A Study of the Predictive Value of Morphometric Assessments in Clinical Outcome in Ovarian Epithelial Malignancy.

by

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Declaration

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Dr Julia Palmer

Abstract

Quantitative pathology as a tool in gynaecological pathology is fairly new. Such techniques allow greater objectivity than histological grading, typing, and residual tumour estimation. This study aims to determine: whether basic morphometry data can predict outcome and chemotherapeutic response, whether newer semi-automated methods of tumour morphometry provide similar results to older methods, and whether advanced image analysis methods can offer further tumour outcome data in ovarian carcinoma.

The study was performed on a well-selected group of serous ovarian carcinomas. Tumour outcome, survival and chemotherapeutic response, were investigated in 132 patients treated with the same platinum containing regimes.

Traditional clinicopathologic parameters, p53 & Bcl2, mitotic activity index (MAI) and angiogenesis determinants were initially investigated. Semi-automated analysis, using immunohistochemically based techniques, were applied to estimate volume percentage epithelium (VPE) and nuclear morphometric parameters. Syntactic structure analysis including, minimum spanning tree, and neighbourhood features, was also investigated.

Multivariate analysis revealed residual disease status, FIGO stage, MAI, VPE, equivalent nuclear diameter, and angiogenesis parameters to be strong prognosticators for overall and disease free survival. Residual disease status, VPE, nuclear length and angiogenesis parameters were found significant predictors of chemotherapy response. Angiogenesis parameters, as determined by semi-automated image analysis techniques, were found overall to be the strongest prognosticators.

Morphometric data can predict outcome and chemotherapeutic response in ovarian serous carcinoma. Semi-automated morphometry techniques provide similar results to older methods, and advanced image analysis can offer further outcome data. The rationale for the application of semi-automated and automated detection is that it may provide an unbiased sampling of a lesion and possibly a more representative estimate of areas that a human expert might label. Such determined, quantitative pathological findings were found to have important value in predicting prognosis in ovarian carcinoma and, if not to supersede, certainly to add to classical prognostic factors.

Abbreviations

a	-anaplastic
B	-Brenner
cc	-clear cell
CE	-Coefficient of Error
CI	- Confidence Interval
CK	- Cytokeratin
CT	- Computerised Tomography
DAB	-3,3'-diaminobenzidine chromogen solution
df	-degrees of freedom
DFS	-Disease Free Survival
ED	-Equivalent Diameter
EMA	-Epithelial Membrane Antigen
EOC	-Epithelial Ovarian Carcinoma
Exp(B)	-Hazard Function
FIGO	-International Federation of Gynaecologic Oncology
FR	-Fullness Ratio
GF	-Growth Fraction
GOG	-Gynaecological Oncology Group
H&E	-Haematoxylin & Eosin
HMFG	-Human Milk Fat Globulin
IDS	-Interval Debulking Surgery
IgG	-Immunoglobulin G
IHC	-Immunohistochemistry
IMVD	- Intra-tumoural Microvessel Density
KDA	- Kilo Daltons
Length MST	- Length of Minimum Spanning Tree
m	-mucinous
MAI	-Mitotic Activity Index
Max MST	-Maximum Length of Minimum Spanning Tree
MI	-Mitotic Index
mi	-mixed differentiation
MIB 1	-monoclonal antibody against Ki67
MIB 1 LI	-MIB 1 Labelling Index
Min MST	-Minimum Length of Minimum Spanning Tree
MNA	-Mean Nuclear Area
MNV	-Mean Nuclear Volume
MRI	-Magnetic Resonance Imaging
MST	-Minimum Spanning Tree
MVD	-Microvessel Density

Abbreviations (continued)

NB	-Nuclear Breadth
NL	-Nuclear Length
NP	-Nuclear Perimeter
NR	-Nuclear Roundness
n/s	-Not Specified
o	-other differentiation
OHSS	-ovarian hyperstimulation syndrome
OS	-Overall Survival
P	-probability
PAP	-Peroxidase-anti-Peroxidase
PI	-Proliferation Index
PFS	-Progression Free Survival
r	-The correlation coefficient (Pearson), r, ranges from -1 to 1.Based on assumption that both x and y values follow Gaussian distribution, at least with proximity
r^2	-coefficient of determination (r squared). It is has a value that ranges from zero to one, and is the fraction of the variance in the two variables that is shared.
s	-serous
sdMST	-standard deviation Minimum Spanning Tree
sdNA	-standard deviation Nuclear Area
SE	-standard error
Spearman	-non-parametric correlation. (Based on ranking the 2 variables, makes no assumption about distribution of the values).
SSA	-syntactic structure analysis
TBS	-Tissue Binding Substrate
tcc	-transitional cell carcinoma
TSp-1	-thrombospondin-1
u	-undifferentiated
un	-unclassified
VEGF	- vascular endothelial growth factor
VPE	-Volume Percentage Epithelium
WHO	-World Health Organisation
XFCP	- the X co-ordinate of the feature count point
YFCP	- the Y co-ordinate of the feature count point

Chapter 1. Introduction: Traditional Clinicopathologic Features Used as Predictive Factors

1.1 Preface: Ovarian carcinoma and the need for reliable prognosis

Ovarian cancer is the most common gynaecological cancer in the UK with 6,900 newly diagnosed cases and 4,600 deaths each year. It is the fourth most common cancer death in women and accounts for 6% of all female cancer deaths. The 5year survival for all female cancers combined is approximately 43%, yet the 5year survival for ovarian cancer is 29.2%. [1] Prognosis is poor, as patients tend to present with advanced disease, with earlier stage disease being relatively asymptomatic.

With the advent of combined platinum-based chemotherapy in 1975, a significant influence upon survival in ovarian cancer was achieved, but little improvement has occurred since. In principle mortality could be reduced by more aggressive treatment of those at risk of disease recurrence, but therapeutic strategies such as platinum chemotherapy agents have considerable side effects (see appendix 1). Hence a reliable means of distinguishing between those patients at risk and those not is needed.

Prognostic indicators are factors defined at a given time point which give information on subsequent clinical outcome. Predictive factors give information useful in the selection of patients likely to benefit from a specific treatment such as targeted systemic therapy. [2] Prognostic tools should ideally allow an individualised therapy that optimises survival and minimises the risk of severe side effects. [3] A reliable prognostic, or indeed predictive tool, may enable accurate patient selection, and select only those patients who will benefit from therapy. [4] We must consider that patients with very progressive ovarian cancer may have only a very short term survival (FIGO stage IV < 5%, 5yr survival) and, that in spite of their good overall prognosis, a considerable number of FIGO stage I patients will die from recurrent disease (20 – 40% in different series). [5] Reliable prognostic tools might

therefore enable us to select out those short-term survivors and offer a much less toxic palliative therapy and select in those early stage patients who will benefit from more aggressive therapy, who, would perhaps have traditionally not been felt to benefit. As current therapeutic results in ovarian cancer are reached at the expense of severe side effects, with most patients undergoing radical treatment, knowledge of predictive factors could lead to a better selection of patients who will benefit from aggressive therapy. [2]

Prognostic and predictive factors have been widely studied in ovarian cancer as a means of predicting both patient survival and chemotherapeutic outcome. Evidence is available that, in ovarian cancers, the prognostic value of morphometric and cytometric features, either alone or in combination, and as multivariate combinations with, for example, FIGO stage exceeds that of classical pathological features. This thesis is concerned with the development and application of such techniques to ovarian cancer data in order to attempt to predict both patient survival and response to platinum-based chemotherapeutic regimes. In addition advanced digital imaging techniques will be employed to study tumour architecture and nuclear morphology to determine whether such procedures can add further to the predictive value of routine techniques in such tumours. In this introductory section I shall discuss traditional clinicopathologic factors and their prognostic ability in ovarian carcinoma.

1.2 Traditional Prognostic Indicators in Ovarian Epithelial Malignancy

Knowledge of prognostic factors may help to explain and perhaps diminish the unchanged high mortality in epithelial ovarian cancer and perhaps be used to select patients for adjuvant cytotoxic therapy or rather provide clinicians with guidelines for decisions on treatment strategies.

Traditional prognostic factors include clinical factors such as patient age, tumour stage and extent of residual disease post primary laparotomy, serum markers such as Ca125 and

classic pathologic features of histological type and grade.

1.2.1 Patient age at presentation

Advanced ovarian cancer (stage III & IV) in the >50yrs age group has been associated with a higher probability of death, [7] with multivariate analysis showing increased age as a predictor of death during the first 5 years of diagnosis and patients > 65yrs having a 2 fold higher risk of death compared with those patients <45yrs. [8] Age has also been shown as an independent prognostic factor of survival with patients under 65yrs surviving significantly longer than those >65yrs, but this observation may have also resulted from poorer performance status. [9] Results from the gynaecological oncology group (GOG) trial data (n=2123) showed patients older than 69yrs exhibited significantly poorer survival than those younger, even after correction for stage, residual disease and performance status. [10] A significant reduction in survival has also been noted among patients 80yrs or older as compared to their younger counterparts, but it appears that conservative treatments contributed to the decreased survival of older ovarian cancer patients. [11]

A study of women in the ≤ 25 yrs age group with grade I or grade II tumours showed excellent prognosis, those with grade III tumours showed a lesser improvement on 5 yr survival. [12] Five year survival, median survival and progression free survival have been found better in younger patients, as compared to older patients, with younger women having significantly better survival when adjusted for stage. [13] When considering however only a sub-population of women with late stage ovarian cancer the 5yr survival rate for young women was found to be 22.9%, a number in keeping with the literature for post menopausal women suggesting that young age does not confer an improved prognosis. [14] Age was also not found to be an independent prognostic factor in Stage IV disease [15], nor as an independent prognostic factor for ovarian carcinoma in women younger than 40yrs.

[2]

It is not clear why younger women might have better outcome than older patients. It is questionable whether age is an independent prognostic factor, or whether better performance status, earlier presentation, more aggressive tumour biology, tumour grade, impaired immunologic response or inability to tolerate more intensive treatment may bias the prognostic significance of age.

1.2.2 Tumour stage

Appropriate staging of a tumour is to define categorical descriptors that allow general conclusions to be made regarding the likelihood of relapse and survival for an individual patient. [2] It is widely accepted that staging of ovarian cancer is performed by the FIGO (International Federation of Gynaecology & Obstetrics) system. This system stages ovarian tumours largely on the basis of tumour site and the presence of metastases where, stage I tumours have tumour growth limited to the ovaries, stage II tumours have growth involving one or both ovaries with pelvic extension, stage III have tumour involving one or both ovaries with peritoneal implants outside the pelvis and / or positive retroperitoneal or inguinal nodes and stage IV tumours involve distant metastases (see table 1a: FIGO staging for primary carcinoma of the ovary). Staging in ovarian cancer is based on laparotomy findings, histological assessment and cytology of peritoneal fluid (including washings taken in the absence of clinical ascites). [17] Devised in 1974, the FIGO system was later revised in 1985 to reflect an improved understanding of tumours' natural history and the prognostication value of parameters such as tumour rupture, surface excrescences, positive peritoneal cytology and size of abdominal implants. [18]

FIGO staging is widely used not only as a tool for predicting 5 year survival but also as a means of decision making regarding further treatment with chemotherapeutic agents. Numerous studies [18-21,22,23] have evaluated patient survival with regard to tumour

stage. Despite some minor discrepancies, these studies, on the whole, have shown reasonable correlation, with approximate 5yr survival estimations in stage I tumours being 70-80%, stage II 40-60%, stage III 10-40% and Stage IV 2-10%. (See Table 1b: FIGO staging and 5yr survival in ovarian carcinoma). Tumour stage is generally regarded as one of the most important variables for prognosis. Surgical staging is a fairly extensive process being best performed by gynaecological oncologists. Importantly FIGO staging should not be altered on the basis of clinical response or indeed after treatment has ensued. In the event that surgical intervention is inappropriate, staging may be performed using imaging techniques such as computerised tomography (CT) or magnetic resonance imaging (MRI) but formal tissue diagnosis should be obtained (e.g. biopsy) prior to commencing chemotherapeutic treatments.

