particular area of the tumour, since the track lengths are not identical) are typically reported. In addition, since the distribution of the measurements in an individual patient may not be a normal distribution (and in fact usually is not), the variance of the overall mean of the patient oxygen levels was also determined.  

3) The hypoxic proportion (HP) or fraction of oxygen readings below a certain level (e.g. 5mmHg (HP5)). HP5 has been defined as a poor prognostic marker in cervical cancer when ≤50% and so has become a common figure to report in the literature.  

4) HP5 reporting has also been extended to a pO2 of 10mmHg (HP10) because of the often quoted figure of 10mmHg being critical at which tissue hypoxia induces sub-cellular changes in tissues. HP5 has also been lowered to a pO2 of 2.5mmHg (HP2.5) in an attempt to delineate extremely hypoxic environments.  

5) Pooled HP5 or HP10 may then be compared against different patient characteristics such as age, grade, stage, type and tumour volume.  

In summary, after consideration of the literature I elected to report on:  

1) The mean of oxygen measurements in a tumour  
2) The mean of the means of all tracks through a tissue obtained and the mean of all of the individual measurements in each tumour is then presented for analysis  
3) The proportion or fraction of oxygen readings below 10mmHg (HP10), 5 mmHg (HP5), or 2.5mmHg (HP2.5)  
4) Pooled data such as the mean or HP10, was then be compared against different patient characteristics such as age, grade, stage, type and tumour volume.
3.4.3 Probe cleaning and sterilisation

Each probe was cleaned and sterilised as per manufacturer recommended guidelines. Particular attention was paid to educating the Central Sterilising Services Department staff on handling, cleaning and sterilisation of probes to ensure no damage occurred. In brief, probes were immediately and gently hand cleaned after use in humans. Gas sterilization was then performed with pure ethylene oxide concentration 300 g/m²; at a temperature of 54 °C; with relative humidity being 100 % and a gassing time of four hours. Probes were packaged in appropriate heat stable sterile wrapping on trays and stored until next required. The minimum degassing time was 48 hours. The actual probe unit was cleaned with alcohol after each use and the unit covered with a sterile drape before each use to reduce contamination of the patient or the probe unit.

3.4.4 Equipment specifications and limitations

The POS although licensed for use in humans is not able to be used laparoscopically. Therefore, again I was not able to use the probe in situations where a laparoscopic radical nephrectomy was being conducted. I felt that the clinical decision pathway should not be influenced by the study. In other words, the investigators were not allowed to place a patient in the study group until the type of surgery (open radical or partial or laparoscopic) had been decided on clinical grounds- tumour size, imaging assessment of the tumour, patient co-morbidities, anaesthetic assessment and finally patient choice. In this way surgeons were blinded from selecting patients for more open nephrectomies in the study period that would have suited results but potentially compromised patient care. Ultimately, only patients having open radical or partial nephrectomy were able to have POS measurements taken.
Table 3-1 Monitoring specifications of the Polarographic Oxygen Sensor

<table>
<thead>
<tr>
<th>Specification</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity (nA/kPa)</td>
<td>0.25</td>
</tr>
<tr>
<td>Linearity (decades-measured)</td>
<td>4</td>
</tr>
<tr>
<td>Stability (± %/hr)</td>
<td>0.1</td>
</tr>
<tr>
<td>Min. Zero Current (nA)</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Temperature Coef.(%/°C.)</td>
<td>2.9</td>
</tr>
<tr>
<td>Temperature Range (°C.)</td>
<td>0-50</td>
</tr>
<tr>
<td>Response Time (Sec/decades of response)</td>
<td>20/4</td>
</tr>
<tr>
<td>Polarizing Voltage (V)</td>
<td>0.55</td>
</tr>
<tr>
<td>Membrane Thickness (mm)</td>
<td>0.05</td>
</tr>
<tr>
<td>Membrane Material</td>
<td>Teflon</td>
</tr>
<tr>
<td>Membrane Retainer</td>
<td>Teflon cylinder</td>
</tr>
<tr>
<td>Body Material</td>
<td>Ceramic</td>
</tr>
<tr>
<td>Electrolyte</td>
<td>0.9% NaCl buffer+chelator</td>
</tr>
<tr>
<td>Storage</td>
<td>Wet/Dry</td>
</tr>
</tbody>
</table>

1nA(nanoamp)=10⁻⁹ amps, kPa=kilopascal
3.4.5 Ethical elements related to polarographic oxygen probe use in humans

The ethical element of any clinical study is not to compromise patient care. As such, it was not always possible to conduct PET studies in all patients. Some required urgent nephrectomy due to symptoms and some patients travelling from long distances also were not suitable. However, other data was extremely useful and patients themselves did not wish to be excluded and were willing to comply with other facets of the study.

With direct oxygen probe measurements it is important to stress that these have only ever been conducted in the percutaneous situation (head and neck cancer, sarcomas, breast, glioma), per vagina (cervical) or transrectally (prostate). No studies of intra-abdominal visceral tumours have ever been attempted. Nephrectomy although safe, may cause significant intra-abdominal haemorrhage. Where this type of haemorrhage occurred, readings were to be abandoned and not conducted in order to not compromise patient care.
3.5 Non-invasive Imaging Studies of Hypoxia in Humans with RCC using Combined Positron Emission Tomography and CT (PET-CT)

3.5.1 Introduction

PET uses radioisotopes that undergo positron emission decay. Positrons are emitted from the unstable radionuclide and interact with an electron in the surrounding tissues, resulting in an annihilation reaction with subsequent release of two, 511-kcV photons in opposite directions. A sophisticated ring detector surrounding the patient then detects the coincident photons and registers the interactions in the image\textsuperscript{543}. Several different isotopes are available for use in PET scanning and examples of the most frequently used are summarised in the table below. A cyclotron is required to produce the radioisotopes. By tagging radioisotopes to naturally occurring substances in the human body, PET scanning is able to supply unique information on the metabolic activity of a tissue.\textsuperscript{522}

To summarise the PET process, a radiopharmaceutical consisting of a radiotracer (e.g. $^{18}\text{F}$) and a substrate (e.g. analogue of glucose such as fluorodeoxyglucose) is formed in a cyclotron. This radiopharmaceutical (e.g. $^{18}\text{F}$-Fluorodeoxyglucose or $^{18}\text{F}$-FDG) is then injected into the bloodstream, given time to distribute and is metabolised according to substrate properties. A CT scan and PET study are undertaken in succession on the same machine (PET-CT). PET records radioactivity which is collated and processed. The extent of radiopharmaceutical uptake may then be compared between sites and organs to ascertain if a particular functional process (e.g. excessive metabolism of glucose as occurs in a rapidly dividing tumour) is occurring. Thus concurrently obtained anatomical and functional data for an individual is assessed by a nuclear medicine physician at a computer console in the context of a given clinical history.

The Centre for PET studies at our institution, the Austin Hospital, is facility at consisting of a medical cyclotron with dedicated radiochemistry facilities and a whole body PET-CT.
scanner and a whole body PET scanner. A brief overview of the methods in production of radiopharmaceutical, equipment used and data processing as well as the specifics of the radiopharmaceuticals and their protocols within this thesis will now be detailed.

### 3.5.2 Radiopharmaceutical Production for PET studies

The medical cyclotron (Table 3-2) at our institution is a Cyclone 10/5 from IBA (Louvain-la-Neuve, Belgium). This negative ion design machine accelerates $\text{H}^-$ ion to 10 MeV and $\text{D}^-$ ion to 5 MeV. At the extraction radius, the negative particles are stripped of their electrons by passing through a very thin carbon foil and the resulting positively charged ions ($\text{H}^+$ or $\text{D}^+$) are bent outwards to the target ports, by the magnetic field. Up to 50mA of proton and 30mA of deuteron beam intensity can be extracted onto a single target or divided between two oppositely mounted targets. The cylindrical magnet return yoke consisting of 15 cm of steel acts as the primary radiation shield and in addition the machine is enclosed inside a cylindrical shielding system consisting of 68 cm thickness of boron-doped water. Experimental measurements indicate that the cyclotron shielding, together with the 60 cm thick concrete wall of the vault, is sufficient to keep the radiation dose level outside the cyclotron vault to a safe level. In public areas, measurements of neutron and gamma dose rates were 0.007 mSv/h and 0.24 mSv/h respectively.\textsuperscript{615,616} The cyclotron produces all radiopharmaceuticals for the Austin Hospital and other sites in Australia and New Zealand.
Table 3-2 Specifications of the Austin Hospital cyclotron

<table>
<thead>
<tr>
<th>Cyclotron:</th>
<th>IBA Cyclone 10/5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Installation Date:</td>
<td>1992</td>
</tr>
<tr>
<td>Beam:</td>
<td>50mA 10MeV proton</td>
</tr>
<tr>
<td></td>
<td>30mA 5MeV deuteron</td>
</tr>
<tr>
<td>Targetry:</td>
<td>Five targets (2 x $^{18}$F, $^{11}$C, $^{13}$N &amp; $^{15}$O)</td>
</tr>
<tr>
<td>Hot-cell:</td>
<td>Ten lead-shielded hot-cells</td>
</tr>
<tr>
<td>Radiochemistry:</td>
<td>Five radiochemistry for $^{18}$F-nucleophilic substitution; two for $^{11}$C-methylation, one for $^{15}$O-water production and one for radioactive gas delivery.</td>
</tr>
<tr>
<td>Clean Room:</td>
<td>One clean room facility for sterile production of $^{18}$FDG</td>
</tr>
<tr>
<td>Radiopharmacy:</td>
<td>Standard equipment for quality control (Radio-HPLC, Radio-TLC, GC, GC-MS)</td>
</tr>
</tbody>
</table>
3.5.2.1 Radiolabelling of compounds

Radiolabelling of compounds involves considerable amounts of radioactivity and must be performed by remote control in lead-shielded hot-cells. Two types of hot-cells have been installed within the laboratory: two large hot-cells with viewing area mainly used for research and development; and eight small shielded hot-cells used to house automated radiochemistry modules for routine radiopharmaceutical production. Manufacture and installation of the lead-shielded hot-cells were performed by Bucek Industries (Geelong, Australia).\(^{615,616}\)

\(^{18}\)F-radiolabelling is performed in fully automated synthesis modules. Five modules are currently in operation including two from IBA (Ion Beam Applications, Belgium), one from EBCO Technologies (Richmond, BC, Canada), one from Coincidence SA (Belgium) and one from GE (General Electric Healthcare, UK) \(^{11}\)C-radiolabelling and \(^{15}\)O-radiolabelling will not be discussed.\(^{615,616}\)

3.5.2.2 Radiolabelling quality control

Quality-control systems for short-lived radiopharmaceuticals has been recognised as extremely important since the 1960s.\(^{617}\) In particular, there are biological quality controls to ensure safe and sterile agents for injection and secondly, the ability to have reproducible and stable radiotracers. All steps of radiolabelling in our institution followed accepted international conventions and standards including those of the United States Pharmacopoeia; typical specifications for radiopharmaceuticals include radionuclidic purity, radiochemical purity, chemical purity, pH, particle size, sterility, pyrogenicity (or bacterial endotoxin), specific activity, osmolality and stability.\(^{615,618}\) Additional quality control measures were used including measuring syringes containing radiopharmaceutical before and after injection.

3.5.3 Administration of radiopharmaceutical

Patients were given specific instructions on fasting status if appropriate. Intravenous access was obtained with a cannula after skin preparation and the radiotracer was injected. All radiopharmaceuticals had radioactivity assessed in the giving syringe (10 ml, plain) prior to
and after intravenous administration to ensure the whole radiopharmaceutical dose was injected. No cases of failed IV injection occurred in any of the studies in this thesis. No cases of allergic reaction or other medical event were noted.

### 3.5.4 PET-CT machine utilised in the studies

The initial patients enrolled in the study had pure PET combined with scanned CT images acquired at a separate time. PET images were obtained using the ECAT 951/31R PET scanner (Siemens/CTI Inc., Knoxville, TN). As outlined, images using data from different time periods and on different machines is not the most ideal way to localise anatomical and functional imaging but PET-CT was extremely new at the commencement of this thesis and I was fortunate to have the machine updated early in my study to a combined PET-CT scanner.

Thus the remaining human studies in the thesis utilized a combined PET-CT machine (Gemini PET and Dual slice Philips MX 8000 CT, Philips Electronics North America Corporation, New York, NY). This scanner allowed multidetector row helical CT scanning. This machine was consistent throughout the study. All protocols (Appendix A) were developed in conjunction with the PET centre at our institution and followed for all patients. No deviations were reported and no scan failures recorded.

### 3.5.5 Data Processing

Emission and transmission data are acquired by the PET and PET-CT scanner hardware and stored on the acquisition computer sub-system. Coincidence events are stored in acquisition memory in either histogram or listmode format.

In histogram mode, events are incrementally binned whilst in listmode, the binning stage is performed off-line. The binning stage converts the event from the coincident detector pairs to a line of response to give an angle and offset of the event.

This so-called ‘sinogram’ representation of the data corresponds to sets of 2-dimensional projections of the tracer distribution. Whole body scans are performed by acquiring multiple
‘beds’ where the patient is scanned sequentially by moving the bed to cover the region of interest.

After emission and transmission acquisition for each bed are acquired, the data are reconstructed using dedicated high performance hardware. The transmission data are used to generate attenuation correction data to be applied to the emission data. The reconstruction of the attenuation corrected emission data gives a volume where each voxel represents the concentration of radio-isotope at that location in the volume. A range of algorithms are used from analytic methods such as FBP (Filtered Back Projection) to Iterative methods such as OSEM (Ordered Subsets Expectation Maximisation) and RAMLA (Row Action Maximum Likelihood Algorithm). Essentially, PET scans were obtained using the three dimensional (3D) acquisition mode and were reconstructed using standard 3D reconstruction algorithms.

3.5.6 Standardised Uptake Values

In addition to a visual analysis, the accurate interpretation of PET images requires knowledge of the physiological distribution of the tracer compound in normal organs and the standardized uptake value (SUV) of these organs. As a consequence, a semi-quantitative analysis of the PET data using the SUV is required. The SUV is used widely in the measurement of static FDG accumulation in tissues. The SUV is not a true kinetic rate constant, so it is often referred to as a semi-quantitative measure.

The SUV serves as a normalized target-to-background measure and it takes into account the differences between normalising for body weight, lean body mass and body surface area. In our study I used the most prevalent SUV calculation, which is as follows:

\[
\text{SUV} = \frac{\text{mCi/ml (decay-corrected) in tissue}}{\text{mCi of tracer injected/body weight (g)}}
\]

It is important to appreciate that physiological radionuclide accumulation can be significant in some organs and that this may occasionally mimic pathology. Recognizing this physiological activity is essential for the accurate interpretation of a whole-body PET investigation.
Standardised uptake values (SUVs) depend on acquisition, reconstruction and region of interest (ROI) parameters. ROI are drawn onto the computer screen by the nuclear medicine physician or where it exists based on the anatomical (usually obtained by CT) and clinical information.

SUV may be recorded on the basis of the maximum reading (SUV\text{max}) in the ROI, a mean or a minimum. Most tumour studies use SUV\text{max}, including urological malignancies, and that is what I reported on in this thesis. It is also the method I have most experience with at our institution and the usefulness of SUV\text{max} has been supported in a recent meta-analysis of imaging for lung tumours.

In recent studies the SUVs of cancer lesions and normal organs were comparable between the modalities of stand-alone PET and combined PET-CT, supporting the usefulness of PET/CT-derived SUVs for quantification of tumour metabolism and the ability to compare between the two modalities.

**3.5.7 PET and PET-CT image analysis.**

In imaging PET, standardised uptake values (SUV) have become important as they help quantify the amount of radioisotope uptake in a tissue. The maximum (SUV\text{max}) or mean (SUV\text{mean}) levels may be measured but more often than not SUV\text{max} is reported in tumour studies. Regions of interest (ROI) within tumours are highlighted, have the SUV\text{max} calculated by computer and then have this radioisotope uptake level compared to a background tissue, preferably a normal contralateral organ or muscle or blood.

In this thesis, all PET studies were analysed first by myself and then again by two nuclear medicine physicians blinded to each other and then reviewed with a consensus SUV calculated, when required. Firstly, images were initially assessed visually to identify any potential regions of increased uptake, particularly away from the area of interest (which may have identified metastases). Background SUV were calculated in the normal tissues being
studies (i.e. kidney) so that there was internal correction and controls for each patient and not an arbitrary figure for an organ. So, for most studies, the kidney with no pathology had normal tissue and ROI were drawn away from the collecting system where radiotracer accumulates so that normal SUV could be established. The kidney with pathology, using the CT acquired on the day (or prior in the case of stand-alone PET) had ROI drawn over the kidney tumour, again avoiding the collecting system, with SUV calculated. Thus a ratio of SUV from tumour: normal was created.

3.5.8 Radionuclides used in PET

PET scanning supplies unique information on metabolic activity of a tissue by tagging radioisotopes to natural substances in the body. The most commonly used radionuclide in PET scanning combines the radioisotope Fluorine-18 (\(^{18}\text{F}\)) with the D-glucose analogue: 2 fluoro-2-deoxy-D-glucose (\(^{18}\text{F}-\text{Fluorodeoxyglucose or }^{18}\text{F-FDG}\)). This is also the most common radiotracer used in tumour imaging. However, it is not suitable for all tumours (particularly for RCC) and one of the objectives of this thesis was to characterise tumour hypoxia with an appropriate radiotracer and to investigate other radiotracers based on increased cell turnover.

Radioimmunoscintigraphy using positron emitters (immuno-PET) for tumour visualisation has developed because antibodies may now be linked with positron emitters. Monoclonal antibodies (mAb) have been labelled with \(^{111}\text{In}\) or \(^{99m}\text{Tc}\) as radioisotopic imaging agents in prostate, colorectal and ovarian carcinoma. This is discussed later (section 3.13).

3.5.9 \(^{18}\text{F-Fluorodeoxyglucose (}^{18}\text{F-FDG)}\)

\(^{18}\text{F-FDG}\) is the most commonly used radionuclide in PET scanning. Tumour imaging with FDG is based on the premise that tumours are more metabolically active than their surrounding tissues and thus will metabolise more glucose. Tumour cells will actively take up and shuttle FDG into glycolysis. Once the cycle has begun, the glucose is
phosphorylated to FDG-6-phosphate after the first step. This metabolite becomes trapped and cannot proceed along the normal pathway of glucose metabolism. Over time, increased amounts of FDG accumulate within malignant cells. This abnormal concentration of FDG in the tumour cells produces a detectable signal that is greater than the background, thus allowing isolation of tumour deposits\textsuperscript{522}.

### 3.5.10 Radiolabelled nitroimidazoles and imaging of tissue hypoxia

Radiolabelled nitroimidazoles (e.g. $^{18}$F-Fluoromisonidazole) have emerged as non-invasive radiotracers for the assessment of tumour hypoxia when delivered in conjunction with PET-CT. Nitroimidazoles become metabolised and trapped within hypoxic yet metabolically active cells\textsuperscript{551} with binding rates reducing the better oxygenated the tissue.

Variation in radiotracer uptake between individuals is to be expected in tissues. In particular, the sensitivity of nitroimidazole imaging for the detection of hypoxic tissue will be determined by several factors including delivery, retention and metabolism of the nitroreductase\textsuperscript{552}. The sensitivity of nitromidazole imaging for the detection of hypoxic tissue will be determined by four factors:\textsuperscript{552}

1. the amount of radiopharmaceutical delivered to the site of hypoxia
2. the fraction that gets past the initial reversible reaction
3. the rapidity of clearance of radioactivity from normoxic cells
4. sufficient duration of retention in hypoxic cells to record high-quality images.

The specificity of nitromidazole imaging will depend on:

1. the contrast between lesion and background
2. the oxygen concentration at which trapping occurs\textsuperscript{552}
3.5.11 $^{18}$F-Fluoromisonidazole ($^{18}$F-FMISO)

Early interest for non-invasive imaging for hypoxia centred around first labelling nitroimidazole compounds with a radioisotope. Iodoazomycin arabinoside is a misonidazole analogue that was labelled with iodine-123 ($^{123}$I-IAZA). It has been trialled in humans with advanced malignancy and although proving feasible and safe, it had only limited utility. It did, however, lead to the development of $^{18}$F-FMISO is the most extensively studied hypoxia-sensitive radiopharmaceutical. It has demonstrated hypoxia in nasopharyngeal and other head and neck tumours, as well as in non-small cell lung tumours. It also has other applications. In our institution, it has been used to demonstrate hypoxic regions of brain in stroke patients whilst other studies have investigated anaerobic infections and myocardial ischaemia with $^{18}$F-FMISO. Even today the most extensively investigated and validated PET radiotracer of hypoxia to date remains $^{18}$F-FMISO. Thus I solely utilised $^{18}$F-FMISO as an hypoxic imaging agent in this thesis.

The advantages of $^{18}$F-FMISO are that it is highly stable after injection. Up to 2 hours after administration of FMISO, no metabolites of it were found in the blood of humans or mice. Uptake of 18F into bone would indicate free fluorine and this has never been observed. Other hypoxic tracers ($^{123}$I-IAZA) have not entered clinical studies or practice to a great degree and have not been studied in this thesis.

3.5.12 $^{18}$F-Fluorothymidine ($^{18}$F-FLT)

Tumour tissues that have high proliferation rates requires high rates of DNA synthesis and tracers such as those based on the nucleic acid thymidine have been developed. The advantages of a proliferative marker over $^{18}$F-FDG is that a proliferation marker (e.g. $^{18}$F-
Fluorothymidine or $^{18}$F-FLT may give better specificity in the assessment of tumours or better accuracy in the evaluation of early response. This is because proliferation is more sensitive than glucose utilisation. 593,594 $^{18}$F-FLT has been used in this thesis.

3.6 Serum Osteopontin Immunoassay in Renal Cell Carcinoma: Human Studies

In essence, immunoassay simply involves the mixing of antigen with antibody followed by the discrimination of bound from free reactants. To assist in this process, either the antigen or the antibody is labelled, most often with an enzyme or radioisotope. The basis of all assays is the formation of a complex between analyte (the substance being analysed) and the analytical reagent (usually antibody but maybe an antigen), followed by an estimation of bound and free components. At almost every step assays may vary so there is much methodological flexibility so different techniques will suit different analytes.637

There are two broad types of immunoassay:

1) Competition (Saturation) immunoassay: an excess of antigen competes for a limiting amount of antibody. Unlabelled antigen inhibits the binding of labelled antigen. A set of standards may be used to construct an inhibition curve from which the amount of an unknown analyte may be determined by the degree of inhibition induced.638

2) Immunometric (excess antibody) immunoassays: Standard amounts of antigen are reacted with excess labelled antibody, then following separation of bound and free reactants, the bound antibody is estimated.639 Theoretically this type of assay may detect one molecule of bound antibody. An advantage of immunometric assays is that two analytical reagents may be used to characterise that analyte as long as it has two distinct epitopes.637 This assay is frequently referred to as a “sandwich assay” and this was the type of assay used in this thesis to determine serum (and plasma) osteopontin levels.
3.6.1 Collection and storage of blood
All blood containers were consistent (Becton, Dickinson and Company Franklin Lakes, NJ USA) and standard methods of collection and storage applied. A 10ml sample of blood was taken (10cc syringe, 21G needle, and plain 10ml collection tube) from study enrolled patients prior to surgery. Blood was allowed to clot at room temperature, and then centrifuged with the serum stored (and later tested for the presence of the tumour marker osteopontin). Further samples were obtained in the same manner from volunteers without RCC or other tumours to act as controls or standards for the assay and each were stored in a similar fashion (at -20°C).

For the last part of the methods with osteopontin, extra samples (10ml sample of blood via a 10cc syringe, 21G needle, anti-coagulated [lithium heparin] 10ml collection tube) were taken for plasma testing of osteopontin levels. Samples were centrifuged to remove plasma and stored (at -20°C) for later testing.

3.6.2 Type of label for the immunoassay
As described, two main types of immunoassay exist (competition or immunometric) and each of these assay systems may utilise radioisotope or enzyme labels. Both types of labelling are used in this thesis.

Radioisotope labelling
For radioisotopes, two main factors contribute to the sensitivity of and accuracy of signal detection. Firstly, the level of background radiation and secondly, the measurable units of activity generated per unit time for each molecule. Sensitivity will be at it greatest when background is near zero and each molecule of bound antibody emits an observable signal. I has been a popular radioisotope because of a low natural background in the environment and biological fluids and tissues and its radioactive disintegration is independent of the chemical or physical nature of the assay in which it is being used.
Biodistribution studies with gamma well counting and PET-CT studies with radiolabelled antibodies rely on this technique as used in this thesis.

**Enzyme labelling**
This has the advantage over radioisotopes in that each enzyme molecule can convert many molecules of substrate to detectable product. With current technology, most enzyme assays are limited by the sensitivity of the detection apparatus (photometer, spectrophotometer) and are potentially limited optical and chemical reproducibility of specific equipment such the micro-tire trays and cuvettes. 637

Enzyme-linked Immunosorbent Assays (ELISA) are common examples using such a method.

**Methods for detection of a label**
Radioisotopes may be detected using traditional gamma well counting devices or gamma cameras in PET machines. Enzymes generally produce coloured products from colourless substrates and as such, a spectrophotometer or colorimeter may be used to determine the amount of bound product.

**3.6.3 Enzyme-linked Immunosorbent Assay (ELISA) for Osteopontin**
Immunometric assays such as ELISA almost invariably require solid-phase systems. They may be performed in many ways but a common arrangement637 and the method used in this thesis was to have antibody in the solid phase to capture antigen which is then detected by a second labeled antibody directed against another epitope on the antigen.
Figure 3-3 A diagrammatic representation of the osteopontin assay

A plate coated with rabbit polyclonal antibody to human osteopontin (OPN) has the human sera with OPN added to the plate. A monoclonal antibody to human OPN labelled with the enzyme horseradish peroxidase (HRP) binds to the OPN allowing identification after further incubation because the enzyme is able to dissolve substrate identifying only bound human OPN molecules after washing.
3.6.4 Radioimmunossay in biodistribution studies using radioisotopes

This is described in detail in section 3.13.

3.6.5 The immunoassay procedure for serum osteopontin

The assay procedure for serum osteopontin that was followed was supplied with the commercial test kit (Assay Designs Inc, Ann Arbor, MI, USA). It has a sensitivity of 2.2 ng/mL and a range of 5 to 320 ng/mL:

All reagents brought to room temperature for 30 minutes prior to opening.
All standards and samples were run in duplicate.

   a. Wash Buffer: Prepare Wash Buffer (Cat# 80-0171) by diluting 25ml of the supplied concentrate with 975 ml of D. water.

   b. Human OPN Standards: Add 500µl of D water to the human OPN Standard (Cat# 80-0291). Let it sit at room temperature for 5 minutes. This solution contains 640ng/ml human OPN.

   c. Preparation of Labeled Antibody Conjugate: Add the entire contents of one bottle of Labeled Antibody Diluent to the vial of OPN Antibody Conjugate. Let it stand at room temperature for 5 minutes and then vortex it gently.

   d. Preparation of Substrate: Just prior to use, add 1 substrate tablet (TMB Tablets Catalog No. 80-0175) to 2.5ml of Substrate Buffer (Catalog No. 80-0173) and mix allowing the tablet to completely dissolve before proceeding. Add 2.75 ml of the Peroxide Solution (Catalog No. 80-0174) to the tablet solution and mix well.

Steps:

1. Wash the plate (Catalog No. 80-0289) by adding 400ml of wash solution (Catalog No. 80-0171; 1:40 dilution with D H₂O, 25 ml + 975 ml water) to each well for 2 washes.

2. Prepare and add the standard (see plan sheet);
3. Prepare and add samples (see plan sheet 1:10, 1:20, 1:40 dilution with assay buffer Cat# 80-0170);
4. Seal the plate and incubate at room temperature for 2 hours;
5. Wash the plate with washing buffer for 6 times on plate washer. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer;
6. Pipet 100µl the Labeled Antibody (Catalog No. 80-0290); Mouse antibody to human OPN conjugated with HRP diluted with Labeled Antibody Diluent (Catalog No. 80-0985), except the Blank (or substrate control).
7. Seal the plate and incubate at RT for 1 hour.
8. Repeat step 5.
9. Add 100µl of the Substrate Solution to each well.
10. Incubate at RT for 30 minutes in the dark;
11. Add 100 ml of Stop Solution (Catalog No. 80-0176);
12. Read the optical density at 450nm and manually subtract the mean optical density of the blank wells from all readings.
13. Calculate sample concentrations from Standard Curve provided by manufacturer

3.6.6 Quality Control of the assay procedure for serum osteopontin

The standard curve created in conjunction with the above method had a linear relationship allowing accurate assessment of the osteopontin levels in all cases. As a means of quality control the samples of serum from experiment 1 were re-tested at the same time as those in experiment 2. This is because the validity of repeat experiments in measuring serum osteopontin has been questioned by some authors. The results were extremely similar with an almost perfect correlation (correlation coefficient 0.98; range -1.0 to 1.0). This gave us the confidence that our assay was reproducible in samples from patients across a period of time.
3.7 Histopathology and Immunohistochemistry: Human Studies

3.7.1 Renal Tissue Specimen Handling and Processing
Handling of and kidney tumour (and normal kidney) specimens was identical in all cases. Fresh tumour and normal renal tissue (2-3cm slices) was obtained from all patients once the specimen (either the whole kidney or a partially excised section) was safely removed from the patient and taken immediately to the laboratory. All human immunohistochemistry was done on the paraffin embedded sections. Tissue was immediately frozen in the event tissue was required to examine for antibodies that did not bind to paraffin embedded tissue or in the event of future DNA or RNA analysis studies (not undertaken as part of this thesis).

As with a previous study where immunohistochemistry was undertaken alongside POS measurements, a special feature of the methodology was to measure from the areas taken for analysis so as to reduce the potential for interference of tumour heterogeneity. In our study, the specimens were taken immediately from the operating theatre for fresh specimen tissue harvest immediately after performing the oxygen readings when the kidney was removed. The actual points of measurement were also noted to ensure that the correct site was harvested from both normal kidney and tumour for analysis.

3.7.2 Histopathology of Renal Tissue
Histopathological analysis was done in the standard fashion with specimens handled routinely once the fresh tissue samples were removed. The surgically removed specimens (once the fresh tissue was taken for processing) were immediately fixed in 10% buffered formalin and then embedded in paraffin. Standard haematoxylin and eosin-stained sections were used for diagnosis as previously described. The specimens were examined by two experienced pathologists for diagnosis and classification of the renal tumours (Table 2-1).

3.8 Immunohistochemistry in Human Studies
Immunohistochemistry is a process utilizing highly selective interaction between an antibody and a target antigen. This then provides a highly specific and sensitive assay for detecting the
presence of an antigen in a variety of tissues. An ideal antibody for immunohistochemical studies is one that displays both a high specificity and high affinity for the antigen of interest and be produced at high titre. However, the specificity and affinity of this primary antibody are not the sole determinants of successful immunohistochemical detection; this is because the primary antibody itself is rarely tagged with a marker for visualization. Instead, the primary antibody bound to its antigen itself is used as the target for a secondary antibody that has been labelled. This is advantageous as it obviates the need to provide a label for every primary antibody as the secondary antibodies react with a whole class of primary antibodies, and as long as they are generated in a species different to that being used by the target antigen, specificity is retained.

There are a number of different antigen detection systems available for use in immunohistochemistry. These range from the use of radiolabelled probes, fluorescent markers to enzymatic activity. This thesis uses only enzymatic detection type methods and these are discussed in detail in each section below.

Controls are important with immunohistochemistry because of the possibilities of varied staining intensity, uniformity, specificity, background, and counterstaining always exist. Thus positive tissue controls containing the antigen under test and a negative control section from each test block were used throughout.

### 3.8.1 Immunohistochemistry in Human Studies: General Methods

Immunohistochemistry was performed to evaluate tissue expression of HIF-1α, VEGF (VEGF-A), CAIX, EGFR, Microvessel Density (MVD), GLUT-1 and Ki67.
HIF-1α is the active component of HIF-1 that is regulated by pVHL and oxygen levels. VEGF and GLUT-1 are known to be over-expressed in hypoxic cells and this is related to increased HIF-1 levels. HIF-1 is also responsible for increased cell proliferation and Ki67 is a marker of this. MVD as assessed by von Willebrand factor is a measure of the vascularity of a tissue specimen. CAIX is a marker of hypoxia and EGFR expression is linked to the hypoxic pathway in tumour cells.

All tissue used in this study was stored as formalin fixed paraffin embedded blocks. When required for immunohistochemistry, tissue was cut into 4µm slices and mounted onto slides. Slides were deparaffinised and rehydrated.

For antigen retrieval and staining processes were followed as in previous studies. Firstly, slides were placed in plastic racks and immersed in 500mL 0.01M citrate buffer. They were then placed in a 700W microwave oven for 10 minutes on “high”. Following this, they were left to stand at room temperature for 20 minutes and then washed in tap water for 5 minutes. They were then transferred to distilled water for immunostaining.

Immunostaining was done by the avidin-biotin complex (ABC) method, which utilises the capability of the glycoprotein avidin to non-immunologically bind 4 molecules of the vitamin biotin. In this method, 3 reagents are used – the primary antibody specific for the tissue antigen; biotin conjugated secondary antibody capable of binding to the first; and a complex of peroxidase-conjugated biotin and avidin which can bind to the biotin on the secondary antibody. Prior to immunostaining, slides were washed in PBS buffer, pH 7.4 for 5 minutes.

The first step was treatment with 3% H2O2 (BDH, Dorset, UK) for 10 minutes at room temperature to eliminate endogenous peroxidase. Slides were then washed with PBS buffer, pH 7.4 for 5 minutes. The next step was incubation in Protein Blocking Agent (Immunon™, Pittsburgh, PA, USA) at 1:1 dilution for 10 minutes at room temperature. Following this,
primary antibody clone was applied to the slides at varying concentrations and times, depending on the antibody in question (Table 3-3).

Slides were then washed in PBS buffer, pH 7.4 for 5 minutes and then incubated at room temperature with biotinylated anti-mouse and/or rabbit IgG for 10 minutes or 30 minutes, depending on the Kit Detection System used. The next step was washing in PBS buffer, pH 7.4 for 5 minutes and then incubating with either ABC reagent or Streptavidin Peroxidase, again depending on the Kit Detection System used (Table 3-4).

Slides were washed in PBS buffer, pH 7.4 for 5 minutes and then incubated in AEC chromogen for 10-20 minutes. Finally, slides were counterstained with Mayer’s haematoxylin (Amber Scientific, Belmont, WA, Australia) and mounted in Crystal Mount® (Sigma, St. Louis, Missouri, USA).
<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Clone</th>
<th>Company</th>
<th>1° Antibody concentration (μg/mL)</th>
<th>1° Antibody incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki67</td>
<td>MIB-1</td>
<td>Immunotech (Marseille, France)</td>
<td>1</td>
<td>30 minutes</td>
</tr>
<tr>
<td>Glut-1</td>
<td>A3536</td>
<td>Dako (Glostrup, Denmark)</td>
<td>22</td>
<td>30 minutes</td>
</tr>
<tr>
<td>VEGF</td>
<td>147</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA, USA</td>
<td>3</td>
<td>16 hours</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>H206</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA, USA</td>
<td>2</td>
<td>16 hours</td>
</tr>
<tr>
<td>VWF (MVD)</td>
<td>F8/86</td>
<td>Dako (Glostrup, Denmark)</td>
<td>15</td>
<td>30 minutes</td>
</tr>
<tr>
<td>EGFR</td>
<td>1005</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA, USA</td>
<td>3</td>
<td>60 minutes</td>
</tr>
<tr>
<td>CAIX</td>
<td>MN75</td>
<td>Gift from Bayer Group Diagnostics, Elkhart, IN, USA with support from J. Zavada Inst of Molecular Genetics, Academy of Sciences of Czech Republic, Prague, Czech Republic.</td>
<td>0.75</td>
<td>45 minutes</td>
</tr>
</tbody>
</table>
Table 3-4 Immunohistochemistry kit detection systems and incubation times

<table>
<thead>
<tr>
<th>Primary antibody (type)</th>
<th>Kit Detection System</th>
<th>Time for Incubation with biotinylated anti-mouse and/or rabbit IgG</th>
<th>Final incubation and Detection System</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki67 murine</td>
<td>LSAB+, Dako, Glostrup, Denmark</td>
<td>10 minutes</td>
<td>Linking solution for antibodies (LSAB) + Streptavidin Peroxidase (10 minutes)</td>
</tr>
<tr>
<td>Glut-1 rabbit</td>
<td>LSAB+, Dako, Glostrup, Denmark</td>
<td>10 minutes</td>
<td>LSAB+ Streptavidin Peroxidase (10 minutes)</td>
</tr>
<tr>
<td>VEGF rabbit</td>
<td>Vectastain Elite, Vector Laboratories, Burlingame, CA, USA</td>
<td>30 minutes</td>
<td>Vectastain ABC reagent (30 minutes)</td>
</tr>
<tr>
<td>HIF-1α rabbit</td>
<td>Vectastain Elite, Vector Laboratories, Burlingame, CA, USA</td>
<td>30 minutes</td>
<td>Vectastain ABC reagent (30 minutes)</td>
</tr>
<tr>
<td>VWF (MVD) murine</td>
<td>LSAB+, Dako, Glostrup, Denmark</td>
<td>10 minutes</td>
<td>LSAB+ Streptavidin Peroxidase (10 minutes)</td>
</tr>
<tr>
<td>EGFR rabbit</td>
<td>-rabbit-Bt, Chemicon, Temecula, CA, USA</td>
<td>30 min</td>
<td>Vectastain ABC reagent (30 minutes)</td>
</tr>
<tr>
<td>CAIX murine</td>
<td>Envision+ HRP conjugated, Dako, Glostrup, Denmark</td>
<td>30 min</td>
<td>Not required.</td>
</tr>
</tbody>
</table>
For assessment of antigen positive cells, slides were first examined at low power (100x) to determine regions of normal tissue and tumour within the section. Slides were then examined at high power (400x), with the assistance of a graticule. Ten areas of tumour and ten areas of adjacent normal tissue were examined. Haematoxylin & Eosin stained slides were examined for the purposes of cell counting.

For HIF-1α, VEGF and GLUT-1, the number of positively stained cells, intensity of staining and pattern of staining were measured. The number of positively stained cells was expressed as a percentage of total number of cells per high power field. The intensity of staining was expressed as negative, weakly positive, moderately positive or strongly positive (-, +, ++ or +++). Expression was considered rich if more than 50% of cells stained moderately or strongly positive, poor if fewer than 25% of cells stained weakly positive and intermediate for everything in between. For Ki67, the number of positively stained cells was counted and the index calculated as a percentage of total number of cells in a high power field.

3.8.2 Analysis of Immunohistochemistry

Immunohistochemistry is the process of detection of antigens in tissue using antibodies. The antibodies can be polyclonal or monoclonal in origin, the monoclonal ones being more specific in nature. However, analysis of immunohistochemical samples is by no means standardised for any particular antibody or process. Processes may be done manually or by using computer programs, each with their own advantages. Further, amongst manual methods varying types of quantitative analysis have been performed from simple gross quantification to more complex counting of cells within fields that I used.

3.8.3 Analysis of Immunohistochemistry- General

Analysis of immunohistochemical samples is by no means standardised for any particular antibody or process. Processes may be done manually or by using computer programs, each
with their own advantages. Further, amongst manual methods varying types of quantitative analysis have been performed.

I elected to use a refined quantitative analysis for adequate sampling with immunohistochemistry based on systems that have been used successfully and validated. Such refined or semi-quantitative analysis using light microscopy-scoring systems has also been utilised, devised and validated in a multi-centre studies comparing immunohistochemistry and POS oxygen electrode measurements in the context of tumour hypoxia as in our study.

A simple method for assessment of staining patterns is to establish gross grades (0, no labelling; 1, labelling of few scattered cells or small groups of cells; 2, labelling of less than 50% of cells; 3, labelling of more than 50% of cells). I rejected this for a more complex but robust method. The presence of positive cells in tissues was quantified by calculating the number of positive cells in ten randomly chosen microscopic fields under high power (X400) magnification with the assistance of a graticule over the total cells in the field and expressed as a percentage as described previously. The total cells in each field were first determined with the assistance of a graticule under high power (400x) on standard haematoxylin and eosin-stained sections. Matched fields were used for all specimens studied (controls and tumours) and with haematoxylin and eosin-stained or immunohistochemically analysed sections. In other words, fields one through ten in the histopathology (standard haematoxylin and eosin-stained) and immunohistochemistry was the same for each antibody used for staining. I elected to use this process in line with previous studies to reduce subjectivity and allow analysis of the same regions within tumours. As in other studies edge effects and necrotic and stromal areas were ignored on sections. In summary, sections were analysed for the percentage of positive cells based on matched fields. A percentage of cells staining positive was obtained for each field within each section for analysis. The matching of fields further allowed for the calculation of cell density in
fields. Specific issues relating to the sub-analysis and calculations of MVD, and Ki-67 are discussed below.

