Angiogenesis in Superficial Transitional Cell Carcinoma of the Human Urinary Bladder

A thesis presented for the degree of

Medicinae Doctor

from

The University of Leicester

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Abstract

Angiogenesis in Superficial Transitional Cell Carcinoma of the Human Urinary Bladder

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Aims

To determine whether microvessel density (MVD) could be measured in papillary superficial transitional cell carcinoma of the human urinary bladder in a simple, meaningful and reproducible way. Also, to determine whether angiogenesis, as measured by MVD, at presentation is related to subsequent recurrence or progression of superficial bladder cancer and its relationship to known angiogenic cytokines.

Methods

Immunohistochemical techniques were used to measure MVD (using CD34), vascular endothelial growth factor (VEGF) thrombospondin-1 (TSP-1)(using both TSP Ab-4 and TSP Ab-7 antibodies), thymidine phosphorylase (TP) and p53 in 5μm sections of formalin-fixed, paraffin-embedded samples of primary superficial bladder cancer. A computer image analysis system was devised to measure MVD and compared to a standard manual technique. The percentage expression of VEGF, TSP-1, TP and p53 was recorded. MVD and the cytokines were correlated with outcome using binary logistic regression analysis.

Results

The database included 293 cases of primary superficial bladder cancer. The computer image analysis system was able to measure MVD in superficial bladder cancer and correlated strongly with manual counting (r=0.95).

In a multivariable analysis, MVD (p<0.0001), TSP (Ab-7) tumour staining (p=0.001), decreased TSP (Ab-4) perivascular staining (p=0.007), tumour stage (p<0.0001), multiplicity (p=0.01), age (p=0.003) and aspirin consumption (p=0.004), were all independent predictive factors for superficial bladder cancer progression.

MVD in this study showed a positive correlation with p53 and the pro-angiogenic factor TP. Low perivascular TSP staining correlated with increased MVD.

Conclusion

This study has shown that MVD can be measured in superficial bladder cancer. A method has been described that is accurate, reproducible and time efficient.

This study has also shown, for the first time, that increasing MVD at presentation is a significant risk factor for subsequent progression to muscle invasive disease.
“amara lento tempera risu”
Onely because the Cancer aboue all Tumors hath most need of the actual Cautery, if a man may come to apply it; and because the fashion of a Crab doth represent the horrid forme of that Ulcer, whence also it hath his name, you have here a Crab figured to make unto you (as it were) a representation of a Cancer.

Frontispiece
Declaration

I hereby declare that this thesis is the work of my own hand. It is a record of work planned and carried out by myself excepting those instances specifically referred to and gratefully acknowledged. All previous work quoted has been fully accredited and referenced. This work was carried out in Leicester between 1999 and 2003.

Permission was granted to me by the Local Ethical and Research Committee to use archived paraffin embedded human tissue and to consult the patients' hospital records.

There are no financial conflicts of interest regarding this work.

Jonathan Charles Goddard

Leicester

2004
Acknowledgements

I am most grateful for the tremendous amount of help and support my two supervisors have given me whilst working on this thesis; Mr Roger Kockelbergh for his continual gentle support, for sharing his amazing comprehensive knowledge of the urological literature and for his careful and so detailed reading and re-reading of all I have written and Dr Ken O’Byrne for his enthusiasm, for supplying me with a constant flow of suggestions and for his ever-present faith that it would all work.

I would like to acknowledge the help given to me by the pathology departments of all three hospitals in Leicester; in particular, the Specials Lab., led by Diana Cullen and Lisa Wheatley who patiently taught me the techniques of immunohistochemistry and explained each time just why it had gone wrong; Caroline at Leicester General Hospital who helped so much when I started out building my database and Jason who cut most of my sections. At the Glenfield Hospital, I gratefully acknowledge the help of Dr Louise Jones who guided me through the grading of my slides and acted as a blind observer to check my technique. I am particularly grateful for the help of Professor Peter Furness who allowed me to use his digital pathology laboratory and gave me the original computer macros from which mine are derived (and indeed helped me re-write them).
I am very thankful for the help of Nick Taub from the University department of statistics for his patient explanations and advise.

I also acknowledge the help and support of Mr Chris Sutton who worked with me so closely particularly in the early stages of antibody work up when we shared normal tissue and mixed our runs for extra controls. Much more so I am grateful for his support over the last few years; for pushing me forward when I was flagging.

Finally, but most important of all, I would like to thank Kerenza, my wife, for still being my wife despite all this.
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<td>Ang</td>
<td>Angiopoetin</td>
</tr>
<tr>
<td>AWMVD</td>
<td>Area weighted microvessel density</td>
</tr>
<tr>
<td>β</td>
<td>Correlation coefficient for regression</td>
</tr>
<tr>
<td>BCDR</td>
<td>Blood capillary density ratio</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guerin</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BTA</td>
<td>Bladder tumour antigen</td>
</tr>
<tr>
<td>CAIA</td>
<td>Computer assisted image analysis</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge coupled device</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster determinant</td>
</tr>
<tr>
<td>cdk</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CIAS</td>
<td>Computer aided image analysis</td>
</tr>
<tr>
<td>cis</td>
<td>Carcinoma in situ</td>
</tr>
<tr>
<td>COMP</td>
<td>Cartilage oligomeric matrix protein</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CSVTCG</td>
<td>Cysteine-serine-valine-threonine-cysteine-glycine repeats</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>2dD1P</td>
<td>2-Dexy-D-ribose-1-phosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees centigrade</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid</td>
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<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>FVIIIRA</td>
<td>Factor VIII related antigen</td>
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<td>HCL</td>
<td>Hydrochloric acid</td>
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<td>HIF</td>
<td>Hypoxia inducing transcription factor</td>
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<td>Hue, saturation, intensity value colour image</td>
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<td>HPF</td>
<td>High powered field</td>
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<tr>
<td>HR</td>
<td>Hazard ratio</td>
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<tr>
<td>HSV</td>
<td>Hue, saturation, intensity value colour image</td>
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ilMD Image analysis microvessel density
IL Interleukin
IP₃ Inositol 1, 4, 5-triphosphate
Kb Kilobytes
kDa Kilodaltons
l litre
LOH Loss of heterozygosity
LUTS Lower urinary tract symptoms
M molar
MAGS Microscopic angiogenic grading system
mg Milligram
mIMD Manually counted microvessel density
ml Millilitre
mm Millimetre
mM milimolar
mmHg Millimetres of mercury
MMP Matrix metalloprotinase
mRNA Messenger ribonucleic acid
MVA Microvascular area
MVAC Methotrexate, Vinblastine, Doxorubicin and Cisplatin chemotherapy
MVD Microvessel density
μl microlitre
μm Micrometres
NHS National Health Service
NIH National Insitute of Health
NiSO₄ Nickel sulphate
NMP22 Nuclear matrix protein 22
NSAIDs Non steroidal anti-inflammatory drugs
NVES Number of vessels per square millimetre
OMVD Optimised microvessel density
PA Plasminogen activator
PAI-1 Plasminogen activator inhibitor -1
PBS Phosphate buffered saline
PDECGF Platelet derived endothelial cell growth factor
PDGF Platelet derived growth factor
PECAM Platelet endothelial cell adhesion molecule
PIGF Placental growth factor
pRb Product of retinoblastoma gene
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<thead>
<tr>
<th>Acronym</th>
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<tr>
<td>r</td>
<td>correlation coefficient</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RGB</td>
<td>Red Green Blue colour image</td>
</tr>
<tr>
<td>SBC</td>
<td>Superficial bladder cancer</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation from the mean</td>
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<tr>
<td>TBS</td>
<td>Modified TRIS buffered saline</td>
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<tr>
<td>TCC</td>
<td>Transitional Cell Carcinoma</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of matrixmetalloprotinase</td>
</tr>
<tr>
<td>TNFa</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TP</td>
<td>Thymidine Phosphorylase</td>
</tr>
<tr>
<td>TRIS</td>
<td>Modified Trisma base buffered saline</td>
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<tr>
<td>TSP</td>
<td>Thrombospondin</td>
</tr>
<tr>
<td>TURBT</td>
<td>Transurethral resection of bladder tumour</td>
</tr>
<tr>
<td>TVA</td>
<td>Total area occupied by vessels</td>
</tr>
<tr>
<td>UEA-1</td>
<td>Ulex europaeus agglutinin I</td>
</tr>
<tr>
<td>UK</td>
<td>The United Kingdom</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase type Plasminogen activator</td>
</tr>
<tr>
<td>uPAR</td>
<td>Urokinase type Plasminogen activator receptor</td>
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<tr>
<td>USA</td>
<td>The United States of America</td>
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<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
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<td>VPF</td>
<td>Vascular permeability factor</td>
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<td>VSD</td>
<td>Vascular surface density</td>
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Chapter 1

Introduction
1.1.0 Superficial Bladder Cancer

1.1.1 The Epidemiology of Superficial Bladder Cancer

Cancer of the urinary bladder is the fourth most common cancer in men and the eighth commonest in women (Greenlee, Murray, Bolden, & Wings. 2000) its incidence in the United Kingdom is 18.6/100,000 (Parkin & Muir. 1992). There were 3890 cases in women and 9710 in men in 1996 with a mortality of 1710 and 3300 respectively (1998 data) (Office for National Statistics. 2001). The incidence of bladder cancer is increasing, a phenomenon more marked in men than in women (Napalkov, Maisonneuve, & Boyle. 1997). Bladder cancer is three times more common in men than in women (Boring, Squires, Tong, & Montgomery. 1995). Bladder cancer can occur at any age, even in childhood, but it is most common in late middle age and in the elderly; the median age of occurrence being 69 years in men and 71 years in women (Lynch & Cohen. 1995). Younger patients tend to have lower grade and stage tumours which are less likely to recur (Fitzpatrick & Reda. 1986). The disease is more common in western countries such as the UK or the USA than in eastern countries like Japan (Morrison, Buring, Verhoek, et al. 1984). Italy has the highest incidence of bladder cancer with rates of incidence ranging between 30.3 and 34/100000 (Parkin & Muir. 1992). Bladder cancer, particularly squamous cell carcinoma is more common in areas where bilharzia infection is found. In Egypt, where bilharzia is endemic, 75% of bladder cancers are squamous (El-Bolkainy, Mokhtar, Ghoneim, & Hussein. 1981). Bladder cancer is twice as
common in white males compared to black males in the USA (Bethesda MD. 1987).

1.1.2 The Aetiology of Bladder Cancer

1.1.2.1 Tobacco

Cigarette smokers have a two to four fold measured risk of developing bladder cancer (Morrison, Buring, Verhoek, et al. 1984; Burch, Rohan, Howe, et al. 1989; Clavel, Cordier, Boccon-Gibod, & Hemon. 1989). An estimated 50% of bladder cancer in the UK is attributable to smoking (Moolgavkar & Stevens. 1981). It is associated with between 25% and 60% of cases of bladder cancer in industrialised countries in general (Morrison, Buring, Verhoek, et al. 1984). The number of cigarettes smoked (>20 per day), the duration of the habit (>30 years) and the use of black, as opposed to blond tobacco, all increase the risk (D'Avanzo, Negri, & La Vecchia. 1990). The presence of nitrosamines and 2-naphtylamine, both known bladder carcinogens, in cigarette smoke has been put forward as a causative link (Auerbach & Garfinkel. 1989).

1.1.2.2 Occupational Exposure

At the end of the nineteenth century, an association was noted between bladder cancer and occupational exposure to aniline dyes. This association was described in three patients by a German clinician, Rehn (Rehn L. 1895). Many chemicals have been associated with bladder cancer; the majority are aromatic amines. There are a large number of occupations that have been associated with their use and thus linked to increased bladder cancer risk, table 1.1. In a 1954
### Table 1.1 Occupational Risks in Bladder Cancer

<table>
<thead>
<tr>
<th>Chemicals associated with increased risk of bladder cancer</th>
<th>Occupations associated with increased risk of bladder cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Naphthylamine</td>
<td>Car workers</td>
</tr>
<tr>
<td>4-Aminobiphenyl</td>
<td>Painters</td>
</tr>
<tr>
<td>Benzidine</td>
<td>Lorry drivers</td>
</tr>
<tr>
<td>Chlornaphazine</td>
<td>Drill press operators</td>
</tr>
<tr>
<td>4-Chloro-o-toluidine</td>
<td>Leather workers</td>
</tr>
<tr>
<td>O-Toluidine</td>
<td>Metal workers</td>
</tr>
<tr>
<td>4,4′-Methylene bis (2-chloroanuline)</td>
<td>Machinists</td>
</tr>
<tr>
<td>Methylene dianiline</td>
<td>Dry cleaners</td>
</tr>
<tr>
<td>Benzidine derived azo dyes</td>
<td>Paper manufacturers</td>
</tr>
<tr>
<td>Phenacetin containing analgesics</td>
<td>Rope makers</td>
</tr>
<tr>
<td></td>
<td>Dental technicians</td>
</tr>
<tr>
<td></td>
<td>Barbers</td>
</tr>
<tr>
<td></td>
<td>Beauticians</td>
</tr>
<tr>
<td></td>
<td>Hairdressers</td>
</tr>
<tr>
<td></td>
<td>Physicians</td>
</tr>
<tr>
<td></td>
<td>Clothes makers</td>
</tr>
<tr>
<td></td>
<td>Plumbers.</td>
</tr>
</tbody>
</table>


study, workers in the British dye industry were shown to have risk rates 10 to 50 times higher than expected after exposure to 2-naphthylamine and benzidine (Case, Hosker, & McDonald. 1954). Occupational exposure may account for upto 33% of bladder cancer (Cole, Hoover, & Friedell. 1972). Onset of the disease can be separated by 15 to 40 years from exposure (Cohen & Johansson. 1992).

1.1.2.3 Chronic Infection

Chronic cystitis has also been shown to predispose to bladder cancer. This is seen in patients with long term in-dwelling catheters and patients with bladder stones. Between 2% and 10% of paraplegics with long term catheters are likely to develop bladder cancer, particularly of a squamous cell type (Kantor, Hartge, & Hoover. 1984; Locke, Hill, & Walzer. 1985). It has been proposed that micro organisms produce nitrite and N-nitroso compounds which are carcinogenic to the urothelium (Tricker, Mostafa, Spiegelhalder, & Preussmann. 1989).

1.1.2.4 Bilharzia

Bilharzia infection due to Schistosoma haematobium is a major cause of squamous cell bladder cancer worldwide but also leads to an increase in transitional cell carcinoma (TCC) (Lucas. 1982). Schistosoma haematobium is a blood fluke found in the Middle East and Africa. The infection is acquired in fresh water where the cercariae penetrate human skin. They migrate into the pelvic veins where they mature into adult worms. The worms reproduce and their
eggs cross into viscera such as the bladder. The eggs are excreted in urine and if this is passed into fresh water the eggs hatch and the subsequent miracidium penetrate into fresh water bulinus snails. Within the snails the miracidium undergo asexual reproduction and more circariae are excreted into the fresh water supply.

1.1.2.5 Other Risk Factors

Phenacetin, a paraaminophenyl derivative used as an analgesic, has been linked to the development of bladder cancer. Its structure is similar to that of aniline dyes. Large amounts need to be taken over a long period of time for it to lead to bladder cancer and there is a long latent period before onset. The association with phenacetin and upper urinary tract TCC however is much stronger than in lower tract disease (McCredie, Stewart, Ford, & MacLennan. 1983; Piper, Tonascia, & Metanoski. 1985).

The chemotherapeutic agent, cyclophosphamide has also been shown to increase the risk of bladder cancer. The risk is dose dependent with relative risks of 2.4, 6.0 and 14.5 for patients receiving cumulative doses of less than 20g, 20-49g and more than 50g (Kaldor, Day, & Kittelmann. 1995; Lucas. 1982; Travis, Curtis, Glimelius, et al. 1995). Another anti-neoplastic drug used in the 1960’s, chlornaphazine, has also been related to bladder cancer. It is chemically related to 2-naphthylamine, fortunately it was not widely used (Lucas. 1982; Thiede & Christensen. 1969).
Pelvic radiotherapy for management of cervical carcinoma increases the risk of TCC of the bladder by two to four times (Kaldor, Day, & Kittelmann. 1995). These tumours tend to be high grade and invasive rather than superficial at presentation (Quilty & Kerr. 1987).

1.1.3 The Molecular Biology of Bladder Cancer

Bladder cancer is associated with complex chromosomal changes. The number of chromosomes in bladder cancer cells can range from 42 to 78 (Sandberg & Berger. 1994). Genetic changes leading to bladder cancer consist of a combination of oncogene activation, loss of tumour suppressor genes and amplification or overexpression of normal cell cycle and growth proteins.

Proto-oncogenes code for proteins controlling cell replication and growth. Functional mutations of these genes may lead to incorrect or overproduction of their protein products and hence a derangement in cell growth or duplication. Other genetic mutations lead to down-regulation or altered function of tumour suppressor genes. Tumour suppressor genes form part of the normal control system for cell reproduction. They also code for proteins which halt or delay the cell cycle in the event of DNA damage to allow repair or, in the case of irreparable damage, institute programmed cell death or apoptosis. Loss of tumour suppressor function is usually by deletion of one allele and mutation of the other (Cordon-Cardo. 1998). The suppressor genes that have been most commonly associated with bladder cancer are p53 (on chromosome 17p), the
retinoblastoma gene (on chromosome 13q) and genes on chromosome 9 producing p14, p15 and p16.

Several mutated proto-oncogenes (oncogenes) have been associated with bladder cancer. Mutation of the ras oncogene family leads to expression of an abnormal gene product in bladder cancer. The frequency of ras mutation in bladder cancer is unclear and lies between 6% and 44% (Knowles. 1999).

The ERBB2 gene at 17q21 is also overexpressed in bladder cancer leading to increased production of its protein product. The ERBB2 product is similar to epidermal growth factor receptor (EGFR). Overexpression of ERBB2 occurs in bladder cancers of higher grade and stage (Mellon, Lunec, Wright, Horne, Kelly, & Neal. 1996). Overexpression of ERBB2 has been found in the absence of gene amplification implicating post transcriptional mechanisms.

The protooncogene CCND1, located at 11q13, is overexpressed in TCC of the bladder (Richter, Jiang, Gorog, et al. 1997). Its product, cyclin D1, is associated with TCC recurrence and is involved in the retinoblastoma gene (Rb) pathway. The Rb gene is deleted in 30% of TCCs (Knowles. 1999); it is a tumour suppressor gene and its loss is associated with tumour progression and decreased survival (Cairns, Proctor, & Knowles. 1991). Its product (pRb) normally phosphorylates E2F, a protein which regulates the cell cycle. Loss of Retinoblastoma gene or blocking of its product results in unregulation of E2F and leads to uncontrolled proliferation.
Deletions of chromosome 9 are the most frequent genetic event in bladder cancer. They occur in between 30% and 70% of cases, even in early disease (Cairns, Shaw, & Knowles. 1994). The areas deleted lie in the INK4A and INK4B loci on 9p21. This area is associated with the tumour suppressor gene CDKN2A which codes for the p16 cell cycle inhibitory protein. This inhibits the cyclin dependent kinases (cdk), cdk4 and cdk6. Overexpression of p16 leads to cell cycle arrest at G1. However, loss of p16 allows the cyclin dependent kinases cdk4 and cdk6 to phosphorylate the Rb gene product. This in turn prevents its normal complexing with E2F. E2F thus released allows the progression of the cell cycle into the DNA synthesis (S) phase. Therefore, loss of p16 disrupts the Rb gene pathway allowing progression of the cell cycle. 9p21 also codes for two other cell cycle regulators, p14ARF and p15 (Knowles. 1999). The p14ARF gene is part of the p53 pathway of cell cycle regulation.

The p53 tumour suppressor gene is the most frequently altered gene in human cancer (Harris & Hollstein. 1993). Loss of heterozygosity (LOH) at its locus, 17p13 with mutation of the remaining allele leads to nuclear accumulation of mutated p53. p53 protein can be inactivated by mechanisms other than p53 gene mutation. Indeed, p53 protein accumulation often occurs in the absence of mutation, and yet still has prognostic value. This correlates with risk of recurrence and decreased survival in bladder cancer (Esrig, Elmajian, Groshen, et al. 1994). Wild type p53 can induce cell cycle arrest in the G1 phase or cause cell death by apoptosis. The p14ARF repressor normally blocks p53 degradation.
by MDM2. Loss of p14ARF allows MDM2 to degrade p53 and loss of this suppressor allows the cell cycle to continue.

Disruption in the normal control of the cell cycle in bladder cancer can occur by multiple means. Overexpression of oncogenes lead to growth stimulation (ras) and increase in cyclin kinase levels. Loss of control of cell cycle progression occurs by deletion of the Rb gene and blockage of its action via loss of p16. Further loss of control and inhibition of apoptosis occurs by loss of function of the p53 pathway.

1.1.4 The Pathology of Bladder Cancer

1.1.4.1 Introduction

There are three main histological types of bladder carcinoma, Transitional Cell Carcinoma (TCC), Squamous Cell Carcinoma (SCC) and Adenocarcinoma. In the United Kingdom more than 90% of bladder cancers are transitional cell carcinoma.

1.1.4.2 Transitional cell carcinoma

Histologically, TCC demonstrates an increased number of epithelial cell layers, papillary folding of the mucosa, loss of cell polarity, abnormal cell maturation from basal to superficial layers, giant cells, nuclear crowding, increased nuclear to cytoplasmic ratio, prominent nucleoli, clumping of chromatin and an increased number of mitoses (Koss. 1975).
1.1.4.3 Pathological Grading of Transitional Cell Carcinoma

Bladder cancer is graded depending upon certain pathological criteria. Well-differentiated, grade 1 (G1) tumors have a thin fibrovascular stalk with a thickened urothelium containing more than seven cell layers, with cells exhibiting only slight anaplasia and pleomorphism. The disturbance of the base-to-surface cellular maturation is mild, and there are only rare mitotic figures.

Moderately differentiated, grade 2 (G2) have a wider fibrovascular core, a greater disturbance of the base-to-surface cellular maturation, and a loss of cell polarity. The nuclear-cytoplasmic ratio is higher, with more nuclear pleomorphism and prominent nucleoli. Mitotic figures are more frequent.

Poorly differentiated, grade 3 (G3) tumors have cells that do not differentiate as they progress from the basement membrane to the surface. Marked nuclear pleomorphism is noted, with a high nuclear-cytoplasmic ratio. Mitotic figures may be frequent.

In 1998 the World Health Organisation and the International Society of Urological pathology published a new consensus classification of TCCs (Epstein, Amin, Reuter, & Mostofi. 1998). This altered, in particular, papillary lesions of lower grades. Mildly dysplastic lesions were classified as Papillary Urothelial neoplasms of Low Malignant Potential. They have an orderly arrangement of cells within papillae with minimal architectural abnormalities
and minimal nuclear atypia. These lesions are only very rarely associated with invasion or metastasis. However, they do have the potential to recur.

Papillary urothelial carcinomas, low grade, are characterized by an overall orderly appearance but with easily recognisable variation of architecture or cytological appearance. There is variation of polarity and nuclear size, shape and chromatin texture. Mitotic figures may be seen. These tumours have a low (<5%) risk of invasion but frequently recur.

Papillary urothelial carcinoma, high grade, have a totally disorganised appearance, even at low power. Cells are irregularly clustered. Chromatin is clumped with frequent nucleoli and mitotic figures are seen at all cell levels. These tumours have a 15% to 40% risk of progression.

1.1.4.4 Pathological Staging of Transitional Cell Carcinoma

The staging of bladder cancer is dependent upon the depth of tumour invasion. This can be assessed by a combination of clinical assessment, endoscopy and imaging or by histological examination when the stage is then prefixed by “p”. Macroscopically, bladder cancer may be papillary, nodular, mixed or flat. Approximately 70% are papillary, 10% are nodular and 20% are mixed.
A major distinction can be made between stages pTa, pT1 and carcinoma in situ (cis) which are classed as superficial bladder cancer (SBC) and stages pT2 and above which represent invasive bladder cancer, table 1.2 & figure 1.1.

At the time of diagnosis, 74% of bladder cancers are superficial, of these 70% are pTa and 30% are pT1 (Ro, Staerkel, & Ayala. 1992). pTa tumours are confined to the mucosa only, pT1 tumours have breached the lamina propria layer into the submucosa.

Carcinoma in situ (cis) of the urinary bladder describes a severe cellular dysplasia confined to the mucosa. It was first described as an entity in bladder cancer by Melicow in 1952 as grossly abnormal areas of epithelium adjacent to bladder cancer in cystectomy specimens (Melicow. 1952) and was soon seen to be a high risk entity (Melicow & Hollowell. 1952). It is not always visible endoscopically but may appear as a red area or velvety patch.

1.1.4.5 Non TCC tumours of the bladder

In England, squamous cell carcinoma only accounts for 1% of bladder cancers. (Costello, Tiptaft, England, & Blandy. 1984) In Egypt, however, it accounts for 75%. Eighty percent of these are associated with Schistosoma haematobium infection (El-Bolkainy, Mokhtar, Ghoneim, & Hussein. 1981). Non-bilharzial squamous cell carcinomas are often caused by chronic irritation from long term catheters, bladder stones or chronic urinary infections.
Table 1.2  Staging of bladder cancer

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>The primary tumour cannot be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>There is no evidence of primary tumour</td>
</tr>
<tr>
<td>Cis</td>
<td>Carcinoma in situ</td>
</tr>
<tr>
<td>Ta</td>
<td>Non invasive papillary carcinoma only in the mucosa</td>
</tr>
<tr>
<td>T1</td>
<td>Tumour invades the subepithelial connective tissue</td>
</tr>
<tr>
<td>T2</td>
<td>Tumour invades muscle</td>
</tr>
<tr>
<td>T2a</td>
<td>Tumour invades superficial muscle, the inner half</td>
</tr>
<tr>
<td>T2b</td>
<td>Tumour invades deep muscle, the outer half</td>
</tr>
<tr>
<td>T3</td>
<td>Tumour invades perivesical tissue</td>
</tr>
<tr>
<td>T3a</td>
<td>Microscopically</td>
</tr>
<tr>
<td>T3b</td>
<td>Macroscopically (extravesical mass)</td>
</tr>
<tr>
<td>T4a</td>
<td>Tumour invades prostate, uterus or vagina</td>
</tr>
<tr>
<td>T4b</td>
<td>Tumour invades pelvic wall or abdominal wall</td>
</tr>
</tbody>
</table>

{Sobin L & Wittekind Ch 1997 ID: 1113}
Figure 1.1  Staging of Bladder Cancer

A. Superficial disease with no invasion of the muscle
B. Invasive disease invading, in this case through (pT3b) the muscle
C. Carcinoma in situ may appear as a red area on the mucosa
Histologically, there are characteristic keratinized islands containing eccentric aggregates of cells called squamous pearls.

Adenocarcinoma accounts for less than 2% of bladder cancer (Kantor, Hartge, Hoover, & Fraumeni, Jr. 1988). It may be primary vesical, urachal or metastatic. Primary vesical adenocarcinoma occurs around the trigone and on the lateral walls, commonly in response to chronic infection (Nielsen & Nielsen. 1983). Most are mucin producing but all variants of enteric adenocarcinoma may occur. Urachal tumours are very rare. They are separate from bladder urothelium and have a poor prognosis (Johnson, Hodge, Abdul-Karim, & Ayala. 1985). The most common adenocarcinomas are metastatic from rectum, stomach, endometrium, breast, prostate and ovary but they constitute only 0.26% of bladder tumours (Klinger. 1951).

1.1.5 Pathways to progression in superficial bladder cancer

It was previously believed that SBC was the earliest step on a downhill ladder to invasive bladder cancer, (Jewett & Strong. 1946) suggesting that all SBC’s had the capability to progress (Koss. 1998). However, only 15% to 30% of SBC will progress to muscle invasive disease.

It now appears likely that there are two pathways to invasive bladder cancer, progression from superficial disease or more commonly progression from cis (Spruck, Ohneseit, Gonzalez-Zulueta, et al. 1994). There is both clinical and molecular support for this theory.
Of pT1 tumours, 25% progress, whereas only 5% of pTa tumours are likely to do so (Sandberg & Berger. 1994). Less than 2% of pTa tumours are of high grade (G3) whereas pT1 tumours are more commonly high grade (Jordan, Weingarten, & Murphy. 1987; Koss. 1998). pTa tumours are less likely to be associated with cis than pT1 tumours (Kakizoe, Matumoto, Nishio, Ohtani, & Kishi. 1985; Kakizoe, Matumoto, Nishio, Ohtani, & Kishi. 1985). Well differentiated tumours of the urinary bladder very rarely progress to invasive carcinoma unless accompanied by cis. Two separate pathways have therefore been proposed for progression to invasive carcinoma of the bladder, an aggressive pathway and a superficial pathway. Those patients who present with invasive disease are likely to have gone down the aggressive pathway with no early superficial disease but perhaps with subclinical cis. The majority of patients pass down the superficial pathway and most will not progress beyond the superficial stage. Some (15%-30%) will continue down this pathway and develop invasive bladder cancer.

A proposed chronology of low risk superficial bladder cancer is supported by differential mutation of genes in the two pathways (Spruck, Ohneseit, Gonzalez-Zulueta, et al. 1994). \textbf{figure 1.2.} Chromosome 9p and 9q deletions occur in up to 34% of papillary pTa tumours but are rare in cis (12%). Mutation of the p53 suppressor gene is found in invasive bladder cancer where it is a marker of poor prognosis, (Esrig, Spruck, Nichols, et al. 1993; Esrig, Elmajian, Groshen, et al. 1994) it is also found in cis (63%) however, it is less commonly seen in pTa SBC (3%). A larger number of genetic defects are seen in
Figure 1.2  A model for TCC progression based on histopathological observations and molecular data.  

Normal urothelium

Hyperplasia

Atypia/G1

Hyperplasia

Atypia/G2

Atypia

Superficial papillary (Ta)

Carcinoma in situ

T1- invading lamina propria

Muscle invasive carcinoma (>T2)

9p/9q-?

TP53

9p/9q- (34%)

TPS3 (3%)

10-20%
pT1 tumours however and p53 mutation is more common (32%). Chromosome 9 deletions are more common in pT1 disease than in both cis and pTa tumours (59%). In pT1 disease there are gains in 1q, 8q, 3p, 3q, 5p, 6p and 10p, losses of 2q, 8p and 11p and amplification of 1q, 3p, 6p, 8p, 8q, 10p, 11q, 12q, 13q, Xp and Xq (Knowles. 1999). Muscle invasive bladder cancer has further mutations with a 51% mutation rate of p53.

1.1.6 Natural History of Superficial Bladder Cancer

SBC is a polychronotropic disease. It may present and recur at multiple sites and at multiple times anywhere in the urothelium. Upto 70% of SBC will recur, but only between 15% and 30% progress to muscle invasive disease (Lutzeyer, Rubben, & Dahm. 1982).

1.1.6.1 Recurrence of Superficial Bladder Cancer

There is a recurrence of between 30% and 90% of SBC after initial transurethral resection (Herr. 1997). The presence of multiple tumours, early recurrence, larger tumours and concomitant cis at presentation all increase the risk of tumour recurrence (Parmar, Freedman, & Hargreave. 1989). Millan-Rodriguez et al evaluated the risk factors for recurrence in 1,529 patients with primary SBC. They found that multiplicity (OR=2.0), tumours larger than 3cm (OR=1.65) and the presence of cis (OR=1.6) were all significant risk factors for subsequent tumour recurrence (Millán-Rodriguez, Chéchile-Toniolo, Salvador-Bayarri, Palou, & Vicente-Rodríguez. 2000).
Lamina propria invasion (pT1) and grade 3 disease at presentation also increase the risk of tumour recurrence. In a series of 249 SBC’s at three years follow up, recurrence rate for G1 disease was 50%, for G2, 59% and G3, 80%. In the same series, patients presenting with pT1 disease saw a recurrence rate of 75% at three years compared to 48% in pTa disease (Heney, Ahmed, Flanagan, et al. 1983).

Early recurrence tends to predict future recurrence rate. Fitzpatrick et al studied the natural history of pTa G1 and G2 tumours. Those who did not recur by three months had an 80% chance of remaining tumour free whereas those who did develop another tumour within three months had a 70% chance of future recurrences (Fitzpatrick, West, Butler, Lane, & O’Flynn. 1986). Follow up in this study was upto 10 years and although some studies have shown that recurrence is rare after a long tumour free interval, it does occur. Tumours of greater than 10g resection weight also had a higher risk of recurrence. Herr et al reported 15 year progressions of 39% in G3pTa disease and 56% in G3pT1 disease. Thompson et al followed 20 patients who had remained disease free for five years and found 7 (35%) subsequently progressed (Herr. 1997; Thompson, Campbell, & Kramer. 1993).

1.1.6.2 Progression of superficial bladder cancer

Longitudinal studies of SBC treated by transurethral resection or fulguration alone have shown that increased grade, lamina propria invasion (pT1), the presence of \textit{cis}, size and multiplicity all increased the risk of
progression (Millán-Rodríguez, Chéchile-Toniolo, Salvador-Bayarri, Palou, & Vicente-Rodríguez. 2000). In The National Bladder Cancer Group study, 207 patients were followed up for a median of 39 months. Progression rate of grade 1 disease was 2%, grade 2 11% and grade 3 45%. Only 4% of cases presenting with pTa disease progressed whereas 30% of pT1 disease progressed. Tumours >5cm showed 35% progression, those <5cm showed 9% (Heney, Ahmed, Flanagan, et al. 1983). In a study of 178 G1pTa tumours only three (2%) developed muscle invasion (Prout, Barton, Griffin, & Freidell. 1992). Fitzpatrick et al followed 414 patients with pTa grade 1 or 2 disease and saw a progression to muscle invasion in 5% (Fitzpatrick, West, Butler, Lane, & O’Flynn. 1986) whereas Thompson et al found a 35% (7 out of 20) progression rate after a five year disease free interval; the numbers however were small (Thompson, Campbell, & Kramer. 1993).

The presence of *cis* predicts a poorer prognosis with at least 50% progression to invasive disease (Herr. 1997). Carcinoma in situ increases the risk of death from bladder cancer by three fold (Millán-Rodríguez, Chéchile-Toniolo, Salvador-Bayarri, Palou, & Vicente-Rodríguez. 2000).

Millan-Rodriguez et al grouped patients in to three risk groups dependent on clinico-pathological prognostic factors. Low risk tumours were G1pTa or solitary G1pT1, intermediate risk tumours were multiple G1pT1 or G2pTa or solitary G2pT1’s, high risk tumours were multiple G2pT1, any G3 disease or the presence of *cis* (Millán-Ro, M, Millán-Rodríguez, et al. 2000).
1.1.6.3 Explaining the polychronotropic nature of SBC

The presence of several tumours widely separated across a large area of urothelium and the appearance of recurrent tumours at new sites has led to the concept of a "field change" throughout the urothelium lending it a pre-neoplastic nature (Harris & Neal. 1992).

An alternative theory is a monoclonal origin of bladder cancer with all subsequent tumours being derived from the same original cell. Recurrences and metachronous tumours could be explained by cell implantation or seeding or possibly local migration. This theory is supported in a study by Sidranski et al who found X chromosome inactivation in 13 bladder cancers from four female patients undergoing cystectomy revealed inactivation of the same X chromosome in tumours in the same bladder but a random pattern of inactivation in normal mucosa (Sidransky, Frost, Von Eschenbach, Oyasu, Preisinger, & Vogelstein. 1992). There are often identical genetic mutations in both synchronous and metachronous tumours in the same patient, for example, p53 mutation (Xu, Stower, Reid, Garner, & Burns. 1996; Takahashi, Habuchi, & Kakehi. 1998). It is however, more difficult to fit this theory to cases of very late recurrence after a long tumour free period, although these could be new primary tumours.

1.1.7 Clinical Presentation

The majority of SBC patients present with painless haematuria. Haematuria is either gross or microscopic; gross haematuria is more likely to indicate the presence of bladder cancer. Lower Urinary Tract Symptoms (LUTS)
such as frequency, dysuria and urgency may be initial presenting symptoms. These are particularly associated with \textit{cis}. The incidental finding of SBC occurs when cystoscopic examination is carried out for another indication, for example, prior to Transurethral resection of the prostate.

1.1.8 The Management of Superficial Bladder Cancer

1.1.8.1 Endoscopic

Endoscopic transurethral resection of superficial bladder tumours provides tissue for histological examination and excises the tumour. It is important not only to remove the macroscopic tumour but also to resect the area beneath it at least into detrusor muscle. The pathologist must be provided with muscle to determine whether the tumour is superficial or invasive. In cases where muscle is not provided a further resection is required. In high grade tumours or pT1 disease early resection of the tumour base or scar is recommended to ensure all disease has been removed and to confirm the staging. In a series by Herr et al, 25\% of tumours that were resected were understaged (Herr. 1997). For some tumours endoscopic resection is the only treatment required (Herr. 1997). Follow up by cystoscopy with or without urine cytology is however, mandatory.

1.1.8.2 Intravesical therapy

Intravesical adjuvant chemotherapy and immunotherapy reduces recurrence of SBC (Hall. 1997). A single instillation of intravesical agents, Epirubicin or Mitomycin-C, is superior to transurethral resection alone
(Oosterlink, Kurth, Schroder, Bultinck, Hammond, & Sylvester. 1993; Tolley, Parmar, Grigor, et al. 1996). A six week course of intravesical chemotherapy can be used to reduce recurrence in intermediate or high risk tumours (Oosterlink, Lobel, Jakse, Malmstrom, Stockle, & Sternberg. 2001).

Intravesical bacillus Calmette-Guerin (BCG) is used if there is associated cis, multiple tumours (more than four), high grade, advanced stage (pT1) disease or failure of chemotherapy. The mechanism of action appears to be immune related and an initial six week course is given followed by booster courses. Maintenance schedules provide a significant improvement in time to recurrence (Lamm, Crawford, Blumenstein, et al. 2000). Maintenance BCG schedules lasting at least one year are associated with delayed progression, compared with induction BCG schedules (Sylvester RJ et al, 2002).

Some advocate early radical cystectomy for high risk superficial disease (Esrig, Freeman, Stein, & Skinner. 1997).

1.1.8.3 Surveillance

The fact that between 30% and 90% of superficial bladder cancers recur after initial resection (Herr. 1997) necessitates careful surveillance of treated SBC. Various methods have been attempted to predict which bladder cancers will recur or progress. Urine cytology is often used as an adjunct to cystoscopy. It has a high predictive value for high grade disease or cis but not low grade disease and cannot be used as a substitute for cystoscopy (Ro, Staerkel, & Ayala.
1992). Bladder tumour antigen (BTA) detects human compliment factor H-related protein. Its sensitivity is between 40% and 72% but again it is less sensitive in low grade tumours (Ellis, Blumenstein, & Ishak. 1997). Nuclear Matrix Protein 22 (NMP22) is a protein found in urothelium but is 10 times more common in TCC. It has also been used as a marker for recurrent disease. Again it is highly sensitive in high grade disease (70%-80%) but not in low grade tumours (Soloway, Briggman, Carpinito, et al. 1996). The gold standard therefore remains the check cystoscopy. Often a day case procedure, performed under local anaesthetic, with a flexible cystoscope, it is still an invasive intervention for each patient and represents a considerable financial load.

1.1.9 Prognosis

Superficial bladder cancer has an overall mortality rate of 4 per 100,000. When graded into low, intermediate and high risk groups, Millan-Rodriguez et al found the mortality rates for SBC were 0%, 1% and 10% respectively (Millán-Ro, M, Millán-Rodríguez, et al. 2000).

1.1.10 Summary

Bladder cancer is a common disease. At presentation, the majority of cases are superficial. A large proportion of cases of SBC recur after initial treatment. A small but significant number of cases will subsequently progress to muscle invasive disease which has a considerable mortality. At present an intensive surveillance program for SBC follow up is required which is costly and not without morbidity. There are only a limited number of prognostic indicators
available at disease presentation which may help to predict the subsequent disease course. Neither together nor alone can these substitute regular check cystoscopies for SBC management.
1.2.0 Angiogenesis

1.2.1 Introduction

Angiogenesis is the formation of new blood vessels from pre-existing vessels by sprouting and can be a normal biological process. The term angiogenesis was first coined by Hertig in 1935 (Hertig. 1935). It differs from vasculogenesis, which is the appearance of new vessels from blood islands. It was previously believed that, unlike angiogenesis, vasculogenesis does not contribute to repair and disease in post-embryonic life (Battegay. 1995). This has now been challenged and it is clear that vasculogenesis does play some part although vessels may be immature (Carmeliet. 2000). Angiogenesis may be physiological or pathological. It occurs in endothelial cells during normal cell turnover but this turnover is slow, the life span of an endothelial cell being around 1000 days (Denekamp. 1993). Angiogenesis also occurs as part of the wound healing process (Arnold & West. 1991) and in the endometrium during menstruation, where the cell turnover may be as high as four days (Rogers, Abberton, & Susil. 1992; Torry & Rongish. 1992). Angiogenesis, along with vasculogenesis, is required for growth and development in the embryo (Risau. 1991).

Pathological angiogenesis occurs in conditions where the disease process involves new vessel growth, for example, diabetic retinopathy, psoriasis and rheumatoid arthritis (Folkman & Klagsburn. 1987). Angiogenesis also plays a major part in the growth and progression of cancer.
1.2.2 Angiogenesis in Cancer

Ambroise Paré (1510-1590), the Sixteenth Century French surgeon, discussed the use of cautery in the management of cancers in his book, *The Practise of Chirurgery, and the use of Three and Fifty instruments*. In the English translation of 1634, the word cancer and the word crab were freely interchanged (Paré. 1634). Indeed, at the end of his description he included a picture of a crab so the practitioner might know how to recognise a cancer, *frontispiece*. The derivation of the word cancer, in its medical context, stems from the similarity of this disease (particularly cancer of the breast) to the body and projecting legs of a crab. This singular appearance represents the profound and abnormal vascularity of these tumours. Thus, the association with cancers and their peculiar blood supply has long been recognised.

The luxuriant vascularity of many solid tumours is not purely a consequence of malignant growth; it is a necessity for that growth.

In 1971, Judah Folkman proposed that these vessels were absolutely essential for tumour growth and that angiogenesis was a fundamental requirement for the growth, progression and metastasis of cancer (Folkman. 1971).

It is now accepted that a neoplasm, like the developing embryo, requires oxygen and nutrition for continued growth and survival. Tumour induction and development to a very small size, some early in-situ carcinomas contain fewer
than $10^6$ cells (Folkman. 1990), may proceed without a specific blood supply. Once a tumour is 2 to 3mm in size it can no longer survive by diffusion alone for provision of nutrients and removal of waste products. For a tumour to continue to grow, invade locally and metastasise, a blood supply must be provided. A neoplastic cell line, therefore, must be able to induce angiogenesis in order to survive and develop into a cancer (Folkman. 1990). The first evidence for this was based on animal model experiments.

In 1941, Harry Greene published a study on heterologous transplantation of mammalian tumours (Greene. 1941). He described the transplantation of rabbit adenocarcinomas (H-31) into the anterior chambers of guinea pig eyes. A pinkish colouration was seen in the tumour after around two weeks indicating successful tumour take; this represented early neovascularisation. Visible vascularisation was usually seen by the third week. Occasionally, tumour growth was delayed (up to 110 days). Greene noted that this latent period was associated with a lack of vascularisation.

In 1945, Algire and Chalkley specifically looked at the vascular reactions of benign and malignant tissue implanted into a transparent skin-flap chamber of a mouse (Algire, Chalkley, & Legallais. 1945). Growth of implanted mammary gland carcinomas only began after new capillary sprouts appeared in adjacent tissue. When compared to wound healing and implantation of benign tissue they observed the tumour implants had an outstanding capacity to elicit continued growth of new capillaries from the host. Neovascularisation occurred much
sooner in tumour implants (three days) than with wound healing or benign tissue implants (six days).

In 1966, Folkman et al developed an isolated organ perfusion system to provide a vascular bed for the study of tumour growth (Folkman, Cole, & Zimmerman. 1966). They found that after a week and a half there was loss of endothelial cells. After this time tumour implants grew only to 2-3mm and no further but expand rapidly after transplantation onto mice (Gimbrone, Leapman, Cotran, & Folkman. 1972a; Folkman. 1990).

Gimbrone et al investigated the existence of a fundamental relationship between angiogenesis and the ability of a tumour to grow malignantly. They found that prevention of neovascularisation led to tumour dormancy (Gimbrone, Leapman, Cotran, & Folkman. 1972a). Brown-Pearce rabbit epithelioma was implanted into the eyes of male New Zealand rabbits. Tumour was placed either on the iris or in the avascular anterior chamber of the eye. Tumour growth and vascularity were observed.

In the iris implants, a characteristic growth pattern was seen. For the first five days there was only slow growth of tumour. At between five and eight days, vessels became visible within the tumour implant. This was associated with significant increase in size and was followed by exponential growth of the tumour to fill the entire anterior chamber. Once the eye was filled with tumour, the rate of growth deteriorated, but growth continued, if left, until rupture of the
globe. This growth pattern is described by a sigmoid curve revealing the tri-phasic growth of an early pre-vascular phase, a rapid growing vascular phase and a slower late growth phase. The rate of growth of the vascular phase was ten times that of the pre-vascular phase.