Table 1a: International Federation of Gynaecology & Obstetrics: Staging for Primary Carcinoma of the Ovary

Stage 1: Growth limited to the Ovaries.
Stage 1a: Growth limited to one ovary, no ascites. No tumour on the external surface; capsule intact. **1b:** Growth limited to both ovaries; no ascites. No tumour on the external surface; capsules intact. **1c:** Tumour either Stage 1a or 1b, but with tumour present on surface of one or both ovaries; or with capsule ruptured; or with ascites present containing malignant cells or with positive peritoneal washings
Stage 2: Growth involving one or both ovaries with pelvic extension.
Stage 2a: Extension and / or metastases to the uterus and / or tubes. **2b:** Extension to other pelvic tissues. **2c:** Tumour either Stage IIa or Stage IIb, but with tumour present on surface of one or both ovaries; or with capsule(s) ruptured; or with ascites present containing malignant cells or with positive peritoneal washings.
Stage 3: Tumour involving one or both ovaries with peritoneal implants outside the pelvis and / or positive retroperitoneal or inguinal nodes. Superficial liver metastasis equals stage 3. Tumour is limited to the true pelvis, but with histologically proven malignant extension to the small bowel or omentum.
Stage 3a: Tumour grossly limited to the true pelvis with negative nodes, but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces. **3b:** Tumour involving one or both ovaries with histologically confirmed implants of abdominal peritoneal surfaces none exceeding 2cm in diameter. Nodes are negative. **3c:** Abdominal implants greater than 2cm in diameter and / or positive retroperitoneal or inguinal lymph nodes.
Stage 4: Growth involving one or both ovaries with distant metastases. If pleural effusion is present, there must be positive cytology to allot a case to stage 4. Parenchymal liver metastases equals stage IV.

Table 1b: FIGO staging and 5yr survival in ovarian carcinoma

Study	FIGO[19] 1979	Sigurdsson [20] 1983	Einhorn [21] 1985	Nguyen [18] 1993	FIGO [23] 1998
n	4892	494	770	5156	?
5 Year Survival					
Stage 1	72%	82%		88.9%	
1a 1a1 1a2			80% 86% 75%		
Stage 1b	62.5%	75%	60%		71.3%
1b1 1b2			67% 55%		
Stage 1c	57.4%	83%	59%		79.2%
Stage 2				57.1%	
2a 2b 2c	52.2% 37.5% 37.5%	79% 39% 61%	75% 51% 41%		66.6% 55.1% 57.0%
Stage 3	10.8%	8%	11%	23.8%	
3a 3b 3c					41.1% 24.9% 23.4%
Stage 4	4.6%	2%	4%	11.6%	11.1%

1.2.3 Residual Disease

The early local spread and metastasis of ovarian cancer results in complete tumour resection at laparotomy being often not possible in view of for example, site of metastases, and involvement of adjacent structures. The extent of residual disease following primary debulking surgery is considered an important prognosticator in advanced ovarian cancer with numerous studies reporting the completeness of primary cytoreductive surgery to positively influence survival.

Initial surgical options in advanced ovarian cancer include biopsy alone, limited resection of the primary tumour and aggressive cytoreduction. [24] The goal of surgical debulking is to leave the smallest volume of residual disease possible [25] and the primary

purpose of cytoreductive surgery is to prolong median survival. [26] Primary cytoreductive surgery has been defined as a surgical procedure in which the aim is to achieve a residual tumour mass of <2cm in total and sub-optimal cytoreductive surgery as when residual tumour mass exceeds 2cm. [27]

The concept of cytoreductive surgery for advanced ovarian cancer has been present since the 1930's, [28] but it was Griffith's [29] in the 1970's who was credited with first indicating that improved survival in advanced ovarian cancer was related to the diameter of the largest residual tumour. In the 1980's Wharton [30] showed that patients undergoing partial tumour removal had survival no better than patients undergoing biopsy alone.

Cytoreductive surgery allows histological confirmation of the diagnosis and proper disease staging. [31] In addition to relief from direct tumour insult, bulk resection may result in an improved response to post-operative chemotherapeutic agents. [29] Large tumour masses with poor blood supply are less likely to receive adequate chemotherapy doses, [32] thus removal of necrotic tumour mass may provide improved delivery of therapeutic agents, and improved drug availability to metastases, [33] as well as remove chemo-resistant cells. Tumour reduction surgery may stimulate remaining tumour cells into active cell division therefore enhancing susceptibility to chemotherapeutic cell kills, [25] and may reduce the number of chemotherapy cycles required to eliminate disease and diminish development of subsequent drug resistance. [33]

Multiple series have reported completeness of primary cytoreductive surgery to independently influence the survival of patients with advanced epithelial ovarian cancer. In bulky disease, patients uncommonly survive at 5 years, and it has been suggested that survival varies inversely with the volume of residual tumour. [29]

Critics argue that the survival advantage associated with minimal residual disease has more to do with the inherent biologic predisposition of the tumour than with the

predisposition and skill of the surgeon. [26,33-34] Optimal and complete cytoreduction is more easily achieved for small tumours [33] but the rate of complete cytoreduction is related to case selection and surgical technique. [24] It is questionable whether large volume tumours are biologically different and more aggressive and it is suggested that residual tumour bulk does not influence survival as it is due to the fact of tumour biology allowing easier resection, [35] i.e. the apparent improvement in survival is a result of limited tumour growth which in fact makes maximal resection feasible. [29] In fact it has been demonstrated that patients with tumour seedlings <1cm have a significantly inferior survival compared with patients with bulk residual disease. [26]

Peri-operative morbidity and quality of life must be considered when debating the benefits of maximal cytoreductive surgery. Multi-organ resection may be required to achieve maximal cytoreduction in some cases with a high rate of complications found. [31] Therefore consideration is needed of whether the added risk of higher morbidity and mortality outweighs the potential survival benefit to the patient.

Primary debulking surgery remains the gold standard in the management of advanced ovarian cancer. Secondary cytoreductive surgery involves repeat surgical effort after first line chemotherapy to further debulk the cancer. Secondary cytoreductive surgery may be complex with an associated increase in morbidity and mortality although patients with no residual disease have been found to have a 70% relative risk reduction compared to those with disease > 1cm. [36] If the extent of residual disease is regarded as being a significant prognosticator for survival then, interval debulking surgery (IDS), ' a surgical procedure with debulking intent preceded and followed by chemotherapy,' [37] should be considered in patients with sub-optimal primary surgery. IDS after primary chemotherapy in patients with advanced stage ovarian cancer seems to offer the same chance of survival as does initial debulking surgery but with a possible reduction in morbidity. [27,38-39]

Although the volume of residual tumour after debulking surgery appears to relate well to the clinical outcome there is no accurate measure and the critical volume for long-term survival remains unknown. That is the precise definition of 'what is optimal surgery' has not been established. Present methods of quantifying residual tumour are imprecise [26] and the maximal diameter of residual disease is a very crude estimate. [32] The gynaecological oncology group (GOG) definition of optimal cytoreduction is that in which the largest residual tumour nodule measures <1cm or less, [40] however a survey of 393 gynaecological oncologists showed the definitions of optimal cytoreductive surgery chosen varied markedly. [41] Therefore there may be variation in the meaning of optimal cytoreduction and caution needs to be taken in its interpretation with regards to survival benefit.

1.2.4 The Value of Ca125 in Epithelial Ovarian Cancer Management

In 1981 Bast developed a murine monoclonal antibody that reacted with a glycoprotein antigen (Ca125) derived from a human ovarian tumour cell line using a mouse somatic cell hybridisation technique. [42] Ca125 is a high molecular weight glycoprotein found in the coelomic epithelium [43] and is found distributed in the endothelium of the fallopian tubes, endometrium, endocervix and ovary and in the mesothelial cells of the pleura, peritoneum and pericardium. [44] Ca125 was found raised in 1% of the general population when a level of 35u/ml was taken as the upper limit of normal. [44] It has been found to be raised in 29% of cancers other than ovarian including breast, lung, pancreas and colon, [45] and in other benign conditions such as cirrhosis with ascites, acute pancreatitis, pelvic inflammatory disease, endometriosis, [45] ovarian hyperstimulation syndrome (OHSS), pregnancy and lymphoma. [46] In fact a large study of 22,000 healthy participants found that a serum Ca125 elevation was a risk factor for death not only from gynaecological cancers, but also from a wide spectrum of benign and

malignant diseases. [47]

Ca125 is raised in >80% of epithelial ovarian carcinomas where the upper limit of normal is regarded as 35u/ml and in 74% when 65u/ml. [44,48] The upper limit of normal values of Ca125 is therefore largely regarded as 35u/ml, which is clearly able to distinguish sera from healthy individuals and ovarian cancer patients. [48] Other studies however have used 65u/ml as the upper limit of normal, which does not cause substantial effect on sensitivity.

Approximately 20% of epithelial ovarian carcinoma patients do not express Ca125 (especially mucinous tumours comprising 5-10% of all EOC). [49] Ca125 has been found to be raised in only 50 – 55% of stage I tumours [46,50] and 42% FIGO II (but positive in 87% FIGO III & IV). [50] Therefore a normal Ca125 does not exclude tumour. It is commonly accepted that serum Ca125 levels are used only to monitor the course of disease in patients who had positive Ca125 levels before surgery. A study however of 56 Ca125 negative tumours showed excellent correlation of serum Ca125 with the development of recurrent disease [50] indicating that patients with a low Ca125 ovarian carcinoma marker level perhaps should have Ca125 tumour marker levels monitored during and after follow-up treatment.

The prognostic significance of pre-operative Ca125 levels is controversial. Significantly longer survival has been suggested when pre-op levels are <65u/ml. [44,51-53] In stage I epithelial ovarian cancer pre-operative Ca125 levels have significantly predicted survival on both univariate and multivariate analysis. [54] Initial Ca125 levels however have also been found to be of no prognostic significance. [45,55-56]

It has been suggested that high pre-operative Ca125 values indicate advanced disease and thus poor response to following cytotoxic therapy, [51] with Ca125 reflecting a larger tumour burden.[45] It has therefore been postulated that pre-operative serum Ca125

levels correlate with the probability of attaining optimal cytoreduction as mean Ca125 levels in optimally cytoreduced patients has been shown lower than in patients sub-optimally debulked, [57] but as no correlation has been found between ovarian mass volume and Ca125 levels, [58] the effects of ascites, ovarian mass volume and peritoneal carcinomatosis on Ca125 levels should be known before pre-operative prediction of optimal respectability in advanced cases is considered.

The pre-chemotherapy level of Ca125 in itself has been correlated with progression rate and the probability of progression within three years, [59] but immediately post surgery, Ca125 levels have been found to be elevated at highest 5-14 days post surgery and to normalise between three and four weeks after surgery. [60] Therefore use of Ca125 as a tumour marker in the early post-operative period may well be of only limited value. Elevated post-operative levels however have corresponded with post-operative residual tumour bulk resulting in poor prognosis. [51]

In general, with raised Ca125 levels, patients have shown greater tumour bulk than those with low values, [46,61] with strong associations seen between the size of residual tumour mass and the pre-chemotherapy level of Ca125. [49,56,59,62-63] In patients with residual tumour mass of >2cm, post-operative Ca125 levels may reflect tumour burden and perhaps should not be used as independent prognostic factors. [51,62]

Falling Ca125 levels have long been associated with tumour regression or responding tumours. Decreasing Ca125 levels have correlated with objective regression of ovarian carcinoma in 95% of cases [64)] and when halving of antigen levels was considered to be significant, Ca125 levels have correlated with clinical course in 93%. [64] Either a 50% decrease after 2 samples or a 50% decline over 3 samples (28 day gap in each sample) is generally accepted as a responding patient. [43-44,61] An average ten fold fall in Ca125 level from pre-treatment to post-treatment has been found both in complete and partial

responses. [49] Patients with a short serum half-life of Ca125 have shown a significantly better probability of survival with an orderly trend to better survival with shorter half-life [65]

Ca125 has shown to be a valid marker for recurrence in the follow up period, and of considerable value in the monitoring of effect during treatment. [66] Rising levels are indicative of poor tumour response to chemotherapy, with Ca125 levels being useful in demonstrating disease persistence following completion of chemotherapy. If the Ca125 level remains abnormal there is a >95% certainty that residual disease is present. [61] A rising Ca125 level may indicate progressive disease during initial chemotherapy (20% progression during initial treatment). [44]

Ca125 kinetics, defined as the rate at which Ca125 levels decrease during chemotherapy, [63] has been used as predictors of prognosis. A half-life of <20 days has been correlated with longer survival compared with a half-life of > 20 days correlating with significantly shorter survival with patients having 1.8 times longer survival. [63] When considering patients with FIGO stage III and IV ovarian carcinoma median survival was found to be significantly greater in patients with a shorter half-life. [62]

Although Ca125 has not been found a significant predictor of progression free survival (PFS) immediately after surgery, Ca125 and PFS after one cycle, and throughout primary chemotherapy, was found a significant predictor of survival. [49] After 2 courses of chemotherapy patients with a Ca125 <35u/ml were found more likely to achieve complete remission and better prognosis than if levels >35u/ml, i.e. gave the greatest discrimination between patients alive at 12 months and those who did not respond that long. [52] Fayers et al [67] found the best predictive measurement of Ca125 was the value prior to the 3rd dose of chemotherapy. Patients in whom the Ca125 levels did not normalise until the third cycle had 30% less chance of survival compared with those with response in Ca125 levels after

the first 2 cycles of induction chemotherapy. [63] An abnormal level Ca125 after 3 courses of chemotherapy is indicative of a very bad prognosis. [66]

Therefore if serial Ca125 measurements and Ca125 half-life are able to provide early information with regard to the effectiveness of induction chemotherapy it is possible to adapt individual therapies and modify early treatments avoiding early cytotoxic effects and development of drug resistance.