A semi-quantitative scoring system has been devised and validated in a multi-centre study comparing the clinical use of pimonidazole and Eppendorf oxygen electrode measurements as measures of tumour hypoxia.\textsuperscript{190,648} Tumour sections were initially scanned at x 25 magnification so that the distribution of staining could be assessed. At x 200 magnification, the sections were then analysed field-by-field, from top left to bottom right of the section. Each field was assigned a score of I to 4, representative of the approximate area of Immunostaining (0=0%; 1= 0—5%; 2 = 5—15%; 3 = 15—30%; 4 = >30%). To counteract the effect of variations in staining intensity, only areas of unequivocal staining were included. Areas of necrosis, stroma, normal epithelium and distinct edge effects were ignored. The overall scores used in that study to calculate pimonidazole binding across the tumour was derived from the average score, for a fields, from either one or all available biopsies. Where only one biopsy was used for analysis, this was selected at random from those available.

Microscope and software
The compound microscope used throughout (Carl Zeiss Jena GmbH, Jena, Germany) with the software and camera used compatible for later photomicrograph examination: 10x ocular (eyepiece) lens and variable objective lenses (10x, 20x and 40x) giving total magnification of 100x, 200x and 400x. The fields of view were calibrated such that at 10x: 4.15mm\(^3\); 20x: 1.13mm\(^3\) and 40x: 0.28mm\(^3\).

3.8.4 Analysis of Immunohistochemistry-Specific
HIF-1\(\alpha\), VEGF, Glut-1, VEGFR, EGFR, CAIX -67 staining
For assessment of antigen positive cells, slides were first examined at low power (100x) to determine regions of normal tissue and tumour within the section. Slides were then examined at high power (400x), with the assistance of a graticule. Ten areas of tumour and ten areas of
adjacent normal tissue were examined. Haematoxylin & Eosin stained slides were examined for the purposes of cell counting.

For HIF-1α, VEGF and Glut-1, the number of positively stained cells, intensity of staining and pattern of staining were measured. The number of positively stained cells was expressed as a percentage of total number of cells per high power field. The intensity of staining was expressed as negative, weakly positive, moderately positive or strongly positive (-, +, ++ or +++). Expression was considered strongly positive if more than 50% of cells (that were positive) stained moderately or strongly positive, poor if fewer than 25% of cells stained weakly positive and intermediate for everything in between. For Ki67, the number of positively stained cells was counted and the index calculated as a percentage of total number of cells in a high power field (and is discussed in detail next section). MVD will be discussed shortly.

For VEGFR, EGFR, CAIX being receptor molecules, the number of positively stained cells (or cell membranes), were measured. The number of positively stained cells was expressed as a percentage of total number of cells per high power field.

Cell Density
A gross measure of cell proliferation is to calculate the density of cells per high power field. Tumours cells are usually reproducing at a greater rate than normal cells. This is typically expressed as cells per mm² or cells per high power field.¹⁷⁴

Ki-67 staining (Proliferation index)
Similar to previous studies, the proliferation index (Ki-67 antigen labeling index) was determined by counting a total of 10 randomly chosen fields, at 400x magnification. Thus the mean percentage value of all slides representing each tumour was used for further evaluation.³⁴¹
Attempts may be made to stratify tumours based on different cut offs for overexpression were set at for example, 1%, 5% and 10% of positively stained nuclei. 167 I reported on raw numbers (using the formula: cells positive for Ki-67 staining/ total cells x 100) and compared tumours with normal tissue. The value of sub-stratifying really is only of benefit when following patients for prognosis.341

More strict definitions of calculating proliferation index have been described where the immunohistochemical detection of Ki-67 has been graded using an index method of quantification.653-655 This method requires counting of at least 1000 tumour nuclei at 1000x magnification. In other studies the semiquantitative assessment of Ki-67-reactive cells has been equally effective in separating tumour cells (for example into non-metastatic and metastatic) This method of quantification is faster and more practical for routine use by investigators and pathologists.442

EGFR Methods

EGFR expression in colorectal cancer, usually evaluated by immunohistochemistry, is actually based on heterogenous data, considering tumour stage or survival rates. Results are variable due to differences in evaluation criteria between studies.656

Measuring Microvessel density (MVD)

Intratumoral microvessel density (MVD) may be determined using antibodies to vWF298,380,455,456,474, CD31454 or CD34456,457. I chose vWF in the human and CD31 in the xenograft studies to outline vessels because of ease of use of the antibodies and matching of antibody to tissue used because vWF could not be used easily on frozen xenograft specimens but CD31 could.657

The specific methods I used have been utilised in the past. This consisted of firstly determining the number of positively staining vessels in ten randomised fields221 in human
studies or all fields observed in animal xenograft studies (so I could compare MVD to other markers on a field-by-field basis in any one experiment).

The second method was the neovascular "hot spot"\textsuperscript{447,474} technique for quantification of angiogenesis. Sections are first scanned at low power (100x; 4.15mm\textsuperscript{3}) to determine the areas with the maximal number of small vessels (or most highly dense with blood vessels) within the tumour or tissue being studied. Marked areas on each section are then analysed by counting the vessels in three randomly selected high magnification fields (x 400; 0.28mm\textsuperscript{3}) within the marked area (or "hot spots"), and calculating the average number of vessels.\textsuperscript{456} The number calculated in the three fields is then averaged to give the mean "hot spot" score for each section.

Strict criterion for counting of vessels was used whereby individual microvessels were counted. Branches arising from vessels were counted as individual vessels. Any brown-staining endothelial cell or endothelial cell cluster that was clearly separate from adjacent microvessels and tumour cells was considered a single countable microvessel.\textsuperscript{178} Areas of haemorrhage, necrosis and ulceration were avoided. For each case, the mean microvessel count for the three fields assessed was calculated.\textsuperscript{221} A microvessel was defined as any single endothelial cell or group of endothelial cells stained with a discernable vessel lumen that was clearly separate from adjacent microvessels. Other studies\textsuperscript{178} do not require the presence of a lumen but I felt it necessary as has been the case with other investigators.\textsuperscript{221} for a structure to be defined as a microvessel.

### 3.9 MATERIALS AND METHODS –XENOGRAFT STUDIES

Materials and methods specific to xenograft experiments conducted in animals will now be outlined.
3.10 Cell line used in all experiments

SK-RC-52, a CAIX expressing human RCC cell line, was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in 175-cm² plastic flasks (Nunc; Nunc, Roskilde, Denmark) and maintained in log-phase growth in RPMI 1640 (Trace Biosciences, Sydney, Australia) supplemented with 5% FCS (MultiSer; Trace Biosciences, Sydney, Australia), 100 units/ml penicillin, 100mg/ml streptomycin, 0.25 ml/l insulin, 2mM glutamine, and essential amino acids. Cells were cultured at 37°C in a 5% CO2 incubator (Forma Scientific, Inc., Marietta, Ohio) and passaged with 0.05% EDTA/PBS (BDH Chemicals, Merck, Melbourne, Australia). Cell viability in all experiments, as determined by trypan blue exclusion, exceeded 90%.

3.10.1 Animal model

In summary, the SK-RC-52 human RCC xenograft model was established from a primary tumour by serial subcutaneous transplantations in nude mice after implantation of cells from culture suspended in reconstituted basement membrane (Matrigel) as previously described. Tumour pieces (approximately 2mm³) were transplanted subcutaneously beneath the nuchal fold of 6 week-old athymic BALB/c, nu/nu mice (Animal Resources Centre, Perth, Australia).

Establishing SK-RC-52 Renal Cell Carcinoma xenografts

To establish SK-RC-52 human RCC xenografts in the study population, a tumour was grown subcutaneously (s.c.) after implantation of cells from culture suspended in reconstituted basement membrane (Matrigel) as previously described. Three further generations of mice then had SK-RC-52 tumours serially transplanted (2mm³) from the initial tumour and grown s.c. using sterile techniques under a sterile fumehood into a small s.c. pocket fashioned between the shoulder blades as described previously.

Transplantation was conducted in mice with 10 week tumours greater than 8mm diameter after being anaesthetised with an intraperitoneal injection of xylocaaine hydrochloride.
(20mg/ml, Troy laboratories Pty Ltd Smithfield, Australia) and ketamine (100mg/ml, Parnell laboratories Alexandria, Australia) diluted in normal saline at 0.1ml solution per gram body weight (1 part ketamine, 1 part xylocaine, 8 parts normal saline). Skin overlying the tumour was prepared with 10% povidone-iodine solution and the tumour exposed by dissection with scissors and forceps under a sterile hood. The surgical incision was closed with a single stitch (6-0 absorbable Dacron). Mice used for tumour donation were later sacrificed using sevofluorane after the tumour was harvested. One mouse tumour was selected to become the donor tumour from which the fifty mice had s.c xenografted tumours implanted as described. Thus all tumours in the experiment were derived from the same donor tumour and cell line. Tumour volume was calculated by the formula \(V = \frac{1}{2} \times \text{length} \times \text{width}^2\), where length was the longest axis and width the measurement at right angles to the length.

Food and water were provided for the mice ad libitum and their health was monitored daily. Mice were maintained in autoclaved microisolator cages housed in a positive pressure containment rack (Thoren Caging Systems, Inc., Hazelton, PA). Mice were identified by earmarks and were weighed weekly.

Typically, depending on the experiment, being either in vivo biodistribution, PET or direct oxygen tension studies (or a combination of one or more) were initiated 10 weeks after inoculation and all tumours used in the studies were derived from a single donor tumour.

### 3.10.2 Ethical decisions animal experiments

Animal care and experiments followed the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes endorsed by the National Health and Medical Research Council and was approved by our institutional animal ethics committee.
3.11 Direct measurements of oxygenation in tumours and normal tissue: Xenograft Studies

3.11.1 The Time-resolved Luminescence-Based Optical Probe (Oxylite Probe)

In all animal studies tumour pO\textsubscript{2} was measured with a two-channel time-resolved luminescence-based optical oxygen-sensing probe (Oxylite\textsuperscript{TM} 2000, Oxford Optronix, Oxford, UK) or oxylite probe. It measures pO\textsubscript{2} by determining the O\textsubscript{2}-dependent fluorescent lifetime of ruthenium chloride.\textsuperscript{176} The ruthenium chloride is immobilized at the tip of the fibre-optic probe. The monitoring specifications of the probe are outlined in Section XX. The probes (230 um outside diameter) were precalibrated by the manufacturer (±0.7 mmHg or < ±10\% of actual pO\textsubscript{2}, whichever was greater). To further ensure correct pO\textsubscript{2} readings in the experiments the probe was checked in normal saline and again in animals just sacrificed to ensure a 0mmHg recording as previously described.\textsuperscript{177,661} Additionally, I conducted control measurements in the right leg muscle of each animal for comparison with tumour measurements.\textsuperscript{662} New probes were used for each experiment.
These figures demonstrate how the oxylite probe appears once connected to its base hardware system with oxygen readings and temperature on the monitor display (above). A close-up of the probe (below) demonstrating the oxygen sensor and thin copper temperature probe that act as one unit.
Polarographic Oxygen Sensor Probing in Xenografts

Animals had anaesthetic administered prior to oxygen tension studies as previously described. Briefly, intraperitoneal injection of anesthetic with xylocaine hydrochloride (20mg/ml, Troy laboratories Pty Ltd Smithfield, Australia) and ketamine (100mg/ml, Parnell laboratories Alexandria, Australia) was undertaken. These anaesthetic agents were chosen because they do not decrease the hypoxic cell fraction. Body core temperatures were maintained in animals by a heated pad at 37.5°C. Tumour temperature was measured by a fine thermocouple that was attached to the oxylite probe giving automatic temperature compensation.

Determining track length and number of tracks

As for POS probe measurements, oxygen tension measurements are typically made along a number of tracks within the tumour. Often the track length, number and location are determined arbitrarily by the investigator. The track length used in our studies covered the length of the tumour. Necrotic tissue was avoided so as not to adversely affect the pO2 measurements.

Advancing the probe within tissue and recording data

Again, as in the human studies with the POS, it is important to stress that due to technical difficulties with POS data in the past, it is imperative that measurements are not contaminated by measurements in stroma or necrosis. This was made possible by again avoiding necrotic areas of tumour based careful examination of tumours and experience or when possible utilising images from the PET-CT scan.

The probes were inserted into the tumours by puncturing with a 23 gauge intravenous needle, inserting the oxylite probe retrograde into the sheath as per manufacturer recommendations. This ensured minimal tissue and probe trauma. The probe was then fixed with a micromanipulator and at each location, pO2 was recorded after several minutes stabilisation for a minimum of 10 s, and an average pO2 over the interval was calculated later. For each
track it is necessary to let the probe stabilise or equilibrate to eliminate movement artifact and get a true tissue reading. The probe was then withdrawn and reinserted at a new location allowed to stabilise over several minutes to ensure quality readings.\(^8\)

Each tumour had 3 tracks with the probe and a minimum of 100 measurements, summarised via computer software (WinDaq Data Acquisition Software, DATAQ Instruments Inc., Akron, OH, USA). Mice were sacrificed by exposure to sevofluorane. They were dissected prior to gamma well counting for the biodistribution analysis study only or the tumour only was removed and frozen for later analysis. Integrated temperature compensation for pO\(_2\) measurements was done automatically because from probes with inbuilt integrated temperature sensors.\(^{63}\)

In each animal the hind leg thigh muscle was also probed as a tissue for comparison with the tumour. It was essential to make a small skin incision to ensure that the probe was in muscle (and not just subcutaneous tissues or skin). The reading was 0mmHg ensuring probe calibration. Each animal was euthanased at the end of the experiment as discussed.
Firstly, the cannula (22G) is introduced into the tumour after avoiding necrotic areas (top left). The plastic sheath remains in the tumour and the probe is passed in (top right) to the sheath from the opposite way. The sheath is then removed and then the probe is in the tissue (bottom left). The probe is then gently placed back into the periphery of the tumour to begin recording pO₂ measurements (bottom right).
Reporting of probe data

There is no universal means of recording or reporting data that is obtained from oxylite probes which is a similar situation to POS probes as discussed above. Nevertheless, there is some consensus on what baseline data to obtain based on previous studies and this includes similar data to what recorded for human studies:

1) The mean of oxygen measurements in a tumour
2) The mean of the means of each track through a tissue obtained and the mean of all of the individual measurements
3) The proportion or fraction of oxygen readings below 10mmHg (HP10), 5 mmHg (HP5), or 2.5mmHg (HP2.5)
4) Pooled data such as the mean or HP10, was then be compared against different patient characteristics such as age, grade, stage, type and tumour volume.

3.11.2 Equipment specifications and limitations

The oxylite probe is not licensed for use in humans and thus is not able to be used in human studies. Table 3-5 summarises the specifications of the oxylite probe used in this thesis.
### Table 3-5 Monitoring specifications of the oxylite probe

<table>
<thead>
<tr>
<th>Mode of operation</th>
<th>Fluorescence quenching</th>
<th>Thermocouples input</th>
</tr>
</thead>
<tbody>
<tr>
<td>Units</td>
<td>mmHg/kPa</td>
<td>°C</td>
</tr>
<tr>
<td>Range</td>
<td>0-100 mmHg (30-44°C)</td>
<td>20–50 °C</td>
</tr>
<tr>
<td>Resolution</td>
<td>0.1 mmHg</td>
<td>0.1 °C</td>
</tr>
<tr>
<td>Accuracy</td>
<td>0.7 mmHg / 10% reading</td>
<td>0.2 °C</td>
</tr>
<tr>
<td>Response Time</td>
<td>&lt; 10 seconds</td>
<td>1 second</td>
</tr>
<tr>
<td>Output Range</td>
<td>0-5 V (0–100 mmHg)</td>
<td>0–5 V (0-50 °C)</td>
</tr>
<tr>
<td>Output Resolution</td>
<td>0.05 mmHg (2.5 mV)</td>
<td>0.025 °C (2.5 mV)</td>
</tr>
<tr>
<td>Default Averaging</td>
<td>5 s</td>
<td>None</td>
</tr>
<tr>
<td>Dimensions</td>
<td>90 x 290 x 260 mm</td>
<td></td>
</tr>
<tr>
<td>Weight</td>
<td>2 Kg</td>
<td></td>
</tr>
<tr>
<td>Power</td>
<td>93 - 240 VAC; 47 - 63 Hz</td>
<td></td>
</tr>
<tr>
<td>Storage Temp.</td>
<td>5°C - 50°C</td>
<td></td>
</tr>
<tr>
<td>Operating Temp.</td>
<td>10°C - 35°C</td>
<td></td>
</tr>
<tr>
<td>Operating Humidity</td>
<td>0 - 70% (non condensing)</td>
<td></td>
</tr>
</tbody>
</table>
3.12 Imaging Studies of Hypoxia with Combined Positron Emission Tomography and CT in Mouse Xenografts (PET-CT)

3.12.1 Introduction
Specific small animal PET-CT machines now exist but none was in operation at our facility or in Australia at the time of undertaking this thesis. The same combined PET-CT machine as used in the human studies was utilized as in other animal studies.666 The radiopharmaceuticals were prepared using the onsite cyclotron as outlined.

3.12.2 Administration of radiopharmaceutical in mice
Mice were fasted at least 3 hours prior to injection of radiotracer (e.g. $^{18}$F-FDG). In order to inject $^{18}$F-FDG intravenously (IV), animals were warmed and a needle inserted into a tail vein. After at least 4 hours of fasting, 100μCi or 0.1 mCi of radiopharmaceutical ($^{18}$F-FDG, $^{18}$F-FMISO, and $^{18}$F-FLT). All radiopharmaceuticals had radioactivity assessed in the giving syringe (2 ml, plain) prior to and after intravenous administration to ensure the whole radiopharmaceutical dose was injected. No cases of failed IV injection occurred in any of the studies in this thesis. No cases of allergic reaction or other medical event were noted. For all radiopharmaceuticals, tumour bearing mice are anaesthetized 1 hour after tracer injection and scanned for 30 minutes in LIST mode. One hour later, under anaesthesia whole-body CT images with

3.12.3 PET-CT machine utilised in the studies
All animal studies in the thesis utilized a combined PET-CT machine (Gemini PET and Dual slice Philips MX 8000 CT, Philips Electronics North America Corporation, New York, NY). This machine was consistent throughout the studies (Figure 3-6). All protocols (outlined below) were developed in conjunction with the PET centre at our institution and followed for all animals. No deviations were reported and no scan failures recorded.
3.12.4 Imaging Studies in Mice

The PET-CT scan experimental set-up was similar to methods used described previously and utilised at our institution for prior studies with considerable experience with such imaging. The protocol for PET-CT was similar to other studies (Appendix B). Mice were fasted at least 3 hours prior to injection of radiotracer (e.g. $^{18}$F-FDG). In order to inject $^{18}$F-FDG intravenously (IV), animals were warmed and a needle inserted into a tail vein.

Animals had induction anesthetic via intraperitoneal injection of xylocaine hydrochloride (20mg/ml, Troy laboratories Pty Ltd Smithfield, Australia) and ketamine (100mg/ml, Parnell laboratories Alexandria, Australia). All animals then received maintenance isofluorane inhalation anesthesia (1.5% mixed with air) with animals on warming mats.

Groups of six mice were positioned on a styrofoam (Dow Chemical Co., Midland, MI) base directly on the flat-bed table of the PET-CT (Figure 3-6). Mice were not physically immobilized to a computer-controlled motorised gantry capable of precisely moving the animals. The CT images were obtained first (5-min) and then a 30-min PET acquisition was performed on the whole-body of each mouse using a three-dimensional (3D) acquisition mode. Transmission images were also acquired and measured attenuation correction was performed. Images were reconstructed using a standard 3D reconstruction algorithm.
Figure 3-6 The PET-CT machine used for both human and animal studies

*The PET-CT machine used in this thesis (above). A group of six mice being simultaneously studied (below).*
3.12.5 Imaging Studies of Renal Cell Carcinoma in xenografts

The various PET protocols for the radiopharmaceuticals used are included in Appendix B.

3.12.6 Xenograft Specimen Handling

Handling of human RCC xenograft specimens was consistent in all cases. Xenografts were excised from the mice, embedded in Tissue Tek ornithine carbamyl transferase compound (Sakura Finetek, Torrance, CA), frozen in liquid nitrogen, and stored at -80°C. Prior to immunostaining, five-micrometer sections were cut and stored wrapped in aluminium foil at -80°C if not stained in the same session as cutting.

At x 200 magnification, the sections were then analysed field-by-field, from top left to bottom right of the section.

3.12.7 Osteopontin and EGFR Immunohistochemistry

Slides were removed from the freezer and allowed to warm to room temperature (RT). They were then fixed in fixed in acetone for ten minutes and then air dried. The first step was treatment with 3% H₂O₂ (BDH, Dorset, UK) for 10 minutes at room temperature to eliminate endogenous peroxidase. Prior to immunostaining, slides were then washed with PBS buffer, pH 7.4 for 5 minutes. The primary antibody being either Osteopontin (Assay designs Inc., Ann Arbor, MI, rabbit, USA at dilution 10µg/mL) or EGFR (Chemicon, Temecula, CA, USA, rodent, at dilution 2.5µg/mL) was added. A group of control slides also had the subclass control rat IgG2a antibody added, (Dako, Glostrup, Denmark at dilution 2.5µg/mL). At all times a further group of slides were negative controls with no primary antibody added.

All slides were then incubated for 60 min at RT. Slides were washed with PBS buffer, pH 7.4 for 5 minutes and then incubated with the secondary antibody, an anti-rabbit-horseradish peroxidase antibody (Dako, Glostrup, Denmark) for 30 min and bound antibody detected with incubation in AEC substrate solution (0.1mol/l acetic acid, 0.1mol/L sodium acetate, 0.02mol/L AEC, and 0.03% 11202) for 10-20 minutes.
Finally, slides were counterstained with Mayer’s haematoxylin (Amber Scientific, Belmont, WA, Australia) and mounted in Crystal Mount® (Sigma, St. Louis, Missouri, USA).

Using this method cells were stained for EGFR and osteopontin expression. This method was modified slightly using a biotinylation kit for CD34 staining on endothelial cells for the calculation of MVD (see below).

### 3.12.8 CAIX Immunohistochemistry

Immunostaining of xenograft tumours was again done by the avidin-biotin method as outlined above. Briefly, it relies on the capability of the glycoprotein avidin to non-immunologically bind molecules of the vitamin biotin. Three reagents are used – the primary antibody specific for the tissue antigen; biotin conjugated secondary antibody capable of binding to the first; and a complex of peroxidase-conjugated biotin and avidin which can bind to the biotin on the secondary antibody.

Dako ark peroxidase animal research kit (Dako Corporation Carpinteria, CA, USA) avidin-biotin and peroxidase methodologies were used. This system was used to minimize reactivity of secondary anti-mouse antibody with endogenous immunoglobulin that may be present in the specimen mouse. Prior to application of the primary antibody to the specimen, the antibody is labelled using the biotinylation reagent, a modified biotinylated anti-mouse immunoglobulin. The primary antibody and biotinylation reagent are mixed in solution resulting in binding of biotinylated secondary antibody to the primary antibody.

Slides were removed from the freezer and allowed to warm to room temperature (RT). They were then fixed in fixed in acetone for ten minutes and then air dried. The first step was treatment with 3% H$_2$O$_2$ (BDH, Dorset, UK) for 10 minutes at room temperature to eliminate endogenous peroxidase. Prior to immunostaining, slides were then washed with PBS buffer, pH 7.4 for 5 minutes.
Prior to use, the primary antibody being anti-CAIX (Dako, Glostrup, Denmark, rodent) was prepared using the Kit Detection System (Dako, Glostrup, Denmark) by incubating it with the biotin reagent with PBS buffer, pH 7.4, as a diluent for 15 mins. Protein blocking agent was then added for 5 mins and the biotinylated primary antibody was then ready to use at a dilution of 2.5μg/mL and added to the slides. A group of slides were negative controls with no primary antibody added.

All slides were then incubated for 60 min at RT. Slides were washed with PBS buffer, pH 7.4 for 5 minutes and then incubated with the secondary antibody, an anti-rabbit-horseradish peroxidase antibody (Dako, Glostrup, Denmark) for 30 min. Slides were washed with PBS buffer, pH 7.4 for 5 minutes and then incubated with streptavidin peroxidase from the Kit Detection System (Dako, Glostrup, Denmark) for 15 mins. Bound antibody was detected with incubation in AEC substrate solution (0.1 mol/l acetic acid, 0.1 mol/L sodium acetate, 0.02 mol/L AEC, and 0.03% H₂O₂) for 7 minutes.

Finally, slides were counterstained with Mayer’s haematoxylin (Amber Scientific, Belmont, WA, Australia) and mounted in Crystal Mount® (Sigma, St. Louis, Missouri, USA).

3.12.9 Microvessel density using CD31 Immunohistochemistry

Immunostaining of xenograft tumours was again done by the avidin-biotin method as outlined above. Briefly, it relies on the capability of the glycoprotein avidin to non-immunologically bind molecules of the vitamin biotin. Three reagents are used – the primary antibody specific for the tissue antigen; biotin conjugated secondary antibody capable of binding to the first; and a complex of peroxidase-conjugated biotin and avidin which can bind to the biotin on the secondary antibody.
Slides were removed from the freezer and allowed to warm to room temperature (RT). They were then fixed in acetone for ten minutes and then air dried. The first step was treatment with 3% H$_2$O$_2$ (BDH, Dorset, UK) for 10 minutes at room temperature to eliminate endogenous peroxidase. Prior to immunostaining, slides were then washed with PBS buffer, pH 7.4 for 5 minutes.

Prior to use, the primary antibody being CD31 (BD biosciences, San Jose, CA, USA, rodent) was prepared using the Kit Detection System (Dako, Glostrup, Denmark) by incubating it with the biotin reagent with PBS buffer, pH 7.4, as a diluent for 15 mins. Protein blocking agent was then added for 5 mins and the biotinylated primary antibody was then ready to use at a dilution of 20µg/mL and added to the slides. A group of slides were negative controls with no primary antibody added.

All slides were then incubated for 60 min at RT. Slides were washed with PBS buffer, pH 7.4 for 5 minutes and then incubated with the secondary antibody, an anti-rabbit-horseradish peroxidase antibody (Dako, Glostrup, Denmark, 3µg/mL) for 30 min. Slides were washed with PBS buffer, pH 7.4 for 5 minutes and then incubated with streptavidin peroxidase from the Kit Detection System (Dako, Glostrup, Denmark) for 30 mins. Bound antibody was detected with incubation in AEC substrate solution (0.1 mol/L acetic acid, 0.1 mol/L sodium acetate, 0.02 mol/L AEC, and 0.03% H$_2$O$_2$) for 7 minutes.

Finally, slides were counterstained with Mayer’s haematoxylin (Amber Scientific, Belmont, WA, Australia) and mounted in Crystal Mount® (Sigma, St. Louis, Missouri, USA).

**Analysis of Immunohistochemistry-Xenograft Studies**
As discussed in section 3.8 above, I elected to use a refined quantitative analysis for adequate sampling with immunohistochemistry based on systems that have been used successfully and validated. The presence of positive cells in tissues was quantified by calculating the number of positive cells in each section analysed field-by-field, from top left
to bottom right of the section. This was done under high power (X400) magnification and then compared to the total cells in each matched field as counted under haematoxylin and eosin staining. Then each field was expressed as a percentage of cells staining positively as described previously.440,649.

This was a slight variation on the human studies as outlined above where ten randomly chosen microscopic fields were used to gather data. It became feasible to examine the whole tumour section as this method had been undertaken in previous xenograft studies involving hypoxia231 and the sections were much smaller than the human specimens making it physically possible to count fields over each whole section. Again, I used matched fields for all specimens studied (controls and tumours). Also, as discussed above, edge effects and necrotic and stromal areas were ignored on sections.182,652

In summary, sections were analysed for the percentage of positive cells based on matched fields. This also allowed for the calculation of cell density. Specific issues relating to the calculation of MVD, Ki-67 are discussed below.

3.13 Biodistribution Studies of Carbonic Anhydrase IX Expression in a xenografted RCC model with 124I-G250 Monoclonal Antibodies

3.13.1 Analytical grade reagents and dose calibration

All analytical grade reagents, except when stated, were obtained from Merck Pty. Ltd. (Melbourne, Australia). 124I in 0.02 N NaOH [10mCi/ml] was purchased from Advanced Nuclide Technologies (Voorhees, NJ). Radioactivity was measured either with a dose calibrator, Biodex Atomlab-100 (Brookhaven, NY) or a shielded Cobra II automated gamma counter (Canberra- Packard, Melbourne, Australia).
3.13.2 Monoclonal antibodies

The generation and characterization of the original murine mAb cG250 and its humanization have been described previously. Humanized cG250 (Wilex AG, Munich, Germany) was obtained whilst humanized anti-A33 mAb (huA33), which specifically recognizes the A33 antigen of colon cancer, and does not bind G250-expressing cells, was produced in the Biological Production Facility (Ludwig Institute for Cancer Research, Melbourne, Victoria, Australia). Both were conjugated to $^{124}$I as described below. $^{124}$I was purchased from (Ritverc GmbH, St. Petersberg, Russia).

3.13.3 Radiolabelling and Quality Assurance

An aliquot of 0.18 ml 0.5 M potassium phosphate buffer, pH 7.5 was added to 0.09 ml of $^{124}$I in 0.05N NaOH (1080 µCi). An aliquot of 0.15 ml of this mixture was added to 60 µl of chG250 antibody (5 mg/ml) followed by 2 iodogen glass coated beads. After 10.0 mins, labelling mixture removed and purified on a 5 ml Sephadex G50 column equilibrated in saline. The control antibody huA33 was similarly radiolabelled and purified. Radiochemical purity of labelled antibody was analysed by instant-thin layer chromatography-silica gel (ITLC-SG) using 10% w/v trichloroacetic acid as solvent. Radioactivity bound to antibody remains at the origin whereas free $^{124}$I migrated with the solvent front. Radiolabelling was performed on the day of injection into mice. Radiochemical purity was assessed prior to injection with the percentage of unbound radionuclide content was determined by ITLC-SG developed using 10% TCA solution.

3.13.4 Immunoreactive Assays and Receptor Expression

Lindmo and Scatchard Assays

The immunoreactivity of cG250 after radiolabelling was determined using the assay of Lindmo et al. This cell-based assay consisted of incubating 20ng radiolabelled antibody with increasing concentrations of SK-RC-52 ranging from 0 to 6.0 x 10⁶ cells in 1.0 ml
medium. The cells were incubated for 45 min at room temperature with continuous mixing. After 45 min at room temperature, the cells were centrifuged and washed 3 times with medium to remove unbound antibody and pellets were measured in a gamma counter (Cobra II Model 5002, Auto-gamma; Canberra-Packard). Standards were measured at the same time as pellets and percentage binding of radiolabelled cG250 to SK-RC-52 cells was calculated. The immunoreactivity was determined by plotting the double reciprocal plot for binding against cell concentration using GraphPad Prism 4.0 software (GraphPad Inc., San Diego, CA, USA). Scatchard analysis was used to determine the affinity constant ($K_a$) and number of antigen binding sites/ cell as previously described.  

Fluorescence-activated cell sorting (flow cytometry) of receptor expression
Cells were either left pure or incubated with primary antibody (cG250) or a negative control antibody (IgG1) for assessment of CAIX expression. Analysis was done using an Epics Elite ESP (Beckman Coulter, Hialeah, FL, USA) by observing a minimum of 20,000 events and data analysed using EXPO (version 2) for Windows.

Serial dilutions starting from 10µg/ml unlabeled G250 to 0.01 were mixed with 20ng labelled $^{124}$I-cG250. The antibody mixture was added to 3.0 x $10^6$ cells SK-RC-52 cells and incubated at room temperature with continuous mixing. After 45 min incubation, cells were washed and counted as described above for Lindmo assay. The IR fraction was taken into account in calculating the amount of free, reactive antibody $[(100 - 2 \% \text{ bound})/100 \times \text{ total antibody} \times \text{IR fraction}]$, and specific binding (nM; total antibody 3 % bound) was graphed against specific binding/reactive free. The association constant was determined from the negative slope of the line. The number of G250 molecules bound per cell was derived by: $[(X\text{-intercept of Scatchard plot (nM)/1000} \times (6.023 \times 10^{23}))$/number of cells used in the assay (12 x $10^6$)].
3.13.5 Serum Stability

The stability of radioconjugates in the blood circulation of mice was also determined. Serum was obtained at days 0, 3 and 7 post-injection. An aliquot of serum was spotted on an ITLC-SG strip and developed using 10% w/v trichloracetic acid as solvent. For immunoreactivity assay concentrations of radiolabelled antibody in mouse serum were estimated from the blood-time activity curve of the biodistribution study and used in the cell-binding assay.

3.13.6 Biodistribution Studies of $^{124}$I-cG250

Biodistribution studies were performed 10wk after tumour induction, at which time the mice weighed 11–23 g. The general cG250 biodistribution kinetics in the current tumour model was investigated using $^{124}$I-cG250. Mice (n=40) were injected intravenously with 5MBq $^{124}$I-cG250 (25 mg) in 200mL PBS 1 0.5% HAS on day 0. Groups of mice (n= 5) with a mean (± SD) tumour volume of 629 ± 195 mm$^3$ were sacrificed on Day 0 (2h), 1, 2, 3, 5, 7, 10 and 14 after injection of radiolabelled antibody. Control groups of mice (n = 5) bearing SK-RC-52 xenografts received a single injection of radiolabelled $^{124}$I-huA33 on the same day and were sacrificed on days 0 (2h) and 3.

Mice were sacrificed after having invasive oxygen measurements and in most cases PET imaging (both outlined below) by exposure to sevofluorane. Mice were bled via cardiac puncture, and blood was collected into heparinised tubes. Tumours and organs (skin, liver, spleen, intestine, stomach, kidneys, brain, bone [femur], lungs, and heart) were immediately removed, blotted dry, and weighed (Sartorius Basic Balance, Ratingen Germany). All samples were counted in a dual chamber gamma scintillation counter (Cobra II, Auto-gamma; Packard Instruments) using a dual tracer program with standard windows set for the isotope as described previously. Standards prepared from the injected material were counted each time with tissues and tumours, enabling calculations to be corrected for the isotope physical decay. Results of radiolabelled antibody distribution over time were
expressed as the percentage of injected dose per gram tissue (%ID/g) and as tumour-to-blood ratios.

**Pharmacokinetics of $^{124}$I-cG250**

A three-compartment model of the concentration of antibody ($^{124}$I-cG250) in serum, peripheral tissue and tumour with bolus IV injection, no lag time and mixed macro- and micro-parameters was fitted to the measured concentration antibody from the serum and tumour compartments.

Fitting was performed by minimizing the sum of the weighted, squared residuals using the Nelder-Mead method of the general-purpose optimisation function in statistical programme R (version 2.2.0, The R Foundation for Statistical Computing, Madison, WI, U.S.A.). The variances of the measured concentrations were used to weight the residuals. The sum of the square of the residuals for the tumour compartment was further scaled by a factor of 2, so that both compartments contributed approximately equally to the total error estimate.

The Area Under Curve (AUC) and the maximum concentration (Cmax) in the serum and tumour compartments were calculated from the parameter estimates. The volume of the serum compartment (V1) and the clearance from the serum compartment were also calculated.

**3.13.7 Autoradiography for CAIX expression**

Immediately after sacrifice, an SK-RC-52 tumour from one mouse at time points 24, 48, 72, and 120 h in the $^{124}$I biodistribution study was excised, frozen, and sectioned on a cryomicrotome (Zeis Microm HM 5000, Melbourne, Australia). Sequential 5-mm sections were used for direct autoradiography and for H&E staining. Tissue sections were placed on silane-coated glass slides and allowed to dry. Glass slides were placed face down in contact with standard radiography film. The glass slides and film were enclosed in a cassette and

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stored at −80°C. Slides and film were taken up at varying time intervals, and film was
developed in a standard automatic film processor.

3.14 Studies of the effects of therapeutic monoclonal antibodies on tumour oxygenation in an RCC xenograft model

Antiangiogenic agents have been used in RCC and as described. The newer TKI have shown promise as agents in the treatment of RCC. To understand more about the oxygenation of tumours and what effect anti-angiogenic agents may have on oxygenation in RCC, I used RCC xenografts in therapeutic studies with anti-angiogenic agents; in this case, monoclonal antibodies (mAb).

To explore with potential for targeting receptors overexpressed in RCC I decided to focus on mAb targeting antigens:
- CAIX- mAb cG250
- EGFR- mAbs c528 and c806
- VEGF- mAb bevacizumab (Avastin, Roche Pharmaceuticals Division, Basel, Switzerland)

3.14.1.1 Anti-CAIX antibodies- cG250
The mAb cG250 recognizes CAIX that as alluded to, is highly expressed on the cell surface of almost all RCC. The targeting of RCC expressing the G250 antigen has been studied in both murine and chimeric G250 mAb with significant uptake in CAIX antigen-positive cells versus antigen-negative cells.146,598

3.14.1.2 Anti EGFR antibodies- c528 and c806
The mAb c806 is a novel EGFR antibody with significant antitumour activity that recognizes different types of EGFR: the de2-7 EGFR and a subset of the wild type (wt) EGFR when overexpressed (it does not bind the wt EGFR expressed in normal tissues).142 The mAbs c528 is more general and recognises only the wt EGFR.676
3.14.1.3 **Bevacizumab**

A mAb against VEGF and has been discussed elsewhere (Section 2.8.4)

### 3.15 Animal model and RCC xenograft techniques

Previously described in section 3.10.

### 3.16 Monoclonal antibody production

At our institution I had access to facilities engineering monoclonal antibodies and had experience in using them in prior studies (Ludwig Institute for Cancer Research, Biological Production Facility Austin Hospital, Heidelberg, Victoria, Australia). The process of engineering the antibodies in our institution has been described elsewhere and will not be discussed further in this thesis. Our facility manufactured cG250, c528, c806. Bevacizumab was surplus from a previous experiment within our institution where it was obtained and supplied by the manufacturer (Roche Pharmaceuticals Division, Basel, Switzerland).

#### 3.16.1 Monoclonal antibody dose and administration

All mAb were administered intravenously into the tail vein of mice and were suspended in phosphate buffer solution (PBS). The mAb cG250, c528 and c806 were administered at 1 mg; 3 times per week for 2 weeks as per our institutional standard protocol. When mAb were combined (c528 and c806 only) they were both administered in a dose of 1mg; 3 times per week. Bevacizumab 400 ug/100ul was administered once per week.

#### 3.16.1.1 Control for experiments

PBS was injected into a tail vein in control animals as this was the agent used as the conduit for the mAb. It was administered 3 times per week for 2 weeks.
3.16.2 Tissue oxygenation measurements
Again the oxylite probe was used. Different time-points over approximately a 2 week period were chosen to monitor the effects of therapy on tumour oxygenation in the xenograft model.

3.17 Xenografting tumour beneath the renal capsule using modern surgical equipment

*In vivo* growth of experimental tumours is an important aspect of research into factors affecting tumour behaviour, in order to determine the pathophysiological significance of the transformed phenotype displayed by tumour cells in vitro e.g. motility and invasion. It is important to have appropriate *in vivo* models with which to assess tumour cell behaviour. Reproducible models are a necessity because as a general rule it is often more difficult to control the growth of cells following implantation into experimental animal hosts compared to in vitro experiments, where growth conditions and cell numbers are tightly controlled.

The most widely applied method for growth of tumour cells in vivo is with subcutaneous implantation. This offers obvious advantages with ease of access and speed of deployment as well as observation but may not be suitable for all cell types in all models. The subrenal capsule, because of its rich vascular supply was considered a valuable target for the implantation of tumour cells. Human tumours as diverse as prostate cancer, ovarian cancer, melanoma, colon cancer, sarcoma, lung cancer and renal cell cancer have been grown under the renal capsule in mice. Such models have been used extensively in the investigation of tumour response to chemotherapeutic agents and when doing so are known as the subrenal capsule assay. Tumour implantation under the renal capsule minimises lag phase before measurable growth that is required for induction and proliferation of supportive vasculature compared to subcutaneous models.