Tumour implants in the avascular anterior chamber failed to vascularise, remaining small pearly white masses. On careful inspection these implants underwent a biphasic growth pattern. During the first six to eight days there was slight increase in size to four times their initial volume. The final mean volume was $0.56 \text{mm}^3$ ($\pm 0.24 \text{SD}$) and none attained a volume greater than $1 \text{mm}^3$. This growth corresponded to the pre-vascular phase of the iris implants. Microscopic examination of the anterior chamber tumours at 16, 34 and 44 days confirmed their avascularity.

Reimplantation of anterior chamber tumours into the irises of fresh rabbits caused the tumours to grow in a vascularising tri-phasic pattern. Human retinoblastomas, which metastasise to the avascular anterior chamber of the eye, follow a similar pattern of avascularity and growth restriction.

Gimbrone et al then further advanced this model by implanting within the cornea as well as on the iris and in the anterior chamber (Gimbrone, Leapman, Cotran, & Folkman. 1973b). Corneal implants were placed either peripherally, close to the blood supply or centrally, further from a potential blood supply. The iris and anterior chamber implants grew as before. Centrally placed corneal
implants remained avascular for up to five weeks growing only slowly. At two to three weeks, neovascularisation of the iris vessels was seen. Peripherally placed corneal implants became vascularised within seven to twelve days. After that time they grew rapidly. Interestingly, vessel proliferation was also seen in the iris on the opposite side to the tumour implant.

They concluded that cellular contact was not necessary between tumour cells and host vessels for tumour neovascularisation to occur. They suggested that the tumour cells may be producing a mediator that stimulated vascularisation.

Similar results were seen in a different animal model by Knighton et al in 1977 (Knighton, Ausprunk, Tapper, & Folkman. 1977). A 1mm x 1mm x 2mm implant of Walker 256 carcinoma was injected into the chorioallantoic membrane of a chicken egg through a window in the shell. The same window was used to observe vessel growth. The tumours exhibited pre-vascular and vascular growth phases. Neovascularisation always occurred at 72 hours. Unlike the previous studies this experiment placed the tumours in an area surrounded by healthy vessels. Even so, tumour growth still did not occur until the tumour was penetrated by host vessels.

Based on this work, Folkman in 1971, concluded that, “The growth of solid neoplasms is always accompanied by neovascularisation” (Folkman. 1971). He stated this evidence indicated that tumour cells have a stimulating influence
on endothelial cells, noting that, capillaries grow towards tumours from the host unlike skin grafts which send out capillaries. He also importantly stated that the vessels have a modulating influence on tumours. He described an early diffusion stage (pre-vascular) of minimal tumour growth and, after vessel growth, a faster perfusion period of tumour growth. He summarised by proposing the use of anti-angiogenesis as novel cancer therapy.

1.2.3 The Process of Angiogenesis

The process of angiogenesis is a continuum. It can however, be conveniently divided into a series of stages for descriptive purposes, *figure 1.3*. Much of this information was elucidated by Ausprunk et al in 1977 (Ausprunk & Folkman. 1977). The new vessel buds arise from venules. The basement membrane of the venule is first degraded and the surrounding extra cellular matrix altered to support new vessel growth. Tumour cells cause a variety of proteases and collagenases to be secreted which break down the membrane and alter the matrix (Folkman & Klagsburn. 1987). Endothelial cells then move towards an angiogenic stimulus by cell proliferation and cell migration. The cells elongate to form a vessel sprout. At first this is a solid projection of cells. The endothelial cells then curve and develop a lumen to form a tube. Two tubes join forming a capillary loop and blood then flows. Pericytes align along the base of the sprout with subsequent vessel formation occurring from the apex of the loop. The vessels are stabilised by mural smooth muscle cells. These, somewhat artificial divisions of a continuous biological process allow the study of angiogenesis and angiogenically active substances by comparing the stage at
Figure 1.3 The Process of Angiogenesis

Figure 1.3.1 A vessel bud forms from a pre-existing venule. The local extracellular matrix is broken down to allow sprout growth.

Figure 1.3.2 Solid cores grow out towards the tumour cells drawn by chemotactic cytokines.

Figure 1.3.3 The vessel sprouts join to form an arcade, a lumen forms and blood flows.
which they act. It also allows some thought as to how to inhibit angiogenesis for therapeutic ends.

1.2.4 The Control of Angiogenesis

Angiogenesis is controlled, as in all molecular systems, by a balance of promoter and inhibitor substances. For a tumour to induce neovascularisation angiogenic factors must be present and at the same time there must be inhibition of anti angiogenic factors which normally hold vessels in the quiescent state. In normal tissues angiogenesis is rare; endothelial cell turnover approaches 1000 days (Denekamp. 1993).

For new vessel growth to occur, a pro-angiogenic phenotype must be produced (Bouck, Stellmach, & Hsu. 1996). An increase in angiogenic factors may stimulate endothelial cells directly, activating neovascularisation, or may act indirectly on the surrounding extra cellular matrix rendering it more pro-angiogenic. Stimulation of stromal fibroblasts produces further pro-angiogenic factors. Other angiogenic factors are chemotactic to macrophages or mast cells which themselves may release further pro-angiogenic substances. Generation of a pro-angiogenic phenotype also involves the suppression of antiangiogenic factors found in normal tissues. Thus, the sum of the activities of pro- and anti-angiogenic molecules induces new vessel growth.

One of the first events in the development of the new vessels bud is an increase in vascular permeability. Microvascular hyperpermeability leads to a
profound alteration in the extra cellular matrix transforming it from an antiangiogenic to a pro-angiogenic phenotype. The increased permeability allows the extravasation of plasma proteins including fibrinogen; this clots forming a fibrin gel in the extra cellular matrix. This fibrin provides a medium which attracts and supports the growth of endothelial cells and fibroblasts contributing to angiogenesis (Dvorak, Harvey, Estrella, Brown, McDonagh, & Dvorak. 1987; Dvorak, Brown, Detmar, & Dvorak. 1995).

The most potent vasodilator known to man is Vascular Endothelial Growth Factor (VEGF) a major pro-angiogenic factor. VEGF causes increased vascular permeability by the opening of vesiculo-vacuolar organelles, grape-like clusters of interconnecting smooth membrane bounded vesicles and vacuoles extending across the entire thickness of the venular endothelial cytoplasm (Feng, Nagy, Hipp, Dvorak, & Dvorak. 1996). Vascular permeability is also increased by the pro-angiogenic factor, thymidine phosphorylase (TP), also known as Platelet Derived Endothelial Cell Growth Factor (PDECGF). This occurs indirectly by stimulation of mast cells which release histamine (Gruber, Marchese, & Kew. 1995). TP is also chemotactic to endothelial cells drawing them towards the tumour (Toi, Taniguchi, Yamamoto, Kurisaki, Suzuki, & Tominaga. 1996).

Increased vascular permeability leads to destabilisation of the cell to cell connections of endothelial cells. This is further promoted by angiopoetin-2 (Ang-2) via inhibition of the Tie-2 signalling pathway. It also allows migration of
endothelial cells by redistribution of the platelet endothelial cell adhesion molecule (PECAM-1) (Gale & Yancopoulos. 1999).

This increase in permeability and membrane destabilisation is balanced by the action of angiopoietin (Ang-1) which acts as a ligand to the endothelial receptor Tie-2. This is a natural inhibitor of permeability and stabilises the membrane preventing excessive leakage (Thurston. 2000).

For a vessel sprout to project from the venule a change must occur in the surrounding extra cellular matrix, it must develop an angiogenic sympathetic phenotype. Matrix metalloprotinases (MMP’s) degrade the matrix proteins and allow new vessel growth; the degraded matrix releases further growth factors. Once again this pro-angiogenic process is balanced by Tissue Inhibitors of Matrix Metalloprotinases (TIMP-1 and TIMP-2) which inhibit it. Multiple factors lead to endothelial cell sprouting which consists of both proliferation and migration. Both VEGF and TP are mitogens as is basic fibroblast growth factor. Basic Fibroblast Growth Factor (bFGF) was the first angiogenic cytokine to be identified (Gospodarowicz. 1976). VEGF appears to be an initiator of endothelial proliferation but requires the input of other factors to complete the process by stabilisation of endothelial cell groups (Gale & Yancopoulos. 1999). This sprouting is inhibited by the antiangiogenic factors angiostatin, endostatin and antithrombin III (Carmeliet. 2000). These substances inhibit both the stimulated proliferation and migration of the endothelial cells.
Once a sprout has formed, the cells metamorphosise to form a vessel lumen. VEGF is a promoter of lumen formation, particularly in combination with Ang-1 (Suri, McClain, Thurston, et al. 1998). Thrombospondin (TSP-1) however, is an inhibitor of lumen formation.

Once formed, the new vessel must be stabilised. Endothelial survival molecules such as VEGF, Ang-1 and $\alpha_5\beta_3$ act to prevent endothelial apoptosis by tumour necrosis factor alpha (TNF$\alpha$), angiostatin and TSP-1 (Varner, Brooks, & Cheresh. 1995). These survival factors suppress the degenerative effects of p53, p21, p16, p27 and BAX but promote survival and anti-apoptotic factors such as P13 kinase/Akt, p42/44 mitogen activated protein kinase, Bcl-2, A1 and Survivin (Carmeliet. 2000).

Vessel stabilization is also achieved by the surrounding and supporting mural smooth muscle cells. VEGF promotes mural cell accumulation and Ang-1 and Tie-2 promote the interaction of smooth muscle cells with the endothelium (Gale & Yancopoulos. 1999). Transforming Growth Factor Beta (TGF$\beta$) further stabilises the vessel by inhibiting endothelial proliferation and inducing smooth muscle cell differentiation. It also stimulates the repair of the extra cellular matrix.

The control of angiogenesis in tumours is closely linked with tissue oxygen tension. Hypoxia and other stimuli cause inflammatory and tumour cells to generate angiogenic molecules (Shweiki, Itin, Soffer, & Keshet. 1992; Plate,
Breier, Millauer, Ullrich, & Risau. 1993). Tissue hypoxia triggers the release of Hypoxia inducible transcription factors (HIF) –1 and –2. These in turn induce the expression of VEGF, the VEGF receptors (VEGFR-1 and VEGFR-2), Ang-2, nitric oxide synthase, Transforming Growth Factor β-1 (TGF β-1), Platelet Derived Growth Factor –BB (PDGF-BB), endothelin-1, interleukin-8, cyclooxygenase-2 (COX-2) and Tie-1 (Blancher & Harris. 1998; Carmeliet, Dor, Herbert, et al. 1998; Maxwell, Dachs, Gleadle, et al. 1997).

1.2.5 Summary

Angiogenesis is the growth of new vessels from pre-existing venules and is a complex biological phenomenon under tight control in normal tissues by the balance of pro- and anti-angiogenic factors. For a neoplasm to grow beyond 2-3mm in size it must have a blood supply. Tumours are able to induce blood vessel growth by angiogenesis. Angiogenesis is a fundamental part of cancer biology.
1.3.0 The Measurement of Angiogenesis

1.3.1 Historical Studies of Angiogenesis measurement

In the Eighteenth Century, John Hunter (1728-1793) studied the seasonal growth of the antlers of the Fallow Deer (Cervus dama). He noted the early appearance of new blood vessels to supply the growing antlers, observing “a soft membranous pulp shooting out from this knob [the early sprouting antler from the deer’s skull], which is extremely vascular” (Hunter. 1861b). In general, he remarked that vessels, “would appear to have more powers of perfecting themselves, when injured, than any other part of the body; for their use is almost immediate and constant, and it is they which perform the operation of restoration on the other parts, therefore they themselves must first be perfect.” (Hunter. 1861a). As such he was a pioneer in the study of angiogenesis.

On 23rd October 1907, Professor E. Goldmann of Freiburg gave a lecture to The Royal Society of Medicine in London (Goldmann. 1907). In it he outlined his work and theories on the associations of cancers and blood vessels. He recorded the phenomenon that the normal regular distribution of blood vessels is disturbed by the invading growth of a cancer leading to chaotic irregularity. He noted the extensive new formation of vessels in the growing tumour, particularly at the periphery.

In experiments on mice, he injected Indian Ink into their vasculature which, he said, enabled him to study the number, form and width of new vessels.
He described a common feature of transplanted experimental tumours. As soon as they begin to grow, “a great commotion is produced in the system of surrounding blood vessels.” He felt the degree of this vascular reaction was related to the power of the host tissue to react against the cancer.

In 1927, Warren Lewis published a descriptive study of the vascular patterns of tumours in laboratory mice models (Lewis. 1927). Under ether anaesthesia, 50-100ml of 3% Indian Ink in Locke solution was injected into the aorta via an incision in the heart. He used the method previously described by Goldmann (Lindgren & Docent. 1945). Tumours were fixed in formalin and sections dehydrated and cleared in Eyclashymer fluid before being mounted in balsam. These specimens allowed Lewis to describe the vascular characteristics of a fibrosarcoma, a spindle cell sarcoma, a round cell sarcoma, a breast adenofibroma and an adenocarcinoma.

He concluded that different tumours had different vascular patterns and suggested these characteristics may be useful in diagnosis. He also noted from studies of tumour necrosis, that the blood vessels did not determine the growth of the tumour; rather, the tumour determined the growth and pattern of vessels.

Lingren and Docent studied the angio-architecture of human tumours acquired at surgery or post-mortem (Lindgren & Docent. 1945). Frozen sections, cut at 200μm, were stained with Lepehne’s stain which stains erythrocytes brown-black; no counterstain was used. This achieved a map of vessels on a pale
background. Adjacent sections of tissue were fixed in paraffin and stained with Haematoxylin and Eosin, Weingert’s stain, Gieson-Hansen’s stain or modified Mallory stain for comparison of the cyto-architecture.

The authors claimed their method gave similar results to dye injection but with less distortion of vessels due to rupture or dilatation caused by the pressure of dye injection. For study, the sections were photographed at x25 magnification with a Busch’s Citophot camera. X-ray angiography was also used to study tumour vessels.

Lindgren and Docent concluded that tumour vessels develop from pre-existing capillaries in a process which differed from foetal vasculogenesis. They quote Goldman’s theory that tumours exert an angiogenic influence and suppose that this could be due to certain substances produced by the tumours. They found that the vascular architecture “differed greatly from the normal tissue” in less differentiated tumours. They also felt that the tumour stroma affected the tumour vessel development. Although increasing grade of tumour was related to decreased vascular organisation no mention is made of vessel number or density in this context.

In 1945, Algire and Chalkley studied the vascular reaction of host tissue to tumour growth (Algire, Chalkley, & Legallais. 1945). Vessels were studied in mice by the use of a transparent chamber implanted in a skin flap. They quantified vascularity, defining it as, “the percentage of the volume measured
that consists of functional vessels.” The method used was based on geometric probability. They applied Chalkley’s method of repeated random presentation of a point to the vascular pattern, counting the number of hits (that is when a point falls upon a vessel) (Chalkley. 1943). The vascular level was obtained by measurement in 100 microscopic fields and represents the vascularity of the tissues.

They compared vascular growth in wounds, in implanted benign tissue and in implanted sarcomas and carcinomas. New vessel growth was seen at three days in cancers and six days in benign lesions. They concluded that the tumours produced a specific substance which induces vessel growth.

The question of quantitative measurement of angiogenesis was re-addressed by Brem et al in 1972 (Brem, Cotran, & Folkman. 1972). Working with Judah Folkman, he aimed to devise a reliable method of measuring angiogenesis in histological tissue.

They expressed angiogenesis as a summation of three vascular characteristics: vasoproliferation, endothelial cell hyperplasia and endothelial cell cytology. An equation was devised to give a microscopic angiogenesis grading system (MAGS).

\[ K_n N + K_e E + K_x X = \text{MAGS} \]
KnN represents vasoproliferation, KeE represents endothelial cell hyperplasia and KcX represents endothelial cell cytology.

In KnN, N is the number of vessels in a high powered (x500) field (1.10^5 μm²). As this varies between microscopes, the constant Kn was used to correct for this. Slides were scanned at low magnification and the most vascular area was chosen for analysis. This was felt to represent the best estimate of the neovascularising potential of the tumour. An effort was also made to choose an area where the majority of vessels were cut in cross section. Each vessel (that is, a lumen with a surrounding endothelial process) was counted, although a maximum score was set at 40. This was not further explained.

The endothelial cell hyperplasia score was calculated using Ke as a constant set at three and E as the number of endothelial cells lining the cross section of the most hyperplastic capillary. Vessels with more than 20 cells were not used and the maximum value of E was 10 making a maximum KeE of 30.

When calculating endothelial cytology, Kc was a constant, allowing the maximal cytological change of regenerating endothelium to correspond to maximal vasoproliferation and maximal hyperplasia and was set at six. X stood for the cytological appearance of the most primitive group of endothelial cells with a hyperplastic zone. A score of 0-5 was given depending upon the atypia of the cells.
Although this appears complex, the authors claimed its lack of complexity and its rapidity as an advantage. They stated, it took an experienced observer ten minutes to assess a section.

Once again, in 1985, Mlynek et al state there is a lack of a reliable and readily available method for measuring vascularisation of malignant tumours and offer one (Mlynek, van Beunigen, Leder, & Streffer. 1985). Vessels were identified by staining alkaline phosphatase present in endothelial cells. Fushin B was used which produced red staining. Some cross staining was seen with neutrophils and newly formed stroma. Arterioles and arterial capillaries stained but larger vessels and veins did not.

At first, both total length of vessels and number of cross sectioned vessels were measured. All areas of the section were assessed (range 105 – 331). To aid vessel measurement, photomicrographs were taken and put together to form a picture of the section at x98 magnification. As good correlation was found between vessel length and number of cross sectioned vessels (no data given), it was felt sufficient to count vessels in cross section only. Only 100 areas, each being 0.09mm² of tumour were counted in each section. They state that statistically it was found that evaluation of 100 fields sufficed to obtain representative results but no details of their calculation are given.
Using this method they found that normal mucosa (4.74 +/- 1.27) was richer in vessels than corresponding colorectal carcinoma (2.06 +/- 1.43) (p=0.002).

Noel Weidner et al quantified angiogenesis in 49 specimens of breast carcinoma to correlate with metastasis (Weidner, Semple, Welch, & Folkman. 1991). Vessels were identified using a polyclonal antibody to Factor VIII (FVIII). This staining technique was previously described by Pinkus et al who did not use it to perform vessel counts (Pinkus, Etheridge, & O'Conner. 1986). Weidner et al assessed a single area of one section taken from one embedded tumour block. This area was chosen by scanning the section at low magnification (x40 or x100) to detect the most dense area of vessels.

The subjective density of vessels in this area was given a grade from 1 to 4 (low to high). At higher magnification, the number of stained vessels were counted. This value was expressed as microvessel density (MVD) per high powered field (x200 or x400). Any separate area of staining was considered to be a single vessel. The presence of an obvious lumen or red blood cells was not required for confirmation. Counting was performed simultaneously by two observers using a double headed microscope. It took 5 – 10 minutes per case. For validation a third observer rescored the cases producing correlations of r=0.61 for subjective grading scores, r=0.59 for MVD at x200 magnification and r=0.62 for MVD at x400 magnification.
Weidner's technique of counting in the most vascular area (subsequently called the hotspot) has become the most frequently used model for MVD estimation.

1.3.2 The use of Microvessel Density in Cancer Prognosis

Angiogenesis as represented by a micro vessel count has been shown to be a major prognostic factor in many cancers.

Brem et al after devising their MAGS system for vessel quantification suggested it may be useful to compare vascularity with other tumour properties including metastatic potential (Brem, Cotran, & Folkman. 1972). The first quantitative evidence that the intensity of angiogenesis in a human tumour may predict progression was reported by Srivastava et al in melanoma in 1986 (Srivastava, Laidler, Hughes, Woodcock, & Shedden. 1986). In 20 cases, vessels were identified using Ulex europaeus agglutinin I (UEA-I). Vessel counts were performed using the IBAS-2 image analysis system in four fields in the tumour, at the tumour-dermal junction and in adjacent normal dermis. There was a positive correlation between the percentage of area occupied by vessels at the tumour-dermal junction and the thickness of melanoma (r=0.687, p<0.002). The thickness of malignant melanoma is related to prognosis. There was no direct correlation between vascularity and clinical outcome in this study.

Weidner et al then examined angiogenesis in 49 cases of carcinoma of the breast in 1991 (Weidner, Semple, Welch, & Folkman. 1991). Thirty patients had
metastases and 19 patients did not. As previously described, (section 1.3.1) the number of vessels (stained with polyclonal antibody against FVIIIRA) were counted at x200 and x400 magnification and subjectively graded. The area for counting was the most vascular seen at low magnification, again subjective. They used a Cox logistic regression to look at the association of metastasis with potential prognostic factors. Those patients with metastases were found to have 101 vessels per x200 field and 37 per x400 field. Those patients without metastases had 45 per x200 field and 17 per x400 field, p=0.003 (x200) and p=0.004 (x400) respectively. With each 10 vessel increase in MVD they found a 1.59 (1.19-2.12) increased risk of metastasis, p=0.003. A significant difference was also found between cases that were subjectively graded, p<0.001. In the multivariate regression analysis, subjective microvessel grade at x200 provided the best estimate of relative risk (r=0.8, p<0.0001).

The association of breast cancer outcome with angiogenesis measured by MVD was confirmed by Bosari et al and Horak et al in 1992 and in an editorial by Weidner in 1998 he was able to quote 29 articles of MVD measurement in breast cancer. In 1993, Wakui et al found that a high vascularity as measured by a blood capillary density ratio (BCDR) was related to high Gleason grade in cases of non metastatic prostate cancer (Wakui, Furusato, Itoh, et al. 1992). In 1993, Mikami et al found that high MVD (with FVIIIRA) staining was associated with increasing nodal status and stage in head and neck cancers (Mikami, Tsukuda, Mochimatsu, Kokatsu, Yago, & Sawaki. 1993). In the same year, Gasparini et al also looking at angiogenesis in head and neck cancers found
that high MVD (measured with antibody to CD31) was associated with metastasis (Gasparini, Weidner, Maluta, et al. 1993). In the multivarient analysis, MVD and stage were predictive factors.

Macchiarini et al were the first group to find that increased MVD correlated with metastases and tumour size in a series of 87 T1N0M0 non small cell lung carcinomas (Macchiarini, Fontanini, Hardin, Squartini, & Angeletti. 1992). Again, MVD was the only variable in the multivariate analysis to predict metastasis.

Further positive correlation between MVD and outcome were seen in rectal tumours (Saclarides, Speziale, Drab, Szeluga, & Rubin. 1994) and gastric cancer (Maeda, Chung, Takatsuka, et al. 1995) and in ovarian (Hollingsworth, Kohn, Steinberg, Rothenberg, & Merino. 1995) and cervical cancers (Wiggins, Granai, Steinhoff, & Calabresi. 1995).

1.3.3 Microvessel Density in Bladder Cancer

The first group to measure angiogenesis by MVD in bladder cancer were Dickinson et al in 1994 (Dickinson, Fox, Persad, Hollyer, Sibley, & Harris. 1994). They examined 45 cases of invasive bladder cancer. All cases were transurethral resection specimens of first presentation tumours. All these patients presented primarily with invasive bladder cancer (G2pT2 n=2, G3pT2 n=7, G3pT3 n=36) and none had progressed from a previous superficial bladder cancer. Vessels were identified in 8μm sections with antibody against CD31.
Staining was enhanced with nickel chloride against a light haematoxylin counterstain.

Microvessels (defined as capillaries and small venules) were counted in three areas of high vascular density; these were identified at x40 magnification. Counting was performed using a Chalkley graticule at x100 magnification. The graticule was placed so the maximum number of random dots were positioned over vessels. The vascular count for each tumour was created by the sum of the three Chalkley counts. In three tumours the available area was too small to perform counts in this manner so a subjective assessment was made placing each in a high or low MVD category. MVD scores were subjected to a cut point analysis to give a binary cut off of high versus low. In 39 patients, DNA ploidy was also assessed. Cases were categorised as diploid or aneuploid.

Overall, the counts ranged from 16 to 32 (median 24). The cut point analysis dichotomised the count at greater or less than 21. A MVD greater than 21 indicated a poorer survival, \( p=0.019 \). This was also seen in the proportional hazard model, \( p=0.026 \). Vascularity in the study was not related to stage \( (p=0.219) \), grade \( (p=0.467) \), histological pattern \( (p=0.705) \) or ploidy \( (p=1.000) \). The hazard ratio for poor survival with a MVD count greater than 21 was 2.5 \( (1.1 - 5.9) \).

It was noted that papillary transitional cell carcinomas were not included in this study as a pilot study had found it impossible to perform MVD
measurement using this method. The authors stated that in these tumours areas of neovascularisation could not be defined with any degree of confidence. That is, papillary tumour hotspots could not be defined subjectively by the observers.

In 1995, Jaeger et al also measured MVD in bladder cancer (Jaeger, Weidner, Chew, et al. 1995). They examined 41 randomly selected cases of invasive bladder cancer (pT2 n=4, pT3 n=30, pT4 n=7). Five patients had squamous cell carcinoma not transitional cell carcinoma. All samples were taken from radical cystectomy specimens with pelvic lymphadenectomies. MVD was compared to the presence or absence of lymph node metastases.

Microvessels were identified with antibodies against FVIIIRA and were counted in the area of highest neovascularisation. These areas were chosen at x40 magnification in separate routinely stained Haematoxylin and Eosin sections. Only one area was assessed and counts were performed at x200 magnification giving a field of 0.74mm$^2$. Any brown stained endothelial cells were considered to be positive and the presence of vessel lumen or erythrocytes were not required for identification. As not all hotspots filled the x200 (0.74mm$^2$) field, a 10 x 10 square ocular grid was also used in each case, giving a field of 0.16mm$^2$.

In the 27 patients without lymph node metastases MVD was 56.2 (SD 29.5) per x200 field and the grid count was 28.6 (SD 14.4). In the 14 patients with lymph node metastases the MVD was 138.1 (SD 37.9) per x200 field and
74.7 (SD 14.7) with the grid. MVD counts correlated with the presence of node metastases, \(p<0.0001\). No statistical relationship was found between MVD and stage, \(p=0.44\). As grid and area counting correlated well, \(r=0.972\), they claimed the grid could be used as a more convenient and effective method of counting.

Also in 1995, Bernard Bochner et al found MVD to be an independent prognostic factor in patients with invasive bladder cancer (Bochner, Cote, Weidner, et al. 1995). In tissue from 164 radical cystectomies for TCC, 5\(\mu\)m sections were stained with anti CD34 monoclonal antibodies to label vessels. Weidner's hotspot method was used, a single area being assessed by three observers (one of whom was Weidner) at x200 magnification. A good interobserver correlation was found, \(r=0.62\), with more than 90% of counts within 10% between observers. MVD was divided into three predetermined groups, MVD<65, MVD 65-99 and MVD >99. Although the authors confirmed these groups were predefined, they do not explain how they determined these divisions. Of the 164 tumours 115 were lymph node negative. Of these, 28 were pT1, 15 were pT2, 73 were pT3 and 9 were pT4. The stage breakdown of the 49 node positive patients was not given.

MVD was associated with an increased risk of both disease recurrence, \(p<0.0001\), and reduced survival, \(p=0.0007\). The five year probability of disease recurrence was 19% (95% CI=8-29) for low MVD tumours, 56% (95% CI=43-69) for intermediate MVD tumours and 68% (95% CI=55-81) for high MVD tumours. The five year survival probabilities were, 68% (95% CI=56-81) for low MVD tumours, 44% (95% CI=30-57) for intermediate MVD tumours and 34%
(95% CI=21-47) for high MVD tumours. Using MVD as a continuous variable in a Cox hazard model it was related to increased disease recurrence, \( p < 0.001 \) and decreased overall survival, \( p = 0.003 \). MVD was not associated with stage or grade of tumour. In a multivariate analysis including grade, stage and nodal status, MVD was found to be a significant predictor of disease recurrence and survival, \( p < 0.0001 \).

The cases of pT1 disease were analysed separately. There was a trend towards higher MVD and decreased risk of recurrence, but this was not statistically significant. No comment was made on the ease or difficulty of MVD measurement in this subgroup. However, it was noted that as bladder cancer diagnosis is by transurethral resection, analysis of MVD in cystectomy specimens must take this into account. Specifically, previous resections causing areas of inflammation and healing may be associated with non-cancer hotspots. The heterogeneity of bladder tumours with respect to vascularity was also noted making careful examination of all tumour to select a hotspot critical.

In 1997, the same group examined MVD in association with immunohistochemical p53 status (Bochner, Esrig, Groshen, et al. 1997). In a series of 161 tumours they found that patients with tumors exhibiting no evidence of p53 alterations those with low microvessel counts demonstrated 3% recurrence and 88% survival, compared to 43% recurrence and 59% overall survival for patients with intermediate vessel counts and 61% recurrence and 43% overall survival for patients with the highest vessel counts (\( P < 0.001 \) and \( P \))
An association between angiogenesis and \( p53 \) status was seen \((P = 0.05)\); however, 27\% of the tumors that showed no evidence of \( p53 \) alterations exhibited high microvessel counts, and 26\% of tumors with evidence of \( p53 \) alterations had low microvessel counts.

The same group also examined the relationship between MVD, \( p53 \) and thrombospondin \((\text{Grossfeld, Ginsberg, Stein, et al. 1997})\). Although in this study MVD was not examined with respect to outcome, an increase in MVD was found to correlate with a decrease in TSP expression. In an analysis of variance, both \( p53 \) status and TSP were associated with MVD. However, after adjustment for TSP status, \( p53 \) no longer remained significantly associated with MVD suggesting a link between these two factors.

Philp et al, in 1996, quantified angiogenesis in a series of 113 consecutive bladder tumours with a minimum follow up of 12 years \((\text{Philp, Stephenson, & Reed. 1996})\). As this was an unselected series it contained superficial as well as invasive bladder cancers. The authors state that 59\% of cases were \( pTa \), however, in the table of results, this is quoted as stage \( pT1 \) \((67 \text{ of 113 patients})\).

The 5\( \mu \text{m} \) sections were labelled with anti CD31 antibody against endothelial cells and antibody to collagen IV in the basement membrane giving double staining against a malachite green counterstain. MVD counting was performed in random areas throughout the sections. The areas were determined
using a random number generator set to the graduations of the microscope stage scale. The number of areas to be counted was determined using a method of cumulative mean and was set at 12. Counting was performed at 160x magnification (field size not given) and was carried out in all blocks of each tumour. Each section took seven minutes to count. Intra-observer (r=0.825) and inter-observer (r=0.424) variation was assessed.

Univariate analysis found mean MVD (p=0.001) and stage (p=0.002) to be significant predictors of death from bladder cancer. There was no significant correlation between MVD and grade (p=0.07) but there was with stage (p=0.01) and vascular invasion (p=0.007). Multiple regression analysis found a combination of stage and MVD a significant prognostic factor for the relative risk of death from bladder cancer, p=0.004.

This method of counting aimed to provide greater objectivity than the subjective hotspot method. It was again noted that there was marked heterogeneity of vascularity within individual tumours. Although the multiple regression analysis suggested MVD to be additive to stage in the prognosis of bladder cancer, the Kaplan Meier curves suggested this was minimal.

In an abstract published by Chopin et al in 1996, they describe a study in which they were unable to find a statistical correlation between MVD and outcome (Chopin, Popov, Hoznek, LeFrere, Bellot, & Abbou. 1996). In 49 cases of node negative bladder cancer treated by cystectomy vessels were stained with
anti FVIII:RA. Adjacent frozen sections were stained for p53. Both p53 and vessels were assessed using an eyepiece graticule at x400 magnification in three random fields per slide. No further details are given. No statistical difference was found in MVD in cases that did or did not progress. There was no correlation between MVD and p53. A Kaplan Meier plot for survival did not demonstrate adverse progression free survival for high or low angiogenic tumours.

Dinney et al investigated angiogenesis by MVD measurement in 54 pT1 TCCs (Dinney, Babkowski, Antelo, et al. 1998). They chose pT1 tumours because of their unique hallmark of biological aggressiveness in superficial disease, that is, their invasion of the lamina propria. Their intent was to determine if MVD could be used in the patient selection of pT1 disease for early cystectomy.

The 54 cases were stained with anti-FVIII:RA antibody and MVD was assessed in two separate ways. Areas with the highest number of vessels (hotspots) were identified at x125 magnification and MVD was counted in six fields (0.75mm²) contiguous to that area at x500 magnification. The mean of the MVD counts of two independent scorers were used. If these two differed by more than 25%, a third score was performed and the best two used. If none were close the mean of all three scores were used. MVD was expressed as vessels per mm². A second method was also employed as the authors anticipated problems scoring the vessels in papillary disease due to the vascular cores. Eight fields were counted (x200 magnification) at the interface between tumour and lamina
propria. These areas were felt to possibly represent the areas of most intense angiogenic activity.

There was poor correlation between MVD and outcome with both methods used (p value not given). The authors note again the great heterogeneity of vessel distribution between and within tumours. Hotspots were seen well away from tumours (these were not counted) and within the fibrovascular cores. The latter may just then represent the normal histopathology of papillary TCC.

Hawke et al measured MVD in 42 cystectomy specimens using anti FVIIIRA antibody (Hawke, Delahunt, & Davidson. 1998). They also compared MVD of these with MVD of the original transurethral resection specimens in 29 cases. Hotspots were identified at x40 magnification. Counts were performed at 200x magnification (0.94mm²). The authors state that the highest field count obtained for each specimen was designated the MVD. This suggests counts were performed in multiple areas within each section. This is not explicitly stated and a number of areas assessed is not given. MVD counts were then designated high or low around the mean of (80.8).

Heterogeneity was again noted. High areas of vascularity were observed adjacent to areas of necrosis. Normal bladder had lower MVD (2-16 vessels per high powered field). Lamina propria adjacent to cis had significantly higher MVD (28-125), p=0.0025. The cancer specific survival for patients with higher
MVD was less than for those with low MVD, p=0.013. Although there was an increasing trend between MVD and stage, this was not statistically significant.

In the 29 transurethral resection specimens, the median MVD was 100 (51-155). With cases divided around this median, high MVD patients again had a decreased survival, p=0.04. There was a poor correlation between transurethral resection MVD and cystectomy MVD, r=0.21. The authors felt this was due to the heterogeneity of vessel distribution in TCC.

Chaudhary et al performed a pilot study of vessel staining on five cases of bladder cancer using anti FVIIIRA, anti CD31 and anti CD34 (Chaudhary, Bromley, Clarke, et al. 1999). Anti FVIIIRA antibodies failed to stain the vessels; both CD31 and CD34 worked well. The authors found CD31 generally superior. They proceeded to use CD31 to stain a series of 88 cases of bladder cancer. All cases were from cystectomy specimens performed for invasive disease. However, on review, 11 had pTa disease, 22 had pT1, 14 had pT2, 24 had pT3 and 9 had pT4. Thirty three cases were salvage cystectomies after radiotherapy. Three separate hotspots (found at 100x magnification) were counted using a Chalkley graticule. The mean of the three Chalkley counts was expressed as the MVD.

Univariate analysis of MVD (split above and below the median of 3.4) revealed an association with high MVD and poor prognosis, p=0.02 and MVD and higher stage, p=0.0002. In a Cox model MVD was entered as a continuous
variable. In a univariate analysis it was found to be a predictor of worse outcome, \( p=0.03 \). In a multivariate model along with, stage, grade and previous radiotherapy, it was no longer significant, \( p=0.13 \). It regained significance after adjustment for stage, \( p=0.02 \). The authors concluded that the association was likely to represent low study numbers rather than MVD being a manifestation of stage or grade.

In 1999, Ozer et al examined the significance of angiogenesis in 20 G3pT1 cases of superficial bladder cancer (Ozer, Mungan, Tuna, Kazimoglu, Yorukoglu, & Kirkali. 1999). Vessels were identified with monoclonal antibody against FVIIIRA. Vessel analysis was performed in the most representative area of the section. This area represented the best histopathological appearance of G3pT1 disease and was not a hotspot. The results from ten of these areas were compared with ten randomly selected areas and no difference was seen on paired t-test (results not given). Two pathologists scored the sections; no statistically significant difference was found between their scores or their selection of areas (results not given).

Section images were viewed on a computer monitor via a video camera giving a final magnification of x525. An 11 x 11 line optical grid was superimposed over the image. The vascular surface density (VSD) was then calculated using the formula:

\[
VSD = \frac{\sum \ln\ 2.121}{\text{Istr} \cdot \text{Lr}}
\]
Istr is the number of grid points superimposed on the stroma. In is the counts of intersections between test lines and vessel walls, and Lr is the line length. VSD is the vascular surface area of vessels per unit tissue volume.

The number of vessels per square millimetre of stroma (NVES) was also calculated:

\[
\text{NVES} = \frac{N}{121} \cdot \frac{1}{Istr}
\]

N is the number of vessels within the measured field. The NVES is therefore the equivalent of the MVD count.

There was no difference in NVES (p=0.37) or VSD (p=0.80) between patients who recurred and patients who progressed. However, there was a difference in both NVES (p=0.002) and VSD (p=0.01) between patients who neither recurred nor progressed and those patients who either recurred or progressed. There was no correlation between the vascular parameters and cis, BCG treatment, tumour size or tumour number. However, VSD was associated with morphology of tumours, p=0.01. Solid tumours were more likely to recur or progress, p=0.07. The authors proposed that this was due to the higher nutritional need of solid compared to papillary tumours.

The same group went on to examine 80 superficial papillary bladder tumours in 2000 (Sagol, Yorukoglu, Sis, et al. 2001). Although in this study they
identified vessels with anti-CD31, they used the same MVD analysis system. Of the 80 cases, 32 were pTa and 48 were pT1.

No statistical difference was seen in VSD or NVES between cases that recurred and cases that did not. Although vascularity increased with grade, \( p=0.019 \), there was no correlation with stage.

When pTa and pT1 tumours were analysed separately using logistic regression, VSD values were higher in pTa tumours with coexisting cis, \( p<0.001 \). Also, higher NVES values correlated with early recurrence in pTa tumours. After performing further evaluation using predictive values for vascularity the authors suggested measurement of vascularity was not diagnostically worthwhile.

Inoue et al investigated MVD, bFGF, VEGF and IL-8 in 55 patients with invasive bladder cancer in a treatment programme consisting of radical cystectomy and neoadjuvant MVAC chemotherapy (Inoue, Slaton, Karashima, et al. 2000). Transurethral resection samples were available for 55 patients and cystectomy specimens were available for 51 patients. However, matched transurethral resection and cystectomy samples were only available in 31 patients.

In situ mRNA hybridisation analysis was used to quantify bFGF, VEGF, and IL-8. For MVD analysis, 5μm sections were labelled with monoclonal antibody against CD34. Five hotspots were counted at x100 magnification and a
The mean of these was used as MVD count. The counts were rendered dichotomous at the median for statistical analysis.

In the transurethral resection specimens, median MVD was 30 (16-62) per x100 field. High MVD correlated with recurrence, \( p=0.015 \). A univariate Cox proportional hazards model confirmed a high MVD was a significant predictor of disease recurrence, \( p=0.023 \). This was not found at the multivariable analysis where only VEGF expression predicted recurrence, \( p=0.012 \). There was a significant correlation between MVD and expression of bFGF, \( p=0.03 \), but not with VEGF or IL-8.

In the cystectomy specimens, MVD remained a predictor of tumour recurrence in the univariate, \( p=0.010 \), and multivariate analysis, \( p=0.048 \). In a subset of patients with residual disease after cystectomy (number not given), high MVD (>32, the median in this group) was a significant predictor of disease progression, \( p=0.0091 \).

In 31 cases both the transurethral resection specimen and the cystectomy specimen were available for analysis. These cases allowed assessment of MVD before and after MVAC chemotherapy. There was however no difference in MVD in this subset.

In 2000, Krupski et al published a prospective study examining 31 consecutive patients with suspected TCC at initial endoscopy (Krupski,
Moskaluk, Boyd, & Theodorescu. 2000). Endothelial cells were labelled using the same method as Bostwick et al in prostate cancer; FVIIIRA antibodies were used with a light Eosin Y counterstain (Bostwick, Wheeler, Blute, et al. 1996).

MVD was measured using an optimised microvessel density (OMVD) calculation, also described by Bostwick. Images were captured digitally at a resolution of $512 \times 480 \times 8$ pixels; this could be converted to true area at $6.13 \times 10^7 \text{mm}^2$ per pixel. Grey scale images were converted to binary (black or white). Area and perimeter measurements were then made of the microvessels and were expressed per unit area of tissue. Bostwick’s original OMVD algorithm was given a heavier weighting for cross sectional blood vessel area, biasing the measurement towards larger vessels. The reason for this was not given. This produced an Area Weighted MVD (AWMVD). EGFR and p53 status were also measured and a BTA TRAK urine test performed.

Only 22 of the 31 patients were found to have TCC after the resected areas were analysed; 14 of these were superficial, 8 had invasive disease. One of the 22 had insufficient tissue for analysis leaving only 21 in the study.

A threshold value of 61 for OMVD was set which predicted 7 out of 8 cases with invasive disease. A threshold of 1100 for AWMVD only identified 3 out of the 8 patients who developed invasive disease.
In a univariant analysis MVD was not predictive of stage. Using a Furnival-Wilson algorithm of best fit of the variables for a predictive model of stage, all variables together gave an $R^2$ value of 0.75 (perfect fit = 1.0). When analysed separately, grade, BTA TRAK and OMVD and AWMVD had predictive values nearly equal to this. The authors conclude that grade is the best predictor of stage but that MVD may offer extra information for prediction of tumour aggressiveness.

A more detailed analysis of vascularity in bladder cancer was carried out by Korkolopoulou et al (Korkolopoulou, Konstantinidou, Kavantzas, et al. 2001). The authors felt that measurement of MVD alone may overlook other parameters in the investigation of angiogenesis in histological tissues. They studied 115 patients with TCC. Only cases with solid or a mix of papillary and solid tumour growth were included. Six cases with predominantly papillary architecture were excluded. The authors stated that accurate assessment of vascularity is not possible in papillary tumours quoting Dickinson et al (Dickinson, Fox, Persad, Hollyer, Sibley, & Harris. 1994). Vessels were identified with antibody to CD31.

The area of greatest vascularity was found at x40 magnification and the image saved as a JPEG file (24-bit) at 1550 x 1070 pixels. As well as MVD, the total area occupied by vessels (TVA) and several morphometric parameters were measured. For each countable microvessel: major axis length, minor axis length, area, perimeter, compactness ($\text{perimeter}^2 / \text{area}$), shape factor ($4\pi \times \text{area} / \text{perimeter}^2$), and other.
perimeter$^2$), and Feret diameter ($\sqrt{4 \times \text{area} / \pi}$). Intraobserver ($r=0.75, p=0.8$) and interobserver ($r=0.84, p=0.9$) differences were not significant.

No vascular characteristic correlated significantly with grade but MVD increased with tumour stage, $p=0.049$. Area, major axis, perimeter and Feret diameter were found to be significantly higher in pT2 tumours compared to pT1, $p=0.033$. However, these variables were also higher in pT2 tumours compared to pT3, $p=0.022$ and pT4, $p=0.042$ tumours. The authors suggested that this was in agreement with the concept of angiogenesis initiation being associated with hyperfusion and the formation of wide sinusoidal spaces and that this coincided with transition to early invasive cancer (pT2).

In superficial tumours (pT1), higher TVA ($p=0.0277$) and major axis length ($p=0.0428$) correlated with poorer survival. In multivariate analyses, high TVA, as a dichotomous variable (cut off $41.2 \mu m^2$) and increased compactness predicted poor outcome.

In invasive tumours, as well as grade and stage, MVD ($p=0.0147$) and compactness ($p=0.0085$) were associated with decreased survival in a univariate analysis. Tumour relapse however, was associated with lower values of major axis ($p=0.0035$), minor axis ($p=0.0397$), perimeter ($p=0.0095$), Feret diameter ($p=0.0332$) and TVA ($p=0.0003$). Multivariate analysis selected stage and decreased TVA ($p=0.0007$) as indicators of poor disease free survival. This unusual finding the authors relate to the large vessel area seen in pT2 tumours.
At the Annual meeting of the European Association of Urology in 2003, Stavropoulos et al presented a study of MVD, VEGF immunoreactivity and p53 staining in a series of 127 superficial bladder cancers (Stavropoulos, Tsimaris, Ioachim, et al. 2003). The patients were followed up for a median of 36 months during which time there was a 73.4% recurrence and a 7.3% (8 patients) progression rate. In the abstract, no details of the evaluation methods of MVD or VEGF or p53 immunostaining were given.

A significant correlation was seen between MVD and VEGF \( (p=0.019) \). There was no correlation between MVD and p53 or VEGF and p53. No significant association was seen between VEGF or MVD and recurrence or progression.

The number of patients progressing (8) in this study is small and no details of MVD measurement are given. If a hot spot method was used it would be unlikely to work well with superficial disease due to the difficulty in determining areas to count in this heterogeneous tumour (Bochner, Cote, Weidner, et al. 1995; Dickinson, Fox, Persad, Hollyer, Sibley, & Harris. 1994; Hawke, Delahunt, & Davidson. 1998).

1.3.4 The limitations of Microvessel Density Measurement

Although the majority of studies of tumour angiogenesis show a positive relationship between MVD and tumour growth and spread, this is not a universal finding. High MVD has been found to be a predictor of poor outcome in many
Buuyounouski, Bagiella, et al. 1999). Indeed, Kohler et al found that a high MVD correlated with a better outcome in renal cell carcinoma (Kohler, Barth, Siebel, Gerharz, & Bittinger. 1996). One reason for this discrepancy may lie in the method of MVD assessment.