Early detection of disease recurrence is the primary goal of patient follow-up, after completion of therapy. [59] Although Ca125 may generally be regarded as a poor predictor of long-term prognosis it is generally regarded as an accurate predictor of tumour progression or recurrence. A rising Ca125 level in patients whose Ca125 level has previously normalised indicates progression, but a single elevation in the absence of confirmatory data should not be accepted.[61]

Traditionally there has been no specific guideline on the percentage increase in Ca125 level, which should be accepted as evidence of disease progression. A >25% rise in Ca125 level was shown to indicate progressive disease, [59] but disease progression is generally reflected as a doubling of levels in 80% - 90% of cases [43-44,49,55, 68-71] with two consecutively elevated levels being strongly suggestive of progressive disease. Progression, defined as doubling by the North Thames ovary trial, has shown a positive predictive value of 80% - 98% with regard to disease progression. [44-45,68-70] A significantly positive correlation between Ca125 half-life and disease progression rate has also been reported with a half-life of 20 days or more being associated with a progression rate of 3.2 times higher than if <20 days. [59]

A rising Ca125 may be the first indication of relapse preceding clinical detection of progression in 55% - 95% of cases [43-44,59,68-70] with Ca125 increase preceding clinical relapse (i.e. lead time) by a median of 3 – 4 months. [44,56,70-71] Therefore pre-clinical

detection of recurrent disease in post-treatment follow up may encourage the clinician to implement higher imaging for tumour progression or recurrence, may encourage implementation of therapy in those previously untreated, or additional therapy / further active therapy which may improve survival.

Several studies have investigated the use of Ca125 in conjunction with second look surgery i.e. repeat laparotomy following chemotherapy. A rising Ca125 is associated with tumour progression at second look laparotomy in 94% - 100% of cases. [43,51,59,70,72] Thus elevated levels, help to establish the disease status of patients and probably indicates the presence of intraperitoneal tumour. A negative Ca125 (Ca125 <35u/ml) however may, or may not be, associated with complete clinical response at surgical restaging [64] and so does not obviate the need for second look laparotomy. A negative Ca125 has been found as giving a false negative rate in approximately 40% - 50% of cases. [43,51,54,59,61,69,72] Therefore second look surgery is probably not indicated where Ca125 levels are increased, but when Ca125 is normal the need for second look surgery is certainly not eliminated and may perhaps be regarded as obligatory.

1.2.5 Histological Type

Histological typing depends on the constituent cell types of the tumour. The majority of ovarian tumours are of common epithelial type comprising serous (60%), mucinous (15-20%), endometrioid (10%), clear cell (5%) and Brenner / un-differentiated (>5%).

Histological sub-types in ovarian cancer are known to be associated with different epidemiological risk factors [73-77] and are in some instances felt to have varying prognosis. Serous tumours tend to present at an older age [73] and have a greater tendency to spread to the upper part of the abdomen, [15] mucinous tumours tend to present at a lower stage and grade to other types and be associated with a better prognosis, [20,79-80] and endometrioid

tumours tend to present as well to moderately differentiated. [17] Mucinous and clear cell tumours presenting at late stages however are generally accepted to result in adverse outcome because of their poor chemotherapy response. [81-82]

Assignment of tumour cell type has been fraught with reproducibility problems because it is performed by simple microscopic examination and so includes a subjective element: histopathologists often disagree. Discriminating between moderate to poorly differentiated serous and endometrioid ovarian carcinomas is difficult due to the fact they are closely related Mullerian tumours. Poorly differentiated mucinous carcinomas may focally resemble serous carcinomas, and discrimination between moderately differentiated endometrioid and mucinous carcinomas may also be difficult. [17] Studies concerning reproducibility have shown varying results, [83-86] with intraobserver reproducibility (70.6% - 87.3%) tending to be better than interobserver reproducibility (60% - 68%). Agreement is generally improved following panel discussions [87] with differences in typing felt largely due to tumour cell heterogeneity and difficulty discriminating between serous and undifferentiated tumours. [83] (See table 1c: Reproducibility of Histological Type in Ovarian Carcinoma)

Table 1c: Reproducibility of Histological Type in Ovarian Carcinoma

Study [ref]	**Year**	**Population (n)**	**N° of Observers**	**Type Agreement (Intraobserver)**	**Type Agreement (Interobserver)**
Hernandez [83]	1984	34	2 Observers (a & b)	Observer a.70.6% Observer b.73.5%	60%
Baak [84]	1986	198	4 Observers (1,2,3 & 4)	n/s	Round 1 4 = 29.8% 3 = 20.7% 2 = 22.3% 1 = 27.2% / Round 2 4 = 36.4% 3 = 20.2% 2 = 33.3% 1 = 10.1%
Brugghe [85]	1995	102	3 Observers (a,b,& c)	Observer b. 87.3% Observer c. 79.4%	61% (observer a vs b vs c)
Lund [86]	1991	221	2 Observers (a & B)	n/s	68%

Table 1c illustrates studies of reproducibility for histological sub-type in ovarian carcinoma. Studies show varying results. Intraobserver reproducibility tends to be better than interobserver reproducibility, yet marked disagreement may be observed.

Histological type is generally considered to be of limited prognostic value but, as there seems to be inherent differences in the biological behaviour of the various types of epithelial ovarian cancer, including grade and stage of presentation, likelihood of metastases and chemotherapeutic response, it seems probable to assume different histological sub-types may be associated with different prognoses. Therefore to exclude any influence that histological sub-type may confer on patient outcome prognostic factors in epithelial ovarian cancer should perhaps be investigated on a type specific basis.

1.2.6 Histological Grade

Differentiation means the extent to which a tumour resembles, histologically, its cell or tissue of origin i.e. it determines the tumour grade. Grading is an attempt to assign a rough numerical value to the extent of histological deviation from normal, [88] and again is somewhat subjective.

As histological grading in epithelial ovarian malignancy is considered by many pathologists and clinicians to have prognostic significance, grading is widely performed. [83,87,89-92] Therapeutically, a difference between grades 1 to grade 3 may result in decision for use of adjuvant treatments such as chemotherapy.

Problems arise in tumour grading as there are no universally accepted standardised systems of grading for any tumour site. [90] Numerous systems of grading however do actually exist but are based on varying criteria. Broder's 1926, [93] Czernobilsky 1977, [94] and Russell 1979 [95] graded ovarian malignancy in the clinical context, the FIGO grading system [96] is based on architectural features, the World Health Organisation (WHO) system is based on architectural and nuclear features, and the Shimzu system [97] is based on architectural grade, nuclear pleomorphism and mitotic activity. Pathologists may therefore rely upon and establish their own grading criteria thus giving the subjective element.

If pathologists establish their own criteria, the prognosis of tumours assigned to a particular grade by different pathologists may show considerable differences. [87] Differences in grading, resulting in marked intra- and interobserver variability, have shown univariate prognostic implications for survival in ovarian cancer. [84-85] Several studies [83-87,98] (see table 1d: Reproducibility of Histological Grade in Ovarian Carcinoma) have reported significant differences of opinion between pathologists when grading the same tumours. Considerable intra and interobserver variability has been established. Intra-observer variability has generally been shown to be smaller than inter-observer variability. [86] Evaluation of a greater number of features has not seemed to produce better internal consistency in diagnosis [87] and studies have shown the more observer pairs the greater the disagreement. [83] Agreement on grade between centres has also been shown to be poor,[85] and complete agreement between observers rare. [87]

Table 1d: Reproducibility of Histological Grade in Ovarian Carcinoma

Study [Ref]	Year	(n)	Observer Number	Grading Agreement (Interobserver)		Grading Agreement (Intraobserver)
Hernandez [83]	1984	34	2 (a&b)	66%		Observer a = 63.2% Observer b = 78.0%
Baak [84]	1986	198	4 (a,b,c & d)	**Round 1** d =18.7 c =30.8 b =34.4 a =16.1	**Round 2** d =32.8 c =40.0 b =26.6 a =0.6	Observer a = 86.2% Observer b = 63.6% Observer c = 78.0% Observer d = 62.0%
Haapasalo [99]	1990	75	2 (compared 3 methods)	Method a 74.6% Method b 73.3% Method c 81.3%		n/s
Brugghe [85]	1995	102	2	n/s		75.5%
Lund [86]	1991	221	2	41%		X

Table 1d illustrates studies of reproducibility for histological grading in ovarian carcinoma. Studies show varying results. Intraobserver reproducibility tends to be better than interobserver reproducibility, yet marked disagreement may be observed.

Due to this diagnostic discrepancy and deemed importance of grade, more objective methods are required as accepted prognostic factors should in the least be reproducible.

1.2.7 Study Aims

Quantitative pathology as a tool in gynaecological pathology is fairly new, though the techniques used are generally well established and accepted in other fields. Such techniques allow greater objectivity than histological grading, typing, estimation of residual tumour etc. and have been shown in ovarian tumours to have considerable prognostic importance. Previous studies have, however, suffered from poor patient selection and trial design and have often included poorly selected tumours, which have themselves different outcomes to the main tumours which were under study.

Most previous work on morphometry and stereology in ovarian tumours has relied upon manual measurements, which are reproducible, but very time consuming, and require specialist knowledge of tissue appearances and the ability by the operator to separate tumour and non-tumour tissues. To date the best work on outcome in ovarian tumours has originated from northern Europe, though for a number of reasons such techniques have not been widely used elsewhere. Studies by Baak et al have shown that, in cisplatin treated patients, strong prognostic information can be obtained from the measurement of a range of nuclear characteristics, the mitotic index and the volume percentage epithelium. Mathematical scores have been proven of value in advanced ovarian cancer and it has been suggested that such techniques should be used extensively. On the whole this study aims:-

1. To determine whether basic morphometry data can predict outcome and chemotherapeutic response in ovarian serous carcinoma.

2. To determine whether newer semi-automated method of tumour morphometry provides similar results to older methods.

3.To determine whether advanced image analysis methods can offer further tumour

outcome data etc in serous ovarian cancer.

The study intends to determine, whether the morphometric prognostic variables delineated by other workers hold when performed in a large routine teaching hospital setting and provide prognostic information, which is useable and useful for clinical oncology.

The study will be initially performed on carefully selected group of serous carcinomas of the ovary. Tumour outcome, survival and chemotherapeutic response will be investigated in patients treated with the same platinum containing regimes, after histological confirmation of diagnosis, and where good follow up and survival data are available.

Outcomes will be primarily investigated using traditional prognostic parameters including:- clinicopathologic features, apoptotic markers (p53 & bcl2), mitotic activity and angiogenesis determinants. Semi-automated analysis, using immunohistochemically based techniques, will also be applied to estimate volume percentage epithelium and nuclear morphometric parameters, In recent years spatial and textural analysis in tumours has expanded dramatically and there are considerable expectations that it will have a greater predictive value and higher reproducibility than other simpler morphometric techniques. The effectiveness of various spatial textural features (e.g. those derived from certain grey level transformations of a group of neighbouring image pixels) will be investigated.

A search of morphometric parameters will be made to attempt to define any that predict clinical outcome in a hope that the results will allow more accurate prediction of outcome and therapeutic response in serous ovarian cancer and be useable for patient selection in earlier tumours. The methodology would also allow more accurate assessment of clinical trial data.

The end point of the study would be essentially to provide ovarian prognostic scores or morphometric data allowing prediction of likely outcome in individual ovarian serous carcinoma cases and to help to define those patients likely to benefit from chemotherapy with platinum containing or taxol containing regimes.

This MD considers clinicopathologic features, apoptotic markers, mitotic activity, volume percentage of epithelium, nuclear morphometric parameters, spanning tree parameters and angiogenesis determinants in separate chapters, including an introduction to each parameter, methodology, results, and discussion. A final analysis of all parameters will be performed followed by final conclusions.

Chapter 2: Common Methodology

2.1 Patients

This study assessed 184 patients who underwent initial surgery for ovarian cancer at University Hospitals Coventry & Warwickshire NHS Trust and Birmingham Women's Hospital NHS Trust, UK, from July 1994 to July 2002. Tumour stage and histological diagnosis of each case were determined according to the criteria of FIGO and The World Health Organisation (WHO). Tumours were graded as well (G1), moderate (G2) or poorly (G3) differentiated by an experienced pathologist. Extent of disease residuum was taken as < or >2cm as determined by the operator with all surgical procedures being undertaken by a gynaecological oncologist. Survival was taken as date of primary surgery to date of death or study closure. Cause of death was determined from patient records or via the West Midlands Cancer Registry.