Traditional models of subcapsular growth involve injection of cells under the renal capsule. Slight modifications to this technique have involved the suspension of cells
in collagen gel solution prior to implantation. Early tissue transplant models employed dissection of tissue using a scalpel (of approximately \( 1 \text{mm}^3 \)) with trochars used to place tissue under the renal capsule after making a 3mm incision in the convex surface of the kidney. More recently, tumours have been transplanted after being cut into cubes with pouches meticulously dissected under the renal capsule. All these models are labour intensive and time consuming.

The method I developed and present here has never been described in the literature.

3.17.1 Justification for developing a new model

A prerequisite for obtaining reproducible and reliable results is that only viable tumour tissue should be implanted. Subcutaneous human tissue xenografts have been previously demonstrated to be successfully grown using other models of implantation into the kidney, however it has been difficult to repeatedly implant a constant volume of tumour tissue due to inherent difficulties in measuring such tissue volume prior to grafting. This is important, as comparison of results of treatment is dependent upon a constant initial tumour volume. Surgical precision of tumour implantation also is considered a key factor in establishing transplant grafts beneath the renal capsule.

Automated biopsy systems have been in common use in surgery for over 25 years. Epidural needles have been in use for over 40 years. However, both instruments have undergone considerable refinement. The ergonomics of biopsy devices have improved considerably, with spring-loading and availability of different biopsy size and needle lengths making them more attractive to use. Also, visualisation of the biopsy core prior to transplantation allows visibly necrotic tissue to be avoided, thus conserving tissue rather than discarding whole pieces when cores are not taken.

Finally, the automated biopsy device may be used in conjunction with any tumour model requiring xenografting, not just the renal subcapsular model. Although I did not biopsy
tumours below 8mm using the 16G automated biopsy gun, further studies would be required to establish the minimal tumour size needed to use such a device. Furthermore, smaller biopsy devices are commercially available and could be used to obtain biopsies from smaller tumours.

Epidural needles are precision instruments and do not compare to trochars used in the past that were merely a perforator enclosed in a tube used to deploy tissue fragments. Contemporary needles are easy to hold, often have directed tips and come with introducers. They are also extremely sharp and do not require renal capsule perforation with other instruments as was necessary in the past.

The particular epidural needle used in this model was chosen because its hollow fitted the biopsied tumour graft snugly and it has a plastic (rather than metal) trochar that allows deployment of the tumour graft completely from the needle tip without tissue trauma. Finally, the needle tip is slightly curved allowing for easy puncturing of the capsule, again aiding precise deployment of the graft.

3.17.2 Animals and Cell Lines in Renal Cell Carcinoma Xenograft Studies
These have been discussed in section 3.10.

Establishing SK-RC-52 human Renal Cell Carcinoma Xenografts
Two donor tumours from mice were ultimately used for transplantation and had their tumour harvested by biopsy gun and applied either subcapsularly (n=12) or subcutaneously (n=12).

The donor mice had human xenografted tumours grown subcutaneously after implantation of RCC cells from culture (SK-RC-52 cell line) suspended in reconstituted basement membrane (Matrigel) as previously described (see section 3.10). At 8 weeks two mice with similar tumour volumes and diameters (being greater than 8mm) were anaesthetised with an intraperitoneal injection of xylocaine hydrochloride (20mg/ml, Troy laboratories Pty Ltd Smithfield, Australia) and ketamine (100mg/ml, Parnell laboratories Alexandria, Australia)
diluted in normal saline at 0.1ml solution per gram body weight (1 part ketamine, 1 part xylocaaine, 8 parts normal saline). Skin overlying the tumour was prepared with 10% povidone-iodine solution and the tumour exposed by dissection with scissors and forceps under a sterile hood. Mice used for tumour donation (n=2), one for the subcapsular group and one for the subcutaneous group, were later sacrificed using sevofluorane after the tumour was harvested. The other mice (n=10) were used in other studies.

A 16 gauge (16G), 6cm length spring-loaded, automatic biopsy gun with handle (Achieve™ Programmable Biopsy System, Cardinal Health, Inc., Dublin, USA) is used to obtain the tumour tissue from the host mouse. As outlined in Figure 3-7, firstly, the gun is set so that the distal 2cm working element of sheath and trochar are both retracted. The gun is then fired once adjacent to and touching the tumour mass with the needle entering the mass. A second firing then moves the sharp sheath over the trochar obtaining the tumour graft (figure 1C) and the needle is gently removed from the tissue. The biopsy core obtained is 1.6mm diameter (16G) and the length is equivalent to the maximal length of the host tumour at the biopsy site (between 8 and 10mm). Digital calipers are used to measure a 2mm section for grafting, and sectioning is then undertaken with a scalpel. Cores taken are placed in normal saline until used for transplantation. The cores are essentially cylinders and their volume may be accurately estimated by using the appropriate formula (volume of a cylinder in mm$^3$ = π x radius$^2$ x length). As the biopsy needle sheath has a radius of 0.8mm and the length is two millimetres, grafts are consistently 4.02mm$^3$

Further immunocompromised mice (n=12) were then anaesthetised. A small incision (approximately 8-10mm is made in the right flank after laying the animal on its side and preparing the skin with 10% povidone-iodine solution. Using gentle pressure from the forefinger and thumb the kidney is extruded out of the body cavity incision and exteriorized. Once the kidney is exposed it is ready to receive the transplant from the first donor mouse.
Figure 3-7 The automatic biopsy gun and a diagram of the method of use

The automatic biopsy gun with sheath withdrawn over needle ready for firing (top). Buttons A and D are used to deploy the needle and then sheath respectively. The sheath is now withdrawn (immediately below) revealing the biopsy needle with indentation to accept the biopsy core. Harvesting of the tumour graft (lower panels): A) Biopsy gun placed adjacent to dissected tumour touching the mass, ready to fire. B) The trochar is deployed and C) the sheath is then deployed cutting the biopsy. D) Finally the sheath and trochar are removed with the sheath moved back revealing tumour biopsy.
Using an epidural needle to insert tumour xenografts beneath the renal capsule

The tumour graft is first carefully inserted into a 16G epidural needle (Epidural Minipack, SIMS Portex Ltd, Hyde, UK) with blunt forceps being careful not to damage the tissue (Figure 3-8). This needle was chosen because it is hollow, fitted the biopsied tumour graft snugly and has a plastic trochar that allows deployment of the tumour graft completely from the needle tip The needle tip was also slightly curved allowing for easy puncturing of the capsule. The needle is placed under the renal capsule and the trochar introduced to deploy the graft beneath the capsule with minimal trauma. Sterile gauze is held over the needle puncture to ensure coagulation and graft retention. A 4-0 dissolving suture containing braided co-polymer of glycolic and lactic acid (Vicryl Rapide, Ethicon, Somerville, USA) is used to close the wound. At all times animals are heated to maintain core body temperature.

Food and water were provided for the mice ad libitum and their health was monitored daily. Animal care and experiments followed the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes endorsed by the National Health and Medical Research Council and was approved by our institutional animal ethics committee. Mice were weighed weekly.

The second donor mouse then had its tumour harvested by automated biopsy gun as above. This mouse was from the same initial pool of mice tumours as that used for the subrenal capsule donation. However, this second group (n=12) had the tumour implants placed subcutaneously using standard procedures for comparison as described previously. At five and ten weeks half (n=6) of each of the subcapsular and subrenal groups mice were sacrificed with the kidneys and tumours harvested. Tumours (n=24) were measured in two planes with digital calipers to calculate volume (length x width^2/2). Tumours were then frozen with liquid nitrogen. Histologic assessment involved analysis of 5-micron tissue sections that were obtained using a microtome with hematoxylin and eosin staining of slides.
Figure 3-8 The epidural needle and diagram of xenograft transplant

Upper panels: The epidural needle (trochar) then the epidural needle demonstrating its plastic introducer. Finally the graft (arrow) being placed within the needle ready for transplanting. Lower panels: Grafting the tumour biopsy- A) The graft is placed in the epidural needle which is inserted under the renal capsule. B) The tumour graft is then deployed by pushing gently on the blunt introducer. C) Finally, the trochar and introducer are removed leaving the graft in place.
3.18 Statistical analysis and other computer software used

Throughout the thesis, unless otherwise specified, continuous data were analysed using a computer package (Graphpad Prism 4.0, GraphPad Software, San Diego, CA, USA or StatCrunch 3.0, Copyright 2002-2004, Integrated Analytics LLC, USA) with 95% confidence intervals used to determine the significance between the tumour model groups. Graphs were created by computer package (Graphpad Prism 4.0, GraphPad Software, San Diego, CA, USA or Microsoft Excell 2003, Microsoft Corporation, Washington, DC, USA).

For assistance with some diagrams and to create graphics, graphics software was used (Smartdraw trial version 2005, Smartdraw.com, San Diego, CA, USA). Word processing conducted using Microsoft word (Version 1997-2003; Microsoft Corporation, Washington, DC, USA). Endnote (version XI, Thomson Reuters, Carlsbad, CA, USA) was used for collating references.

3.18.1 Reporting

The statistics are reported with standard statistical tests where required: mean, median, standard deviation, standard error of the mean and 95% confidence intervals in human studies. The standard deviation (SD) and 95% confidence intervals (95% C.I.) of the mean are reported. In animal studies because of the larger numbers the mean and standard error of the mean (SEM) has been utilised as per convention.

It is generally considered that if two 95% CI ranges do not overlap, the difference is statistically significant with \( p < 0.05 \). However, if they do overlap then the difference between the two means may or may not be statistically significant.\(^{691}\) This is not rigorous enough so where relationships between defined parameters was analysed, for differences between two groups the nonparametric test (Mann-Whitney) was used and across groups the nonparametric test (Kruskall Wallis) was used due to an assumption of a non-Gaussian distribution. If they were paired the nonparametric Wilcoxon signed rank test was used.
Finally, correlation coefficients (logistic regression) are also reported to establish relationships. In testing for correlation between the mean of data groups, Pearson’s correlation coefficient ($r^2$) was used and has a range of $-1.0$ (perfect negative correlation) to $1.0$ (perfect positive correlation) with $r=0$ indicating the two variables do not vary at all together. A value of $r^2 > 0.8$ is considered a strong correlation, $<0.5$ a weak correlation and $0.5 > r^2 < 0.8$ is a moderate correlation.

If $r^2$ is far from zero, there are some possible explanations: 1) Changes in the X variable lead to changes in the Y variable; 2) Changes in the Y variable lead to changes in the X variable; 3) Changes in another variable influence both X and Y and finally 4) that the correlation occurred by chance. That is why it is necessary to do a two-tailed p value in order to determine the chance that this would occur. The p value answers the question: If there really is no correlation between X and Y, what is the chance that a random sampling would result in a correlation coefficient as far from zero (or further) as observed in this experiment? Another way of consolidating the role of the p value is that a p value less than 0.05 means that the correlation is unlikely to occur by chance in greater than 95% of occasions if repeated.

The $r$ value is typically squared to create $r$ squared ($r^2$) so that if the value for example is 0.59, then 59% of the variance in X can be explained by variation in Y and vice versa. That is how the $r$ value is reported in this thesis, as $r^2$ with two-tailed P value.

Within-patient variation for each immunohistochemical parameter is important and is accounted for by examining patient means rather than individual data entry points (each field being ten per patient for each stain). Inter-patient variability is best assessed by means (with SD and 95% C.I)

The determination of sample size requires statistical criteria but also practical and clinical considerations. In this study there were a limited number of patients with suitable RCC
available to study (e.g. laparoscopic cases could not be used because the equipment was only suitable for open cases). We also knew that technical and clinical failures would occur based on other studies (e.g. need to progress to nephrectomy because of bleeding). We also took notice of prior studies in other organs where fewer than ten patients were tested with POS where a significant result occurred (see Chapter 1). Overall five is the smallest number of patients likely to demonstrate a mean 15mmHg difference between tumor and normal tissue pO2. However, if we look further at the number of readings below 5mmHg (HP5), then the number of patients falls to four as the number of readings per tumor is over 100.
Chapter 4
RESULTS AND DISCUSSION: HUMAN STUDIES

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4.1 Human Studies: Overview

Patients with suspected RCC were recruited from urology outpatient clinics at the Austin Hospital, Heidelberg, Victoria, Australia from end of 2001 until 2004. Patients were enrolled after being consented for the study that had been approved by the Austin Health Human Research Ethics Committee. All patients provided written informed consent prior to participation. I aimed to recruit approximately fifty patients.

In total, 53 patients agreed to the study, one of whom withdrew prior to completing any study procedures (Table 4-1). The study essentially had four components:

1) Surgery with storage of tissue for histology and immunohistochemical studies
2) PET studies with $^{18}$F-Fluorodeoxyglucose and $^{18}$F-Fluoromisonidazole
3) Polarographic oxygen electrode sensor (POS) studies of tumour and normal renal tissue
4) Serum osteopontin levels (added to the protocol part way through the study)
Table 4-1 Summary of study participants and steps completed for the study

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<tr>
<th>Total Patients</th>
<th>Surgery on Tumours</th>
<th>Surgical Procedures</th>
<th>Tumours with Histology</th>
<th>PET Studies</th>
<th>POS</th>
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<tr>
<td>Total</td>
<td>Total</td>
<td>Radial Nephrectomy</td>
<td>Partial Nephrectomy</td>
<td>Laparoscopic Nephrectomy</td>
<td>(¹⁸F-FDG and ¹⁸F-FMISO)</td>
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<td>1</td>
<td>Withdrew</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>1</td>
<td>No surgery</td>
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</tr>
<tr>
<td>1</td>
<td>Biopsy only</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1</td>
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<tr>
<td>48</td>
<td>Unilateral nephrectomy or partial nephrectomy</td>
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<td>31</td>
<td>6</td>
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<td>Unilateral nephrectomy with 2 tumours</td>
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<td>-</td>
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<td>S2 Procedures</td>
<td>S1 Nephrectomies</td>
<td>S4 Tumours</td>
<td>S3 Patients</td>
</tr>
</tbody>
</table>
4.1.1 Reporting of Results

The statistics reporting is described in section 3.18. In brief, nonparametric tests (Mann-Whitney) were used and across groups the nonparametric test (Kruskall Wallis) was used due to an assumption of a non-Gaussian distribution. For the linear regression correlations, the $r^2$ classifications are: $r^2 <0.5$ weak, moderate $r^2>0.5$ but $<0.8$ and $r^2 >0.8$ a strong correlation.

4.1.2 Ethical decisions and patient enrolment

It was anticipated that in recruiting patients not all would complete all parts of the study, the constant step being an analysis of tissue comparing normal to tumour tissue for immunohistochemical markers of angiogenesis and hypoxia. PET studies, direct oxygen measurements and serum osteopontin then followed in order of importance. The addition of serum osteopontin to the protocol occurred later in the study and is now part of an ongoing further study that has just commenced (see section 3.5.12).

In any clinical trial it is important not to compromise patient care. As such, it was not always possible to conduct PET studies in all patients. Some required urgent nephrectomy due to symptoms and some patients travelling from long distances also were not suitable. However, other aspects of data collected were clearly useful and patients themselves did not wish to be excluded and were willing to comply with other facets of the study.

In practice up to 30% of tumors suspected of being RCC on imaging will be benign (e.g. oncocytomas, fat poor AML). Thus, information on any benign tumors is important for imaging (POS readings, PET studies) so we may better understand then preoperatively with an opportunity to distinguish benign from malignant with further information. Biopsying of tumors is not universal and so characterizing tumors non-invasively becomes even more important (e.g. with immunohistochemistry if hypoxia responsive elements are profiled). Finally, oncocytomas locally invade surrounding tissues and many consider them not purely benign and thus characterizing them is important. For these reasons we elected to include some data on benign tumours.
4.2 Direct measurements of oxygenation in tumours and normal tissue: Human Studies

4.2.1 Patient Demographics

The patient demographics are outlined in Table 4-2. The patient who withdrew before completing any part of the study is not included in any further analysis leaving 52 patients enrolled and completing at least one key component of the study (Mean age 61, median age 62, range 30-77). There were 29 females and 23 males. Median ECOG performance status was 1 (range 0-4). One patient failed to complete any part of the study apart from the FMISO and FDG PET studies, declining nephrectomy due to surgical risks related to poor performance status (ECOG performance status 4) and was lost to follow-up from our hospital. This left 51 patients who had a total of 54 tumours.

Of the 51 patients with 54 tumours all were diagnosed histologically. However, when considering immunohistochemistry not all were able to be processed. In one patient, bilateral oncocytomas were demonstrated after partial nephrectomies on each kidney some eight weeks apart. In the same patient, a small papillary tumour was discovered incidentally on histopathology. This also occurred in one patient with a large clear cell RCC who also had a small incidental papillary tumour. Hence one patient provided three tumours and another two. However, only the primary lesions (two oncocytomas and one clear cell RCC) underwent immunohistochemistry. Further, a patient with metastatic disease had histology confirming clear cell RCC and a tumour on imaging in the right kidney but no nephrectomy was performed so renal tissue was not analysed immunohistochemically. Finally, one patient had no obvious tumour on visual inspection and no normal kidney on that side hence all tissue obtained went to diagnostic pathologist to exclude a small tumour in block dissections with no tissue for study. The final histology was benign disease. Thus, in total, 49 patients provided 50 renal tumours and normal renal tissue for immunohistochemical analysis.
The tumour type, serum biochemistry (urea and electrolytes) and full blood counts are summarised in Table 4-3.
### Table 4-2 Patient Demographics and Tumour Characteristics on all patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Demographics and Tumour Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age (mean, range in brackets)</td>
</tr>
<tr>
<td></td>
<td>Sex (M=male, F=female)</td>
</tr>
<tr>
<td></td>
<td>Side (R=right, L=left)</td>
</tr>
<tr>
<td></td>
<td>Max Length (mean cm)</td>
</tr>
<tr>
<td></td>
<td>Volume of Tumour (cc)</td>
</tr>
<tr>
<td>ALL (n=52)</td>
<td>60.85 (31-77)</td>
</tr>
<tr>
<td></td>
<td>29F, 23M</td>
</tr>
<tr>
<td></td>
<td>24R, 28L</td>
</tr>
<tr>
<td></td>
<td>6.29</td>
</tr>
<tr>
<td></td>
<td>187.18</td>
</tr>
<tr>
<td>RCC (n=39)</td>
<td>60.62</td>
</tr>
<tr>
<td></td>
<td>22F, 17M</td>
</tr>
<tr>
<td></td>
<td>18R, 21L</td>
</tr>
<tr>
<td></td>
<td>6.60</td>
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<tr>
<td></td>
<td>212.92</td>
</tr>
<tr>
<td>RCC clear (n=32)</td>
<td>61.5 (31-77)</td>
</tr>
<tr>
<td></td>
<td>17F, 15M</td>
</tr>
<tr>
<td></td>
<td>15R, 17L</td>
</tr>
<tr>
<td></td>
<td>6.55</td>
</tr>
<tr>
<td></td>
<td>221.46</td>
</tr>
<tr>
<td>RCC-non clear (n=7)</td>
<td>56.57 (33-74)</td>
</tr>
<tr>
<td></td>
<td>5F, 2M</td>
</tr>
<tr>
<td></td>
<td>3R, 4L</td>
</tr>
<tr>
<td></td>
<td>6.80</td>
</tr>
<tr>
<td></td>
<td>175.14</td>
</tr>
<tr>
<td>Non-RCC Malignancy (n=2)</td>
<td>56 (43-69)</td>
</tr>
<tr>
<td></td>
<td>1F, 1M</td>
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<td></td>
<td>0R, 2L</td>
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<td></td>
<td>9.25</td>
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<tr>
<td></td>
<td>321.88</td>
</tr>
<tr>
<td>TCC (n=5)</td>
<td>65 (51-75)</td>
</tr>
<tr>
<td></td>
<td>3F, 2M</td>
</tr>
<tr>
<td></td>
<td>1R, 3L</td>
</tr>
<tr>
<td></td>
<td>4.60</td>
</tr>
<tr>
<td></td>
<td>33.53</td>
</tr>
<tr>
<td>Oncocytoma (n=4)</td>
<td>65.75 (60-73)</td>
</tr>
<tr>
<td></td>
<td>1F, 3M</td>
</tr>
<tr>
<td></td>
<td>2R, 2L</td>
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<tr>
<td></td>
<td>3.05</td>
</tr>
<tr>
<td></td>
<td>18.84</td>
</tr>
<tr>
<td>Other Benign (n=2)</td>
<td>50.00</td>
</tr>
<tr>
<td></td>
<td>2F</td>
</tr>
<tr>
<td></td>
<td>2R, 0L</td>
</tr>
<tr>
<td></td>
<td>13.50</td>
</tr>
<tr>
<td></td>
<td>471.00</td>
</tr>
</tbody>
</table>
Table 4-3 Serum Electrolyte and Haematological Parameters on all patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Serum Biochemistry</th>
<th>Full Blood Count</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na⁺ (mmol/L 135-145)</td>
<td>K⁺ (mmol/L 3.5-5.0)</td>
</tr>
<tr>
<td>ALL (n=52)</td>
<td>140.44</td>
<td>4.26</td>
</tr>
<tr>
<td>RCC (n=39)</td>
<td>140.19</td>
<td>4.32</td>
</tr>
<tr>
<td>RCC clear (n=32)</td>
<td>140.19</td>
<td>4.32</td>
</tr>
<tr>
<td>RCC-non clear (n=7)</td>
<td>142.71</td>
<td>4.09</td>
</tr>
<tr>
<td>Non-RCC Malignancy (n=2)</td>
<td>140.50</td>
<td>4.35</td>
</tr>
<tr>
<td>TCC (n=5)</td>
<td>139.40</td>
<td>4.34</td>
</tr>
<tr>
<td>Oncocytoma (n=4)</td>
<td>138.00</td>
<td>4.00</td>
</tr>
<tr>
<td>Other Benign (n=2)</td>
<td>144.00</td>
<td>4.15</td>
</tr>
</tbody>
</table>
Staging was based on pathological specimens in all but one patient with T1 disease, (T1N0M1). This patient had histologically proven metastatic disease to the spine (4th lumbar vertebra) and did not go on to have a nephrectomy but instead had spinal stabilisation surgery and chemotherapy. The diagnosis for this patient is thus based on biopsy and radiological findings rather than definitive nephrectomy histology. This left 31 patients with clear cell RCC for immunohistochemical analysis of normal and renal tumour tissue. The majority of these patients had T1 disease (n=14), with five patients with stage 2 and 12 patients stage 3 disease. Metastases to lymph nodes were only demonstrated in two patients, both with stage T3 disease (penetrating beyond the renal tissue and into surrounding fat): one T3bN2M0 and one T3bN1M0.
Table 4-4 The different sub-types of RCC at low (10x) and high (400x) power magnification
4.2.2 Polarographic Oxygen Sensor (POS) Measurements

From the original fifty patients having surgery, eleven patients having laparoscopic nephrectomise were not eligible. Intraoperative POS measurements were able to be obtained with confidence in 11 surgical procedures from both normal kidney and renal tumour from the remaining 39 patients having open surgery (Table 4-1). Readings were attempted on 26 patients; thirteen had no attempt made because of software difficulties that could not be resolved. Fifteen cases resulted in no usable measurements because of: two cases being abandoned when the probe was ready due to intraoperative difficulties (excessive bleeding resulting in urgent nephrectomy); five cases abandoned due to an inability to adequately calibrate the probe due to software malfunction; four cases were abandoned because the probe although appearing calibrated would not read and finally four patients had readings taken but were excluded because the probe could not be calibrated post readings to ensure reliability of the POS readings available for analysis.

Ten patients had RCC and one TCC. A mean of 105 measurements were taken in tumour tissue and 94 in normal tissue in 1-2 tracks per tissue (Figure 4-1).
Figure 4-1 Polarographic oxygen sensor readings from a study patient

A solitary POS track measurement is displayed (A) reflecting the heterogeneity of normal and renal tissue. A summary histogram (B) displays the data once all tracks have been measured.
Table 4-5 Polarographic Oxygen Sensor measurement: mean and median values

<table>
<thead>
<tr>
<th></th>
<th>Normal pO$_2$ (mmHg) ± SD</th>
<th>Median pO$_2$ (mmHg)</th>
<th>Tumour pO$_2$ (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCC (n=10)</td>
<td>Normal: 44.95±36.42</td>
<td>Normal: 38.6</td>
<td>Tumour: 10.8</td>
</tr>
<tr>
<td></td>
<td>(10.9-113.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCC- Clear Cell</td>
<td>49.14±34.27</td>
<td>39.5</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>(14-106.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCC- Non-clear Cell</td>
<td>40.76±42.03</td>
<td>25.2</td>
<td>15.4</td>
</tr>
<tr>
<td></td>
<td>(10.9-113.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCC (n=1)</td>
<td>7.10</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 4-3 Polarographic Oxygen Sensor Measurements by Hypoxic Fraction in Renal Cell Carcinoma patients (mean ± 95% CI)

Table 4-6 Polarographic Oxygen Sensor measurements: Hypoxic Fraction

<table>
<thead>
<tr>
<th></th>
<th>HP_{10} (%)±SD</th>
<th>HP_{5} (%)±SD</th>
<th>HP_{2.5} (%)±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±95% C.I</td>
<td>Mean ±95% C.I</td>
<td>Mean ±95% C.I</td>
</tr>
<tr>
<td>Tumour RCC (n=10)</td>
<td>21.55±17.61</td>
<td>16.99±15.71</td>
<td>13.21±12.02</td>
</tr>
<tr>
<td></td>
<td>21.55±12.60</td>
<td>16.99±111.24</td>
<td>13.21±8.61</td>
</tr>
<tr>
<td></td>
<td>53.36±32.69</td>
<td>47.44±31.69</td>
<td>33.05±31.32</td>
</tr>
<tr>
<td></td>
<td>53.36±23.39</td>
<td>47.44±22.67</td>
<td>33.05±22.41</td>
</tr>
<tr>
<td></td>
<td>16.76±16.83</td>
<td>12.76±16.83</td>
<td>11.46±13.93</td>
</tr>
<tr>
<td></td>
<td>16.76±20.9</td>
<td>12.76±17.42</td>
<td>11.46±17.30</td>
</tr>
<tr>
<td></td>
<td>58.32±37.03</td>
<td>53.12±36.64</td>
<td>42.80±42.28</td>
</tr>
<tr>
<td></td>
<td>58.32±45.98</td>
<td>53.12±45.05</td>
<td>42.80±52.49</td>
</tr>
<tr>
<td></td>
<td>26.34±18.91</td>
<td>21.22±17.73</td>
<td>14.95±11.12</td>
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<tr>
<td></td>
<td>26.34±27.89</td>
<td>21.22±22.42</td>
<td>14.95±14.78</td>
</tr>
<tr>
<td></td>
<td>48.40±31.18</td>
<td>41.76±28.90</td>
<td>23.30±13.48</td>
</tr>
<tr>
<td></td>
<td>48.40±43.81</td>
<td>41.76±36.6</td>
<td>23.30±20.82</td>
</tr>
<tr>
<td></td>
<td>30.3</td>
<td>24.2</td>
<td>79</td>
</tr>
</tbody>
</table>

RCC Clear Cell (n=5)

RCC Non-clear Cell (n=5)

TCC (n=1)
4.2.2.1 **Mean Oxygen Level**

Figure 4-2 and Table 4-6 above summarise the tumour POS measurements. The mean oxygen level between tumour groups represents the mean of the means as is convention in other studies, with normal tissue and tumours ranging from 10.9-113.3 mmHg (median 38.6 mmHg) and 0-24.9 mmHg (median 10.8 mmHg) respectively.

4.2.2.2 **Hypoxic Fraction**

Figure 4-2 and Table 4-6 above summarise the tumour POS measurements by hypoxic fraction (percentage of readings below or equal to a nominated oxygen level): either 2.5, 5 or 10 mmHg being HP\(_{2.5}\), HP\(_{5}\) and HP\(_{10}\) respectively) demonstrated a trend (but not significant) between tumour and normal tissue at hypoxic fractions HP\(_{2.5}\) and HP\(_{5}\) and HP\(_{10}\).

4.2.2.3 **Relationship between Oxygen Measurements and other parameters**

On analysis (Table 4-7), no significant correlative relationship between normal or tumour tissue mean values could be made with tumour volume, maximal length, grade or stage. Also, no relationship was identified between any patient immunohistochemical marker, specifically endogenous markers of hypoxia (HIF-1\(\alpha\), CAIX and VEGF). Finally, serum biochemistry and haemoglobin were not related to tumour oxygen level. On analysis, no significant correlative relationship between normal renal tissue and the above mentioned parameters was made (table not shown).
### Table 4-7 Relationship between mean tumour oxygen level and other parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean pO$_2$ Tumour RCC -all (n=10)</th>
<th>Mean pO$_2$ RCC -clear cell (n=5)</th>
<th>Mean pO$_2$ RCC -other (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r^2$</td>
<td>p value</td>
<td>$r^2$</td>
</tr>
<tr>
<td>HP$_{10}$ (%&lt;10mmHg Tumour)</td>
<td>0.75</td>
<td>&lt;0.001</td>
<td>0.97</td>
</tr>
<tr>
<td>HP$_{5}$ (%&lt;5mmHg Tumour)</td>
<td>0.78</td>
<td>&lt;0.001</td>
<td>0.99</td>
</tr>
<tr>
<td>HP$_{2.5}$ (%&lt;2.5mmHg Tumour)</td>
<td>0.62</td>
<td>0.01</td>
<td>0.77</td>
</tr>
<tr>
<td>Age</td>
<td>0.08</td>
<td>0.44</td>
<td>0.07</td>
</tr>
<tr>
<td>Sex</td>
<td>0.03</td>
<td>0.63</td>
<td>0.05</td>
</tr>
<tr>
<td>Tumour Volume</td>
<td>0.01</td>
<td>0.74</td>
<td>0.10</td>
</tr>
<tr>
<td>Tumour Grade</td>
<td>0.12</td>
<td>0.32</td>
<td>0.07</td>
</tr>
<tr>
<td>Pathological Stage</td>
<td>0.05</td>
<td>0.54</td>
<td>0.13</td>
</tr>
<tr>
<td>ECOG</td>
<td>0.00</td>
<td>0.87</td>
<td>0.13</td>
</tr>
<tr>
<td>Serum Sodium</td>
<td>0.00</td>
<td>0.95</td>
<td>0.06</td>
</tr>
<tr>
<td>Serum Potassium</td>
<td>0.20</td>
<td>0.20</td>
<td>0.36</td>
</tr>
<tr>
<td>Serum Chloride</td>
<td>0.03</td>
<td>0.63</td>
<td>0.23</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>0.02</td>
<td>0.70</td>
<td>0.45</td>
</tr>
<tr>
<td>Serum Urea</td>
<td>0.00</td>
<td>0.95</td>
<td>0.77</td>
</tr>
<tr>
<td>Serum Creatinine</td>
<td>0.01</td>
<td>0.80</td>
<td>0.37</td>
</tr>
<tr>
<td>Serum Haemoglobin</td>
<td>0.00</td>
<td>0.89</td>
<td>0.28</td>
</tr>
<tr>
<td>White Cell Count</td>
<td>0.38</td>
<td>0.06</td>
<td>0.03</td>
</tr>
<tr>
<td>Platelets</td>
<td>0.07</td>
<td>0.47</td>
<td>0.22</td>
</tr>
<tr>
<td>$^{18}$FDG SUVmax Tumour</td>
<td>0.05</td>
<td>0.61</td>
<td>0.23</td>
</tr>
<tr>
<td>$^{18}$FDG ratio Tumour: Normal</td>
<td>0.03</td>
<td>0.66</td>
<td>0.04</td>
</tr>
<tr>
<td>$^{18}$FMISO SUVmax Tumour</td>
<td>0.06</td>
<td>0.65</td>
<td>0.01</td>
</tr>
<tr>
<td>$^{18}$FMISO ratio Tumour: Normal</td>
<td>0.18</td>
<td>0.39</td>
<td>0.38</td>
</tr>
<tr>
<td>MVD Tumour</td>
<td>0.01</td>
<td>0.77</td>
<td>0.25</td>
</tr>
<tr>
<td>MVD Hotspot T</td>
<td>0.08</td>
<td>0.44</td>
<td>0.09</td>
</tr>
<tr>
<td>HIF1-a Tumour</td>
<td>0.00</td>
<td>0.91</td>
<td>0.24</td>
</tr>
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<td>VEGF Tumour</td>
<td>0.03</td>
<td>0.66</td>
<td>0.27</td>
</tr>
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<td>VEGFR-1 Tumour</td>
<td>0.03</td>
<td>0.62</td>
<td>0.00</td>
</tr>
<tr>
<td>CAIX Tumour</td>
<td>0.04</td>
<td>0.59</td>
<td>0.00</td>
</tr>
<tr>
<td>EGFR Tumour</td>
<td>0.00</td>
<td>0.85</td>
<td>0.05</td>
</tr>
<tr>
<td>Glut-1 Tumour</td>
<td>0.19</td>
<td>0.21</td>
<td>0.16</td>
</tr>
<tr>
<td>Ki-67 Tumour</td>
<td>0.16</td>
<td>0.26</td>
<td>0.16</td>
</tr>
<tr>
<td>Cell Density Tumour</td>
<td>0.02</td>
<td>0.73</td>
<td>0.00</td>
</tr>
</tbody>
</table>
4.2.3 Discussion of POS measurement results

Although the integrated approach to delineating tumour oxygen status with the “gold standard” POS equipment can be a powerful tool in tumour studies, it has its limitations.\textsuperscript{175,178,192} Unfortunately the POS manufacturer became insolvent during the study leading to software difficulties that could not be resolved despite many attempts with software upgrades and new parts. Although our institution had a service contract, I was at the mercy of an overseas company that ran into financial difficulties.

Technical failures around 10% have been reported in the past.\textsuperscript{192} This highlights the extremely complex nature of polarographic oxygen sensing equipment and the need to have adequate servicing within one’s own country. I was limited because no other company would service the device (hardware equipment worth over AUD$100,000) because of potential problems in any event that the parent company manufacturing the device became solvent and warranties could not be guaranteed with unauthorised work.

Despite these difficulties with the probe I did achieve reliable readings in eleven patients with renal tumours. A mean of 105 measurements were taken in tumour tissue and 94 in normal tissue in 1-2 tracks per tissue. The reason for the slight discrepancy between the tumour and normal tissue in total measurements was that on most occasions the tumour was deeper than the normal parenchyma. As the probe measurements were based on the perceived size of the tumour and parenchyma on CT, it is reasonable that the measurements be set to be slightly less than the maximal kidney parenchymal thickness to avoid damaging surrounding structures and to ensure only kidney tissue is measured. Also, the ability to puncture normal anatomical structures, in particular vessels, it was necessary to be slightly conservative in the parenchyma.

In the RCC patients, the oxygenation in normal renal tissue was significantly greater than in the tumour tissue (p<0.0039). This is a key finding of this thesis and demonstrated that not only does hypoxia exist in RCC, the degree is such that compared to normal tissue the
processes occurring at a sub-cellular level are likely to be vastly different. Certainly, the ranges of pO\(_2\) are heterogenous in tumour tissue and to a degree in normal tissue, but this is to be expected given findings in other tumours.

Examined more closely, the patients with clear cell RCC compared to those with non-clear cell RCC had lower pO\(_2\) within tumours, ranging from 0-19.3 mmHg (median 4.6 mmHg) and 14.24±7.56 mmHg (median 15.4 mmHg) respectively. Although this is an interesting trend, the low numbers in each group provide insufficient statistical power to be confident that the difference is real.

4.2.3.1 Oxygen tension in normal renal tissue

Under resting conditions, around 20% of the cardiac output in humans perfuses the kidneys, organs that constitute only about 0.5% of the human body mass. This rate of blood flow, approximately 400 mL/100 g of tissue per minute or 1 L/min, is much greater than that observed in other vascular beds ordinarily considered to be well perfused, such as heart, liver, and brain. From this prodigious blood flow, only a small quantity of urine is formed (about 1mL/min). Although the metabolic energy requirement of urine production is great—about 10% of basal O\(_2\) consumption—examination of the renal arteriovenous O\(_2\) difference reveals that blood flow far exceeds metabolic demands.

Despite the many studies and investigations linking oxygenation and hypoxia to renal blood flow, data describing the actual pO\(_2\) in human renal parenchyma are almost non-existent. Small surface micro-electrodes have been used in only a single study to measure the pO\(_2\) on the surface of a single transplant kidney where the pO\(_2\) was found to have a mean of 30mmHg. In humans, the median value for oxygen partial pressure is between 40 and 60 mmHg in normal subcutaneous tissue using POS.

Our study found the mean pO\(_2\) to be around 45mmHg in normal renal parenchyma but with a variable pO\(_2\) range (10.9-113.3 mmHg, median 38.6 mmHg). This heterogeneity is consistent
with animal studies measuring \( pO_2 \) in kidneys.\(^{694}\) The results are also consistent with the single finding in the transplant kidney and considering that I was dealing with native kidneys, the slightly higher result is not unexpected.

An alternative explanation for the findings in normal tissue is that the probe is erratic and unreliable- this seems unlikely given the vast number of studies that have been published demonstrating similar findings in other organs. Also, the heterogeneity in the kidney makes sense in normal tissue considering the anatomical arrangement of vessels. In simple terms, the probe entered smaller and larger blood vessels (well oxygenated arteries, less well oxygenated veins) as it passed between different types of renal tissue outer cortex and inner medulla (different configurations of tissue and different levels of oxygenation considering the functional anatomy of the nephrons). It is possible also to have hit collecting tubules which would have very low levels of oxygen. Thus the range of \( pO_2 \) measurements observed fits well into this pattern. Again, as has been found in other studies it is the global assessment rather than individual point measurements that are important. In fact, it would be quite unexpected if the oxygen tension measurements were all similar given the anatomical arrangement of the kidney and function of the probe to be able to determine oxygen status in minute areas.

Thus, I may conclude that the \( pO_2 \) in normal human renal parenchyma is heterogenous but similar in range to subcutaneous tissues, but at the lower end of this range being closer to 40mmHg- despite the high blood flow and oxygen availability to the renal tissue. This probably reflects the variability in regional blood flow within the kidney (e.g. cortex versus medulla)\(^{694}\) and gives us a bench mark with which to compare oxygen levels in tumour tissue.

The degree of heterogeneity in control tissues as experienced in our study is almost universal with that found in other tissues (Appendix E). Previous reports most commonly used muscle tissue as a control because this is accessible percutaneously; our study used native kidney.
One other study\textsuperscript{223} used an intra-abdominal organ (pancreas), and found similar pO\textsubscript{2} ranges from 24.3 to 92.7 mmHg and median 51.6 mmHg in seven patients.

\textbf{4.2.3.2 Oxygen tension in renal tumour tissue}

Oxygen studies in human renal tumour tissue have not been previously performed. In our patients, the mean pO\textsubscript{2} in RCC tissue was 11 mmHg, with a wide range. Considering the original understanding of the term “hypoxia” in this thesis, a “typical” tumour will have a median pO\textsubscript{2} oxygen partial pressure of approximately 10 mmHg. By definition, half the cells have oxygen levels less than 10 mmHg and half have oxygen levels greater than 10 mmHg. This breakpoint is considered significant, as cells with oxygen levels of less than 10 mmHg demonstrate higher levels of radiation resistance.\textsuperscript{32}

Overall, the result in RCC is extremely close to the strict definition of hypoxia. This is the first time tissue hypoxia has been demonstrated by POS in RCC. I believe I was able to sample data adequately from the tumours. I state this with confidence as it has been demonstrated that only as few as twenty-four measurements per tumour are needed to classify a tumour as hypoxic or not.\textsuperscript{183} The studies conducted far exceeded this minimal number of measurements.

Comparing clear cell and non clear RCC there was a trend towards lower oxygen levels in tumours and also a greater proportion of measurements lower than 2.5 mmHg but neither was significant in this sample.

\textbf{4.2.3.3 Oxygen tension in RCC compared to other tumours}

Other tumours studied more than once fall into six broad categories as outlined in the background chapter (Table 2-6). The tumour pO\textsubscript{2} broadly ranges from 10-21.5 mmHg. In kidney, I found a level of around 10 mmHg, which fits within the range presented by other human tumours. In the same studies using POS, normal tissue oxygen levels ranged 14-45 mmHg, and the levels of around 38 mmHg in the kidney fits again into this spectrum. As with
other tumours, there was intra and inter-tumour heterogeneity, reflecting other tumours. There was one case of transitional cell carcinoma (TCC) or urothelial carcinoma in our study that was hypoxic but the whole kidney also recorded normal levels that were somewhat low. As this was a more extensive tumour one may conclude perfusion to the whole kidney was disrupted but this would need to be outlined in further studies of TCC.