This may be due, at least in part, to the difficulties in measuring angiogenesis (Hall, Fish, Hunt, Goldin, Guillou, & Monson. 1992; Van Hoef, Knox, Dhesi, Howell, & Schor. 1993; Axelsson, Ljung, Moore, et al. 1995). There are certainly a great number of techniques which have been used to measure MVD in histopathological specimens. In a review of MVD measurement in urological tumours 41 different methods of MVD measurement were identified in a series of 48 papers (Goddard, Sutton, Berry, O'Byrne, & Kockelbergh. 2001).

The methods of MVD counting differ in many aspects, however the technique may be divided into several distinct processes to allow clearer analysis of the variations. Immunohistochemical staining techniques are used to identify the microvessels but several different vessel wall antigens are being used as markers. The area of tumour that is counted differs between researchers. Many count in areas seen to have high vascularity at low magnification, the “hotspot” method. Alternatively, some count in randomly selected areas or in specifically defined areas, such as the tumour edge. Once the area to be counted has been determined there are different ways of performing that count and finally, once the count is done, numerical expression of MVD can be shown in different ways.
1.3.4.1 Vessel Identification

Historically, Goldmann and Lewis identified vessels for study with Indian Ink (Goldmann. 1907; Lewis. 1927). Subsequently immunohistochemical techniques have allowed vessel identification by labelled antigen-antibody binding to components of the vessel endothelium.

Antibodies against CD31, CD34 and FVIIIRA, all expressed by endothelial cells, are the most commonly used. FVIIIRA is involved in platelet adhesion and aggregation, CD31 is associated with platelet adhesion and CD34 is involved in leucocyte adhesion and endothelial cell migration (Martin, Green, Renshaw, et al. 1997).

The suitability of these antibodies as reliable markers of microvessels has been examined in both urological and non-urological tumours. Goulding et al. compared CD31 and CD34 in breast cancer finding CD34 “more crisp and easy to interpret.” (Goulding, Abdul, Robertson, et al. 1995). Martin et al compared CD31, CD34 & FVIIIRA in breast looking at optimum concentration and concluding CD34 was superior (Martin, Green, Renshaw, et al. 1997). De Jong found FVIIIRA had low sensitivity but high specificity when compared to CD31. CD31 was 100% sensitive and 95% specific (de Jong, van Diest, & Baak. 1995). Gasparini et al and Toi also found CD31 more sensitive than FVIIIRA (Gasparini, Weidner, Bevilacqua, et al. 1994; Toi, Kashitani, & Tominaga. 1993). however, Weidner concludes both are reliable markers of MVD (Weidner. 1995). Giatromanolaki et al examined FVIIIRA and CD31 in non-
small cell lung cancer finding that CD31 had several advantages, being more sensitive for highlighting small, immature microvessels or single endothelial cells (Giatromanolaki, Koukourakis, Theodossiou, et al. 1997).

In prostate cancer, Bettencourt et al compared FVIII:RA and CD34 finding CD34 superior (Bettencourt, Bauer, Sesterhenn, Connelly, & Moul. 1998), whereas Offersen et al, unable to obtain acceptable staining with CD31 used FVIII:RA (Offersen, Borre, & Overgaard. 1998). Bochner et al found that CD34 identified the small-calibre microvessels associated with neovascularisation in bladder cancer more efficiently than FVIII:RA (Bochner, Nichols, Groshen, Skinner, & Cote RJ. 1995) and Gelb et al, looking at MVD in renal cell carcinoma, used both CD31 and FVIII:RA and found MVD for both correlated closely (Gelb, Sudilovsky, Wu, Weiss, & Medeiros. 1997).

1.3.4.2 Selection of Microvessel Counting Area – Hotspots Methods

The most widely used and quoted method, is that described by Weidner previously discussed (section 1.3.1) (Weidner, Semple, Welch, & Folkman. 1991). This identifies “hotspots” in the tumour, areas of high vascular density identified under low power magnification. MVD counts are then performed in these areas at high power.

The hotspot is thought to correlate best with clinical outcome because of its relationship to the process of haematogenous spread (Weidner, Semple, Welch, & Folkman. 1991; Weidner, Folkman, Pozza, et al. 1992). McCulloch et
al measured the shedding of malignant cells in 16 breast cancer patients and found a correlation with MVD (p=0.024) as measured using the hotspot method supporting an association between counts in the hotspot and metastasis (McCulloch, Choy, & Martin. 1995). Martin et al (Martin, Green, Renshaw, et al. 1997) compared the standard immunohistochemical technique of Weidner et al (Weidner, Semple, Welch, & Folkman. 1991) with microangiography in breast tumours. A correlation was found between the vascularity as measured by immunohistochemistry and the vascularity of the tumour as a whole as estimated by microangiography (p<0.0003). The immunohistochemical technique however, varied from Weidner in that ten areas were counted rather than one. In some tissues the areas of highest vessel density are at the invasive edge of the tumour suggesting that these areas represent the most aggressive disease and thus might best predict its behaviour. However, in other tissues, such as the prostate, the most vascular area is in the centre of the tumour (Siegal, Yu, & Brawer. 1995).

Determining what is a hotspot within a section of tissue may not be obvious (Bosari, Lee, DeLellis, Wiley, Heatley, & Silverman. 1992; Axelsson, Ljung, Moore, et al. 1995). Vermeulen et al and Simpson et al suggested this may be due to lack of observer experience and could be helped by training (Vermeulen, Verhoeven, Hubens, et al. 1995; Simpson, Ahn, Battifora, & Esteban. 1996). Dickinson et al encountered difficulty measuring MVD in superficial bladder cancer due to the papillary nature of the tumour and were thus obliged to assess only invasive solid tumours (Dickinson, Fox, Persad, Hollyer, Sibley, & Harris. 1994). Within the hotspot technique there are further
variations. Weidner counts one area in a single hotspot [personal communication]. In the methods examined here between one and 40 hotspots were chosen and between one and 20 areas were counted within these hotspots. The hotspot method however does provide a convenient area in which to perform a vessel count.

1.3.4.3 Selection of Microvessel Counting Area – Random Methods

Philp et al determined MVD in bladder cancer in randomly chosen areas. Using the method of cumulative mean twelve was found to be the optimum number of areas to be counted. Counts were performed manually and correlation was found between high MVD and poor survival (Philp, Stephenson, & Reed. 1996). Interestingly, perhaps as recognition of hotspots was not required, superficial papillary tumours could be included in their data unlike the study by Dickinson et al (Dickinson, Fox, Persad, Hollyer, Sibley, & Harris. 1994). Random selection of areas for counting has also been used in several studies (Chopin, Popov, Hoznek, LeFrere, Bellot, & Abbou. 1996; Bigler, Deering, & Brawer. 1993; Brawer, Deering, Brown, Preston, & Bigler. 1994; Lissbrant, Stattin, Damber, & Bergh. 1997; Offersen, Borre, & Overgaard. 1998; Ozer, Mungan, Tuna, Kazimoglu, Yorukoglu, & Kirkali. 1999; Kohler, Barth, Siebel, Gerharz, & Bittinger. 1996). Lissbrant et al and Offersen et al counted in both random areas and within hotspots and found a significant association between the two methods (Lissbrant, Stattin, Damber, & Bergh. 1997; Offersen, Borre, & Overgaard. 1998). Offersen found only hotspot MVD and not random area counting was associated with survival.
1.3.4.4 Counting the vessels

What constitutes a countable vessel may lead to much unconscious bias (Axelsson, Ljung, Moore, et al. 1995). Rules must therefore be followed which precisely define what may and may not be counted. For example Weidner et al in their seminal paper stated that in counting, any separate area of staining was considered to be a single vessel. The presence of an obvious lumen or red blood cells was not required for confirmation (Weidner, Semple, Welch, & Folkman. 1991). The grids and counting aids used by some allow a smaller area to be counted and may allow other parameters such as vessel area to be calculated. Kohler et al used a 121 point lattice composed of 11 vertical and 11 horizontal lines. (Periplan x10, leitz, Wertlar, Germany) This method checks the vessels are distributed randomly and measures the absolute vascular surface area in a defined volume of tumour (Kohler, Barth, Siebel, Gerharz, & Bittinger. 1996).

Researchers have also used a subjective estimation of the vascularisation of the tumour (eyeballing) (Mooteri, Rubin, Leurgans, Jakate, Drab, & Saclarides. 1996). This method is fast and has compared favourably with other techniques (Fox, Leek, Weekes, Whitehouse, Gatter, & Harris. 1995).

1.3.4.5 Influence of the investigator

The influence of the investigator on accurate and reproducible MVD counts has been examined with regard to counting and choosing hotspots. The widely used hotspot technique requires accurate identification of the most vascular area of the tumour, but has been correlated with outcome in a wide
variety of malignancies. Hansen et al examined the inter and intra observer variation on the selection of hotspots in a series of 40 breast carcinomas. The investigators were instructed to identify the three hottest spots on a section from each tumour. The individual investigators correctly re-identified the three hotspots in only 10 out of the 40 cases (25%). The interobserver identification of the three hotspots was even less reproducible. (4 out of 40, 10%). Vermeulen et al found that with prior training on 20 sections the interobserver variability of hotspot selection in breast cancer fell to acceptable levels ($r>0.7; p<0.01$) (Vermeulen, Libura, Libura, et al. 1997).

In the 48 urological papers studied in the review (Goddard, Sutton, Berry, O'Byrne, & Kockelbergh. 2001), intra or inter observer variability for selecting hotspots was not formally examined in any. However, in 20 studies, multiple observers performed microvessel counts. In seven of these the counts were performed simultaneously by two observers using a double headed microscope. The counts were performed independently by two observers in 16 papers. In one of these a third observer was used if the first two counts differed by more than 25% (Dinney, Babkowski, Antelo, et al. 1998) and in a further study a third observer was used in 20% of cases (Bochner, Cote, Weidner, et al. 1995). In four studies, the observers’ counts were compared to computer counts and not to each other. Nine studies assessed interobserver variation. All of these found a statistically significant correlation between the observers.
1.3.4.6 What constitutes the MVD value

The treatment of the MVD value has been subjected to many manipulations. Actual values being used by some, mean values by others. The mean of several slides, the mean of three, five or ten counted areas, the sum of several areas, or the highest value has been used. This prevents comparison of studies unless raw data is also provided. Offersen found a significant association between MVD and death in prostate cancer when the maximum value was used but the results were not significant if the mean was used (Offersen, Borre, & Overgaard. 1998). This emphasises the need for standardisation of techniques; unless the field size is stated or the result expressed per unit area comparison of results between papers is difficult.

1.3.4.7 Treatment of MVD data

In different studies, one or more threshold values of MVD have been selected about which patients were distributed into differing prognostic groups. Some have used the mean, (Chopin, Popov, Hoznek, LeFrere, Bellot, & Abbou. 1996; Lissbrant, Stattin, Damber, & Bergh. 1997) and others the median (Borre, Offersen, Nerstrom, & Overgaard. 1998; Grossfeld, Ginsberg, Stein, et al. 1997; Hawke, Delahunt, & Davidson. 1998). MVD as a continuous variable has also been used (Edwards, Cox, Andi, et al. 2001).

Bostwick et al devised a scoring system in prostate cancer using the sum of log PSA, log MVD and Gleason score. The MVD as a continuous variable has also be used (Gelb, Sudilovsky, Wu, Weiss, & Medeiros. 1997).
1.3.4.9 Heterogeneity within tumours

Not all studies of MVD have found a correlation between vascularity and outcome. One possible reason for this may be that a sampling error has occurred during the selection of pathological material examined. The majority of reviewed methodologies rely on a single 5\(\mu\)m section to represent the angiogenic potential of the tumour as a whole. Several authors have attempted to address this problem. De Jong et al examined 10 cases of breast cancer taking four sections 100\(\mu\)m apart from between two and four blocks from each tumour. MVD was assessed in two ways, firstly, a hotspot method and then counts were performed in ten high power fields in two or three systematically spread areas within the section. For the latter method, the coefficient of variation from different areas within the same section was 17.1\% (range 0.7 – 52.1\%), between sections from the same block was 14.7\% (range 0.5 – 41.9\%) and between blocks from the same tumour was 25.8\% (range 9.9 – 44.6\%). The hotspot method showed less variation with the coefficient of variation for sections from the same block being 11.1\% (range 0.7 – 29.5\%) and for different blocks from the same tumour 24.2\% (5.7 – 54.9\%) (de Jong, van Diest, & Baak. 1995). Van Hoef et al achieved similar results when they examined sections from two blocks of 41 cases of breast carcinoma. There was a 71-78\% concordance between MVD counts between blocks (Van Hoef, Knox, Dhesi, Howell, & Schor. 1993).

Martin et al, in a series of breast carcinoma patients compared the MVD assessment of three sections from three blocks from each tumour. When the patients were divided into high or low MVD groups an 85\% correlation was
achieved between sections from different blocks. In addition they compared
MVD with microangiography: a mixture of barium, gelatin and formalin was
injected into fresh mastectomy specimens and the vascularity on subsequent
mammograms was assessed subjectively by two investigators and checked by a
third. A statistically significant correlation was found between the vascularity as
measured by immunohistochemistry and the vascularity of the tumour as a whole
as estimated by microangiography (p<0.0003). This suggests that despite tumour
heterogeneity, the technique of selecting a single section from a block of tumour
is a valid representation of the tumour as a whole in carcinoma of the breast

This great heterogeneity in MVD counting has also been recognised in
other tumours (Fox, Leek, Weekes, Whitehouse, Gatter, & Harris. 1995; de Jong,
have attempted to redress the problem by suggesting a standardised technique.
They proposed the use of anti CD31 as a vessel marker, the selection of a hotspot
in viable tumour tissue at x10 magnification and counting with a Chalkley
graticule in a field size approximately the same as the hotspots in that tumour
type. They suggest assessment by two observers with a third if the counts differ
by more than 10% (Vermeulen, Gasparini, Fox, et al. 1996).

Although concern regarding the great variation in methodology has been
voiced, as yet, no standard technique has been adopted either in urology or in

If angiogenesis is to be used as an index of cancer growth or progression there must be accurate methods of assessing angiogenic activity in biological systems. Performing microvessel counts can be difficult because there may be great heterogeneity within tumours and indeed within individual sections (Weidner, Semple, Welch, & Folkman. 1991; Bosari, Lee, DeLellis, Wiley, Heatley, & Silverman. 1992; Van Hoef, Knox, Dhesi, Howell, & Schor. 1993; de Jong, van Diest, & Baak. 1995). Poorly vascularised tumours in particular have a greater inter-observer variation when vessel counting is performed manually (van der Laak, Westphal, Schalkwijk, et al. 1998).

1.3.5 Summary

Microvessel counting is the standard measurement of angiogenesis (Weidner, Semple, Welch, & Folkman. 1991). Microvessel counts can be performed in several different ways. The simplest is subjective "eyeballing" of the slides and classifying them into high density or low density counts. This is of course highly subjective. The most widely used method of MVD measurement is by counting in hotspots (Weidner, Semple, Welch, & Folkman. 1991). Using this method a definite relationship between angiogenesis and outcome has been demonstrated in many tumours. The choice of a single hotspot, is subjective and a learned skill and the assessment of these "hot spots" in tumours with heterogeneous vascular density can be a difficult task (Barbareschi, Gasparini,
Morelli, Forti, & Dalla. 1995). Thus MVD counting in superficial bladder cancer has proved difficult and given conflicting results.

To date, sixteen papers have examined MVD in bladder cancer, *table 1.3*. Of these, four have analysed superficial disease alone although eight papers have included superficial disease along with invasive samples. Dickinson et al, the first to measure MVD in bladder cancer did not include superficial samples as they found the papillary architecture too difficult to analyse. Korkolopoulou et al, although including superficial samples only included those with solid architecture. Bochner et al in 1995 analysed superficial disease separately but found no correlation with outcome.

Of the four papers looking purely at MVD in superficial bladder cancer, Dinney et al, Stavropoulos et al and Sagol et al found no correlation between MVD and outcome. Ozer et found that superficial tumours that recurred or progressed had significantly higher MVD.
Table 1.3  The Measurement of Microvessel Density in Bladder Cancer

<table>
<thead>
<tr>
<th>Paper</th>
<th>Patient No</th>
<th>Origin of tissue</th>
<th>Stage of tumours</th>
<th>Vessel Stain</th>
<th>Counting Method</th>
<th>Association with MVD found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dickinson 1994</td>
<td>45</td>
<td>TUR</td>
<td>T2 – T3</td>
<td>CD31</td>
<td>3 Hotspots &amp; Chalkley grid</td>
<td>Poor survival</td>
</tr>
<tr>
<td>Jaeger 1995</td>
<td>41</td>
<td>Cystectomy</td>
<td>T2 – T4</td>
<td>Factor VIII</td>
<td>1 Hotspot &amp; 10 x 10 grid</td>
<td>Lymph node metastases</td>
</tr>
<tr>
<td>Bochner 1995</td>
<td>164</td>
<td>Cystectomy</td>
<td>T1 – T4</td>
<td>CD34</td>
<td>1 Hotspot</td>
<td>Recurrence and poor survival</td>
</tr>
<tr>
<td>Chopin 1996</td>
<td>49</td>
<td>Cystectomy</td>
<td>T2+</td>
<td>Factor VIII</td>
<td>Random</td>
<td>No correlation</td>
</tr>
<tr>
<td>Philp 1996</td>
<td>113</td>
<td>TUR</td>
<td>T1 – T4</td>
<td>CD31</td>
<td>12 Random areas</td>
<td>Poor survival when combined with stage</td>
</tr>
<tr>
<td>Bochner 1997</td>
<td>161</td>
<td>Cystectomy</td>
<td>T1 – T4</td>
<td>CD34</td>
<td>1 Hotspot</td>
<td>Recurrence and survival independent of p53 status</td>
</tr>
<tr>
<td>Grossfeld 1997</td>
<td>160</td>
<td>Cystectomy</td>
<td>T1 – T4</td>
<td>CD34</td>
<td>1 Hotspot</td>
<td>Inverse correlation with Thrombospondin</td>
</tr>
<tr>
<td>Hawke 1998</td>
<td>42</td>
<td>Cystectomy &amp; TUR</td>
<td>T1 – T4</td>
<td>Factor VIII</td>
<td>1 Hotspot</td>
<td>Poor survival but not in multivariate analysis</td>
</tr>
<tr>
<td>Dinney 1998</td>
<td>54</td>
<td>TUR</td>
<td>T1</td>
<td>Factor VIII</td>
<td>6 areas around a Hotspot or 8 areas at the invasive edge</td>
<td>No correlation</td>
</tr>
<tr>
<td>Chaudhary 1999</td>
<td>88</td>
<td>Cystectomy</td>
<td>Ta – T4</td>
<td>CD31</td>
<td>3 Hotspots &amp; Chalkley grid</td>
<td>Poor survival</td>
</tr>
<tr>
<td>Paper</td>
<td>Patient No</td>
<td>Origin of tissue</td>
<td>Stage of tumours</td>
<td>Vessel Stain</td>
<td>Counting Method</td>
<td>Association with MVD found</td>
</tr>
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<td>------------------</td>
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<td>-----------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>Ozer 1999 (10)</td>
<td>20</td>
<td>TUR</td>
<td>T1</td>
<td>Factor VIII</td>
<td>Most representative area &amp; 11x11 grid</td>
<td>Tumour recurrence</td>
</tr>
<tr>
<td>Sagol 2000</td>
<td>80</td>
<td>TUR</td>
<td>Ta - T1</td>
<td>CD31</td>
<td>Most representative area &amp; 11x11 grid</td>
<td>Tumour grade and cis</td>
</tr>
<tr>
<td>Inoue 2000</td>
<td>55</td>
<td>Cystectomy &amp; TUR</td>
<td>T2 – T4</td>
<td>CD34</td>
<td>5 Hotspots</td>
<td>Tumour recurrence</td>
</tr>
<tr>
<td>Krupski 2000</td>
<td>31</td>
<td>TUR</td>
<td>Ta – T3</td>
<td>Factor VIII</td>
<td>OMVD</td>
<td>Predictor of stage</td>
</tr>
<tr>
<td>Korkolopoulou 2001</td>
<td>115</td>
<td>TUR</td>
<td>T1 – T4</td>
<td>CD31</td>
<td>Computer analysis of multiple variables in Hotspot</td>
<td>Vessel area in T1 tumours correlated with poor survival</td>
</tr>
<tr>
<td>Stavropoulos 2003</td>
<td>127</td>
<td>TUR</td>
<td>T1 – T1</td>
<td>Not given</td>
<td>Not given</td>
<td>Correlated with VEGF but not with outcome</td>
</tr>
</tbody>
</table>

TUR = transurethral resection of bladder tumour  
OMVD = Optimised Microvessel Density {Bostwick, Wheeler, et al. 1996 ID: 2905}
1.4.0 Angiogenic Factors

1.4.1 Vascular Endothelial Growth Factor

Vascular Endothelial Growth Factor (VEGF) was described independently in 1989 by Gospodarowicz et al (Gospodarowicz, Abraham, & Schilling, 1989) Ferrara et al (Ferrara & Henzel, 1989) and Keck et al (Keck, Hauser, Krivi, et al. 1989) as an angiogenic growth factor which induced endothelial cell mitogenesis. It was subsequently found to be identical to the previously described Vascular Permeability Factor (VPF) (Dvorak, Senger, & Dvorak, 1983).

The VEGF family currently includes six known members: VEGF, VEGF-B (VEGF Related Factor), VEGF-C (VEGF related protein), VEGF-D (c-fos-induced growth factor) VEGF-E and Placental Growth Factor (PIGF) (Narko, Enholm, Makinen, & Ristimaki, 1999). They are all dimeric glycoproteins with homologous amino acid sequences shared with platelet derived growth factor (PDGF) and bind to similar tyrosine kinase growth factor receptors (Cox G, Jones, Walker, Steward WP, & O'Byrne, 2000).

The VEGF-B gene is located on chromosone 11q13 (Paavonen, Horelli-Kuitunen, Chilov, et al. 1996; Olofsson, Pajusola, von Euler, Chilov, Alitalo, & Eriksson, 1996). Alternative splicing results in the production of two isoforms, VEGF-B_{167} and VEGF-B_{186}. VEGF-B_{167} appears to be the most prevalent isomer. The exact function of VEGF-B remains to be determined. It does not
appear to undertake a major role in the development of the vascular system, as VEGF-B knockout mice are viable and fertile (Olofsson, Jeltsch, Eriksson, & Alitalo. 1999). However, VEGF-B does form heterodimers with VEGF and may therefore modulate its signalling (Olofsson, Pajusola, Kaipainen, et al. 1996).

The VEGF-C gene is located on chromosome 4q34 (Olofsson, Pajusola, von Euler, Chilov, Alitalo, & Eriksson. 1996; Paavonen, Horelli-Kuitunen, Chilov, et al. 1996). VEGF-C stimulates the migration of endothelial cells and increases vascular permeability (Joukov, Pajusola, Kaipainen, et al. 1996). It is a relatively weak endothelial cell mitogen but it stimulates the proliferation of lymphatic endothelial cells (Joukov, Sorsa, Kumar, et al. 1997).

VEGF-D is structurally related to VEGF-C. VEGF-D mRNA is found most abundantly in heart, lung, skeletal muscle, colon, and small intestine (Achen, Jeltsch, Kukk, et al. 1998).

VEGF-E isomers are viral homologues of VEG-F encoded by different strains of the Orf virus (Ogawa, Oku, Sawano, Yamaguchi, Yazaki, & Shibuya. 1998).

PlGF was isolated from a term placenta cDNA library; hence the name. It is a 149-amino-acid-long protein with 53% homology to the platelet-derived growth factor-like region of human VEGF (Maglione, Guerriero, Viglietto, Delli-Bovi, & Persico. 1991). The PlGF gene is located on chromosome 14 and
has two isomers PIGF_{129} and PIGF_{152} (Maglione, Guerriero, Viglietto, et al. 1993). Both isomers are weakly mitogenic for endothelial cells (Ziche, Maglione, Ribatti, et al. 1997). PIGF forms a heterodimer with VEGF and may modulate VEGF induced angiogenesis (Cao, Chen, Zhou, et al. 1996).


VEGF_{165} is the most predominant iso-form and is produced by both normal and malignant cells. It is a basic homodimeric glycoprotein demonstrating heparin binding capacity in vitro and heparan sulphate binding in vivo hence its affinity for the extra cellular matrix (ECM) (Houck, Leung, Rowland, Winer, & Ferrara. 1992). In contrast, VEGF_{121} is weakly acidic, has no heparin binding characteristics and therefore is freely diffusible. VEGF_{145} binds to heparin with an affinity similar to that of VEGF_{165} (Poltorak, Cohen, Sivan, et al. 1997) having an additional independent heparin binding site (Neufeld, Tessler, Gitay-Goren, Cohen, & Levi. 1994). VEGF_{189} and VEGF_{206} are more basic and have greater affinity for heparin than VEGF_{165} and VEGF_{145} (Houck, Leung, Rowland, Winer, & Ferrara. 1992). Because of this VEGF_{189} and
VEGF\textsubscript{206} are almost completely sequestered by the ECM (Park, Keller, & Ferrara. 1993).

1.4.1.1 *Vascular Endothelial Growth Factor Receptors*

To date, two VEGF tyrosine-kinase receptors have been identified, VEGFR-1 (flt-1) (de Vries, Escobedo, Ueno, Houck, Ferrara, & Williams. 1992) and VEGFR-2 (KDR flk-1) (Terman, Dougher-Vermazen, Carrion, et al. 1992). The receptors are present both on vascular endothelium (Joukov, Pajusola, Kaipainen, et al. 1996; Joukov, Sorsa, Kumar, et al. 1997) and non vascular epithelium. The binding of VEGF to the receptors causes dimerisation with phosphorylation of their cytoplasmic tyrosine kinase domains. This provides a binding site for phosphoinositide-specific phospholipase C which is phosphorylated to form inositol 1, 4, 5-triphosphate (IP\textsubscript{3}) and 1,2-diacylglycerol (Berridge. 1993). Interaction between IP\textsubscript{3} and its receptor leads to a three to fourfold increase in intracellular calcium (Brock, Dvorak, & Senger. 1991).

1.4.1.2 *Vascular Endothelial Growth Factor functions*

As discussed previously (section 1.2.4), VEGF is one of the most potent vasodilators known (Dvorak, Brown, Detmar, & Dvorak. 1995). Chronic exposure to VEGF leads to a change in the vascular endothelial phenotype from contiguous to fenestrated endothelium as seen in the kidney and brain (Kubitza, Hickey, & Roberts. 1999).
Microvascular hyperpermeability leads to a profound alteration in the extracellular matrix transforming it from an antiangiogenic to a proangiogenic phenotype. VEGF induces the expression of urokinase type and tissue type plasminogen activators (PA’s), PA inhibitor-1 and metalloprotinase interstitial collagenase (Pepper, Ferrara, Orci, & Montesano. 1992) leading to further alteration of the ECM. The conversion of Prothrombin to Thrombin stimulates Metalloproteinase-2 (MMP-2) activation which in turn further breaks down the ECM (Zucker, Mirza, Conner, et al. 1998).

VEGF is a potent mitogen for endothelial cells (Ferrara & Henzel. 1989) inducing vessel sprouting from rat aorta embedded in a collagen gel (Nicosia, Nicosia, & Smith. 1994). Activation of the VEGFR-2 receptor leads to endothelial cell proliferation while activation of both VEGFR-1 and VEGFR-2 receptors lead to endothelial cell migration (Seetharam, Gotoh, Maru, Neufeld, Yamaguchi, & Shibuya. 1995; Waltenberger, Claesson-Welsh, Siegbahn, Shibuya, & Heldin. 1994; Yoshida, Anand-Apte, & Zetter. 1996; Barleon, Sozzani, Zhou, Weich, Mantovani, & Marme. 1996).

VEGF is vital for angiogenesis. In gene knockout experiments deletion of VEGF or VEGF receptors leads to gross vascular abnormalities and perinatal death (Carmeliet, Ferreira, Breier, et al. 1996).

VEGF inhibits cell apoptosis (Katoh, Tauchi, Kawaishi, Kimura, & Satow. 1995) inducing the expression of antiapoptotic factors such as bcl-2
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(Gerber, Dixit, & Ferrara. 1998; Nor, Christensen, Mooney, & Polverini. 1999). This may explain the positive effect of VEGF on endothelial cell survival (Gerber, McMurtrey, Kowalski, et al. 1998; Alon, Hemo, Itin, Pe'er, Stone, & Keshet. 1995).

1.4.1.3 Regulation of Vascular Endothelial Growth Factor

The VEGF gene can be induced by hypoxia in a number of cell types including endothelial and smooth muscle cells (Namiki, Brogi, Kearney, et al. 1995; Stavri, Hong, Zachary, et al. 1995). Endothelial cells respond to oxygen tensions below 70mmHg. Hypoxia in the cell leads to a redox state with the generation of free radicals. This activates the transcription factors Hypoxia Inducible Factor-1 (HIF-1) and NF-κB which in turn induce the expression of hypoxia-related genes (Huang, Arany, Livingston, & Bunn. 1996; Koong, Chen, Mivechi, Denko, Stambrook, & Giaccia. 1994). HIF-1 induces VEGF expression by binding to a cis element and increasing VEGF mRNA transcription (Levy, Levy, Wegner, & Goldberg. 1995; Minchenko, Salceda, Bauer, & Caro. 1994).

1.4.1.4 Vascular Endothelial Growth Factor in Bladder Cancer

In 1993, Brown et al performed in situ hybridisation on two cystectomy specimens for VEGF mRNA and VEGFR-1 and -2 (Brown, Berse, Jackman, et al. 1993). They also studied 13 nephrectomy specimens, one of which contained TCC in the renal pelvis. All the TCC's labelled strongly for VEGF mRNA. Labelling was particularly strong in areas adjacent to necrosis.
Chapter 1 Introduction

VEGFR-1 and -2 were found to be expressed on tumour endothelium. VEGF was also detected on vessels by immunohistochemistry but not VEGF mRNA. This suggests that the VEGF was made elsewhere, for example in tumour cells and then bound to vessels via its receptors.

O'Brien et al investigated VEGF mRNA expression in 45 primary bladder tumours and eight specimens of normal bladder using RNase protection analysis (O'Brien, Cranston, Fuggle, Bicknell, & Harris. 1995). Specimens were acquired by transurethral resection, seven were pTa, 21 pT1 and 17 were invasive.

Expression of VEGF was 3-fold higher in TCC than in normal bladder (p<0.0004). VEGF was 10-fold higher in superficial tumours than normal bladder (p<0.0002) and 4-fold higher in superficial tumours than in invasive tumours (p<0.0006). Expression in invasive tumours however was only 2-fold higher than normal bladder (p<0.001).

Of the 19 patients with superficial disease, 18 had a check cystoscopy at three months. Ten patients developed a recurrence. The VEGF level was 10-fold higher in those patients who recurred than in those who did not (p<0.002).

The same group found that high VEGF mRNA expression was associated with poor prognosis in pT1 bladder tumours (Crew, O'Brien, Bradburn, et al. 1997). In 55 pT1 tumours, VEGF mRNA (p<0.001) and VEGF protein
(p<0.0001) expression was found to be higher in superficial bladder cancer than in normal bladder. The VEGF protein to VEGF mRNA ratio however, was 4-fold higher in tumours. This suggested a post-transcriptional regulation of VEGF in these tumours.

VEGF mRNA was also found to be higher in those cases that recurred within six months (p<0.001) and those cases that progressed (p=0.02). It was also shown that tumours with VEGF mRNA levels above the median were at significantly higher risk of early recurrence (p=0.001; HR 3.09, 95% CI 1.54-6.20) and progression (p=0.02; HR 5.33, 95% CI 1.16-24.4).

Tumours that had abnormal p53 expression had a 3-fold greater VEGF mRNA expression and a 2-fold VEGF protein expression. Tumours with high VEGF mRNA expression and abnormal p53 had the poorest prognosis for recurrence (p<0.001) and progression (p=0.04).

In a multivariate analysis, VEGF mRNA expression was an independent prognostic factor for early recurrence in pT1 bladder cancer (p=0.02; HR 1.12, 95% CI 1.02-1.22).

In the study by Inoue et al VEGF expression was compared along with b-FGF and IL-8 expression to MVD and outcome in invasive bladder cancer (Inoue, Slaton, Karashima, et al. 2000). In a series of 55 cases of invasive bladder cancers that were treated by MVAC chemotherapy and cystectomy in
situ mRNA hybridisation analysis was used to measure VEGF. In 51 cases both pre-treatment and cystectomy specimens were available. In the pretreatment group, VEGF mRNA showed no correlation with MVD (p=0.230). Nine patients were rendered pT0 after transurethral resection and M-VAC. In this group VEGF mRNA was significantly lower, p<0.05. VEGF expression correlated with disease recurrence, p=0.032 and a Cox univariate analysis confirmed that it was a predictive factor for progression, p=0.010. In the multivariate analysis, VEGF alone was a predictive factor for recurrence, p=0.012. VEGF expression in the cystectomy specimens (post MVAC) however, was not a predictive factor for disease outcome. Indeed VEGF levels were higher in post chemotherapy cystectomy specimens than in pre-treatment biopsies. As VEGF protects cells from hypoxia it was considered that the higher levels in the post chemotherapy residual tumours represented the cells thus protected.

In 185 cases of superficial bladder tumour, Chow et al examined VEGF immunostaining (Chow, Liu, Chan, Cheng, & Tzai. 1999). They used a polyclonal antibody against VEGF_{165} (Calbiochem, Cambridge, MA, USA). Grading was into three groups, <5% staining (+), 5-25% (++) , >25% (+++). VEGF staining was seen in the extracellular matrix of both cancer and controls. VEGF was seen in the endothelial cells and cytoplasm only in tumor cells. VEGF was found to localise more adjacent to the basement membrane.
VEGF staining was related to stage (p=0.01) and grade (p=0.03) but was higher in pTa than in pT1 disease (p=0.001). VEGF did not however correlate with tumour recurrence or survival.

An increase in urinary VEGF is also found in patients with bladder tumours. Crew et al measured VEGF by enzyme linked immunosorbent assay in the urine of 261 patients (Crew, O'Brien, Bicknell, Fuggle, Cranston, & Harris. 1999). Urinary VEGF was higher in patients with bladder cancer (p<0.0001). It was also higher in patients with active bladder cancer than those undergoing bladder cancer surveillance with clear cystoscopies (p<0.0001). Levels were lower in patients with pTa disease (p<0.01) compared to invasive disease however, there was no difference in levels between pT1 and invasive disease. Urinary VEGF increased with increased frequency of superficial bladder cancer recurrence, p<0.001.

In a study by Jeon et al, VEGF was measured in the urine of 350 patients having transurethral resection of bladder tumours (Jeon, Lee, & Chang. 2001). This study also found a higher urinary VEGF concentration in patients with bladder tumours than normal controls, p=0.003. There was no difference between pTa and pT1 tumours (p=0.12) but VEGF was higher in patients that subsequently recurred, p=0.001.
1.4.1.5 Vascular Endothelial Growth Factor Summary

VEGF is a major promoter of angiogenesis and plays a major part in its control. Crew et al hypothesised that p53 regulates angiogenesis via Thrombospondin and VEGF (Crew, O'Brien, Bradburn, et al. 1997). However, they found VEGF was related to poor prognosis irrespective of whether p53 was positive or negative. This may be due to a problem with detection of p53 or because p53 regulates angiogenesis via other factors such as TSP more than VEGF.

In both invasive and superficial bladder cancer, increased levels of VEGF have been shown to correlate with a poor outcome. Only one study has studied VEGF expression in superficial bladder cancer using immunohistochemistry (Chow, Liu, Chan, Cheng, & Tzai. 1999). In this study, no correlation was found between VEGF and outcome.

1.4.2 Thymidine Phosphorylase

Thymidine Phosphorylase (TP) was originally identified in 1987 as Platelet Derived Endothelial Cell Growth Factor (PDECGF), a factor that stimulated the in vitro growth of endothelial cells (Miyazono, Okabe, Urabe, Takaku, & Heldin. 1987; Furukawa, Yoshimura, Sumizawa, Haraguchi, & Akiyama. 1992; Moghaddam & Bicknell. 1992). TP consists of two identical subunits, each with a molecular weight of 55kDa (Desgranges, Razaka, Rabaud, & Bricaud. 1981).
TP is pro-angiogenic but unlike many angiogenic factors it lacks a heparin binding domain and a secretion peptide. TP catalyses the breakdown of thymidine to thymine. Although its exact proangiogenic mechanism is unknown, it is chemotactic to endothelial cells (Moghadam, Zhang, Fan, et al. 1995). Ishikawa et al found that xenografts from transformed fibroblasts that were transfected with TP formed more vascular tumours than controls (Ishikawa, Miyazono, Hellman, et al. 1989). Also, when TP was transfected into the breast carcinoma line MCF-7, the tumours grew more rapidly (Moghadam, Zhang, Fan, et al. 1995).

One possible pathway for TP induced angiogenesis is via the induction of oxidative cell stress. Brown et al showed that TP overexpressing cell lines induced oxidative stress after addition of thymidine. The 2-Deoxy-D-ribose-1-phosphate (2dD1P) produced from the thymidine by the action of TP, it was suggested, generated oxygen free radicals. Thymidine catabolism by TP increased production of VEGF, IL-8 and MMP-1. Thus, TP may be angiogenic by induction of other proangiogenic factors (Brown, Jones, Fujiyama C, Harris, & Bicknell. 2000).

1.4.2.1 Thymidine Phosphorylase in Bladder Cancer

In a paper by O’Brien et al, expression of TP by RNase protection analysis was found to be 40-fold higher in TCC than in normal bladder (p<0.01) (O’Brien, Cranston, Fuggle, Bicknell, & Harris. 1995). Although there was no significant difference in TP expression between superficial tumours and normal
bladder, expression in invasive tumours was 260-fold higher than in normal bladder (p<0.0003) and 33-fold higher than in superficial tumours (p<0.0001). This paper showed that VEGF and TP, although both raised in bladder cancer, were differentially expressed; VEGF was higher in superficial bladder cancer and TP higher in invasive tumours.

In a further study O’Brien et al examined 105 primary bladder carcinomas by Western Blotting and immunohistochemistry for TP expression (O’Brien, Fox, Dickinson, et al. 1996). Tumours were graded positive for TP if they had greater than 55% stained tumour cells of moderate or higher intensity. In this study, staining of TP was also seen in the stroma, in inflammatory cells and in the endothelium of vessels.

TP expression in the 52 cases analysed by Western Blotting was 5-fold higher in tumours than in normal bladder. Expression was 15-fold higher in invasive bladder cancers than in normal bladder and 8-fold higher in invasive cancer compared with superficial. TP immunostaining was seen in 27% of tumours. Immunostaining correlated well with Western analysis for TP expression (r=0.53, p<0.06).

Although TP tumour staining was related to grade (p<0.02), there was no relationship to stage or p53 status. TP tumour immunostaining was not related to survival. There was no record of TP staining in other tissue areas being correlated with outcome.
MVD was estimated by Chalkley counting in three hotspots in CD31 labelled invasive tumours only. They found a significant but inverse relationship between endothelial TP expression and MVD (expressed as a dichotomous variable) (p<0.01).

Kubota et al examined TP activity in a series of bladder cancers by determining the conversion rate of 5’ deoxy-5-fluorouridine to 5-fluorouracil, a function of TP. In 37 bladder cancers and 8 normal bladder samples, TP activity was seen to be higher in the cancer (p=0.0002). Activity was also higher in invasive than in superficial cancers (p=0.04) and higher with high grade disease (p=0.0008) (Kubota, Miura, Moriyama, et al. 1997).

Arima et al examined bladder cancer from 108 cystectomy specimens. TP activity measured by spectrophotometry in 37 TCC’s and 12 adjacent areas of normal urothelium was 10.2-fold higher in TCC than normal bladder (p=0.002). They also examined the expression of TP by immunohistochemistry grading cases TP-positive if they contained more than 5% of parenchymal cells stained (a notable difference from the method of O’Brien et al). They found a significant correlation between TP positivity and grade (p=0.014), stage (pT1 versus pT2-pT4) (p=0.002) and lymph node metastasis (p=0.003).

Using a Kaplan Meier estimation, TP positive carcinomas were found to have poorer survival (p<0.001). TP expression was also seen to be a risk factor
for survival in a univariate (p=0.001) and multivariate (p=0.047) analysis (Arima, Imazono, Takebayashi, et al. 2000).

1.4.2.2 Thymidine Phosphorylase summary

TP expression is associated with high stage and high grade disease and poor outcome in bladder cancer. TP is known to be proangiogenic and has been the proposed mechanism for this association. However, MVD in bladder cancer has been shown in one study to be inversely related to TP expression.

1.4.3 Thrombospondin

1.4.3.1 The Structure of Thrombospondin

In 1971 Baenziger and Majerus identified a 190 kdal polypeptide selectively released from human platelets by the action of thrombin, designated thrombin-sensitive-protein (Baenziger, Brodie, & Majerus. 1971). This was subsequently found to be one subunit of a larger, 450kdal, protein and renamed thrombospondin (Lawler, Slayter, & Coligan. 1978). The molecule is located in the α granules of platelets and has also been termed glycoprotein-Ig or glycoprotein-G.

Thrombospondin is a large, multifunctional, extra cellular, membrane bound glycoprotein that can influence endothelial cell function in vitro and angiogenesis both in vitro and in vivo (Good, Polverini, Rastinejad, et al. 1990; Murphy-Ullrich & Hook. 1989; Rastinejad, Polverini, & Bouck. 1989).
Thrombospondin, now designated Thrombospondin-1 (TSP-1), has been found to be one of five members of a gene family. The thrombospondins can be divided into two groups. TSP-1 and thrombospondin-2 (TSP-2) are trimeric glycoproteins while thrombospondin-3 (TSP-3), thrombospondin-4 (TSP-4) and thrombospondin-5 (TSP-5), also known as Cartilage Oligomeric Matrix Protein (COMP) are pentamers (DiPietro. 1997).

TSP-1 consists of three identical disulphide linked 1152 amino acid chains (Lawler & Hynes. 1986). Each 145 kdal chain is divided into six separate domains consisting of repeating homologous amino acid sequences. The two globular terminal domains are joined by a central stalk (Qian, Wang, Rothman, Nicosia, & Tuszynski. 1997), Figure 1.4. The amino terminal globular domain is heparin binding while the carboxy domain is involved in cell adhesion and chemotaxis. The stalk may be sub divided into five distinct domains:

1. Disulphide bonding domain
2. Procollagen homology domain
3. Type I (properdin) repeat domain
4. Type II (epidermal growth factor) repeat domain
5. Type III (Calcium binding sequence calmodulin) repeat domain

These distinct domains interact with different cell surface receptors and macromolecules conferring upon TSP-1 its multifunctional nature.
Figure 1.4  The Structure of Thrombospondin-1
Although originally thought to be only a product of platelets, TSP-1 is now known to be produced by a wide variety of cell types including endothelial cells, fibroblasts, smooth muscle cells, keratinocytes, macrophages and neutrophils (DiPietro. 1997).

1.4.3.2 Thrombospondin is antiangiogenic

There is a wealth of experimental and clinical evidence to suggest that TSP is antiangiogenic and that by this method holds tumours in check.

In 1989, Rastinejad et al found that a 140 kDal glycoprotein inhibited bFGF stimulated angiogenesis in the classical rodent corneal pocket assay (Rastinejad, Polverini, & Bouck. 1989). This glycoprotein was subsequently shown to be a fragment of TSP (Good, Polverini, Rastinejad, et al. 1990).

Volpert et al studied the angiogenic progression of human fibroblasts cultured from Li Fraumeni patients. He found that angiogenesis was due to the loss of TSP inhibition (Volpert, Dameron, & Bouck. 1997). Activation of angiogenesis was found to be a two stage process. The first was dependent on the loss of both alleles of wild-type p53 which caused a drop of at least 20-fold in secreted thrombospondin and a fourfold increase in secreted VEGF. Angiogenic activity increased again upon transformation by activated ras via a further twofold increase in VEGF. Changes in relative levels of VEGF mRNA were sufficient to account for changes in secreted protein levels and in overall angiogenic activity. These studies demonstrate that an angiogenic phenotype
able to support tumorigenicity can arise in a step-wise fashion in response to both oncogene activation and tumor suppressor gene loss and involve both a decrease in the secretion of inhibitors and the sequential ratcheting up of the secretion of inducers of angiogenesis (Volpert, Dameron, & Bouck. 1997).

Growth of human skin carcinoma cells implanted into mice was suppressed by reintroduction of a lacking chromosome 15. The TSP gene is located on chromosome 15q15 (Bornstein. 1992) and transfection with TSP causes similar inhibition of tumour growth. This effect was reversed by TSP-1 antisense oligonucleotides. TSP was found in high concentration at the tumour-stromal border suggesting that TSP deposition may act as an antiangiogenic barrier to tumour expansion (Bleuel, Popp, Fusenig, Stanbridge, & Boukamp. 1999).

Streit et al examined human squamous cell carcinoma lines A431 and SCC-13 transfected to overexpress TSP-1. Tumour growth was inhibited in A431 and abolished in SCC-13 in xenograft models but there was no apparent effect on cell proliferation, anchorage dependent and independent growth rates and susceptibility to apoptosis by serum withdrawal (Streit, Velasco, Brown, et al. 1999).