2.2 Coefficient of Error

Coefficients of error (CE) were used to calculate the number of parameters, and fields of vision, required for measurements to attain adequate sample sizes. The CE expresses standard error (SE) as a percentage of the mean. (CE =(SE/mean) X100%). When repeated measurements reach a value of ≤5% a high degree of measurement precision is indicated, thus when measurement's remained stable over several fields (≤5% variation), this was accepted as a representative sample.

2.3: Immunohistochemistry - Peroxidase Anti-Peroxidase Technique

Immunohistochemical studies were performed using a standard peroxidase anti-peroxidase technique.

1. Take sections to water (i.e. de-wax and re-hydrate).
2. Block endogenous peroxidase in hydrogen peroxide / Optimax wash buffer 50/50

(20 minutes).

3. Wash with Optimax wash buffer (5 minutes).
4. Incubate in normal horse serum diluted 1:100 in TBS (20 minutes) – to block non-specific antibody binding.
5. Incubate with primary antibody at appropriate dilution (60 minutes).
6. Wash with Optimax wash buffer pH 7.4 (2 X 5 minutes).
7. Incubate with appropriate secondary biotinylated antibody diluted 1:50 (30 minutes) – i.e. labels primary antibody with secondary antibody.
8. Wash with TBS (2 X 5 minutes).
9. Incubate with horseradish peroxidase streptavidin (30 minutes) – labels secondary antibody.
10. Wash with TBS (2 X 5 minutes)
11. Incubate in DAB peroxidase substrate solution (4 minutes) – visualises secondary antibody.
12. Rinse in water and counterstain as required (30 seconds)
13. Wash well in tap water
14. Dehydrate, clear and mount.

2.4 Statistical Analysis

Statistical analysis was performed using SPSS® for Windows, Version 13.0, Copyright b c 2003, SPSS inc., USA. Intra and inter-observer reproducibility was assessed with linear regression analysis. For a better understanding of the biologic meaning all individual parameters were analysed for correlations with clinicopathologic features again by linear regression analysis. Overall survival (OS) - time between date of first operation and death, or time to study closure, and disease free survival (DFS) - time between initial surgery and detectable increase in disease on CT scan, (Univariate Cox model) was

performed on both individual and clinicopathologic parameters to evaluate their individual prognostic value. Multivariate OS and DFS analysis (Cox Forward stepwise likelihood ratios with OS or DFS time as the dependent factor) was performed to test whether combinations of features could improve the prognostic value of individual features.

To evaluate the ability of covariates to predict response to chemotherapy multi-logistic regression analysis was performed (forward stepwise likelihood ratios) using, as per WHO criteria, no response (no decline or increase in Ca125 levels), partial response (up to 50% reduction in Ca125 levels) and complete remission (normalisation of Ca125 levels at 6 months) as dependent variables and measurement parameters of interest as covariates. Individual parameters were primarily entered into the model, followed by consideration of individual and clinicopathologic parameters. Analysis was performed using mean values for each measurement parameter. P values <0.05 were regarded as being significant.

2.5 Prediction of Chemotherapy response

Ca125 data was available for 69 cases, 5 of which did not receive primary chemotherapy so results of 64 patients were analysed. All patients were treated with primary carboplatin or carboplatin / paclitaxel regimes.

Complete remission was achieved in 60% of patients, partial response in 34% and no response in 6%. As there was only a small number of cases in the no response group, these cases were combined with the partial response group and analysed together i.e. no/partial response (Group 1, n =26) versus complete response (Group 2, n =38).

Chapter 3: Clinicopathologic Parameters: Results

3.1 Introduction

Clinicopathologic parameters were analysed as to their prognostic and predictive ability with regard to overall survival (OS), disease free survival (DFS), and prediction of chemotherapy response. Analysis was performed to ensure that base clinicopathologic results, in this group of patients, were comparable with prior study findings.

3.2 Results

3.2.1 Patients

Of an initial 184 identified cases of serous ovarian cancer, 132 cases were investigated. 52 cases (28%) were excluded from analysis as these cases were regarded as being arguably metastatic in origin, borderline tumours, of mixed histological sub-type or technically unsatisfactory (poor preservation, small tumour sample etc). Of those analysed 16% were stage I, 14% stage II, 63% stage III and 7% stage IV, as determined by FIGO. Seventeen percent were grade 1, 40% grade 2 and 43% grade 3 as determined by WHO. Thirty five percent had an estimated disease residuum of <2cm and 65%> 2cm as defined by the operator. Mean age was 61.4 years (range 26-82 years). Ca125 pre-operative levels were available for evaluation in 91 cases. Post-operative Ca125 levels were taken at varying intervals following surgery and therefore disregarded for further survival analysis in this study. Ascites was regarded as either being present or absent as documented by the operator or as assessed by histocytopathology. All surgery was performed in tertiary referral centres by recognised gynaecological oncologists hence operator dependent variables were also disregarded in this study. Overall 5year survival was 26%. (See Table 3a:Summary of Clinicopathologic Data.)

Table 3a: Summary of Clinicopathologic Data (n=132)

Parameter	n	%	Alive (%)	5-Year Survival (%)
Age				
<65	79	60	25	22
>65	53	40	26	23
Ca125	91			
>35	82	90	33	26
<35	9	10	33	22
Ascites				
Present	87	66	3	8
Absent	45	34	62	47
Residual Disease				
<2cm	46	35	50	41
>2cm	86	65	13	12
FIGO Stage				
I	21	16	62	67
II	19	14	42	47
III	83	63	14	18
IV	9	7	11	11
Tumour Grade				
1	22	17	59	64
2	53	40	19	23
3	57	43	19	21

3.2.2 Correlations Between Clinicopathologic Parameters

Linear regression analysis of the clinicopathologic parameters parameter's found tumour grade, FIGO stage, extent of residual disease, and ascites to strongly correlate. Tumour grade also correlated with patient age. Pre-operative Ca125 levels were found to correlate with FIGO stage, ascites, and extent of residual disease. (See Table 3b: Correlations of Clinicopathologic Parameters).

Table 3b: Correlations Between Clinicopathologic Parameters

	Pre-op Ca125			Ascites			Age			Grade			Stage			Residual Disease		
	r	r^2	p	r	r^2	p	r	r^2	p	r	r^2	p	r	r^2	p	r	r^2	p
Pre-op Ca125				0.2087	0.04355	0.0471	-0.034	0.001141	0.7506	0.1876	0.0352	0.0749	0.2429	0.05898	0.0204	0.3214	0.1033	0.0020
Ascites	0.2087	0.04355	0.0471				0.1611	0.02596	0.0649	0.3943	0.1555	<0.0001	0.5619	0.3157	<0.0001	0.5473	0.2996	<0.0001
Age	-0.034	0.001141	0.7506	0.1611	0.02596	0.0649				0.1822	0.03318	0.0366	-0.548	0.003001	0.5327	0.09123	0.008322	0.2982
Grade	0.1876	0.03521	0.0749	0.3943	0.1555	<0.0001	0.1822	0.03318	0.0366				0.3729	0.1391	<0.0001	0.4200	0.1764	<0.0001
Stage	0.2429	0.05898	0.0204	0.3943	0.1555	<0.0001	-0.5478	0.003001	0.5327	0.3729	0.1391	<0.0001				0.6278	0.3941	<0.0001
Residual Disease	0.3214	0.1033	0.0020	0.5473	0.2996	<0.0001	0.09123	0.008322	0.2982	0.4200	0.1764	<0.0001	0.6278	0.3941	<0.0001			

Table 3b illustrates correlations found between clinicopathologic parameters.
Tumour grade, FIGO stage, residual disease status, and presence of ascites were found to strongly correlate. Tumour grade also correlated with patient age, and pre-operative Ca125 levels were found to correlate with FIGO stage, ascites, and extent of residual disease.
p values <0.05 were regarded as significant

3.2.3 Survival Analysis

3.2.3.1 Overall Survival Analysis

OS data was available for all 132 patients. P values below 0.05 were regarded as significant.

Clinicopathologic variables were analysed using the univariate Cox model. Of the clinicopathologic data, FIGO stage (p=<0.01), tumour grade (p=0.002), pre-operative Ca125 levels (p=0.016), presence of ascites (p=<0.01) and extent of disease residuum (p=<0.01) were found to be significant predictors of OS. Age (p=0.065) was found to be insignificant. (Cox overall survival curves are illustrated in Figure 3.1).

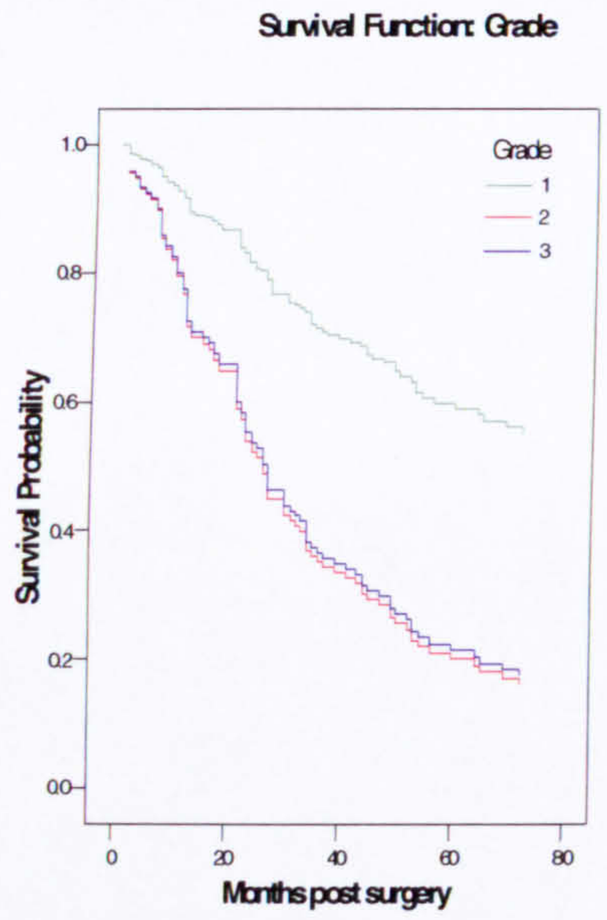
To estimate the simultaneous influence of prognostic factors all clinicopathologic variables were entered into Cox regression analysis. Analysis showed extent of disease residuum (p=<0.01) to be a significant predictor for OS. The other factors did not retain independent prognostic significance. (See table 3c & 3d for univariate and multivariate analysis of clinicopathologic data).

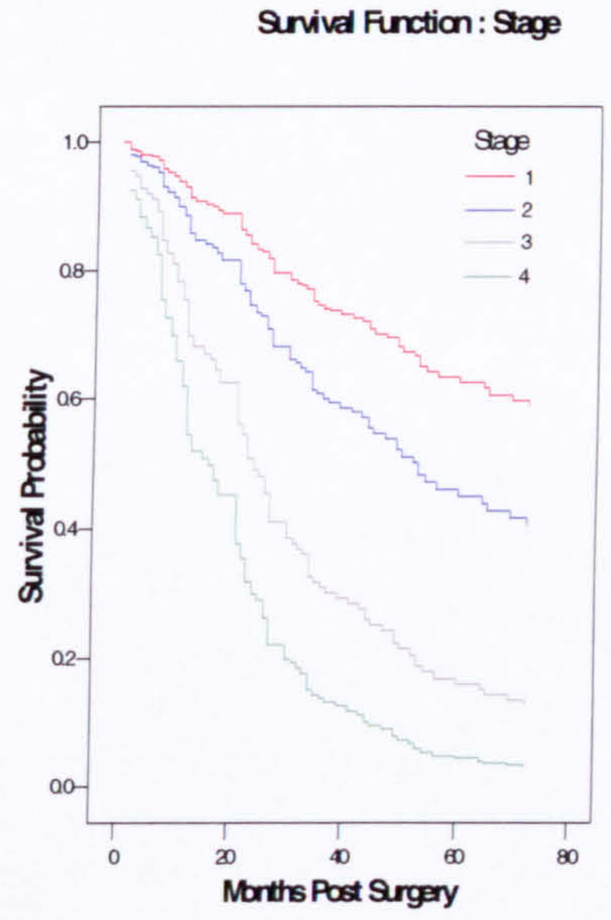
3.2.3.2 Disease Free Survival Analysis

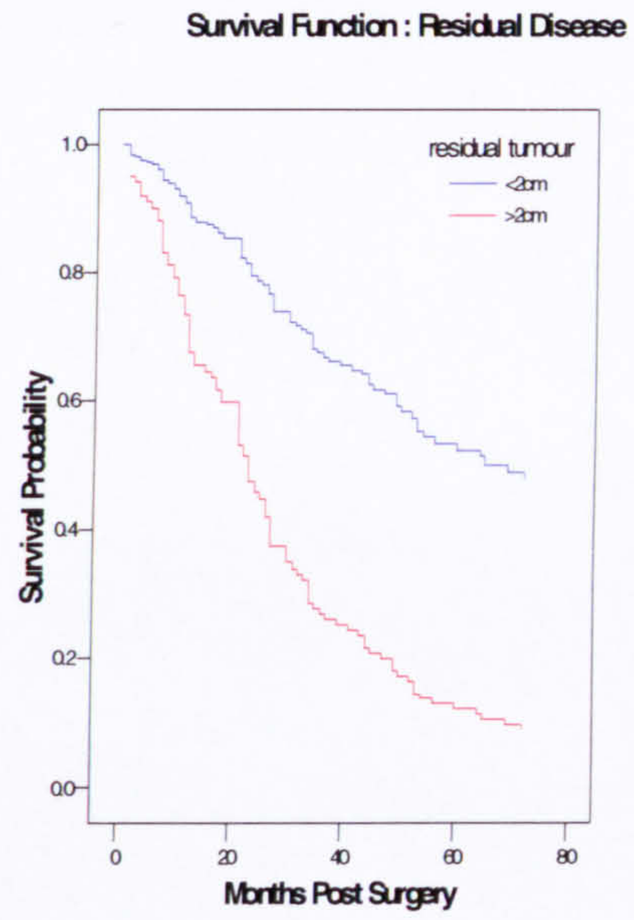
DFS data was available for 94 cases. P values below 0.05 were regarded as significant.