4.2.4 Surgery and impact on Future Direct Oxygen Measurements

With direct oxygen probe measurements it is important to emphasise that these have almost exclusively been conducted in the percutaneous situation that previous studies used techniques where electrodes were placed into deep tissues by passing them through the skin or orifice: (head and neck cancer, sarcomas, breast and brain), per vagina (cervical) or transrectally (prostate). This is in contrast to direct placement in the context of surgery as I have done. Nephrectomy although safe, may cause significant intra-abdominal haemorrhage. This is a factor and in two situations in this study although haemorrhage occurred and was easily controlled and not related to probe use, readings had to be abandoned in order to not compromise patient care. This will remain a paramount concern in any study of critical organs such as the kidney.

Surgical techniques are important, as they directly influence not only patient outcome but our ability to perform certain studies. In the past, only open surgery was available to patients needing radical nephrectomy. This involved an incision through the flank, abdomen or even chest in larger tumours. Although this type of surgery is still performed and remains suitable for larger tumours (>8cm), laparoscopic surgery has evolved in the past decade. This involves three or four small trochar incisions and a small extraction incision offering the new standard of care with small to medium-sized tumours. There are fewer transfusions, reduced morbidity and shorter hospital stay. However, being performed laparoscopically means that open exposure of a wound through the skin with the whole kidney on display does not occur. Thus it is impossible to conduct, for example, oxygen measurements with an electrode probe as these instruments are not designed to be passed through laparoscopic ports.
Nephron sparing surgery via partial nephrectomy involves removing the tumour with an adequate margin of normal tissue and repairing the kidney (i.e. leaving the “good” kidney behind and removing only the tumour). Such nephron preserving surgery is an absolute indication in those with a solitary kidney or those with impending renal disease to avoid dialysis where possible. Again, this surgery is evolving with it typically being done via a conventional open approach but more experience is being gathered for laparoscopic techniques. Again, this has implications for future studies where laparoscopic oxygen tension measuring instruments would need to be constructed as they currently do not exist.

### 4.2.5 Summary of Polarographic Oxygen Sensor Measurements and Renal Cell Carcinoma

Hypoxia, as suspected by surrogate markers over the years, does exist in RCC. Like other tumours RCC and normal kidney tissue are both heterogenous with respect to point oxygen levels; however, taken in a global context RCC tumour tissue is hypoxic and normal tissue normoxic. Measurements may be conducted safely with probes although the technology is heavily reliant on technical software and backup to ensure reliable measurements in any environment. Thus as already alluded to in the literature, although probing tumours is still considered the “gold standard” it is unlikely to be commonplace because of the challenging technical aspects and the invasive nature of the microelectrode particularly with deep-seated neoplasms such as the kidney.
4.3 Non-invasive Imaging Studies of Hypoxia in Humans with RCC using Combined Positron Emission Tomography and CT (PET-CT)

4.3.1 Imaging Studies of Renal Cell Carcinoma in Patients

In all, 44 patients underwent study with $^{18}$F-FDG whilst 34 underwent $^{18}$F- FMISO studies (Table 4-7 and Table 4-8, Figure 4-4 and Figure 4-5). One patient having both studies did not undergo surgery for medical reasons leaving 43 patients underwent study with $^{18}$F-FDG whilst 33 underwent $^{18}$F- FMISO studies. The discrepancy is due to timing of surgery whereby surgery had to take place before there was time to complete the $^{18}$F- FMISO study or even the $^{18}$F-FDG in a smaller number.

Overall, considering all RCC there was a statistically significant difference between tumour and normal renal tissue uptake (p=0.017) for $^{18}$F-FDG as calculated by maximal standardised uptake volume ($SUV_{\text{max}}$) but not so for $^{18}$F-FMISO (p = 0.4224).

When considering just clear cell RCC, the $^{18}$F-FDG there was again statistically significant difference between tumour and normal renal tissue uptake (p=0.0232) as calculated by maximal standardised uptake volume ($SUV_{\text{max}}$) but not so for $^{18}$F-FMISO (p = 0.9845).
Table 4-8 RCC Results of all the PET studies undertaken with $^{18}$F-FDG and $^{18}$F-FMISO

<table>
<thead>
<tr>
<th>$^{18}$F-FDG</th>
<th>SUV Max ±SD</th>
<th>SUV Max ±95% C.I (range)</th>
<th>$^{18}$F-FMISO</th>
<th>SUV Max ±SD</th>
<th>SUV Max ±95% C.I (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Tumour</td>
<td>Normal</td>
<td>Normal</td>
<td>Tumour</td>
<td>Normal</td>
</tr>
<tr>
<td>ALL SCANS</td>
<td>43</td>
<td>1.7 ± 0.54 2.42 ± 1.69</td>
<td>1.42 ± 0.68 1.44 ± 0.22 (0.6-2.8) (0.5-8.9) (0.33-3.23)</td>
<td>33</td>
<td>1.41 ± 0.6 1.41 ± 0.21 1.41 ± 0.21 (0.49-3.19) (0.49-3.19) (0.49-3.19)</td>
</tr>
<tr>
<td>RCC (all)</td>
<td>27</td>
<td>1.55 ± 0.55 2.06 ± 1.29</td>
<td>1.37 ± 0.63 1.37 ± 0.24 (0.6-7.5) (0.5-3.3) (0.63-3.20)</td>
<td>23</td>
<td>1.21 ± 0.38 1.21 ± 0.15 1.21 ± 0.15 (0.5-1.95) (0.5-1.95) (0.5-1.95)</td>
</tr>
<tr>
<td>RCC (clear)</td>
<td>22</td>
<td>1.52 ± 0.51 2.11 ± 1.34</td>
<td>1.44 ± 0.67 1.44 ± 0.28 (0.9-7.5) (0.6-2.8) (0.63-3.20)</td>
<td>18</td>
<td>1.24 ± 0.38 1.24 ± 0.17 1.24 ± 0.17 (0.5-1.95) (0.5-1.95) (0.5-1.95)</td>
</tr>
<tr>
<td>RCC (non-clear cell)</td>
<td>5</td>
<td>1.69 ± 0.78 1.85 ± 1.1 1.03 ± 0.18 (0.6-2.6) (0.5-3.3) (0.8-1.3)</td>
<td>5</td>
<td>1.11 ± 0.4 1.11 ± 0.35 1.11 ± 0.35 (0.5-1.57) (0.5-1.57) (0.5-1.57)</td>
<td></td>
</tr>
</tbody>
</table>
Table 4-9 Non-RCC Results of all the PET studies undertaken with $^{18}$F-FDG and $^{18}$F-FMISO (non-RCC)

<table>
<thead>
<tr>
<th>TCC</th>
<th>Normal</th>
<th>Tumour</th>
<th>Tumour: Normal</th>
<th>Normal</th>
<th>Tumour</th>
<th>Tumour: Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.21 ± 0.35</td>
<td>5.39 ± 2.72</td>
<td>2.35 ± 0.91</td>
<td>4</td>
<td>2.25 ± 0.82</td>
<td>2.65 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>(1.82-2.6)</td>
<td>(2.29-8.9)</td>
<td>(1.26-3.40)</td>
<td></td>
<td>(1.26-3.19)</td>
<td>(1.26-4.37)</td>
</tr>
<tr>
<td>Oncocytoma</td>
<td>4</td>
<td>1.87 ± 0.31</td>
<td>2.05 ± 0.46</td>
<td>1.09 ± 0.09</td>
<td>4</td>
<td>1.29 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>(1.68-2.13)</td>
<td>(1.61-2.46)</td>
<td>(1.0-1.1)</td>
<td></td>
<td>(1.28-1.4)</td>
<td>(1.07-1.22)</td>
</tr>
<tr>
<td>Other benign tumours</td>
<td>2</td>
<td>2.05 ± 0.19</td>
<td>2.23 ± 0.26</td>
<td>1.1 ± 0.23</td>
<td>1</td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td>(1.91-2.18)</td>
<td>(2.04-2.41)</td>
<td>(0.94-1.26)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
<td>1</td>
<td>2.17</td>
<td>2.20</td>
<td>1.00</td>
<td>1</td>
<td>2.67</td>
</tr>
</tbody>
</table>
Figure 4-4 Summary of the PET data in Renal Cell Carcinoma patients only for SUV\textsubscript{max} of \textsuperscript{18}F-Fluorodeoxyglucose (\textsuperscript{18}F-FDG) and \textsuperscript{18}F-Fluorodeoxyglucose (\textsuperscript{18}F-FDG) PET Patient Study Data in all Renal Cell Carcinomas.

Figure 4-5 Summary of the PET data in Renal Cell Carcinoma patients only for Tumour: Normal ratios of uptake.
Figure 4-6 $^{18}$F-FDG uptake by tumour type

$^{18}$F-Fluorodeoxyglucose PET Patient Study Data
$SUV_{max}$ and Tumour:Normal Ratio in Renal Tumours

<table>
<thead>
<tr>
<th>Group</th>
<th>$SUV_{max}$ mean±95% C.I</th>
<th>Tumour: Normal (Background) mean±95% C.I</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCC-all</td>
<td>2</td>
<td>2.4</td>
</tr>
<tr>
<td>RCC-clear</td>
<td>2</td>
<td>2.0</td>
</tr>
<tr>
<td>RCC-other</td>
<td>2</td>
<td>2.2</td>
</tr>
<tr>
<td>TCC</td>
<td>2</td>
<td>2.5</td>
</tr>
<tr>
<td>Oncocytoma</td>
<td>2</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Figure 4-7 $^{18}$F-FDG PET-CT study

$tumour$ (arrow), low is black (e.g. in a necrotic tumour) with non-contrast CT (left), PET “thermal” images alone (centre) and then combined PET-CT (right) with the uptake in the context of the anatomy.
4.3.2 $^{18}$F-Fluorodeoxyglucose ($^{18}$F-FDG)

Figure 4-6 highlights the results of the $^{18}$F-FDG studies in groups with at least 4 patients. There were some small but not significant differences. Some patients had excellent uptake (Figure 4-7 and Figure 4-8) but this was not universal. Comparing all RCC the difference between maximal uptake in tumour and normal approached significance ($p=0.06$). If this was narrowed to just those with clear cell RCC the difference was significant ($p=0.02$).

TCC appeared to be much more $^{18}$F-FDG avid than RCC. Oncocytomas were not distinguishable from RCC which is interesting because they behave more as benign tumours rather than malignant- although they still have cell turnover and have local growth which probably explains the similar uptake to RCC.
Figure 4-8 $^{18}$F-FDG PET-CT studies more RCC patients from the study

Axial and coronal $^{18}$F-FDG thermal PET study with a non-contrast CT (left), PET “thermal” (centre) and combined PET-CT (right) highlights the ability of PET not only demonstrate weak uptake in a primary renal tumour (arrow pointing to kidney) but to demonstrate more definitively a metastatic deposit (arrow pointing to spine). In this case the metastatic deposit was confirmed in L4 by biopsy and was clear cell RCC.

This $^{18}$F-FDG PET study with a non-contrast CT (left), PET “thermal” (centre) and combined PET-CT (right) highlights the ability of PET to assess locally advanced disease (arrow pointing to the increased uptake in the extensive tumour) with no evidence for uptake in other areas.
4.3.3 Analysis of $^{18}$F-FDG maximal uptake compared to RCC tumour parameters and immunohistochemistry

Against tumour parameters such as grade, stage, maximal length or tumour volume for all types of RCC (Table 4-10) there was a moderate correlation between $^{18}$F-FDG uptake with grade ($r^2=0.29; p=0.01$). Immunohistochemistry also yielded no significant relationships apart from a moderate correlation between $^{18}$F-FDG uptake and Ki-67 expression ($r^2=0.58; p<0.0001$). Focusing on tumour: normal ratio there was one weak correlation with cell density only ($r^2=0.24; p=0.02$)

Similarly there were no significant correlations recorded against $^{18}$F-FDG uptake and RCC clear cell histology (full data not shown). There was a moderate correlation towards increased uptake with grade only ($r^2=0.60; p=0.11$). Again immunohistochemistry also yielded no significant relationships apart from a trend toward Ki-67 expression ($r^2=0.64; p=0.23$). In particular Glut-1 expression did not correlate with $^{18}$F-FDG uptake ($r^2=-0.006; p=0.98$).

4.3.4 Analysis of $^{18}$F-FDG uptake and direct Polarographic Oxygen Sensor measurements (all RCC)

In the $^{18}$F-FDG group whom had POS measurements, the RCC median pO$_2$ was 9.7mmHg (range, 0-19.3mmHg) whilst in normal tissue it was 40.8mmHg (range 14-106.2mmHg).

$^{18}$F-FDG uptake in tumours versus oxygen parameters are summarised in Table 4-10. In terms of correlation between $^{18}$F-FDG maximal uptake in tumours versus median oxygen level (all RCC, n=10), there were no trends identified.

There were no correlations noted in the tumour group at POS measurements of <10mmHg, <5mmHg and <2.5mmHg. Also, no correlations were found for the tumour: normal ratios.
Table 4-10 Correlation of $^{18}$F-FDG and $^{18}$F-FMISO uptake compared to other parameters for all RCC

<table>
<thead>
<tr>
<th>Parameter</th>
<th>FDG SUV$_{\text{max}}$</th>
<th>FDG T:N</th>
<th>FMISO SUV$_{\text{max}}$</th>
<th>FMISO T:N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r^2$</td>
<td>p value</td>
<td>$r^2$</td>
<td>p value</td>
</tr>
<tr>
<td>Median Tumour</td>
<td>0.12</td>
<td>0.37</td>
<td>0.06</td>
<td>0.52</td>
</tr>
<tr>
<td>$H_P_{10}$ (%&lt;10mmHg Tumour)</td>
<td>0.11</td>
<td>0.39</td>
<td>0.13</td>
<td>0.35</td>
</tr>
<tr>
<td>$H_P_{5}$ (%&lt;5mmHg Tumour)</td>
<td>0.06</td>
<td>0.51</td>
<td>0.09</td>
<td>0.44</td>
</tr>
<tr>
<td>$H_P_{2.5}$ (%&lt;2.5mmHg)</td>
<td>0.00</td>
<td>0.99</td>
<td>0.01</td>
<td>0.85</td>
</tr>
<tr>
<td>Tumour Type</td>
<td>0.02</td>
<td>0.54</td>
<td>0.00</td>
<td>0.79</td>
</tr>
<tr>
<td>Tumour Volume</td>
<td>0.14</td>
<td>0.09</td>
<td>0.06</td>
<td>0.28</td>
</tr>
<tr>
<td>Tumour Grade</td>
<td>0.29</td>
<td>0.01</td>
<td>0.13</td>
<td>0.11</td>
</tr>
<tr>
<td>Pathological Stage</td>
<td>0.09</td>
<td>0.17</td>
<td>0.14</td>
<td>0.09</td>
</tr>
<tr>
<td>MVD</td>
<td>0.00</td>
<td>0.97</td>
<td>0.10</td>
<td>0.17</td>
</tr>
<tr>
<td>MVD Hotspot</td>
<td>0.02</td>
<td>0.58</td>
<td>0.00</td>
<td>0.81</td>
</tr>
<tr>
<td>Cell density</td>
<td>0.00</td>
<td>0.86</td>
<td>0.24</td>
<td>0.02</td>
</tr>
<tr>
<td>HIF-1-α</td>
<td>0.16</td>
<td>0.08</td>
<td>0.08</td>
<td>0.21</td>
</tr>
<tr>
<td>VEGF</td>
<td>0.02</td>
<td>0.53</td>
<td>0.04</td>
<td>0.39</td>
</tr>
<tr>
<td>VEGFR-1</td>
<td>0.01</td>
<td>0.68</td>
<td>0.03</td>
<td>0.44</td>
</tr>
<tr>
<td>Ki-67</td>
<td>0.58</td>
<td>$&lt;0.0001$</td>
<td>0.09</td>
<td>0.18</td>
</tr>
<tr>
<td>Glut-1</td>
<td>0.18</td>
<td>0.06</td>
<td>0.00</td>
<td>0.92</td>
</tr>
<tr>
<td>CAIX</td>
<td>0.00</td>
<td>0.94</td>
<td>0.01</td>
<td>0.67</td>
</tr>
<tr>
<td>EGFR</td>
<td>0.04</td>
<td>0.37</td>
<td>0.00</td>
<td>0.78</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>0.07</td>
<td>0.22</td>
<td>0.00</td>
<td>0.81</td>
</tr>
<tr>
<td>FDG SUV$_{\text{max}}$ Tumour</td>
<td>0.43</td>
<td>0.00</td>
<td>0.00</td>
<td>0.05</td>
</tr>
<tr>
<td>FDG SUV$_{\text{max}}$ Normal tissue</td>
<td>0.40</td>
<td>0.0001</td>
<td>0.03</td>
<td>0.42</td>
</tr>
<tr>
<td>FDG T:N</td>
<td>0.43</td>
<td>0.0001</td>
<td>0.03</td>
<td>0.42</td>
</tr>
<tr>
<td>FMISO SUV$_{\text{max}}$ Tumour</td>
<td>0.33</td>
<td>0.01</td>
<td>0.21</td>
<td>0.03</td>
</tr>
<tr>
<td>FMISO SUV$_{\text{max}}$ Normal tissue</td>
<td>0.38</td>
<td>0.0001</td>
<td>0.08</td>
<td>0.21</td>
</tr>
<tr>
<td>FMISO T:N</td>
<td>0.05</td>
<td>0.30</td>
<td>0.22</td>
<td>0.03</td>
</tr>
</tbody>
</table>
4.3.5 Discussion of $^{18}$F-Fluorodeoxyglucose ($^{18}$F-FDG) in RCC

As expected based on previous studies, there was a modest but discernable difference between $^{18}$F-FDG SUV$_{\text{max}}$ in normal and tumour tissue for all RCC. When focusing on clear cell RCC, the difference was significant. Such findings support previous studies at our institution and reinforce the difficulties with PET studies in the renal tract and why $^{18}$F-FDG will probably remain a staging rather than diagnosing tool in RCC. Figure 4-8 highlights this point in particular where uptake in the metastatic deposit to L4 far outweighs uptake in the primary tumour in the right kidney. In other words, CT scan alone with and without contrast provides anatomical information of the diagnosis of the tumour itself with PET adding little data. However, when attempting to stage the patient looking for visceral or bony metastatic deposits, PET seems to be useful- this is because there is no background uptake by normal structures in these areas as opposed to the urinary tract where there is excretion of radioisotope giving the appearance of background uptake making it difficult to distinguish between normal and abnormal tissues.

As outlined, several factors make interpretation of PET radiotracer activity in the kidney difficult. Firstly, radiotracers are excreted through the glomeruli but are not reabsorbed by the tubules and so accumulate in the collecting system. Radiotracer uptake levels of up to an SUV$_{\text{max}}$ of 22 have been reported in the kidney with $^{18}$F-FDG. Secondly, retention of urine in the collecting system of the kidneys that is aided, but not eliminated by adequate hydration. Finally, previous studies of $^{18}$F-FDG at our institution that radiotracer uptake is only moderate in most kidneys, but may vary.

I was not able to demonstrate a relationship between $^{18}$F-FDG SUV$_{\text{max}}$ and tumour oxygenation by direct POS measurements. This is not surprising considering the variability of uptake in prior studies of other tumours with known hypoxia. Furthermore the basic biology is not necessarily directly linked- $^{18}$F-FDG uptake is linked to glucose metabolism of
cells whilst hypoxic cells by definition use alternate methods of energy creation such as anaerobic metabolism or altered levels of glucose utilisation. Hypoxia is a general factor affecting glucose metabolism yet it is also true that some hypoxic tumours can have modest glucose metabolism, whereas some highly metabolic tumours are not hypoxic, showing discordance in tracer uptake that can be tumour type specific. There is evidence from experimental data using human cancer cell lines that hypoxic conditions lead to increased FDG uptake. In RCC, this relationship has been more difficult to establish. RCC has been demonstrated to have differential Glut-1 expression between patients. Further in the same study there was no correlation with Glut-1 immunoreactivity and FDG-PET positivity. This was a similar finding to our results.

Certainly in RCC our data support the contention that $^{18}$F-FDG PET cannot reliably differentiate hypoxic from normoxic tumours. As proposed, other factors affecting $^{18}$F-FDG in an hypoxic tumour environment, such as acute versus chronic hypoxia may render $^{18}$F-FDG unsuitable to studying hypoxia with PET.

### 4.3.6 $^{18}$F-Fluoromisonidazole ($^{18}$F-FMISO)

Figure 4-9 highlights the results of the $^{18}$F-FMISO studies in groups. $^{18}$F-FMISO uptake in RCC renal tumours was almost identical between the tumour and normal tissues (Figure 4-10). This held true for RCC clear cell and all other types. In TCC there was a trend toward increased uptake in tumour tissue (not significant) and because an increase in uptake occurred in both normal and tumour tissue ensuring the tumour: normal ratio remained low. In the oncocytoma group the results were similar to the RCC group.
Figure 4-9 $^{18}$F-FMISO uptake by tumour type

$^{18}$F-Fluoromisonidazole PET Patient Study Data
$SUV_{\text{max}}$ and Tumour:Normal Ratio in Renal Tumours

<table>
<thead>
<tr>
<th>Group</th>
<th>$^{18}$F-MISO SUV$_{\text{max}}$ mean±95% C.I. or Ratio mean±95% C.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCC-all</td>
<td></td>
</tr>
<tr>
<td>Rcc-clear</td>
<td></td>
</tr>
<tr>
<td>RCC-other</td>
<td></td>
</tr>
<tr>
<td>TCC</td>
<td></td>
</tr>
<tr>
<td>Oncocytoma</td>
<td></td>
</tr>
</tbody>
</table>

Legend:
- Normal Tissue
- Tumour Tissue
- Tumour:Normal (Background)
Figure 4-10 $^{18}$F-FMISO PET-CT study

An $^{18}$F-FMISO PET-CT study with a non-contrast CT (left), PET “thermal” (centre) and combined PET-CT (right) highlighting the modest uptake of this radioisotope in a primary renal tumour (arrow pointing to the RCC in inferior right kidney)
4.3.7 Analysis of $^{18}$F-FMISO maximal uptake compared to tumour parameters and immunohistochemistry

Table 4-10 demonstrates that against tumour parameters such as grade, stage, maximal length or tumour volume there were no significant correlations recorded for $^{18}$F-FMISO uptake and all types of RCC. Immunohistochemistry also yielded no significant relationships. Focusing on tumour: normal ratio there were no correlations with tumour parameters.

Similarly there were few significant correlations recorded against $^{18}$F-FMISO uptake and RCC clear cell type against tumour parameters such as grade, stage, maximal length or tumour volume (full data not shown). However, immunohistochemistry yielded increased uptake with increasing MVD (0.60; $p=0.012$). This is not surprising given the great “hypoxia paradox” as outlined in section 2.6.5: in summary, that paradoxically, even in the presence of a blood supply a tumour can remain hypoxic. So although more vessels are generally present, they are of a poorer quality and this is not assessed by MVD.

4.3.8 Analysis of $^{18}$F-FMISO uptake and direct Polarographic Oxygen Sensor measurements (All RCC)

In the $^{18}$F-FMISO group whom had POS measurements, the RCC median $pO_2$ was 9.7mmHg (range, 0-11.5mmHg) whilst in normal tissue it was 47.10mmHg (range 37.6-106.2mmHg).

$^{18}$F-FMISO uptake in tumours versus oxygen parameters are summarised in Table 4-10. In terms of correlation between $^{18}$F-FMISO maximal uptake in tumours versus median oxygen level (all RCC n=6), there was weak inverse correlation ($r^2=-0.24$; $p>0.05$). Again trends were noted in the tumour group whereby in the POS measurements of <10mmHg, <5mmHg and <2.5mmHg there were correlations but not significant. In the tumour: normal ratio there were some significant correlations at <10mmHg, <5mmHg (Figure 4-11).
Figure 4-11 Correlations between $^{18}$F-FMISO uptake and Hypoxic Fraction

**Correlation between $^{18}$F-FMISO SUV Tumour:Normal ratio and HP$_5$ in clear cell RCC patients having oxygen probe measurements (n=7)**

**Correlation between $^{18}$F-FMISO SUV Tumour:Normal ratio and HP$_{10}$ in clear cell RCC patients oxygen probe measurements (n=7)**
4.3.9 Discussion of $^{18}$F-Fluoromisonidazole ($^{18}$F-FMISO) in RCC

$^{18}$F-FMISO is the most extensively studied hypoxia-sensitive radiopharmaceutical. In our institution, it has been used to demonstrate hypoxic regions of brain in stroke patients and tumours. $^{18}$F-FMISO PET scanning has demonstrated tumour hypoxia in solid tumours rising from 30% of soft tissue tumours to nearly 100% in head and neck tumours (Table 2-14). Importantly, serial PET scans have demonstrated a correlation between patients who responded to treatment and those whose tumours showed a reduction in hypoxic fractions by the end of treatment. 

Variation in radiotracer uptake between individuals is to be expected in tissues. In particular, the sensitivity of nitroimidazole (e.g. $^{18}$F-FMISO) imaging for the detection of hypoxic tissue will be determined by four factors: 1) amount of radiopharmaceutical delivered to the site of hypoxia; 2) the fraction that gets past the initial reversible reaction; 3) the rapidity of clearance of radioactivity from normoxic cells; 4) sufficient duration of retention in hypoxic cells to record high-quality images. The specificity of nitromidazole imaging will depend on the contrast between lesion and background the oxygen concentration at which trapping occurs.

When considering radiotracer uptake in tissue, one must be mindful of variable retention and in particular, the variation in kidney tissue. Evidence from previous studies suggests that $^{18}$F-FMISO uptake in tumour is heterogenous and only SUV$_{\text{max}}$ and a ratio with normal tissue are considered for hypoxia measurements. Previous studies have used blood as a surrogate for normal tissue because oxygen levels may be representative and found maximal normal uptake reported in the range of 1.2-19.38, median 3.95 for $^{18}$F-FMISO. This highlights the variability of uptake in an individual and why a ratio of tumour to normal is potentially, although not always, more accurate for assessing uptake in tumour tissue rather than individual SUV$_{\text{max}}$ levels alone. It also supports our study where normal renal tissue had a variable range (0.5-2.6).
The definition of what constitutes positive $^{18}$F-FMISO uptake indicating hypoxia on PET imaging is not standardised in the literature. Several studies (including this one) have compared the uptake of $^{18}$F-FMISO in the tumour to normal parenchyma, giving a ratio for individual patients in the studies using themselves as controls. Other studies have based analysis on animal studies where ratios for normal healthy tissue uptake of $^{18}$F-FMISO compared to plasma were below 1.3 in 90% of cases. Thus a conservative selected “threshold” tumour: plasma ratio of 1.4 was proposed for human studies. This figure has been modified to 1.2 in a recent study of soft tissue sarcomas. A “threshold” tumour: plasma ratio has the advantage of offering consistency for comparison across studies but gives little room for individual variation in uptake of $^{18}$F-FMISO in different tissues. As outlined above uptake in tissue groups (e.g. the kidney) may be affected by many variables and using plasma as a comparison tissue may not reflect such diversity in tissue uptake hence I compared uptake in the tumour to the normal contralateral kidney to allow for individual variation.

The results of our study indicate that $^{18}$F-FMISO uptake in RCC is mild in contrast to other cancers studied e.g. sarcomas, gliomas, head and neck cancers (Table 2-6). There is limited reported experience in the literature on hypoxia studies in RCC. One possible reason as to the results of the current study may be that RCC is not as hypoxic a tumour as other tumours, despite sharing the clinical behaviour (e.g. resistance to therapy) such as head and neck cancer, which have significant hypoxia.

This is supported by our invasive probe measurements indicating that RCC do contain areas of hypoxia, although there was considerable variation in the $pO_2$ levels within the tumours. I am confident that the renal tumours studied were viable and not necrotic from the areas we sampled, which would influence $^{18}$F-FMISO uptake. This was supported by the MVD measurement that was markedly increased in the RCC compared to normal parenchyma. Necrotic tissue would have no MVD. Thus the results of this study are consistent with previous studies indicating that $^{18}$F-FMISO uptake requires hypoxia <10mmHg to demonstrate retention. The median $pO_2$ was just
at the threshold for $^{18}$F-FMISO uptake at a median of 9.7mmHg. This may be at the cusp of where once could expect reasonable uptake. The threshold of imaging increased $^{18}$F-FMISO uptake may need to be breached by a larger population or amount of tumour tissue at low oxygen tension. The ability of $^{18}$F-FMISO to predict response to therapy$^{560}$ has clearly been demonstrated and $^{18}$F-FMISO uptake has been shown to be influenced by therapy.$^{559}$

Ultimately, the biological significance of $^{18}$F-FMISO uptake in RCC is still to be determined. I could not find a correlation with median tumour $pO_2$ or any other hypoxic parameters. Clearly factors have influenced the ability of RCC to uptake the radiotracer. It has been demonstrated in vitro in four cell lines that very low oxygen levels are required for substantial uptake of FMISO by cells.$^{703}$ The oxygen level inhibiting binding FMISO binding by 50%, relative to binding under anoxic conditions, varied from 720-2300 ppm (0.55-1.75mmHg).$^{553,554}$ These levels were achieved in RCC but globally the $pO_2$ was higher. Also against this contention is that $^{18}$F-FMISO uptake is considered to represent a global value for macroscopic tumour parts$^{203}$; in other words, a heterogenous tumour (like RCC) should overall still show uptake compared to background tissue across the whole tumour if it is occurring. This is what has driven investigators to believe it is adequate to evaluate the state of oxygenation in tumours.

Another reason put forward for the limitation of $^{18}$F-FMISO is that it has a relatively long plasma clearance half-life, so that the signal-to-noise ratio (the ability to distinguish the $^{18}$F-FMISO in tumour from background) obtained in PET images is relatively small. Therefore, considerable effort has been made to develop nitroimidazoles with either increased specific tumour uptake or faster clearance (e.g. $^{18}$F-FAZA).$^{704}$ Only time will tell if future hypoxic tracers are more suited to RCC. A combination of factors appears at play and in reality it is
Figure 4-12 Correlations between $^{18}$F-FDG and $^{18}$F-FMISO uptake

Correlation between $^{18}$F-FDG and $^{18}$F-FMISO SUV Tumour:Normal ratio in clear cell RCC patients having both PET studies (n=22)

Correlation between $^{18}$F-FDG SUV$_{max}$ and $^{18}$F-FDG SUV$_{max}$ in clear cell RCC only patients having both PET studies (n=22)
unlikely $^{18}$F-FMISO will play a large role in non-invasively determining RCC hypoxia or
diagnosis in the future.

4.3.10 Discussion of $^{18}$F-Fluoromisonidazole ($^{18}$F-FMISO) and $^{18}$F-Fluorodeoxyglucose
($^{18}$F-FDG) in Renal Cell Carcinoma

In our studies there were some weak but significant correlations made between the amount of
$^{18}$F-FDG uptake and $^{18}$F-FMISO in the same tumours in the same patients (Table 4-10). This
is also depicted in Figure 4-12. As summarised (section 2.11.10.4) the majority of studies
have found no correlation between $^{18}$F-FMISO and $^{18}$F-FDG uptake so this result is not
surprising. Thus our findings support the concept that no strong relationship exists between
uptake of the two radioisotopes in RCC. This likely indicates as in other tumours that
different sub-cellular mechanisms are contributing to their uptake and that if $^{18}$F-FMISO is
accumulating in hypoxic areas, then glucose metabolism appears only weakly related as
evidenced by the degree of $^{18}$F-FDG uptake.
**4.3.11 $^{18}$F-Fluorothymidine**

The approved protocol aimed to recruit 50 patients for the study. The first patient was recruited as a pilot (and is reported on in this thesis) and the rest of the study then passed to another principal investigator for completion at this institution.

In the first case enrolled in the study, a 59-year-old gentleman presented with haematuria, a longstanding diverticulum and calculi. The postero-medial wall of the diverticulum was thickened and suspicious for tumour but computed tomography was not conclusive. An $^{18}$F-FLT scan (PET-CT) was performed demonstrating extremely high uptake in the posterior wall of the cyst (Figure 4-13 $^{18}$F-FLT PET-CT study in the patient from the study with TCC) suspicious for neoplasm. The $SUV_{max}$ for the lesion was 21.48 (normal $\leq 1.0$). Nephrectomy was performed revealing a diverticulum with tumour in the posterior wall (Figure 4-14): high-grade papillary transitional cell carcinoma (Figure 4-15). $^{18}$F-FLT may in the future be a useful tracer delineating renal masses and this is being specifically investigated in our centre as part of a new study.

**4.3.12 Discussion of $^{18}$F-Fluorothymidine**

$^{18}$F-FLT PET studies may in the future be a useful tool in delineating renal masses and this is being specifically investigated in our centre as part of a new study. This first case was a transitional cell carcinoma and not an RCC so further data will clearly need collecting before any conclusions may be drawn on the ability of this radioisotope as a diagnostic and staging tool.
Figure 4-13 $^{18}$F-FLT PET-CT study in the patient from the study with TCC

An $^{18}$F-FLT PET-CT “thermal” study with the centre axial panel best depicting the tumour uptake with the arrow demonstrating the thickened postero-medial wall on CT (left), its increased uptake with PET (centre) and on combined PET-CT (right).
Figure 4-14 Pathology specimen of the kidney with the cyst opened immediately after nephrectomy. The postero-medial wall with the bumpy/granular area (arrow) is the carcinoma which showed considerable uptake on the PET study.

Figure 4-15 Histopathology with H&E staining at low power (left) and high power (right) demonstrating high grade transitional cell carcinoma.
4.3.13 Overall summary of PET in RCC

$^{18}$F-FDG and $^{18}$F-FMISO are of limited usefulness for diagnosis of RCC. This is predominantly because of the excretion issues and difficulty with discerning true tissue uptake from background emission. $^{18}$F-FMISO does not appear to be a reliable radiopharmaceutical to detect hypoxia in patients with RCC. $^{18}$F-FLT needs further analysis and this is being undertaken at our institution. Other radiotracers such as Divgi et al\textsuperscript{599} using $^{124}$I-cG250 appeared to identify clear cell RCC accurately in 26 patients which is promising but needs further data in order to validate its usefulness.
4.4 Indirect hypoxia marker: Serum Osteopontin Immunoassay in RCC

Several indirect or surrogate markers of hypoxia have been investigated as outlined and in this thesis we began collecting blood to measure serum and plasma osteopontin.

4.4.1 Results: Osteopontin in patients with Renal Cell Carcinoma

A sub-group of 32 patients from the initial study group had 33 samples of serum stored for analysis of serum osteopontin. The serum of six healthy controls was available for analysis. This included 22 patients with clear cell RCC, one each with chromophobe, collecting duct and papillary RCC (one patient bilateral renal tumours with a sample taken prior to each nephrectomy), four with oncocytoma, one with chronic pyelonephritis, two with transitional cell carcinoma and one with a metanephric adenoma. As this study is concerned predominantly with clear cell RCC, the results of other study patients are not presented.

Figure 4-16 explores the relationship of clear cell RCC to grade and stage. Levels of osteopontin were also increased in the 22 patients with RCC being 34.05 ±17.40 ng/mL (mean± 95% C.I.; range 5-188) compared to controls at 17.00 ±11.4 ng/mL (range 8-40) but this was not statistically significant. Serum osteopontin was greater at 46.93 ±26.07 ng/ml (mean± 95% C.I.; range 5-188) in higher Fuhrman grade (3-4) clear cell RCC than in lower grade (1-2) tumours at 14.63 ± 9.92 ng/mL and this was statistically significant (p<0.042). Regarding stage, there appear to be a stepwise correlation between Stages. In particular, stage 1 tumours 18.58 ± 10.95 (mean± 95% C.I.) had a lower level than stage 3 at 66.29 ± 44.08 ng/mL and this is significant (p= 0.0104). There was no significant difference between stage 1a and 1b (not shown).
Figure 4-16 Serum Osteopontin in clear cell Renal Cell Carcinoma compared to controls and tumour parameters

Serum Osteopontin levels in clear cell Renal Cell Carcinoma

- RCC Clear Cell
- Normal Controls
- Fuhrman Grade 1-2
- Fuhrman Grade 3-4
- Osteopontin Stage 1
- Osteopontin Stage 2
- Osteopontin Stage 3

n=22
n=6
n=8
n=14
n=12
n=2
n=7

Serum Osteopontin ng/ml (mean ± 95% C.I.)
4.4.1.1 Analysis of Serum Osteopontin compared to tumour parameters and immunohistochemistry

There were some significant moderate correlations recorded between osteopontin, $^{18}$F-FMISO uptake and tumour parameters such as grade ($r^2=0.51; p=0.014$) and stage ($r^2=0.53; p=0.011$) but not for tumour volume ($r^2=0.26, p=0.26$). This is an interesting finding as this supports the theory that osteopontin may be linked to tumour aggressiveness but not size. There may also be a critical point in the growth and development of a tumour whereby the hypoxia is increasing and osteopontin increases perhaps even prior to the tumour dramatically increasing in size. Presumably necrotic areas do not produce osteopontin which is another reason why size and osteopontin may not correlate. Clearly larger numbers of tumours would be needed to make more definitive comments about such relationships.

Immunohistochemistry yielded only two significant correlations with osteopontin: weak for HIF-1α ($r^2=0.49; p=0.005$) and moderate for Ki-67 ($r^2=0.57; p=0.007$). In particular CAIX expression ($r^2=-0.14; p=0.54$) had no significant correlation.

4.4.1.2 Analysis of Serum Osteopontin and direct Polarographic Oxygen Sensor measurements

In terms of correlation between serum osteopontin in tumours versus median oxygen level, there was a moderate inverse correlation in the 6 patients with RCC ($r^2 = 0.78, p= 0.068$), which would be worthy of study in a larger series if this was feasible.

4.4.2 Comparison of Serum to Plasma osteopontin

The serum and plasma osteopontin levels (n=10) were similar (mean± 95% C.I.) being 40.30 ±35.88 ng/mL and 49.10 ±42.74 ng/mL respectively and were well correlated ($r^2=0.988; p<0.001$). Although not completely surprising it is reassuring ad this does have implications for research where only plasma or serum may be stored. Both serum and plasma levels have been measured in tumours in the past and the assay is available to do both. Caution with results as assays may differ.
4.4.3 Discussion Serum Osteopontin in RCC

Our study suggests that serum osteopontin may be a useful tumour marker in RCC. Serum osteopontin levels appear raised in RCC patients prior to nephrectomy. This study demonstrates that there appears to be a relationship between osteopontin and stage of tumour but not bulk. There was also a trend to increasing levels with increasing grade and stage. All of these findings need to be supported in a larger series, preferably with case-controls.

Plasma osteopontin has been studied in one retrospective series where high serum osteopontin levels were associated with distant metastases and poor survival in RCC patients. They concluded that osteopontin may be a tumour marker to monitor new treatment strategies in patients with advanced RCC but needs evaluation in prospective studies. No studies of serum osteopontin in RCC patients have been published. Our findings support these and are certainly the first evidence that osteopontin has a role in the non-metastatic population.

The numbers of patients having both osteopontin levels and POS measurements was too small in this study to make any conclusions in support of recent evidence that serum osteopontin and tumour hypoxia may be linked. However, it is possible that osteopontin may be involved in the progression of RCC through angiogenesis promotion. Osteopontin may have other associations with tumourigenesis such as in bone where it has a role in adhesion, migration and cell survival and these were not part of this thesis. In immunohistochemical studies, RCC has demonstrated expression of osteopontin in 72% of cases. Further, osteopontin has also been found to reflect cancer stage in RCC, with high expression associated with a higher stage.

Like all blood markers, there has been recent caution with results of serum osteopontin assays because they may differ between laboratories. Thus I used serum as standard in all methods except for the last experiment where I compared plasma to serum levels.
However, there are situations where only serum or plasma may be used due to collection technicalities and processes. That is why it was important to also validate the results of plasma versus serum osteopontin levels. This may have implications in the future where biobanking of patient materials, such as serum and plasma, has become commonplace in large oncologic centres. Knowing the levels are equivalent gives confidence to test whatever material has been stored.