TSP has also been shown to act as an inhibitor of angiogenesis in normal physiology. The endometrial cycle is a rare example of active angiogenesis in the non pathological setting. Iruela-Arispe et al linked TSP-1 to the inhibition of
vessel formation in the later (secretory) stage of the endometrial cycle. In 46 human endometrial specimens TSP-1 protein was identified in the basement membrane of capillaries during the secretory phase with abundant TSP-1 mRNA detected by in situ hybridization. Contrasting low levels were seen in the proliferative phase. Levels of TSP-1 mRNA and protein were increased after incubation with progesterone and the levels of TSP-1 present in progesterone-stimulated cultures were sufficient to inhibit the migration of endothelial cells in vitro an effect nullified by anti-TSP antibodies (Iruela-Arispe, Porter, Bornstein, & Sage. 1996).

TSP has also been examined in experimentally produced benign disease in animal models. Progression of an experimental goitre in the rat is accompanied by an increase in neovascularisation, fibroblast growth factor-2 (FGF-2) and transforming growth factor-beta 1 (TGF beta 1). TSP-1 immunoreactivity in the control rat thyroid was found in the stroma and in the endothelial cells, while weak follicular cell staining was also present. In the goitrous rat thyroid the TSP immunoreactivity was present after 1 week of treatment in the endothelial cells and most follicular cells, whilst stroma localisation was weak. After week 2 of treatment the endothelial cell and stromal localisation was no longer apparent, although a follicular localisation was still present. Immunoreactivity was present in the cytoplasm of a minority of the follicular cells in control rat thyroids, while their nuclei were unstained. These results show that in the hyperplastic thyroid, increases in FGF-2 and TGF beta 1,
and decreases in TSP1 accompany angiogenesis (Patel, Hill, Eggo, Sheppard, Becks, & Logan. 1996).

TSP may bind to proangiogenic factors preventing them attaching to their target molecules. TSP binds to bFGF via its 140fDal carboxy terminal fragment. This binding is blocked by heparin. The TSP blocks bFGF binding to endothelial cells at concentrations which inhibited endothelial cell proliferation (although not migration) (Taraboletti, Belotti, Borsotti, et al. 1997).

1.4.3.3 Thrombospondin and Vascular cord formation

Another possible site of TSP action on angiogenesis inhibition is the formation of endothelial cells into vascular cords or tubes (Canfield & Schor. 1995). Endothelial cells plated on the surface of a two-dimensional substratum form a cobblestone monolayer at confluence, whereas cells plated within a three-dimensional gel matrix elongate into a sprouting morphology and self-associate into tube-like structures. Canfield in 1990 demonstrated that production of TSP by proliferating endothelial cells grown in a three dimensional collagen matrix was markedly decreased compared to identical cells grown in two dimensions (Canfield, Boot-Handford, & Schor. 1990).

In a murine brain endothelial cell line that can either proliferate or form tube-like structures, a fragment of TSP (4N1K peptide) derived from the C-terminal domain inhibited tubule formation but not cell proliferation. The effect
was blocked by antibody to 4N1K (Kanda, Shono, Tomasini-Johansson, Klint, & Saito. 1999).

Tolsma et al also found that TSP-1 prevented cultured capillary endothelial cells forming into cords. It blocked the migration of endothelial cells and vascular smooth muscle cells, but not that of fibroblasts, neutrophils or keratinocytes. Inhibition of TSP with antibodies allowed migration towards bFGF, cord formation and cavity formation by the fusion of vesicles (Tolsma, Stack, & Bouck. 1997).

The influence of TSP-1 on cord formation may be concentration dependent as a permissive angiogenic effect of TSP-1 has also been demonstrated on endothelial cells. Tube formation of bovine endothelial cells in collagen gel was induced at low TSP-1 concentration but inhibited at high concentration. In addition, TSP stimulated MMP9 activity and this was also concentration dependent. Antibody blockade of either MMP9 or TSP1 inhibited invasion and tube formation (Qian, Wang, Rothman, Nicosia, & Tuszynski. 1997). Tolsma et al showed that high level of TSP-1 blocked endothelial cell cord formation but TSP had no stimulatory effect on the activity of the soluble gelatinases or plasminogen activator (Tolsma, Stack, & Bouck. 1997).

Similarly, Di Petro et al studied the effect of endogenously produced TSP on cord formation in bovine aortic endothelial cells grown on a three dimensional matrix. They found that endothelial cells with reduced TSP production (via anti
sense RNA transfection) formed capillary like cords at half the rate of controls. However, the decrease in TSP-1 production had no observable effect upon cell morphology or growth when the cells were grown in two dimensional conditions (DiPietro, Nebgen, & Polverini. 1994).

1.4.3.4 Thrombospondin may modulate angiogenesis via the Extra cellular Matrix

Modulation of the ECM by TSP is suggested by its action as a matrix enzyme. Hogg et al reported that TSP may function as a protease inhibitor and thus influence angiogenesis by effecting ECM turnover and composition (Hogg. 1994). The location of TSP within the tissues may thus influence its action on angiogenesis; this location is dependent upon receptor binding.

1.4.3.5 CD36 appears to be the receptor that inhibits angiogenesis

CD36 is a transmembrane glycoprotein composed of a single polypeptide chain, which interacts with thrombospondin, collagens type I and IV, oxidized low density lipoprotein, fatty acids, anionic phospholipids, and erythrocytes parasitized with Plasmodium falciparum. Its expression is restricted to a few cell types, including monocyte/macrophages. In these cells, CD36 is involved in phagocytosis of apoptotic cells, and foam cell formation by uptake of oxidized low density lipoprotein (Armesilla, Calvo, & Vega. 1996).

CD36 is also found on microvascular endothelial cells. CD36 mediates the inhibitory effects of thrombospondin-1 on endothelial cells. This effect (the
migration of cultured microvascular endothelial cells) is blocked by anti-CD36 antibodies. Furthermore, other molecules which stimulate the CD36 receptor mimic TSP-1’s anti-angiogenic effects (Dawson, Pearce, Zhong, Silverstein, Frazier, & Bouck. 1997).

Increased expression of the TSP receptor CD36 correlated with decreased stromal vascularisation and a better outcome in colon cancer (Tsuchida, Kijima, Tokunaga, et al. 1999).

1.4.3.6 Thrombospondin as a promoter of adhesion & growth of cancer cells

In a serum-free model Nicosia et al evaluated TSP-containing fibrin and collagen matrices for their capacity to support angiogenesis and cell growth from explants of rat aorta. TSP promoted dose-dependent growth of microvessels and fibroblast-like cells. The TSP effect was due in part to cell proliferation. The effect was TSP-specific because TSP preparations adsorbed with anti-TSP antibody showed no activity. TSP did not promote angiogenesis directly since no TSP-dependent growth of isolated endothelial cells could be demonstrated. Rather TSP directly stimulated the growth of aortic culture-derived myofibroblasts which in turn promoted microvessel formation when cocultured with the aortic explants. Angiogenesis was also stimulated by myofibroblast-conditioned medium. These results indicate that matrix-bound TSP can indirectly promote microvessel formation through growth-promoting effects on myofibroblasts and that TSP may be an important stimulator of angiogenesis and

Chandrastaken et al showed that TSP-1 is pro-adhesive and chemotactic via the integrin alpha-3-beta-1 for several breast carcinoma cell lines. Albo et al showed that TSP and TGFβ both increased expression of urokinase type Plasminogen activator (uPA) and its receptor uPAR in pancreatic cancer cells and in a Boyden chamber, both TSP and TGFbeta increased cell invasion by 3.5 and 4.5 fold respectively. Blocking antibodies to uPA and uPAR blocked this invasion indicating that TSP and TGFβ mediate pancreatic tumour cell invasion through upregulation of the plasminogen/plasmin system (Albo, Berger, & Tuszynski. 1998).

In breast cancer, thrombospondin and TGFβ-1 function similarly to increase cell-associated uPA and cell-secreted Plasminogen activator inhibitor-1 (PAI-1). Suggesting that thrombospondin may not only function as an adhesive molecule, but through a mechanism involving the activation of TGFβ-1, may modulate cell surface protease expression. In addition, these observations suggest that thrombospondin and TGFβ-1 could promote metastasis by increasing uPA-mediated cell invasion, whereas through the action of PAI-1, also protect blood-born tumor emboli from destruction by host fibrinolytic enzymes. Thrombospondin and TGFβ-1 also induced a more flattened and spread appearance in the cells with no effect on proliferation (Arnoletti, Albo, Granick, et al. 1995).
1.4.3.7 **Thrombospondin has also been associated with poor outcome in Cancer**

In human gallbladder carcinoma TSP-1 immunoreactivity was seen in stromal rather than cancer cells and was observed more frequently in higher grade and metastatic cancer (Ohtani, Kijima, Dowaki, et al. 1999).

Similarly with squamous cell carcinoma of the oesophagus TSP immunoreactivity correlated with a higher grade of disease and lymph node and venous involvement (Oshiba, Kijima, Himeno, et al. 1999).

A major TSP cell adhesive domain, Cysteine-serine-valine-threonine-cysteine-glycine (CSVTCG) has been shown to play a role in tumor cell metastasis. CSVTCG was found in breast carcinoma but not in benign tissue and normal breast. Increasing expression of stromal TSP and the CSVTCG-specific TSP receptor in ductal epithelium correlates with neoplastic transformation. Both malignant and benign breast tissue stimulated surrounding capillaries to express the TSP receptor, whereas only carcinoma has the capacity to stimulate surrounding nonendothelial stromal cells, such as myofibroblasts, to secrete a TSP-rich matrix that may contribute to the desmoplastic stromal reaction characteristic of ductal carcinoma tumor. The TSP-rich matrix may then promote tumor cell attachment, migration, and angiogenesis. The receptor-rich capillary endothelium may promote the cell adhesive interactions important in tumor intravasation (Tuszynski & Nicosia. 1994). Increasing amounts of receptor for
this domain significantly correlated with poor outcome in a series of breast carcinomas (Roth, Reiver, Granick, Rothman, Nicosia, & Tuszynski. 1997).

Head and neck tumour sections have also been specifically stained for CSVTCG-specific TSP receptor. Patients with CSVTCG-specific TSP receptor had high microvessel density and a poor survival whereas patients with low staining had low microvessel counts and remained disease-free for at least 2 years (Arnoletti, Albo, Jhala, et al. 1994). Nathan et al looking at plasma levels of thrombospondin in patients with gynaecological malignancies found higher levels in cancer patients compared to controls.(Nathan, Hernandez, Dunton, et al. 1994) The CSVTCG region in TSP is the same as the Type 1 repeats (Catimel, Leung, el Ghissasi, Mercier, & McGregor. 1992).

1.4.3.8 Thrombospondin in Bladder Cancer

In 1997 Grossfeld et al identified TSP-1 expression by immunohistochemistry in a series of 163 radical cystectomy specimens. Although 63 patients had organ confined disease (pT1 – pT3a), the number of superficial bladder cancers (pT1) was not given or analysed separately.

The antibody they used was MA-II recognising an epitope in the amino-terminal. In order to get this to work, antigen retrieval was performed by microwaving in TRIS / HCL buffer at pH1. The MVD (by hotspot counting) and p53 status were also measured.
Only extracellular TSP staining was graded. The authors felt that cytoplasmic and nuclear staining represented artefact secondary to the harsh antigen retrieval technique. In a previous paper describing the optimum antigen retrieval technique for this (MA-II) TSP antibody, distribution of TSP staining in frozen sections was seen in the stroma and surrounding vessels. No comment on perivascular staining was made in the 1997 study. Evaluation of TSP staining included grading of the amount and intensity of stromal staining and cases were graded as low, moderate or high. For the purposes of statistical analysis, moderate and high staining were grouped together.

Grossfeld et al found no association between TSP staining and grade or stage. However, low TSP staining was associated with increased probability of disease recurrence (p=0.009) and decreased survival (p=0.023).

In a multivariable analysis with grade, stage, lymph node status and TSP, TSP expression was found to be an independent predictive factor for disease recurrence (p=0.002) and survival (p=0.01). TSP expression was not independent of p53 status. There was in fact a significant association between TSP expression and p53 status (p=0.001).

In 160 of the 163 cases, MVD counts were performed; it was not clear if the cases omitted were superficial, or why counts could not be carried out. MVD counts were either high or low, dichotomised at the median of 79 vessels per high powered field (HPF). Low TSP expression was associated with a high
MVD count (p=0.001). This association was retained when MVD was expressed as tertiles. MVD as a continuous variable was not analysed.

In an analysis of variance, p53 status (p=0.015) and TSP expression (p=0.0006) were significantly associated with MVD. TSP remained significant after adjustment for p53 (p=0.008). However, when p53 was adjusted for TSP it did not (p=0.35). The authors suggested that p53 influences TSP levels and TSP expression impacts on MVD.

In 1998 Campbell et al investigated the relationship between TSP and angiogenesis in bladder cancer (Campbell, Volpert, Ivanovich, & Bouck. 1998). Serum from four bladder cancer cell lines (RT4 (low grade), HT1376, UMUC-3 and TCC-S) induced endothelial cell migration in an in vivo assay; serum from normal urothelial cell lines did not. High grade bladder cancer cell lines induced neovascularisation in a rat cornea model; normal urothelium did not. Furthermore, media from normal urothelial cells blocked angiogenesis stimulated by bFGF, VEGF and the bladder cancer cell line media. An antibody against TSP (A4.1) blocked this antiangiogenic activity suggesting that the normal urothelial cells inhibited angiogenesis via TSP. Indeed, medium from normal urothelial cells became pro-angiogenic when TSP was blocked. This suggested that both malignant and non malignant cell lines were proangiogenic unless TSP was present to block this.
In this experimental series VEGF and bFGF were the primary stimulators of angiogenesis in bladder tumour cell lines and high levels of VEGF were secreted by normal urothelial lines. Bladder cancer cell lines secreted much lower levels (<=5.8%) of TSP compared to normal urothelium.

Campbell et al also examined TSP expression by immunohistochemistry in frozen sections of bladder tumours. They found strong staining of TSP in normal urothelium but little or no staining in tumour cells. TSP staining was seen in stroma and basement membrane and also in endothelial cells which they used as an internal positive control. Only six cases of superficial bladder cancer and ten cases of invasive bladder cancer were evaluated.

1.4.3.9 Thrombospondin Summary

Thrombospondin is a complex molecule with multiple functions. Its part in tumour growth and progression is complicated apparently acting as tumour suppressor through its antiangiogenic properties but also as tumour promoter by stimulation of cell growth. Its contradictory nature may be due to its multiple epitope binding sites and its varying actions may be promoted via different TSP receptor molecules. In some models, the actions of TSP are concentration dependent. The location of TSP, matrix bound or intracellular, may also influence its action. Although TSP is regarded as a classical antiangiogenic molecule that holds the process of neovascularisation in check, this would appear to be an oversimplification and its true place in cancer biology has not yet been established.
1.4.4 p53

Mutation of the p53 tumour suppressor gene is the commonest genetic defect in human cancers, including bladder cancer. The normal (wild type) p53 gene encodes for a 393 amino acid nuclear protein. Wild type p53 suppresses cellular proliferation.

Cellular stress, including hypoxia, heat and DNA damage, leads to accumulation of p53 protein product in the nucleus. P53 acts as a transcription factor for several key enzymes leading to inhibition of the cell cycle or cellular apoptosis. For example, production of p53 may lead to an increase in p21 which in turn leads to phosphorylation of cyclin dependent kinases and inhibition of the cell cycle. P53 also leads to an increase in BAX and a decrease in bcl-2 promoting cellular apoptosis.

P53 is closely associated with the regulation of angiogenesis. It acts as a transcription factor for VEGF, bFGF and TSP. Mutation of p53 may therefore lead not only to a decrease in the regulation of cell proliferation but also uncontrolled angiogenesis.

1.4.4.1 p53 and outcome in bladder cancer

Mutation of p53 has been shown to predict poor prognosis in superficial bladder cancer (Casetta, Gontero, & Russo. 1997; Sarkis, Dalbagni, Cordon-Cardo, et al. 1993; Sarkis, Dalbagni, & Cordon-Cardo. 1994; Serth, Kuczyk, & Bokemeyer. 1995). Not all studies have shown this however (Gardiner, Walsh, &
Accumulation of p53 has also been associated with a poorer response to intravesical treatment with both BCG (Lacombe, Dalbagni, Zhang, et al. 1996a; Zlotta, Noel, & Fayt. 1999; Lacombe, Dalbagni, Zhang, et al. 1996b) and chemotherapy (Tzai, Chow, Lin, Yang, & Tong. 1998).

In a study of 243 patients undergoing cystectomy for bladder cancers of all stages (pTa-pT4), Esrig et al found that p53 mutation was the only independent predictor of disease progression (Esrig, Elmajian, Groshen, et al. 1994).  

1.4.5 Angiogenic Factors – Summary

Angiogenesis is controlled by a complex balance of promoter and inhibitor factors. Interaction and feedback occurs between these factors which influences the angiogenic phenotype.

In cultured fibroblasts the switch to such an angiogenic phenotype was found to correspond with a loss of wild type p53 and to be a result of reduced expression of TSP (Dameron, Volpert, Tainsky, & Bouck. 1994). Mutation of p53 is thought to play a major part in the control of angiogenesis possibly via TSP.

A series of malignant melanomas which expressed mutant p53 Grant et al found lower mean TSP-1 levels and a higher MVD. Metastatic tumours had a
higher incidence of mutant p53, lower TSP and high MVD (Grant, Kyshtoobayeva, Kurosaki, Jakowatz, & Fruehauf. 1998). Also in colorectal cancer the level of TSP-1 gene expression showed a significant inverse relationship with p53 status suggesting that p53 mutation may result in loss of TSP-1 expression (Tokunaga, Nakamura, Oshika, et al. 1998).

Nishimori et al isolated a gene product of p53 which contains 5 TSP type -1 repeats. The protein product of this gene inhibited neovascularisation in the rat cornea. This gene was found to be absent in 9 out of 10 glioblastomas suggesting a role in antiangiogenesis via p53 & TSP in this tumour (Nishimori, Shiratsuchi, Urano, et al. 1997).

In the study by Campbell et al an inverse correlation was found between p53 and TSP suggesting a controlling influence over angiogenesis by p53 and via TSP. However, in a subsequent study by the same group, they found that p53 had no influence over the secretion of TSP in bladder cancer cell lines or on angiogenesis. The authors felt that angiogenesis via TSP was regulated by other factors and p53 was more involved with later progression from superficial to invasive disease. They found that hypoxia was the major regulating factor of angiogenesis in bladder cancer via upregulation of VEGF (Campbell, Volpert, Ivanovich, & Bouck. 1998).

VEGF is one of the major proangiogenic factors in cancer. In several studies a reciprocal relationship is seen between VEGF and TSP as pro and anti
angiogenic factors. In a small study of 28 cases of squamous cell lung cancer, the 14 non smokers were found to have lower MVD, lower VEGF and higher levels of TSP compared to the matched 14 smokers (Volm, Koomagi, & Mattern. 1999).

Morelli et al found that serum from patients with breast or GI cancers was either stimulatory or inhibitory to endothelial cells. VEGF levels were raised in 45% of stimulatory serum and high TSP appeared to play a part in the inhibition of endothelial cell growth (Morelli, Lazzerini, Cazzaniga, et al. 1998).

In a series of 60 endometrial cell carcinomas expression of TSP-1 was found to be more strongly expressed in those tumours which weakly expressed VEGF, although this did not reach significance (Salvesen & Akslen. 1999).

TSP may also influence Thymidine Phosphorylase. In a study by Hogg et al, TSP-1 was seen to bind to Platelet Derived Endothelial Cell Growth Factor or TP. This was inhibited by heparin. Anti-TSP-1 antibody decreased binding of TP to its receptor on rat aorta. It is possible therefore that TSP-1 may assist TP in its binding to its receptor on vascular smooth muscle cells (Hogg, Hotchkiss, Jimenez, Stathakis, & Chesterman. 1997).

In superficial bladder cancer, overexpression of TP is seen and associated with progression to invasive disease whilst VEGF is lower in superficial than in invasive disease. Loss of TSP in two small studies was associated with poor
prognosis and p53 influences angiogenesis but it is not clear whether this is via TSP or VEGF control.

Chemokines, small chemotactic cytokines, can also modulate tumour behaviour by regulation of tumour-associated angiogenesis. They can stimulate or inhibit proliferation and chemotaxis of endothelial cells. Where a tumour secretes greater amounts of a pro-angiogenic chemokine (e.g. interleukin 8) than an angiostatic chemokine, angiogenesis is stimulated. An excess of angiostatic chemokines (e.g. interferon-γ-inducible protein 10 (IP-10)) inhibits neovascularisation, leading to tumour necrosis. (Frederick & Clayman, 2001)
1.5.0 Image Analysis

1.5.1 Introduction

There are three potential reasons for using computerised image analysis for MVD measurement. Firstly, time; computers are able to perform tasks in fractions of a second. Using a computer system to count objects like vessels will speed the process. This is particularly useful in a situation where multiple measurements are required. The system will be governed by the degree of automation; the greater the amount of human input required to assess a vessel, area or case, the slower the process will become.

Secondly, a computer is totally objective. A computer will only do what it is instructed to do and it will do this in the same way every time. It therefore removes some subjectivity from MVD counting. The amount of subjectivity remaining in a system will again depend upon the degree of human input. Subjectivity is related to choice. Thus, in a manual counting system a choice is made regarding which area is to be counted and also what constitutes a countable vessel within that chosen area. If a computerised image analysis system is allowed to make the choice governed by strict repeatable rules, it will be objective. The programming will determine how that choice is made but the more automation that is achieved within the program the more reproducible it will be.
Thirdly, a computerised image analysis system is able to measure other parameters than number of vessels. For example, measurements of area, perimeter and vessel shape. These are possible using manual techniques but are very time consuming.

Thus, a computerised image analysis system for the measurement of MVD allows more counts to be performed in a set time, allows greater objectivity and therefore greater accuracy and gives the opportunity to explore more detailed parameters of tissue vascularity. It also makes a tedious and time consuming process into something which would be more acceptable in a clinical setting.

1.5.2 Digital Images

Digital images consist of two dimensional arrays of dots. On a computer monitor these are called pixels. The colour or shade of each individual pixel determines the picture as a whole. Each pixel has a value. It is this value that determines its colour, shade or intensity; this value can be measured. The simplest colour combination is black and white made of black or white pixels. The value of a black pixel is 0 and a white pixel 255. The binary image produced is referred to as 1-bit. A shaded picture can be created by varying the distribution and thus the density of the individual black and white pixels.

A more complex picture may be created by using different shades of grey. The number of shades may vary from one to infinity (depending upon the
power of the computer and equipment used) but the human eye can comprehend no more than 256 different shades. Images using 256 different shades are referred to as 8-bit. A picture composed of varying shades of grey is referred to as greyscale.

Colour images are created by using pixels of varying colours. The commonly used colour combination is red, green and blue (RGB). Each colour may be represented in one of 256 shades. The image will therefore be \((3 \times 8 =)\) 24-bit. A second way of creating a coloured image uses different degrees of hue (colour), saturation (a scale of variation between grey and pure colour) and brightness or intensity value; termed HSV or HIS (Furness. 1997). *figure 1.5.*

**1.5.3 Acquisition of digital images**

The view down a microscope is an analogue image dependent upon the varying wavelengths of the coloured light seen. This image can be recorded using a still or video camera. Video cameras used on microscopes contain light sensitive charge coupled devices (CCD) to detect the intensity of light viewed. Each CCD chip in a video camera has 800 x 600 sensors. To improve the image multiple chips may be used, for example a 3-chip camera has three.

The image captured on a video camera is still analogue, expressed as a continuous variation in voltage on monitor lines. A framegrabber converts a chosen image from the video into a digital (bitmap) image on the computer.
**Figure 1.5 Digital Images**

**Figure 1.5.1** "Two Frigates, Gabriel Bay" Colour Image (RGB) 24 bit. File size 898 Kb This image is composed of pixels each being one of 256 shades of red, green or blue.

**Figure 1.5.2** "Two Frigates, Gabriel Bay" Greyscale Image. 8-bit. File size 300Kb. The pixels in this image are each one shade of 256 shades of grey.
Figure 1.5.3  "Two Frigates, Gabriel Bay" Black and white 1-bit image. Thresholded automatically using Adobe Photoshop 5.0. Pixel value for thresholding was 128. File size 38.5KB. Pixels are either black or white; shading depends on the distribution of the pixels.
1.5.4 Analysis of images

Image analysis software enables measurement of multiple factors within a digital image. Measurements depend upon the distribution, number and individual value of the pixels in the image. The size of an area of black pixels on a white background is a simple binary calculation: are the pixels black or white and how many black pixels are there?

Measurements of this type in greyscale or colour images will also depend on what shade or colour the pixels are. In an object of varying colour or shade its size and shape depends upon the definition of where its edge lies. Whereas in a black and white picture this is clearly defined, in a greyscale or colour object this depends on what is deemed to be background and what is foreground (or object), figure 1.6.

An image analysis program can be made to determine what constitutes an object. The instruction to determine what is a foreground object or vessel and what is background is called thresholding. The threshold is the colour or shade of grey that lies between foreground and background. If the value of a pixel is higher than the threshold it is foreground if it is lower it is background (or vice versa depending on whether the objects are black or white). In MVD counting, if the threshold is too high some objects, or vessels, will be lost, if it is too low, objects that are not vessels will be included. The setting of the threshold is therefore critical, figure 1.7.
Figure 1.6  “Two Frigates, Gabriel Bay” Close up of the mizzen mast in colour and 1-bit black and white reveals how the distribution of the individual coloured pixels and black and white pixels create the picture in each case. It is also clear that the edge of the sails are not as clearly defined in the coloured picture as in the black and white, although, due to threshold correction to a 1-bit image, some of the sail area has been lost.
Figure 1.7 Thresholding of Digital Images

Figure 1.7.1 Black and white image manually thresholded at a pixel value of 100. Although the picture is still recognisable some objects have been made white and lost.

Figure 1.7.2 Black and white image manually thresholded at a pixel value of 200. Here, although some objects are now retained (for example the ensign on the frigate on the left) other features are lost in the black background.
Once an image has been defined measurements of it can easily be made. The size of the individual objects can be measured in terms of area or perimeter or axis. The number of discrete foreground objects can be counted (as in a MVD count). Counts and area can be expressed relative to other measured areas in the image, for example area of vessels per total area or number of vessels in the area of the tumour. As an image analysis system measures the value of each individual pixel the intensity of colour can be measured. This can be used to assess the intensity of an immunohistochemical stain.

1.5.5 Use of image analysis in MVD measurement

An image analysis system must be given rules for MVD counting just as in manual counting. The computer must replicate what is performed in the manual count.

In manual counting systems, rules are followed to define what is a countable vessel. Commonly, a countable vessel consists of any stained area, it does not have to have a lumen and does not have to contain blood cells to be called a vessel. When counting in multiple areas it is usual to omit vessels falling on two of the four perimeters as this prevents over counting. Vessels counts are performed only in areas of interest; this may be tumour or tissue adjacent to tumour. During a manual count the observer has to decide what is a countable vessel; in image analysis these definitions have to be carefully programmed into the computer. It is easier for the human eye and brain to decide what does or
does not represent a vessel. However, this decision can also introduce bias and subjectivity.

Briefly, an image analysis program must be able to recognise what is a countable vessel and then count the number of vessels in a given area. To do this the computer has to:

1. Recognise vessels
2. Not count objects that are not vessels,
3. Recognise when a vessel is fragmented and not count its separate pieces,
4. Recognise separate vessels when they are touching and not count them as one,
5. Only count vessels in specified tissue (for example, tumour),
6. Not count vessels that fall on either of two specified margins of the field
7. Express the count per unit area.

One way of defining vessels in an image analysis system is to manually mark each vessel on an image. Once marked the computer can then count or measure the vessels. This of course takes time and is again subjective.

In order to automatically count the number of objects in a picture, those objects must be carefully defined. Digital images can be manipulated to allow
Chapter 1 Introduction

the computer to more easily define what is a foreground object from what is background. This can be thought of as cleaning or preparing the images. Image manipulation involves changing the value of individual pixels depending upon the values of their neighbours (Furness. 1997). Images can be cleaned or smoothed by removing stray pixels that are not part of the important image but would falsely alter image analysis (background noise). Images can be sharpened to make the areas of interest cleaner and easier to identify. To achieve this, techniques of erosion and dilatation are used.

If all black pixels adjacent to white pixels are deleted (eroded), stray pixels (noise) will be removed. The outer layer of pixels from larger objects will also be removed, to replace these, a dilate function is used. Pixels are added to black pixels with black neighbours. The size of the original object is then restored. An erode function which removes all black pixels except those with two black neighbours will skeletonize an object (Furness. 1997).

Once vessels have been defined as individual foreground objects on a separate background it is easy for an image analysis system to measure them in whatever way is required.

1.5.6 Use of computerised image analysis for MVD measurement in human cancers

There have been many attempts to use image analysis to calculate MVD and other aspects of vascularity in human tumours. These are summarised in
table 1.4. Not all papers detail how the image analysis was performed or what equipment was used. Examination of the available methodologies in these studies however, reveals the different solutions that have been applied to overcome the problems associated with image analysis.

The earliest paper describing the use of computerised image analysis in the measurement of MVD in a human cancer was that of Srivastava et al (Srivastava, Laidler, Davies, rgan, & Hughes. 1988). Vessels in malignant melanomas were identified using UEA-1 and analysed using an IBAS-2 semi automatic image analysis system by Kontron Ltd, West Germany. Unfortunately, no details of the image analysis system or technique used were given in the paper. Image analysis programs by Kontron have been used by other investigators although not necessarily in the same way. Smolle et al for example, used the Kontron Vidas software to assess vascularity in melanoma (Smolle, Soyer, Hofmann-Wellenhof, Smolle-Juettner, & Kerl. 1989). Vessels were identified with monoclonal antibody BMA 120. The authors listed the steps used in image analysis as;

- digitalisation of grey value TV image,
- shading correction, selection of area of interest,
- median filtering with a 3x3 matrix,
- normalization of grey value histogram,
- interactive linking of artificially separated structures,
- non linear low pass filtering,
<table>
<thead>
<tr>
<th>Paper</th>
<th>Tissue</th>
<th>Vessel stain</th>
<th>Software</th>
<th>Colour or Black &amp; White capture</th>
<th>Technique</th>
<th>Compared to manual</th>
<th>Reproducible</th>
</tr>
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</table>
Table 1.4 Summary of studies using Image Analysis for MVD measurement (Cont.)

<table>
<thead>
<tr>
<th>Study</th>
<th>Tissue</th>
<th>Antigen</th>
<th>Imaging System</th>
<th>Color Camera</th>
<th>Hot Spot, Random Grid</th>
<th>Manual Thresh &amp; Erasing</th>
<th>Automated Conversion &amp; Reconstruction</th>
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<tbody>
<tr>
<td>Study Description</td>
<td>Tissue</td>
<td>Staining Method</td>
<td>Image Analysis Method</td>
<td>Correlation</td>
<td>Interobserver</td>
<td>Outcome</td>
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<tr>
<td>Study</td>
<td>Tissue</td>
<td>Staining/Methodology</td>
<td>Outcome</td>
<td>Results</td>
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<tr>
<td>Study</td>
<td>Tissue</td>
<td>Antibody</td>
<td>Analysis</td>
<td>Correction</td>
<td>Interobserver, Cohen's kappa</td>
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<tr>
<td>Hannen-Egied et al. (2002)</td>
<td>Tongue</td>
<td>CD34</td>
<td>VidasPlus (Kontron GmbH, Eching, Germany)</td>
<td>RGB converted to HSI, Interactive correction of artifacts</td>
<td>Interobserver, Cohen's kappa = 0.78</td>
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<tr>
<td>Hannen et al. (2001)</td>
<td>Tongue</td>
<td>CD34</td>
<td>not given</td>
<td>Colour analysis</td>
<td>RGB converted to HSI, Interactive correction of artifacts</td>
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<tr>
<td>Hannen et al. (2001)</td>
<td>Tongue</td>
<td>CD34</td>
<td>VidasPlus (Kontron GmbH, Eching, Germany)</td>
<td>Colour analysis</td>
<td>Good correlation, p&lt;0.01</td>
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<tr>
<td>Herbst et al. (1998)</td>
<td>Renal</td>
<td>CD31</td>
<td>Quantimet 500 QWin (Leica, Germany)</td>
<td>Hotspots, Thresholded, Manual verification of vessels</td>
<td>ns</td>
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</tbody>
</table>

Note: CD34 Vidasplus (Kontron GmbH, Eching, Germany) Colour analysis RGB converted to HSI, Interactive correction of artifacts Interobserver, Cohen's kappa = 0.78
<table>
<thead>
<tr>
<th>Study</th>
<th>Tissue</th>
<th>Antibody</th>
<th>Imaging Method</th>
<th>Analytic Tool</th>
<th>Correlation Measure</th>
<th>Result</th>
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<tr>
<td>Nga Tran Kim, Nicolas Elie, Benoit Plancoulaine, Paulette Herlin, and Michel Coster. An original approach for quantification of blood vessels on the whole tumour section. Analytical cellular pathology 25 (2):63-75, 2003.</td>
<td>Ovarian</td>
<td>FVIII-RAg</td>
<td>Aphelion software v 3.0 (Adcis, France)</td>
<td>Colour analysis</td>
<td>Manually drawn areas of interest.</td>
<td>Qt=75% ns</td>
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### Table 1.4  Summary of studies using Image Analysis for MVD measurement (Cont.)

<table>
<thead>
<tr>
<th>Study Description</th>
<th>Tumour Type</th>
<th>CD34</th>
<th>CD31</th>
<th>Image Analysis System</th>
<th>Colour Capture</th>
<th>Photographic and scanned images</th>
<th>Manual tracing of vessels</th>
<th>Reliability Coefficient</th>
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<tr>
<td>Prognostic implications of microvessel morphology in diffuse astrocytic neoplasms.</td>
<td>Astrocytoma</td>
<td>CD34</td>
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<td>Image Scan (Jandel Scientific, Erkrath, Germany)</td>
<td>Colour capture</td>
<td>Photographed and scanned in.</td>
<td>Manual tracing of vessels</td>
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<td>Reliability coefficient 0.8% - 4.1%</td>
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<tr>
<td>Evaluation of tumour angiogenesis as a prognostic marker in malignant mesothelioma.</td>
<td>Mesothelioma</td>
<td>CD34 &amp; CD31</td>
<td></td>
<td>Vidas (Kontron GmbH, Eching, Germany)</td>
<td>Colour analysis</td>
<td>RGB converted to HSI. Interactive correction of artifacts</td>
<td>ns</td>
<td>n=0.736 ns</td>
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<tr>
<td>Quantification of angiogenesis by a computerized image analysis system in renal cell carcinoma.</td>
<td>Renal</td>
<td>CD34</td>
<td></td>
<td>IBAS (Kontron, Munich, Germany)</td>
<td>B&amp;W</td>
<td>Hotspots found with H&amp;E sections.</td>
<td>Manual threshold for each case</td>
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<tr>
<td>Study</td>
<td>Organ</td>
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<td>Method</td>
<td>Notes</td>
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<tr>
<td>Study</td>
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<td>Correlation</td>
<td>Significance</td>
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<td>Study</td>
<td>Tissue</td>
<td>Image Analysis System</td>
<td>Colour Capture Method</td>
<td>Manual Tracing/Computerized Pixel Counting</td>
<td>Reliability Coefficient</td>
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### Table 1.4  Summary of studies using Image Analysis for MVD measurement (Cont.)

<table>
<thead>
<tr>
<th>Study Description</th>
<th>Tissue</th>
<th>Antibody</th>
<th>Imaging System</th>
<th>Color/Filter</th>
<th>Analysis Method</th>
<th>Interobserver Variation</th>
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Table 1.4  Summary of studies using Image Analysis for MVD measurement (Cont.)

<table>
<thead>
<tr>
<th>Study</th>
<th>Site</th>
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<th>Software/Methodology</th>
<th>Correlation coefficient</th>
<th>Significance</th>
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Table 1.4  Summary of studies using Image Analysis for MVD measurement (Cont.)

<table>
<thead>
<tr>
<th>Study</th>
<th>Location</th>
<th>Stain</th>
<th>Camera</th>
<th>Brown stain conversion</th>
<th>Measurements</th>
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<tr>
<td>S. Wakui, M. Furusato, T. Itoh, H. Sasaki, A. Akiyama, I. Kinoshita,</td>
<td>Prostate</td>
<td>Vimentin</td>
<td>Olympus-Avio</td>
<td>Brown stain was</td>
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<tr>
<td>T. Tokuda, S. Aizawa, and S. Ushigome. Tumour angiogenesis in prostatic</td>
<td></td>
<td></td>
<td>SP500 (Olympus</td>
<td>converted to red and</td>
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<tr>
<td>carcinoma with and without bone marrow metastasis: a morphometric</td>
<td></td>
<td></td>
<td>Optical)</td>
<td>red area measured</td>
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<tr>
<td>C. Burger. Quantitative immunohistological analysis of the microvasculature in untreated human glioblastoma multiforme.</td>
<td></td>
<td>IV</td>
<td>GmbH, Eching,</td>
<td>erasing</td>
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<tr>
<td>Computer-assisted image analysis of whole-tumor sections. Journal of</td>
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<td>Germany)</td>
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<tr>
<td>Ruiter. Quantitative analysis of microvascular changes in diffuse astrocytic neoplasms with increasing grade of malignancy.</td>
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<td>IV</td>
<td>GmbH, Eching,</td>
<td>erasing</td>
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<tr>
<td>Automatic quantification of microvessel density in urinary bladder carcinoma.</td>
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<td>transfer, average</td>
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<td>histogram to</td>
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<td>threshold. Automatic</td>
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<td>vessel identification.</td>
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<td>Compared with manual</td>
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<td>thresholding</td>
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<td></td>
<td>Reproducible, r=0.76</td>
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<td>Study</td>
<td>Tissue</td>
<td>Antibody</td>
<td>Staining</td>
<td>Method</td>
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grey level-based segmentation,
automated filling of holes,
automated measurement of segmented objects.

This list summarises the usual steps required for MVD measurement using image analysis. That is, the conversion of the analogue to a digital one, determining countable vessels by removing background noise and cleaning images, thresholding images into 1-bit black and white files and finally image analysis assessment.

Techniques may vary at multiple points in this image analysis cascade. In particular, there is a spectrum of automation seen varying from a fully automated system to one requiring considerable human intervention.

In the study by Smolle et al, manual input was required to correct the image before analysis but the computer was then able to measure several variables with ease. In this instance, input was required to manually join separated vessel parts before allowing the computer to measure the vascular area therefore human subjectivity remains. The majority of systems have required some degree of manual input; this has been applied in a variety of ways.

Wesseling et al chose to use image analysis in an attempt to remove interobserver variation and account for vascular heterogeneity in glioblastomas (Wesseling, van der Laak, de Leeuw, Ruiter, & Burger. 1994; Wesseling, van
der Laak, Link, Teepen, & Ruiter. 1998). This method required several manual inputs both to set thresholds and to erase non vascular areas. The interobserver variation for the system was $r^2 = 0.9$, although it was not compared to manual counting.

Manual thresholding was also used by Lee et al in renal cell carcinoma (Lee, Jung, & Kim. 2000), breast carcinoma (Lee, Kim, Jung, Kim, Park, & Lee. 2001; Lee, Kim, Jung, Kim, Lee, & Park. 2002) and cervical neoplasia (Lee, Kim, Jung, Lee, & Chang. 2002; Lee, Kim, Jung, Lee, & Chang. 2002). The same group have also examined colonic carcinoma (Song, Lee, Kim, et al. 2002). Counting was performed using IBAS image analysis software in renal cell carcinoma and SIS GmbH software in breast and cervical tissue. An adjacent section of tumour stained with haematoxylin and eosin was used to manually select hotspots.

Strieth et al used a Leica Quantimet 600Win-Image Analysis System looking at squamous cell carcinoma of the skin (Strieth, Hartschuh, Pilz, & Fusenig. 2000; Strieth, Hartschuh, Pilz, & Fusenig. 2002). Although the vessels were automatically detected by the system, any contaminating dark area had to be removed manually. Arora et al also used this system in breast (Arora, Joshi, Nijhawan, Radotra, & Suresh. 2002).

Vacca et al used a mix of manual counting and computer manipulation to calculate the microvessel area in multiple myeloma (Vacca, Ribatti, Roccaro,
Frigeri, & Dammaco. 2001; Vacca, Ribatti, Roccaro, Ria, Palermo, & Dammaco. 2001). Vessels were counted manually with the aid of a 484 point reticule. The KS-300 Zeiss image analysis software was used to normalize this planimetric derived area with respect to non tumour tissue. The same group used this system to measure MVD in B cell chronic lymphocytic leukaemia (Molica, Vacca, Ribatti, et al. 2002).

Ridell et al used a Leica Q Win measurement program to measure vascularity in B cell non Hodgkins Lymphoma (Ridell & Norrby. 2001). Stained vessels on the colour images were marked on the screen and the number and area of vessels was recorded. Similarly, Kamijo et al used microvessel surface area to predict radiosensitivity in laryngeal carcinoma (Kamijo, Yokose, Hasebe, Yonou, Hayashi, & Ebihara. 2001). Vessels labelled with CD31 were outlined manually on the computer screen. Using Zeiss KS-300 software the image was converted to binary and the number, area and perimeter of vessels was calculated.

Wester et al from Uppsala in Sweden presented both semiautomatic and fully automatic image analysis systems for MVD measurement in bladder cancer (Wester, Ranefall, Bengtsson, Busch, & Malmstrom. 1999). They used mixed vessel staining with CD31 and CD34. 756x572 pixel colour images with 3x256 grey levels were captured. In the semi automatic system, vessels were labelled manually and the computer counted them and measured the endothelial area. In the automatic system, all that was required of the operator was to choose the
field for analysis. The computer then counted the vessels and measured the area of stained endothelial cells and also total vessel area (including the lumen).

The details of the image analysis system were better described by Ranefall et al in an earlier paper (Ranefall, Wester, Busch, & Malmstrom. 1998). A mixture of CD34 and CD31 was used to identify vessels with a Lightgreen counterstain. The specimens thus had very little colour which would allow the initial use of greyscale images; despite this the authors chose to capture in RGB colour. A principle component transfer was performed to convert these to greyscale. An average component histogram was used to threshold. All objects of less than 30 pixels were erased, to remove noise. The objects were then digitally cleaned. Gaps were filled and touching vessels were separated by cleaving thin peninsulars that joined objects. The lumen of hollow objects (vessels) were filled leaving solid vessels.

The semiautomatic system was reproducible for counting \( r=0.88 \) and measured area \( r=0.72 \). If exactly the same image was assessed on two separate occasions the reproducibility was \( r=0.97 \) and \( r=0.88 \). That this was not completely correlated presumably represents human error in this semi automated system.

The fully automated system was less reproducible for counting \( r=0.53 \) and area \( r=0.65 \). This improved when only high quality vessel staining was
measured to $r=0.73$ and $r=0.87$ respectively. The two methods were also closely correlated for counts ($r=0.72$) and areas ($r=0.75$).

All that was required of the observer in the automatic system was to select the area to be analysed. Selection of a hotspot allows for subjectivity but measurement in random areas removes this.

The same group evaluated microvessel density in thyroid carcinoma (Kavantzas, Tseleï, & Davaris. 2002) and superficial bladder cancer (as discussed in section 1.3.3) (Korkolopoulou, Konstantinidou, Kavantzas, et al. 2001). This time a video camera was used to capture the image and Image Scan software by Jandel Scientific was used to analyse the pictures.

Although several authors have used both manual counting methods and image analysis in the same study, few have statistically correlated the two. Kumar-Singh et al used a Vidas Kontron image analysis system (Kumar-Singh, Vermeulen, Weyler, et al. 1997). CD34 and CD31 were used to identify vessels in malignant mesothelioma, hot spots were selected and vessel density was measured manually (mIMD) and automatically (iIMD). The microvessel area was also calculated (MVA). No further details are given regarding the actual method of counting individual vessels using the image analysis system. In this method images were captured using a blue filter to enhance contrast and a five pixel filter removed noise. The image was then thresholded with vessels being seen as white on a black background. The correlation between mIMD and iIMD
was $r=0.736$, although the image analysis count (iIMD) overestimated compared to mIMD. iIMD however was more closely correlated with outcome.

Bigler et al counted twenty fields manually using two observers and a double headed microscope and compared this to the computer count for the same area. No significant difference was found but there was a slight positive bias in the image analysis system of $+2.8\%$ (SD 7.9) (Bigler, Deering, & Brawer. 1993). In the Brawer et al paper of 1994 correlation between computer and manual counting in 28 fields from eight tumours was $R^2=0.978$ ($p<0.001$) (Brawer, Deering, Brown, Preston, & Bigler. 1994). This method demonstrates both manual “retouching” and automatic “blurring” to correct the image for analysis.

Use of the OPTIMAS software in bone marrow biopsies was described by Rajkumar et al (Rajkumar, V, Fonseca, Ansell, Witzig, Gertz, & Greipp. 1998) in an abstract in 1998 and elaborated upon in a paper by Mesa et al in 2000 (Mesa, Hanson, Rajkumar, V, Schroeder, & Tefferi. 2000). They claimed a significant correlation between this method and manual counting but specific data is not given. Callagy et al used both a semiautomatic image analysis system and a manual counting method to assess vascularity in breast cancer (Callagy, Dimitriadis, Harmey, Bouchier, & Leader. 2000). The two methods showed no correlation when assessed by Kendall’s tau correlation ($0.13$) or a Bland Altman plot. Neither did MVD measured in this paper correlate with VEGF.
Mirecka et al used both manual hotspot counts and computer assisted image analysis (CAIA) in pancreatic ductal carcinoma (Mirecka, Libura, Libura, et al. 2001). Analysis was by the IPS Imal 512 software. Images were transformed into pseudocolour then binary using a threshold. Number of vessels per field, area of vessels and perimeter of vessels were measured. Manual and computer counts statistically correlated, $r=0.68$, $\leq0.05$.