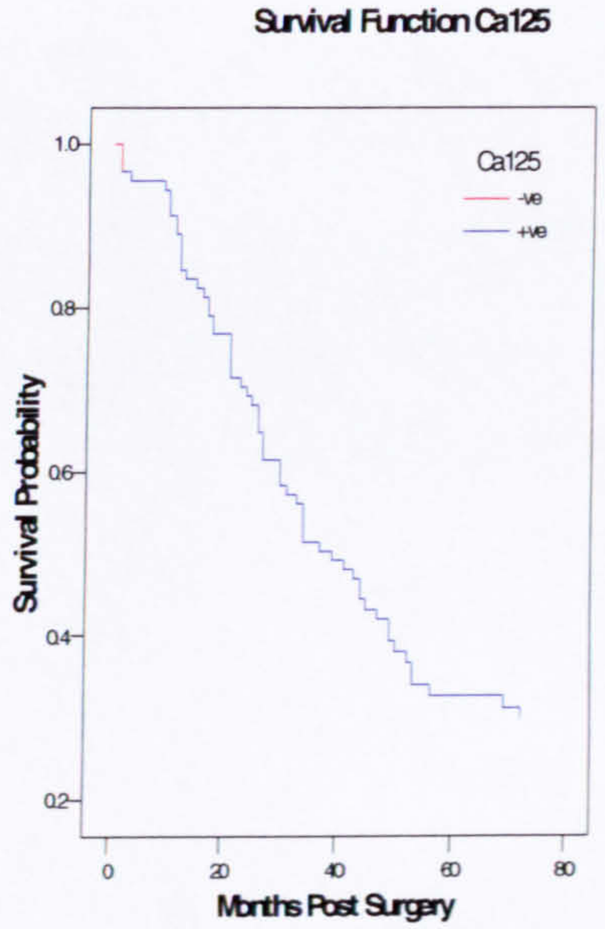
Clinicopathologic variables were analysed using the univariate Cox model. Of the clinicopathologic data FIGO stage (p=<0.01), tumour grade (p=0.001), pre-operative Ca125 levels (p=0.004), presence of ascites (p=<0.01) and extent of disease residuum (p=<0.01) were found to be significant predictors of DFS. Age (p=0.722) was found to be insignificant. (DFS curves are illustrated in Figure 3.2).

Figure 3.1: COX Univariate Curves: Clinicopathologic Data & Overall Survival









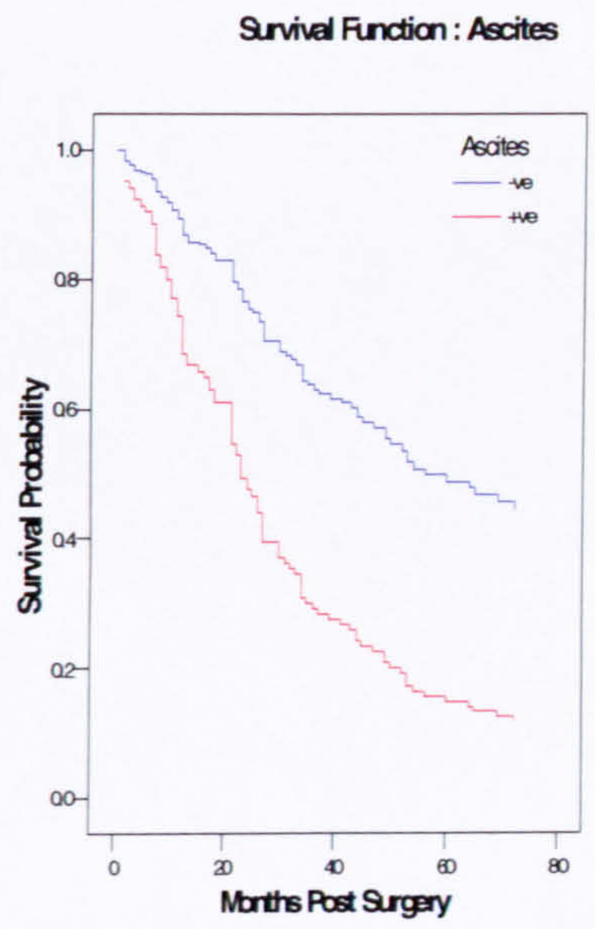
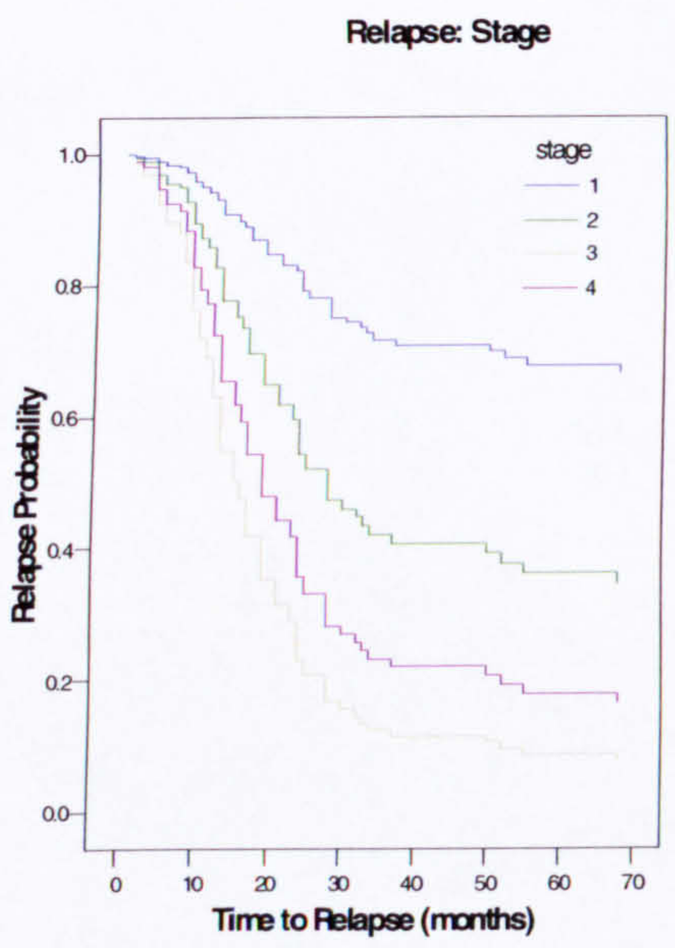
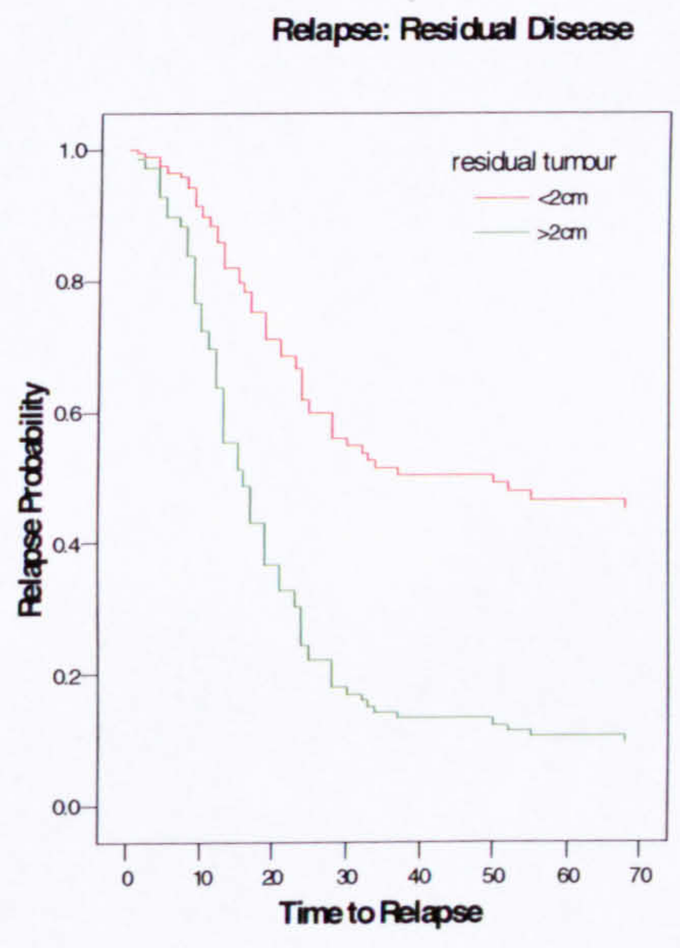
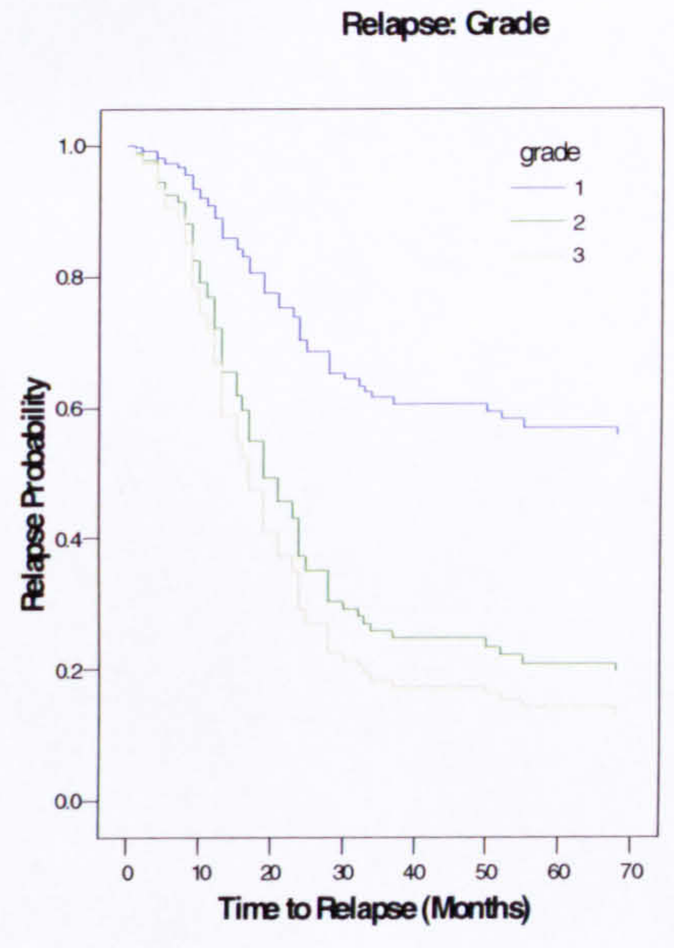
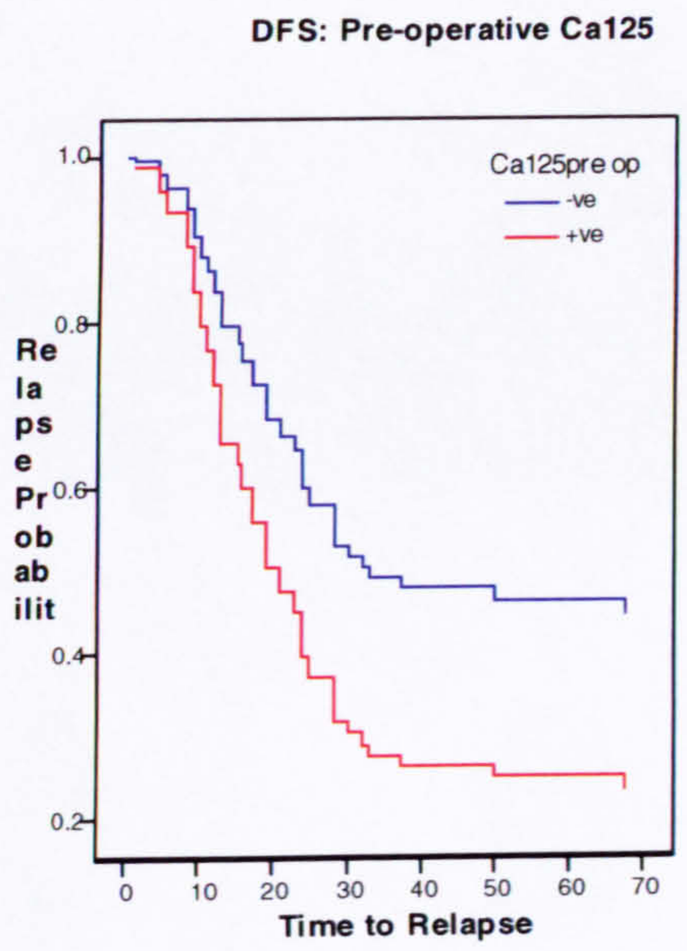