Serum osteopontin may become a tumour marker for RCC in the future. With time, it may also have a role in prognosis and follow-up of RCC patients, which our laboratory is currently investigating in the non-metastatic setting. The reason for proposing these two scenarios evolves from recent studies in metastatic RCC where high plasma osteopontin levels have been associated with distant metastases and poor survival in RCC patients and in head and neck tumours where osteopontin has correlated well with tissue hypoxia and worse prognosis. Also, immunohistochemical studies of clear cell RCC have demonstrated overexpression of osteopontin appears to be involved in the progression of RCC. Future studies should examine not only the role of serum osteopontin in prognosis, but also any relationship between serum levels and tissue expression. Finally, serum osteopontin may have a role in the identification of hypoxic in RCC, which has already been investigated in head and neck cancers.
4.5 Histopathology and Immunohistochemistry: Human Studies

4.5.1 Histopathology of Renal Tissue
The subtypes of RCC and other tumours encountered are summarised in Table 4-11. One patient had a small incidental 8mm papillary RCC encountered after the specimen was processed but this was not included in the immunohistochemical analysis because the lesion was too small and taking a sample would have precluded histopathological diagnosis. In any case it was the larger adjacent lesion, which was subsequently found to be an oncocytoma which prompted the surgery and this was processed for immunohistochemistry.

4.6 Immunohistochemistry in Human Studies: General Results
The staining found was of moderate (++) to high (+++) intensity on all occasions so the analysis was kept to the percentage of cells staining rather than including intensity that appeared not to differentiate tumours.

4.6.1 Cell Density
Cells per high power field were increased in the RCC group (356.05± 50.16; mean±95%C.I.) compared with the normal renal tissue (328.69±31.17). The difference was marginally increased between tumour and normal if only clear cell RCC are assessed (361.64±50.16 versus 326.56±31.17).
Table 4-11 Summary of cell density and immunohistochemical markers in all malignant tumour patients (benign and oncocytoma not included; n=45)

<table>
<thead>
<tr>
<th>PATIENTS</th>
<th>Cell Density</th>
<th>Ki-67</th>
<th>MVD</th>
<th>MVD Hotspot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Tumour</td>
<td>Normal</td>
<td>Tumour</td>
</tr>
<tr>
<td>RCC (all) (n=38)</td>
<td>Mean</td>
<td>328.69</td>
<td>356.05</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>98.04</td>
<td>157.77</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>31.17</td>
<td>50.16</td>
<td>0.22</td>
</tr>
<tr>
<td>RCC clear (n=31)</td>
<td>Mean</td>
<td>326.56</td>
<td>361.64</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>93.55</td>
<td>155.33</td>
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<tr>
<td></td>
<td>95% CI</td>
<td>32.93</td>
<td>54.68</td>
<td>0.26</td>
</tr>
<tr>
<td>RCC non-clear (n=7)</td>
<td>Mean</td>
<td>338.13</td>
<td>331.27</td>
<td>0.19</td>
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<tr>
<td></td>
<td>SD</td>
<td>124.03</td>
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<td></td>
<td>95% CI</td>
<td>91.88</td>
<td>132.48</td>
<td>0.15</td>
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<tr>
<td>Non-RCC Malignancy n=2</td>
<td>Mean</td>
<td>331.10</td>
<td>838.35</td>
<td>0.14</td>
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<td></td>
<td>SD</td>
<td>81.46</td>
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<td></td>
<td>95% CI</td>
<td>112.90</td>
<td>914.05</td>
<td>0.28</td>
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<tr>
<td>TCC n=5</td>
<td>Mean</td>
<td>291.94</td>
<td>506.90</td>
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</tr>
<tr>
<td></td>
<td>SD</td>
<td>70.97</td>
<td>48.26</td>
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<tr>
<td></td>
<td>95% CI</td>
<td>62.21</td>
<td>42.31</td>
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<table>
<thead>
<tr>
<th>PATIENTS</th>
<th>Glut-1</th>
<th>CAIX</th>
<th>EGFR</th>
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</thead>
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<tr>
<td>RCC (all) (n=38)</td>
<td>Mean</td>
<td>Normal</td>
<td>Tumour</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>2.52</td>
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<tr>
<td></td>
<td>95% CI</td>
<td>3.64</td>
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<td>RCC clear (n=31)</td>
<td>Mean</td>
<td>1.16</td>
<td>2.64</td>
</tr>
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<td></td>
<td>SD</td>
<td>2.27</td>
<td>5.92</td>
</tr>
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<td>95% CI</td>
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<td>Mean</td>
<td>1.18</td>
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<td></td>
<td>SD</td>
<td>3.61</td>
<td>6.30</td>
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<td></td>
<td>95% CI</td>
<td>4.88</td>
<td>5.52</td>
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<tr>
<td>Non-RCC Malignancy n=2</td>
<td>Mean</td>
<td>3.61</td>
<td>4.09</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>3.51</td>
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</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>3.82</td>
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<td></td>
<td>SD</td>
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</tr>
<tr>
<td></td>
<td>95% CI</td>
<td>1.75</td>
<td>8.78</td>
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Figure 4-17 Study patient with clear cell RCC and all immunohistochemical studies.

The H & E panel represents low (10x) and high power (400x) for control and tumour whilst the remainder have controls versus normal or tumour tissue as indicated at the bottom of the panels. Only Glut-1 failed to be expressed in this tumour. MVD hotspot refers to focusing on areas with vessels rather than standard MVD which is a random.
Figure 4-18 Immunohistochemical studies in clear cell RCC group (mean ratio of expression ±95% C.I.)

Immunohistochemistry in Renal Cell Carcinoma
Clear Cell Group (n=31)

- KI-67
- MVD
- MVD Hotspot
- GLUT-1
- HIF1α
- VEGF
- VEGFR-1
- CAIX
- EGFR

- Normal Tissue
- Tumour Tissue
4.6.2 RCC Clear Cell Group

There was significantly increased expression of the endogenous markers of hypoxia being CAIX, HIF-1α, and VEGF (Figure 4-18). It is important to distinguish hypoxia as read by the gold standard POS measurements and surrogate endogenous markers such as those outlined. Endogenous markers in themselves cannot define hypoxia but they are either associated in the known pathways of hypoxia at a sub-cellular level or they have been associated with POS hypoxia in other tumours. As researchers only ever get a static view of such markers, it is difficult in some circumstances to know if they are the cause or the effect or part of the process. In any case, the endogenous markers play a role in helping us to understand hypoxia at a cellular rather than global level, as the POS with its probing of multiple areas does.

VEGFR-1 expression comparing tumour to normal was greater but just outside significance. There was significantly increased expression of EGFR, Ki-67 and also MVD and MVD hotspot measures between expression between tumour and normal tissue. With cell density, there was a trend (not significant) to a greater number of cells in the clear cell RCC per high power field compared to normal. Similarly there was a trend to greater Glut-1 expression in tumours but this was not significant. An example of the immunohistochemical studies for a single patient with clear cell RCC is given in Figure 4-17.

When combining the other RCC types together, comparisons may be made even though the groups are of a different size with the clear group much larger (n=31) than the others combined (n=7). EGFR and Glut-1 expression were similar in both groups. MVD was similar but MVD hotspot was increased in the RCC clear group over others approaching significance (15.4±2.50 and 11.81±2.29 respectively; mean±95%C.I). In brief, the hotspot measure allows the observer to focus on areas which appear to be the most vascularised before counting vessel density as opposed to standard MVD which is a random process (full explanation in methods section 3.8.4). Ki-67 and cell density had a trend (not significant) toward greater expression in the RCC group. The same was true for CAIX, HIF-1α, VEGF and VEGFR-1.
4.6.3 Relationship of Immunohistochemical markers and cell density all Renal Cell Carcinomas

There were several significant correlations between immunohistochemical markers Table 4-12. The majority of these were weak (i.e. $r^2<0.5$ or $>-0.5$ less than 50% chance of a true correlation) apart from VEGF and its receptor and relationship to HIF1-α, so a further discussion will be undertaken of the sub-group of clear cell RCC in the next section where correlations were extremely similar yet stronger and it is a more homogenous group.

4.6.4 Relationship of Immunohistochemical markers and cell density in clear cell RCC group only

Table 4-13 summarises the correlative relationships between clear cell RCC, cell density and immunohistochemical markers. These will be discussed in greater detail and for the following clear cell RCC analysis, the breakdown of stages was: Grades 1-2 n= 14; Grades 3-4 n=17; Stage 1 n=17 (stage 1a, n=7; 1b n=7), Stage 2 n=4 and stage 3 n=10.
Table 4-12 All Renal Cell Carcinomas with correlative relationships for immunohistochemistry.

<table>
<thead>
<tr>
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<th>GLUT-1</th>
<th>MVD Ratio</th>
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<th>VEGF</th>
<th>VEGFR-1</th>
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4.6.4.1 Ki-67 or Proliferation index

Ki-67 expression had a weak but significant correlation with HIF-1α (Table 4-13). Ki-67 expression also trended toward greater expression by stage and grade (Figure 4-19) but none were significant.

These results are not surprising given that Ki-67 antigen has had mixed reports of association and non-association with prognosis in RCC. However, Ki-67 expression has been considered an indicator of biologic aggressiveness in RCC and this may explain the trend with grade and stage. The weak correlation with HIF-1α may indicate that tumours with more cells dividing are in the process of requiring more vasculature or that they are becoming more hypoxic due to lack of adequate vasculature.

4.6.4.1 MVD and MVD hotspot

MVD hotspot as expected correlated with MVD because they are related; hotspot merely attempts to create the highest MVD score by focusing on areas with obvious proliferation of vessels under low power before counting under high power whereas standard MVD does not. MVD correlated with cell density and EGFR but no other correlation between these two measures of vascular density and other immunohistochemical markers was confirmed (Table 4-13). This finding does not support earlier work whereby expression of HIF-alpha proteins was associated significantly with up-regulation of VEGF mRNA and protein and increased MVD. However, the results may explained given that up-regulation of HIF-alpha in clear cell RCC has been found to involve increased mRNA as well as protein expression, suggesting that both VHL-dependent and VHL-independent mechanisms are involved. In other words, depending on what sub-cellular mechanisms are at play and at what cycle the tumours are at in their growth, the expression of VEGF and HIF-alpha proteins may not always be reflected in MVD.
Figure 4-19 Ki-67 or Proliferation Index adjusted for clear cell Renal Cell Carcinoma stage and grade

Ki 67 expression or Proliferation Index in clear cell Renal cell carcinoma

[Graph showing mean ratio of expression of immunohistochemical marker (%) ± 95% CI for Ki-67 Stage 1a, Ki-67 Stage 1b, Ki-67 Stage 1, Ki-67 Stage 2, Ki-67 Stage 3, Ki-67 Grade 1-2, and Ki-67 Grade 3-4.]
A further point: RCC has always been considered a highly vascular tumour by morphologic standards but high MVD is not equivalent to high perfusion rates. Thus, in comparison with normal renal tissue, RCCs are poorly perfused, hampering adequate delivery of nutrients and potentially oxygen.\textsuperscript{709} So one cannot align MVD with perfusion, and relationships of perfusion to hypoxia and further stimulation of angiogenesis are not well understood.

Tumour tissue has large leaky blood vessels, impaired blood flow patterns, neovascularisation, high interstitial fluid pressures, and aberrant lymphoid vessels. Impaired diffusion of molecules, including oxygen and monoclonal antibodies.\textsuperscript{166} These phenomena are also inconsistent with time, so different parts of the tumour will have varying levels of molecules depending on access by diffusion driven molecules that will vary accordingly.\textsuperscript{167}

The two different methods of MVD analysis yielded similar results (Table 4-13) in that there was no particular difference between stages and stage 1a had fewer vessels than stage 1b. However, in terms of grade, the higher grades had greater MVD in the hotspot group compared to the ratio group. This most likely reflects a selection bias (inherent in searching for a “hotspot” within a tumour but may also reflect that the more aggressive tumours are more active with respect to angiogenesis, but in a heterogenous fashion. The finding of increasing MVD with grade also supports the finding that MVD has been associated with aggressiveness in RCC being increased with more aggressive phenotypes.\textsuperscript{465,466}

The implications of MVD have been suggested before in that increased angiogenesis may be a predictor of who may need closer follow-up and potentially respond to adjuvant therapy.\textsuperscript{456} This is particularly relevant in the TKI era and clinicians await more data to support routine MVD in selecting patients for such therapies.
Figure 4-20 Microvessel Density (MVD) by ratio and hotspot methods adjusted for clear cell Renal Cell Carcinoma stage and grade.

MVD ratio and MVD Hotspot measures in clear cell Renal Cell Carcinoma.

Mean Ratio of Expression of Immunohistochemical Marker (%) ±95% C.I.
4.6.4.2 HIF-1α, VEGF, VEGFR-1 and CAIX

These three markers correlated moderately or strongly each with one another (Table 4-13). The only other correlation was between HIF-1α and Ki-67.

Figure 4-21 summarises HIF-1α expression. There was trend toward greater expression in higher grade tumours. Stage although variable for the second, had a significant difference between stage one 30.64 ± 14.98 (mean±95%C.I.) and stage three at 59.25 ± 17.95 (p<0.047). The difference between Stage 1a and 1b was also approaching significance (p=0.055). I may hypothesise that triggers of HIF-1α expression occur later in the process of tumour growth and potentially in more aggressive tumours. There is potential for the expression to then diminish over time. This is feasible given that in other studies HIF-1α expression in RCC has been analysed by Western blot in tissue where there was no association between HIF-1α and stage or grade. Further, tissue microarray studies have found lower HIF-1α levels in higher stage tumours. The story is by no means complete regarding HIF-1α. Our understanding of the key oncogenes that are enacted in malignancies such as RCC are limited and these are likely to affect the expression of HIF-1α in addition to hypoxia.

What is more consistent is the link between HIF-1α protein expression in clear cell RCC and VHL mutations is compelling. Those RCC harbouring VHL mutations or hypermethylation have over-expression of HIF-1α. HIF-1α expression has further been correlated directly with higher levels of VEGF production. That was also the case in our study where there was a strong correlation.

The results of VEGF expression and its receptor are intriguing (FIGURE 4-22). Although VEGF expression decreased substantially in higher compared to lower grade tumours, within low grades and high grades the receptor expression stayed unchanged. Furthermore, VEGF expression was relatively consistent by stage but the receptor expression increased
Figure 4-21 HIF-1α expression adjusted for clear cell Renal Cell Carcinoma stage and grade

HIF-1α Expression in RCC Clear Cell Summary
Figure 4-22 VEGF and VEGF-R1 expression adjusted for clear cell Renal Cell Carcinoma stage and grade

VEGF and VEGF-R1 Expression in Clear Cell RCC

Mean Ratio of Expression of Immunohistochemical Marker (%) ±95% C.I.
dramatically with stage. This may reflect tumours that are growing and are more active and that pathways involved in angiogenesis rather than expressing more VEGF are able to utilise the available VEGF more efficiently by expressing more receptors.

Previous studies of immunohistochemical expression of VEGF are conflicting although supportive of VEGF expression being 3-37 fold increases in clear cell RCC VEGF expression compared to normal parenchyma. Some studies demonstrated a correlation between serum VEGF and tumour expression of VEGF whereas other did not show a significant correlation. Other studies have also demonstrated that tumour expression of VEGF was not correlated with tumour MVD, and the former but not the latter was prognostic of outcome in cancer patients.

Regarding VEGF-R1 expression, VEGF-R1, but not VEGF-R-2, is expressed in epithelial and stromal cells of clear cell RCC. This was supported in our study. As indicated, there was a strong correlation ($r^2=0.90$, $p<0.001$) between VEGF and VEGF-R1 expression and this is reflected in similar relationships between grade and stage.
Figure 4-23 CAIX expression adjusted for clear cell Renal Cell Carcinoma stage and grade
Figure 4-24 CAIX staining from two different study patients

The CAIX has notable change from normal renal tissue (N) to tumour tissue (T) where expression was great (red staining)
As shown in Figure 4-23, grade and stage appear unrelated to CAIX expression although there was prominent staining (Figure 4-25).

**No significant relationship between CAIX and other parameters could be made (Table 4-13).**

This is somewhat surprising considering the other endogenous hypoxic markers of HIF-1α and VEGF correlated. However, it is hypothesised that in the earlier stages of tumour progression, noxious conditions such as hypoxia or ischemia induce CAIX expression as an adaptation to confer proliferation advantage for tumour growth and spread; however, when this malignant potential is attained in the later stages of tumour growth, continued CAIX expression is no longer a requirement. An alternative hypothesis that the cumulative effects of genetic lesions involved in cancer progression could alter the pathways of hypoxia response and therefore affect CAIX expression.

When comparing to grade and stage, our findings in relation to CAIX are in keeping with the current literature. For example, I found there was really no relationship between CAIX expression and stage as reported in the past. Furthermore there was no significant difference between Fuhrman grade and CAIX expression and this is supported in the literature. On all these points, it must be remembered that studies often conflict regarding stage and progression demonstrated whereby CAIX expression has been demonstrated to be inversely proportional (lower expression confers better prognosis).

Additional studies will be needed to determine whether genetic changes underlie differences in CAIX expression in the primary tumours and in metastatic lesions.

Overall, the endogenous hypoxic markers (VEGF, its receptor VEGF-R1 and HIF-1α) but not CAIX appeared to correlate. No negative correlations between CAIX and other markers or falling expression in relation to stage and grade were demonstrated. Such findings would
have some support in the literature given data finding that tumours expressing lower levels of CAIX appear to have a better prognosis. There is new thought emerging that CAIX may not indeed be such a reliable hypoxic marker, particularly when compared to direct POS measurements. Furthermore, although CAIX has been variable regarding expression and prognosis, most demonstrated that lower expression was best, but even that is being challenged.

4.6.4.3 EGFR
The only correlation was with MVD (Table 4-13). In Figure 4-25, EGFR expression appears variable between Stage 1a (1.96 ±2.64) and 1b (52.58± 17.31 tumours (mean ± 95% C.I; p=0.001) but the significance difference did not hold between higher stages. Higher grade was trending toward greater expression but not significantly.

4.6.4.1 Glut-1
No significant relationship between Glut-1 and other immunohistochemical parameters was made (Table 4-13). Figure 4-26 represents Glut-1 expression in clear cell RCC and it being higher in higher grades but not significantly and variable according to stage. The results of our $^{18}$F-FDG uptake in PET studies also dovetail with our Glut-1 expression results where there were no significant correlations recorded but a weak trend towards increased uptake with grade. This is in contrast to previously reported data whereby clear cell RCC had high expression of Glut-1 and there was a significant correlation with HIF-1α. It is possible that with the type of hypoxia in RCC chronic rather than acute other factors affect glucose metabolism apart from traditional hypoxia pathways e.g. vascularisation, cell density or proliferation, may
Figure 4-25 EGFR expression in clear cell Renal Cell Carcinoma according to stage and grade

**EGFR Expression in RCC Clear Cell Summary**

<table>
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<tr>
<th>EGFR Stage 1a</th>
<th>EGFR Stage 1b</th>
<th>EGFR Stage 1</th>
<th>EGFR Stage 2</th>
<th>EGFR Stage 3</th>
<th>EGFR Grade 1-2</th>
<th>EGFR Grade 3-4</th>
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Mean Ratio of Expression of Immunohistochemical Marker (%) ±95% C.I.
become more relevant.\textsuperscript{717,718} The variability of expression may also be related to variable oncogene expression and their influence on glucose metabolism\textsuperscript{39,85,711-713}

### 4.6.5 Cell Density

The total cells per high power field were increased in the RCC group (356.05±50.16; mean±95% C.I.) compared with the normal renal tissue (328.69±31.17).

This confirms the higher cell turnover in tumours but that it is not significantly different from normal tissue.

The difference was marginally increased between tumour and normal if only clear cell RCC are assessed (361.64±50.16 versus 326.56±31.17).
Figure 4-26 Glut-1 expression adjusted for clear cell Renal Cell Carcinoma stage and grade

GLUT-1 expression in RCC Clear Cell Summary

Mean Ratio of Expression of Immunohistochemical Marker (%) ±95% C.I
4.6.6 Immunohistochemistry in Human Studies: Summary

Of the endogenous hypoxia markers, VEGF (and its receptor VEGF-1) and HIF-1α expression correlated but not CAIX. This reflects recent work suggesting that CAIX may not be as reliable a marker of hypoxia as first thought.

EGFR was unable to differentiate higher grade from lower and within stage only demonstrated limited expression in the lowest stage tumours, being 1a. As a target for future therapies in RCC it would seem to be possible and this requires further study.

MVD correlated with cell density and EGFR but no other correlation between these two measures of vascular density and other immunohistochemical markers was made. This is somewhat surprising considering the proposed mechanisms whereby hypoxia stimulates angiogenesis via the VHL-HIF axis. Of the methods, the hotspot techniques suggested higher number of vessels with higher grade but this was not a significant association.

Ki-67 as an index of tumour proliferation was only correlated with HIF-1α. GLUT-1 had no correlations with cell density or any other immunohistochemical parameters. At least in RCC, Glut-1 cannot be recommended as a reliable endogenous marker for hypoxia.

Regarding the other factors, the relationships found were consistent with other tumours. Hypoxia and its association to tumour biology is far more complex than the VHL-HIF axis. The findings in this study add to our knowledge of the endogenous markers of hypoxia and may contribute in the future to patient selection for radiation therapy, clinical trials or even drug development.
Chapter 5
RESULTS AND DISCUSSION: ANIMAL STUDIES

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5.1 Animal Studies: Overview

Due to our initial results in human studies of RCC detecting hypoxia, I felt it imperative to explore the relationships between hypoxia, imaging and immunohistochemistry in a xenograft model. Our laboratory had extensive experience with both animals and imaging and the expertise to label monoclonal antibodies if required, as well as culturing SK-RC 52 RCC cell line and using targeted agents in xenograft models.

Our xenograft studies essentially had five components:

1) Time-resolved Luminescence-Based Optical Probe (Oxylite Probe) studies of tumour compared to normal tissue
2) PET studies with $^{18}$F-Fluorodeoxyglucose ($^{18}$F-FDG), $^{18}$F-Fluoromisonidazole ($^{18}$F-FMISO) and $^{18}$F-fluorothymidine ($^{18}$F-FLT)
3) Biodistribution Studies of Carbonic Anhydrase IX (CAIX) Expression in a xenografted RCC model with $^{124}$I-G250 Monoclonal Antibodies and immunohistochemical studies
4) Studies of the effects of therapeutic monoclonal antibodies on tumour oxygenation in an RCC xenograft model
5) Xenografting tumour beneath the renal capsule using modern surgical equipment

5.1.1 Reporting of Results

The statistics reporting is described in section 3.18 and is conducted in a similar fashion to the human studies. Standard error of the mean (SEM) is routinely reported. Further, where relationships between defined parameters was analysed, for differences between two groups the nonparametric test (Mann-Whitney) was used and across groups the nonparametric test (Kruskall Wallis) was used due to an assumption of a non-Gaussian distribution. For the linear regression correlations, the $r^2$ classifications are: $r^2 <0.5$ weak, moderate $r^2>0.5$ but $<0.8$ and $r^2 >0.8$ a strong correlation.
5.1.2 Animal model for RCC Xenograft Studies

Figure 5-1 and Figure 5-2 are representative examples of a group of SK-RC-52 xenografted human tumours with the growth curves throughout the experiments and the corresponding total animal mass.
Figure 5-1 Tumour volume in a cohort from the xenograft experiments

![Tumour Volume from time of xenografting with RCC (SK-RC-52) in a representative cohort](image)

Figure 5-2 Total mass of animals in a cohort from the xenograft experiments

![Total Animal Mass from time of xenografting with RCC (SK-RC-52) in a representative cohort](image)
5.2 Direct measurements of oxygenation in tumours and normal tissue: Animal Studies

The following section documents the first series of experiments measuring tissue oxygenation in a xenograft model of human RCC. These types of measurements were also conducted as components of other investigations and as such will be mentioned in the other sections where appropriate.

5.2.1 Time-resolved Luminescence-Based Optical Probe (Oxylite Probe) Measurements

Reliable oxylite probe readings were available in all mice with xenografted renal tumours. An example of a single track through a tumour and normal tissue is given (Figure 5-3). In this case, the two traces represent normal tissue above and tumour xenograft tissue below. On the left axis in both is the actual mean pO\textsubscript{2} recorded by the probe in the centre (35.82 mmHg in normal tissue and 6.10 mmHg in tumour tissue) with the range of the scale above and below this. This recording is of a single track. The flat line represents the calibration phase and the blue arrow represents the beginning of recording normal tissue pO\textsubscript{2}; the red arrow represents the beginning of recording for tumour tissue pO\textsubscript{2} for that track. As may be observed the oxygen levels are fairly stable in the tissue with the line only moving within a small range in any single track. Multiple measurements are taken in each track and it is these repeated measurements in multiple tracks (different parts of the periphery of the tumour avoiding necrotic areas) are taken to calculate the overall tumour oxygen status. Three tracks were taken through each xenografted tumour. Technical failures only occurred on two occasions due to probe dysfunction.
Figure 5-3 Example of a recording of an oxylite probe measurement in a single track of a study xenograft

NORMAL TISSUE TRACE : MUSCLE

TUMOUR TRACE : SK-RC-52 RCC XENOGRAFT

The mean partial pressure of oxygen displayed in the left axis whilst the bottom axis is time. The arrows (blue normal, red tumour) represent the beginning of recording of the tissue $pO_2$. This tumour was hypoxic ($pO_2$ of 6.10 mmHg) compared to normal tissue ($pO_2$ of 35.82 mmHg).
In the first experiments (n=51) tumours were studied by oxylite probe. The animal weights and tumour volumes were 16.56g (median 16.5; 9.67-22.5) and 700.6mm$^3$ (median 532.6; range 201.6-1991.1) respectively.

Although I did not measure central necrotic tumour areas and only the periphery it is important to have a large spread of tumour sizes so that I could examine more closely the relationship between tumour size and hypoxic yet viable tissue. In this way the larger tumours are more representative of larger RCC in humans. However, timing was difficult and on one occasion only did I realise after performing volume calculations that one mouse unfortunately had its tumour grow to just above the accepted limit of 2000mm$^3$. The mouse’s weight was appropriate and it had an equivalent quality of life to the other animals. I subsequently excluded any further measurements from this particular xenograft as it really should not have been in the cohort.

5.2.1.1 Mean Oxygen Level

Figure 5-4 summarises the tumour oxylite probe measurements. The oxygen levels ranged from 19.70- 57.80 mmHg (mean 36.82mmHg; median 36.01mmHg) in normal muscle tissue to 0.66-38.6mmHg (mean 16.42mmHg; median 14.3mmHg) in tumour tissue.

5.2.1.2 Hypoxic Fraction

The hypoxic fraction (percentage of readings in a given tumour or normal tissue below or equal to a nominated oxygen level: 2.5mmHg, 5mmHg or 10mmHg being HP$_{2.5}$, HP$_{5}$ and HP$_{10}$ respectively) demonstrated a difference between tumour and normal tissue at hypoxic fractions HP$_{2.5}$ and HP$_{5}$ and HP$_{10}$ (Figure 5-5). Normal muscle had no recorded oxygen levels below 10mmHg. Thus the graph demonstrates significant differences between tumour tissue and normal tissue. It also highlights that over a third of measurements were in the hypoxic range below 10mmHg. Just over 5% of measurements were in the extremely hypoxic range below 2.5mmHg. Again, heterogeneity was noted as it was in our human studies.
Figure 5-4 Summary of invasive oxygen measurements in tumour versus muscle in xenografts

Renal Tumour versus Normal pO2
SK-RC-52 Transplants (n=53)

Partial Pressure pO2 (mmHg)
Mean ± SEM

Normal Tissue  Tumour Tissue

Figure 5-5 Invasive oxygen measurements in normal tissue and xenografts by hypoxic fraction

Hypoxic Fraction (%) ± SEM

Normal  Tumour

HP_{2.5} (% ≤ 2.5mmHg)  HP_{5} (% ≤ 5mmHg)  HP_{10} (% < 10mmHg)
5.2.1.3 Relationship between Oxygen Measurements and Tumour Volume

On analysis (Figure 5-6), although heterogeneity existed, a weak but significant correlative relationship between tumour tissue pO\textsubscript{2} and tumour volume was established ($r^2=0.14$; $p=0.006$). This is one of the first tumours to do so and this has only been found in squamous cell carcinoma in a study by Urano et al\textsuperscript{177}, and a more gross increase in hypoxic fraction as tumour volume increased has been demonstrated in breast carcinomas by Sorensen et al.\textsuperscript{572}

Other relationships will be discussed in their relevant sections.

5.2.1.1 Oxygen measurements in subrenal grafted tumours

A full description of the results of the new model for subrenal xenografted SK-RC-52 tumours will be given in section 5.7. Here I will summarise the sub-group of tumours (n=4) that had oxylite probe measurements done in the tumour, adjacent renal tissue and normal muscle. The oxygen levels ranged from 19.40-34.07 mmHg (mean 27.84; median 28.12) in normal muscle tissue; 19.66-25.40 mmHg (mean 21.10; median 23.40) in mouse kidney; and 8.08-13.02 mmHg (mean 11.97; median 10.23) in tumour tissue.
Figure 5-6 Relationship between tumour volume and $pO_2$ in the xenograft models

Renal Tumour versus Normal $pO_2$
SK-RC-52 Transplants (n=51)

Figure 5-7 Comparison of oxygen tension measurements of xenografts in the biodistribution study
5.2.2 Oxygen tension measurements from the biodistribution studies

As part of this thesis I also conducted other experiments where I measured the pO$_2$ of tumour and normal tissue in mice. This is detailed in the results section 5.5: biodistribution studies of CAIX expression in a xenografted RCC model with $^{124}$I-G250 mAb.

The results of the invasive oxygen measurements se results are included here for completeness (Figure 5-7) so that all oxygen measurements in xenografts are in the one section.

The oxygen tension in normal tissue (muscle) was 35.08 ± 7.48mmHg (mean ± SD) or 35.08 ± 2.41mmHg (mean ±95% CI), being significantly greater compared to xenograft SK-RC-52 tumours at 5.02 ± 3.48mmHg (mean ± SD) or 5.02 ± 1.12mmHg (mean ±95% CI) (Figure 1). The tumour: normal tissue pO$_2$ ratio (mean ± 95% CI) for all tumours was 0.15:1.0 (± 0.03). The oxygen measurements (%; mean ± SD) for HF$_{2.5}$, HF$_5$ and HF$_{10}$ were 46 ±30, 66 ±0.3 and 83 ±25 respectively for all tumours.

When comparing tumour volume to oxygen tension, a weak but significant inverse correlation was demonstrated ($r^2=-0.33; p=0.04$) but only when compared to HF$_{2.5}$ and not mean tumour pO$_2$, HF$_5$ or HF$_{10}$. When comparing the mean tumour pO$_2$ of the first group (day 0, n=5) with the last group (day 14, n=5) there was a trend toward reduced pO$_2$ (8.82 ± 4.65 and 4.27± 1.62 respectively; mmHg, Mean ± 95% CI) and a significant difference between HF$_{10}$ existed between groups (53.6 ± 31.12 and 91.2± 12.7mmHg) respectively. With the same two groups from day 0 and 14, there was a trend toward increased tumour size over time (705.26 ±204.99 and 586.69 ± 146.91 (mm$^3$) but this was not significant.

On the day of maximal $^{124}$I-cG250 uptake, day 3, there was a trend towards correlation between pO$_2$ and %ID/g on biodistribution but this was not significant ($p=0.08$).
5.2.3 Discussion of Oxylite Probe Measurement results

Technical failures were not an issue with the oxylite probe as compared to the POS measurements in humans. Reliable readings were confidently taken from almost all animals studied with only two technical failures that required changing of probes only throughout the experiments.

In the RCC SK-RC-52 xenografts the mean pO$_2$ of oxygen in normal renal tissue was significantly greater than in the tumour tissue.

5.2.3.1 Oxygen tension in mouse renal tissue

Although not a core objective of the experiments, I did measure the pO$_2$ in a small cohort of animals. Our study found the mean pO$_2$ to have a mean of 21.10 mmHg in normal renal parenchyma but with a variable pO$_2$ range from 19.66-25.40 mmHg (median 23.4 mmHg). This heterogeneity is consistent with animal studies measuring pO$_2$ in kidneys$^{694}$ and similar to the level; of 30mmHg in a study of 17 mice.$^{720}$ The levels are slightly lower than what I established in normal human kidney tissue.

5.2.3.2 Oxygen tension in xenografted renal tumour tissue

In mice, the oxygen partial pressure of xenografted human RCC is unknown. The only data available on RCC has been conducted on a murine cell line. Oxygen studies in renal tumour tissue have only previously been performed in a murine model in a total of six mice. Ziemer et al.$^{665}$ studied RCC (RENCA, murine) where a mean pO$_2$ (±SD) of 24.8 (±17.9) was found the HP$_{10}$ at 15% with HP$_3$ not reported. The relationship to tumour size was not assessed. The RCC (RENCA) xenograft tumours (n=6) tended to be heterogenous in their oxygenation with some severely hypoxic, some moderately hypoxic and some well oxygenated regions.$^{665}$ The only RCC study was with a murine cell line (RENCA) where xenograft tumours (n=6) tended to be heterogenous in their oxygenation with some severely hypoxic, some moderately hypoxic and some well-oxygenated regions.$^{665}$ Our tumours tended to be more
consistently hypoxic and this may reflect the human cell line rather than murine. Also, our numbers were far greater and the lack of power due to small numbers studied in the previous publications may have may explain the discrepancy between their results and ours.

Thus, I may conclude that the pO$_2$ in human renal xenografted RCC is heterogenous but hypoxic when compared to normal tissue (muscle). Again, as in our human studies, the degree of heterogeneity in control tissues as experienced in our study is almost universal. For the most part, muscle tissue is used as a control because this is accessible percutaneously whereas I used native kidney.

In our xenograft studies the mean pO$_2$ in RCC tissue was 14mmHg. Considering the original understanding of the term “hypoxia” in this thesis, a “typical” tumour will have a median pO$_2$ oxygen partial pressure of approximately 10mmHg. By definition, half the cells have oxygen levels less than 10mmHg and half have oxygen levels greater than 10mmHg. This breakpoint is considered significant, as cells with oxygen levels of less than 10mmHg start to become radiation resistant.$^{32}$

In some ways this result is not surprising in light of our human studies. This is because heterogeneity was found within tumours and normal tissue, but moreso in the tumours as was the case in the xenograft model. Further, the RCC xenografts are hypoxic, but slightly more than in the clinical studies. Finally, the overall oxygen tension measurements in the RCC xenograft model fall under the often quoted 10mmHg definition of hypoxia, whilst the clinical studies found the result at about 10mmHg.

This is the first time tissue hypoxia has been demonstrated by POS in a human RCC xenograft model.
5.2.4 Oxylite probe and Future Direct Oxygen Measurements

A model of the oxylite probe suitable for direct oxygen probe measurements in humans has not been developed. Thus it remains a tool for use in animal surgery and experiments only.

5.2.5 Summary of oxylite probe Oxygen Sensor Measurements and Human Renal Cell Carcinoma in xenografts

Hypoxia, as demonstrated in our human studies does exist in RCC. Once again, as with other tumours it is heterogenous. Measurements may be conducted safely and detection of minute-minute changes is possible with the oxylite probe because of continuous measurements as opposed to the POS system that takes point measurements and moves through the tissue. The oxylite probe averages measurements of pO$_2$ over a volume that contains vascular and interstitial components that equalises over a longer period of time whilst the POS microelectrode measures a more localised pO$_2$ by conducting a redox reaction at the probe and then moving forward through tissue. This subtle difference aside, the similarities and discrepancies in pO$_2$ measurements between the two systems have been investigated with tumour-bearing animals with similar results obtained with both systems for the mean and median pO$_2$ values, and the distributions of pO$_2$ values within the interval 0 < pO$_2$ < 40 mmHg (the range important for defining tumour hypoxia) were found to be statistically equivalent. Other studies using animal tumours have demonstrated the utility of oxylite probes and POS and our results add considerably to this body of literature.

It is also important to acknowledge that experimental tumours of different cell lines may show individual and characteristic pO$_2$ fluctuation patterns. The pO$_2$ fluctuations may result in regions with acutely hypoxic cells. The kinetics of the acute hypoxia may differ among tumours of different lines, individual tumours of the same line, and different regions within the same tumour. Thus our conclusions only relate to the SK-RC-52 cell line. It is intended at our institution to measure other human RCC cell lines but this thesis forms the first body of work to conduct any measurements in xenografts at all (and in human RCC in patients)
and it was not intended to delineate the oxygen levels in a range of different cell lines but to examine one used often and one that our laboratory was experienced in handling.
5.3 Non-invasive Imaging Studies of Hypoxia in Xenografts with RCC using Combined Positron Emission Tomography and CT (PET-CT)

Combined PET-CT was utilised throughout the various xenograft studies. However, specifically large groups of tumours (n=12) were investigated using the radioisotopes:

1) $^{18}$F-FDG, $^{18}$F-FMISO and $^{18}$F-FLT
2) Further studies utilising $^{124}$I-cG250 will be discussed later in section 5.5.

5.3.1 Imaging Studies of Renal Cell Carcinoma in xenografts

In all, 3 groups (n= 12 each group) of mice were studied using the radioisotopes $^{18}$F-FDG, $^{18}$F-FMISO and $^{18}$F-FLT. As may be noted in Figure 5-8, uptake of the radioisotope was visible on the images created with no real need to calculate SUV because the tumour uptake is so much greater than the background. This is true in almost every instance in each of the xenografts displayed (an exception is the mouse illustrated on the right for the $^{18}$F-FMISO experiment shown in Figure 5-8). Summaries are provided for each radioisotope in the following sections.

It is important to highlight that the background SUV was corrected to one in all cases so that where a tumour had uptake this is in reference to a SUV$_{\text{mean}}$ or SUV$_{\text{max}}$ of one. Thus the tumour: background ratio is effectively the same as the SUV measured in the tumour. This is an acceptable method when uptake in the background is extremely poor compared to the region of interest. There was no point in calculating the SUV$_{\text{mean}}$ or SUV$_{\text{max}}$ in a mouse kidney considering the xenografted tumour is human and nowhere near the kidney.
Figure 5-8 Xenograft PET study images using $^{18}$F-FDG, $^{18}$F-FMISO and $^{18}$F-FLT

![Xenograft PET study images using $^{18}$F-FDG, $^{18}$F-FMISO and $^{18}$F-FLT](image)

Arrows indicate the subcutaneously xenografted tumours

Table 5-1 Summary of the PET study results in xenografts

<table>
<thead>
<tr>
<th>Radioisotope</th>
<th>SUV Max ± SEM</th>
<th>SUV Mean ± SEM</th>
<th>Volume ± SEM (mm$^3$)</th>
<th>Hot-spot recovery coefficients (HSRC) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{18}$F-FDG</td>
<td>48.04 ± 12.91</td>
<td>19.68 ± 5.44</td>
<td>574.05 ± 110</td>
<td>0.31 ± 0.04</td>
</tr>
<tr>
<td>$^{18}$F-FMISO</td>
<td>54.17 ± 17.4</td>
<td>36.43 ± 11.92</td>
<td>619.87 ± 180.22</td>
<td>0.36 ± 0.03</td>
</tr>
<tr>
<td>$^{18}$F-FLT</td>
<td>56.1 ± 17.5</td>
<td>33.73 ± 11.29</td>
<td>495.05 ± 59.1</td>
<td>0.38 ± 0.02</td>
</tr>
</tbody>
</table>
Figure 5-9 Comparison of SUV_{max} and SUV_{mean} for different radioisotopes (n=12 each group)
Again, in support of the SUV calculations used in this study the hot-spot recovery coefficients (HSRC) were also calculated. HSRC reflects the fraction of the true radioactivity in the region of interest that is actually measured in the region of interest (“hot spot”), when compared to background radioactivity. In other words, when measuring “hot lesions” (i.e. the tumour in this case) in a cold background (usually with poor background uptake) the HSRC is some way of having quality control that what you calculate in the region of interest appears reasonable. In this study, the HSRC were remarkably similar between the radioisotopes as one would hope and this is related to the positron energy of 18F (and not the vehicle such as FMISO or FDG). This consistency means comparisons across the radioisotopes are more likely to be valid and reproducible.

Finally, the quality control was excellent with a significant correlation ($r^2 = 0.89; p< 0.0001$) between $SUV_{max}$ and $SUV_{mean}$ across all radioisotopes (see Figure 5-10).

The PET studies conducted with a three different radioisotopes yielded significant uptake within the tumour is as evidenced by the dramatic $SUV_{max}$ and $SUV_{mean}$ calculated. Positive results were apparent in almost all tumours studied. The tumour volumes were not significantly different despite a trend to slightly smaller measured tumour volumes in the $^{18}$F-FLT group.