Mooteri et al identified vessels with FVIIIRA in colorectal hepatic metastases and primary colonic tumours (Mooteri, Rubin, Leurgans, Jakate, Drab, & Saclarides. 1996). Using an image analysis system, they measured blood vessel to tumour surface area ratio, vessel wall thickness and the optical density of stained vessels. Manual vessel counts were also performed and an objective vascularity score was awarded in each section (1-4). No comparison was made between computer and manual estimations however, computer generated measurements were not found to be related to outcome in this study.

Goulding et al used CD34 to identify vessels in breast cancer when comparing manual and computerised methods (Goulding, Abdul, Robertson, et al. 1995). The SEESCAN (Kontron electronic GmbH, Eching, Germany) system was used for colour image analysis in an interactive image analysis system. Manual vessel counting was carried out in three fields in a hotspot. Once again the two methods were not correlated but neither was found to have a significant association with disease outcome.
Fox et al compared image analysis to manual hotspot counts, Chalkley counting and subjective appraisal of slides grading them into low, medium or high grades. Vessels were identified with CD31 in specimens of breast carcinoma (Fox, Leek, Weekes, Whitehouse, Gatter, & Harris. 1995). NIH Image 1.52 was used for analysis. Both manual outlining and automated pixel analysis was carried out.

There was a significant correlation between subjective vascular grade and computerised luminal area (p=0.008), luminal perimeter (p=0.03), microvessel number (p=0.04) but not stained area (p=0.195). There was also a significant correlation between Chalkley count and computerised luminal area (p=0.00005), luminal perimeter (p=0.00005), microvessel number (p=0.0007) and stained area (p=0.001). Correlation coefficient between manual MVD count and computer calculated microvessel number was r=0.57 (p=0.0009).

Kohlberger et al directly compared manual MVD counting using the hotspot method with computerised image analysis (Kohlberger, Obermair, Sliutz, et al. 1996). FVIIIRA was used to mark vessels. A Quantimet 600 image analysis program was used and colour images captured as RGB were converted to HSI images. Vessel outlines were traced manually on the images using the computer mouse. The images were then converted to binary and analysed. The microvessel area measured by the computer correlated poorly with manual MVD, r=0.24 (CI -0.02 – 0.46)
Hannen et al used several techniques to improve the accuracy of the MVD count in squamous carcinoma of the tongue (Hannen, van-der-Laak, Manni, et al. 2001). They also applied an improved immunohistochemical protocol based on the catalysed reporter deposition (CARD) technique to enhance staining (Hannen-Egied, van-der-Laak-Jeroen, Manni, et al. 2002). This uses a signal amplification system based on horseradish peroxidase catalysed reporter deposition of biotinylated tyramine at sites of immunoreactivity.

Using the value of pixels, those of less than 0.2 were negative and those greater than 0.7 were positive; eight adjacent pixels were connected as one object. These were dilated twice, lumen were filled in and then objects were eroded twice to bring them back to their original size. Objects smaller than 50 pixels were erased. The resulting objects could be projected onto the original colour picture so they could be corrected (for example non tumour areas removed) before analysis. The improved staining methods were used to give maximum contrast between foreground and background. This makes computer differentiation easier. Again, background noise (objects less than 50 pixels) was removed, erode and dilate functions defined vessels and manual corrections were made using a companion image. Reproducibility of this interactive correction was good, r>0.75, p<0.01 but manual input was still required and two separate stained sections were required for each case.

In 1992, Brawer et al used an image analysis system to examine vascularity in benign and malignant prostate tissue (Brawer, Bigler, & Deering.
1992). They identified vessels with FVIIIRA enhanced with nickel chloride and applied a “light” counterstain of methyl green. As with Hannen et al this was an attempt to give a good contrast between foreground and background. The image analysis system was more comprehensively described by Bigler et al in a later paper from the same group (Bigler, Deering, & Brawer. 1993).

Capture of images can be in colour or monochrome. Often colour images are converted to greyscale and then thresholded to black and white. Occasionally, colour image analysis is performed with thresholding based on a particular colour pixel value.

A computer assisted morphometrical approach was used by Wakui et al to calculate the blood capillary density ratio (BCDR) in prostate carcinoma (Wakui, Furusato, Itoh, et al. 1992). They used an Olympus-Avio SP500 image analysis system to capture coloured images. The brown staining of vimentin was digitally converted to red. Staining of non capillary areas was removed but details of this technique are not given. The same system was used to relate prostatic volume to vascularity (Furusato, Wakui, Sasaki, Ito, & Ushigome. 1994).

Wong et al used a Microscale TM/TS image analysis software package to calculate vascularity in thyroid tumours (Wong, Willott, Kendall, & Sheffield. 1999). They used colour images and thresholded between the brown staining of the endothelium and the blue/purple background.
Both RGB colour images and HSI images have been used for image analysis. In the paper by Hannen et al although captured images were 24-bit RGB colour, they were converted to HSI for analysis which has been said to be superior for image analysis (van der Laak. 2000). Kohlberger et al found that using RGB images made it difficult to separate red stained vessels from cell nuclei and therefore used HSI images (Kohlberger, Obermair, Sliutz, et al. 1996).

Colour can also be used to influence the contrast of captured images even though they are subsequently analysed in greyscale. Canete et al used an Olympus CUE-2 image analysis system in neuroblastomas (Canete, Navarro, Bermudez, Pellin, Castel, & Llombart. 2000). To increase the contrast between the brown stained vessels and the green staining nuclei a 436nm filter was applied to the microscope. The images were captured with a black and white camera and then converted to greyscale.

Most methods use greyscale images for analysis. In several studies, a group from the Mayo clinic have used an optimised microvessel density (OMVD) system (Biostage, Bard) (Bostwick, Wheeler, Blute, et al. 1996; Gettman, Bergstralh, Blute, Zincke, & Bostwick. 1998; Gettman, Pacelli, Slezak, et al. 1999).
Images were captured and area and perimeter measurements made using raw pixel data at $6.13 \times 10^{-7}$ mm$^2$/pixel. An automated algorithm reduced the 256-grey-level image to binary and then isolated the individual microvessels.

Schoell et al devised a computer aided image analysis system (CIAS) to measure MVD in ovarian cancer (Schoell, Pieber, Reich, et al. 1997). Images were captured in greyscale and a low pass filtered image was then subtracted from the captured image. The image was then thresholded into a binary image. They expressed vessels as greyscale value 255 (white) on a greyscale value zero (black) background. All pixels with a value of 255, that is white represented a vessel and were counted.

The majority of systems use video cameras to capture images, these are then converted to digital images using a frame grabber. Pavlopoulos et al however used a different method of image capture. They digitally scanned photomicrographs of hotspots in colorectal carcinoma (Pavlopoulos, Konstantinidou, Agapitos, Kavantzas, Nikolopoulou, & Davaris. 1998). They used this method in astrocytoma, chronic myeloid leukaemia and in myelodysplasia (Korkolopoulou, Apostolidou, Pavlopoulos, et al. 2001; Korkolopoulou, Patsouris, Kavantzas, et al. 2002; Korkolopoulou, Viniou, Kavantzas, et al. 2003).
1.5.7 Image Analysis Summary

Digital image analysis has been used in MVD counting in order to speed the process and to make it more objective. Several techniques have been used to make the systems as accurate as possible. Improved vessel staining methods have been used to make the contrast between dark vessels and light background as marked as possible. The staining has been darkened using enhancers such as nickel (Bigler, Deering, & Brawer. 1993; Brawer, Bigler, & Deering. 1992) or the CARD method (Hannen, van-der-Laak, Kerstens, Cuijpers, Manni, & de-Wilde. 2001). The background counterstain has been lightened or simply omitted (Simpson, Ahn, Battifora, & Esteban. 1996).

A varying amount of manual input has been used in the systems ranging from choosing areas to count in to manually drawing around vessels to be measured. Conversion of the images to binary by thresholding has been done manually for individual images or each case. Alternatively, thresholding has been set and performed automatically (Wester, Ranefall, Bengtsson, Busch, & Malmstrom. 1999). Thresholding has been set using greyscale images or colour images (Goulding, Abdul, Robertson, et al. 1995; Hannen, van-der-Laak, Kerstens, Cuijpers, Manni, & de-Wilde. 2001; Kohlberger, Obermair, Sliutz, et al. 1996; Wong, Willott, Kendall, & Sheffield. 1999).

Correction or cleaning of images is done using filters for small particle noise (Hannen, van-der-Laak, Kerstens, Cuijpers, Manni, & de-Wilde. 2001) or manually to remove large non-vessel objects (Strieth, Hartschuh, Pilz, &
Fusenig. 2000; Wesseling, van der Laak, de Leeuw, Ruiter, & Burger. 1994). Image manipulation was also performed to separate or join vessels as appropriate using simple blur filters (Brawer, Deering, Brown, Preston, & Bigler. 1994) or more complex erode and dilate procedures (Wester, Ranefall, Bengtsson, Busch, & Malmstrom. 1999).

Some image analysis systems have attempted to emulate manual counting to produce a number of vessels per unit area, others have examined other, related, vascular parameters such as vessel area. There have been some attempts to correlate computerised and manual MVD counting some showing a good correlation (Fox, Leek, Weekes, Whitehouse, Gatter, & Harris. 1995; Mirecka, Libura, Libura, et al. 2001; Wester, Ranefall, Bengtsson, Busch, & Malmstrom. 1999) and others not (Callagy, Dimitriadis, Harmey, Bouchier, & Leader. 2000; Goulding, Abdul, Robertson, et al. 1995; Kohlberger, Obermair, Sliutz, et al. 1996).

Image analysis systems could be of great use in angiogenesis assessment. Repetitive counts performed in multiple areas of multiple cases demand automation and computer analysis would remove the subjectivity that has been blamed for the failure of some MVD counting studies. However, in order for an image analysis system to be clinically useful it must be reproducible and robust.
1.6.0 Hypothesis & Summary

1.6.1 Hypothesis

Microvessel density can be measured in paraffin embedded specimens of papillary superficial transitional cell carcinoma of the human urinary bladder in a meaningful and reproducible way.

1.6.2 Aims & Objectives

To determine whether MVD could be measured in papillary SBC. Also, to determine whether angiogenesis, as measured by MVD, at presentation is related to subsequent recurrence or progression of superficial bladder cancer and its relationship to known angiogenic cytokines. These cytokines were Vascular Endothelial Growth Factor (VEGF), Thymidine Phosphorylase (TP), Thrombospondin (TSP) and also p53.

Secondary objectives were to create a retrospective database of SBC cases and use it to examine the clinicopathological risk factors associated with SBC individually and in association with MVD. Clinicopathological risk factors recorded and examined were, age at presentation, gender, occupation, regular use of aspirin or NSAIDS, smoking status, multiplicity of tumours, stage, grade, presence of cis, date of first check cystoscopy, time to first recurrence, time to progression, grade and stage of recurrences, administration of intravesical chemotherapy, administration of intravesical BCG therapy, date and cause of death, length of follow up.
1.6.3 Results Summary

The database included 293 cases of primary superficial bladder cancer. The computer image analysis system was able to measure MVD in superficial bladder cancer and correlated strongly with manual counting ($r=0.95$).

In a multivariable analysis, MVD ($p<0.0001$), TSP (Ab-7) tumour staining ($p=0.001$), decreased TSP (Ab-4) perivascular staining ($p=0.007$), tumour stage ($p<0.0001$), multiplicity ($p=0.01$), age ($p=0.003$) and aspirin consumption ($p=0.004$), were all independent predictive factors for superficial bladder cancer progression.

MVD in this study showed a positive correlation with p53 and the pro-angiogenic factor TP. Low perivascular TSP staining correlated with increased MVD.

In conclusion, this study has shown that MVD can be measured in superficial bladder cancer. A method has been described that is accurate, reproducible and time efficient. This study has also shown, for the first time, that increasing MVD at presentation is a significant risk factor for subsequent progression to muscle invasive disease.
Chapter 2

Materials and Methods
2.0.0 Materials and Methods

2.1.0 Identification of subjects

The computerised pathology database for The University Hospitals of Leicester NHS Trust was searched using the parameters; Transitional Cell Carcinoma and the years 1994 and 1995. The resulting list was used to retrieve the histology reports for the patients identified on the archived microfiche database. These reports were individually examined to select patients with superficial bladder cancer diagnosed after transurethral resection. The subsequent list of patients was then cross referenced with the computerised database to determine if this was their first presentation. All cases with previous histopathological records of TCC were excluded.

Within the group delineated there were insufficient cases that subsequently progressed to muscle invasion so a second group were obtained. All cases of Invasive TCC were identified from the total pathology computerised database (1981 to 2000). The corresponding histology reports were individually examined to determine which patients had initially presented with superficial disease and subsequently progressed to invasive bladder cancer.

2.2.0 Collection of Clinical Data

The hospital notes for all patients in the study sample were acquired. The notes were examined and clinico-pathological data was recorded, table 2.1. Pathology reports were not reviewed in this study. The notes were also examined
Table 2.1  Clinico-pathological data retrieved from hospital notes

<table>
<thead>
<tr>
<th>Patient Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surname</td>
</tr>
<tr>
<td>Forename</td>
</tr>
<tr>
<td>Hospital Identification Number</td>
</tr>
<tr>
<td>Date of birth</td>
</tr>
<tr>
<td>Age at presentation</td>
</tr>
<tr>
<td>Gender</td>
</tr>
<tr>
<td>Occupation</td>
</tr>
<tr>
<td>Drugs</td>
</tr>
<tr>
<td>Smoking status</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tumour data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date of first resection</td>
</tr>
<tr>
<td>Size of largest tumour</td>
</tr>
<tr>
<td>Number of tumours</td>
</tr>
<tr>
<td>Stage</td>
</tr>
<tr>
<td>Grade</td>
</tr>
<tr>
<td>Presence of cis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Post operative data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date of first check cystoscopy</td>
</tr>
<tr>
<td>Time to first recurrence</td>
</tr>
<tr>
<td>Time to progression</td>
</tr>
<tr>
<td>Grade and stage of recurrences</td>
</tr>
<tr>
<td>Intravesical Chemotherapy</td>
</tr>
<tr>
<td>Intravesical BCG therapy</td>
</tr>
<tr>
<td>Date and cause of death</td>
</tr>
<tr>
<td>Length of follow up</td>
</tr>
</tbody>
</table>


for evidence that this was the first presentation of superficial TCC. Where this was not so, these patients were excluded.

With this information, a list was prepared of the histology department identification numbers for the blocks containing transurethral resection of bladder tumour (TURBT) specimens from these patients who first presented with superficial TCC in the years 1994 and 1995 and for the initial specimens of TCC for those patients who initially presented with superficial disease and subsequently progressed to muscle invasion.

2.3.0 Preparation of tissue for analysis

Formalin fixed paraffin embedded blocks of the chosen case specimens were retrieved from the pathology stores of the pathology departments of the Leicester General Hospital, The Leicester Royal Infirmary and The Leicester Glenfield General Hospital. These blocks had been previously fixed, by these departments, according to standard protocol. Briefly, TURBT specimens were placed in formalin/saline immediately after resection and fixed for 24 to 72 hours. Specimens were then embedded in wax blocks. After sectioning for clinical purposes the remaining blocks were stored in darkness at room temperature.

For this study, 5μm sections were cut with a microtome and floated onto silane coated glass slides. Slides were then incubated at 56°C overnight before use. Due to the small amount of available tissue in many cases in this study, all
microtomy was performed by the staff of the Specials laboratory, Leicester Royal Infirmary.

2.4.0 Primary antibodies

2.4.1 Primary antibody against Cluster Determinant 34 (CD34)

The anti CD34 purified mouse monoclonal antibody was purchased from Dako (Ely, England). The clone QB-END/10 (IgG1κ) was used, it is a class II monoclonal antibody recognising the CD34 antigen expressed on immature haematopoietic stem/progenitor cells, small vessel endothelial cells, embryonic fibroblasts and some cells in foetal and adult nervous tissue (Nishio, Tada, Hashiyama, Hirn, Ingles-Esteve, & Suda. 1997).

2.4.2 Primary antibody against Cluster Determinant 31 (CD31)

The primary antibody against CD31 was purchased from Dako (Ely, England). Clone JC/70A (IgG1κ) was used which binds to CD31 antigen expressed on all contiguous endothelium (Muller. 1997).

2.4.3 Primary antibodies against Vascular Endothelial Growth Factor

The primary antibody against VEGF used was VG-1; this was a kind gift from Professor Adrian Harris, Imperial Cancer Research Fund, Institute of Molecular Medicine (Oxford, England). VG-1 has been shown to detect the 121, 165 and 189 VEGF isoforms in routinely fixed specimens (Turley, Scott, Watts, Bicknell, Harris, & Gatter. 1998).
2.4.4 Primary antibody against Thymidine Phosphorylase (TP)

Primary antibody against Thymidine Phosphorylase (TP) was a kind gift from Professor Adrian Harris, Imperial Cancer Research Fund, Institute of Molecular Medicine (Oxford, England). The TP clone P-GF.44C is a monoclonal antibody raised against recombinent TP. It stains routinely processed tissue effectively (Fox, Moghaddam, Westwood, et al. 1995).

2.4.5 Primary antibodies against Thrombospondin –1 (TSP-1)

Primary antibody against thrombospondin-1 Ab-1, clone 46.4 was purchased from Oncogene Research Products (Cambridge, England). Clone 46.4 is a monoclonal purified (IgG1κ) anti-mouse antibody. It reacts with a tertiary structure dependent epitope.

Primary antibody against thrombospondin-1 Ab-4, clone A6.1 was purchased from Neomarkers (Fremont, CA., USA). The clone A6.1 was a monoclonal (IgG1κ) anti-mouse antibody. Its epitope localises to the collagen Type-V binding domain of TSP-1 (Dixit, Galvin, O'Rourke, & Frazier. 1986), figure 1.4.

Primary antibody against thrombospondin-1 Ab-7, clone HB8432 was purchased from Neomarkers (Fremont, CA., USA). The clone HB8432 is a monoclonal (IgG2a) anti-mouse antibody. Its epitope localises to the EGF like repeats (type II) in the stalk region of TSP-1 (Jaffe, Ruggiero, Leung, Doyle, McKeown-Longo, & Mosher. 1983) figure 1.4.
2.4.6 Primary antibody against p53

The primary antibody against p53 was purchased from Novacastra Laboratories Ltd. (Newcastle, England). The clone DO7 recognises a denaturation resistant epitope between amino acids 1 and 45 in wild type and mutant p53 protein (Vojtesek, Bartek, Midgley, & Lane. 1992).

2.5.0 Developing kits

The StreptABComplex/HRP Duet, Mouse/Rabbit (ABC) kit was manufactured by Dako (Ely, England). It amplifies the primary antibody reaction by the sequential application of biotinylated antibodies to mouse or rabbit immunoglobulins followed by a complex of streptavidin and biotinylated horseradish peroxidase.

- Reagent A contains 1ml of streptavidin in 0.01 mol/l PBS, 15mmol/l NaN₃, pH 7.2.
- Reagent B contains 1ml of biotinylated horseradish peroxidase in 0.01 mol/l PBS, 15mmol/l NaN₃, pH 7.2.
- Reagent C contains 1ml biotinylated, affinity-isolated goat antibody to mouse/rabbit immunoglobulins in 0.01mol/l PBS, 15mmol/l NaN₃, pH 7.2.

The EnVision+ kit was also manufactured by Dako (Ely, England). It is a two step immunohistochemical technique based on a horseradish peroxidase labelled polymer conjugated with secondary antibodies. The kit contains:
1. Peroxidase blocking solution of 0.03% hydrogen peroxide and sodium azide.

2. Peroxidase labelled polymer conjugated to goat anti mouse immunoglobulins in Tris-HCl buffer.

3. DAB which is mixed with a buffered substrate of pH 7.5 with hydrogen peroxide and a preservative.

**2.6.0 Buffers and solutions**

Buffers and stock solutions were made from analytical grade chemicals and stored in appropriate conditions until use.

**2.6.1 Phosphate buffered saline (PBS)**

1. 600ml of distilled water was added to a one litre beaker with a magnetic flea and placed on the magnetic stirrer.

2. Each of the following chemicals were weighed out in turn and added to the water. Each chemical was fully dissolved before the next was added.

   2.0g Potassium Dihydrogen Orthophosphate (BDH Ltd. Poole, England)

   13.7g Disodium Hydrogen Orthophosphate (BDH Ltd. Poole, England)

   80.0g Sodium chloride (BDH Ltd. Poole, England)

3. The pH of the solution was measured and titrated to pH7.6 as required.
4. The solution was transferred into a 10 litre container and made up to 10 litres with distilled water.

5. PBS is stored at room temperature and has a shelf life 6 months.

2.6.2 Modified Tris buffered saline (TBS)

1. 500ml of distilled water was added to a one litre beaker with a magnetic flea and placed on the magnetic stirrer.

2. Each of the following chemicals were weighed out in turn and added to the water. Each chemical was fully dissolved before the next was added.

   71.0g Trisma base (50mM Hydroxymethyl Aminomethane) (ICN Biochemicals Inc., Ohio, USA)
   85.0g Sodium chloride (BDH Ltd. Poole, England)

3. The pH of the solution was measured and titrated to pH7.6 using concentrated hydrochloric acid in the fume cupboard.

4. The solution was returned to the magnetic stirrer and each of the following chemicals were weighed out in turn and added to the water. Each chemical was fully dissolved before the next was added.

   4.0g Magnesium Chloride (BDH Ltd. Poole, England)
   10g Bovine serum albumin (BDH Ltd. Poole, England)
6. The solution was transferred into a 10 litre container half filled with distilled water and then made up to 10 litres with more distilled water.

5. TBS is stored at room temperature and has a shelf life of 6 months.

2.6.3 1M Acetate Buffer pH 6.0

1. 500ml of distilled water was added to a one litre beaker with a magnetic flea and placed on the magnetic stirrer.

2. 68.04g of Sodium Acetate-3- hydrate (BDH Ltd. Poole, England) was weighed out and added to the water and fully dissolved.

3. The pH of the solution was measured and titrated to pH 6.0 using glacial acetic acid as required.

5. 1M acetate buffer is stored at room temperature and has a shelf life of 6 months.

2.6.4 10mM Citrate buffer pH 6.0

1. 500ml of distilled water was added to a one litre beaker with a magnetic flea and placed on the magnetic stirrer.

2. 10.5g of citric acid crystals (BDH Ltd. Poole, England) were added to the solution and stirred until dissolved.

3. Still stirring, the solution was titrated to pH 6.0 with 2M sodium hydroxide.

4. The solution was transferred to a 5 litre volumetric flask and made up to 5 litres with distilled water.
5. Citrate buffer is stored at room temperature and has a shelf life of 6 months.

2.6.5 TRIS / EDTA Buffer pH 9.0

1. 2 litres of distilled water was added to a 3 litre conical flask with a magnetic flea and placed on the magnetic stirrer.

2. Each of the following chemicals were weighed out in turn and added to the water. Each chemical was fully dissolved before the next was added.

12.12g Trisma base (50mM Hydroxymethyl Aminomethane) (ICN Biochemicals Inc., Ohio, USA)

1.48g EDTA (Ethylenediaminetetra-acetic acid) (BDH Ltd. Poole, England)

3. Still stirring, the solution was titrated to pH 9.0 with concentrated hydrochloric acid.

5. TRIS / EDTA buffer is stored at room temperature and has a shelf life of 6 months.

2.6.6 Copper sulphate solution

1. 700ml of distilled water was added to a one litre beaker with a magnetic flea and placed on the magnetic stirrer.

2. Each of the following chemicals were weighed out in turn and added to the water. Each chemical was fully dissolved before the next was added.
4.0g Copper sulphate (BDH Ltd. Poole, England)  
7.2g Sodium chloride (BDH Ltd. Poole, England)

4. The solution was transferred into a 1 litre volumetric flask and made up to 1 litre with distilled water.
5. Copper sulphate solution is stored at room temperature in a Durnan bottle and has a shelf life of 6 months.

2.6.7 TRIS / HCl buffer pH 1.0
1. 500ml of 0.1M TRIS solution was added to a one litre conical flask with a magnetic flea and placed on the magnetic stirrer.
2. 150ml of 1M HCl was slowly added to the solution in a fume cupboard.
3. The solution was titrated to pH 1.0 using 1M HCl.

2.7.0 Immunohistochemical staining

Two techniques were used for immunohistochemical staining. Identification of vessels with CD34 was carried out using the StreptABComplex/HRP Duet, Mouse/Rabbit (ABC) kit (Dako Ely, England) and staining of VEGF, TP, TSP-1 and p53 was carried out using the EnVision+ kit (Dako Ely, England).

2.7.1 The DAKO ABC technique.

CD34 negative controls omitted the primary antibody. Normal human colon tissue was used as positive controls.
2.7.1.1 Dewaxing of slides

Slides were placed in metal slide racks, each holding 25 slides; all slides were orientated in the same way. Slides were warmed in an oven at 60°C for 5 minutes to ensure good bonding of sections to the silane slides.

The slides were then taken to water (rehydrated) through a series of graded alcohols. Slide trays were placed in 300ml dishes of xylene, three times, 99% alcohol, 99% alcohol again and 95% alcohol for 5 minutes in each. The trays were drained on blotting paper between each dish of alcohol to minimise contamination. The slides were then washed in running tap water for 2 minutes.

2.7.1.2 Blocking of endogenous peroxidase

Slides were placed in a solution of 6% hydrogen peroxidase to block endogenous peroxidase activity and agitated with a magnetic stirrer for 10 minutes. The slides were then washed in running tap water for 2 minutes.

2.7.1.3 Blocking of non-specific staining with normal serum

Slides were then equilibrated in TBS, section 2.6.5, with agitation on a magnetic stirrer for 2 minutes. Individual slides were then removed from the metal slide tray and excess buffer was wiped off with a folded tissue. Great care was taken to skirt the section leaving it in a square of buffer solution. Slides were then placed in a humidity chamber. Care was taken not to allow the slides to touch and this was carried out with some rapidity to prevent the sections drying out.
100μl of normal goat serum (Dako, Ely, England) at a dilution of 1:20 made up in TBS was applied to each slide. Care was taken to ensure the sections were flooded with serum. The slides were incubated in the humidity chamber for 10 minutes.

2.7.1.4 Application of primary antibody

Slides were removed individually from the humidity chamber and drained by placing the edge of the slide on blotting paper. They were then returned to the chamber. Primary CD34 antibody was applied to each section at a dilution of 1:50 in TBS. The dilution of CD34 primary antibody had been previously optimised and been found to be successful by the staff of the Specials Laboratory, Leicester Royal Infirmary. The amount of CD34 primary antibody solution applied to the slide was 120μl. Initially 100μl was used but this did not flood some of the larger specimens. The slides in their humidity chambers were incubated overnight in darkness in a cold room at 4°C.

2.7.1.5 Application of the secondary antibody

The slides were removed from the humidity chambers and replaced in the metal slide racks, again ensuring all slides were similarly orientated. The slides were then washed in PBS, section 2.6.1, with agitation in a magnetic stirrer for 20 minutes.

Individual slides were then removed from the metal slide trays, excess buffer was wiped off and they were placed in a humidity chamber. 120μl of
secondary antibody solution was applied to each slide; the sections were flooded. The secondary antibody was biotinylated goat anti-mouse at a dilution of 1:200 TBS. This was solution C in the Dako ABC kit. The slides were incubated at room temperature in the humidity chamber for 30 minutes.

### 2.7.1.6 Application of the ABC tertiary solution

The slides were removed from the humidity chambers and replaced in the metal slide racks. The slides were then washed in PBS with agitation in a magnetic stirrer for 20 minutes. Individual slides were then removed from the metal slide trays, excess buffer was wiped off and they were placed in a humidity chamber. 120μl of the DAKO A&B tertiary reagent from the DAKO ABC kit was applied to each slide ensuring the section was flooded.

### 2.7.1.7 Preparation and application of the DAB solution

The chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB) was used to develop the CD34 staining and was enhanced with nickel sulphate (NiSO₄) in 1M acetate buffer.

In a pilot experiment CD34 staining was carried out to determine the most suitable enhancement for the vessel staining. Sections of superficial bladder cancer not included in the final data analysis were mounted on silane slides and immunohistochemistry was performed with CD34 as described above. CD34 vessel staining was enhanced with:

1. CD34 + DAB alone
2. CD34 + DAB + Copper sulphate, section 2.6.6.

3. CD34 + DAB + Nickel sulphate (infra vida)

It was clear that CD34 with DAB enhanced with either nickel sulphate or copper sulphate was superior to no enhancement and that nickel sulphate enhancement was superior to copper sulphate enhancement. Counterstaining was required to navigate around the section. The choice of counterstain is discussed below.

The slides were removed from the humidity chambers and replaced in the metal slide racks. The slides were then washed in PBS with agitation in a magnetic stirrer for 20 minutes. Individual slides were then removed from the metal slide trays, excess buffer wiped off, then placed in a humidity chamber. Slides were then flooded with a 1M acetate buffer solution at pH6, section 2.6.3. The slides were then drained before application of the chromagen.

Initially, 0.5g of NiSO₄ was dissolved in 50ml of 1M acetate buffer a frozen aliquot of DAB (5mg of DAB in 0.5ml of PBS) was thawed and added to the solution. This was then filtered into a Hellendahl jar and 150μl of 3% hydrogen peroxide was added. Slides were stood in the Hellendahl jar for 10 minutes.

As the Hellendahl jar only allowed exposure of five slides at a time, this method was changed. The identical solution was prepared but slides were
drained and suspended on glass rods over a sink designated for use with DAB. The DAB – NiSO₄ solution was poured through a filter and allowed to drop onto each of the slides. Tissue sections were flooded. The slides were left exposed to the chromagen for 10 minutes.

After exposure to DAB and NiSO₄, the slides were thoroughly washed with distilled water applied with a washing bottle over a sink designated for use with DAB. A slow jet of water was directed to the end of each slide, taking care to avoid damaging the tissue.

2.7.1.8 Counterstaining

A counterstain was required to allow navigation around the section. However, as vessel counting was to be performed by computer image analysis, background staining was required to be as light as possible to give the most marked contrast between the foreground stained vessels and the light background. The type of counterstain used and the exposure time of the section to the stain both determine the final contrast.

A pilot experiment was performed to determine the most suitable counterstain for this study. Sections of superficial bladder cancer not included in the final data analysis (but all from the same case) were mounted on silane slides and immunohistochemistry was performed with CD34 as described above. Counterstains used were Mayer’s haematoxylin, methyl green, light green, methylene blue and neutral red.
Of these, methyl green and haematoxylin were found to be the most suitable. These two stains were further analysed with respect to exposure time to the counterstain. Methyl green was subsequently found to give a blotchy unreliable counterstain and so haematoxylin was chosen over it. Slides were exposed to haematoxylin for 10 seconds, 5 seconds and 1 second. A one second exposure to Mayer's haematoxylin was found to give sufficient background staining for navigation and allow computer image analysis.

In summary, slides were removed from the glass rods over the DAB sink and replaced in the metal slide racks, again ensuring all slides were similarly orientated. Trays were immersed in Mayer's haematoxylin for 1 second and then instantly plunged into running tap water until all blue staining had cleared. Counterstaining was performed in a fume cupboard.

2.7.1.9 **Dehydration and application of the cover-slips**

The slides were then dehydrated through a series of graded alcohols. Slide trays were placed in 300ml dishes of 95% alcohol, 99% alcohol, 99% alcohol again and finally xylene, three times, for 5 minutes in each. The trays were drained on blotting paper between each dish of alcohol to minimise contamination.

Coverslips were applied using an automated coverslipping machine (Baytec). Automated coverslipping led to less air bubble contamination than coverslipping by hand and allowed a more even application of adhesive, giving
less variation in a series of slides. These points decreased contamination errors due to coverslapping marks during computer image analysis. It was also less time consuming.

2.7.1.10 Evaluation of CD31

The StreptABComplex/HRP Duet, Mouse/Rabbit (ABC) kit (Dako Ely, England) kit was also used to evaluate CD31 vessel staining. Sections of superficial bladder cancer not included in the final data analysis (but all from the same case) were used, negative controls omitted the primary antibody and normal human colon and human haemangioma were used as positive controls.

Staining was carried out as above, section 2.7.1, except, after the dewaxing of the slides the sections to be stained with CD31 were pretreated with trypsin predigestion for antigen retrieval, section 2.7.3.5. Primary CD31 antibody was applied at concentrations of 1:10 and 1:20. It was compared to CD34 at concentrations of 1:50, 1:20 and 1:10. Slides were also stained with a mixture of CD31 (1:20) and CD34 (1:20).

2.7.2 The DAKO EnVision technique

The DAKO EnVision developing kit was used with the primary antibodies against VEGF, TP, TSP-1 and p53. The basic methodology of the kit will be described and the variations for each antibody technique will then be individually appended.
2.7.2.1 Preparation of reagents for the DAKO EnVision+ developing technique.

The EnVision+ DAB+ substrate-chromogen solution is prepared. To each 1 ml of EnVision buffered substrate, 1 drop of EnVision DAB+ chromogen is added. The solution is mixed on a bench agitator and left at room temperature until required.

2.7.2.2 Preparation of slides using the DAKO EnVision+ developing technique.

Paraffin sections (4μm) were mounted on Silane slides and were dewaxed in a warming oven and taken to water through a series of graded alcohols as described in section 2.7.1.1. After the slides have been washed in running tap water for 5 minutes antigen retrieval may be performed if required. This is further discussed below.

2.7.2.3 Blocking of endogenous peroxidase using the DAKO EnVision+ developing technique.

The rack of slides is then washed in TBS with agitation on a magnetic stirring bench for 5 minutes. Excess buffer surrounding the tissue is carefully wiped off each slide and the slides placed in a humidity chamber, section 2.7.1.3.

EnVision blocking solution is then added to each slide. Sufficient liquid is used to cover the whole tissue area (usually two drops). The slides are then incubated at room temperature for 10 minutes.
2.7.2.4 Application of primary antibody using the DAKO EnVision+ developing technique.

Slides are re-racked and washed in TBS with agitation for 5 minutes. Excess buffer surrounding the tissue is carefully wiped off each slide and the slides placed in a humidity chamber, section 2.7.1.3.

Sufficient primary antibody should be used to cover the whole tissue area. In this study, 120μl was used. The slides were then incubated at room temperature in the humidity chamber for 30 min.

2.7.2.5 Application of secondary complex using the DAKO EnVision+ developing technique.

Slides were re-racked and washed in TBS with agitation for 5 minutes. Excess buffer surrounding the tissue is carefully wiped off each slide and the slides placed in a humidity chamber, section 2.7.1.3. Two drops (or sufficient liquid to cover the whole tissue area) of EnVision secondary complex were applied to each slide. The slides were then incubated at room temperature for 30 min.

2.7.2.6 Application of the DAKO EnVision+ DAB solution.

Slides were re-racked and washed in TBS with agitation for 5 minutes. Excess buffer is drained from the slides by pressing one edge into blotting paper and the slides are placed on glass staining racks over a sink, section 2.7.1.7. Care was taken not to allow the slides to dry out.
Sufficient EnVision+ DAB Solution, section 2.7.2.1 was applied to each slide flooding the whole tissue. The slides were incubated at room temperature for 10 minutes before being washed thoroughly with distilled water over the designated DAB sink, section 2.7.1.7.

2.7.2.7 Application of counterstain.

Slides were returned to their racks and immersed in a dish of 300ml of Mayer's haematoxylin for 5 seconds and then immediately washed in running tap water for 3 minutes.

2.7.2.8 Dehydration and application of the cover-slips using the DAKO EnVision+ developing technique.

Slides were dehydrated through a series of graded alcohols and coverslips applied as described in section 2.7.1.9.

2.7.3 Antigen retrieval techniques

2.7.3.1 Microwave method

After dewaxing and dehydrating slides as in section 2.7.2.1, slides were transferred into a plastic rack; care was taken to orientate the slides in the same way. The racks were placed in a container of 300ml of buffer. Buffers used were TRIS / EDTA pH 9.0 buffer, section 2.6.5 or 10mM Citrate buffer, pH 6.0, section 2.6.4, or TRIS /HCl pH 1.0 buffer, section 2.6.7. The container used was a double tray allowing two slide racks, each in a separate 300ml area, to be processed at the same time. The slides were microwaved at full power (750W)
for 15 minutes. The container was then suspended in a dish of running tap water until quite cool (this usually took 20 minutes). Water did not come into contact with the slides until they were cool.

2.7.3.2 Pressure cooking method

After dewaxing and dehydrating slides as in section 2.7.2.1, slides were transferred into a metal rack missing one space between consecutive slides; care was taken to orientate the slides in the same way.

1.5 litre of 10mM citric acid buffer pH 6.0, section 2.6.4, was boiled in the prestige pressure cooker model 6189 with the lid unlocked, on a hot plate. The adjacent sink was filled with cold water.

When the buffer was seen to be boiling, the slide racks were lowered into the buffer solution. The lid was then locked. The large pressure indicator was observed until it rose to the second indicator position, at this point the hotplate was switched off. The slides were allowed to cook from this point for 5 minutes.

The pressure cooker was then plunged into the sink of cold water and the steam was released. Once the pressure was down, the lid was removed and the slides allowed to cool. This usually took about 20 minutes.
2.7.3.3 The Menarini microwavable pressure cooker

The Menarini pressure cooker (A. Menarini Diagnostics, Wokingham, England) allowed sections to be microwaved under pressure. Sufficient citrate buffer (11), section 2.6.4, was added to the pressure cooker to cover a tray of slides.

The liquid was then microwaved in the Menarini pressure cooker, with the lid off, until boiling. This usually took 20 minutes at full power in the 750W microwave oven.

The slides in a plastic tray were placed in the pressure cooker and the lid was secured. The cooker was microwaved at full power, 750W, until the yellow pressure marker on the pressure cooker was raised, usually 2 minutes. The microwave was allowed to continue for 4 minutes. The pressure cooker was removed from the microwave and plunged into a sink of cold water until cool, about 20 minutes.

2.7.3.4 Protease digestion

A dish containing 300ml of PBS was placed in a water bath and warmed until 37°C. The PDS was titrated to pH 7.4. Pronase E (Sigma-Aldrich) was added at a concentration of 1mg/ml and the solution was stirred well. The slides, in a slide tray, were placed in the Pronase solution in the water bath for 15 minutes (in initial experiments 10 minutes was used but was insufficient). Slides were washed in running tap water for 5 minutes.
2.7.3.5 **Trypsin digestion**

A dish containing 300ml of distilled water was placed in a water bath and warmed until 37°C. 0.3g of Trypsin (DIFCO, USA) and 0.36g of Calcium Chloride (BDH Ltd, Poole, England) was added. The solution was titrated to pH7.8 with 0.1M sodium hydroxide. Slides were placed in a plastic tray and incubated in the solution at 37°C for 10 minutes. Slides were then washed in running tap water for 5 minutes.

2.7.4 **Immunohistochemical staining of VEGF**

Immunohistochemical staining of the formalin fixed paraffin embedded sections of TCC was carried out using the primary antibody VG-1. Work up of this staining technique was carried out on sections of superficial bladder cancer not included in the final data analysis. Negative controls were superficial bladder cancer without the application of primary antibody. Positive controls were normal human renal tissue.

Initially the streptavidin – biotin peroxidase complex (ABC) kit (Dako, Ely, England) was used. The VEGF staining was of poor quality.

Antigen retrieval was then carried out on the VEGF stained sections in an attempt to improve the staining. Two methods were used, microwave antigen retrieval in TRIS / EDTA pH 9.0 buffer, section 2.6.5, (this method was used by the Institute of Molecular Medicine Laboratory, Oxford, England, with VG-1) and microwave antigen retrieval in Citrate buffer, pH 6.0, the standard technique.
of the Specials Laboratory, Leicester Royal Infirmary. Using the Oxford technique, more VEGF staining was seen but staining was also seen on the negative controls. Using the citrate buffer, staining was again poor.

Staining was repeated using the EnVision+ developing kit (Dako, Ely, England). This was the developing method used by the Institute of Molecular Medicine Laboratory, Oxford, England, with VG-1; Oxford developed using Fast red, in this study DAB was used. The Oxford group used the VG-1 antibody neat. We used a series of diluted concentrations to further exclude non-specific staining.

The EnVision+ technique was used as described above, section 2.7.2. Antigen retrieval was carried out using pressure cooking of sections in TRIS / EDTA pH 9.0 for 3 minutes, section 2.7.3.2, or microwaving of sections in TRIS / EDTA pH 9.0 for 15 minutes, section 2.7.3.1.

A second primary antibody against VEGF (Santa Cruz, USA) was compared with VG-1. This was developed using the EnVision kit for polyclonal antibodies again using both retrieval techniques.

2.7.5 Staining of Thymidine Phosphorylase

The primary antibody against Thymidine Phosphorylase was the clone P-GF44.C (Oxford). The EnVision+ kit was used to develop the staining as described above, section 2.7.2. Negative controls did not have the primary
antibodies applied. Positive controls were human tonsil. The work up experiments for TP staining used human tonsil and sections of superficial bladder cancer not included in the final data analysis.

The TP (Oxford) primary antibody was initially assessed at three concentrations, 1:1, 1:2 and 1:4. The recommended concentration used by the Institute of Molecular Medicine Laboratory, Oxford, England, was 1:2. Four different antigen retrieval techniques were used, microwaving, section 2.7.3.1, pressure cooking, section 2.7.3.2, the microwavable pressure cooker, section 2.7.3.3 and no retrieval. Strong staining was seen with all techniques so the experiment was repeated with the primary antibody diluted at 1:20. Comparison was made between microwave antigen retrieval and no antigen retrieval.

2.7.6 Staining of Thrombospondin

TSP-1 Ab-7 (LabVision) was optimised on normal human tonsil. Dilutions of 1:50, 1:100, 1:150 and 1:200 were used with the EnVision+ kit. Antigen retrieval was by microwaving in citrate buffer pH 6.0, section 2.7.3.1. Staining with Ab-7 appeared to work well and a dilution of 1:150 was best. However, when used on bladder cancer sections no staining was seen.

Two further TSP-1 antibodies were therefore evaluated. TSP-1 Ab-4 (LabVision) and TSP-1 Ab-1 (Oncogene research products). Both were run with normal human tonsil and normal human colon using the EnVision+ kit. Runs were made with no antigen retrieval and microwave antigen retrieval. The
recommended dilution for Ab-4 was 1:50 – 1:100; therefore a dilution of 1:50 was used. The recommended dilution of Ab-1 was 1:10 – 1:100, therefore dilutions of 1:10 and 1:50 were used. No staining was seen with either antibody using either technique.

Staining was carried out with TSP-1 Ab4 antibody pre-treated with pronase for 10 minutes as antigen retrieval, section 2.7.3.4. Endothelial staining was observed in the normal colon and sporadic staining of macrophages was seen in the normal tonsil.

TSP-1 staining was repeated with all three primary antibodies pretreated with pronase for 15 minutes. Ab-1 was used at a dilution of 1:10, Ab-4 at a dilution of 1:50 and Ab-7 at a dilution of 1:100. Normal human tonsil, normal human colon, normal human kidney and sections of superficial bladder cancer not included in the final data analysis were used. With Ab-4, there was endothelial cell and some extracellular staining. Staining was not seen with the antibodies Ab-1 or Ab-7.

Following the work of Grossfeld et al (Grossfeld, Shi, Ginsberg, et al. 1996; Grossfeld, Ginsberg, Stein, et al. 1997), the experiment was repeated using antigen retrieval by microwaving in TRIS / HCL buffer at pH 1.0, section 2.7.3.1. Using this method good staining was seen with the antibody Ab-7.
2.7.7 Staining of p53

The technique for staining p53 using the streptavidin – biotin peroxidase complex (ABC) kit (Dako, Ely, England) was already well established in the Specials Laboratory, Leicester Royal Infirmary, however, the EnVison+ kit was found to give cleaner results and was therefore used. The technique is as described in 2.7.2 except that slides were not initially dewaxed in the warming oven, but proceeded straight to the graded alcohols. Antibody retrieval was by pressure cooking in citrate buffer, section 2.7.3.2. The primary antibody was used at a dilution of 1:100; this had been previously optimised and been found to be successful by the staff of the Specials Laboratory, Leicester Royal Infirmary. Negative controls omitted the primary antibody and positive controls were breast tissue from cases known to be positive for p53.

2.8.0 Measurement of Microvessel Density

2.8.1 Selection of areas for vessel counting

Random areas were selected throughout the tumour sections for vessel counts. Areas not containing tumour tissue were rejected. In an initial pilot experiment the random areas were chosen by a computerised random number generator.

2.8.1.1 Identification of random areas

Each slide was cleaned using a lint free tissue and placed on the microscope stage. An image of the section was focussed at a magnification of x200. The stage was then moved forwards until the most inferior part of the
section was in the optical field. At this point a reading was taken from the Vernier scale of the microscope stage. The stage was then moved backwards until the most superior part of the section was seen in the optical field. The Vernier reading was taken again. This was repeated moving the stage left and right and measurements of the positions of the edges of the section was noted. The four coordinates representing the size and position of the tumour section on the slide were then entered into a random number generator created in Microsoft Excel. This generated 100 random and different positions for each slide within the boundaries of the section. This list could then be printed out and used to find the random areas on each section. **Table 2.2** is an example of the random number generator; only 50 areas are shown. This method proved to be extremely tedious and time consuming and therefore a second more usable method was devised.