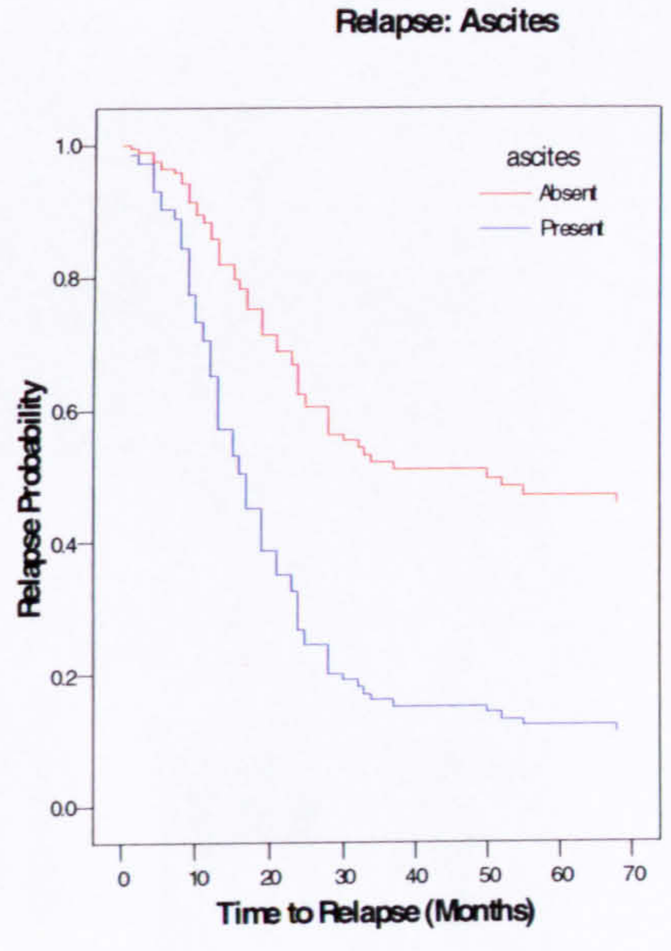
Figure 3.2: COX Univariate Curves: Clinicopathologic Data & DFS











To estimate the simultaneous influence of prognostic factors all clinicopathologic variables were entered into Cox regression analysis. This analysis showed FIGO stage (p=<0.01) to be a significant predictor for DFS. Age was of borderline significance (p=0.059). The other factors were not of additional prognostic relevance. (See table 3c & 3d for univariate and multivariate analysis of clinicopathologic data).

Table 3c: Univariate Analysis of Clinicopathologic Parameters

Overall Survival (OS)					Disease Free survival (DFS)			
Parameter	**n**	**Mean Survival Time (months)**	**% Alive (n)**	**P Value**	**Parameter**	**% Alive (n)**	**Mean Relapse Time (months)**	**P Value**
FIGO Stage					**FIGO Stage**			
Stage I	**21**	**64.9 (8-123)**	**62% (13)**	**<0.01**	**Stage I**	**19**	**69.1 (19-123)**	**<0.01**
Stage II	**19**	**49.53 (7-96)**	**35% (8)**	**<0.01**	**Stage II**	**15**	**40.73 (8-96)**	**0.037**
Stage III	**83**	**31.65 (1-106)**	**15% (12)**	**0.003**	**Stage III**	**56**	**23.52 (1-100)**	**0.417**
Stage IV	**9**	**22.56 (1-104)**	**11% (1)**	**0.158**	**Stage IV**	**4**	**36.5 (9-104)**	**0.555**
Tumour Grade					**Tumour Grade**			
Grade 1	**22**	**56.55 (6-123)**	**59% (13)**	**0.002**	**Grade 1**	**20**	**60.5 (9-123)**	**0.001**
Grade 2	**53**	**34.26 (1-119)**	**19% (10)**	**0.004**	**Grade 2**	**33**	**33.76 (1-119)**	**0.001**
Grade 3	**57**	**34.72 (1-106)**	**19% (11)**	**0.846**	**Grade 3**	**41**	**25.93 (4-96)**	**0.384**
Age	**132**			**0.065**	**Age**	**94**		**0.722**
Ascites					**Ascites**	**94**		**<0.01**
Absent	**45**		**47% (21)**	**<0.01**				
Present	**87**		**15% (14)**					
Pre-op Ca125	**91**			**0.016**	**Pre-op Ca125**	**94**		**0.004**
Residual Disease					**Residual Disease**			
<2cm	**46**	**28.29 (1-104)**	**50% (23)**	**<0.01**	**<2cm**	**40**	**51.63 (1-123)**	**<0.01**
>2cm	**86**	**58.69 (1-123)**	**13% (11)**		**>2cm**	**54**	**24.48 (2-104)**	

Table 3c illustrates results of univariate Cox survival analysis (forward logistic regression). FIGO stage, tumour grade, residual disease status, ascites status, and pre-operative Ca125 levels were found significant predictors of both OS (time between date of first operation and death, or time to study closure), and DFS (time between initial surgery and detectable increase in disease on CT scan). Patient age at presentation was not significant.
p values <0.05 were regarded as significant.

Table 3d: Multivariate Analysis of Clinicopathologic Data

Overall Survival						Disease Free survival					
Parameter	**df**	**Sig.**	**Exp (B)**	**95% CI Lower**	**95% CI Upper**	**Parameter**	**df**	**Sig.**	**Exp (B)**	**95% CI Lower**	**95% CI Upper**
Residual Disease	1	**<0.01**	.272	.150	.494	**FIGO Stage**	3	**<.01**			
Tumour Grade	2	.097				**Stage(1)**	1	**.001**	.090	.021	.391
Grade(1)	1	.115				**Stage(2)**	1	.019	.198	.051	.767
Grade(2)	1	.053				**Stage(3)**	1	.348	.566	.173	1.859
FIGO Stage	3	.091				**Age**	1	1	.059		
Stage(1)	1	.106				**Tumour Grade**	2	2	.145		
Stage(2)	1	.206				**Grade (1)**	1	1	.050		
Stage(3)	1	.065				**Grade (2)**	1	1	.374		
Ascites	1	.118				**Ascites**	1	1	.195		
Ca125 pre op	1	.499				**Residual Disease**	1	1	.183		
Age	1	.143				**Ca125 preop**	1	1	.077		

Table 3d illustrates results of multivariate Cox survival analysis (forward logistic regression). Overall residual disease status and FIGO stage were found to retain independent significance for OS (time between date of first operation and death or time to study closure), and DFS (time between initial surgery and detectable increase in disease on CT scan) respectively.
p values <0.05 were regarded as significant.

3.2.4 Prediction of Chemotherapy Response

3.2.4.1 Patients

All patients were treated with primary carboplatin or carboplatin / paclitaxel regimes. (See table 3e for distribution of clinicopathologic data and chemotherapy response).

In the no/partial response group 4% were FIGO stage I, 15% FIGO II, 73% FIGO III and 8% FIGO IV as compared to the complete response group where 31% were FIGO stage I, 29% FIGO II, 37% FIGO III and 3% FIGO IV. In the no/partial response group distribution of tumour grade was 8% grade one, 31% grade two and 61% grade three as compared to the complete remission group of 42%, 32% and 26% in grade 1-3 respectively. Fifteen percent of the no/partial response group had <2cm disease residuum as compared to 61% in the complete remission group, and 85% of the no/partial response group had >2cm disease residuum as compared to 39% in the complete remission group. The no/partial response group was also twice as likely to have ascites as compared to the complete

response group. Mean survival in the no response group was 16 months with no patents alive at 5yrs, in the partial response group 32.8 months with nine percent alive a 5yrs and in the complete response group 63.34 months with sixty one percent alive at 5yrs. (See table 3e for distribution of clinicopathologic parameters and chemotherapy response).

3.2.4.2 Prediction of Chemotherapy Response - Results

Univariate logistic regression analysis found tumour grade (p=0.02), FIGO stage (p=0.004), extent of residual disease (p=<0.01) and presence of ascites (p=0.004) to be significant predictors of chemotherapy response, with correct overall classification to chemotherapy response group being 70.3%, 68.8%, 68.8% and 67.2% respectively.

Table 3e: Distribution of Clinicopathologic Parameters and Response to Chemotherapy

Clinicopathologic Parameter	No Response (n=4)	Partial Response (n=22)	Complete Response (n=38)
Mean Age (range) yrs	57 (46-63)	63 (46-82)	60 (26-82)
Tumour Grade: **1** **2** **3**	 1 0 3	 1 8 13	 16 12 10
FIGO Stage: **I** **II** **III** **IV**	 0 1 2 1	 1 3 17 1	 12 11 14 1
Residual Disease: **<2cm** **>2cm**	 2 2	 2 20	 23 15
Ascites: **Present** **Absent**	 3 1	 16 6	 14 24
Survival			
Alive (%) **Mean (months)**	0% 16.25 (7-24)	9% 32.8(7-72)	61% 63.34(10-123)

Table 3e illustrates the distribution of clinicopathologic parameters and response to chemotherapy For prediction of chemotherapy response where:-
No response = no decline or increase in Ca125 levels. Partial response = up to 50% reduction in Ca125 levels, and Complete remission = normalisation of Ca125 levels at 6 months.

Multivariate logistic regression analysis found extent of residual disease (p=0.0005) a significant predictor of chemotherapy response with a correct overall classification to chemotherapy response group of 66.1%. The remaining parameters failed to retain independent significance. (See Table 3f for logistic regression analysis and chemotherapy response).

Table 3f: Clinicopathologic Data: Logistic Regression Analysis and Chemotherapy Response

Parameter	df	Sig.	Exp(B)	95.0% C.I .for EXP(B)		Overall Correct Classification
				Lower	Upper	
Univariate Analysis						
Tumour Grade	2	.010				68.8%
Grade(1)	1	.003	12.800	2.412	67.918	
Grade(2)	1	.151	2.400	.728	7.917	
Constant	1	.244	.625			
FIGO stage	3	.025				68.8%
Stage(1)	1	.048	24.000	1.028	560.178	
Stage(2)	1	.209	5.500	.385	78.573	
Stage(3)	1	.761	1.474	.121	17.913	
Constant	1	.571	.500			
Residual Disease	1	.001	8.433	2.420	29.383	70.3%
Constant	1	.253	.682			
Ascites	1	.006	4.653	1.566	13.822	67.2%
Constant	1	.386	.737			
Age	1	.858				
Pre-op Ca125	1	.289				
Multivariate Analysis						
Residual Disease	1	.009	5.500	1.524	19.849	66.1%
Age	1	.858				
Grade	2	.173				
Grade(1)	1	.069				
Grade(2)	1	.680				
Stage	3	.194				
Stage(1)	1	.249				
Stage(2)	1	.192				
Stage(3)	1	.045				
Ascites	1	.106				
Pre-op Ca125	1	.289				

Table 3f illustrates univariate and multivariate logistic regression analysis of clinicopathologic parameters and chemotherapy response. Residual disease status was found to retain independent significance. p values <0.05 were regarded as being significant.