Tumour $^{18}$F-FDG uptake might be reduced by increases in tumour oxygenation and thus may provide a means to further enhance $^{18}$F-FDG functional imaging Chan. However, despite these theoretical suppositions, there is very little convincing data for this point and $^{18}$F-FDG has been a disappointing marker of tissue hypoxia.
Figure 5-10 Correlation between $SUV_{\text{max}}$ and $SUV_{\text{mean}}$ for all radioisotopes studied

Correlation between $SUV_{\text{max}}$ and $SUV_{\text{mean}}$ within SK-RC-52 xenografts for all radioisotopes ($^{18}$F-FDG, $^{18}$F-FMISO and $^{18}$F-FLT; n=36)

- Individual xenograft

Figure 5-11 Comparison of two methods to measure tumour volume- traditional calipers on the tumour versus PET-CT calculated volume

Correlation between volume of tumour as measured by calipers versus that calculated from PET images for all PET studied SK-RC-52 xenografts (n=36)

- Individual xenograft
Overall, considering all radioisotopes and xenografted RCC there was no statistically significant difference between 18F-FDG, $^{18}$F-FMISO and 18F-FLT considering standardised uptake volume (either SUV$_{\text{max}}$ or SUV$_{\text{mean}}$).

5.3.1.1 Correlation between tumour volume calculations

Volume for tumours was calculated using 3 methods: 1) measurement with calipers on the mass, PET-CT and high-resolution PET-CT. There was a good correlation between caliper volume and PET-CT ($r^2=0.60$; $p<0.0001$; Figure 5-11). As expected the two different PET methods correlated relatively well also ($r^2=0.75$; $p<0.0001$). For calculations as with humans, we rely on the caliper measured method.

5.3.2 Analysis of Radioisotope uptake and oxylite probe invasive oxygen measurements

There were four readings out of 36 that were not possible (two due to animal expiration prior to reading, two due to inability of the probe computer software to recognise the probe). This left 32 xenografts using radioisotopes $^{18}$F-FDG (n=11), $^{18}$F-FMISO (n=11) and $^{18}$F-FLT (n=10).

The results of oxygenation were very similar to those of our experiments in section 5.2. There was a statistically significant difference between tumour pO$_2$ and normal tissue pO$_2$ ($p<0.0001$; Mann-Whitney). These results (Table 5-2) will be discussed further in each individual radioisotope section to follow.
Table 5-2 Summary of the oxygen parameters of the tumours used in the PET studies

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Volume (mm³)</th>
<th>Normal Tissue Mean pO₂ (mmHg)</th>
<th>Tumour Tissue Mean pO₂ (mmHg)</th>
<th>Tumour mean pO₂:Normal pO₂ ratio</th>
<th>HP₂₅</th>
<th>HP₅</th>
<th>HP₁₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SEM</td>
<td>570 ±1.72</td>
<td>36.82 ±1.72</td>
<td>12.25 ±1.10</td>
<td>35.45 ±3.67</td>
<td>0.05</td>
<td>0.22</td>
<td>0.45</td>
</tr>
<tr>
<td>Median</td>
<td>240.50</td>
<td>35.82</td>
<td>11.39</td>
<td>29.86</td>
<td>0.00</td>
<td>0.00</td>
<td>0.45</td>
</tr>
<tr>
<td>Range</td>
<td>151.4-202.9</td>
<td>19.4-56.3</td>
<td>2.89-25.8</td>
<td>6.62- 96.72</td>
<td>0.0-</td>
<td>0.44</td>
<td>0-1.0</td>
</tr>
</tbody>
</table>
However, considering hypoxic fractions or the proportion of measurements below certain levels (HP_{2.5}, HP_{5} and HP_{10}) there was only a weak correlation noted between tumour volume and HP_{2.5} (r^2=0.44; p=0.03).

This result is interesting as it suggests that the larger the tumours, the greater the likelihood of having extremely hypoxic regions. Regarding the other relationships, it is not surprising that no relationships between SUV and tumour oxygenation exist as {^{18}}F-FDG is not considered by most to be a true hypoxic marker.

5.3.3 {^{18}}F-Fluorodeoxyglucose (F-FDG)

Examining {^{18}}F-FDG more closely, there was excellent correlation between SUV_{\text{max}} and SUV_{\text{mean}} (r^2=0.88; p<0.0001; Figure 5-12). This emphasises the ability of PET-CT in xenograft models to obtain information related to maximal uptake as well as mean that is reliable and reproducible. This is mainly because the whole graft is tumour and the graft is cutaneous with no interference from nearby organs. This is in contrast to the kidney where background uptake within normal kidney interferes and so the SUV_{\text{mean}} becomes less important than the SUV_{\text{max}}.
Figure 5-12 Correlation between SUV\textsubscript{mean} and SUV\textsubscript{max} for \( ^{18} \text{F-FDG} \)

Correlation between \( \text{SUV}_{\text{max}} \) and \( \text{SUV}_{\text{mean}} \) within SK-RC-52 xenografts for \( ^{18} \text{F-FDG} \) (n=12)

Table 5-3 \( ^{18} \text{F-FDG} \) uptake in all RCC xenografts compared to Oxygen Measurements with \( \text{SUV}_{\text{max}} \) correlations given

<table>
<thead>
<tr>
<th>( ^{18} \text{F-FDG} )</th>
<th>Mean ±SEM</th>
<th>Median</th>
<th>Range</th>
<th>( \text{SUV}_{\text{Max}} )</th>
<th>( r^2 )</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{SUV}_{\text{max}} )</td>
<td>50.88 ± 13.79</td>
<td>33.57</td>
<td>7.92 - 163.8</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>( \text{SUV}_{\text{mean}} )</td>
<td>20.36 ± 5.92</td>
<td>12.96</td>
<td>1.2 - 70.84</td>
<td>0.96</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Tumour Volume (mm\textsuperscript{3})</td>
<td>575 ± 120.5</td>
<td>532.60</td>
<td>151.4 - 1370</td>
<td>0.20</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>Normal Tissue Mean ( \text{pO}_2 ) (mmHg)</td>
<td>31 ± 1.94</td>
<td>29.43</td>
<td>19.4 - 40.83</td>
<td>0.12</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>Tumour Tissue Mean ( \text{pO}_2 ) (mmHg)</td>
<td>9.68 ± 1.38</td>
<td>9.10</td>
<td>4.19 - 17.74</td>
<td>0.09</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>Tumour ( \text{pO}_2 ): Normal ( \text{pO}_2 )</td>
<td>33.96 ± 6.35</td>
<td>23.72</td>
<td>10.59 - 72.11</td>
<td>0.13</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>HP\textsubscript{2.5}</td>
<td>0.01 ± 0.01</td>
<td>0.00</td>
<td>0 - 0.11</td>
<td>0.04</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>HP\textsubscript{5}</td>
<td>0.30 ± 0.09</td>
<td>0.33</td>
<td>0 - 0.78</td>
<td>0.06</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>HP\textsubscript{10}</td>
<td>0.62 ± 0.11</td>
<td>0.67</td>
<td>0 - 1</td>
<td>0.08</td>
<td>0.41</td>
<td></td>
</tr>
</tbody>
</table>
5.3.3.1 Analysis of $^{18}$F-FDG uptake and Oxylite probe invasive oxygen measurements

Data for $^{18}$F-FDG uptake in tumours versus oxygen parameters are summarised in Table 5-3. There was no apparent correlation between $^{18}$F-FDG maximal uptake in tumours versus mean or median oxygen level (all RCC, n=11).

However, considering hypoxic fractions or the proportion of measurements below certain levels (HP$_{2.5}$, HP$_{5}$ and HP$_{10}$) a weak correlation was noted between tumour volume and HP$_{2.5}$ ($r^2=0.44$; p=0.03). This means that the larger the tumour, the more likely it is to have areas of oxygenation below 2.5mmHg. This may indicate that as the tumour grows more regions of lower hypoxia are recruited. Alternatively, the lower hypoxia may be driving growth. This finding compliments other data (section 2.6) discussed regarding the tumour aggressiveness and potential for a poorer prognosis, the lower the oxygen level and the larger the tumour.

Comparing $^{18}$F-FDG SUV$_{\text{max}}$ to other oxygenation parameters or tumour volume yielded no other correlations.

5.3.4 Discussion of $^{18}$F-Fluorodeoxyglucose ($^{18}$F-FDG) in a Xenograft model

As expected based on previous studies, there was obvious uptake of $^{18}$F-FDG in tumour tissue for the human RCC xenograft model. Such findings support the results from our human studies but clearly the uptake was more pronounced considering background renal excretion of $^{18}$F-FDG was not an issue. Again, this finding supports previous studies at our institution and reinforces the difficulties with PET studies in the renal tract and why $^{18}$F-FDG will probably remain a staging rather than diagnostic tool in RCC.

As in the human studies, I was not able to demonstrate a relationship between $^{18}$F-FDG SUV$_{\text{max}}$ and tumour oxygenation by direct oxygen measurements. These points are discussed in section 4.3.5.
In human cancer cell lines there is some evidence from experimental data that hypoxic conditions lead to increased FDG uptake. However, I found no such relationship in RCC. This may be explained partly at least because RCC has been demonstrated to have inconsistent GLUT-1 expression. This finding lead to the conclusion that RCC has a low potential for metabolising glucose. However, this tells us little of the relationship between glucose metabolism and hypoxia in RCC. There is no data to support the premise that they are related in this thesis or from prior studies, as none exist. Finally, factors affecting $^{18}$F-FDG in an hypoxic tumour environment, such as acute versus chronic hypoxia may render $^{18}$F-FDG unsuitable to studying hypoxia with PET.

In summary, regarding relationships between SUV and tumour oxygenation, it is not surprising that no relationships exist as $^{18}$F-FDG is not an hypoxic marker in RCC. Some authors continue in pushing it forward as an hypoxic marker. This is because of a perceived relationship whereby increased tumour glycolysis is stated to be driven by activation of HIF-1 through tumour hypoxia. According to this theory, the degree of $^{18}$F-FDG uptake by tumours might indirectly reflect the level of hypoxia, obviating the need for more specific radiopharmaceuticals for hypoxia imaging. They request further evaluation of FDG uptake by various tumour types and I have demonstrated that as a utility in RCC our data supports that $^{18}$F-FDG PET cannot reliably differentiate hypoxic from normoxic tumours.
5.3.5 $^{18}$F-Fluoromisonidazole ($^{18}$F-FMISO)

Examining $^{18}$F-FMISO more closely, there was perfect correlation between $SUV_{\text{max}}$ and $SUV_{\text{mean}}$. ($p<0.0001$, $r^2=1.00$; Figure 5-13)

5.3.5.1 Analysis of $^{18}$F-FMISO uptake and Oxylite probe invasive oxygen measurements

$^{18}$F-FMISO uptake in tumours versus oxygen parameters are summarised in Table 5-4. No correlation was observed between $^{18}$F-FMISO maximal uptake in tumours versus mean or median oxygen level.

5.3.6 Discussion of $^{18}$F-Fluoromisonidazole ($^{18}$F-FMISO) in RCC

Although $^{18}$F-FMISO is the most extensively studied hypoxia-sensitive radiopharmaceutical, I was unable to delineate any relationships between oxygen parameters in the xenografted tumour and its uptake. In our human studies, there was a modest increase in identifying hypoxic regions of RCC but not enough to make it a reliable radioisotope for this purpose.

The definition of what constitutes positive $^{18}$F-FMISO uptake indicating hypoxia is generally only incremental above background tissue uptake (with a ratio of 1.2 to 1.4). The SUV recorded here were far greater than this $38.75 \pm 3.16$ (mean mmHg ± SEM) considering the background uptake was negligible. This fits with the ability of $^{18}$F-FMISO to localise to the tumours where hypoxia was present as evidenced by the low $pO_2$ readings and in particular, the fact that 30% of measurements were below 10mmHg ($HP_{10}$).

The two major differences between our PET studies with $^{18}$F-FMISO in humans were: firstly, the issue of background uptake and that the overall uptake was not particularly impressive in humans yet in the xenografts there was essentially no background uptake and the uptake was impressive; secondly the level of hypoxia was greater in the xenografts that in the human studies. This highlights the complexity of hypoxia imaging.
Figure 5-13 Correlation between SUV<sub>max</sub> and SUV<sub>max</sub> for <sup>18</sup>F-FMISO

Correlation between SUV<sub>max</sub> and SUV<sub>mean</sub> within SK-RC-52 xenografts for <sup>18</sup>F-FMISO (n=12)

Table 5-4 <sup>18</sup>F-FMISO uptake in RCC compared to Oxygen Measurements

<table>
<thead>
<tr>
<th>&lt;sup&gt;18&lt;/sup&gt;F-FMISO</th>
<th>Mean ±SEM</th>
<th>Median</th>
<th>Range</th>
<th>SUV&lt;sub&gt;Max&lt;/sub&gt;</th>
<th>r²</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUV&lt;sub&gt;Max&lt;/sub&gt;</td>
<td>54.17 ± 17.4</td>
<td>28.76</td>
<td>0.83 - 184.6</td>
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<td></td>
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</tr>
<tr>
<td>SUV&lt;sub&gt;Mean&lt;/sub&gt;</td>
<td>36.43 ± 11.92</td>
<td>19.74</td>
<td>0.45 - 123</td>
<td>1.0 &lt; 0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumour Volume (mm&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>619.9 ± 180.2</td>
<td>353</td>
<td>222.1 - 2029</td>
<td>0.0</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td>Normal Tissue Mean pO&lt;sub&gt;2&lt;/sub&gt;(mmHg)</td>
<td>38.75 ± 3.13</td>
<td>36.57</td>
<td>25.16 - 56.3</td>
<td>0.03</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>Tumour Tissue Mean pO&lt;sub&gt;2&lt;/sub&gt; (mmHg)</td>
<td>12.59 ± 1.50</td>
<td>12.14</td>
<td>7.32 - 21.9</td>
<td>0.09</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>Tumour pO&lt;sub&gt;2&lt;/sub&gt;: Normal pO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>31.44 ± 5.23</td>
<td>32.36</td>
<td>0 - 72.69</td>
<td>0.06</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>HP&lt;sub&gt;2.5&lt;/sub&gt;</td>
<td>0.01 ± 0.01</td>
<td>0</td>
<td>0 - 0.11</td>
<td>0.08</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>HP&lt;sub&gt;5&lt;/sub&gt;</td>
<td>0.08 ± 0.06</td>
<td>0</td>
<td>0 - 0.67</td>
<td>0.03</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>HP&lt;sub&gt;10&lt;/sub&gt;</td>
<td>0.30 ± 0.09</td>
<td>0.11</td>
<td>0 - 0.67</td>
<td>0.05</td>
<td>0.52</td>
<td></td>
</tr>
</tbody>
</table>
$^{18}$F-FMISO has been used in many clinical studies already outlined that established it as a validated hypoxic marker in human tumours. $^{18}$F-FMISO is currently being used clinically to help guide doses of radiotherapy in head and neck cancers. Despite the clinical experience, Kelly and Brady recently highlighted the point whereby the relationship between levels of $^{18}$F-FMISO uptake and the underlying oxygen tension are yet to be elucidated. Furthermore, they contend that it is not fully understood how changes in the underlying physiology affect the appearance of uptake. So although $^{18}$F-FMISO favours uptake in hypoxic tissues, researchers are not certain how the uptake relates to the “gold standard” invasive probe measurements.

The most common reasons for discrepancy between direct POS measurements of tissue oxygenation and 18F-FMISO uptake levels have been outlined in detail in section 2.6.8. In summary tumour heterogeneity, necrosis, tumour architecture, angiogenic differences microregional differences in metabolic activity as well as methodological differences between the approaches to global ($^{18}$F-FMISO) versus targeted (POS) measurements all being considered.

There are also precedents for different oxygen measuring methods not correlating. For example, in one study, no correlation was observed between POS measurements and pimonidazole labelling results (an immunohistochemical technique for hypoxia). Both techniques rely on the metabolism of nitroimidazoles and should in theory be closely related; perhaps moreso than $^{18}$F-FMISO and POS. Even so, the two techniques in the prior study gave different information about tumour hypoxia. The authors concluded both measurements were valid but complementary. The same may be stated of our results: the $^{18}$F-FMISO gave the global status of hypoxia but the POS was more precise and thus SUV$_{\text{max}}$ could not be correlated with the actual pO$_2$.

What was established is that the $^{18}$F-FMISO uptake is high in our xenograft human RCC model (SK-RC-52); what was not supported was the accuracy of $^{18}$F-FMISO uptake at
measuring the exact level of hypoxia. The issues raised in this discussion may help to explain why the correlations could not be made in our xenograft model.

Our conclusions already drawn from human studies thus remain relevant. That is that I agree with Serganova et al that $^{18}$F-FMISO may not be the optimal clinical marker of tissue hypoxia, particularly for RCC. That is why they also point out that other non-invasive PET hypoxia markers continue to be developed. However, $^{18}$F-FMISO does have a role in research of hypoxia in RCC. In xenograft models, it delineates uptake and does have a future role in investigating hypoxia.
5.3.7 \( ^{18} \text{F-Fluorothymidine} \)

Examining \(^{18}\text{F-FLT}\) more closely, there was a strong correlation between SUV\(_{\text{max}}\) and SUV\(_{\text{mean}}\) \((r^2=0.88; \ p<0.0001; \text{Figure 5-14})\)

5.3.7.1 Analysis of \(^{18}\text{F-FLT}\) uptake and oxylite probe invasive oxygen measurements

\(^{18}\text{F-FLT}\) uptake in tumours versus oxygen parameters are summarised in Table 5-5. No correlation was observed between \(^{18}\text{F-FLT}\) maximal uptake in tumours versus mean or median oxygen level (all RCC, \(n=10\)).

5.3.8 Discussion of \(^{18}\text{F-Fluorothymidine} \)

Regarding tumour hypoxia, it has been stated that even if researchers are able to identify hypoxic cells in tumours, they cannot distinguish between viable hypoxic cells capable of proliferation or hypoxic cells that are metabolically active.\(^{729}\) Interestingly, \(^{18}\text{F-FLT}\) should be able to assist in this regard. So perhaps not recognised as a direct measure of tumour hypoxia, \(^{18}\text{F-FLT}\) may yet have a role in defining subpopulations of cells that are proliferating and by definition, at least somewhat metabolically active. This may have implications in the future but at present this study was not designed to answer this specific question. Finally, \(^{18}\text{F-FLT PET}\) studies may in the future be a useful tool in delineating renal masses and this is being specifically investigated in our centre as part of a new study.
Figure 5-14 Correlation between $SUV_{\text{max}}$ and $SUV_{\text{mean}}$ for $^{18}$F-FLT

Correlation between $SUV_{\text{max}}$ and $SUV_{\text{mean}}$ within SK-RC-52 xenografts for $^{18}$F-FLT (n=36)

Table 5-5 $^{18}$F-FLT uptake in all RCC xenografts compared to Oxygen Measurements

<table>
<thead>
<tr>
<th>$^{18}$F-FLT</th>
<th>Mean ±SEM</th>
<th>Median</th>
<th>Range</th>
<th>$SUV_{\text{max}}$</th>
<th>$r^2$</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$SUV_{\text{max}}$</td>
<td>53.48 ± 20.49</td>
<td>20.8</td>
<td>7.17- 198.4</td>
<td>--</td>
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<td></td>
</tr>
<tr>
<td>$SUV_{\text{mean}}$</td>
<td>30.44 ± 12.83</td>
<td>12.92</td>
<td>4.43- 133.2</td>
<td>0.88</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Tumour Volume (mm$^3$)</td>
<td>498.2 ± 58.86</td>
<td>458.60</td>
<td>254.8- 856.4</td>
<td>0.02</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>Normal Tissue Mean pO$_2$(mmHg)</td>
<td>41.09 ± 3.07</td>
<td>44.61</td>
<td>26.2 - 51.63</td>
<td>0.12</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>Tumour Tissue Mean pO$_2$ (mmHg)</td>
<td>14.71 ± 2.581</td>
<td>12.48</td>
<td>2.89 - 25.8</td>
<td>0.08</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>Tumour pO$_2$: Normal pO$_2$</td>
<td>38.34 ± 8.35</td>
<td>27.14</td>
<td>6.619- 96.72</td>
<td>0.03</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>HP$^{2.5}$</td>
<td>0.132 ± 0.060</td>
<td>0</td>
<td>0- 0.44</td>
<td>0.30</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>HP$^5$</td>
<td>0.3 ± 0.12</td>
<td>0.165</td>
<td>0- 1</td>
<td>0.25</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>HP$^{30}$</td>
<td>0.43 ± 0.11</td>
<td>0.5</td>
<td>0- 1</td>
<td>0.06</td>
<td>0.48</td>
<td></td>
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</tbody>
</table>
5.3.9 Overall summary of PET in RCC

\[ ^{18}\text{F}-\text{FDG}, \ ^{18}\text{F}-\text{FMISO} \text{ and } ^{18}\text{F}-\text{FLT} \text{ although demonstrating excellent uptake in xenografted SK-RC-52 RCC tumours, are of limited usefulness for quantifying hypoxia. Although this seems plausible for } ^{18}\text{F}-\text{FLT} \text{ and reasonably so for } ^{18}\text{F}-\text{FDG}, \text{ this is particularly surprising for } ^{18}\text{F}-\text{FMISO}, \text{ which has shown promise demonstrating hypoxia in a variety of other tumours.} \]

It may be that other cell lines and other models will need to confirm the results. However, when combined with our human studies, no radioisotope studies are reliably able to distinguish hypoxia in RCC. Possible explanations include the variability of uptake within tumours, the fact that the human and xenograft models both had levels above 10mmHg. However, when the renal excretion issues of the current radioisotopes and difficulty with discerning true tissue uptake from background emission is omitted in the xenograft model, uptake of radiotracers in human RCC appears significant. The difficulty is that the uptake measurements with SUV are not capable of determining exact degree of hypoxia but this will need to be confirmed in other cell lines and in larger numbers and this is currently being undertaken at our institution.
5.4 Immunohistochemistry in Xenograft Studies

The results of the immunohistochemistry studied groups are in Table 5-6 and Table 5-7. As expected, regarding biodistribution of the radiolabelled antibody, the injected dose $^{124}$I-G250/g tumour tissue (%) and the tumour tissue: blood $^{124}$I-G250 ratios were considerably greater on day 0 than by day 14.

The tumour parameters were themselves not significantly different despite higher tumour weights and mass, probably reflecting the numbers in each group. The oxygenation parameters favoured a more hypoxic environment at day 14 and was close to significance for HP10 with the day 14 group at 91.2 ±6.41 (% ± SEM) compared to the day 0 group 53.6 ±15.88 (p=0.06).

5.4.1 Discussion of Immunohistochemistry-Xenograft Studies

Examining the immunohistochemistry results (Table 5-7), indicators of angiogenic activity being MVD and CAIX had greater expression on day 14 when compared to day 0 but not significantly (Figure 5-15). Also, the tumours became larger and trended toward greater hypoxia (although both not significantly) over the same timecourse. Larger numbers may have been required to bring the results to significance and these results sit well with our earlier findings of immunohistochemical evidence of angiogenesis in human RCC.

EGFR was overexpressed by tumours on day 14 compared with the day 0 group. This supports the role of the EGRF pathway in hypoxia and angiogenesis in this RCC xenograft model. This has implications in the therapeutic studies (next section).
Table 5-6 Xenograft tumour parameters in the $^{124}$I-G250 biodistribution experiment

<table>
<thead>
<tr>
<th></th>
<th>SK-RC-S2 RCC Xenografts</th>
<th></th>
<th>Day 0 (n=5)</th>
<th>Day 14 (n=5)</th>
<th></th>
<th>Day 0 (n=5)</th>
<th>Day 14 (n=5)</th>
<th>p value</th>
<th>Day 0 (n=5)</th>
<th>Day 14 (n=5)</th>
<th>p value</th>
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<tbody>
<tr>
<td><strong>Tumour and Oxygen Measurements</strong></td>
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<tr>
<td><strong>Tumour Volume</strong></td>
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<tr>
<td>Mean ±SEM</td>
<td>586.7 ± 74.95</td>
<td>705.3 ± 104.6</td>
<td>0.31</td>
<td>Mean ±SEM</td>
<td>51.2 ± 15.97</td>
<td>66.6 ± 14.11</td>
<td>0.69</td>
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<tr>
<td>Median</td>
<td>653.80</td>
<td>762.00</td>
<td></td>
<td>Median</td>
<td>56.00</td>
<td>56.00</td>
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<tr>
<td>Range</td>
<td>355.2-776.4</td>
<td>325.9-959.8</td>
<td></td>
<td>Range</td>
<td>0-100</td>
<td>33-100</td>
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<td><strong>Weight of Tumour (g)</strong></td>
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<tr>
<td>Mean ±SEM</td>
<td>0.44 ± 0.06</td>
<td>0.73 ± 0.15</td>
<td>0.15</td>
<td>Mean ±SEM</td>
<td>42.2 ± 12.92</td>
<td>35.4 ± 8.96</td>
<td>0.55</td>
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<tr>
<td>Median</td>
<td>0.40</td>
<td>0.88</td>
<td></td>
<td>Median</td>
<td>44.00</td>
<td>33.00</td>
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<tr>
<td>Range</td>
<td>0.28-0.59</td>
<td>0.14-0.97</td>
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<td>Range</td>
<td>0-78</td>
<td>11-67</td>
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<tr>
<td><strong>Tumour pO$_2$ (mmHg)</strong></td>
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<tr>
<td>Mean ±SEM</td>
<td>8.82 ± 2.37</td>
<td>4.27 ± 0.83</td>
<td>0.15</td>
<td>Mean ±SEM</td>
<td>1.844 ± 0.7475</td>
<td>0.782 ± 0.3136</td>
<td>0.31</td>
<td></td>
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<tr>
<td>Median</td>
<td>8.59</td>
<td>4.30</td>
<td></td>
<td>Median</td>
<td>1.11</td>
<td>0.62</td>
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<td>Range</td>
<td>1.58-16.19</td>
<td>2.13-6.44</td>
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<td>0.24</td>
<td>0.27</td>
<td>0.98</td>
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<tr>
<td><strong>Normal pO$_2$ (mmHg)</strong></td>
<td></td>
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<tr>
<td>Mean ±SEM</td>
<td>36.19 ± 4.26</td>
<td>39.88 ± 3.04</td>
<td>0.84</td>
<td>Mean ±SEM</td>
<td>22.67 ± 6.758</td>
<td>13.04 ± 8.65</td>
<td>0.42</td>
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<tr>
<td>Median</td>
<td>35.31</td>
<td>44.25</td>
<td></td>
<td>Median</td>
<td>25.98</td>
<td>4.89</td>
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<tr>
<td>Range</td>
<td>26.63-47.23</td>
<td>30.66-45.59</td>
<td></td>
<td>Range</td>
<td>2.748</td>
<td>2.189</td>
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<tr>
<td><strong>Tumour pO2: Normal pO2</strong></td>
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<tr>
<td>Mean ±SEM</td>
<td>16.3 ± 4.34</td>
<td>11.55 ± 2.99</td>
<td>0.55</td>
<td>Mean ±SEM</td>
<td>1.844 ± 0.75</td>
<td>0.782 ± 0.31</td>
<td>0.31</td>
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<tr>
<td>Median</td>
<td>18.63</td>
<td>9.43</td>
<td></td>
<td>Median</td>
<td>1.11</td>
<td>0.62</td>
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<tr>
<td>Range</td>
<td>4.81-29.96</td>
<td>4.81-18.66</td>
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<td>Range</td>
<td>0.24-4.56</td>
<td>0.27-1.98</td>
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<tr>
<td><strong>HP$_{10}$ (pO$_2$&lt;10mmHg)</strong></td>
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</tr>
<tr>
<td>Mean ±SEM</td>
<td>53.6 ± 15.88</td>
<td>91.2 ± 6.41</td>
<td>0.06</td>
<td>Mean ±SEM</td>
<td>22.67 ± 6.76</td>
<td>13.04 ± 8.65</td>
<td>0.42</td>
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</tr>
<tr>
<td>Median</td>
<td>56.00</td>
<td>100.00</td>
<td></td>
<td>Median</td>
<td>25.98</td>
<td>4.89</td>
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<tr>
<td>Range</td>
<td>0-100</td>
<td>67-100</td>
<td></td>
<td>Range</td>
<td>2.75-43.24</td>
<td>2.189-47.42</td>
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</table>
Table 5-7 Immunohistochemistry and $^{124}$I-G250 biodistribution parameters in the $^{124}$I-G250 biodistribution study

<table>
<thead>
<tr>
<th>SK-RC-52 RCC Xenografts</th>
<th>Day 0 (n=5)</th>
<th>Day 14 (n=5)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>G250 Biodistribution</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injected Dose $^{124}$I-G250/g tumour tissue (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ±SEM</td>
<td>9.574 ± 1.14</td>
<td>1.998 ± 0.2874</td>
<td>0.01</td>
</tr>
<tr>
<td>Median</td>
<td>8.34</td>
<td>2.09</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>8.014-14</td>
<td>1.321-2.763</td>
<td></td>
</tr>
<tr>
<td><strong>Tumour Tissue: Blood $^{124}$I-G250</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ±SEM</td>
<td>0.3035 ± 0.04228</td>
<td>69 ± 21.1</td>
<td>0.01</td>
</tr>
<tr>
<td>Median</td>
<td>0.30</td>
<td>64.57</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0.22-0.4566</td>
<td>19.39-140.3</td>
<td></td>
</tr>
<tr>
<td><strong>Immunohistochemistry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MVD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ±SEM</td>
<td>7.28 ± 2.101</td>
<td>9.707 ± 3.294</td>
<td>0.69</td>
</tr>
<tr>
<td>Median</td>
<td>6.30</td>
<td>9.31</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>1.7-13</td>
<td>1.909-21.04</td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ±SEM</td>
<td>58.04 ± 11.71</td>
<td>86.25 ± 1.893</td>
<td>0.03</td>
</tr>
<tr>
<td>Median</td>
<td>58.18</td>
<td>88.46</td>
<td></td>
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<tr>
<td>Range</td>
<td>26-86</td>
<td>78.96-89.06</td>
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<tr>
<td>CAIX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ±SEM</td>
<td>61.27 ± 9.732</td>
<td>83.79 ± 4.464</td>
<td>0.10</td>
</tr>
<tr>
<td>Median</td>
<td>69.00</td>
<td>86.82</td>
<td></td>
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<tr>
<td>Range</td>
<td>36-87</td>
<td>72.22-95.31</td>
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</tbody>
</table>
Figure 5-15 Immunohistochemistry with CAIX expression

In this xenograft the CAIX expression at low power (A) with the junction between highly expressed areas in the periphery and areas beginning to but not yet necrotic demonstrated by the arrow. MVD at high power (B) where the red stained areas represent endothelium of blood vessels when a lumen is confirmed, such as where the arrow is pointing.
5.5 Biodistribution Studies of Carbonic Anhydrase IX Expression in a xenografted RCC model with $^{124}$I-G250 Monoclonal Antibodies

5.5.1 In vitro Properties of $^{124}$I-cG250

The radiolabelling efficiency (LE) for $^{124}$I-cG250 was 66.8% with a specific activity of 1.1uCi/ug. For $^{124}$I-huA33, the LE was 52.2 % and specific activity of 1.02 µCi/ug. After chromatographic purification, greater than 97% of $^{124}$I was bound to protein as determined by thin layer chromatography.

Labelled antibody retained the ability to bind CAIX expressing cells (SK-RC-52) on flow cytometry (Figure 5-16), and had an antibody immunoreactivity of 95% (i.e. the percentage of the conjugated antibody is able to bind to the relevant antigen). Such a high level of immunoreactivity ensures quality control. Scatchard analysis of binding of $^{124}$I-cG250 to SK-RC-52 RCC cells determined the binding capacity of cells with the value to be $4 \times 10^5$ antibody molecules bound per cell using standard curve-fitting software (Figure 5-17).

The apparent association constant ($K_a$) was also calculated giving a value of $2.18 \times 10^9$ M$^{-1}$. Serum stability of radioconjugate by thin-layer chromatographic analysis indicated 97%, 82% and 81%, respectively, of $^{124}$I was bound to protein on days 0, 3 and 7. The immunoreactivity of $^{124}$I-cG250 in mouse serum incubated ex-vivo at these times after injection was 95%, 81% and 78%, respectively.
Figure 5-16 Flow cytometry from the biodistribution study

*Flow cytometry indicating the proportion of cells binding SK-RC-52 with cG250 versus no antibody and control antibody (KM-871). FL2-H=Flow Cytometry cell binding*
5.5.2 Biodistribution of $^{124}$I-cG250

$^{124}$I-labelled biodistribution study results are presented in Table 5-8. One mouse died from the $^{124}$I-cG250 day 5 group (tumour embolus), leaving a total of 39 mice for analysis. The %ID/g of the isotope in SK-RC-52 xenografts peaked at $23.45 \pm 5.07$ (mean ± SD) 48 h after antibody injection and was maintained for a further two days ($19.43 \pm 4.31$ and $10.64 \pm 5.64$ %ID/g, respectively). The tumour: blood ratio for $^{124}$I-labelled-cG250 was $4.94 \pm 2.13$ %ID/g on day 3 and reached $30.7 \pm 21.7$ %ID/g in SK-RC-52 on day 7 when blood clearance had occurred. The control antibody $^{124}$I-huA33 had very low tumour uptake (Table 5-8 and Figure 5-18).
Table 5-8 Results of the biodistribution studies

<table>
<thead>
<tr>
<th>% Injected Dose/Gram tissue</th>
<th>$^{124}$I-cG250 (Mean ±SD)</th>
<th>Control $^{124}$I-huA33 (Mean ±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLOOD</td>
<td>Day 0: 32.2 ±5.5</td>
<td>Day 0: 48.5 ±16.3</td>
</tr>
<tr>
<td></td>
<td>Day 2: 12.2 ±4.3</td>
<td>Day 2: 16.3 ±3.7</td>
</tr>
<tr>
<td></td>
<td>Day 3: 4.8 ±2.4</td>
<td>Day 3: 5.7 ±1.3</td>
</tr>
<tr>
<td></td>
<td>Day 7: 0.5 ± 0.5</td>
<td>Day 7: 5.2 ±0.6</td>
</tr>
<tr>
<td>TUMOUR (SK-RC-52)</td>
<td>Day 0: 9.6 ±2.3</td>
<td>Day 0: 5.7 ±1.3</td>
</tr>
<tr>
<td></td>
<td>Day 2: 23.5 ±5.1</td>
<td>Day 2: 13.2 ±1.6</td>
</tr>
<tr>
<td></td>
<td>Day 3: 19.4 ±4.3</td>
<td>Day 3: 5.9 ±1.7</td>
</tr>
<tr>
<td></td>
<td>Day 7: 7.4 ±1.8</td>
<td>Day 7: 13.2 ±1.6</td>
</tr>
<tr>
<td>LIVER</td>
<td>Day 0: 10.9 ±3.2</td>
<td>Day 0: 8.8 ±1.4</td>
</tr>
<tr>
<td></td>
<td>Day 2: 7.0 ±2.2</td>
<td>Day 2: 4.2 ±1.0</td>
</tr>
<tr>
<td></td>
<td>Day 3: 3.4 ±1.2</td>
<td>Day 3: 0.97 ±0.6</td>
</tr>
<tr>
<td></td>
<td>Day 7: 0.3 ±0.2</td>
<td>Day 7: 0.19 ±0.0</td>
</tr>
<tr>
<td>KIDNEY</td>
<td>Day 0: 9.2 ± 2.6</td>
<td>Day 0: 0.29 ±0.1</td>
</tr>
<tr>
<td></td>
<td>Day 2: 3.29 ±0.8</td>
<td>Day 2: 0.4 ±0.0</td>
</tr>
<tr>
<td></td>
<td>Day 3: 1.8 ±0.8</td>
<td>Day 3: 0.60 ±0.2</td>
</tr>
<tr>
<td></td>
<td>Day 7: 0.2 ±0.2</td>
<td>Day 7: 0.30 ±0.1</td>
</tr>
<tr>
<td>BONE</td>
<td>Day 0: 6.9 ± 6.8</td>
<td>Day 0: 0.29 ±0.1</td>
</tr>
<tr>
<td></td>
<td>Day 2: 2.16 ±0.8</td>
<td>Day 2: 0.4 ±0.0</td>
</tr>
<tr>
<td></td>
<td>Day 3: 1.3 ±0.5</td>
<td>Day 3: 0.60 ±0.2</td>
</tr>
<tr>
<td></td>
<td>Day 7: 0.1 ±0.0</td>
<td>Day 7: 0.27 ±0.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TISSUE/BLOOD RATIO</th>
<th>Day 0</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 0</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUMOUR (SK-RC-52)</td>
<td>0.3 ±0.1</td>
<td>2.6 ±2.2</td>
<td>4.9 ±2.1</td>
<td>30.7 ±21.7</td>
<td>0.13 ±0.1</td>
<td>0.3 ±0.1</td>
</tr>
<tr>
<td>LIVER</td>
<td>0.3 ±0.1</td>
<td>0.6 ±0.2</td>
<td>0.9 ±0.6</td>
<td>0.97 ±0.6</td>
<td>0.19 ±0.0</td>
<td>0.3 ±0.0</td>
</tr>
<tr>
<td>KIDNEY</td>
<td>0.3 ±0.0</td>
<td>0.30 ±0.1</td>
<td>0.38 ±0.0</td>
<td>0.60 ±0.2</td>
<td>0.29 ±0.1</td>
<td>0.4 ±0.0</td>
</tr>
<tr>
<td>BONE</td>
<td>0.2 ±0.2</td>
<td>0.2 ±0.0</td>
<td>0.27 ±0.1</td>
<td>0.40 ±0.04</td>
<td>0.1 ±0.0</td>
<td>0.2 ± 0.0</td>
</tr>
</tbody>
</table>

The top half of the table represents the actual percentage of injected dose of radioisotope per gram of tissue. The bottom half summarises the tissue: blood results.
Figure 5-18 Biodistribution data of $^{124}$I-cG250 and control $^{124}$I-huA33 on day 3
5.5.3 $^{124}$I-cG250 PET Imaging

Localisation of $^{124}$I-cG250 in BALB/c mice bearing SK-RC-52 xenograft as determined by PET/CT imaging was clearly apparent (Figure 5-19 and Figure 5-20). At 2 h, some tumour localization was evident, with the images also showing cardiac blood pool activity. At 24 h there was definite localization of antibody to the CAIX-expressing xenograft. Over time with decreasing blood pool activity, the uptake in the SK-RC-52 tumour was more clearly defined.

5.5.4 Correlation between biodistribution and PET studies

There was a significant correlation between tumour uptake with SUV$_{\text{mean}}$ and SUV$_{\text{max}}$ quantified by PET/CT and %/ID $^{124}$I-cG250 measured with a gamma counter (Figure 5-21).
Figure 5-19 Complete panel of coronal PET-CT images of biodistribution of $^{124}$I-cG250.

The complete panels of coronal PET-CT images of biodistribution of $^{124}$I-cG250 in SK-RC-52 xenografts in a BALB/c nude mouse day 3 post injection. Standard black and white PET (top left) with uptake in the xenograft in right shoulder region easily seen on CT (top right). “Thermal” PET images (bottom left) are then co-registered creating the $^{124}$I-cG250 PET/CT image (bottom right).
Figure 5-20 PET-CT images of biodistribution of $^{124}$I-cG250 in a SK-RC-52 xenograft

The “thermal” PET-CT images of biodistribution of $^{124}$I-cG250 in SK-RC-52 xenograft in a BALB/c nude mouse day 3 post injection. A) CT demonstrates the xenograft in right shoulder region; B) $^{124}$I-cG250 PET scan demonstrates uptake in xenograft (arrow) and uptake in the thyroid; C) Co-registered $^{124}$I-cG250 PET/CT image.
The tumour uptake with $\text{SUV}_{\text{mean}}$ (A) and $\text{SUV}_{\text{max}}$ (B) quantified by PET/CT is compared to the biodistribution measured as the percentage of injected dose (%ID) $^{124}\text{I}$-cG250 measured with a gamma counter.
5.5.5 Pharmacokinetics

The calculated pharmacokinetic parameters for the two-compartment model were: $\alpha$ half-life- 2.59 hr, $\beta$ half-life- 40.5 hr; V1- 1.685mL; C1- 0.08148 ml/hr and Serum Area Under Curve- 65.8ng*hr/ml. The estimated pharmacokinetic parameters for the three-compartment model are shown in Table 5-9.