In the second method, each slide was cleaned using a lint free tissue and placed on the microscope stage. The image of the section was blurred using the focus control and the stage was moved randomly by the user in two directions. The focus was set so the difference between stained section and clear glass slide could be just discerned but no impression of the vascularity of the section was given. This method was far quicker and therefore more usable in a clinical setting.

**2.8.1.2 Calculation of number of random areas required**

The number of random areas required to be counted in order to give an accurate representation of the vascularity of the whole section was calculated
Table 2.2 Random number generator

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using the method of cumulative mean. Repeated manual vessel counts were carried out in random areas in a slide. The mean values of these counts were plotted against the number of areas counted. Means were cumulative, thus the first two counts were summed and divided by two and plotted against 2, subsequent counts were added and to the sum and divided by the number of counts. This gives a curve that at some point flattens. The number of counts required when the curve flattens suggests the number of random areas to be counted to give a representative vascularity for that section. This was repeated in 30 sections.

2.8.2 Computer Hardware and software used

Images were viewed using a JVC KYF50 3-chip colour video camera connected to a Nikon Elipse E800 microscope and captured with a Scion cg-7 frame-grabber attached to an Apple Macintosh G–3 computer, figure 2.1. These images were imported into the program NIH Image. This software was download free from the National Institute of Health website. (http://rsb.info.nih.gov/nih-image/) This software enables computerised measurement of captured images.

2.8.3 Manual counting of vessels

Manual counting of CD34 stained microvessels was carried out on the computer monitor screen and within the program NIH image. Images from the microscope were captured live and in colour using the scion cg-7 frame grabber. The image was frozen from a live video image to a still screen shot by clicking on it once with the computer mouse, figure 2.2.1. To allow direct comparison
Figure 2.1 Computer, microscope and camera for image analysis
Figure 2.2  MVD Counting

Figure 2.2.1 An area of tumour is captured in colour. Note the dark vessel staining and very light background.

Figure 2.2.2 The same image converted to greyscale.
Figure 2.2 MVD Counting (cont.)

Figure 2.2.3 Each vessel has been marked, manually, with a black blob. The vessel touching the bottom edge is not, by convention, counted.

Figure 2.2.4 The computer counts the manually marked vessels.
Figure 2.2.5 The area of tumour has been drawn around. The computer counts the number of vessels within that lassoed area. The count can then be expressed per unit area of tumour. The computer does not count the vessel touching the bottom edge.
with the computer image analysis the image was converted to greyscale, figure 2.2.2. This was done using a computer macro within NIH image, appendix 1, line 49.

Any black stained area was considered to be a vessel. There was no necessity for the presence of an obvious lumen or recognisable blood cells for vessel identification. Each area of black staining was considered a separate vessel even if it was obviously the same vessel looping throughout the section. Vessels which fell on the bottom edge of the screen or on the right edge of the screen were not counted. Vessels which fell on the top or left edges were counted.

To prevent the counting of vessels more than once and to make recording of manual counts easier, faster and more accurate the image analysis program was used. Each countable vessel was marked with a computerised blob of black paint, figure 2.2.3. This was applied using the paintbrush tool of NIH image and was applied to the picture using a single click of the mouse.

A macro within NIH image counted the number of marked vessels on the screen and displayed the result in the output window. The macro is described in appendix 1, line 334. As this technique did not involve any recognition analysis by the computer it was 100% accurate and did not influence the manual count in any way, figure 2.2.4.
2.8.4 Macro for computer image analysis vessel counting

A computer macro was written instructing the computer, on command, to convert the captured colour image to greyscale and subtract from it a blank background image. The macro then allowed the computer to count the number of dark foreground objects (vessels) on the screen. The system of counting vessels using the image analysis system is described.

2.8.4.1 Capture image

The image of the randomly chosen area seen at x200 magnification was also seen live on the monitor of the Macintosh computer. Capture was in colour using a KYF50 3-chip colour analogue video camera (JVC). The picture was digitalised using a Scion cg-7 frame-grabber. The image was projected in a window within the NIH Image program and frozen to a still RGB 24-bit colour digital image by a single mouse click.

2.8.4.2 Convert to greyscale

The colour image was then converted to a greyscale image using a macro within NIH Image, appendix 1, line 49. The light source in the microscope is never totally even and one side of the section was illuminated to a different extent than the other. This had the potential to invalidate the image analysis system, it being founded on contrast. A background palette was therefore digitally subtracted from the image; this removed the light imbalance from the picture. Before an image analysis session was commenced, a blank field image
was recorded, appendix 1, line 100. This could then be subtracted from the active image window, appendix 1, line 125.

2.8.4.3 Copy

To enable manual counting and automated image analysis counting to take place on exactly the same randomly chosen area, once the area was captured and converted to greyscale an exact digital replica was made. Any number of image copies could easily and quickly be made. This was particularly useful when assessing techniques many times on the same image. The macro used for this is seen in appendix 1, line 425.

2.8.4.4 Convert to bitmap

Conversion from a greyscale image to a 2-bit black and white bitmap is carried out using the command “MakeBinary”, see appendix 1, line 25. This is incorporated in several sections of the macro. The threshold level is required to determine which pixel will become foreground and which will become background in the 2-bit image. The threshold is one of the variables (var) quoted at the beginning of the macro series, see appendix 1, line 0. Once a threshold was determined (see 2.8.5.1) its value could be entered permanently as a macro constant, appendix 1, line 6.
2.8.4.5 Dilate and erode

Image manipulation to separate touching vessels and join fragmented vessel pieces used a series of pixel additions and deletions called a binarytweak. This is seen in appendix 1, line 23.

2.8.4.6 Count number of vessels.

The vessel counting macros allowed the number of foreground objects to be counted in different situations. Vessels could be counted and expressed as number per whole screen area, appendix 1, line 193 or in a manually defined area, appendix 1, line 237. This allowed the vessels to be expressed per unit area of tumour tissue. This is particularly relevant in superficial bladder cancer specimens because of their papillary architecture and the fragmented nature of surgical specimens.

Manual definition was carried out using a lasso tool in NIH image. This allows an area to be defined by the operator using the mouse to draw a perimeter around a given area. The area within this perimeter is measured, only vessels within this perimeter are counted and the count can then be expressed per this area, figure 2.2.5.

To account for the magnification of the source images by the various lenses of the microscope, macros were written into the program to allow true measurements on the captured image. Without this all captured images would be treated as the same size on the screen irrespective of their source magnification.
Therefore at the beginning of each image analysis session, the program was set to match the microscope magnification. This was invariably x200. With a set x10 eyepiece the objective was set to x20, appendix 1, lines 464 – 542.

2.8.4.7 Step by step summary of counting technique

The following method describes the actual detailed use of the image analysis system to count vessels both manually and by computer step by step. It includes the initial set up of the macros and constants as if beginning an image analysis session. Instructions in italic represent the opening of menus within computer programs. Numbers and letters in square brackets represent short codes written into the macros to speed up the technique.

1. Turn on the Apple Macintosh computer.
2. Turn microscope & camera on.
3. After cleaning the selected slide with a lint free tissue, place it on the microscope stage.
4. Open the NIH Image 1.62a program.
5. Special / Load macros
   Load the macro “vessel counting 4”
6. Special / Setup output window [O]
   This opens a window in which results of vessel counts are displayed.
7. Special / Obtain image [I]
   This opens the first live image of the section.
8. Focus microscope at x200 and set the light level.
Chapter 2 Methods

9. **Special / set objective to x20** [2]
   This corrects measurements to the x200 magnification.

10. Move platform so specimen is not visible, but still on the slide.

11. Click on image to freeze it

12. **Special / Save blank field** [J]
    
    Return
    
    Return
    
    This saves an image of a blank background field which will subsequently be digitally subtracted from the images to remove any background shadowing.

13. Find the random area on the slide section, see 2.8.1.

14. **Special / Obtain Image** [I]

15. Click on image to freeze it.
    This is the image to be analysed in colour.

16. **Special / Colour to greyscale and subtract** [N]
    This is the image to be analysed in greyscale.

17. **Special / Duplicate window** [D]
    An exact duplicate is made to allow manual and computer counting to be performed on identical areas.

18. Double click on paintbrush & set size to 18 (first time only)
    The paintbrush tool is used to manually mark vessels. The default size has to be increased so the image analysis counting system does not ignore it as a background blemish.
19. Perform manual count by placing the paintbrush tool over each vessel and clicking once.

20. *Special / Count Blobs greyscale whole screen [P]*

   The computer performs the manual count and displays the result on the output window.

21. Delete window [X]

   This removes the manual count window and brings the duplicate window up for image analysis.

22. Using the lasso tool mark out the area of tissue in the window.

   This allows vessels to be expressed per unit area of tissue.

23. *Special / Count vessels greyscale manual area definition [C]*

   This performs a computerised image analysis count of the vessels within the lassoed area. If tissue fills the whole image and manual definition is not required the vessels will be counted in the whole screen. The vessel count is displayed in the output window.

24. Delete window [X]

25. Goto 12

   The count is then repeated for the next randomly chosen area from point 12. The system is only required to be set up once at the beginning of each counting session.

   Using the shortcut keys written into the macro, counting can be carried out quite quickly. If the command “speed count lassooed vessels – greyscale” is used or the shortcut key press [G], the vessel count is performed, the window...
deleted and the next live image opened. Thus, during a standard vessel counting session an area may be counted by the following actions only:

1. Find random area and focus image.
2. Click once on image.
3. \( \text{[N]} \)
4. \( \text{[D]} \)
5. Click on vessels (the manual count).
6. \( \text{[P]} \)
7. \( \text{[X]} \)
8. Lasso the tissue if required.
9. \( \text{[G]} \)

The next live field will then be ready. If manual counting is not used this is even faster:

1. Find random area and focus image.
2. Click once on image.
3. Lasso the tissue if required.
4. \( \text{[G]} \)

2.8.4.8 Vessel counting data storage

For each tissue section analysed the output window was then saved as a text file onto a electronic storage ZIP disc (Iomega). Although the analysis was
carried out on an Apple Macintosh computer, if files were saved using the file extension ".txt" they could be easily transferred to a PC for data analysis.

As the cumulated data for each section was in a text file the data was continuous and not tabulated. In order to analyse the data it was imported into Microsoft Excel using the ":" as a separator. These markers were inbuilt into the output window macro just for this purpose. A macro within Microsoft excel was written to collate the data into a usable form.

2.8.5 Matching Computer counting to manual counting

The manual counting of vessels was considered the gold standard and image analysis was compared to this. As with any counting technique rules were established to allow reproducibility between users. A vessel was defined as any separate area of dark staining. The presence of a lumen or blood was not required. Any vessel falling on the lower edge or right hand border of the screen was not counted, but those falling on the top edge or left hand border were. Vessels were counted only in areas of tumour tissue. The computer macros controlling the automatic image analysis were programmed to make the computer counts match manual counts as closely as possible.

2.8.5.1 Setting the threshold

Within the macro, several variables were set to increase the accuracy of image analysis compared to manual counting. The Threshold was set. This sets a level of recognition, or contrast, for the computer to determine the difference
between foreground objects and the background. For each tumour type, the threshold was increased until the image analysis count was equal to the manual count. Once a threshold level was set this was repeated in ten areas of tumour to assess the reliability.

2.8.5.2 Erode and dilate functions

To overcome the rule that each separate dark stained object is counted as one vessel a series of erode and dilate functions were set up. These may be thought of as tidying the vessels to make the count cleaner. This, to some extent, prevents adjacent vessels being counted as one and single fragmented vessels being counted as many (Ranefall, Wester, Busch, & Malmstrom. 1998). Due to the nature of the immunochemical staining, this could never be perfect. The image analysis system was therefore made to match the manual count. A single layer of pixels was eroded from each solid object. The number of layers of pixels added and re-eroded was then determined experimentally and the results rechecked for reproducibility.

2.8.5.3 Particle sizes

The size of particles (vessels) that the computer recognises and counts was also set. The minimum particle size was set to exclude any background contamination whilst not excluding small vessels. This was carried out for each tumour type.
The maximum particle size was set to an infinitely large number so as not to exclude a count when all vessels in a field were contiguous and thus count as one.

### 2.8.5.4 Establishing reliability

Once a model was created within the macro its reliability and reproducibility was assessed by performing manual and image analysis counts in 30 randomly chosen areas in 30 cases in each of the tumour types.

### 2.9.0 Grading of immunohistochemical staining

For each immunohistochemical stain, 24 slides chosen at random from the study cases were examined simultaneously with a consultant histopathologist. Based on the staining patterns of these slides a grading system was created. Major staining patterns were noted and percentage staining of each type was measured. In some cases, intensity of staining was measured. In some cases a score of amount of staining of a particular type was given.

For each immunohistochemical stain, 24 slides were scored twice to assess intraobserver variability. Also, 24 slides were graded by two separate individuals to assess interobserver variability.

### 2.9.1 Vascular Endothelial Growth Factor (VEGF)

Staining of VG-1 (Oxford) was seen in tumour cells and in the underlying stroma. The amount of tumour cell staining was measured as a percentage of
tumour on the section as a whole. The intensity of VEGF staining of tumour cells in each section was scored as weak, moderate or strong. The amount of VEGF staining in the stroma was also graded as weak, moderate or strong.

2.9.2 Thymidine Phosphorylase (TP)

Thymidine Phosphorylase staining was seen in the tumour cells, in the stroma, in inflammatory cells and in endothelial cells. The percentage of tumour cell staining was measured in each section. The intensity of tumour cell staining was also measured. The intensity was given a grade of weak, moderate or strong. The extent of TP staining in the stroma was given a score of 0 (no staining), 1 (small amount of staining), 2 (moderate amount of staining), 3 (extensive staining). The intensity of stromal staining was scored as weak, moderate or strong for each section. The amount of inflammatory cell staining was scored as focal, moderate or extensive. The amount of endothelial cell staining was scored as 0 (no staining), 1 (small amount of staining), 2 (moderate amount of staining) or 3 (large amount of staining).

2.9.3 Thrombospondin –1 (TSP-1)

With both TSP-1 Ab-4 and TSP-1 Ab-7 immunohistochemistry staining was seen around the vessels, in the epidermis, in the tumour cells and in the stroma. Each slide was assessed to determine the amount of staining of that type within that tissue as a percentage of the whole section. For each staining pattern, a grade of the intensity of staining was given. Intensity was graded, weak, moderate or strong.
2.9.4 p53

The staining of p53 is scored as positive or negative. In superficial bladder cancer, a variety of levels of p53 staining have been used to denote positivity, commonly 5% and 20%. In this study the overall percentage of p53 stained tumour cells was measured as a continuous variable but cut offs were also applied at 5% and 20% for analysis.
Chapter 3

Results
3.0.0 Results

3.1.0 The Database

The computerised search of the pathology database for The University Hospitals of Leicester NHS Trust identified 731 pathology specimens of TCC received in the years 1994 and 1995. When the individual pathology reports of these cases were examined it was found that of these, 556 (76.06%) contained superficial bladder cancer.

After examining the medical notes of these 556 cases, 308 were found to represent recurrent disease and were excluded. Therefore 248 cases of apparent primary superficial bladder cancer were identified from the years 1994 and 1995. Of these 248 cases, 25 (10%) subsequently progressed to muscle invasive or metastatic disease.

There were 711 cases of invasive bladder cancer identified from the pathology databases between 1981 and 2000. After removing duplicates and those cases that presented as denovo invasive disease, 86 cases of invasive bladder cancer were identified that had presented initially as superficial disease. These were combined with the 248 cases and give a total of 334 cases identified for the study. Definition of SBC relied upon the original pathology reports.

Of the 334 cases of superficial bladder cancer identified, a further 41 cases were subsequently excluded from the study. In 16 cases no pathological
specimen was available, 11 were found to be duplicated in the database, 11 were found to have had previous tumours resected in other hospitals, in two no hospital notes were available and in one the patient moved areas immediately after the first resection.

Therefore, of the 1442 cases examined, 293 were suitable for inclusion in the final database. These cases were primary presentations of TCC found at TURBT with the first pathological specimen available for analysis and the clinical casenotes available for inspection. A summary of the collation of the database is given in table 3.1.

### 3.2.0 Epidemiology of Superficial Bladder cancer in the database

Of the 293 cases in the database, the median age was 72 years (24 – 95 years). There were 225 (76.8%) male patients and 68 female. From the information in the database, 146 (49.8%) patients were recorded, as being smokers or ex-smokers, 29 (9%) were taking aspirin and 20 (6.8%) were taking regular non steroidal anti-inflammatory drugs (NSAIDs).

At presentation, 174 (59.4%) tumours were stage pTa and 119 (40.6%) were pT1. Only 18 (6.1%) cases were recorded as having *cis* present in the specimen. Ninety (30.7%) cases at presentation were graded as grade 1, 129 (44%) as grade 2 and 74 (25.3%) as grade 3, *table 3.2*. In 90 (30.7%) cases there was a record in the casenotes (either written or pictorial) of tumour multiplicity at first resection. In 13 (4.4%) cases it was not possible to ascertain multiplicity.
Table 3.1. A summary of the collation of the database.

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Table 3.2. Stage and grade distribution in the whole database

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</tr>
<tr>
<td>pT1</td>
<td>9</td>
<td>57</td>
</tr>
<tr>
<td>Total</td>
<td>90</td>
<td>129</td>
</tr>
</tbody>
</table>
In 79 (27%) patients there was a record of intravesical chemotherapy being given. In 45 (15.4%) cases there was a record of intravesical BCG being given. Over this long period, indications for treatment and protocols changed with advancing clinical evidence. A single protocol was not used. Present protocols are irrelevant.

The median length of follow up to the time of death or when last seen was 59 months (1 to 280 months). There were 32 patients who had less than 12 months follow up.

Of the 293 patients in the database, 124 (42.3%) had a subsequent tumour recurrence of the same stage or less. 76 (25.9%) patients progressed to muscle invasive disease (≥pT2). 93 (31.7%) patients had neither recurrence nor progression. In 11 cases there was progression from stage pTa to stage pT1. The median time to tumour recurrence was 7 months (2 to 126 months). The median time to progression to muscle invasive disease was 22 months (2 to 171 months). In 7 patients progression to muscle invasive disease was noted at the first check cystoscopy. It is possible that in these patients the specimen from the initial resection may have been understaged.

The epidemiological parameters of the three groups, cases that neither recurred or progressed, cases that recurred only and cases that progressed to muscle invasive disease are given in table 3.3.
### Table 3.3 Epidemiological parameters in the three groups

<table>
<thead>
<tr>
<th></th>
<th>Cases that neither recurred or progressed (n=93)</th>
<th>Cases that Recurred (n=124)</th>
<th>Cases that progressed to muscle invasion (n=76)</th>
<th>Total (n=293)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Median Age (Range) yrs.</strong></td>
<td>73 (42-94)</td>
<td>69 (24-95)</td>
<td>73 (41-95)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Male (%)</strong></td>
<td>72 (77)</td>
<td>95 (77)</td>
<td>58 (76)</td>
<td>225</td>
</tr>
<tr>
<td><strong>Female (%)</strong></td>
<td>21 (23)</td>
<td>29 (23)</td>
<td>18 (24)</td>
<td>68</td>
</tr>
<tr>
<td><strong>Stage pT1a (%)</strong></td>
<td>64 (69)</td>
<td>84 (68)</td>
<td>26 (34)</td>
<td>174</td>
</tr>
<tr>
<td><strong>Stage pT1 (%)</strong></td>
<td>29 (31)</td>
<td>40 (32)</td>
<td>50 (66)</td>
<td>119</td>
</tr>
<tr>
<td><strong>Grade 1 (%)</strong></td>
<td>38 (41)</td>
<td>39 (31)</td>
<td>13 (17)</td>
<td>90</td>
</tr>
<tr>
<td><strong>Grade 2 (%)</strong></td>
<td>39 (42)</td>
<td>59 (48)</td>
<td>31 (41)</td>
<td>129</td>
</tr>
<tr>
<td><strong>Grade 3 (%)</strong></td>
<td>16 (17)</td>
<td>26 (21)</td>
<td>32 (42)</td>
<td>74</td>
</tr>
<tr>
<td><strong>cis (%)</strong></td>
<td>4 (4)</td>
<td>6 (5)</td>
<td>8 (11)</td>
<td>18</td>
</tr>
<tr>
<td><strong>Multiple Tumours (%)</strong></td>
<td>18 (19)</td>
<td>43 (35)</td>
<td>29 (38)</td>
<td>90</td>
</tr>
<tr>
<td><strong>Intravesical Chemotherapy given (%)</strong></td>
<td>15 (16)</td>
<td>46 (37)</td>
<td>18 (24)</td>
<td>79</td>
</tr>
<tr>
<td><strong>Intravesical BCG given (%)</strong></td>
<td>5 (5)</td>
<td>26 (21)</td>
<td>14 (18)</td>
<td>45</td>
</tr>
<tr>
<td><strong>Smokers (%)</strong></td>
<td>39 (42)</td>
<td>74 (60)</td>
<td>33 (43)</td>
<td>146</td>
</tr>
<tr>
<td><strong>On regular Aspirin (%)</strong></td>
<td>11 (12)</td>
<td>16 (13)</td>
<td>2 (3)</td>
<td>29</td>
</tr>
<tr>
<td><strong>On regular NSAIDs (%)</strong></td>
<td>4 (4)</td>
<td>10 (8)</td>
<td>6 (8)</td>
<td>20</td>
</tr>
<tr>
<td><strong>Median Follow up (range) mths.</strong></td>
<td>54 (0-79)</td>
<td>63 (5-280)</td>
<td>39.5 (1-181)</td>
<td>-</td>
</tr>
</tbody>
</table>

Percentage values are within groups.
3.2.1 Statistical analysis of clinico-pathological parameters with respect to outcome.

3.2.1.1 Factors associated with tumour recurrence

When compared with cases that neither recurred nor progressed (n=93), those cases that recurred (n=124), were younger (p=0.018), were more likely to smoke (p=0.010), were more likely to have had tumour multiplicity at presentation (p=0.014) and received intravesical chemotherapy (p=0.001) or BCG (p=0.001) more frequently. The length of follow up in the cases that recurred was significantly longer than in the cases that did not recur or progress (p=0.0000003). These differences are detailed in table 3.4.

Clinicopathological parameters were entered individually into a binary logistic regression analysis model examining risk of disease recurrence. Lower median age (p=0.016), multiple tumours at presentation (p=0.024), smoking (p=0.010) and receiving intravesical chemotherapy (p=0.002) or BCG (p=0.005) were all identified as risk factors for recurrence, table 3.5.

3.2.1.2 Factors associated with tumour progression

When compared with the cases that neither recurred nor progressed (n=93), in the group that progressed (n=76), there were significantly less patients recorded as regularly taking aspirin (p=0.026). The stage at presentation (p=0.00001) and the grade at presentation (p=0.00004) were higher. Tumour multiplicity at presentation was more common(p=0.001) in the group that progressed. These differences are detailed in table 3.6.
Table 3.4 Clinico-pathological variables displaying a significant difference when comparing the cases that neither recurred or progressed with the cases that recurred

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases that neither recurred or progressed (n=93)</th>
<th>Cases that Recurred (n=124)</th>
<th>Difference (Mann Whitney U test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Age (Range) yrs.</td>
<td>73 (42-94)</td>
<td>69 (24-95)</td>
<td>p=0.018</td>
</tr>
<tr>
<td>Smokers</td>
<td>39 (42%)</td>
<td>74 (60%)</td>
<td>p=0.010</td>
</tr>
<tr>
<td>Multiple Tumours</td>
<td>18 (19%)</td>
<td>43 (35%)</td>
<td>p=0.014</td>
</tr>
<tr>
<td>Intravesical Chemotherapy given</td>
<td>15 (16%)</td>
<td>46 (37%)</td>
<td>p=0.001</td>
</tr>
<tr>
<td>Intravesical BCG given</td>
<td>5 (5%)</td>
<td>26 (21%)</td>
<td>p=0.001</td>
</tr>
<tr>
<td>Median Follow up (range) mths.</td>
<td>54 (0-79)</td>
<td>63 (5-280)</td>
<td>p=0.0000003</td>
</tr>
</tbody>
</table>
Table 3.5  Binary logistic regression of the risk of tumour recurrence for the clinicopathological parameters in the database

<table>
<thead>
<tr>
<th></th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.970</td>
<td>0.946-0.994</td>
<td>0.016</td>
</tr>
<tr>
<td>Gender</td>
<td>0.929</td>
<td>0.485-1.781</td>
<td>0.824</td>
</tr>
<tr>
<td>Increasing Stage</td>
<td>1.118</td>
<td>0.622-2.011</td>
<td>0.709</td>
</tr>
<tr>
<td>Increasing Grade</td>
<td>1.253</td>
<td>0.848-1.850</td>
<td>0.258</td>
</tr>
<tr>
<td>The presence of cis</td>
<td>1.030</td>
<td>0.269-3.951</td>
<td>0.966</td>
</tr>
<tr>
<td>Multiple tumours</td>
<td><strong>2.111</strong></td>
<td><strong>1.104-4.035</strong></td>
<td><strong>0.024</strong></td>
</tr>
<tr>
<td>Intravesical Chemotherapy given</td>
<td><strong>2.849</strong></td>
<td><strong>1.452-5.589</strong></td>
<td><strong>0.002</strong></td>
</tr>
<tr>
<td>Intravesical BCG given</td>
<td><strong>4.255</strong></td>
<td><strong>1.543-11.732</strong></td>
<td><strong>0.005</strong></td>
</tr>
<tr>
<td>Smokers</td>
<td><strong>2.092</strong></td>
<td><strong>1.197-3.656</strong></td>
<td><strong>0.010</strong></td>
</tr>
<tr>
<td>On regular Aspirin</td>
<td>1.141</td>
<td>0.497-2.612</td>
<td>0.756</td>
</tr>
<tr>
<td>On regular NSAIDs</td>
<td>1.925</td>
<td>0.573-6.463</td>
<td>0.289</td>
</tr>
</tbody>
</table>

Statistically significant results are shown in bold type.
Table 3.6  Clinico-pathological variables displaying a significant difference when comparing the cases that neither recurred or progressed with the cases that progressed.

<table>
<thead>
<tr>
<th></th>
<th>Cases that neither recurred or progressed (n=93)</th>
<th>Cases that progressed to muscle invasion (n=76)</th>
<th>Difference (Mann Whitney U test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>On regular Aspirin</td>
<td>11 (12%)</td>
<td>2 (3%)</td>
<td>p=0.026</td>
</tr>
<tr>
<td>Stage pTa</td>
<td>64 (69%)</td>
<td>26 (34%)</td>
<td>p=0.00001</td>
</tr>
<tr>
<td>Stage pT1</td>
<td>29 (31%)</td>
<td>50 (66%)</td>
<td>p=0.00004</td>
</tr>
<tr>
<td>Grade 1</td>
<td>38 (41%)</td>
<td>13 (17%)</td>
<td></td>
</tr>
<tr>
<td>Grade 2</td>
<td>39 (42%)</td>
<td>31 (41%)</td>
<td></td>
</tr>
<tr>
<td>Grade 3</td>
<td>16 (17%)</td>
<td>32 (42%)</td>
<td></td>
</tr>
<tr>
<td>Multiple Tumours</td>
<td>18 (19%)</td>
<td>29 (38%)</td>
<td>p=0.001</td>
</tr>
</tbody>
</table>
When compared with all cases that did not progress (n=217), those cases that subsequently progressed to muscle invasive disease (n=76) had significantly higher median age (p=0.025) and were less likely to take aspirin (p=0.014). Those cases that subsequently progressed had a higher stage (p=0.0000002), grade (p=0.00004) and were more likely to have multiple tumours (p=0.025) at presentation. These differences are detailed in table 3.7.

In a binary logistic regression analysis, a higher median age (p=0.015), not taking aspirin (p=0.026), high tumour stage at presentation (p=0.0000005), high tumour grade at presentation (p=0.00006) and tumour multiplicity (p=0.026) were all predictive factors for progression to muscle invasive disease. The results for these parameters are given in table 3.8.

When the cases that had less than 12 months follow up and the cases that apparently progressed at the first check cystoscopy were removed from the database there were 254 cases remaining. Understaging in some cases would give a false impression of the number of cases that actually progressed. A short follow up period may give insufficient time for an event (recurrence or progression) to occur. Therefore a corrected database of these 254 patients was also analysed.

In the corrected database, in a binary logistic regression model for recurrence, stage (p=0.015), grade (p=0.006), multiplicity (p=0.004) and intravesical chemotherapy (p=0.009) and BCG (p=0.012) remained significant risk factors, table 3.9. For progression there remained a significant difference in
Table 3.7  Clinico-pathological variables displaying a significant
difference when comparing the cases that progressed with those
that did not progress.

<table>
<thead>
<tr>
<th></th>
<th>Cases that did not progress (n=217)</th>
<th>Cases that progressed to muscle invasion (n=76)</th>
<th>Difference (Mann Whitney U test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>On regular Aspirin</td>
<td>27 (12%)</td>
<td>2 (3%)</td>
<td>p=0.014</td>
</tr>
<tr>
<td>Stage pTa</td>
<td>148 (68%)</td>
<td>26 (34%)</td>
<td>p=0.0000002</td>
</tr>
<tr>
<td>Stage pT1</td>
<td>69 (32%)</td>
<td>50 (66%)</td>
<td>p=0.00004</td>
</tr>
<tr>
<td>Grade 1</td>
<td>77 (35%)</td>
<td>13 (17%)</td>
<td>p=0.00004</td>
</tr>
<tr>
<td>Grade 2</td>
<td>98 (45%)</td>
<td>31 (41%)</td>
<td></td>
</tr>
<tr>
<td>Grade 3</td>
<td>42 (19%)</td>
<td>32 (42%)</td>
<td></td>
</tr>
<tr>
<td>Multiple Tumours</td>
<td>61 (28%)</td>
<td>29 (38%)</td>
<td>p=0.025</td>
</tr>
</tbody>
</table>
Table 3.8  Binary logistic regression of the risk of tumour progression for the clinicopathological parameters in the database

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.032</td>
<td>1.006-1.059</td>
<td>0.015</td>
</tr>
<tr>
<td>Gender</td>
<td>0.965</td>
<td>0.521-1.786</td>
<td>0.909</td>
</tr>
<tr>
<td>Stage</td>
<td>4.123</td>
<td>2.371-7.170</td>
<td>0.0000005</td>
</tr>
<tr>
<td>Grade</td>
<td>2.156</td>
<td>1.484-3.132</td>
<td>0.00006</td>
</tr>
<tr>
<td>cis</td>
<td>2.435</td>
<td>0.924-6.419</td>
<td>0.072</td>
</tr>
<tr>
<td>Multiple Tumours</td>
<td>1.902</td>
<td>1.078-3.353</td>
<td>0.026</td>
</tr>
<tr>
<td>Intravesical Chemotherapy given</td>
<td>0.794</td>
<td>0.433-1.455</td>
<td>0.455</td>
</tr>
<tr>
<td>Intravesical BCG given</td>
<td>1.355</td>
<td>0.677-2.711</td>
<td>0.391</td>
</tr>
<tr>
<td>Smokers</td>
<td>0.706</td>
<td>0.417-1.195</td>
<td>0.195</td>
</tr>
<tr>
<td>On regular Aspirin</td>
<td><strong>0.190</strong></td>
<td><strong>0.044-0.820</strong></td>
<td><strong>0.026</strong></td>
</tr>
<tr>
<td>On regular NSAIDs</td>
<td>1.243</td>
<td>0.460-3.359</td>
<td>0.668</td>
</tr>
<tr>
<td>Time to first recurrence</td>
<td>1.015</td>
<td>0.996-1.033</td>
<td>0.116</td>
</tr>
</tbody>
</table>

Statistically significant results are shown in bold type.
Table 3.9  Binary logistic regression of the risk of tumour recurrence for the clinicopathological parameters in the corrected database (n=245)

<table>
<thead>
<tr>
<th></th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.994</td>
<td>0.970-1.018</td>
<td>0.603</td>
</tr>
<tr>
<td>Gender</td>
<td>1.006</td>
<td>0.521-1.944</td>
<td>0.985</td>
</tr>
<tr>
<td>Stage</td>
<td>2.076</td>
<td>1.149-3.749</td>
<td>0.015</td>
</tr>
<tr>
<td>Grade</td>
<td>1.716</td>
<td>1.168-2.520</td>
<td>0.006</td>
</tr>
<tr>
<td>cis</td>
<td>1.390</td>
<td>0.438-4.410</td>
<td>0.576</td>
</tr>
<tr>
<td>Multiple Tumours</td>
<td>2.653</td>
<td>1.373-5.127</td>
<td>0.004</td>
</tr>
<tr>
<td>Intravesical Chemotherapy given</td>
<td>2.465</td>
<td>1.258-4.832</td>
<td>0.009</td>
</tr>
<tr>
<td>Intravesical BCG given</td>
<td>3.519</td>
<td>1.324-9.354</td>
<td>0.012</td>
</tr>
<tr>
<td>Smokers</td>
<td>1.492</td>
<td>0.868-2.565</td>
<td>0.148</td>
</tr>
<tr>
<td>On regular Aspirin</td>
<td>0.682</td>
<td>0.297-1.568</td>
<td>0.368</td>
</tr>
<tr>
<td>On regular NSAIDs</td>
<td>1.275</td>
<td>0.398-4.090</td>
<td>0.682</td>
</tr>
</tbody>
</table>
the age \((p=0.037)\), stage \((p<0.0001)\), grade \((p=0.004)\), presence of multiplicity \((p=0.017)\) and aspirin consumption \((p=0.040)\), table 3.10.

### 3.2.2 Summary of Clinopathological variables in the database

In summary, the database included 293 cases of primary superficial TCC of the human urinary bladder. Of these 124 cases recurred and 76 cases progressed to muscle invasive disease. In the cases that recurred, the patients were older at presentation and were more likely to be smokers. They were more likely to present with multiple tumours and were more likely to receive intravesical therapy.

Those cases that subsequently progressed were also older at presentation. They were less likely to take regular aspirin. Cases that progressed had a higher initial stage and grade and were more likely to have multiple tumours. Presenting tumour stage, grade and multiplicity were predictive factors for progression whereas taking aspirin was associated with a decreased risk.

Once patients with short follow up and possible understaging were removed the database contained 254 patients. In this corrected database, stage, grade, multiplicity and intravesical chemotherapy and BCG remained significant risk factors for tumour recurrence. Stage, grade, multiplicity, aspirin consumption and age were significantly related to progression.
Table 3.10  Binary logistic regression of the risk of tumour progression for the clinicopathological parameters in the corrected database (n=245)

<table>
<thead>
<tr>
<th>Odds ratio</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.031</td>
<td>1.002–1.061</td>
</tr>
<tr>
<td>Gender</td>
<td>1.234</td>
<td>0.590–2.581</td>
</tr>
<tr>
<td>Stage</td>
<td>4.171</td>
<td>2.257–7.710</td>
</tr>
<tr>
<td>Grade</td>
<td>1.826</td>
<td>1.214–2.745</td>
</tr>
<tr>
<td>cis</td>
<td>2.490</td>
<td>0.904–6.863</td>
</tr>
<tr>
<td>Multiple Tumours</td>
<td>2.135</td>
<td>1.139–4.003</td>
</tr>
<tr>
<td>Intravesical Chemotherapy given</td>
<td>0.786</td>
<td>0.406–1.522</td>
</tr>
<tr>
<td>Intravesical BCG given</td>
<td>1.260</td>
<td>0.588–2.700</td>
</tr>
<tr>
<td>Smokers</td>
<td>0.708</td>
<td>0.395–1.271</td>
</tr>
<tr>
<td>On regular Aspirin</td>
<td>0.239</td>
<td>0.055–1.039</td>
</tr>
<tr>
<td>On regular NSAIDs</td>
<td>0.454</td>
<td>0.100–2.057</td>
</tr>
</tbody>
</table>
3.3.0 Microvessel Density Results

This section relates firstly to the staining of microvessels and the optimum methodology chosen. This is followed by a description of the final and superior computer counting technique devised. Results are given showing that MVD can be measured in SBC. Finally, using this technique, the MVD of SBC in the database is related to outcome, clinicopathological variables and other angiogenic indicators.

3.3.1 Chromogen Enhancement

The DAB chromagen when used alone enhanced the CD34 staining with an even brown hue. Enhancement with copper sulphate gave a darker staining, and enhancement with nickel sulphate gave an almost black stain, figure 3.1.

The three types of chromogen enhancement were subjected to vessel counting in 30 random areas by manual and computer counting. Spearman rank correlation of the counts for DAB alone was \( r^2 = 2.43, p=0.195 \); for DAB and copper sulphate, \( r^2 = 0.565, p=0.001 \) and for DAB and nickel sulphate, \( r^2 = 0.834, p=0.000001 \).

3.3.2 Counterstain selection

Methyl green and Mayer’s Haematoxylin produced a clear and even counterstain. Light green left dark blobs of colour which confounded the image analysis counting system. Neutral red gave a good pale contrast but was unreliable to reproduce as was Methylene blue. In practice Methyl green did not
Figure 3.1 Comparison of enhancers of CD34 vessel staining

Figure 3.1.1 CD34 staining with DAB chromagen alone. Superficial bladder cancer. x200

Figure 3.1.2 CD34 staining with DAB and copper sulphate enhancement. Superficial bladder cancer. x200
produce a consistent counterstain and was therefore considered inferior to Mayer's Haematoxylin, \textit{figure 3.2}.

When the different counterstains were subjected to comparison by vessel counting manually and by computer, Haematoxylin was found to be superior. Spearman rank correlation of the counts were, Haematoxylin, $r^2 = 0.834$, $p=0.000001$; Methylene blue, $r^2 = 0.438$, $p=0.016$; Methyl green, $r^2 = 0.688$, $p=0.00003$; Neutral red, $r^2 = 0.455$, $p=0.012$; Light green, $r^2 = 0.685$, $p=0.00003$.

Comparison of the different exposure times to Mayer's Haematoxylin found that the original one second exposure was superior. Spearman rank correlation of manual versus computer counts were; 10 second exposure, $r^2 = 0.465$, $p=0.010$; 5 second exposure, $r^2 = 0.146$, $p=0.440$ and one second exposure, $r^2 = 0.834$, $p=0.000001$, \textit{figure 3.3}.

\textbf{3.3.3 Comparison of CD31 and CD34 vessel staining}

Both CD31 and CD34 identified vessels. CD34 staining was superior to CD31 staining. The mixture of CD31 and CD34 also gave good vessel staining. However, it was felt that this added nothing extra to CD34 staining alone. \textit{Figure 3.4}. 

\textbf{184}
Figure 3.2  Counterstains

Figure 3.2.1  Methylene blue

Figure 3.2.2  Neutral Red
Figure 3.3  Correlation of Manual and computer vessel counts in 30 random areas; CD34 enhanced with DAB and Nickel sulphate with a one second Haematoxylin counterstain.

Manual Count

Computer Count

$r^2 = 0.834,$

$p = 0.000001$
Figure 3.4  Comparison of CD34 and CD31 vessel staining.
(x200)

Figure 3.4.1  CD34. Dilution 1:10 Superficial bladder cancer.
(x200)

Figure 3.4.2  CD34. Dilution 1:20 Superficial bladder cancer.
(x200)
Figure 3.4.3 CD31. Dilution 1:10 Superficial bladder cancer. (x200)

Figure 3.4.4 CD31. Dilution 1:20 Superficial bladder cancer. (x200)
Figure 3.4 Comparison of CD34 and CD31 vessel staining.
(cont.) (x200)

Figure 3.4.9 CD31. Positive control. Superficial bladder cancer. (x200)

Figure 3.4.10 CD31. Negative control. Superficial bladder cancer. (x200)
3.3.4 Computer macro enhancement

3.3.4.1 Determination of the optimum Threshold level

Whilst setting up the computer macro it became apparent that the setting for the threshold would be around 100. This was confirmed by performing a manual count and then multiple computer counts with the threshold varying between 60 and 130 and increasing in steps of 5. The computer count matched the manual count at a threshold of 100, figure 3.5. This was repeated in 10 different areas which confirmed a threshold of 100.

3.3.4.2 Determination of the optimum Minimum & maximum particle size to be recognised

The maximum particle size was set at 2000000 a very large number, so as not to exclude a count if all vessels in a field were contiguous. The minimal particle size was set at 200 pixels. This was calculated empirically by observation of counts in 10 areas in 10 different tumours. Different minimal particle sizes were used to remove all background noise without missing any small vessels.

3.3.4.3 Setting up of the Erode & Dilate function

In a single tumour field, erode and dilate functions were gradually increased until the manual count and computer count were the same and contiguous vessels had been separated and fragmented vessels joined. Once an erode-dilate combination had been devised it was used in 10 areas in 10 different tumours. The erode-dilate combination was corrected until it gave a correct result.
Figure 3.5  Calculating the optimum threshold level for computer counting.

Figure 3.6  Calculating the number of random areas to be sampled using the technique of cumulative mean.
for all these areas. The final combination was: a single erode followed by five
dilates and then three erodes, appendix 1, line 24.

3.3.4.4 Determination of the number of random areas to be counted using the
method of Cumulative mean

Cumulative mean graphs were drawn for 30 random areas in 20 cases. Both manual and computer counts were performed. The curves flattened by the
time the counts were in the twenties. The number of random areas to be counted
was therefore set at 30. figure 3.6.

3.3.4.5 Comparison of manual and computer vessel counting

In 210 cases both manual and computer counts were performed. A
spearman rank correlation coefficient comparing the two methods showed a
good correlation (r=0.95). A Bland-Altman plot was also drawn. This showed a
good grouping of points adjacent to the x axis. There is a slight deviation to the
positive, which indicates that overall the computer tends to overestimate the
count to a small degree. figure 3.7.

The reliability of the computerised image analysis model was assessed by
recounting 12 cases. The two counts were performed separated by several
months to ensure there was no recognition bias. In each case 30 areas were
counted giving 360 area counts each time. Comparison of these 740 counts with
a Spearman's rank test found a good correlation between them, \( r^2 = 0.874 \),
p=0.0002.
Figure 3.7  Bland-Altman plot of Manual vessel count and Computer vessel count in 210 cases.
Chapter 3

Results

The median time taken to assess the MVD in 30 random areas in a section was 4 minutes 30 seconds when the computer image analysis was used. For those cases where both manual counts and computer counts were performed the median time taken to assess a single section was 13 minutes and 48 seconds.

3.3.5 Results of MVD measurement in superficial bladder cancer

The median MVD count (as measured by computerised image analysis) in all the 293 sections was 9.24 vessels per high powered field (HPF) at x200 magnification. The range was 2.88 – 47.63 HPF⁻¹. The maximum vessel count in each case ranged from 7.0 to 101.0 HPF⁻¹ (median 28.0 HPF⁻¹). The median MVD expressed per unit area was 73.56mm², range 24.26 – 372.26mm².

In the cases also counted manually, the median MVD was 8.06 HPF⁻¹, range 2.26 – 50.53 HPF⁻¹. The maximum recorded manual counts ranged from 7.0 – 112.0 HPF⁻¹ (median 22.0 HPF⁻¹). When expressed per unit area the manual MVD was 62.52mm², range 19.31 – 392.06mm².

The median number of areas counted in each section of tissue was 31. This ranged from 5 to 34. In 20 (6.8%) cases the available tissue was too small to allow 30 random areas to be selected. In these cases counts were performed in all tumour areas. The number of areas counted in these 20 cases ranged from 5 to 22, median 13.5. There were less than 10 areas in 3 cases, between 10 and 20 areas in 14 cases and over 20 areas in 3 cases. Of these 20 cases 8 subsequently progressed to muscle invasive disease and 12 did not.
In each case the mean area of tumour was measured. The size of the visible magnified field seen on the computer monitor was $142410.3125\mu m^2$. The mean area measured on each section ranged from $32502.09\mu m^2$ to this maximum size.

The median MVD of those cases that progressed to muscle invasive disease was $13.10$ HPF$^{-1}$ ($6.57 - 47.63$ HPF$^{-1}$). When expressed per area it was $109.57mm^2$ ($53.47 - 372.26mm^2$). The maximum counts ranged from $17 - 101$ HPF$^{-1}$ (median $35$ HPF$^{-1}$).

For those cases that did not progress the median MVD was $8.54$ HPF$^{-1}$ ($2.90 - 31.67$ HPF$^{-1}$), this was $67.70mm^2$ ($24.26 - 336.37mm^2$) per unit area. Maximum counts ranged from $8$ to $90$ (median $27$).

The median MVD of those cases that neither recurred or progressed was $8.10$ HPF$^{-1}$ ($3.66 - 31.67$ HPF$^{-1}$) and $63.37mm^2$ ($33.34 - 336.37mm^2$) when expressed per area. The maximum MVD in these cases ranged from $8$ to $90$ HPF$^{-1}$ (median $27$ HPF$^{-1}$).