3.3: Discussion

Results of the clinicopathologic analysis were as expected. On the whole extent of disease residuum retained independent prognostic significance for both overall survival and prediction of chemotherapy response. FIGO stage retained independent prognostic significance for disease free survival. Grouped analysis of tumours into low stage (FIGO I & II) or high stage (FIGO III & IV), or low-grade (grade 1) and high-grade (grade 2 and 3) did not improve prognostic significance. Therefore FIGO stage (I-IV), tumour grade (1-3), presence or absence of ascites, pre-operative Ca125 levels (< or >35u/ml) and extent of disease residuum (< or >2cm) were analysed as categorical variables.

Chapter 4: Expression of Oncogene / Apoptotic Regulators - p53 and Bcl-2

4.1: Introduction

4.1.1 Background

The cell cycle is a string of events that results in duplication of genetic material and segregation of these 2 copies into 2 daughter cells. The cell cycle has 4 stages: G_1 (preparation for entering DNA synthesis), S (DNA synthesis occurs), G_2 (cell assembles machinery for distributing the newly replicated chromosomes equally to the two daughter cells), M (mitosis stage - formation of 2 daughter cells, chromosomes condense, nuclear envelope dissolves). Non-cycling cells are said to be in G_0 (growth arrest) phase.

The rate of cell accumulation is not only dependent on cell proliferation, but also on the rate of physiological cell death. [99] Apoptosis, or genetically programmed cell death, is a process in which a cell actively degrades itself, forming membrane-bound bodies which are phagocytosed by other cells without stimulating inflammatory processes. Apoptosis provides an efficient mechanism for eliminating cells that are unwanted for some reason and may furthermore be of significance for keeping cell numbers at constant levels in different organs. [100]

A major feature of cancer is the inappropriate division of cells. It is well recognised that many human cancers arise as a result of the accumulation of genetic mutations that occur during DNA replication with normal cellular proliferation. [101] Apoptosis is tightly linked to cell cycle controls, [102] and loss of apoptosis pathways, by mutation or abnormal expression of genes, appears to be an important contributor to the development of tumours by contributing to the survival of cells in inappropriate physiological situations. [102] Apoptosis is controlled by regulators, which have either an inhibitory effect on programmed cell death (anti-apoptotic) or block the protective effect of inhibitors (pro-

apoptotic). Numerous apoptotic markers have been identified but this study will concentrate upon p53, which plays a pivotal role in inducing apoptosis, [103] and Bcl-2 an apoptosis inhibitor.

4.1.2 p53

P53 is a 53 kDa nuclear phosphoprotein, [104-5] whose gene, TP53, is located on the short arm of chromosome 17. [106] The p53 protein binds to specific regions of DNA and inhibits inappropriate cellular proliferation through activating expression of a range of genes, particularly p21. Its action suppresses cell growth, controls the G_1 checkpoint response to DNA damage, [102] and controls entry into s-phase of the cell cycle. [107] It also plays a key role in activation of apoptosis through induction of bax. Loss of p53 suppressor function thus renders the cells susceptible to uncontrolled growth, with mutation or deletion of p53 believed to result in uncontrolled cell proliferation, and failure of apoptosis. The p53 gene is shown to acquire mutations during development of many human malignancies [105] and mutation of the p53 gene is a common genetic alteration in human cancer, [102] with most mutations occurring in exons 5-8. [108] It is thought that p53 protein alterations attributable to missense mutations, nonsense or frameshift mutations provide a selective advantage for clonal expansion of neoplastic cells. [108] In ovarian cancer p53 mutation is the most frequently observed molecular alteration, reported in approximately 50-80% of cases. [101,106,109-110]

Mutated p53 accumulates in the cell and is readily detected by IHC techniques because of the increased stability of the mutated form of the protein [107] and the resultant proteins having a significantly increased half-life. [104,111] Because wild-type p53 is quickly degraded, it cannot be detected by IHC in normal cells, therefore p53 positivity usually corresponds to a dysfunctional protein, with a mutation of the gene in most cases. [112] 10-20% of p53 mutations may occur outside exons 5-9 and these mutations do not lead to over

accumulation of protein. [101] IHC does not detect nonsense or frameshift mutations because these will result in truncated proteins, [111] but immunostaining has a sensitivity of approximately 90% for detecting missense mutations in exons 5-9 of the p53 gene in ovarian cancers. [101] Over-expression of p53 and occurrence of mutation of the p53 gene are significantly correlated events. [105,108-109]

4.1.3 Bcl-2

Bcl-2, so called because of its over-expression in B cell lymphoma, was the first gene identified (1988) as part of the apoptotic process. [107,113] It is an integral inner mitochondrial membrane protein of relative molecular mass 26 kDa [114] encoded by chromosome 18. [107] Bcl-2 is expressed in tissues with rapidly proliferating and differentiating cells [113] with its main effect being prolongation of cell survival by avoidance of apoptosis, [115] i.e. it is an apoptotic inhibitor or inhibitor of programmed cell death. This is achieved by forming heterodimers with pro-apoptotic proteins such as bax. Over-expression of Bcl-2 contributes to neoplastic-transformation. [116]

4.2 Methods

4.2.1 Immunostaining Techniques

IHC studies were performed on 4-6μm formalin-fixed, paraffin-embedded tissue using the peroxidase-anti peroxidase (PAP) complex technique. Areas representative of the invasive component of the tumour were selected from archival sections stained with H&E. Two sections containing areas of worst atypia were selected and immunostained from each case. Sections were immunostained using the monoclonal antibody p53, (DAKO Monoclonal Mouse Anti-Human p53 Protein, Clone DO-7, Code No M 7001), and Bcl-2 (Monoclonal Mouse Anti-Human BCL Oncoprotein, Clone 124, Code No 0887). A positive and negative control slide was included in each staining run. Haematoxylin was used as a counter-stain. Sections were again washed then dehydrated, cleaned and

mounted.

4.2.2 p53 Assessment

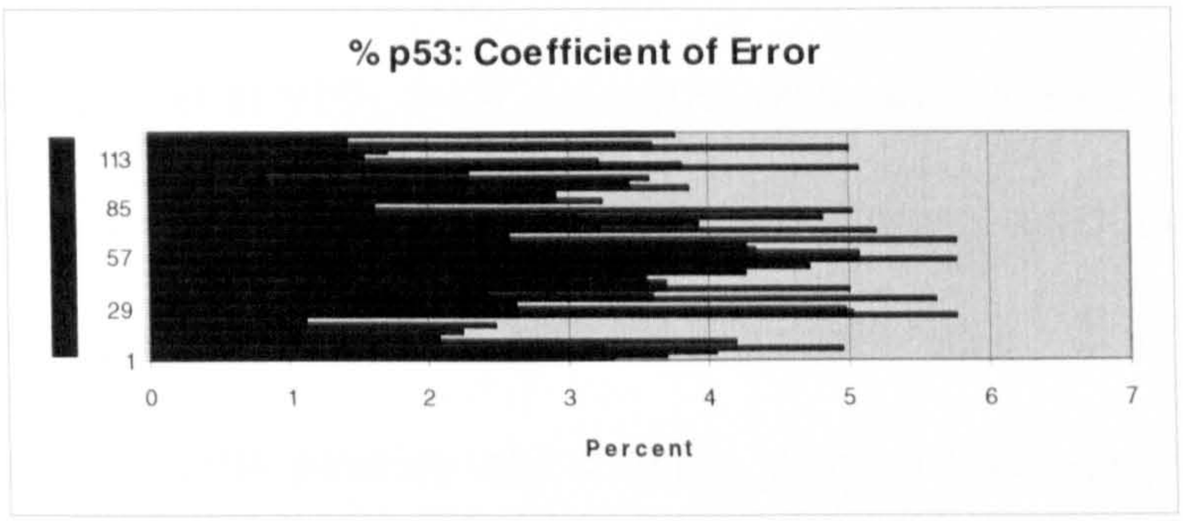
Sections containing areas of worst atypia were initially re-cut and stained with p53 using a haematoxylin counter-stain. Sections were scanned, in parallel to original tumour sections, for areas containing the highest p53 staining. Areas containing the highest p53 staining were selected and assessments made, in a meandering technique, at a final magnification of 400X. Staining for p53 was regarded as being positive when strong and widespread granular staining of the tumour cell nuclei was found. Tumours were assessed as to being either p53 positive or negative, graded as 1-4 depending on staining intensity, and p53 positive nuclei were counted and expressed as a percentage of total nuclei within the field of vision (percent p53 expression). Coefficients of error (CE) were calculated to ensure adequate measurement fields were attained (≤5% variability) for percent expression of p53, and up to 20 fields were counted per case, encompassing a count of approximately 1000 nuclei per case (See Fig 4.1: Percent Expression of p53 Coefficient of Error).

4.2.3 Bcl-2 Assessment

Sections containing areas of worst atypia were initially re-cut and stained with Bcl-2 using a neutral red counter-stain. Sections were scanned, in parallel to original tumour sections, for areas containing the highest Bcl-2 staining. Areas containing the highest Bcl-2 staining were selected and assessments made in a meandering technique at a final magnification of 400X. Staining for Bcl-2 was regarded as being positive when tumour cells exhibited strong staining of the cytoplasmic membrane with strong granular perinuclear cytoplasmic staining. Bcl-2 was assessed as being either positive or negative and graded as 1-4 depending on staining intensity. Initially Bcl-2 positive nuclei were counted and expressed as a percentage of total nuclei within the field of vision but this technique proved difficult due to marked heterogeneity of staining thus Bcl-2 was assessed

by positivity and staining grade alone over a maximum of 20 fields per case

Fig 4.1: Percent Expression of p53 – Coefficient of Error



CE ≤5% Indicates a high level of measurement precision. Twenty visual fields were counted per case, encompassing a count of approximately 1000 nuclei per case

4.3 Results

Analysis was performed using mean values for each parameter. All 132 cases were examined for p53 & Bcl-2 measurement parameters. P values <0.05 were regarded as being significant.

4.3.1 P53 Immunostaining

P53 staining was confined to the nucleus and p53 positivity was observed in 96% of cases. Only 3 cases were p53 negative and these cases were of mixed grades, stage and differing amounts of residual disease. Mean p53 percent expression was 67% (range 16.3 - 96.6%). P53 grading revealed 10% to be grade one, 20% grade two, 24% grade three and 46% grade four.

Table 4a: Mean p53 & Bcl-2 Measurement Parameters and Clinicopathologic Data

Parameter	p53 percent expression	p53 Grade	p53 Positive (%)	Bcl-2 grade	Bcl-2 positive
FIGO Stage					
FIGO I	49.58	2	95	2	52
FIGO II	61.88	3	96	3	35
FIGO III	78.47	4	96	3	42
FIGO IV	73.54	3	100	1	22
Grade					
Grade 1	46.31	2	91	3	32
Grade 2	68.85	3	94	3	45
Grade 3	67.1	4	100	3	42
Residual Disease					
<2cm	58.04	3	95	2	45
>2cm	67.1	4	96.5	3	40

4.3.2 P53 expression and relation to clinicopathologic data

Positive p53 staining was found in 95%, 96%, 96% and 100% of tumours at FIGO stage I-IV respectively and in 91%, 94% and 100% of tumour grades 1-3 respectively. For extent of residual disease, those tumours with <2cm disease residuum, showed 95% of cases with positive p53 staining and 97% in tumours with >2cm disease residuum. However the extent (% cells positive and staining grade) of staining varied with tumour stage and grade (table 4a). Grading of p53 showed FIGO stage I-IV as having mean grades of 2,3,4 and 3 respectively and tumour grades 1,2 and 3 as having mean p53 staining grades of 2, 3 and 4 respectively. For extent of residual disease tumours with a disease residuum of < or > 2cm had mean p53 grades of 3, and 4 respectively.

4.3.3 Bcl-2 immunostaining

Positive staining for Bcl-2 was observed in 42% of cases. Of those positively stained for Bcl-2, 15% were Bcl-2 grade 1, 31% grade 2, 26% grade 3 and 28% grade 4.

4.3.4 Bcl-2 expression and relation to clinicopathologic data

Positive Bcl-2 staining was found in 52%, 35%, 42% and 22% of tumours at FIGO stage I-IV respectively and in 32%, 45% and 42% of tumour grades 1-3 respectively. For extent of residual disease, those tumours with <2cm disease residuum, showed 45% of cases with positive Bcl-2 staining and 40% in tumours with >2cm disease residuum. Grading of Bcl-2 showed FIGO stage I-IV as having mean grades of 2, 3, 3 and 3 respectively and all tumour grades as having mean Bcl-2 staining grades of 3. For extent of residual disease, tumours with a disease residuum of < 2cm had a mean Bcl-2 staining grade of 2 and those > 2cm had a mean Bcl-2 grade of 3. Table 4a illustrates mean p53 and Bcl-2 parameters and their relationship with clinicopathologic data.