Regarding pharmacokinetics, the three-compartment model generally fits both the serum and tumour data very well (Figure 5-22). However, the last time point in the tumour data is somewhat under-estimated by the model and more antibody is retained in tumour than can be accounted for by a three-compartment model. This suggests that the model requires an additional tumour compartment that traps antibody, though there is probably insufficient data to accurately estimate parameters. Consequently, the three-compartment model most likely provides slight under-estimates of the exposure (Cmax and Area under the curve (AUC)) of tumour to antibody. None-the-less the tumour AUC, 536.1µg.hr/L, is substantially higher than the serum AUC, 61.37µg.hr/L. The estimated volume of the central compartment of 1.766 L. suggests that some initial rapid kinetics occur before the first blood sample.
Table 5-9 Estimated pharmacokinetic parameters for the three-compartment model

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$ half-life</td>
<td>hr</td>
<td>2.545</td>
</tr>
<tr>
<td>$\beta$ half-life</td>
<td>hr</td>
<td>28.40</td>
</tr>
<tr>
<td>$\gamma$ half-life</td>
<td>hr</td>
<td>81.19</td>
</tr>
<tr>
<td>$k_{13}$ half-life</td>
<td>hr</td>
<td>7.504</td>
</tr>
<tr>
<td>$k_{31}$ half-life</td>
<td>hr</td>
<td>65.55</td>
</tr>
<tr>
<td>A</td>
<td>ug/L</td>
<td>1.678</td>
</tr>
<tr>
<td>B</td>
<td>ug/L</td>
<td>1.047</td>
</tr>
<tr>
<td>C</td>
<td>ug/L</td>
<td>0.1048</td>
</tr>
<tr>
<td>$V_1$</td>
<td>L</td>
<td>1.766</td>
</tr>
<tr>
<td>Cl</td>
<td>L/h</td>
<td>0.08148</td>
</tr>
<tr>
<td>Cmax</td>
<td>ug/L</td>
<td>2.831</td>
</tr>
<tr>
<td>Tumour $C_{max}$</td>
<td>ug/L</td>
<td>1.011</td>
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<tr>
<td>Serum AUC</td>
<td>ug.hr/L</td>
<td>61.37</td>
</tr>
<tr>
<td>Tumour AUC</td>
<td>ug.hr/L</td>
<td>536.1</td>
</tr>
</tbody>
</table>

Figure 5-22 Pharmacokinetics for serum (A) versus tumour (B)
5.5.6 Histology, Autoradiography and Immunohistochemistry

Haematoxylin staining indicated that the SK-RC-52 xenografts contained viable tissue peripherally and a small amount in the center of the section, with variable areas of central necrosis and connective tissue (Figure 5-23). Antigen (CAIX) was distributed evenly in areas corresponding to viable tumour cells. Control slides for antigen staining showed no staining in the tumour itself.

$^{124}$I-cG250, assessed by autoradiography, was distributed throughout regions of viable tumour including the central of tumours, with minimal activity in the necrotic areas (Figure 5-23) indicating specific localization of radiolabelled cG250 to tumour cells. A similar distribution of $^{124}$I-cG250 throughout viable tumour cells was seen in all other time points studied (results not shown). On analysis of CAIX expression on days 0 versus 14, there was a trend toward greater expression ($61.27\%\pm19.07$ versus $83.79\%\pm8.75$; mean ± 95% CI), but this was not significant. There was no significant correlation between HP_{2.5} and CAIX expression for either day 0 (p=0.79) or day 14 (p=0.33) groups, nor was there for any other oxygen parameter (mean, HP_{3} or HP_{10}).

In the uptake of $^{124}$I-cG250 achieved in our model was certainly well within the expected range of what has been achieved in other similar studies using RCC xenograft models, including SK-RC-52 (Appendix D).

5.5.7 Monoclonal Antibody PET Imaging with G250 Imaging- Discussion

Necropsy-based biodistribution studies of radiolabelled ligands are usually performed by killing cohorts of animals at various time points after injection, to measure the average activity concentration in organs or tissues of interest. I present a method of quantitative evaluation of radiolabelled antibodies using a clinical PET scanner that may be an attractive option since it would permit simultaneous non-invasive time-dependent biodistribution.
studies of the same group of animals. The present phantom and small-animal measurements suggest that useful \textit{in vivo} biodistribution information may be recovered from murine PET images acquired on a clinical PET scanner. Quantitative murine PET imaging using a clinical scanner may allow faster screening of radiolabelled ligands, with subsequent detailed necropsy studies for those agents that show the most promise. A large-bore clinical scanner offers the possibility of simultaneous imaging and absolute quantitation of tracer uptake for several small animals in a single bed position and increased throughput for the evaluation of PET tracers \textit{in vivo}, which may be of utility in the preclinical evaluation of novel PET ligands.
Figure 5-23 Histology, autoradiography and CAIX staining biodistribution study

SK-RC-52 tumour specimen sections from animals administered $^{124}$I -cG250 and sacrificed on day 0, 2 and 3 with comparative studies of a xenograft tumour from a mouse injected with control $^{124}$I-c huA33 Control (Co): A) H&E staining and B) Autoradiography of the same specimens in column A with dark areas representing high uptake of radiolabelled cG250 (the antibody binding CAIX) and C) CAIX expression assessed by immunohistochemistry.
5.6 Studies of the effects of therapeutic monoclonal antibodies on tumour oxygenation in an RCC xenograft model

To explore with potential for targeting receptors overexpressed in RCC I decided to focus on mAb against: CAIX- mAb cG250; EGFR- mAbs m528 and m806; VEGF- mAb bevacizumab (Avastin™, Roche Pharmaceuticals Division, Basel, Switzerland)

This was based on previous experience within our institute and the known response of RCC to some related agents such as TKI. Our primary objective was to observe the effects of the mAb on tumour oxygenation when compared to controls. Our secondary objective was to examine a small sub-group of human SK-RC-52 xenografted tumours for immunohistochemical parameters to document any differences between the expression of CAIX, EGFR assess vascularity with MVD and osteopontin expression (based on our earlier work in humans.

5.6.1 Results

A total of 50 mice were tested. After transplantation from a donor tumour 47 mice with growing tumours were available (two tumours did not take and one mouse was euthanased after losing weight rapidly secondary to tumour growth). Six groups were created with control (n=10); m806 (n=7); cG250 (n=7); m528 (n=7); cG250 +m528 (n=7) and bevacizumab (n=9).

In summary, the mice were treated with the antibodies and then culled at 16 days after having tumour oxygen measurements. The end point was the tumour pO2. This endpoint was chosen as opposed to observing for a prolonged response or survival because I wanted to know if the tumour pO2 was altered by the different treatment regimes.
5.6.2 Therapeutic Response of Xenografts to Monoclonal Antibodies

The monoclonal antibodies m528, bevacizumab and the combination of m528 and cG250 appeared to have the best tumouristatic effect on the xenografts ahead of m806 and cG250 alone when compared to controls (Figure 5-24 and Figure 5-25).

5.6.3 Tissue Oxygenation Measurements

Again the oxylite probe was used with measurements taken at day 16 prior to culling. This was done to monitor the effects of therapy on tumour oxygenation in the xenograft model.

The results of tumour parameters and comparisons with oxygenation are presented in three tables: Table 5-10, Table 5-11 and Table 5-12.
Figure 5-24 SK-RC-52 Xenograft Tumour Volume by Therapeutic Group

Renal Cell Carcinoma (SK-RC-52) Xenograft Therapy Study Summary Data
SK-RC-52 Tumour Volume by Therapeutic Group Normalised so all groups start may be easily compared from the time point (same data as in Figure 5-25)
Figure 5-26 Summary of oxygen measurements in normal and tumour tissue by group

Renal Cell Carcinoma (SK-RC-52) Xenograft Therapy Study
Oxygen Measurements: Normal versus Tumour tissue

Tissue pO₂ (mean ± SEM)

- Normal Tissue
- Tumour Tissue
Table 5-10 Part I. Summary of xenograft therapy groups with tumour characteristics at day 16 post therapy

<table>
<thead>
<tr>
<th>Tumour Volumes Pre Therapy (g)</th>
<th>Control</th>
<th>cG250</th>
<th>m528</th>
<th>cG250 + m528</th>
<th>Bevacizumab (Avastin)</th>
<th>m806</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ±SEM</td>
<td>270.1 ± 42</td>
<td>261.4 ± 71.19</td>
<td>254.9 ± 27.74</td>
<td>209.8 ± 24.26</td>
<td>338.4 ± 44.06</td>
<td>361.3 ± 66.19</td>
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<td>Median</td>
<td>252.00</td>
<td>232.70</td>
<td>236.10</td>
<td>200.90</td>
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<td>333.20</td>
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<tr>
<td>Range</td>
<td>118.2-477.3</td>
<td>64.64-648.9</td>
<td>148.4-372.2</td>
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<tr>
<td>Compared to Control-p value</td>
<td>—</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<tr>
<th>Tumour Volumes Post Therapy (g)</th>
<th>Control</th>
<th>cG250</th>
<th>m528</th>
<th>cG250 + m528</th>
<th>Bevacizumab (Avastin)</th>
<th>m806</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ±SEM</td>
<td>775.3 ± 89.72</td>
<td>582.6 ± 80.95</td>
<td>273.2 ± 28.95</td>
<td>299.1 ± 39.24</td>
<td>445.7 ± 68.76</td>
<td>704.9 ± 50.54</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>Median</td>
<td>817.00</td>
<td>677.40</td>
<td>231.70</td>
<td>282.30</td>
<td>427.40</td>
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<td>Range</td>
<td>421.5-1187</td>
<td>286.8-818.3</td>
<td>211.2-408.6</td>
<td>205.6-508.6</td>
<td>143.2-763.9</td>
<td>572.8-966.4</td>
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</tr>
<tr>
<td>Compared to Control-p value</td>
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<td>0.0001</td>
<td>0.0007</td>
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<table>
<thead>
<tr>
<th>Tumour Size Potpie Therapy (%)</th>
<th>Control</th>
<th>cG250</th>
<th>m528</th>
<th>cG250 + m528</th>
<th>Bevacizumab (Avastin)</th>
<th>m806</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ±SEM</td>
<td>318.5 ± 30.34</td>
<td>276.8 ± 39.23</td>
<td>112.6 ± 14.62</td>
<td>145.9 ± 14.3</td>
<td>131 ± 12.16</td>
<td>227 ± 30.91</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>Median</td>
<td>326.80</td>
<td>262.50</td>
<td>9.282</td>
<td>138.90</td>
<td>117.60</td>
<td>214.50</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>191-454.2</td>
<td>104.4-443.6</td>
<td>84.86-180.2</td>
<td>91.1-199.5</td>
<td>81.72-204.6</td>
<td>87.5-348.5</td>
<td></td>
</tr>
<tr>
<td>Compared to Control-p value</td>
<td>—</td>
<td>0.6009</td>
<td>0.0001</td>
<td>0.0002</td>
<td>&lt;0.0001</td>
<td>0.1088</td>
<td></td>
</tr>
</tbody>
</table>
Table 5-11 Part II. Summary of xenograft therapy groups animal weights pre and post therapy

<table>
<thead>
<tr>
<th>Animal Weight Pre therapy (g)</th>
<th>Control</th>
<th>cG250</th>
<th>m528</th>
<th>cG250 + m528</th>
<th>Bevacizumab (Avastin)</th>
<th>m806</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ±SEM</td>
<td>16.88 ± 1.65</td>
<td>18.4 ± 0.3986</td>
<td>18.31 ± 0.6639</td>
<td>19.48 ± 0.519</td>
<td>18.49 ± 0.5948</td>
<td>17.82 ± 0.5713</td>
<td>0.3494</td>
</tr>
<tr>
<td>Median</td>
<td>18.57</td>
<td>18.43</td>
<td>18.65</td>
<td>19.64</td>
<td>18.74</td>
<td>18.39</td>
<td></td>
</tr>
<tr>
<td>Compared to Control-p value</td>
<td>—</td>
<td>&gt;0.10</td>
<td>&gt;0.10</td>
<td>&gt;0.10</td>
<td>&gt;0.10</td>
<td>&gt;0.10</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Animal Weight Post therapy (g)</th>
<th>Mean ±SEM (mmHg)</th>
<th>Median (mmHg)</th>
<th>Range (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18.33 ± 0.90</td>
<td>17.39 ± 0.63</td>
<td>12.72 - 20.91</td>
</tr>
<tr>
<td></td>
<td>18.09 ± 1.06</td>
<td>19.34 ± 0.41</td>
<td>14.02 - 18.76</td>
</tr>
<tr>
<td></td>
<td>18.93 ± 0.65</td>
<td>18.43 ± 1.22</td>
<td>17.7 - 20.53</td>
</tr>
<tr>
<td></td>
<td>19.36</td>
<td>19.16</td>
<td>14.21 - 20.88</td>
</tr>
<tr>
<td></td>
<td>19.36</td>
<td>19.16</td>
<td>14.21 - 20.88</td>
</tr>
<tr>
<td></td>
<td>11.7 - 21.26</td>
<td>11.7 - 21.26</td>
<td>11.7 - 21.26</td>
</tr>
<tr>
<td>Compared to Control-p value</td>
<td>—</td>
<td>&gt;0.10</td>
<td>&gt;0.10</td>
</tr>
<tr>
<td></td>
<td>&gt;0.10</td>
<td>&gt;0.10</td>
<td>&gt;0.10</td>
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<tr>
<td></td>
<td>&gt;0.10</td>
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<tr>
<td></td>
<td>&gt;0.10</td>
<td>&gt;0.10</td>
<td>&gt;0.10</td>
</tr>
</tbody>
</table>

336
Table 5-12 Part III. Summary of xenograft therapy groups with oxygenation day 16

<table>
<thead>
<tr>
<th>Normal $pO_2$ (mmHg)</th>
<th>Control</th>
<th>cG250</th>
<th>mg528</th>
<th>cG250+mg528</th>
<th>Bevacizumab (Avastin)</th>
<th>m806</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ±SEM</td>
<td>37.09 ± 2.05</td>
<td>38.23 ± 2.06</td>
<td>36.87 ± 2.22</td>
<td>31.54 ± 0.81</td>
<td>31.94 ± 1.10</td>
<td>38.39 ± 2.414</td>
<td>0.0328</td>
</tr>
<tr>
<td>Median</td>
<td>38.81</td>
<td>40.90</td>
<td>39.30</td>
<td>30.05</td>
<td>29.90</td>
<td>36.30</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>22.3 - 45.78</td>
<td>29.9 - 43.53</td>
<td>26.9 - 42.1</td>
<td>29.36 - 34.12</td>
<td>28.81 - 37.13</td>
<td>31.05 - 50.16</td>
<td></td>
</tr>
<tr>
<td>Compared to Control p value</td>
<td>—</td>
<td>0.6691</td>
<td>0.9623</td>
<td>0.0136</td>
<td>0.0172</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

Tumour $pO_2$ (mmHg)

| Mean ±SEM            | 3.04 ± 0.43 | 3.28 ± 0.35 | 14.21 ± 1.66 | 7.90 ± 1.92 | 10.35 ± 1.658 | 2.457 ± 0.6247 | p<0.0001 |
| Median               | 3.20       | 3.18 | 16.77 | 8.75 | 8.79 | 1.70 |
| Range                | 0.61 - 5.22 | 1.66 - 4.39 | 7.9 - 18.29 | 2.57 - 13.43 | 2.98 - 17.8 | 1.12 - 5.72 |
| Compared to Control p value | — | 0.8125 | 0.0001 | 0.1088 | 0.0006 | 0.2698 |

Tumour $pO_2$ mean: Normal

| Mean ±SEM            | 8.89 ± 1.66 | 8.973 ± 1.375 | 39.92 ± 6.015 | 24.62 ± 5.87 | 32.4 ± 5.196 | 6.79 ± 1.94 | p<0.0001 |
| Median               | 8.61       | 8.34 | 41.07 | 25.65 | 27.69 | 4.13 |
| Range                | 1.47 - 19.01 | 3.98 - 14.02 | 18.77 - 67.19 | 8.57 - 42.69 | 10.22 - 54.5 | 2.63 - 16.99 |
| Compared to Control p value | — | 0.8868 | 0.0002 | 0.0431 | 0.0003 | 0.4173 |

Tumour Volumes Pre Therapy (g)

| Mean ±SEM            | 270.1 ± 42 | 261.4 ± 71.19 | 254.9 ± 27.74 | 209.8 ± 24.26 | 338.4 ± 44.06 | 361.3 ± 66.19 | 0.2464 |
| Median               | 252.00     | 232.70 | 236.10 | 200.90 | 343.10 | 331.20 |
| Range                | 118.2 - 477.3 | 64.6 - 648.9 | 148.4 - 372.2 | 152.1 - 322.9 | 175.2 - 529 | 176.7 - 695 |
| Compared to Control p value | — | NS | NS | NS | NS | NS |

Tumour $pO_2$: Tumour Volume (%)

| Mean ±SEM            | 0.46 ± 0.09 | 0.68 ± 0.15 | 5.59 ± 0.96 | 2.81 ± 0.78 | 2.86 ± 0.57 | 0.37 ± 0.11 | p<0.0001 |
| Median               | 0.41       | 0.51 | 4.10 | 2.64 | 3.05 | 0.28 |
| Range                | 0.054 - 0.97 | 0.20 - 1.45 | 3.31 - 8.63 | 0.89 - 6.06 | 0.54 - 5.72 | 0.12 - 0.99 |
| Compared to Control p value | — | 0.3148 | 0.0001 | 0.0004 | 0.0003 | 0.5487 |
The summary statistics in comparing therapeutic groups revealed no significant difference between animal weight pre and post therapy.

Considering tumour volumes, pre-therapy tumour volumes were not significantly different but there was a difference between post therapy groups, particularly with the m528, cG250 +m528 and bevacizumab therapy groups. These groups had significantly smaller tumours than the controls. These differences held true when adjusting for initial volume by having a ratio of post therapy volume to pre therapy volume. This highlights the therapeutic impact of the mAb more as tumourstatic agents rather than tumouricidal. It also matches with other xenograft studies with human RCC using for example EGFR blocking therapies. Importantly, my data confirms clinical data in human trials where the same observations occur with very few complete responses recorded with bevacizumab or any TKI to date.

The oxygenation status of the tumours was also different when comparing to controls (Table 5-12). Firstly, the tumour oxygen status of the controls fell well into the hypoxic range over the therapeutic period. In general the lower levels in the controls may reflect the fact that the tumours were passaged a second time when compared to the first round of experiments indicating more de-differentiation and robustness with the tumour model in these experiments.

In any case, comparing to the therapeutic groups again, in general the different mAb had higher tumour oxygen readings in the groups where the tumours experienced less growth being the m528, cG250 +m528 and bevacizumab therapy although only the m528 and bevacizumab had significant differences compared to controls. When the tumour: normal ratios were compared, which accounts for oxygenation status in the normal tissue and helps correct for differences, all three groups were significantly different than the controls. The tumour oxygen status as a percentage of total tumour volume was also significantly greater in these three groups.
Interestingly, the tumour oxygen status of normal tissue was significantly reduced compared to controls in both the cG250 with m528 and bevacizumab but not the m528 alone group. One explanation is that the mAb are affecting the normal vasculature in a negative manner and perhaps not even in the same manner as within the tumour vasculature. However, one would have expected the m528 alone group to demonstrate a similar change. Nevertheless, the impact of agents is different and the combination may have enhanced normal tissue effects.

5.6.4 Discussion of Therapy Study Tissue Oxygenation Measurements

From these results, I may conclude that in the SK-RC-52 RCC xenograft model over a 16 day period that:

1) The mAb m528 and bevacizumab reduce growth of treatment (cG250 when added to m528 provided no additional benefit and in fact reduced the benefit of m528 alone)

2) The mAb m528 and bevacizumab improve oxygenation in tumours. This may be explained in part by the lack of growth of the tumours and thus the vasculature remaining more normalised. However, when tumour oxygen status is corrected as a percentage of tumour volume, the results were still significant, indicating that it is also likely that the tumour vasculature is altered by the mAb to become more normalised and improve oxygenation, not becoming as hypoxic

3) The combination of mAb cG250 +m806 had little impact on tumour growth or oxygenation in this RCC model
5.6.5 Tissue Immunohistochemistry

Selected tumours (n=5) from controls, the bevacizumab and m528 groups had immunohistochemistry as well as oxylite probe measurements conducted (Table 5-13 and Table 5-14; Figure 5-27).

The results of immunohistochemistry are that firstly, EGFR is expressed in the SK-RC-52 model. There were differences between groups regarding MVD, EGFR expression and osteopontin expression. This was not the case for CAIX expression.

Regarding comparisons between the therapy groups and controls, MVD was significantly increased in the m528 group but not the bevacizumab when compared to controls. However, osteopontin expression in both the m528 and bevacizumab groups demonstrated significantly more than controls. EGFR expression was significantly more in the bevacizumab group than in controls but not for the m528 group. CAIX expression was not significantly different to the controls.
Figure 5-27 Immunohistochemistry of a representative xenograft

Osteopontin

MVD

CAIX
### Table 5-13 Part 1. Immunohistochemistry studies in selected xenograft groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Bevacizumab (anti-VEGF)</th>
<th>m528 (anti-EGFR)</th>
<th>p value (across groups)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normal pO₂ (mmHg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ±SEM</td>
<td>39.71 ± 1.45</td>
<td>36.1 ± 0.68</td>
<td>38.23 ± 1.03</td>
<td>0.0524</td>
</tr>
<tr>
<td>Median</td>
<td>39.91</td>
<td>35.21</td>
<td>39.20</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>29.6 - 46.52</td>
<td>34.94 - 39.05</td>
<td>34.07 - 40.83</td>
<td></td>
</tr>
<tr>
<td>Compared to Control - p value</td>
<td>—</td>
<td>&gt;0.10</td>
<td>&gt;0.10</td>
<td></td>
</tr>
</tbody>
</table>

| **Tumour pO₂ (mmHg)**|         |                         |                  |                         |
| Mean ±SEM            | 2.37 ± 0.14 | 12.65 ± 2.15       | 17.53 ± 1.90     | P<0.0001                |
| Median               | 2.25      | 12.36                  | 18.03            |                         |
| Range                | 1.06-4.38  | 1.25-30.15            | 7.48-31.69       |                         |
| Compared to Control - p value | —       | P<0.0002              | P<0.0001         |                         |

| **Tumour pO₂ mean: Normal (%)**|         |                         |                  |                         |
| Mean ±SEM            | 6.047 ± 0.82 | 34.62 ± 16.51      | 45.41 ± 1.79     | 0.0695                  |
| Median               | 6.21      | 34.62                  | 45.41            |                         |
| Range                | 3.92-7.86  | 18.1-51.13            | 43.61-47.2       |                         |
| Compared to Control - p value | —       | >0.10                  | >0.10            |                         |

| **Tumour Volumes Post Therapy (g)**|         |                         |                  |                         |
| Mean ±SEM            | 845.8 ± 152 | 732.9 ± 31.05      | 648.3 ±32.44     | 0.4083                  |
| Median               | 855.40    | 732.90                 | 648.30           |                         |
| Range                | 485.4-1187 | 701.8-763.9        | 615.9-680.7      |                         |
| Compared to Control - p value | —       | >0.10                  | >0.10            |                         |
Table 5-14 Part II. Immunohistochemistry studies in selected xenograft groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Bevacizumab (anti-VEGF)</th>
<th>m528 (anti-EGFR)</th>
<th>p value (across groups)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MVD (% cells positive)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ±SEM</td>
<td>9.77 ± 0.80</td>
<td>10.07 ± 1.491</td>
<td>15.74 ± 1.58</td>
<td>0.0029</td>
</tr>
<tr>
<td>Median</td>
<td>8.00</td>
<td>6.50</td>
<td>14.00</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>2-27</td>
<td>0-28</td>
<td>3-33</td>
<td></td>
</tr>
<tr>
<td><em>Compared to Control- p value</em></td>
<td>—</td>
<td>0.52</td>
<td>0.0001</td>
<td></td>
</tr>
</tbody>
</table>

| **EGFR (% cells positive)** |       |                          |                  |                         |
| Mean ±SEM                 | 45.1 ± 3.645 | 70.89 ± 4.05            | 50.56 ± 5.751    | 0.0002                  |
| Median                    | 30.00        | 80.00                    | 60.00            |                         |
| Range                     | 10-90        | 20-95                    | 10-90            |                         |
| *Compared to Control- p value* | —             | P<0.0001                | 0.44             |                         |

| **Osteopontin (% cells positive)** |       |                          |                  |                         |
| Mean ±SEM                  | 36.27 ± 3.55 | 47.86 ± 1.808            | 55.19 ± 3.515    | 0.0004                  |
| Median                     | 30.00        | 50.00                    | 60.00            |                         |
| Range                      | 5-95         | 30 70                    | 20 90            |                         |
| *Compared to Control- p value* | —             | 0.01                     | 0.0001           |                         |

| **CAIX (% cells positive)** |       |                          |                  |                         |
| Mean ±SEM                  | 51.57 ± 4.13 | 57.86 ± 2.59            | 55.56 ± 4.45     | 0.7299                  |
| Median                     | 60.00        | 60.00                    | 60.00            |                         |
| Range                      | 10-95        | 30-80                    | 30-95            |                         |
| *Compared to Control- p value* | —             | >0.10                    | >0.10            |                         |
5.6.6 Discussion of Therapy Study Immunohistochemistry groups

EGFR is expressed in the SK-RC-52 model. This has not been previously demonstrated. This finding is intuitive and helps explain the effect of m528 (anti-EGFR) in reducing tumour growth. The finding of EGFR expression being significantly more in the bevacizumab group than in controls but not for the m528 group was noted. It may be that the simplest explanation is that the m528 was not able to downregulate the receptor expression but prevented overexpression. This observation fits well with prior work at our institution whereby when used as single agents, neither mAbs 528 or 806 induced down-regulation of EGFR either in vitro or in vivo in non-RCC human tumour xenograft models. As therapeutic studies examining RCC do not exist, we may observe other xenograft tumour models such as gliomas, whereby tumour response was found to be dependent on the presence of amplified and/or mutated EGFR. Our model had such expression of EGFR and the response again supports these prior findings in other tumours.

Regarding MVD, the results with bevacizumab support prior clinical work in therapeutic effects in clinical studies and a return of the vasculature to a more normal (but not totally) pattern. The MVD pattern was more extreme in the m528 group which supports the muted response in reducing tumour growth.

Interestingly CAIX expression was fairly consistent between the control and both treatment groups. Certainly mAb interfere with sub-cellular mechanisms of angiogenesis but why the bevacizumab did not have greater effect than the m528 group is not explained. Clearly the interplay between different pathways is complex and it may be that the alignment between CAIX expression and tumour growth is less so in this RCC model. Larger studies of CAIX expression would be needed to characterize this further.
Osteopontin is linked to hypoxia and angiogenesis but the relationship is complex. One may have expected the osteopontin expression to stabilise where therapy was successful or possibly increase over the timecourse where it was not or less successful. This is given increased expression and poorer prognosis, at least in the metastatic setting. This is what did occur with the bevacizumab having only a minor but significant increase over controls whilst the increase in the mAb group was greater, where less effect on tumour growth was noted. In the human model, as has been described, serum levels appeared elevated and further studies should compare serum and immunohistochemical expression.

### 5.6.7 Conclusions from Therapy Study

RCC may be like other tumours where it is proposed that the vasculature returns from a chaotic architecture in the hypoxic state to a more normal architecture when adequate treatment inhibiting angiogenesis occurs. This requires more research focusing on vascular architecture but was supported in principle by our findings.

As has been outlined in the past, single EGFR therapeutics do not inhibit all the functions associated with the EGFR; thus, it may be necessary to target the EGFR with multiple therapeutics. This may also involve different temporality of mAb or other TKI. This will need to be investigated in other models and also to further elucidate if oxygen status is a predictor of response.
5.7 Xenografting tumour beneath the renal capsule using modern surgical equipment

Human renal xenografts of RCC were transplanted using an automated biopsy gun to harvest the tumour tissue and an epidural needle with introducer to easily deploy the grafts under the renal capsule. This is a new experimental model and has many applications from renal subcapsular xenografts.

5.7.1 Results

A total of 36 mice were tested. Twelve mice were considered to produce donor tumours available for transplantation of which two mice were ultimately chosen and their tumour harvested by biopsy gun and applied either subcapsularly (n=12) or subcutaneously (n=12). Mice used for tumour donation (n=2), one for the subcapsular group and one for the subcutaneous group, were later sacrificed using sevofluorane after the tumour was harvested. The other mice did not have transplants (n=10) and were used in other studies.

At five and ten weeks half (n=6) of each of the subcapsular and subrenal groups mice were sacrificed with the kidneys and tumours harvested (Figure 5-28). Tumours (n=24) were measured in two planes with digital calipers to calculate volume (length x width²/2). Tumours were then frozen with liquid nitrogen.

A group (n=12) of BALB/c nude mice were used in the experiment. Viable tumour grafts were obtained under the kidney capsules and subcutaneously in ninety percent of the female BALB/c nude mice, as indicated by visual inspection and histology (figure 3). There was clear delineation between the tumour, normal tissue and renal capsule in all specimens. Tumour spillage was not noted in any specimen. Morbidity and mortality was not experienced apart from one mouse that died in the very first subrenal group transplanted. Histologic assessment involved analysis of 5-micron tissue sections that were obtained using a microtome with haematoxylin and eosin staining of slides.
Figure 5-28 Two examples of subcapsular tumour xenografts in situ (left) and ex-vivo (right)

Figure 5-29 Histologic assessment of a human RCC tumour xenograft

Histologic assessment demonstrating the cut frozen specimen (top left) with the tumour extending from beneath the renal capsule. Haematoxylin and eosin staining demonstrates the tumour (T) growing from beneath the capsule (C) of the normal mouse kidney (K) at low and high power.
Growth of the xenografts was relatively slow initially and then accelerated, as did the subcutaneously implanted mice, being consistent with previous experiments involving RCC models (Figure 5-30). Although by ten weeks the tumour volume (mm$^3$) achieved was greater in the subrenal capsular graft model (229.68 ±118.32; mean ± 95% C.I.) compared to the subcutaneous model (163.81 ±43.3.), there was overlap of confidence intervals so statistical significance was not reached (p<0.05).

All tumours were harvested from the same donor mouse in each group (SK-RC-52; Human Renal Cell Carcinoma) with cores (4mm$^3$) taken via automated biopsy for all grafts.

High tumour take rates were achieved in the subcapsular model, and this is to be expected given the rich vascular bed.$^{681}$ Tumour take rates of between 50 and 100% have been reported but depend on the number of cells injected or the method of xenografting, as well as the tumour type grafted.$^{678,682}$ With regard to this subrenal model having a trend toward larger grafts, repeat experiments may yield a statistically significant difference. This, however, was not the aim of this current study that sought primarily to identify an easily reproducible means for subcapsular grafting of a constant volume of malignant tissue. One possible additional explanation for this improved tumour growth is that the subcapsular space may offer reduced contamination by non-cancerous tissue.$^{682}$
Figure 5-30 A graph depicting the growth of human RCC in subcutaneously and subrenal xenografts

Xenograft Tumour Growth
(Renal Cell Carcinoma, SK-RC-52)

Tumour Volume (mm$^3$) [Mean +/- SE Mean]

Timepoint:
- Week 0
- Week 5
- Week 10

Legend:
- Subcutaneous Model
- Subrenal Capsular Model
5.7.2 Discussion

Advantages of implantation under the renal capsule include the well vascularised bed that is available and the ability to graft with smaller numbers of cells than in subcutaneous models. Furthermore, human tumour xenografts, serially transplanted under the renal capsule in immunocompromised mice, possess advantages as a source of tumour tissue. Such xenografts represent a constant and readily available source of material that may be produced in desired quantities. In contrast to implanting patient biopsies, they permit the repetition of experiments on the same fresh, previously untreated tumour cells. Hence the role of the subrenal capsule model in investigating the action of chemotherapeutic agents as described above is significant.

Our model has multiple advantages over that traditionally used, as proposed by Lee and colleagues, where tumour grafts were harvested then cut into small pieces prior to dissecting the renal capsule and placing grafts. The first advantage is one of time. By their own admission, the procedure they propose is time consuming and requires meticulous dissection and thus some training in surgery. Our model requires no meticulous dissection and is quick. Harvesting of the graft takes only seconds and one measurement of core length when using the automated biopsy system. Secondly, the core obtained is consistent with each biopsy ensuring reproducibility. Thirdly, only having to obtain one measurement (length) reduces error with tumour volume measurement and damage to the graft prior to transplantation. Finally, the core is easily placed into the epidural needle, again reducing handling before beneath placed gently beneath the capsule with minimal bleeding by being deployed quickly and accurately.

Clearly any deployment method placing the tumour beneath the renal capsule will cause trauma. The object with any model is to minimize that trauma. I accept that fine spring-loaded scissors to open the renal capsule and a blunt-end Epidural needle, or fine forceps, could be used to introduce the tumour sample under the capsule rather than a sharp needle. Alternate deployment techniques do not detract from the technique of reproducing the
tumour graft size for transplant and in fact could potentially enhance this model. As I appeared to have no issue with trauma using the epidural needle, more studies would be needed to prove the point of trauma although it is a theoretical possibility.

The size of the tumour graft is important as its survival depends on neovascularisation within the graft.\textsuperscript{682} Typically, grafts of 1\,mm\textsuperscript{3} or slightly larger are used.\textsuperscript{681,688} I chose slightly larger grafts given that RCC typically grows slowly and the slightly larger size did not appear to impede growth in our model, presumably due to the rich blood supply of the kidney. The growth of the subrenal model was greater than the subcutaneous model as expected from past models, but would need to be confirmed in further studies and other cell lines for significance in renal carcinoma.

This model may be adapted to almost any type of human or murine tumour model as other renal subcapsular models have been in the past. It may also be used in other organ-specific or subcutaneous models with the same equipment. Serial transplantation of human tumours between animals prior to harvesting and even fresh human tissue placed under the renal capsule has been used.\textsuperscript{680,682,683,688}

In relation to growth of human RCC the preferred and easiest method has been subcutaneous transplantation or inoculation. However, it may be advantageous to implant renal cell carcinoma orthotopically into the mouse kidney as described to reproduce tumour behaviour accurately.\textsuperscript{733,734} Clearly, this model offers an alternative to other subrenal capsule models involving injection or time-consuming and imprecise dissections and is feasible for renal cell carcinoma as well as other tumour models.
5.7.3 Conclusions

I fused modern surgical technology with a well-established model in a bid to allow fast, reproducible and reliable tumour volume delivery beneath the renal capsule. Human renal xenografts of RCC were transplanted using an automated biopsy gun to harvest the tumour tissue and an epidural needle with introducer to easily deploy the grafts under the renal capsule. This model has many applications from renal subcapsular xenografts and may be adapted to both organ-specific and subcutaneous xenograft situations.

In conclusion the ease of use, speed and reproducibility of the model for tumour transplantation under the renal capsule makes it an attractive alternative to previously described models. Clearly, modern surgical equipment may be utilised in the laboratory and allows for the development of new and reliable techniques for transplanting tumours that may then be used for further analysis and study. Even simple measures such as using modern dissolving sutures rather than permanent (e.g. silk) may improve animal care. Surgical researchers and laboratory-based scientists should embrace new techniques and utilise them where appropriate in order to improve the efficiency and reproducibility of tumour transplantation studies.
Chapter 6
EXECUTIVE SUMMARY AND FUTURE DIRECTIONS

6.1 Relevance of the findings to our current understanding of RCC hypoxia and angiogenesis .......................................................... 356
6.2 Implications for research of hypoxia and angiogenesis in RCC .................................. 357
6.3 Implications for clinical practice of patients with RCC .......................................... 359
6.4 Directions for future research ............................................................................. 360
The hypoxic cell hypothesis has dominated radiobiological thinking for many decades.\(^{160,228}\)

In 1988, an editorial in the *International Journal of Radiation Oncology, Biology and Physics* was entitled ‘Seduced by Oxygen’ demonstrating the impact of such thinking.\(^{735}\)

More recently in 2003, Bottaro and Liotta\(^{736}\) revisited the situation in their article in *Nature* titled ‘Out of air is not out of action’ reinforcing interest in the relationship of oxygen with tumours and that hypoxic cells remain viable.

In exploring the topic of hypoxia and angiogenesis in RCC, I outlined several hypotheses. I now reflect on the support each of these given the findings above after completing this thesis:

1. RCC are relatively hypoxic, particularly in the periphery of the tumour, where viable hypoxic cells are most likely to reside.
   
   *Thesis findings:* support this hypothesis

2. In RCC a relationship exists between hypoxia and \(^{18}\)F-FMISO uptake. \(^{18}\)F-FDG uptake may be related to hypoxia in RCC because HIF-1 activates transcription of genes including glucose transporters and glycolytic enzymes, a feature of malignant cells that is the basis of \(^{18}\)F-FDG uptake.
   
   *Thesis findings:* do not support the hypothesis that a direct relationship exists between hypoxia and \(^{18}\)F-FMISO uptake; no direct relationship exists between hypoxia and \(^{18}\)F-FDG; no direct relationship exists between \(^{18}\)F-FMISO and \(^{18}\)F-FDG uptake. In humans marginal \(^{18}\)F-FMISO uptake in RCC whilst in a xenograft model there was significant \(^{18}\)F-FMISO uptake.

3. A correlation exists between the presence of hypoxia and angiogenesis in RCC.
   
   *Thesis findings:* support this hypothesis
In response at least in part to the interest in tumour hypoxia and angiogenesis, the concept of molecular target credentialing in oncology has been advocated by the National Cancer Institute (NCI) of the USA and involves cancer imaging, cancer signatures and molecular targets of prevention and treatment. Hypoxia has been identified as a model for studying the interrelationship of these three areas.\textsuperscript{737} The NCI also outlined a concept that included the three Ds: discovery, development, and delivery where the fields of diagnostic imaging, nuclear medicine, image-guided therapy and radiation therapy can be important contributors to science and health care within this new paradigm.\textsuperscript{738}

Through translational research, this thesis fulfils many of the concepts outlined by the NCI, particularly for discovery and for combining imaging and hypoxia and so is timely in its findings. Prior to the completion of this thesis, our understanding of tumour hypoxia and RCC was limited because:

1. Although oxygen status is important to the pVHL pathway, and hypoxia is constantly referred to in relation to RCC, no one had directly
   a. measured RCC or normal renal tissue oxygen levels
   b. studied oxygen levels in human RCC xenografts
   I now have established that tumour hypoxia in patients with RCC and in human RCC xenografts.

2. No studies have compared tissue oxygen levels with indirect immunohistochemical measurements of hypoxia and angiogenesis: This has now been studied in RCC.

3. We have no non-invasive methods of targeting tumour hypoxia in RCC. In particular, there are no studies examining $^{18}$F-FMISO, the most studied hypoxic PET radiotracer, in RCC. Further, no studies of $^{18}$F-FLT in RCC xenografts or humans with RCC: $^{18}$F-FMISO now studied and established uptake in RCC but limited clinical utility in patients. As expected, $^{18}$F-FDG is limited for primary diagnosis of RCC but also is not a reasonable utility for imaging of hypoxia.
4. No extensive studies of serum osteopontin, a surrogate marker of tumour hypoxia have been conducted in primary RCC or in conjunction with oxygen measurements: encouraging results in patients with primary RCC for the first time.

5. There are no studies of $^{124}$I-cG250 in RCC tumour xenograft models that have examined tumour oxygen levels: now studied with excellent uptake and relationship explored.

6. No therapeutic studies in xenografts of systemic treatments in conjunction with oxygen measurements within tumours: comprehensive analysis of oxygenation and response to therapy in the SK-RC-52 model of human RCC.

7. Orthotopic models for developing xenografts in kidney cancer have not been explored using modern surgical equipment: a new method for transplanting xenograft tumours beneath the renal capsule using modern surgical equipment has been described.

8. No studies of $^{18}$F-FLT in RCC xenografts or humans with RCC: First patient dramatic uptake although this was in TCC. $^{18}$F-FLT appears promising but further studies are ongoing.

After the completion of this thesis, many of these questions have been explored and our understanding has been advanced so that research into RCC may evolve even further in the future.

6.1 Relevance of the findings to our current understanding of RCC hypoxia and angiogenesis

Hypoxia, angiogenesis and functional imaging of RCC with PET-CT have been the major themes of this thesis. We now may state with force that RCC is an hypoxic tumour. Regarding angiogenesis, the results of the immunohistochemistry supports upregulation of the sub-cellular mechanisms driving hypoxia but that the relationship is complex, and
although globally related, the actual pO$_2$ is more often not inversely related to expression of immunohistochemical markers of angiogenesis.