For those cases that recurred but did not progress the MVD was $8.78$ HPF$^{-1}$ ($2.90 - 20.87$ HPF$^{-1}$), $68.35mm^2$ ($24.26 - 200.11mm^2$). The maximum counts ranged from $9$ to $63$ HPF$^{-1}$ (median $27$ HPF$^{-1}$). These results are summarised in **table 3.11**.
Table 3.11  MVD results as assessed by the different techniques

<table>
<thead>
<tr>
<th></th>
<th>Median MVD (range) (HPF\textsuperscript{1})</th>
<th>MVD per unit area (range) (mm\textsuperscript{2})</th>
<th>Maximum Counts (median) (HPF\textsuperscript{1})</th>
<th>Median number of areas assessed per section</th>
<th>Median Area measured (range) (\mu m\textsuperscript{2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases that progressed</td>
<td>13.10 (6.57-47.63)</td>
<td>109.57 (53.47-372.26)</td>
<td>17-101 (35)</td>
<td>31 (5-33)</td>
<td>122521.68 (77931.30-142395.80)</td>
</tr>
<tr>
<td>Total cases that did not progress</td>
<td>8.55 (2.90-31.67)</td>
<td>67.70 (24.26-336.37)</td>
<td>8-90 (27)</td>
<td>31 (13-33)</td>
<td>123574.21 (32502.09-142410.30)</td>
</tr>
<tr>
<td>Cases that neither recurred nor progressed</td>
<td>8.10 (3.66-31.67)</td>
<td>63.37 (33.34-336.37)</td>
<td>8-90 (27)</td>
<td>31 (13-33)</td>
<td>122881.42 (82211.18-142324.30)</td>
</tr>
<tr>
<td>Cases that recurred</td>
<td>8.78 (2.90-20.87)</td>
<td>68.35 (24.26-200.11)</td>
<td>9-63 (27)</td>
<td>31 (15-33)</td>
<td>124279.05 (32502.09-142410.30)</td>
</tr>
</tbody>
</table>
A statistical comparison (Mann Whitney U test) was made between the two groups, recurrence and non recurrence. There was no statistical difference in MVD between these groups.

Examining the cases that progressed and those that did not, a significant difference was seen in MVD, both computer counted (p<0.00001) and manual (p<0.00001), maximum vessel count, both computer (p<0.00001) and manual (p<0.00001), and in MVD per unit area, again seen in both manual (p<0.00001) and computer (p<0.00001) counts. There was no difference in the number of areas counted (p=0.094) or the tissue area measured (p=0.256). Table 3.12.

The MVD count was significantly higher in those cases which presented with pT1 disease (p=0.001), table 3.13. MVD was also associated with grade at presentation. There was a significant difference in MVD between grade 1 and grade 3 disease (p=0.022) but not between grade 1 and 2 disease (p=0.370) or between grade 2 and 3 disease (p=0.067), table 3.14.

There was a significant difference in MVD in those cases identified as having cis at presentation, MVD was higher in these cases (p=0.022). There was no difference in MVD with respect to tumour multiplicity (p=0.119), smoking status (p=0.209) or aspirin consumption (p=0.538), table 3.15.

In a binary logistic regression model, there was no difference in MVD between those cases that recurred and those that did not. However, higher
<table>
<thead>
<tr>
<th></th>
<th>Computer MVD (range) (HPF(^{-1}))</th>
<th>Manual MVD (range) (HPF(^{-1}))</th>
<th>Computer MVD per unit area (range) (mm(^2))</th>
<th>Manual MVD per unit area (range) (mm(^2))</th>
<th>Maximum computer Counts (median) (HPF(^{-1}))</th>
<th>Maximum manual Counts (median) (HPF(^{-1}))</th>
<th>Median number of areas assessed per section</th>
<th>Median Area measured (range) ((\mu m^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cases that progressed</strong></td>
<td>13.10 (6.57-47.63)</td>
<td>11.22 (4.20-50.53)</td>
<td>109.57 (53.47-372.26)</td>
<td>94.79 (34.49-392.06)</td>
<td>17-101 (35)</td>
<td>12-112 (29)</td>
<td>31 (5-33)</td>
<td>122521.68 (77931.30-142395.80)</td>
</tr>
<tr>
<td><strong>Total cases that did not progress</strong></td>
<td>8.55 (2.90-31.67)</td>
<td>7.24 (2.26-29.03)</td>
<td>67.70 (24.26-336.37)</td>
<td>57.74 (19.31-307.90)</td>
<td>8-90 (27)</td>
<td>7-64 (21)</td>
<td>31 (13-33)</td>
<td>123574.21 (32502.09-142410.30)</td>
</tr>
<tr>
<td><strong>Mann Whitney U (p value)</strong></td>
<td>(p&lt;0.00001)</td>
<td>(p&lt;0.00001)</td>
<td>(p&lt;0.00001)</td>
<td>(p&lt;0.00001)</td>
<td>(p&lt;0.00001)</td>
<td>(p&lt;0.00001)</td>
<td>(p=0.094)</td>
<td>(p=2.56)</td>
</tr>
</tbody>
</table>
Table 3.13  Relationship of Computer MVD per unit area and tumour stage at presentation

<table>
<thead>
<tr>
<th></th>
<th>Median MVD (vessels/mm²)</th>
<th>Difference (Mann Whitney U test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage pTa</td>
<td>64.29</td>
<td>p=0.001</td>
</tr>
<tr>
<td>Stage pT1</td>
<td>87.66</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.14  Relationship of Computer MVD per unit area and tumour grade at presentation

<table>
<thead>
<tr>
<th>Grade</th>
<th>Median MVD (vessels/mm²)</th>
<th>Difference (Mann Whitney U test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 1</td>
<td>67.76</td>
<td>p=0.370</td>
</tr>
<tr>
<td>Grade 2</td>
<td>74.29</td>
<td></td>
</tr>
<tr>
<td>Grade 2</td>
<td>74.29</td>
<td>p=0.067</td>
</tr>
<tr>
<td>Grade 3</td>
<td>87.66</td>
<td></td>
</tr>
<tr>
<td>Grade 3</td>
<td>87.66</td>
<td>p=0.022</td>
</tr>
</tbody>
</table>

Table 3.15  Relationship of Computer MVD per unit and other clinico-pathological variables

<table>
<thead>
<tr>
<th></th>
<th>Median MVD (vessels/mm²)</th>
<th>Difference (Mann Whitney U test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No cis</td>
<td>72.38</td>
<td>p=0.022</td>
</tr>
<tr>
<td>cis present</td>
<td>124.85</td>
<td></td>
</tr>
<tr>
<td>Solitary tumour</td>
<td>71.48</td>
<td></td>
</tr>
<tr>
<td>Multiple tumours</td>
<td>75.97</td>
<td>p=0.119</td>
</tr>
<tr>
<td>Non smoker</td>
<td>75.00</td>
<td>p=0.209</td>
</tr>
<tr>
<td>Smoker</td>
<td>71.75</td>
<td></td>
</tr>
<tr>
<td>Not on Aspirin</td>
<td>73.15</td>
<td>p=0.538</td>
</tr>
<tr>
<td>On Aspirin</td>
<td>73.09</td>
<td></td>
</tr>
</tbody>
</table>

Statistically significant results are shown in bold type.
median or maximum MVD measured by either manual counting or image analysis were found to be predictive risk factors for disease progression, table 3.16.

The factors, MVD and MVD per area and maximum MVD count were entered separately into a multivariable binary logistic regression model containing tumour stage, tumour grade, presence of cis and tumour multiplicity. These factors are accepted as risk factors for tumour progression to muscle invasive disease. When entered separately into this model, MVD (p<0.00001), and MVD per area (p<0.00001) were shown to be independent risk factors for tumour progression along with stage and grade. Maximum MVD (p<0.00001) was shown to be an independent risk factors for tumour progression with stage alone. Table 3.17

In the corrected database MVD as measured by whatever method remained a significant risk factor for progression in univariable and multivariable binary logistic regression models.

3.3.6 Summary of MVD results

In summary, using the method of cumulative mean, 30 random areas are required to be counted to calculate the MVD of a section of superficial bladder cancer in this series. The computer image analysis method devised here, when used with suitable vessel identification, correlated very well with manual vessel
Table 3.16  Binary logistic regression analysis for MVD related to tumour progression

<table>
<thead>
<tr>
<th></th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manual MVD count</td>
<td>1.220</td>
<td>1.135-1.311</td>
<td>0.0000001</td>
</tr>
<tr>
<td>Maximum manual count</td>
<td>1.075</td>
<td>1.044-1.106</td>
<td>0.000001</td>
</tr>
<tr>
<td>Computer MVD count</td>
<td>1.236</td>
<td>1.160-1.318</td>
<td>0.00000001</td>
</tr>
<tr>
<td>Maximum computer count</td>
<td>1.072</td>
<td>1.046-1.099</td>
<td>0.0000003</td>
</tr>
<tr>
<td>Manual MVD per unit area</td>
<td>1.019</td>
<td>1.011-1.026</td>
<td>0.000001</td>
</tr>
<tr>
<td>Computer MVD per unit area</td>
<td>1.022</td>
<td>1.015-1.029</td>
<td>0.00000002</td>
</tr>
<tr>
<td>Number of areas counted</td>
<td>1.295</td>
<td>0.910-1.005</td>
<td>0.079</td>
</tr>
<tr>
<td>Median Area measured (μm²)</td>
<td>1.000</td>
<td>1.000-1.000</td>
<td>0.258</td>
</tr>
</tbody>
</table>

Statistically significant results are shown in bold type.
Table 3.17 Multivariable binary logistic regression of MVD and clinicopathological risk factors

<table>
<thead>
<tr>
<th></th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td>2.694</td>
<td>1.302-5.574</td>
<td>0.008</td>
</tr>
<tr>
<td>Grade</td>
<td>1.702</td>
<td>1.016-2.851</td>
<td>0.043</td>
</tr>
<tr>
<td><em>cis</em></td>
<td>0.727</td>
<td>0.196-2.692</td>
<td>0.633</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>1.721</td>
<td>0.882-3.357</td>
<td>0.112</td>
</tr>
<tr>
<td>MVD</td>
<td>1.222</td>
<td>1.140-1.310</td>
<td>0.00000001</td>
</tr>
</tbody>
</table>

Table 3.17.1 Multivariable binary logistic regression containing MVD

<table>
<thead>
<tr>
<th></th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td>2.989</td>
<td>1.449-6.165</td>
<td>0.003</td>
</tr>
<tr>
<td>Grade</td>
<td>1.673</td>
<td>1.004-2.787</td>
<td>0.048</td>
</tr>
<tr>
<td><em>cis</em></td>
<td></td>
<td></td>
<td>0.447</td>
</tr>
<tr>
<td>Multiplicity</td>
<td></td>
<td></td>
<td>0.118</td>
</tr>
<tr>
<td>MVD / area</td>
<td>1.021</td>
<td>1.013-1.029</td>
<td>0.0000001</td>
</tr>
</tbody>
</table>

Table 3.17.2 Multivariable binary logistic regression containing MVD / area

<table>
<thead>
<tr>
<th></th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td>3.052</td>
<td>1.513-6.155</td>
<td>0.002</td>
</tr>
<tr>
<td>Grade</td>
<td>1.565</td>
<td>0.951-2.577</td>
<td>0.078</td>
</tr>
<tr>
<td><em>cis</em></td>
<td>1.490</td>
<td>0.464-4.782</td>
<td>0.503</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>1.815</td>
<td>0.942-3.498</td>
<td>0.075</td>
</tr>
<tr>
<td>Maximum MVD</td>
<td>1.068</td>
<td>1.039-1.097</td>
<td>0.00000001</td>
</tr>
</tbody>
</table>

Table 3.17.3 Multivariable binary logistic regression containing maximum MVD

Statistically significant results are shown in bold type.
counting. The computer image analysis system is considerably faster than manual counting.

A high MVD in superficial bladder cancer was associated with high tumour stage, grade and cis at presentation. MVD was higher at presentation in those cases that subsequently progressed to muscle invasive disease. High MVD was also an independent risk factor for tumour stage progression in univariable and multivariable regression analysis models.

3.4.0 VEGF Results

3.4.1 VEGF expression using the StreptABComplex/HRP Duet, Mouse/Rabbit (ABC) kit

When the VG-1 (Oxford) antibody was developed using the StreptABComplex / HRP Duet, Mouse/Rabbit (ABC) kit, there was unfortunately, very little staining, and it was of poor quality. The results were consistently poor despite several attempts.

3.4.2 VEGF expression after Antigen retrieval

When the VG-1 (Oxford) antibody was developed after using antigen retrieval by microwave technique in TRIS / EDTA pH 9.0 buffer (section 2.7.3.1), more VEGF staining was revealed. There was staining also seen however, on the negative controls.
The attempt was repeated using microwave antigen retrieval in Citrate buffer, pH6.0. Once again, VEGF staining was very poor and continued to be seen on negative controls.

3.4.3 VEGF expression using the DAKO EnVision+ kit

VG-1 staining was far superior using the DAKO EnVision+ kit. Of the three antigen retrieval techniques used, microwaving in TRIS / EDTA pH 9.0 buffer gave the best result. The preferred dilution of the primary antibody supernatant was 1:2, *figures 3.8*.

3.4.4 VEGF expression in superficial bladder cancer

Good clean VEGF staining was seen in the tumour cells, and in the underlying stroma in the sections of superficial bladder cancer, *figure 3.9*. Tumour cell staining was heterogeneous but in some cases it appeared more pronounced adjacent to the basement membrane.

VEGF staining was seen in tumour cells in 269 (91.89%) cases and in the stroma in 272 (92.83%). Tumour cell staining was graded as weak in 104 (35.5%) cases, moderate in 86 (29.4%) cases and strong in 79 (27%) cases. VEGF stromal staining was weak in 81 (27.6%) cases, moderate in 104 (35.5%) cases and extensive in 87 (29.7%) cases.

In a statistical analysis to compare VEGF expression between the cases that recurred and those that did not there was no significant difference with
Figure 3.8  VEGF staining using Microwave antigen retrieval and the EnVision+ kit  (x200)

Figure 3.8.1  VG-1 (1:1) staining, microwave antigen retrieval. Normal Human kidney. (x200)

Figure 3.8.2  VG-1 (1:2) staining, microwave antigen retrieval. Normal Human kidney. (x200)
Figure 3.8. VEGF staining using Microwave antigen retrieval using the EnVision kit (x200)

Figure 3.8.3 VG-1 (1:4) staining, microwave antigen retrieval. Normal Human kidney. (x200)

Figure 3.8.4 VG-1 (1:8) staining, microwave antigen retrieval. Normal Human kidney. (x200)
Figure 3.8  VEGF staining using Microwave antigen retrieval using the EnVision kit (x200)

Figure 3.8.5 Negative control, microwave antigen retrieval. Normal Human kidney. (x200)
Figure 3.9 Distribution of VEGF staining in Superficial bladder cancer

Figure 3.9.1 VEGF cytoplasmic tumour cell staining. Superficial bladder cancer. x200

Figure 3.9.2 VEGF stromal staining. Superficial bladder cancer. x200
respect to tumour cell staining \((p=0.860)\) or stromal staining \((p=0.902)\).
Similarly, there was no significant difference in tumour cell \((p=0.144)\) or stromal staining \((p=0.827)\) between progressed and those cases that did not.

VEGF staining in the tumour cells \((p=0.160)\) or in the stroma \((p=0.810)\) was not found to be an independent risk factor for progression to invasive bladder cancer in a logistic regression model.

The percentage of staining of VEGF in tumour cells was converted from a continuous variable into a staining score. This was identical to the score used to grade VEGF immunostaining used by Chow et al (Chow, Liu, Chan, Cheng, & Tzai. 1999). Staining was graded as 0 for no staining, 1+ for less than 5% staining, 2+ for between 5 and 25% staining and 3+ for greater than 25% staining. Using this score VEGF tumour cell staining approached but did not reach statistical significance in a logistic regression model, \((p=0.050)\).

There was no statistical association between VEGF staining and tumour stage or grade, the presence of cis or tumour multiplicity. In the corrected database the lack of statistical correlation between VEGF and progression of superficial bladder cancer was retained.

### 3.4.5 Summary of VEGF results

Vascular Endothelial Growth Factor immunostaining was widespread, seen in tumour cells in 91.89% and in the stroma in 92.83% of cases in this
series. The amount or intensity of VEGF staining was however, not seen to correlate with standard clinicopathological risk factors. There was no statistical difference in VEGF staining between cases that progressed and cases that did not progress and VEGF was not found to be a prognostic risk factor in this series.

3.5.0 Thymidine Phosphorylase Results

3.5.1 Dilutions and antigen retrieval using TP (Oxford)

Initial experiments using microwaving, pressure cooking, the Menarini microwavable pressure cooker, and no antigen retrieval all gave strong staining with all dilutions.

After repeating the experiment at a dilution of 1:20 with microwaving and no retrieval, this dilution was found to be acceptable. No antigen retrieval was necessary, figures 3.10.

3.5.2 Thymidine Phosphorylase expression in superficial bladder cancer

Thymidine Phosphorylase staining was seen in the tumour cells, the stroma, in inflammatory cells and in endothelial cells in sections of superficial bladder cancer. Figure 3.11.

TP expression was seen in tumour cells in 181 (62.78%) of cases. In 71 cases this was weak, in 55 it was moderate and in 55 it was strong. Stromal staining was seen in 213 (72.69%) cases. In 78 there was only a small amount, in 68 there was a moderate distribution of staining, in 59 cases there was a large
Figure 3.10  TP dilutional experiment at 1:20 using DAKO EnVision+ in Normal human tonsil (x200)

Figure 3.10.1  TP staining macrophages, 1:20 Microwave retrieval. Normal Human tonsil. (x200)

Figure 3.10.2  Negative control Microwave retrieval. Normal Human tonsil. (x200)
Figure 3.10  
TP dilutional experiment. At 1:20 DAKO EnVision+ in Normal human tonsil (x200)

Figure 3.10.3  
TP staining, 1:20 No antigen retrieval. Normal Human tonsil. (x200)

Figure 3.10.4  
Negative control, No antigen retrieval. Normal Human tonsil. (x200)
Figure 3.11  TP staining distribution in superficial bladder cancer (x200)

Figure 3.11.1  Tumour cell staining (x200)

Figure 3.11.2  Stromal staining (x200)
Figure 3.11 TP staining distribution in superficial bladder cancer (x200)

Figure 3.11.3 Inflammatory cell staining (x200)

Figure 3.11.4 Endothelial cell staining (x200)
amount of staining and in 8 cases the staining was widespread and extensive. Stromal staining intensity was graded as weak in 30 cases, moderate in 102 cases and strong in 81 cases. There was TP staining of inflammatory cells in 231 (78.84%) cases. This was focal in 85 cases, moderate in 96 cases and there were many stained cells in 50 cases. Endothelial cell staining was seen in 120 (40.95%) cases. This was focal in 52 cases, moderate in 60 cases and pronounced in 8 cases.

There was no association between TP staining and tumour recurrence. There was however, significantly more TP staining in those cases that progressed to invasive disease. This was apparent with percentage (p=0.003) and intensity (p=0.003) of stromal TP staining, TP expression in inflammatory cells (p=0.008) and TP staining in endothelial cells (p=0.00024). There was no difference however in TP staining seen in tumour cells (p=0.339).

There was a strong correlation between TP staining and tumour stage and grade at presentation. With all methods of TP staining assessment this was present and showed that pT1 disease at presentation was associated with higher TP tumour staining (p=0.001) and intensity (p=0.005), higher stromal TP staining (p<0.00001) and intensity (p<0.00001), and increased staining in inflammatory (p<0.00001) and endothelial cells (p<0.00001).
Higher grade disease at presentation was associated with increased TP stromal staining \( (p=0.0001) \) and intensity \( (p=0.00004) \), and increased TP staining in inflammatory \( (p=0.001) \) and endothelial cells \( (p=0.0002) \).

There was no statistical association between TP staining and the presence of cis or tumour multiplicity at presentation.

In a binary logistic regression analysis TP expression in the stroma was found to be a predictive variable for subsequent progression to invasive disease. Both increasing amount \( (p=0.005) \) and intensity \( (p=0.004) \) of TP stromal staining were found to be predictive. Increased expression of TP staining in inflammatory cells was also a predictive factor for progression \( (p=0.010) \). TP staining of endothelial cells again was the strongest predictive factor for progression to muscle invasive disease \( (p=0.00027) \). The results of the binary logistic regression analysis for all measured TP expression variables are given in Table 3.18. There was no association between TP staining and tumour recurrence.

Each individual TP staining variable was entered separately into the multivariable binary logistic regression model containing tumour stage, tumour grade, presence of cis and tumour multiplicity discussed in section 3.3.5. In this model TP endothelial cell staining was found to be an independent prognostic risk factor tumour progression along with stage. Table 3.19
Table 3.18  Binary logistic regression analysis for all measured TP expression variables

<table>
<thead>
<tr>
<th></th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of TP staining in tumour cells</td>
<td>1.001</td>
<td>0.985-1.017</td>
<td>0.931</td>
</tr>
<tr>
<td>Intensity of TP staining in tumour cells</td>
<td>1.093</td>
<td>0.870-1.373</td>
<td>0.446</td>
</tr>
<tr>
<td>Extent of TP staining in the stroma</td>
<td>1.381</td>
<td>1.101-1.732</td>
<td>0.005</td>
</tr>
<tr>
<td>Intensity of TP staining in the stroma</td>
<td>1.428</td>
<td>1.120-1.821</td>
<td>0.004</td>
</tr>
<tr>
<td>Extent of TP staining in inflammatory cells</td>
<td>1.421</td>
<td>1.088-1.856</td>
<td>0.010</td>
</tr>
<tr>
<td>Extent of TP staining in endothelial cells</td>
<td>1.695</td>
<td>1.276-2.252</td>
<td>0.00027</td>
</tr>
</tbody>
</table>

Statistically significant results are shown in bold type.
Table 3.19  Multivariable binary logistic regression of TP and clinicopathological risk factors

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<th>p value</th>
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<td>Stage</td>
<td>2.783</td>
<td>1.426-5.430</td>
<td>0.003</td>
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<tr>
<td>Grade</td>
<td>1.538</td>
<td>0.965-2.450</td>
<td>0.070</td>
</tr>
<tr>
<td>cis</td>
<td>1.781</td>
<td>0.614-5.165</td>
<td>0.288</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>1.810</td>
<td>0.975-3.360</td>
<td>0.060</td>
</tr>
<tr>
<td>TP endothelial cell staining</td>
<td><strong>1.394</strong></td>
<td><strong>1.004-1.934</strong></td>
<td><strong>0.047</strong></td>
</tr>
</tbody>
</table>

Statistically significant results are shown in bold type.
In the corrected database, the amount (p=0.005) and intensity (p=0.004) of stromal staining and TP staining in inflammatory cells (p=0.016) and endothelial cells (p=0.001) was significantly higher in cases that progressed. TP stromal staining intensity (p=0.005), inflammatory cell (p=0.019) and endothelial (p=0.001) cell staining were significant predictive risk factors for progression in univariable binary logistic regression model. TP staining in the stroma (p=0.047) and TP endothelial cell staining (p=0.030) remained risk factors in the multivariable model containing clinicopathological risk factors.

The amount and intensity of TP endothelial cell staining was correlated with outcome. This could be a function of the number of the MVD so TP staining was divided by MVD for each case to give a representation of the amount of TP per number of vessels. Once corrected in this way TP endothelial cell staining was no longer a predictive risk factor in the univariable binary logistic regression analysis (p=0.891).

3.5.3 Summary of TP results

Thymidine Phosphorylase immunostaining was distributed in tumour cells, the stroma, inflammatory cells and in the endothelial cells of vessels. The amount and intensity of stromal staining and also inflammatory cell staining was higher in cases that subsequently progressed to muscle invasion. However, the relationship was most pronounced with TP staining of vessels which was a predictive factor for progression in univariable and multivariable models. Once corrected for the MVD this was no longer a significant risk factor.
3.6.0 Thrombospondin Results

3.6.1 Initial staining of TSP (Ab7)

Initial staining using TSP (Ab-7) antibody and the DAKO EnVision+ kit produced some staining of macrophages in normal human tonsil. However, there was no evidence of staining in a series of superficial bladder cancers and staining of positive controls, although present was poor.

3.6.2 Results of TSP (Ab4) & TSP (Ab1) staining using DAKO EnVision+

When the new antibodies TSP (Ab-1) and TSP (Ab-4) were used with the using the DAKO EnVision+ kit, no staining was seen using microwave antigen retrieval or no antigen retrieval with either antibody.

3.6.3 Results of TSP-1 staining using pronase predigestion

Staining carried out with TSP-1 Ab4 antibody pre-treated with pronase for 10 minutes as antigen retrieval revealed endothelial staining in the normal colon and sporadic staining of macrophages in the normal tonsil.

When staining was repeated with all three primary antibodies (Ab-1 at a dilution of 1:10, Ab-4 at a dilution of 1:50 and Ab-7 at a dilution of 1:100) pretreated with pronase for 15 minutes, there was endothelial cell and some extracellular staining with TSP (Ab-4), figure 3.12. Staining was not seen with the antibodies Ab-1 or Ab-7.
Figure 3.12  
*TSP (Ab-4) at 1:150, staining with Pronase predigestion for 15 minutes using DAKO EnVision kit. (x200)*

---

Figure 3.12.1  
*TSP (Ab4) staining, Pronase predigestion with endothelial staining. Normal Human Colon. (x200)*

---

Figure 3.12.2  
*Negative control, Pronase predigestion. Normal Human Colon. (x200)*
3.6.4 Results of TSP-1 staining using antigen retrieval by microwaving in TRIS / HCL buffer at pH 1.0

Using this technique good staining was seen with TSP (Ab-7). There was however, considerable distortion of tissue architecture due the aggressive antigen retrieval technique. This technique was not as successful with TSP (Ab-1) or TSP (Ab-4). This technique was therefore only used with TSP (Ab-7). Dilution experiments with TSP (Ab-7) found 1:100 to be the optimum dilution, figure 3.13.

3.6.5 Thrombospondin-1 expression in superficial bladder cancer

With both TSP-1 Ab-4 and TSP-1 Ab-7 immunohistochemical staining was seen around the vessels (perivascular), the basement membrane, in the tumour cells and in the stroma of the superficial bladder cancer. Figures 3.14 & 3.15.

3.6.5.1 TSP (Ab-7) expression

There was perivascular TSP (Ab-7) staining seen in 183 (62.58%) cases. In 62 cases this staining was graded as weak, in 115 cases moderate and in 6 cases strong. TSP (Ab-7) staining was seen in the basement membrane in 198 (67.58%) cases; staining was weak in 75 cases, moderate in 115 cases and strong in 8 cases. Tumour staining was only seen in 53 cases; weak in 21, moderate in 28 and strong in 4 cases. Stromal staining of TSP (Ab-7) was seen in 47 (16.04%) cases; weak in 13 cases, moderate in 28 cases and strong in 6 cases.
Figure 3.13  
*Figure 3.13  TSP (Ab-7) dilutional experiment with microwave retrieval in pH 1 TRIS / HCL buffer. DAKO EnVision+ kit. (x200)*

Figure 3.13.1  
*Figure 3.13.1 TSP (Ab7) staining at 1:100, Normal Human Colon. (x200)*

Figure 3.13.2  
*Figure 3.13.2 TSP (Ab7) staining at 1:150, Normal Human Colon. (x200)*
Figure 3.13  TSP (Ab-7) dilutional experiment with microwave retrieval in pH1 TRIS / HCL buffer. DAKO EnVision^ kit. (x200)

Figure 3.13.3  TSP (Ab7) staining at 1:200, Normal Human Colon. (x200)

Figure 3.13.4  Negative control, TRIS/HCL pH1 buffer. SBC. (x200)
Figure 3.14  TSP (Ab-7) staining distribution in Superficial bladder cancer (x200)

Figure 3.14.1  Perivascular Staining.  
(x200)

Figure 3.14.2  Staining defining the lining of the papillary cores  
(x200)
Figure 3.14  TSP (Ab-7) staining distribution in Superficial bladder cancer (x200)

Figure 3.14.3  Stromal staining (x200)

Figure 3.14.4  Tumour cell staining. (x200)
Figure 3.15  TSP (Ab-4) staining distribution in Superficial bladder cancer (x200)

Figure 3.15.1  Perivascular Staining. (x200)

Figure 3.15.2  Staining defining the lining of the papillary cores (x200)
Figure 3.15  TSP (Ab-4) staining distribution in Superficial bladder cancer (x200)

Figure 3.15.3  Stromal staining (x200)

Figure 3.15.4  Tumour cell staining. (x200)
There was no statistical association between TSP (Ab-7) staining and tumour recurrence. There was a significant difference in the amount of stromal TSP (Ab-7) staining between cases that progressed and cases that did not progress. This was seen in both the percentage of stromal staining (p=0.00043) and in the intensity of stromal staining (p=0.00044).

An increase in TSP (Ab-7) stromal staining (p<0.00001) or intensity (p<0.00001) was significantly associated with a higher stage (pT1) at presentation. TSP (Ab-7) stromal staining (p=0.001) and intensity (p=0.002) were also associated with a higher grade at presentation. There was no association with TSP (AB-7) staining and cis or tumour multiplicity. There was no statistical association between TSP (Ab-7) tumour staining and any clinicopathological variables.

In a binary logistic regression analysis expression of TSP (Ab-7), intensity of tumour cell staining (p=0.025), the stromal staining score (p=0.009) and intensity of stromal staining (p=0.002) all were found to be risk factors for predicting progression to muscle invasive disease, *table 3.20*.

Each individual TSP (Ab-7) staining variable was entered separately into the multivariable binary logistic regression model containing tumour stage, tumour grade, presence of cis and tumour multiplicity discussed in section 3.3.5. In this model increased TSP (Ab-7) tumour cell staining was found to be an
### Table 3.20  Binary logistic regression analysis for TSP (Ab-7) expression related to tumour progression

<table>
<thead>
<tr>
<th></th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of perivascular staining</td>
<td>1.012</td>
<td>0.991-1.033</td>
<td>0.270</td>
</tr>
<tr>
<td>Perivascular staining score</td>
<td>1.079</td>
<td>0.959-1.214</td>
<td>0.208</td>
</tr>
<tr>
<td>Intensity of perivascular staining</td>
<td>1.277</td>
<td>0.959-1.701</td>
<td>0.094</td>
</tr>
<tr>
<td>Percentage of papillary core staining</td>
<td>0.994</td>
<td>0.978-1.012</td>
<td>0.524</td>
</tr>
<tr>
<td>Papillary core staining score</td>
<td>0.946</td>
<td>0.851-1.050</td>
<td>0.298</td>
</tr>
<tr>
<td>Intensity of papillary core staining</td>
<td>0.838</td>
<td>0.627-1.121</td>
<td>0.234</td>
</tr>
<tr>
<td>Percentage of tumour cell staining</td>
<td>1.013</td>
<td>0.989-1.038</td>
<td>0.294</td>
</tr>
<tr>
<td>Tumour cell staining score</td>
<td>1.091</td>
<td>0.945-1.260</td>
<td>0.235</td>
</tr>
<tr>
<td>Intensity of Tumour cell staining</td>
<td><strong>1.480</strong></td>
<td><strong>1.050-2.088</strong></td>
<td><strong>0.025</strong></td>
</tr>
<tr>
<td>Percentage of stromal staining</td>
<td>1.029</td>
<td>0.993-1.067</td>
<td>0.111</td>
</tr>
<tr>
<td>Stromal staining score</td>
<td><strong>1.279</strong></td>
<td><strong>1.063-1.539</strong></td>
<td><strong>0.009</strong></td>
</tr>
<tr>
<td>Intensity of stromal staining</td>
<td><strong>1.679</strong></td>
<td><strong>1.208-2.332</strong></td>
<td><strong>0.002</strong></td>
</tr>
</tbody>
</table>

Statistically significant results are shown in bold type.
independent prognostic risk factor for tumour progression along with stage and grade. *Table 3.21*

In the corrected database tumour staining intensity (p=0.028) and stromal staining (p=0.001) remained significantly different in the two groups. Tumour staining intensity (p=0.008) and stromal staining (p=0.024) were significant risk factors in a univariable analysis. In a multivariable analysis with clinocopathological variables TSP (Ab-7) tumour staining (p=0.021) and intensity (p=0.001) remained risk factors along with stage (p<0.0001) for disease progression. TSP (Ab-7) stromal staining was no longer a risk factor.

### 3.6.5.2 TSP (Ab-4) expression

There was perivascular TSP (Ab-4) staining seen in 158 (53.92%) cases. In 17 cases this staining was graded as weak, in 121 cases moderate and in 20 cases strong. TSP (Ab-4) staining was seen in the basement membrane in 140 (47.78%) cases; staining was weak in 38 cases, moderate in 81 cases and strong in 21 cases. Tumour staining was only seen in 13 cases this was weak in 7 and moderate 6 cases. Stromal staining of TSP (Ab-4) was seen in 31 (10.58%) cases; weak in 2 cases, moderate in 16 cases and strong in 13 cases.

There was a significant difference in the amount of perivascular TSP (Ab-4) staining between cases that progressed and cases that did not progress. This was seen in the percentage of perivascular staining (p=0.004) in the
### Table 3.21 Multivariable binary logistic regression of TSP (Ab-7) and clinicopathological risk factors

<table>
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<th>Odds ratio</th>
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<tbody>
<tr>
<td>Stage</td>
<td>3.182</td>
<td>1.662-6.093</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Grade</td>
<td>1.714</td>
<td>1.070-2.747</td>
<td>0.025</td>
</tr>
<tr>
<td>cis</td>
<td>1.912</td>
<td>0.660-5.544</td>
<td>0.233</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>1.830</td>
<td>0.989-3.389</td>
<td>0.054</td>
</tr>
<tr>
<td>TSP (Ab-7)</td>
<td>1.176</td>
<td>1.002-1.380</td>
<td>0.047</td>
</tr>
</tbody>
</table>

Statistically significant results are shown in bold type.

### Table 3.21.1 Multivariable binary logistic regression containing TSP (Ab-7) tumour staining score

<table>
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<th>95% CI</th>
<th>p value</th>
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<tbody>
<tr>
<td>Stage</td>
<td>3.347</td>
<td>1.734-6.463</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Grade</td>
<td>1.703</td>
<td>1.064-2.726</td>
<td>0.027</td>
</tr>
<tr>
<td>cis</td>
<td>2.086</td>
<td>0.714-6.095</td>
<td>0.179</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>1.861</td>
<td>1.000-3.465</td>
<td>0.050</td>
</tr>
<tr>
<td>TSP (Ab-7)</td>
<td>1.794</td>
<td>1.203-2.678</td>
<td>0.004</td>
</tr>
</tbody>
</table>

### Table 3.21.2 Multivariable binary logistic regression containing TSP (Ab-7) tumour staining intensity

<table>
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<th>p value</th>
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<tbody>
<tr>
<td>Stage</td>
<td>3.347</td>
<td>1.734-6.463</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Grade</td>
<td>1.703</td>
<td>1.064-2.726</td>
<td>0.027</td>
</tr>
<tr>
<td>cis</td>
<td>2.086</td>
<td>0.714-6.095</td>
<td>0.179</td>
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<tr>
<td>Multiplicity</td>
<td>1.861</td>
<td>1.000-3.465</td>
<td>0.050</td>
</tr>
<tr>
<td>TSP (Ab-7)</td>
<td>1.794</td>
<td>1.203-2.678</td>
<td>0.004</td>
</tr>
</tbody>
</table>
intensity of perivascular staining ($p=0.002$) and in the perivascular staining score ($p=0.004$).

There was no association between TSP (Ab-4) perivascular staining and tumour stage or grade. However, there was such an association with TSP (Ab-4) stromal staining. Increased stromal staining ($p=0.01$) and intensity ($p=0.031$) were associated with higher stage (pT1) and grade ($p=0.00003$ & $p=0.0002$ respectively) disease at presentation.

In a binary logistic regression analysis, expression of TSP (Ab-4) in the perivascular area as seen in the perivascular staining score ($p=0.043$) and perivascular staining intensity ($p=0.002$) were risk factors for progression to invasive disease. However, these parameters gave a decreased risk of progression, Table 3.22.

Each individual TSP (Ab-4) staining variable was entered separately into the multivariable binary logistic regression model containing tumour stage, tumour grade, presence of cis and tumour multiplicity discussed in section 3.3.5. In this model reduced TSP (Ab-4) perivascular staining intensity was found to be an independent prognostic risk factor for tumour progression along with stage and grade. Table 3.23.

In the corrected database TSP (Ab-4) perivascular staining ($p=0.008$) and intensity ($p=0.011$) remained significantly different between groups. However,
Table 3.22  Binary logistic regression analysis for TSP (Ab-4) expression related to tumour progression

<table>
<thead>
<tr>
<th></th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>p value</th>
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</thead>
<tbody>
<tr>
<td>Percentage of perivascular staining</td>
<td>0.993</td>
<td>0.966-1.020</td>
<td>0.589</td>
</tr>
<tr>
<td>Perivascular staining score</td>
<td>0.854</td>
<td>0.733-0.995</td>
<td>0.043</td>
</tr>
<tr>
<td>Intensity of perivascular staining</td>
<td>0.688</td>
<td>0.516-0.866</td>
<td>0.002</td>
</tr>
<tr>
<td>Percentage of papillary core staining</td>
<td>0.998</td>
<td>0.979-1.018</td>
<td>0.854</td>
</tr>
<tr>
<td>Papillary core staining score</td>
<td>0.966</td>
<td>0.857-1.088</td>
<td>0.566</td>
</tr>
<tr>
<td>Intensity of papillary core staining</td>
<td>0.948</td>
<td>0.735-1.222</td>
<td>0.679</td>
</tr>
<tr>
<td>Percentage of tumour cell staining</td>
<td>0.731</td>
<td>0.464-1.152</td>
<td>0.177</td>
</tr>
<tr>
<td>Tumour cell staining score</td>
<td>0.309</td>
<td>0.053-1.812</td>
<td>0.193</td>
</tr>
<tr>
<td>Intensity of Tumour cell staining</td>
<td>0.283</td>
<td>0.049-1.637</td>
<td>0.159</td>
</tr>
<tr>
<td>Percentage of stromal staining</td>
<td>1.006</td>
<td>0.974-1.039</td>
<td>0.707</td>
</tr>
<tr>
<td>Stromal staining score</td>
<td>1.129</td>
<td>0.916-1.393</td>
<td>1.393</td>
</tr>
<tr>
<td>Intensity of stromal staining</td>
<td>1.138</td>
<td>0.818-1.582</td>
<td>0.433</td>
</tr>
</tbody>
</table>

Statistically significant results are shown in bold type.
Table 3.23  *Multivariable binary logistic regression of TSP (Ab-4) and clinicopathological risk factors*

<table>
<thead>
<tr>
<th></th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td>3.272</td>
<td>1.691-6.332</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Grade</td>
<td>1.649</td>
<td>1.026-2.649</td>
<td>0.039</td>
</tr>
<tr>
<td><em>cis</em></td>
<td>1.343</td>
<td>0.453-3.981</td>
<td>0.595</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>1.752</td>
<td>0.942-3.258</td>
<td>0.076</td>
</tr>
<tr>
<td>TSP (Ab-4) perivascular staining intensity</td>
<td>0.712</td>
<td>0.532-0.952</td>
<td>0.022</td>
</tr>
</tbody>
</table>

Statistically significant results are shown in bold type.
this was no longer seen as a risk factor for progression in univariable or multivariable binary logistic regression analyses.

3.6.6 Summary of Thrombospondin-1 results

Thrombospondin-1 expression with both primary antibodies was seen in the same areas in the sections; perivascular, lining the papillary cores, in the stroma and in tumour cells. The two antibodies were expressed in the same areas of tissue in individual cases although the intensity of staining and overall amount of staining varied.

TSP (Ab-7) immunostaining in the stroma was more extensive in those cases that progressed to muscle invasion. Stromal staining was also associated with higher stage and grade disease at presentation. Both stroma and tumour cell staining were predictive factors for progression and tumour staining was an independent variable for risk of progression.

Significantly less extensive perivascular TSP (Ab-4) immunostaining was seen in those cases that progressed to muscle invasive disease. Decreased perivascular staining was an independent prognostic risk factor for disease progression in both univariable and multivariable binary logistic regression models. This relationship however, was lost in the corrected database.
3.7.0 p53 Results

3.7.1 Results of p53 staining technique.

Staining of p53 worked well using the DAKO EnVision+ kit at a dilution of 1:100. Antigen retrieval was by pressure cooking in citrate buffer, pH 6.0, figure 3.16.

3.7.2 p53 expression in superficial bladder cancer

Staining by p53 was clean and clear and seen in the nuclei of tumour cells, figure 3.17.

Staining of p53 positive cells was recorded as a continuous variable. As well as analysing it so, two separate sets of data were created with tumours being graded as p53 positive or negative. In the first set, a tumour was deemed positive if the number of p53 stained cells was greater than 5%, in the second set a positive tumour had greater than 20% stained cells.

Overall, 226 (77.13%) cases contained some p53 stained cells. 123 (41.98%) cases were graded positive with a 5% cut off and 56 (19.11%) were positive when a 20% cut off was used.

There was a significant difference in p53 staining between cases that progressed and cases that did not progress. This was seen in all three p53 data sets; percentage p53 staining assessed as a continuous variable (p=0.003), with a 5% cut off (p=0.001) and with a 20% cut off (p=0.011).
Figure 3.16  
*p53 staining. DAKO EnVision* kit with microwave antigen retrieval. (x200)*

Figure 3.16.1  
P53 staining seen in carcinoma of the human breast known to be p53 positive. x200

Figure 3.16.2  
Negative control. Carcinoma of the human breast known to be p53 positive. x200
Figure 3.17  Distribution of p53 staining in human superficial bladder cancer. (x200)

Figure 3.17.1 Strong and widespread p53 staining seen in the nuclei of superficial bladder cancer cells. x200

Figure 3.17.2 Weak p53 staining seen in only a few nuclei of superficial bladder cancer cells. x200
In a binary logistic regression analysis p53 positivity was seen to be a significant prognostic factor in those cases that progressed to muscle invasive disease. This was true however p53 positivity was expressed, *table 3.24*. Using a 5% cut point for p53 positivity was superior with an odds ratio of 2.402 (p=0.001). Therefore in subsequent calculations this is used.

The p53 status of tumours significantly correlated with pT1 stage (p<0.00001) and high grade (p<0.00001) at presentation. The p53 status of the cases remained a significant risk factor for progression in the corrected database.

When p53 was entered into the multivariable binary logistic regression model, it no longer remained an independent risk factor; only stage remained so, *table 3.25*. When grade was removed from the equation p53 retained its significant risk status, *table 3.25.2*.

### 3.7.3 Summary of p53 results

Cases that were positive for p53 mutation were assessed by three different methods, percentage of p53 stained cells as a continuous variable, greater than 5% p53 cell staining and greater than 20% p53 cell staining. All three methods were found to correlate with tumour stage and grade. P53 positivity was seen more in the cases that progressed to muscle invasion and was a predictive risk factor for progression. A 5% cut off value for p53 positive cases gave the highest risk in the binary logistic regression. Only when grade was
Table 3.24  p53 status related to progression

<table>
<thead>
<tr>
<th></th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of p53 staining in</td>
<td>1.014</td>
<td>1.004-1.024</td>
<td>0.005</td>
</tr>
<tr>
<td>tumour cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive p53 status &gt;5%</td>
<td>2.402</td>
<td>1.410-4.092</td>
<td>0.001</td>
</tr>
<tr>
<td>Positive p53 status &gt;20%</td>
<td>2.193</td>
<td>1.184-4.061</td>
<td>0.013</td>
</tr>
</tbody>
</table>
Table 3.25 Multivariable binary logistic regression of p53 and clinicopathological risk factors

<table>
<thead>
<tr>
<th></th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td>2.890</td>
<td>1.490-5.604</td>
<td>0.002</td>
</tr>
<tr>
<td>Grade</td>
<td>1.374</td>
<td>0.846-2.230</td>
<td>0.199</td>
</tr>
<tr>
<td>cis</td>
<td>1.775</td>
<td>0.608-5.183</td>
<td>0.294</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>1.770</td>
<td>0.952-3.291</td>
<td>0.071</td>
</tr>
<tr>
<td>p53</td>
<td>1.746</td>
<td>0.936-3.256</td>
<td>0.080</td>
</tr>
</tbody>
</table>

Table 3.25.1 Multivariable binary logistic regression containing p53 and clinicopathological variables

<table>
<thead>
<tr>
<th></th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td>3.620</td>
<td>1.961-6.681</td>
<td>0.00004</td>
</tr>
<tr>
<td>cis</td>
<td>2.124</td>
<td>0.740-6.094</td>
<td>0.161</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>1.812</td>
<td>0.984-3.338</td>
<td>0.056</td>
</tr>
<tr>
<td>p53</td>
<td>1.967</td>
<td>1.073-3.606</td>
<td>0.029</td>
</tr>
</tbody>
</table>

Table 3.25.2 Multivariable binary logistic regression containing p53 and clinicopathological variables, with Grade removed.

Statistically significant results are shown in bold type.
removed from the multivariable equation was p53 a significant risk factor suggesting a relationship with tumour grade.

3.8.0 Summary of Results for individual angiogenesis factors

A high MVD measured by manual counting or by computer image analysis was found to be significantly associated with progression of SBC to invasive disease. The mean MVD of 30 areas and the maximum value were both associated with poor outcome. The mean MVD measured by computer image analysis was used as the definitive measurement as this was seen to be quick, accurate and reproducible. High MVD (p<0.0001) was an independent risk factor for progression in a multivariable analysis of clinicopathological variables along with stage and grade.

In this study the expression of VEGF measured by immunohistochemical staining was not found to be a significant risk factor in the recurrence or progression of SBC. Neither was there an association with VEGF staining and tumour stage or grade.