The issue of sample size is important to consider as some groups were small and thus it is difficult to draw conclusions, particularly with statistical tests. It is important that this is factored into any interpretation of the data presented in this thesis particularly where with smaller groups equivocal or borderline significant differences found may have increased or decreased with a larger sample size.

### 6.2 Implications for research of hypoxia and angiogenesis in RCC

Although direct measurement of tumour hypoxia using needle electrodes is robust and reproducible, this is an invasive technique that requires special equipment and expertise that is expensive to acquire and maintain. Other limitations, include its invasive nature making it difficult to access tumours, interoperator variability, and the limited number of centres with the necessary equipment and technical expertise. Training and mentoring for our unit was provided by the company manufacturing the probe (Helzel Medical Systems, Germany). Such training is imperative to obtain successful readings; as is having a regular service agent who can solve technical issues. Ultimately, the POS was able to provide enough data in support for our hypothesis of hypoxia in RCC.

Until newer technology is created for use in humans, those considering measuring pO$_2$ in RCC will need to use the POS but this requires local support and regular checks to prevent software malfunctions. The diminishing application of open surgery for RCC will also limit the breadth of tumours available for recording pO$_2$, which will restrict data collection. The oxylite probe in the RCC xenograft models was technically less challenging and will continue to play an important role in investigating oxygenation of RCC.
Although researchers may measure tissue pressures of oxygen or even attempt to further enhance our knowledge of hypoxia by grading tumours based on hypoxic fractions\textsuperscript{212} and this will be important particularly in xenograft models, future efforts in humans may be better off looking at more global non-invasive measures. As has been suggested,\textsuperscript{742} it may not be necessary to provide accurate estimates of tumour median pO\textsubscript{2} or HP\textsubscript{5} in all circumstances. This is because clinicians often use information from categorical variables (like stage) when making management decisions. Thus, it may be sufficient for clinical purposes simply to categorize a tumour as either normoxic or hypoxic, recognizing that this is a very simplified representation of the complex oxygen dynamics that exist in tumours. This may be done with a limited number of POS measurements\textsuperscript{183} or more realistically, in RCC, with non-invasive endogenous or indirect markers or imaging studies. This is because open kidney surgery is declining rapidly and the ability to measure pO\textsubscript{2} directly in the laparoscopic surgical era will remain a challenge due to probe imitations and the new instrumentation required, making non-invasive techniques more attractive.

For RCC, the perfect non-invasive technique to determine hypoxia is yet to be developed. For PET, the ideal hypoxia tracer is one regulated by oxygen exclusively and, as an all-or-nothing response at a well-defined pO2 level, shows rapid and cell-type–independent accumulation in hypoxic cells; and should be cleared completely from well oxygenated tissue at the time of imaging. Present tracers satisfy none of these requirements.\textsuperscript{584} Therefore, better non-invasive markers of hypoxia in RCC need to be developed and work will continue with new radioisotopes. Another argument for assessing oxygenation with novel non-invasive imaging is that indications of global tumour oxygenation and response to treatment may be assessed\textsuperscript{743} and treatment may be followed, rather than invasive or post surgery immunohistochemical techniques needs to be considered and may be more relevant for tumours such as the kidney but this remains to be demonstrated.
Angiogenesis continues to be primarily investigated with immunohistochemistry. However, non-invasive techniques are being developed, particularly with imaging of VEGF and EGFR\textsuperscript{744,745} which may provide future alternatives to \textsuperscript{18}F-FMISO in RCC.

Hypoxia-related proteins such as osteopontin need to be further characterised and their relationship with angiogenesis more completely understood before they enter clinical practice. However, our preliminary steps do indicate the role of osteopontin in primary RCC for the first time and currently larger studies at our institution are attempting to build further on the knowledge gained in this thesis.

6.3 Implications for clinical practice of patients with RCC

With the incidence of RCC is increasing at all stages there is a pressing need for treatment options in patients with locally advanced, recurrent and metastatic disease. A greater understanding of the basic biology of RCC has lead to new treatment options, namely the TKI. It is this development that has spurned an interest in hypoxia and angiogenesis in RCC, the very basis of this thesis.

More accurate and reliable methods of diagnosing, staging and monitoring for response to treatment in RCC are needed using functional and not just anatomical imaging modalities. This thesis has given further support to the concept that \textsuperscript{18}F-FDG and \textsuperscript{18}F-FMISO have only a limited role to play in primary disease diagnosis and prognostication. \textsuperscript{18}F-FLT remains to be supported but is exciting. As such, alternative imaging techniques such as direct targeting of hypoxia-related molecules such as HIF-\textsubscript{1}$\alpha$, is an attractive approach currently tested in preclinical models. For clinical applications, the appropriate combination of hypoxia detection for patient selection with a hypoxia-specific treatment is essential.\textsuperscript{746}
6.4 Directions for future research

The overall aim of this project was to evaluate the relationship between tumour hypoxia and angiogenesis in human RCC. Hypoxia is known to be an important stimulating factor for neovascularisation\textsuperscript{25} and this may correlate with defined markers of angiogenesis in some cancers. However, this relationship had not been studied in RCC. Invasive measurements of pO\textsubscript{2} in human and xenografted tumours determined RCC to be hypoxic. \textsuperscript{18}F-Fluoromisonidazole (\textsuperscript{18}F-FMISO) PET scanning has now been characterised and validated as a marker of hypoxia but one that cannot be readily translated to clinical use because of the subtle differences. PET remains a non-invasive means of measuring tissue pO\textsubscript{2} that may be compared with the invasive Eppendorf readings.

Regarding tumour hypoxia, it has been stated that even if researchers are able to identify hypoxic cells in tumours, researchers cannot distinguish between viable hypoxic cells capable of proliferation or hypoxic cells that are metabolically active.\textsuperscript{729} This remains true and a challenge if researchers are to further understand the complex relationship between hypoxia and angiogenesis in RCC. Finally, the presence of hypoxic tumour cells is therefore regarded not only as an adverse prognostic factor but as an opportunity for tumour-specific treatment. Many challenges await RCC and the story of the relationship it shares with hypoxia and angiogenesis is just beginning.
References

16 Fang J, Yan L, Shing Y, et al. HIF-1alpha-mediated up-regulation of vascular endothelial growth factor, independent of basic fibroblast growth factor, is important...
in the switch to the angiogenic phenotype during early tumorigenesis. Cancer Res 2001; 61:5731-5735


24 Coleman CN, Mitchell JB, Camphausen K. Tumor hypoxia: chicken, egg, or a piece of the farm? J Clin Oncol 2002; 20:610-615


37 Rosen LS. Clinical experience with angiogenesis signaling inhibitors: focus on vascular endothelial growth factor (VEGF) blockers. Cancer Control 2002; 9:36-44
50 Nguyen MM, Gill IS, Ellison LM. The evolving presentation of renal carcinoma in the United States: trends from the Surveillance, Epidemiology, and End Results program. J Urol 2006; 176:2397-2400; discussion 2400
56 Hudes G, Carducci M, Tomczak P. A phase III, randomized, 3-arm study of temsirolimus (TEMSR) or interferon-alpha (IFN) or the combination of TEMSR + IFN in the treatment of first-line poor-prognosis patients with advanced renal cell carcinoma. (Abstract). Proc Am Soc Clin Oncol 2006; 24::2s
69 Lawrentschuk N, Gyomber D, Bolton DM. Renal cell carcinoma following testicular cancer: case report and literature review. Int Urol Nephrol 2006; 38:1-4


95 Roe JS, Youn HD. The positive regulation of p53 by the tumor suppressor VHL. Cell Cycle 2006; 5:2054-2056


369
370


Höckel M, Vaupel P. Biological consequences of tumor hypoxia. Semin Oncol 2001; 28 (suppl 8):36-41


371


Terris DJ. Head and neck cancer: the importance of oxygen. Laryngoscope 2000; 110:697-707


Baumgartl H, Zimelka W, Lubbers DW. Evaluation of PO(2) profiles to describe the oxygen pressure field within the tissue. Comp Biochem Physiol A Mol Integr Physiol 2002; 132:75-85
Olive PL, Banath JP, Aquino-Parsons C. Measuring hypoxia in solid tumors--is there a gold standard? Acta Oncol 2001; 40:917-923
Koch CJ. Measurement of absolute oxygen levels in cells and tissues using oxygen sensors and 2-nitroimidazole EF5. Methods Enzymol 2002; 352:3-31
Bussink J, Kaanders JH, van der Kogel AJ. Tumor hypoxia at the micro-regional level: clinical relevance and predictive value of exogenous and endogenous hypoxic cell markers. Radiother Oncol 2003; 67:3-15
Semenza GL. Involvement of hypoxia-inducible factor 1 in human cancer. Intern Med 2002; 41:79-83


Kwon M, Libutti SK. Advances in understanding angiogenesis through molecular studies. Int J Radiat Oncol Biol Phys 2006; 64:26-32


Carmeliet P. VEGF as a key mediator of angiogenesis in cancer. Oncology 2005; 69 Suppl 3:4-10
Campbell SC. Advances in angiogenesis research: relevance to urological oncology. J Urol 1997; 158:1663-1674
Chrastina A. High cell density-mediated pericellular hypoxia is a crucial factor inducing expression of the intrinsic hypoxia marker CA IX in vitro in HeLa cells. Neoplasma 2003; 50:251-256


Branca MA. Multi-kinase inhibitors create buzz at ASCO. Nat Biotechnol 2005; 23:639


Slichenmyer WJ, Fry DW. Anticancer therapy targeting the erbB family of receptor tyrosine kinases. Semin Oncol 2001; 28:67-79


Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev 2004; 18:1926-1945


379
323 Miller JL. Sirolimus approved with renal transplant indication. Am J Health Syst Pharm 1999; 56:2177-2178
324 Radulovic S, Bjelogrlic SK. Sunitinib, sorafenib and mTOR inhibitors in renal cancer. J BUON 2007; 12 Suppl 1:S151-162
326 Sloan B, Scheinfeld NS. Pazopanib, a VEGF receptor tyrosine kinase inhibitor for cancer therapy. Curr Opin Investig Drugs 2008; 9:1324-1335
330 Khurdayan VK. Take-home message: are we "off target"? Drug News Perspect 2007; 20:345-351
336 Pfizer Oncology Australia. Product information sunitinib (sunitinib malate). West Ryde, Australia, 2006; 1-6

380

Shi YH, Fang WG. Hypoxia-inducible factor-1 in tumour angiogenesis. World J Gastroenterol 2004; 10:1082-1087


Seagroves T, Johnson RS. Two HIFs may be better than one. Cancer Cell 2002; 1:211-213


McMahon G. VEGF receptor signaling in tumor angiogenesis. Oncologist 2000; 5 Suppl 1:3-10

Ferrara N. VEGF as a therapeutic target in cancer. Oncology 2005; 69 Suppl 3:11-16


Hanada N, Lo HW, Day CP, et al. Co-regulation of B-Myb expression by E2F1 and EGF receptor. Mol Carcinog 2006; 45:10-17


385


Levin AA. A review of the issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides. Biochim Biophys Acta 1999; 1489:69-84


Rittling SR, Chambers AF. Role of osteopontin in tumour progression. Br J Cancer 2004; 90:1877-1881


Wu Y, Denhardt DT, Rittling SR. Osteopontin is required for full expression of the transformed phenotype by the ras oncogene. Br J Cancer 2000; 83:156-163


Das R, Mahabeleshwar GH, Kundu GC. Osteopontin induces AP-1-mediated secretion of urokinase-type plasminogen activator through c-Src-dependent epidermal


Standal T, Borset M, Sundan A. Role of osteopontin in adhesion, migration, cell survival and bone remodeling. Exp Oncol 2004; 26:179-184


391
525 Townsend DW, Beyer T. A combined PET/CT scanner: the path to true image fusion. Br J Radiol 2002; 75 Spec No:S24-30


Hockel M, Vaupel P. Biological consequences of tumor hypoxia. Semin Oncol 2001; 28:36-41


Kallinowski F, Brownell AL, Vaupel P, et al. Combined tissue oxygen tension measurement and positron emission tomography studies on glucose utilization in
oncogene-transformed cell line tumour xenografts in nude mice. Br J Radiol 1991; 64:350-359


394


West C, Charnley N. The potential of PET to increase understanding of the biological basis of tumour and normal tissue response to radiotherapy. British Journal Radiology 2005; Supplement 28:50-54


Padhani A. PET imaging of tumour hypoxia. Cancer Imaging 2006; 6:S117-121


396


610 Buerk DG. Measuring tissue PO2 with microelectrodes. Methods Enzymol 2004; 381:665-690
614 Helzel Medical Systems. Maintenance Notes for PHOENIX TOP 300 pO2-fine-needle-probes: Helzel Medical Systems GmbH, Kaltenkirchen, Germany 2002
616 The Centre for PET Studies AH, Australia. PET in more details- facilities. Melbourne: © 2007 Austin Hospital, Austin Health 2007


Liberatore G. Plasticity and regeneration in the nigrostriatal dopaminergic system. Department of Medicine Melbourne, Australia: University of Melbourne, 1998; 218


Hsu S. The use of the avidin-biotin in immunohistochemistry. In: Cuello A, ed. Immunohistochemistry II: John Wiley and Sons, 1993; 126-178


400
401


401


677 Panousis C, Rayzman VM, Johns TG, et al. Engineering and characterisation of chimeric monoclonal antibody 806 (ch806) for targeted immunotherapy of tumours expressing de2-7 EGFR or amplified EGFR. Br J Cancer 2005; 92:1069-1077


402


Stein JH, Fadem SZ. The renal circulation. JAMA 1978; 239:1308-1312

Lubbers DW, Baumgartl H. Heterogeneities and profiles of oxygen pressure in brain and kidney as examples of the pO2 distribution in the living tissue. Kidney Int 1997; 51:372-380


Denko NC. Hypoxia, HIF1 and glucose metabolism in the solid tumour. Nat Rev Cancer 2008


Kelly CJ, Brady M. A model to simulate tumour oxygenation and dynamic [18F]-Fmiso PET data. Phys Med Biol 2006; 51:5859-5873


Martens T, Laabs Y, Gunther HS, et al. Inhibition of glioblastoma growth in a highly invasive nude mouse model can be achieved by targeting epidermal growth factor receptor but not vascular endothelial growth factor receptor-2. Clin Cancer Res 2008; 14:5447-5458


Coleman CN. Linking radiation oncology and imaging through molecular biology (or now that therapy and diagnosis have separated, it's time to get together again!). Radiology 2003; 228:29-35


Corry J, Rischin D. Strategies to overcome accelerated repopulation and hypoxia--what have we learned from clinical trials? Semin Oncol 2004; 31:802-808

Brown JM, Le QT. Tumor hypoxia is important in radiotherapy, but how should we measure it? Int J Radiat Oncol Biol Phys 2002; 54:1299-1301


Appendix A

Imaging Protocols for PET Studies of Renal Cell Carcinoma in Patients

The various PET protocols for the different radiopharmaceuticals ($^{18}$F-FDG, $^{18}$F-FLT and $^{18}$F-FMISO) used included:

<table>
<thead>
<tr>
<th>Parameters of PET and PET-CT in Human Studies (18F-FDG, 18F-FLT and 18F-FMISO)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Whole Body low dose CT parameters:</strong></td>
</tr>
<tr>
<td>- Radial field of view (FOV): 600 mm</td>
</tr>
<tr>
<td>- Slice thickness: 6.5 mm</td>
</tr>
<tr>
<td>- Slice increment: 5.0 mm</td>
</tr>
<tr>
<td>- Pitch: 1.5</td>
</tr>
<tr>
<td>- Rotation time: 0.75 sec</td>
</tr>
<tr>
<td>- Voltage: 140 kVp</td>
</tr>
<tr>
<td>- maS/slice: 30 mA.s</td>
</tr>
<tr>
<td>- Scan length: ~806.5 mm</td>
</tr>
<tr>
<td><strong>PET Parameters:</strong></td>
</tr>
<tr>
<td>- Radial FOV: 576 mm</td>
</tr>
<tr>
<td>- Transmission scan ($^{137}$Cs 20mCi point source) - 1 rotation/bed = 23secs/bed position</td>
</tr>
<tr>
<td>- Emission Scan- 3 mins/bed position (~ 9 bed positions)</td>
</tr>
<tr>
<td>- Scan time approximately 40 minutes</td>
</tr>
</tbody>
</table>
PET protocols $^{18}$F-FDG and $^{18}$F-FMISO Studies:

<table>
<thead>
<tr>
<th>Radiopharmaceutical</th>
<th>$^{18}$F-FDG and $^{18}$F-FMISO Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient Preparation</td>
<td></td>
</tr>
<tr>
<td>$^{18}$F-FDG study: 6 hours fasting</td>
<td></td>
</tr>
<tr>
<td>$^{18}$F-FMISO study: no fasting</td>
<td></td>
</tr>
<tr>
<td>Dose</td>
<td></td>
</tr>
<tr>
<td>$^{18}$F-FDG: 10 mCi</td>
<td></td>
</tr>
<tr>
<td>$^{18}$F-FMISO: 10 mCi</td>
<td></td>
</tr>
<tr>
<td>Radiopharmaceutical Uptake Time</td>
<td></td>
</tr>
<tr>
<td>$^{18}$F-FDG: 60 minutes</td>
<td></td>
</tr>
<tr>
<td>$^{18}$F-FMISO: 120 minutes</td>
<td></td>
</tr>
</tbody>
</table>

$^{18}$F-FLT Study:

<table>
<thead>
<tr>
<th>Radiopharmaceutical</th>
<th>$^{18}$F-FDG and $^{18}$F-flurothymidine ($^{18}$F-FLT) studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient Preparation</td>
<td></td>
</tr>
<tr>
<td>$^{18}$F-FDG study: 6 hours fasting</td>
<td></td>
</tr>
<tr>
<td>$^{18}$F-FLT study: no fasting</td>
<td></td>
</tr>
<tr>
<td>Dose</td>
<td></td>
</tr>
<tr>
<td>$^{18}$F-FDG: 10 mCi</td>
<td></td>
</tr>
<tr>
<td>$^{18}$F-FLT: 10 mCi</td>
<td></td>
</tr>
<tr>
<td>Radiopharmaceutical Uptake Time</td>
<td></td>
</tr>
<tr>
<td>$^{18}$F-FDG: 60 minutes</td>
<td></td>
</tr>
<tr>
<td>$^{18}$F-FLT: 60 minutes</td>
<td></td>
</tr>
</tbody>
</table>
Acquisition/Clinical Mode on Pet Scanner

18F-FDG Study:
- Document patient’s weight, height and BSL for SUV calculations
- If BSL is >10mmol/L, check with investigator before proceeding further
- Position patient on scanner table
- Have the patient raise arms behind head
- Set up standard whole body acquisition
- Aim for the emission scan to start at 60 minutes post-injection
- Emission should start at pelvis first so the bladder is imaged while it is empty
- Scan area must be from mid-meatal to bottom of pelvis/upper thighs
- Cs attenuation must be performed and a low dose CT, if available, should be performed for co-registration with PET

18F-FMISO 18F- FLT Study:
- Document patient’s weight, height and BSL for SUV calculations
- Position patient on scanner table
- Have the patient raise arms behind head
- Set up standard whole body acquisition
- Aim for the emission scan to start at 60 minutes post-injection
- Emission should start at pelvis first so the bladder is imaged while it is empty
- Scan area must be from mid-meatal to bottom of pelvis/upper thighs
- Emission time per bed is 3 minutes
- Cs attenuation must be performed and a low dose CT, if available, should be performed for co-registration with PET
Appendix B

Imaging Protocols for PET Studies of Renal Cell Carcinoma in Xenograft (animal) studies

The PET-CT imaging protocol for xenograft animal studies was:

<table>
<thead>
<tr>
<th>Parameters of PET-CT in Xenograft (animal) Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CT parameters:</strong></td>
</tr>
<tr>
<td>- Radial FOV: 600 mm</td>
</tr>
<tr>
<td>- Axial FOV ~ 20 cm</td>
</tr>
<tr>
<td>- Slice thickness: 0.6 mm</td>
</tr>
<tr>
<td>- Slice increment: 0.6 mm</td>
</tr>
<tr>
<td>- Pitch: 1.5</td>
</tr>
<tr>
<td>- Rotation time: 1.5 sec</td>
</tr>
<tr>
<td>- Voltage: 90 kVp</td>
</tr>
<tr>
<td>- mAs/slice: ~ 150 mA.s</td>
</tr>
<tr>
<td>- Scan length: 216.5 mm</td>
</tr>
<tr>
<td><strong>PET parameters:</strong></td>
</tr>
<tr>
<td>- Radial FOV: 256 mm</td>
</tr>
<tr>
<td>- Axial FOV: 180 mm (1 bed position)</td>
</tr>
<tr>
<td>- Slice thickness: 2 mm</td>
</tr>
<tr>
<td>- Change isotope field to isotope in use</td>
</tr>
<tr>
<td>Scan duration/bed: 30 mins (may alter depending on injection activity and duration of anaesthesia)</td>
</tr>
</tbody>
</table>
Appendix B, Figure 1

Positioning of the group of mice in the PET-CT. Up to six animals were scanned at one time (see figure immediately above). The height of the animals was checked against horizontal and central lasers to determine accurate positioning and image acquisition in every study (below).
\textbf{\textsuperscript{124}I-cG250 Imaging studies.}

PET-CT Imaging was performed in 25 mice in groups of six on Day 0 (2h), 1(24h), 3, 7 and 10 after \textsuperscript{124}I-cG250 injection. All mice were injected with 5 MBq \textsuperscript{124}I-cG250 (25 mg) in 200 mL PBS 1 0.5\% HAS on day 0.

The PET and CT fields of view are pre-determined as per the protocol and confirmed using laser guidance (Appendix B, Figure 1). The width of the field was maintained at 25cm for all studies. All mice were studied on identical foam pieces for consistency. The rationale for the CT settings for animal studies was that a lower voltage would lead to lower energy x-rays and higher absorption for smaller object which allows higher current with a lower signal-to-noise ratio and better quality images.

<table>
<thead>
<tr>
<th>Further Technician Study Protocol for xenograft (animal) PET-CT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acquisition</strong></td>
</tr>
<tr>
<td>- Place radiotherapy bed on scanner table</td>
</tr>
<tr>
<td>- Position mice/rats on bed supine or prone (ensure you select the correct patient position when entering the acquisition details)</td>
</tr>
<tr>
<td><strong>Positioning</strong></td>
</tr>
<tr>
<td>- 6 mice can be imaged in one bed position. Place 3 mice wide and 2 mice in length (up to six with two rows of three). Ensure the width of the mice is no greater than 25cm (see below)</td>
</tr>
<tr>
<td>- Move table into PET scanner and position mice so that the centre laser runs through the centre of the middle mouse or between the two rats</td>
</tr>
<tr>
<td>- Adjust table height so the horizontal laser runs through the middle of the mouse</td>
</tr>
<tr>
<td>- Ensure mice/rats are within the FOV and note down translate position</td>
</tr>
<tr>
<td>- Move table into CT mode and prepare for CT first</td>
</tr>
</tbody>
</table>
## Data Entry and Study Protocol Checklist

- Queue in study with MICE as surname and investigator’s name for the first name
- Enter comment field with name of compound used or disease type i.e. FMISO mice, Pancreatitis rats
- Click on “Body” icon
- Use “Mice-Sv+CT+PET” protocol in the purple protocol area
- Use a CT length of at least 216mm and centre this area over mice/rats
- When setting up the PET scan, enter translation as noted down earlier for start position
- Check correct time per bed is entered (30 mins for one bed position, 15-20/bed for multiple bed scans)
- Once bed moves into PET scanner, turn lasers on and ensure the mice/rats are in the correct position and are within the FOV
- Continually check on mice and let investigator know if they start to twitch so investigator can administer more anaesthesia so they do not wake
- Emission scan will be automatically set to reconstruct concurrently with CT for attenuation correction
<table>
<thead>
<tr>
<th>Author</th>
<th>Human xenografts unless indicated</th>
<th>Number of tumours</th>
<th>Probe</th>
<th>Median $pO_2$(mmHg)</th>
<th>Mean $pO_2$(mmHg)</th>
<th>HP5 (%)</th>
<th>HP10 (%)</th>
<th>Relationship to Tumour Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ziemer et al(^{38})</td>
<td>RCC (RENCA, murine)</td>
<td>6</td>
<td>OP</td>
<td>NA</td>
<td>24.8</td>
<td>NA</td>
<td>NA</td>
<td>22</td>
</tr>
<tr>
<td>Ziemer et al(^{38})</td>
<td>Melanoma</td>
<td>6</td>
<td>OP</td>
<td>NA</td>
<td>37.8</td>
<td>NA</td>
<td>NA</td>
<td>6</td>
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<tr>
<td>Menon et al(^{74})</td>
<td>Melanoma</td>
<td>5</td>
<td>POS</td>
<td>2.0</td>
<td>NA</td>
<td>90</td>
<td>96</td>
<td>NA</td>
</tr>
<tr>
<td>Bruberg et al(^{91})</td>
<td>Melanoma</td>
<td>38</td>
<td>OP</td>
<td>NA</td>
<td>NA</td>
<td>53</td>
<td>73</td>
<td>NA</td>
</tr>
<tr>
<td>Bruberg et al(^{171})</td>
<td>Melanoma</td>
<td>70</td>
<td>OP</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Adam et al(^{94})</td>
<td>Melanoma</td>
<td>4</td>
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<td>NA</td>
<td>66</td>
<td>78</td>
<td>NA</td>
</tr>
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<td>Wachsberger(^{95})</td>
<td>Glioma</td>
<td>12</td>
<td>OP</td>
<td>NA</td>
<td>5</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Chastagnier(^{68})</td>
<td>Glioma</td>
<td>30</td>
<td>POS</td>
<td>9</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Scigliano et al(^{19})</td>
<td>Glioma</td>
<td>10</td>
<td>POS</td>
<td>NA</td>
<td>8.6</td>
<td>NA</td>
<td>75</td>
<td>NA</td>
</tr>
<tr>
<td>Bussink et al(^{71})</td>
<td>Glioma (I)</td>
<td>28</td>
<td>OP</td>
<td>16</td>
<td>11</td>
<td>7</td>
<td>14</td>
<td>NA</td>
</tr>
<tr>
<td>Bussink et al(^{84})</td>
<td>Glioma (II)</td>
<td>38</td>
<td>OP</td>
<td>22</td>
<td>24</td>
<td>21</td>
<td>45</td>
<td>NA</td>
</tr>
<tr>
<td>Adam et al(^{17})</td>
<td>Squamous Cell</td>
<td>25</td>
<td>POS</td>
<td>2.7</td>
<td>NA</td>
<td>73</td>
<td>89</td>
<td>NA</td>
</tr>
<tr>
<td>Urano et al(^{17})</td>
<td>Squamous Cell</td>
<td>12</td>
<td>OP</td>
<td>NA</td>
<td>NA *&lt;2.5 (10-100%)</td>
<td>NA</td>
<td>HP ↑ with ↑ tumour size</td>
<td></td>
</tr>
<tr>
<td>Ressel(^{70})</td>
<td>Squamous Cell</td>
<td>9</td>
<td>POS</td>
<td>NA</td>
<td>25.5</td>
<td>NA</td>
<td>41</td>
<td>NA</td>
</tr>
<tr>
<td>Bussink et al(^{70})</td>
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<td>35</td>
<td>OP</td>
<td>6.4</td>
<td>2</td>
<td>77</td>
<td>63</td>
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<tr>
<td>Sorensen(^{172})</td>
<td>Squamous Cell</td>
<td>7</td>
<td>POS</td>
<td>1</td>
<td>NA</td>
<td>97-99%</td>
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<td>NA</td>
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<tr>
<td>Krishna et al(^{71})</td>
<td>Squamous Cell</td>
<td>14</td>
<td>POS</td>
<td>NA</td>
<td>1.4</td>
<td>58</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Stuben(^{176})</td>
<td>Squamous Cell</td>
<td>38</td>
<td>POS</td>
<td>0.8-1.9</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Sorensen(^{172})</td>
<td>Breast carcinoma</td>
<td>7</td>
<td>POS</td>
<td>7.0</td>
<td>NA</td>
<td>NA</td>
<td>71-90%</td>
<td>NA</td>
</tr>
<tr>
<td>Piert et al(^{71})</td>
<td>Breast carcinoma</td>
<td>5</td>
<td>POS</td>
<td>2.9</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Zanzonico(^{175})</td>
<td>Breast carcinoma (murine)</td>
<td>10</td>
<td>OP</td>
<td>1.5-2.5</td>
<td>5.1-11.5</td>
<td>NA</td>
<td>NA</td>
<td>Larger tumours more hypoxic</td>
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<td>Braun et al(^{82})</td>
<td>Breast carcinoma (murine)</td>
<td>5</td>
<td>POS</td>
<td>7.4 (±4.4)</td>
<td>4.0 (±3.5)</td>
<td>70</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Braun et al(^{94})</td>
<td>Breast carcinoma (murine)</td>
<td>5</td>
<td>OP</td>
<td>7.4 (±4.4)</td>
<td>1.0 (±0.7)</td>
<td>83</td>
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<td>NA</td>
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<tr>
<td>Author</td>
<td>Human xenografts unless indicated</td>
<td>Number of tumours</td>
<td>Probe</td>
<td>Median pO$_2$(mmHg)</td>
<td>Mean pO$_2$ (mmHg)</td>
<td>HP$_3$ (%)</td>
<td>HP$_9$ (%)</td>
<td>Relationship to Tumour Size</td>
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<td>--------------------</td>
<td>-------------------</td>
<td>------------</td>
<td>------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>Iyer et al$^{199}$</td>
<td>Prostate carcinoma</td>
<td>15</td>
<td>POS</td>
<td>7.5</td>
<td>NA</td>
<td>45</td>
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<td>NA</td>
</tr>
<tr>
<td>Yeh et al$^{18}$</td>
<td>Prostate carcinoma</td>
<td>30</td>
<td>POS</td>
<td>2.2</td>
<td>NA</td>
<td>NA</td>
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<td>NA</td>
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<tr>
<td>O’Donoghue$^{199}$</td>
<td>Prostate carcinoma (non-human)</td>
<td>3</td>
<td>POS</td>
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<td>NA</td>
<td>NA 61</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Zazmer et al$^{173}$</td>
<td>Lung</td>
<td>7</td>
<td>OP</td>
<td>1.8</td>
<td>NA</td>
<td>30*(15)</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>Adam et al$^{175}$</td>
<td>Lung</td>
<td>34</td>
<td>POS</td>
<td>2.7</td>
<td>-</td>
<td>72</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>Chastagner$^{198}$</td>
<td>Lung</td>
<td>30</td>
<td>POS</td>
<td>7</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Buchler et al$^{176}$</td>
<td>Pancreatic carcinoma</td>
<td>17</td>
<td>POS</td>
<td>NA</td>
<td>8.75</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Pietr et al$^{194}$</td>
<td>Pancreatic carcinoma</td>
<td>5</td>
<td>POS</td>
<td>0.4</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Zanzonico$^{195}$</td>
<td>Fibrosarcoma (murine)</td>
<td>9</td>
<td>OP</td>
<td>1.42-16.8</td>
<td>7.1-24</td>
<td>NA</td>
<td>NA</td>
<td>NA Larger tumours more hypoxic</td>
</tr>
<tr>
<td>Lyng et al$^{197}$</td>
<td>Cervical</td>
<td>8</td>
<td>POS</td>
<td>3-22</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Adam et al$^{195}$</td>
<td>Colon</td>
<td>41</td>
<td>POS</td>
<td>2.4</td>
<td>-</td>
<td>76</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>Adam et al$^{197}$</td>
<td>Large Cell Carcinoma</td>
<td>5</td>
<td>POS</td>
<td>3.0</td>
<td>-</td>
<td>71</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>Zolzer et al$^{194}$</td>
<td>5 lines (sarcoma x3, gliomas, SCC)</td>
<td>120</td>
<td>POS</td>
<td>NA</td>
<td>5</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Collingridge et al$^{7}$</td>
<td>Sarcoma (murine)</td>
<td>35</td>
<td>POS</td>
<td>1.4 (±0.3)</td>
<td>NA</td>
<td>79</td>
<td>89</td>
<td>NA</td>
</tr>
<tr>
<td>Collingridge et al$^{7}$</td>
<td>Sarcoma (murine)</td>
<td>20</td>
<td>OP</td>
<td>2.8 (±0.5)</td>
<td>NA</td>
<td>75</td>
<td>83</td>
<td>NA</td>
</tr>
<tr>
<td>Zywietz et al$^{18}$</td>
<td>Rhabdomyosarcoma (murine)</td>
<td>32</td>
<td>POS</td>
<td>23</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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</table>
Appendix D

Studies using radiolabelled cG250 in human RCC xenograft models

Part I. - comparison of maximal tumour uptake of the radioisotope in vivo with other studies using radioiodine.

<table>
<thead>
<tr>
<th>Radioisotope</th>
<th>Cell Line</th>
<th>% ID/G (± SD)</th>
<th>Blood</th>
<th>Tumour:Blood</th>
<th>Time point (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tumour</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Radioiodine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>¹²⁵I</td>
<td>SK-RC-52</td>
<td>19.43 ± 4.3</td>
<td>4.94 ± 2.13</td>
<td>14.79 ± 2.40</td>
<td>3</td>
</tr>
<tr>
<td>Brouwers et al⁷⁵⁹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>¹²⁵I</td>
<td>SK-RC-52</td>
<td>14.0 ± 3.0</td>
<td>12.4 ± 3.0</td>
<td>NA</td>
<td>3</td>
</tr>
<tr>
<td>van Dijk et al⁷⁵⁶</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>¹²⁵I</td>
<td>SK-RC-52</td>
<td>13.5 (10.4-18.5)</td>
<td>1.7 (1.1-2.0)</td>
<td>NA</td>
<td>2</td>
</tr>
<tr>
<td>¹²⁵I F(ab’)_2</td>
<td>SK-RC-52</td>
<td>5.1 (4.4-6.2)</td>
<td>0.12 (0.07-0.13)</td>
<td>NA</td>
<td>2</td>
</tr>
<tr>
<td>¹²⁵I Fab’</td>
<td>SK-RC-52</td>
<td>1.1 (0.8-1.4)</td>
<td>0.1 (0.07-0.13)</td>
<td>NA</td>
<td>2</td>
</tr>
<tr>
<td>Brouwers et al⁷⁵⁷</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>¹²⁵I F(ab’)_2</td>
<td>SK-RC-52</td>
<td>2.5 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>2.3 ± 0.2</td>
<td>5</td>
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<tr>
<td>¹³¹I</td>
<td>SK-RC-52</td>
<td>15 ± 2.0</td>
<td>7 ± 1.0</td>
<td>2.3 ± 0.2</td>
<td>5</td>
</tr>
<tr>
<td>Steffens et al⁷⁵⁸</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>¹²⁵I</td>
<td>SK-RC-52</td>
<td>10.7 ± 5.2</td>
<td>2.0 ± 2.0</td>
<td>4.5</td>
<td>3</td>
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</table>
Part II. Studies using radiolabelled cG250 in human RCC xenograft models - comparison of maximal tumour uptake of the radioisotope in vivo with other studies using other radioisotopes.

<table>
<thead>
<tr>
<th>Radioisotope</th>
<th>Cell Line</th>
<th>%ID/G (± SD)</th>
<th>Tumour</th>
<th>Blood</th>
<th>Tumour:Blood</th>
<th>Time point (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Radioiodine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>²¹²⁹I</td>
<td>SK-RC-52</td>
<td>19.43 ± 4.3</td>
<td>4.94 ± 2.13</td>
<td>14.79 ± 2.40</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><strong>Other Radioisotopes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Brouwers et al⁷⁵⁹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>⁷⁷⁷Lu</td>
<td>SK-RC-52</td>
<td>86.9 ± 14.5*</td>
<td>11.3 ± 1.2</td>
<td>NA</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SK-RC-52</td>
<td>113.3 ± 24.7#</td>
<td>13.4 ± 3.8</td>
<td>NA</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Brouwers et al⁷⁵⁷</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>⁹⁰⁸Zr</td>
<td>SK-RC-52</td>
<td>5.0 ± 2.4</td>
<td>1.5 ± 0.2</td>
<td>2.9</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>⁹⁹⁷In</td>
<td>SK-RC-52</td>
<td>4.9 ± 2.9</td>
<td>1.7 ± 0.3</td>
<td>muscle 0.4 ± 0.2</td>
<td>3</td>
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<tr>
<td>FDG</td>
<td></td>
<td>--</td>
<td>--</td>
<td>0.5 ± 0.1</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>Van Schaijk⁷⁶⁰</td>
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<td></td>
</tr>
<tr>
<td>¹¹¹In</td>
<td>SK-RC-52</td>
<td>66 ± 23</td>
<td>NA</td>
<td>NA</td>
<td>3d</td>
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<td>SK-RC-1</td>
<td>251± 90</td>
<td>NA</td>
<td>NA</td>
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<td></td>
<td>NU-12</td>
<td>211 ± 79</td>
<td>NA</td>
<td>NA</td>
<td>3d</td>
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</tr>
<tr>
<td>¹²⁵I</td>
<td>SK-RC-52</td>
<td>**22± 7</td>
<td>NA</td>
<td>NA</td>
<td>3d</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SK-RC-1</td>
<td>**83± 30</td>
<td>NA</td>
<td>NA</td>
<td>3d</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NU-12</td>
<td>137 ± 36</td>
<td>NA</td>
<td>NA</td>
<td>3d</td>
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</tr>
<tr>
<td>Steffens et al⁷⁶⁷</td>
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</tr>
<tr>
<td>⁹⁹mTc-HYNIC</td>
<td>SK-RC-52</td>
<td>92.4±6.4</td>
<td>11.2±1.6</td>
<td>*8±2</td>
<td>2d</td>
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<td>⁹⁹mTc</td>
<td>SK-RC-52</td>
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<td>3.75±0.18</td>
<td>7±2</td>
<td>2d</td>
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<td>9±3</td>
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<tr>
<td>Steffens et al⁷⁶⁸</td>
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<tr>
<td>¹¹¹In-DTPA</td>
<td>SK-RC-52</td>
<td>18.6 ± 6.2</td>
<td>1.5 ± 1.0</td>
<td>13.3</td>
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<td>¹⁸⁶⁰Re-MAG3</td>
<td>SK-RC-52</td>
<td>5.9 ± 4.5</td>
<td>1.5 ± 1.25</td>
<td>4.2</td>
<td>3</td>
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Part III. Studies using radiolabelled cG250 in human RCC xenograft models- comparison of maximal tumour uptake of the radioisotope in vivo with other studies using pre-targeting strategies.

<table>
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<th>Radioisotope</th>
<th>Cell Line</th>
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<th>Blood</th>
<th>Tumour:Blood</th>
<th>Time point (days)</th>
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<td>Tumour:Blood</td>
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<tr>
<td><strong>Radioiodine</strong></td>
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HYNIC= hydrazinonicotinamide  MAG3= mercaptoacetyltriglycine

* $^{177}$Lu-DOTA-cG250  
$^\#$ $^{177}$Lu-SCN-B2-DTPA-cG250  
**pre-targeting used  
^D.A.A. Peptide  
^^^L.A.A. Peptide
## Appendix E

### Human studies using invasive oxygen sensor measurements

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| Parker | 2004 | 55 | 70 | 2 | 40-50 | 4.5 | 60 | 0.2-57.3 |
| Movsas | 2002 | 57 | 100 |            |            |                          |                     |                  |         |        |                          |                       |
| Movsas | 2001 | 59 |            |            |                          |                     |                  |         |        |                          |                       |
| Movsas | 1999 | 12 |            |            |                          |                     |                  |         |        |                          | 10.8                  |
| Movsas | 2000 | 55 | <62=22; >62=33 |            |                          | 9.9 |            |         |        |                          | Muscle 28.6           |
| Movsas | 2000 (Stage T1) | 31 | | | | 12.5 | | | | | | 29.6 |
| Movsas | 2000 (stage T2/3) | 22 | | | | 6 | 21 | 14 | 14 | 27.1 |
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| Evans | Yes | 2004 | 12 | 51 | | | | | | | | |
| Collingridge | | 1999 | 25 | 3 to 5 | | 5 | 68 | 0-40 |
| Cruickshank | | 1994 | 9 | 52 | 6 | 32 | 15 | 60 | (2-42) |
| | | | 7 | 53 | 6 | 32 | 4 | 71 | (0-24) |
| | | | 1 | 54 | 6 | 32 | 14 | 0 | NA |
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Author/s: Lawrentschuk, Nathan Leo

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Date: 2009

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