Increased TP staining in the stroma was associated with SBC progression (p=0.005) in a binary logistic regression model. This was also seen in TP staining in inflammatory cells. This however could represent the extent of the inflammatory response rather than the function of TP. Increased TP staining in
endothelial cells ($p=0.00027$) was also found to be a positive risk factor for progression. This however, was likely to be a function of MVD.

Thrombospondin (Ab-7) staining in the stroma ($p=0.009$) and in tumour cells ($p=0.025$) was a significant risk factor for progression. Tumour cell staining ($p=0.004$) was also an independent risk factor in the multivariable analysis along with stage and grade.

A reduction in TSP (Ab-4) perivascular staining was found to be a significant ($p=0.043$) risk factor in SBC progression. This association was retained in the multivariable analysis ($p=0.022$). There was no association between TSP (Ab-4) perivascular staining and grade or stage. However, interestingly there was an association between increased TSP (Ab-4) stromal staining and both stage ($p=0.01$) and grade ($p<0.001$).

Increased expression of p53 was an independent risk factor in SBC progression. A cut off of 5% staining was found to be the strongest predictor of progression ($p=0.001$). This was only retained as a significant factor in the multivariable analysis when grade was removed from the equation.
3.9.0 Correlation of factors and multivariable analysis

3.9.1 Relationship between angiogenic factors

Although VEGF staining was not found to be a predictive factor in the progression of SBC it was examined for any relationship with MVD. In a multiple regression model, there was no statistical association with MVD.

In a linear regression model, there was a weak ($\beta=0.134$) but significant ($p=0.022$) positive relationship between MVD and TP stromal staining.

There was a significant but weak correlation between high MVD and low TSP(Ab-4) perivascular staining. This was seen with both the amount of staining ($\beta=-0.167$, $p=0.004$) and intensity of staining ($\beta=-0.188$, $p=0.001$).

There was no correlation between TSP (Ab-7) staining in tumour cells or stroma and MVD. There was however, a weak correlation between TSP (Ab-7) perivascular staining and MVD. As with TSP (Ab-4) this was an inverse relationship ($\beta=-0.137$, $p=0.019$). There was a strong positive correlation between TSP (Ab-7) perivascular staining and TSP (Ab-4) perivascular staining ($\beta=0.439$, $p<0.0001$), also between TSP (Ab-7) basement membrane staining and TSP (Ab-4) basement membrane staining ($\beta=0.408$, $p<0.001$). This correlation was not seen in tumour staining or stromal staining.
Staining of p53 was positively correlated with MVD ($\beta=0.134$, $p=0.018$). Again this was a weak, albeit significant correlation. There was no correlation between p53 staining and expression of TSP (Ab-4) or TSP (Ab-7) staining.

3.9.2 Multivariable Analysis

A binary logistic multivariable analysis model was created to examine how the previously demonstrated clinicopathological and angiogenic risk factors behaved with respect to SBC progression. Clinicopathological variables in the equation were: age, the consumption of aspirin, stage, grade, the presence of $cis$, tumour multiplicity, TP stromal staining, TSP (Ab-7) tumour staining, TSP (Ab-4) perivascular staining, p53 staining and MVD.

In this analysis, age ($p=0.009$), aspirin consumption ($p=0.005$), stage ($p=0.012$), multiplicity ($p=0.022$), TSP (Ab-7) tumour staining ($p=0.001$), TSP (Ab-4) perivascular staining ($p=0.011$) and MVD ($p<0.0001$) were all independent predictive factors for SBC progression. Aspirin consumption and TSP (Ab-4) perivascular staining were both inversely proportional to progression. *Table 3.26.*

The model was then reapplied after removal of the non-contributing factors. This revealed that age ($p=0.003$), aspirin consumption ($p=0.004$), stage ($p<0.0001$), multiplicity ($p=0.01$), TSP (Ab-7) tumour staining ($p=0.001$), TSP (Ab-4) perivascular staining ($p=0.007$) and MVD ($p<0.0001$) were all independent predictive factors for SBC progression. Again aspirin consumption
### Table 3.26  Multivariable analysis

<table>
<thead>
<tr>
<th></th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.051</td>
<td>1.013-1.092</td>
<td>0.009</td>
</tr>
<tr>
<td>Aspirin consumption</td>
<td>0.086</td>
<td>0.015-0.483</td>
<td>0.005</td>
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<tr>
<td>Stage</td>
<td>2.984</td>
<td>1.276-6.976</td>
<td>0.012</td>
</tr>
<tr>
<td>Grade</td>
<td>1.604</td>
<td>0.909-2.829</td>
<td>0.103</td>
</tr>
<tr>
<td>cis</td>
<td>0.733</td>
<td>0.158-3.401</td>
<td>0.691</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>2.358</td>
<td>1.131-4.916</td>
<td>0.022</td>
</tr>
<tr>
<td>TP Stromal staining</td>
<td>1.213</td>
<td>0.851-1.729</td>
<td>0.285</td>
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<tr>
<td>TSP (Ab-7) tumour staining</td>
<td>2.332</td>
<td>1.445-3.766</td>
<td>0.001</td>
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<tr>
<td>TSP (Ab-4) perivascular staining</td>
<td>0.626</td>
<td>0.436-0.899</td>
<td>0.011</td>
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<tr>
<td>p53</td>
<td>1.443</td>
<td>0.696-2.994</td>
<td>0.324</td>
</tr>
<tr>
<td>MVD</td>
<td>1.021</td>
<td>1.013-1.030</td>
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### Table 3.26.1  All factors included

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<td>Age</td>
<td>1.058</td>
<td>1.019-1.098</td>
<td>0.003</td>
</tr>
<tr>
<td>Aspirin consumption</td>
<td>0.081</td>
<td>0.014-0.450</td>
<td>0.004</td>
</tr>
<tr>
<td>Stage</td>
<td>5.080</td>
<td>2.445-10.556</td>
<td>0.000013</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>2.595</td>
<td>1.259-5.349</td>
<td>0.010</td>
</tr>
<tr>
<td>TSP (Ab-7) tumour staining</td>
<td>2.293</td>
<td>1.428-3.682</td>
<td>0.001</td>
</tr>
<tr>
<td>TSP (Ab-4) perivascular staining</td>
<td>0.614</td>
<td>0.429-0.877</td>
<td>0.007</td>
</tr>
<tr>
<td>MVD</td>
<td>1.021</td>
<td>1.013-1.029</td>
<td>0.00000041</td>
</tr>
</tbody>
</table>

### Table 3.26.2  Non-contributory factors removed

Statistically significant results are shown in bold type.
and TSP (Ab-4) perivascular staining were both inversely proportional to progression. *Table 3.26.*

When the same model was used with the corrected database once again age (p=0.034), aspirin consumption (p=0.010), stage (p=0.004), multiplicity (p=0.011), TSP (Ab-7) tumour staining (p=0.001), TSP (Ab-4) perivascular staining (p=0.041) and MVD (p<0.0001) were all independent predictive factors for SBC progression. However, in this database, TP stromal staining was also seen to be predictive (p=0.034). *Table 3.27.*

### 3.10.0 Results Summary

#### 3.10.1 Factors associated with SBC recurrence

The clinicopathological variables, decreasing age (p=0.016), tumour multiplicity (p=0.024), the receiving of intravesical chemotherapy (p=0.002) or BCG (p=0.005) and cigarette smoking (p=0.010) were all found to be significant risk factors in SBC recurrence. MVD, VEGF, TP, TSP-1 and p53 were not risk factors for tumour recurrence.

#### 3.10.2 Factors associated with SBC progression

Increasing age (p=0.015), stage (p<0.0001), grade (p<0.0001) and tumour multiplicity (p=0.026) were all significant risk factors for tumour progression to invasive disease. The consumption of regular aspirin decreased the risk (p=0.026) of progression. High MVD (p<0.0001), amount (p=0.005), and intensity (p=0.004) of TP stromal staining, TP inflammatory cell staining
Table 3.27  Multivariable analysis using the corrected database

<table>
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<tr>
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<th>Odds ratio</th>
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<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.045</td>
<td>1.003-1.089</td>
<td>0.034</td>
</tr>
<tr>
<td>Aspirin consumption</td>
<td>0.093</td>
<td>0.015-0.568</td>
<td>0.010</td>
</tr>
<tr>
<td>Stage</td>
<td>3.608</td>
<td>1.523-8.545</td>
<td>0.004</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>2.870</td>
<td>1.268-6.498</td>
<td>0.011</td>
</tr>
<tr>
<td>TP Stromal staining</td>
<td>1.549</td>
<td>1.034-2.322</td>
<td>0.034</td>
</tr>
<tr>
<td>TSP (Ab-7) tumour staining</td>
<td>2.366</td>
<td>1.420-3.944</td>
<td>0.001</td>
</tr>
<tr>
<td>TSP (Ab-4) perivascular staining</td>
<td>0.652</td>
<td>0.443-0.982</td>
<td>0.041</td>
</tr>
<tr>
<td>MVD</td>
<td>1.023</td>
<td>1.014-1.033</td>
<td>0.00000041</td>
</tr>
</tbody>
</table>

Statistically significant results are shown in bold type.
(p=0.010), TP in endothelial cells (p<0.001), TSP (Ab7) tumour staining intensity (p=0.025), TSP (Ab-7) stromal staining score (p=0.009), TSP (Ab-7) intensity of stromal staining (p=0.002) and p53 positivity (p=0.001) were all significant risk factors for SBC progression. TSP (Ab-4) perivascular staining score (p=0.043) and intensity of perivascular staining (p=0.002) were significantly reduced in cases that subsequently progressed. There was no association between VEGF staining and progression.

3.10.3 Correlation of angiogenic and clinicopathological factors

MVD was associated with higher stage (p=0.001) and grade (p=0.022), disease and with cis (p=0.022).

pT1 disease at presentation was associated with TP tumour staining (p=0.001) and intensity (p=0.005), increased stromal TP staining (p<0.00001) and intensity (p<0.00001), and increased staining in inflammatory (p<0.00001) and endothelial cells (p<0.00001). Higher grade disease at presentation was associated with increased TP stromal staining (p=0.0001) and intensity (p=0.00004), and increased TP staining in inflammatory (p=0.001) and endothelial cells (p=0.0002).

An increase in TSP (Ab-7) stromal staining (p<0.00001) or intensity (p<0.00001) was significantly associated with a higher stage (pT1) at presentation. TSP (Ab-7) stromal staining (p=0.001) and intensity (p=0.002) were also associated with a higher grade at presentation.
There was no association between TSP (Ab-4) perivascular staining and tumour stage or grade. However, there was such an association with TSP (Ab-4) stromal staining. Increased stromal staining (p=0.01) and intensity (p=0.031) were associated with higher stage (pT1) and grade (p=0.00003 & p=0.0002 respectively) disease at presentation.

In the multivariable analysis, age (p=0.003), aspirin consumption (p=0.004), stage (p<0.0001), multiplicity (p=0.01), TSP (Ab-7) tumour staining (p=0.001), TSP (Ab-4) perivascular staining (p=0.007) and MVD (p<0.0001) were all independent predictive factors for SBC progression. These factors all remained significant predictors of progression in the corrected database with the addition of TP stromal staining which was also seen to be predictive (p=0.034).
4.0 Discussion

4.1 The Database

The cases of invasive SBC were derived from both the 1994/5 dataset and 1981-2000 dataset. The data was therefore treated as grouped and analysed as such, that is binary logistic regression was chosen as the key statistical test.

With a median age of 72 years and a male to female ratio of 3.3 : 1, the age and gender distribution of the database is equivalent to other studies. Within the total database the percentage of high stage (pT1) and high grade (G3) tumours was greater than expected. This is due to the biasing effect of the group of invasive tumours from the whole database. A further potential source of bias with respect to tumour grade and stage may be the variability in the interpretation of these factors by individual pathologists.

4.1.1 Clinico-pathological parameters and outcome

Risk factors for tumour recurrence in the binary logistic regression model were cigarette smoking (OR 2.092 CI 1.197-3.656, p=0.010) and multiplicity at presentation (OR 2.111 CI 1.104-4.035, p=0.024). This is in line with other studies (Parmar, Freedman, & Hargreave. 1989). Patients that recurred were younger (OR 0.970 CI 0.946-0.994), p=0.016). Patients having intravesical chemotherapy or BCG were also seen to be more likely to recur. However, more high risk cases are treated by intravesical therapy; this therefore could be an association rather than a prognostic risk factor in tumour recurrence.
The follow up period for cases that recurred was significantly longer than for those that never recurred or progressed. This is likely to be due to the limited length of urological surveillance in cases that remain tumour free for some considerable time. The difference, of course, remains a possible confounding factor in the interpretation of this dataset.

Factors associated with an increased risk of progression were a higher age at presentation (OR 1.032 CI 1.006–1.059, p=0.015) and tumour multiplicity (OR 1.902 CI 1.078–3.353, p=0.026). High tumour grade (OR 2.156 CI 1.484–3.132, p<0.001) and stage (OR 4.123 CI 2.371–7.170, p<0.001) at presentation was associated with progression; this is in line with previous studies (Millán-Ro, M, Millán-Rodriguez, et al. 2000).

It is interesting to note that taking regular aspirin appears to be a protective factor in tumour progression in this model (OR 0.190 CI 0.044–0.820, p=0.026). Aspirin has been shown to inhibit carcinogenesis in some bladder cancer cell lines and animal models (Klan, Knispel, & Meier. 1993; Murasaki, Zenser, & Davis. 1984; Yeh, Chung, & Wu. 1999). Thun et al studied the association between aspirin use and fatal cancer (Thun, Namboodiri, Calle, Flanders, & Heath. 1993). Although there was a decreased death rate for aspirin users in cancers of the oesophagus, stomach, colon and rectum, this was not seen in bladder cancer. In a large case control study, Castelao et al found that regular aspirin users were at decreased risk of developing bladder cancer (Castelao, Yuan, Gago-Dominguez, Yu, & Ross. 2000). The consumption of regular aspirin
has not been previously described as a preventative factor for SBC progression. The absolute numbers of patients taking aspirin in this database is small (table 3.3), therefore these results should be viewed with interest and caution. This small sample number represents a significant risk of bias in these results. The small numbers may represent poor recording in the case notes of aspirin use. As it is used commonly as a cardiovascular prophylactic in small dosage, it is often not perceived as important medication and therefore not stated in a medical history. The small numbers in this area of the study give rise to concern over its power to support a true significant result. This however, is the first time in the literature that the regular use of aspirin has been associated with progression in bladder cancer, previous studies have shown only a link to risk of development. A power calculation in this area would not have any basis.

4.2 Measurement of MVD

The limitations of MVD measurement have been discussed in section 1.3.4. The technique devised here addresses these with particular relevance to SBC. Previous papers, which have specifically attempted to measure MVD in SBC, have yielded conflicting results, table 1.3.

Solid tumours present a relatively homogeneous distribution of vascular architecture. It is therefore possible to use a variation of techniques to measure MVD. These techniques are usually derived from that used by Noel Weidner (Weidner, Semple, Welch, & Folkman. 1991). Pathological samples of superficial bladder cancer however, do not present this advantageous
architecture. Due to the papillary nature of these tumours and their piecemeal removal by TURBT they present a heterogeneous surface for analysis. Furthermore, the method of selecting hotspots to determine the most biologically active, and thus most relevant, area is not possible in SBC (Dickinson, Fox, Persad, Hollyer, Sibley, & Harris. 1994).

4.2.1 Identification of vessels

Previous studies have used mainly FVIIIRA, CD31 and CD34 for vessel identification. Bochner et al found that CD34 was superior to FVIIIRA in bladder cancer (Bochner, Nichols, Groshen, Skinner, & Cote RJ. 1995). In this study, CD34 was found to be superior to CD31.

As image analysis was used, the enhancement of staining and maximum contrast was required to improve automated recognition. Careful evaluation of several enhancers and counterstains found nickel sulphate enhanced CD34 and an extremely light haematoxylin counterstain to be superior.

4.2.2 Use of random area selection

As the hotspot technique was not suitable for SBC, random areas were sampled. The number of areas must be balanced between insufficient numbers to be representative and excess counting which is time wasteful. Therefore, the method of cumulative mean was used to determine the minimum number of areas required. The final number of 30 areas compares to the 12 areas used by Philp et al in that they examined a mixed case load of solid and papillary
tumours. Earlier studies using higher numbers give no indication that this number was calculated leading to speculation that it was chosen randomly (Algire, Chalkley, & Legallais. 1945; Mlynek, van Beunigen, Leder, & Streffer. 1985).

This study, along with many others, uses MVD expressed per unit area. An alternative would be to express MVD as a function of cellularity; MVD per number of tumour cells in a given area. It may be possible to adapt a computer image analysis system to count both cells and microvessels. In order to do this separate staining may be required. This in turn may require the use of serial sections. This alternative technique has not been addressed in this thesis.

4.2.3 Use of a computerised image analysis system

The computerised image analysis system was used here not to add to a MVD count with measurement of extra parameters, but to enable multiple areas to be assessed quickly and accurately. The system was therefore designed to reproduce a manual count. It was successful in that the correlation between automated and manual systems was $r=0.95$. This is superior to other methods where a correlation has been performed ($r=0.68 - r=0.86$) (Kumar-Singh, Vermeulen, Weyler, et al. 1997; Mirecka, Libura, Libura, et al. 2001; Strieth, Hartschuh, Pilz, & Fusenig. 2000; Strieth, Hartschuh, Pilz, & Fusenig. 2002).

As a computer was used, repeat counts in the same area were always 100% reproducible. Intraobserver variability was good at $r=0.874$. This again
compares favourably with other techniques, \((r=0.7 - r=0.8)\) (Korkolopoulou, Apostolidou, Pavlopoulos, et al. 2001; Pavlopoulos, Konstantinidou, Agapitos, Kavantzas, Nikolopoulou, & Davaris. 1998; Ranefall, Wester, Busch, & Malmstrom. 1998; Wester, Ranefall, Bengtsson, Busch, & Malmstrom. 1999).

The time taken to measure MVD in 30 areas in a specimen of SBC was 4 minutes 30 seconds. This would be acceptable in clinical practice and compares favourably with other methods such as that by Philp et al. Although they counted in only 12 areas, they used a manual method and each case took seven minutes (Philp, Stephenson, & Reed. 1996). This technique therefore represents an accurate, reproducible and time efficient method of measuring MVD in SBC.

### 4.2.4 MVD and outcome in superficial bladder cancer

Although increased MVD has been associated with poor outcome in bladder cancer since 1994 (Dickinson, Fox, Persad, Hollyer, Sibley, & Harris. 1994), only four papers have attempted to measure MVD in SBC specimens (Dinney, Babkowski, Antelo, et al. 1998; Ozer, Mungan, Tuna, Kazimoglu, Yorukoglu, & Kirkali. 1999; Sagol, Yorukoglu, Sis, et al. 2001; Stavropoulos, Tsimaris, Ioachim, et al. 2003). Dinney et al and Stavropoulos et al found no correlation between MVD and outcome. Ozer et al and Sagol et al working in the same centre both used the same technique for measuring MVD. Ozer et al found that higher vascularity was associated with a group of patients that “recurred or progressed”. They do not give separate data for progression alone. Sagol et al however, found no correlation with recurrence or progression.
This study therefore is the first to show that increased MVD at presentation in SBC is associated with subsequent progression to muscle invasive disease. The failure of previous studies to show this is likely to be due to sampling error. The computer image analysis system used here allows multiple areas to be counted thus giving a representative sample of the MVD throughout the tumour. The maximum MVD count in the tumour areas was also strongly correlated with outcome. Although this is not the same as the hotspot count it indicates that, if hotspot counting was possible in SBC, a similar result may be expected.

4.3 VEGF in Superficial Bladder Cancer

In this study, VEGF staining was seen in tumour cells and was more pronounced adjacent to the basement membrane. This pattern of expression was also found by Chow et al (Chow, Liu, Chan, Cheng, & Tzai. 1999). In this study, as with that by Chow et al, there was no association with progression however it did approach significance.

Although previous studies have found increased levels of VEGF mRNA and VEGF protein using hybridisation techniques are associated with poor outcomes, the only study using immunohistochemistry did not show this.

These results therefore are in line with both studies in that there was a trend to increased VEGF staining in tumours that progressed. However, this, like the study by Chow et al, was not significant. This suggests that
immunohistochemical techniques are not suitable for a prognostic test of VEGF levels in SBC.

4.4 Thymidine Phosphorylase in Superficial Bladder Cancer

The distribution of TP staining was the same as that described by O’Brien et al (O’Brien, Fox, Dickinson, et al. 1996). In the present study, TP staining in the stroma was a significant risk factor in SBC progression. This was also seen by Kubota et al who analysed parenchymal staining (Kubota, Miura, Moriyama, et al. 1997).

There was a strong correlation between TP staining and stage and grade. This was also found by Kubota et al; O’Brien et al found a relationship between TP expression and stage.

Interestingly, endothelial cell staining of TP was strongly associated with progression. This association was lost when divided by MVD. This suggests it represented the number of vessels (i.e. MVD) rather than the expression of TP. In O’Brien’s study there was an inverse relationship with MVD. There was no correlation in this study between MVD and TP. The relationship between TP in inflammatory cells and outcome in this study could represent an increased number of these cells in poor prognosis tumours.
4.5 Thrombospondin in Superficial Bladder Cancer

The biology of TSP is both complex and contradictory. The distribution of TSP-1 staining was comparable with that found by Grossfeld et al (Grossfeld, Ginsberg, Stein, et al. 1997) and Campbell et al (Campbell, Volpert, Ivanovich, & Bouck. 1998). These authors selectively analysed different staining patterns. Grossfeld ignored tumour staining and Campbell used endothelial staining purely as a positive control. In this series, all areas were analysed as it is apparent the location of TSP-1 may influence its action.

The TSP (Ab-7) antibody binds to the Type II ECF like repeats on the TSP-1 molecule. Staining using TSP (Ab-7) in the stroma and tumour cells was associated with an increased risk of progression. TSP (Ab-4) binds to the Collagen type V repeats on the TSP-1 molecule. Decreased endothelial staining with TSP (Ab-4) was associated with a poor outcome. Although it was not apparent from examining the sections, there was a strong correlation between the amount and intensity of TSP (Ab-7) and TSP (Ab-4) staining. This suggests that the two antibodies were detecting a similar TSP distribution. The difference in the risk factors therefore must be related to the detection of TSP. The two antibodies required quite different antigen detection techniques. In particular, TSP (Ab-7) sections were subjected to a harsh retrieval by heating in a pH1 solution. This caused significant tissue distortion. Grossfeld et al using the same technique, used only limited stain analysis because if this.
The biological actions of TSP have been shown in some studies to be anti-angiogenic. The results of TSP labelling with TSP (Ab-4) support this. Low levels of perivascular staining were associated with increased progression and also with high MVD. This upholds the theory that TSP acts as a break to angiogenesis and loss of this break allows tumour progression.

Increased staining of TSP (Ab-7) in the tumour cells and in the stroma however, was associated with a poor prognosis. Again this is supported by the literature; TSP has been shown to be a promoter of cancer growth. These actions are not necessarily via the angiogenic pathway and may be associated with other growth factor pathways. TSP located in the tumour cells may therefore have a different action to TSP found in association with blood vessels. Indeed, in this study there was only an association between perivascular TSP staining and MVD and not TSP located in other areas.

The location of individual TSP molecules is dependent upon TSP receptors. These may also determine the downstream pathways of TSP’s action. CD36 has been associated with TSP’s antiangiogenic properties. Whereas the receptor to the CSVTCG adhesive domain (Type I repeat area) moderates tumour promoter activity.

The conflicting results of the two TSP antibodies in this study may therefore represent different TSP actions being dependent upon TSP binding location. This is supported by the fact that TSP (Ab-4) stromal staining was
positively associated with high stage and grade disease. Also, there was a weak but significant inverse correlation between TSP (AB-7) perivascular staining and MVD. This suggests that both antibodies are detecting TSP in the same areas but in differing amounts. This is more likely due to the nature of the antibody and its retrieval technique than the true expression of TSP in the tissue.

This study suggests that TSP bound adjacent to vessels is functioning in an antiangiogenic way and loss of this type of TSP action is associated with tumour progression. However, TSP in the tumour cells or adjacent stroma may be acting on cellular adhesion and migration and promoting tumour growth and spread. This supports the theory that TSP is a multifunctional molecule with many downstream pathways. Different detection methods used to locate TSP have led to abstracted conclusions in previous studies.

4.6 p53 in Superficial Bladder Cancer

In this study a continuous variable was used as well as 5% and 20% cut offs to determine positivity of p53. All three methods found that p53 was a predictive factor for progression in SBC. There was also a positive correlation with MVD. This supports the theory that p53 plays a part in the upstream regulation of angiogenesis. There was no correlation however with TSP or with VEGF in this study. This may be due to the antibodies used. In a review of the literature Schmitz-Drager et al analysed 43 trials (14 in SBC) which examined the prognostic use of p53 in bladder cancer. They concluded that there was
massive variation in p53 measurement both in antibody used and cut off for positivity (Schmitze-Dräger, Goebell, Ebert, & Fradet. 2000).

4.7 Summary

The multivariable analysis of factors found that the strongest predictor of subsequent progression of SBC was stage (OR 3.608, p=0.004); this supports the findings of the general literature and the validity of the database. Advancing age and tumour multiplicity are also known risk factors in SBC. TP is a known proangiogenic factor and has previously been shown to be a risk factor for SBC progression. TSP has been shown in many studies to be both antiangiogenic and a promoter of cancer. Loss of perivascular TSP (OR 0.652, p=0.041) and increased expression of tumour TSP (OR 2.366, p=0.001) both confer an increased risk of progression. MVD measured by manual or computerised method is a predictor of outcome in SBC. However, computer image analysis MVD is faster and remains an independent predictor of progression in SBC.
Chapter 5

Conclusion
5.0 Conclusion

This study has shown that MVD can be measured in SBC. A method has been described that is accurate, reproducible and time efficient. This method could be applied in clinical practice.

This study has also shown, for the first time, that increasing MVD, measured as a continuous variable, at presentation is a significant risk factor for subsequent progression to muscle invasive disease.

MVD in this study showed a positive correlation with p53, as measured by immunohistochemistry with a 5% cut off for positivity. MVD also showed a positive association with the pro-angiogenic factor Thymidine Phosphorylase. Both p53 and TP were shown to be significant risk factors for progression in SBC in this dataset.

Thrombospondin is a complex molecule with a variety of biological functions. This study has found that decreased TSP staining associated with vessels is an independent risk factor for SBC progression. Low perivascular TSP staining was also correlated with increased MVD. This supports the theory that TSP can act as an antiangiogenic restraint. TSP however, was also seen to be associated with an increased risk of SBC progression when measured in the tumour cells and stroma. This therefore suggests that the distribution of TSP rather than the absolute amount may be important in its action.
Subsequent studies could be directed towards determining the use of MVD as a prospective risk factor in the progression of SBC. In a series of primary SBC, sections of tumour should be stained as described with CD34 and MVD measured. Those measuring the MVD should be blinded to the stage, grade of the tumours and to the clinical outcome. MVD results could be correlated with clinical outcomes after a follow up period of 5 years.

Further information is also required regarding the actions of TSP in SBC. Better determination of its expression in the tissues may be carried out by microdissection and protein analysis.

Angiogenesis is a requirement for cancer progression. As such its measurement can lead to prognostication of the cancer’s behaviour. Angiogenesis is controlled by a complex balance of promoter and inhibitor factors. This balance is between different factors and, it appears, within the function of individual ones.
6.0 References


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Appendix 1

Computer macros


Appendix 1  Computer macros

This is the collection of macros used to automate the image analysis program NIH image. The language is that of the NIH image program and although similar to Pascal is not exactly the same. The title lines of each part are expressed in **bold text**. This is done to allow ease of reading in this appendix and is not used in the working macro.

```pascal
var

  c,pid1,pid2,pid3,pid4,pid5,pid6,scale,count,min,max,i,width,height,thresh:integer;
  maxrad,minrad,marea,pparea:real;
  unit:string;

procedure Initialise;
begin
  10 PutMessage('Initialising constants...');
      c:=2;
      min:=200;
      max:=2000000;
      thresh:=100;
      scale:=1000;
      unit:='mcm';
      SetUser1Label('Particles');
      SetUser2Label('Pt./Area');
      LabelParticles(true);
      OutlineParticles(false);
      IgnoreParticlesTouchingEdge(true);
      IncludelnteriorHoles(false);
  20 SetBinaryCount(3);
      Dilate;
      Dilate;
      Dilate;
      Dilate;
      Dilate;
      SetBinaryCount(5);
      Erode;

procedure binarytweak;
begin
  MakeBinary;
  SetBinaryCount(4);
  Erode;
  30 SetBinaryCount(3);
  Dilate;
  Dilate;
  Dilate;
  Dilate;
  Dilate;
  SetBinaryCount(5);
  Erode;
```

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Appendix 1 Computer Macros

Erode;
Erode;

40  MakeLineROI(0,0,0,575);
Clear;
KillROI;
MakeLineROI(0,0,767,0);
Clear;
KillROI;

end;

50  procedure RGBToGray;
begin
  pid4 := PidNumber;
  Bit24toRGBColor;
  pid5 := PidNumber;
  SetPalette('Grayscale');
  RGBToIndexed('Existing LUT, Dither');
  if c < 1 then begin
    Initialise;
  end;
  SetScale(scale,unit);
  pid3 := PidNumber;
  SelectPic(pid4);
  Dispose;
  SelectPic(pid5);
  Dispose;
  SelectPic(pid3);
end;

70  procedure BackgroundSubtract;
begin
  pid2 := PidNumber;
  if PidExists(pid1)=true then begin
    ImageMath('sub',pid2,pid1,1,0,pid2);
  end else begin
  {Put the next line in curly brackets like these to disable background subtraction};
    PutMessage('Cannot subtract background as blank field image is not open.');
  end;
  SetScale(scale,unit);
end;

macro 'Set up output window [O]'
Appendix 1 Computer Macros

begin
  NewTextWindow('Output',700,200);
  Writeln('Vessel count : ', 'Av. max rad. (mcm) : ', 'Av. min rad. (mcm) : ', 'Area (mcm^2) : ', 'Vessels/mm^2');
  Writeln(');
end;

macro '('-';

macro 'Obtain image [I]';
begin
  StartCapturing;
end;

macro 'Save blank field [J]';
begin
  PutMessage('This assumes you have just captured a near-white image of the background illumination. Leave the resulting window OPEN.');
  RGBToGray;
  SetScale(scale,'mcm');
  SetSaveAs('TIFF');
  SaveAs('Macintosh HD:white');
  pidl:=PidNumber;
end;

macro 'Identify blank field [K]';
begin
  PutMessage('This assumes you now have open a near-white image of the background illumination. Leave this window OPEN.');
  SetScale(scale,'mcm');
  pidl:=PidNumber;
end;

macro '24bit colour to greyscale [L]';
begin
  RGBToGray;
end;

macro 'Greyscale background subtract [M]';
begin
  BackgroundSubtract;
end;

macro 'Colour to grey & subtract [N]';
begin
RGBToGray;
BackgroundSubtract;
end;

macro '(-);

140 macro 'Autocount - colour[A]';

begin
if c<l then begin
    Initialise;
end;
SetScale(scale,'mcm');
ResetCounter;
{Put the next line into curly brackets like these, or delete it, if using
photographic positives};
{Invert};
{Put the next 2 lines line into curly brackets like these, or delete it, if
using greyscale images};
RGBToGray;
BackgroundSubtract;
SetScale(scale,'mcm');
SetThreshold(thresh);
binarytweak;
SetParticleSize(min,max);
150 GetPicSize(width,height);
MakeRoi(0.01*width,0.03*height,0.98*width,0.96*height);
AnalyzeParticles;
count:=rCount;

maxrad:=0;
minrad:=0;
for i:=1 to count do begin
    maxrad:=maxrad+rMajor[i];
    minrad:=minrad+rMinor[i];
end;
maxrad:=maxrad/count;
minrad:=minrad/count;

ResetCounter;
Measure;
pparea:=1000000*(count/rArea[1]);
rUser1[1]:=count;
rUser2[1]:=pparea;
160 pid6:=PidNumber;
Appendix 1 Computer Macros

{ShowResults;}

SelectWindow('Output');
{Change the 8:2 to alter the number of digits before & after the decimal point};
   Writeln(' count,' : ',maxrad:6:4,' :
   ',minrad:6:4,' : ',mArea:6:4,' : ',pparea:8:0);

SelectPic(pid6);
190 Dispose;
StartCapturing;
end

macro 'Count vessels - greyscale, whole screen [B]';

begin
   if c<l then begin
      Initialise;
   end;
   ResetCounter;
   {Put the next line into curly brackets like these, or delete it, if using
   photographic positives};
   {Invert};
   {Put the next line into curly brackets like these, or delete it, if using
   greyscale images};
   {ApplyLUT};
   SetThreshold(thresh);
   binarytweak;
210 SetParticleSize(min,max);
   GetPicSize(width,height);
   {MakeRoi(0.01*width,0.03*height,0.98*width,0.96*height);} 
   AnalyzeParticles;
   count:=rCount;

maxrad:=0;
minrad:=0;
for i:=1 to count do begin
   maxrad:=maxrad+rMajor[i];
220 minrad:=minrad+rMinor[i];
end;
maxrad:=maxrad/count;
minrad:=minrad/count;

   ResetCounter;
   Measure;
   pparea:=1000000*(count/rArea[1]);
   rUser1[1]:=count;
   rUser2[1]:=pparea;

xxx
Appendix 1 Computer Macros

230 {ShowResults;} pid6:=PidNumber;
SelectWindow('Output');
{Change the 8:2 to alter the number of digits before & after the decimal point};
SelectPic(pid6);
end

macro 'Count vessels - greyscale, manual area definition [C]';

240 begin
  if c <1 then begin
    Initialise;
  end;
  ResetCounter;
SetScale(scale,'mcm');
  Measure;
  marea:=rArea[l];
  ResetCounter;
250 SetScale(scale,'mcm');
  {Put the next line into curly brackets like these, or delete it, if using photographic positives};
  {Invert};
  {Put the next line into curly brackets like these, or delete it, if using greyscale images};
  {ApplyLUT};
  SetThreshold(thresh);
  binarytweak;
  SetParticleSize(min,max);
260 AnalyzeParticles;
  count:=rCount;

  maxrad:=0;
  minrad:=0;
  for i:=1 to count do begin
    maxrad:=maxrad+rMajor[i];
    minrad:=minrad+rMinor[i];
  end;
  maxrad:=maxrad/count;
  minrad:=minrad/count;
270
  ResetCounter;
  pparea:=1000000*(count/marea);
  rUser1[1]:=count;
  rUser2[1]:=pparea;
  {ShowResults;}
  pid6:=PidNumber;
SelectWindow('Output');

{Change the 8:2 to alter the number of digits before & after the decimal point};

280 Writeln('count,' : 6:4,':',maxrad:6:4,' :',minrad:6:4,' :',mArea:6:4,' :',pparea:8:0);
SelectPic(pid6);
end;

macro 'Speed Count lassoed vessels - greyscale, [G]';

begin
if c <1 then begin
  Initialise;

290 end;
ResetCounter;
SetScale(scale,'mcm');
Measure;
marea:=rArea[1];
ResetCounter;
SetScale(scale,'mcm');
{Put the next line into curly brackets like these, or delete it, if using photographic positives};
{Invert};
{Put the next line into curly brackets like these, or delete it, if using greyscale images};
{ApplyLUT};
SetThreshold(thresh);
binarytweak;
SetParticleSize(min,max);
AnalyzeParticles;
count:=rCount;

maxrad:=0;
minrad:=0;
for i:=1 to count do begin
  maxrad:=maxrad+rMajor[i];
  minrad:=minrad+rMinor[i];
end;
maxrad:=maxrad/count;
minrad:=minrad/count;

ResetCounter;
pparea:=1000000*(count/marea);
320 rUser1[1]:=count;
rUser2[1]:=pparea;
{ShowResults};
pid6:=PidNumber;
SelectWindow('Output');
{Change the 8:2 to alter the number of digits before & after the decimal point};

xxxii
Writeln('count,' : ',maxrad:6:4,' :
',minrad:6:4,' : ,mArea:6:4,' : ,pparea:8:0);
SelectPic(pid6);
Dispose;
StartCapturing;
end;

macro '(-';

macro 'Count blobs - greyscale, whole screen [P]';
begin
if c<1 then begin
    Initialise;
    ResetCounter;
    {Put the next line into curly brackets like these, or delete it, if using photographic positives};
    {Invert};
    {Put the next line into curly brackets like these, or delete it, if using greyscale images};
    {ApplyLUT;}
    SetThreshold(254);
    SetParticleSize(min,max);
    GetPicSize(width,height);
    {MakeRoi(0.01*width,0.03*height,0.98*width,0.96*height);}
    AnalyzeParticles;
    count:=rCount;
    maxrad:=0;
    minrad:=0;
    for i:=1 to count do begin
        maxrad:=maxrad+rMajor[i];
        minrad:=minrad+rMinor[i];
    end;
maxrad:=maxrad/count;
minrad:=minrad/count;
    ResetCounter;
    Measure;
    pparea:=1000000*(count/rArea[1]);
    rUser1[1]:=count;
    rUser2[1]:=pparea;
    {ShowResults;}
    pid6:=PidNumber;
    SelectWindow('Output');
    {Change the 8:2 to alter the number of digits before & after the decimal point};
    Writeln('count,' : ',maxrad:6:4,' :
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Appendix 1

Computer Macros

'minrad:6:4,' : 'mArea:6:4,' : 'pparea:8:0);
SelectPic(pid6);
end

macro 'Count blobs - greyscale, manual area definition [Q]';

380 begin
  if c < 1 then begin
    Initialise;
  end;
  ResetCounter;
  SetScale(scale,'mcm');
  Measure;
  marea:=rArea[l];
  ResetCounter;
  SetScale(scale,'mcm');
  {Put the next line into curly brackets like these, or delete it, if using photographic positives};
  {Invert};
  {Put the next line into curly brackets like these, or delete it, if using greyscale images};
  {ApplyLUT;}
  SetThreshold(254);
  SetParticleSize(min,max);
  AnalyzeParticles;
  count:=rCount;
  maxrad:=0;
  minrad:=0;
  for i:=1 to count do begin
    maxrad:=maxrad+rMajor[i];
    minrad:=minrad+rMinor[i];
  end;
  maxrad:=maxrad/count;
  minrad:=minrad/count;

390 maxrad:=0;
minrad:=0;
for i:=1 to count do begin
  maxrad:=maxrad+rMajor[i];
  minrad:=minrad+rMinor[i];
end;
maxrad:=maxrad/count;
minrad:=minrad/count;

410 ResetCounter;
pparea:=100000*(count/marea);
rUser1[1]:=count;
rUser2[1]:=pparea;
{ShowResults;}
pid6:=PidNumber;
SelectWindow('Output');
{Change the 8:2 to alter the number of digits before & after the decimal point};
Writeln('count' : 'maxrad:6:4:' :
'minrad:6:4,' : 'mArea1:6:4,' : 'pparea:8:0);
SelectPic(pid6);
end;

xxxiv
macro 'Duplicate Window[D]';
begin
    Duplicate(concat(WindowTitle,' -2'));
end

macro 'Change size & threshold values [V]';
begin
    min:=GetNumber('Area in pixels of SMALLEST particle?',min);
    max:=GetNumber('Area in pixels of LARGEST particle?',max);
    if (min+1)>max then begin
        beep;
        PutMessage('The largest must be bigger than the smallest! Try again.');
        initialise;
        exit;
    end;
    thresh:=GetNumber('Figure for threshold? ',thresh);
    if thresh<1  then begin
        beep;
        PutMessage('The threshold value must be an integer above 0 and below 256. Try again.');
        initialise;
        exit;
    end;
    if thresh>256  then begin
        beep;
        PutMessage('The threshold value must be an integer above 0 and below 256. Try again.');
        initialise;
        exit;
    end;
end

macro 'Reset to default values [R]';
begin
    initialise;
end

macro 'Junk image [X]';
begin
    Dispose;
end
Appendix 1 Computer Macros

470 end;

macro '(':`

macro 'Set scale x1 objective [O]';
begin
  if c <1 then begin
    Initialise;
    end;
  scale:=0.0884;
  SetScale(scale,unit);
end;

macro 'Set scale x2 objective [T]';
begin
  if c <1 then begin
    Initialise;
    end;
  scale:=0.179;
  SetScale(scale,unit);
end;

macro 'Set scale x4 objective [F]';
begin
  if c <1 then begin
    Initialise;
    end;
  scale:=0.355;
  SetScale(scale,unit);
end;

macro 'Set scale x10 objective [1]';
begin
  if c <1 then begin
    Initialise;
    end;
  scale:=0.884;
  SetScale(scale,unit);
end;

macro 'Set scale x20 objective [2]';
Appendix 1 Computer Macros

begin
if c < 1 then begin
  520 Initialise;
  end;
  scale:=1.763;
SetScale(scale,unit);
end;

macro 'Set scale x40 objective [4]';
begin
if c < 1 then begin
  530 Initialise;
  end;
  scale:=3.515;
SetScale(scale,unit);
end;

macro 'Set scale x100 objective [H]';
begin
if c < 1 then begin
  540 Initialise;
  end;
  scale:=8.84;
SetScale(scale,unit);
end;

macro 'Binarytweak';
begin
  binarytweak;
end;

macro 'Checknumbers';
begin
  PutMessage('Threshold',thresh,' Minsize',min,' Max size',max,' Scale',scale);
end;

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Appendix 2

Publications
Appendix 2 Publications

Original articles relating to this thesis


Appendix 3

Presentations
Presentations related to this thesis

   
   Goddard JC, Sutton CD, Furness PN, O’Byrne KJ, Kockelbergh RC, A Lack of Standardization in Microvessel Density Counting in Urological Tumours. Poster

   
   Goddard JC, Sutton CD, O’Byrne KJ, Kockelbergh RC, An Immunohistochemical Investigation to produce reliable, reproducible vessel identification for Microvessel Density Measurement using Image Analysis in Superficial Bladder Cancer. Poster

3. **Trent Urology Group, Leicester** April 2000
   
   Goddard JC, Angiogenesis in Urological Malignancy

   
5. **Urological Research Association, London**  
   January 2001  
   Goddard JC, Sutton CD, Jones JL, O’Byrne KJ, Kockelbergh RC,  
   Immunohistochemical Staining of Thrombospondin-1 relates to Progression of  
   Superficial Bladder Cancer. Poster  

6. **Grand Round, Leicester General Hospital**  
   February 2001  
   Goddard JC, Angiogenesis in Superficial Bladder Cancer  

7. **Scottish Urological Society & Scottish Urological Oncology Society,**  
   **Glasgow**  
   April 2001  
   Goddard JC, Sutton CD, Jones JL, O’Byrne KJ, Kockelbergh RC,  
   Reduction in Thrombospondin-1 Staining is associated with Progression to  
   invasion in Superficial Bladder Cancer. Poster *(Prize Winner)*  

8. **Surgical Research Society, Birmingham**  
   April 2001  
   Goddard JC, Sutton CD, Jones JL, Berry DP, O’Byrne KJ, Kockelbergh  
   RC, Reduction in Thrombospondin 1 expression significantly correlates with  
   disease progression in superficial bladder cancer.  

9. **Surgical Research Society, Birmingham**  
   April 2001  
   Sutton CD, Goddard JC, (presenter) Fumess PN, Berry DP, O’Byrne KJ,  
   Dennison AR, The development of an accurate, reproducible image analysis  
   system to assess Microvessel Density in solid tumours.
10. **Geoffrey Chisholme Prize Presentation at The Royal Society of Medicine, Section of Urology Spring Meeting,**

    **Harrogate**  
    **May 2001**

    Goddard JC, Sutton CD, Jones JL, Furness PN, O’Byrne KJ, Kockelbergh RC, Microvessel Density at presentation in superficial bladder cancer is higher in cases that subsequently progress. *(Prize Winner)*

11. **Sidney King Prize Presentation, Leicester**  
    **June 2001**


12. **British Association of Urological Surgeons Annual Meeting,**

    **Dublin**  
    **June 2001**

    Goddard JC, Sutton CD, Brown R, Kockelbergh RC, pTa Bladder Cancer: is Early Discharge Safe?

13. **British Association of Urological Surgeons Annual Meeting,**

    **Dublin**  
    **June 2001**

    **Goddard JC**, Sutton CD, Jones JL, O’Byrne KJ, Kockelbergh RC, Reduced Immunohistochemical Staining of Thrombospondin-1 is related to Progression in Superficial Bladder Cancer. Poster
14. **British Association of Urological Surgeons Annual Meeting**  
   **Dublin**  
   **June 2001**  

   Thames Research Urologists & Surgical Trainees Meeting.

15. **British Cancer Research Meeting, Leeds**  
   **July 2001**  
   Goddard JC, Sutton CD, Jones JL, O’Byrne KJ, Kockelbergh RC,  
   Reduction in immunohistochemical staining of Thrombospondin-1 predicts 
   progression in superficial bladder cancer. Poster

16. **The Institute of Cancer Studies, Study Day, Leicester**  
   **July 2001**  
   Goddard JC, A Biological Model of Angiogenesis in Superficial Bladder 
   Cancer. Invited lecture.

17. **The Trent Urology Group, Chesterfield**  
    **October 2001**  
    Goddard JC, Counting Blood Vessels in Superficial Bladder Cancer – A 
    Change of Heart. Registrar’s Prize Lecture

18. **The 3rd Institute of Cancer Studies, Study Day,**  
    **Leicester**  
    **June 2003**  
    Goddard JC, Prognostic Factors in Superficial Bladder Cancer 
    Invited lecture.