4.3.5 Reproducibility

For p53 percent expression intraobserver error was calculated by repeating

measurements for ten cases at a 6-month interval. Inter-observer error was calculated over ten cases for each measurement variable. Intra- ($p<0.0001$) and interobserver ($p<0.0001$) reproducibility was excellent for percent p53 expression. (See Table 4b: Reproducibility of percent p53 expression). Two observers simultaneously assessed tumours for p53 and Bcl-2 positive staining and staining grades. A 99% concordance rate was achieved.

Table 4b: Reproducibility Percent p53 Expression

Patient Number	Intra Observer		Inter Observer
	Round 1	Round 2	
Patient 11	86.57	85.542	87.2200
Patient 19	90.553	90.606	92.502
Patient 25	95.1645	92.598	93.6645
Patient 37	83.907	84.325	89.5
Patient 38	75.4855	74.928	80.66
Patient 79	39.4695	43.691	48.1
Patient 154	93.5615	90.8355	90.265
Patient 165	69.6025	69.918	74.2
Patient 169	93.623	92.4175	93.2
Patient 183	91.7335	91.4515	88.9275
r	0.9983		0.9985
r^2	0.9966		0.9772
p	<0.0001		<0.0001
spearman	0.9879		0.8909
2-tailed p	<0.0001		0.0011

Table 4b illustrates reproducibility of percent expression of p53 Ten cases were counted with measurements repeated at a 6 month interval. Excellent reproducibility was found at both at inta- and interobserver level.
p values <0.05 were regarded as significant

4.3.6 Correlation between parameters

On linear regression analysis p53 percent expression strongly correlated with FIGO stage ($p=0.0002$), tumour grade ($p=0.0003$), the presence of ascites ($p=0.015$), and extent of residual disease ($p=0.0053$). Positive p53 staining also correlated with tumour grade ($p=0.0229$) and p53 grade correlated with the presence of ascites ($p=0.0293$). Bcl-2 was not found to correlate with any clinicopathologic parameter. None of the Bcl-2 or p53 variables showed any correlation with one another. (See Table 4c).

4.3.7 Survival Analysis

OS data was available for all 130 cases. DFS data was available for 94 cases. P values below 0.05 were regarded as significant.

4.3.7.1 Significance of single parameters to predict survival / Univariate Analysis

Clinicopathologic variables, p53 and Bcl-2 were analysed using the univariate Cox model. Of the clinicopathologic data, FIGO stage (p=<0.01), tumour grade (p=0.002), pre-operative Ca125 levels (p=0.016), presence of ascites (p=<0.01) and extent of disease residuum (p=<0.01) were found to be significant predictors of OS. Age (p=0.065) was found to be insignificant. None of the p53 or Bcl-2 measurement parameters were found to be significant on univariate analysis for OS. For DFS, FIGO stage (p=<0.01), tumour grade (p=0.001), pre-operative Ca125 levels (p=0.004), presence of ascites (p=<0.01) and extent of disease residuum (p=<0.01) were found to be significant. Percent p53 expression (p=0.012) and p53 grade (p=0.01) were also found to be significant predictors of DFS. The remaining parameters were insignificant.

Expression of p53 and Bcl-2 was combined into four groups and analysed separately. Group 1 comprising p53 –ve & Bcl-2 –ve tumours; group 2, p53 +ve & Bcl-2 – ve; group 3, p53 -ve & Bcl-2 +ve and group 4, p53 +ve & Bcl-2 +ve. Repeat analysis was neither significant for prediction of OS nor DFS.

4.3.7.2 Multivariate Analysis

Multivariate analysis revealed extent of disease residuum to be an independent prognosticator of OS (p=<0.01). Analysis of p53 and Bcl-2 parameters alone revealed p53 percent expression (p=0.013) an independent prognosticator of DFS, with the remaining parameters adding no additional prognostic relevance. Combined analysis of all p53, Bcl-2 and clinicopathologic variables revealed FIGO stage (p=<0.01) to be an independent prognosticator for DFS. See table 4d for univariate and multivariate analysis.

Table 4c: p53 & Bcl-2 – Correlations Between Parameters

Parameter	p53 Percent Expression			p53 Grade			p53 positive			Bcl-2 Grade			Bcl-2 positive		
	r	r^2	p	r	r^2	p	r	r^2	p	r	r^2	p	r	r^2	p
Age	0.090	0.008	0.31	0.06	0.003	0.48	0.07	0.005	0.44	-0.02	0.0006	0.78	-0.05	0.003	0.56
Residual Disease	0.24	0.06	0.0053	0.12	0.02	0.15	0.05	0.003	0.57	0.008	6.632E-05	0.93	-0.06	0.003	0.52
Tumour Grade	0.31	0.09	0.0003	0.14	0.02	0.10	0.2	0.04	0.0229	0.07	0.004	0.46	0.53	0.003	0.55
Ascites	0.21	0.05	0.0145	0.19	0.04	0.0293	0.05	0.002	0.59	-0.04	0.001	0.7	-0.06	0.003	0.52
Ca125 pre-op	0.03	0.001	0.76	0.04	0.002	0.68	-0.14	0.02	0.2	-0.09	0.008	0.4	-0.1	0.01	0.32
FIGO Stage	0.32	0.10	0.0002	0.12	0.02	0.16	0.6	0.003	0.5	-0.01	0.0002	0.9	-0.08	0.007	0.49
p53 percent expression										-0.04	0.002	0.6	-0.08	0.006	0.38
p53 Grade										0.0009	9.85E-07	0.99	-0.2	0.0003	0.86
P53 Positive										0.06	0.004	0.5	-0.03	0.0006	0.77

Table 4c illustrates correlations between p53, bcl-2 and clinicopathologic parameters. Linear regression analysis revealed, p53 percent expression to strongly correlate with FIGO stage, tumour grade, ascites status, and residual disease status. Positive p53 staining also correlated with tumour grade, and p53 grade correlated with ascites status. Bcl-2 was not found to correlate with any clinicopathologic, or p53 parameter.
p values <0.05 were regarded as significant

Table 4d: Univariate and Multivariate Analysis – OS & DFS

Parameter	df	Sig. (p)	Exp (B)	95% CI Lower	95% CI Upper	Parameter	Sig. (p)	Exp (B)	95% Lower CI	95% Upper CI
Univariate Analysis										
Overall Survival (OS)						**Disease Free Survival (DFS)**				
Clinicopathologic Variables						**Clinicopathologic Variables**				
Grade	2	**.008**				**Grade**	**.004**			
Grade(1)	1	**.004**	.344	.168	.705	**Grade(1)**	**.001**	.288	.138	.603
	1					**Grade(2)**	**.384**	.798	.479	1.327
Stage	3	**<.01**				**Stage**	**<.01**			
Stage(1)	1	**<.01**	.151	.056	.403	**Stage(1)**	**.037**	.228	.057	.913
Stage(2)	1	**.003**	.256	.103	.639	**Stage(2)**	**.417**	.592	.167	2.101
	1					**Stage(3)**	**.555**	1.421	.442	4.568
Residual Disease	1	**<.01**	.341	.204	.568	**Residual Disease**	**<.01**	.341	.204	.568
Presence of Ascites	1	**<.01**	.377	.237	.599	**Presence of Ascites**	**<.01**	.358	.212	.605
Pre-operative Ca125	1	**.016**	1.000	1.000	1.000	**Pre-operative Ca125**	**.001**	1.000	1.000	1.000
p53 & Bcl-2										
p53 Percent Expression	1	0.74				**p53 Percent Expression**	**.012**	1.013	1.003	1.024
p53 Grade	1	0.28				**p53 Grade**	**0.01**	1.046	1.011	1.082
p53 Positive	1	0.91				**p53 Positive**	0.216			
Bcl-2 Grade	1	0.88				**Bcl-2 Grade**	0.257			
bcl-2 Positive	1	0.98				**Bcl-2 Positive**	0.658			
p53/Bcl-2 combined	1	0.94				**p53/Bcl-2 combined**	0.607			
Multivariate Analysis										
P53 & Bcl-2 Data										
Nil significant						**p53 Percent Expression**	**.013**	**1.013**	**1.003**	**1.024**
Morphometric & Clinicopathologic Data										
Residual Disease	1	**<.01**	.274	.151	.497	**FIGO Stage**	**<.01**	**2.428**	**1.663**	**3.544**

Table 4d illustrates results of univariate and multivariate Cox survival analysis. Percent expression of p53, and p53 grade were found significant predictors of DFS on univariate analysis, but failed to retain independent significance, being superseded by FIGO stage. None of the p53 parameters were significant for prediction of OS. Bcl-2 parameters were not found to be of prognostic signicance for either OS or DFS.

4.3.8 Prediction of Chemotherapy Response

Of the clinicopathologic data tumour grade (p=0.02), FIGO stage (p=0.004), extent of residual disease (p=<0.01) and presence of ascites (p=0.004) were found significant predictors of chemotherapy response with correct classification to chemotherapy response group being 70.3%, 68.8%, 68.8% and 67.2% respectively. Neither the p53 nor the Bcl-2

parameters showed any significance for prediction of chemotherapy response on univariate analysis. Multivariate analysis revealed extent of disease residuum (p=0.003) to offer independent prognostic ability with an overall correct classification to chemotherapy response group of 70%. (See table 4e for prediction of chemotherapy response results.

Table 4e: Logistic Regression Analysis: p53, Bcl-2 and clinicopathologic data & chemotherapy response

Parameter	df	Sig. (p)	Correct Classification	95% CI Lower	95% CI Upper
Univariate Analysis					
Clinicopathologic Variables					
Grade	2	**.002**	**68.8%**		
Grade(1)				2.412	67.918
Grade (2)				.728	7.917
Stage	3	**.004**	**68.8%**		
Stage(1)				1.028	560.178
Stage(2)				.385	78.573
Stage (3)				.121	17.913
Residual Disease	1	**<.01**	**70.3%**	2.420	29.383
Presence of Ascites	1	**.004**	**67.2%**	1.566	13.822
Pre-operative Ca125	1	.093			
Age	1	.554			
P53 & bcl-2 Variables					
P53 Grade	1	.217			
p53 positivity	1	.235			
p53 percent expression	1	.663			
Bcl-2 grade	1	.718			
Bcl-2 positivity	1	.771			
Multivariate Analysis					
p53, Bcl-2 & Clinicopathologic Data					
Residual Disease	1	**.003**	**70.3%**	1.960	25.738

Table4e illustrates univariate and multivariate logistic regression analysis of p53, bcl-2 & clinicopathologic parameters and chemotherapy response. Neither p53 nor bcl-2 parameters were found to be of significance.

4.4 Discussion

In agreement with prior ovarian carcinoma studies, this study found p53 positivity and over-expression to correlate with advancing FIGO stage, [99,104,106,108,110-111,117-120] worsening tumour grade, [99,101,104,106-109,111,117-122] increased amounts of residual disease,

[99,104,108,11118-120] and the presence of ascites. [120] Considering that p53 gene mutation in ovarian cancer appears with lower frequency in early stage disease, [111] it could be argued that p53 mutation is a late event in ovarian carcinogenesis, this is however, contradicted by the generally large levels of positivity found at all stages. As p53 over-expression is more likely found in tumours with increased amounts of residual disease, and in disseminated disease, it may be indicative of a more aggressive nature in p53 positive tumours. The fact that only 3 tumours were found to be p53 negative in this study was not surprising as p53 over-expression is observed more frequently in serous tumours than in any other histological sub-types, [101,110,119,121,123] with serous type being associated with moderate to high p53 expression, [109] and this study also has a higher number of patients presenting with advanced stage disease.

Bcl-2 over-expression is also reportedly seen most frequently in serous ovarian tumours [107] yet, using strict criteria for assessment, 58% of tumours were found to be Bcl-2 negative in this study. In fact, in agreement with prior study data, Bcl-2 was not found to correlate with any clinicopathologic [118] or p53 parameter.

P53 accumulation is felt generally to be associated with adverse prognosis, [99] yet prior study findings are contradictory. In early stage ovarian carcinoma positive staining for p53 has been associated, in some studies, with significantly worse survival rates [107] and p53 expression status has been found both a strong negative prognosticator [120] and an independent prognostic factor for survival. [123] When considering all stages of ovarian carcinoma, p53 over-expression on the whole has shown prognostic significance on univariate analysis, [103-104,111,118,124,] but often failed to retain independent prognostic significance on multivariate analysis. [120-121] Like this study however, numerous prior investigations have also found no correlation between p53 and OS. [106,108,110,115,123,125-7]

Bcl-2 up-regulation has occasionally been related to better clinical outcome in ovarian cancer and has been reported as a marker of poor prognosis. [128] Decreased apoptosis correlating to high Bcl-2 expression should in theory increase cell mass and decrease cell loss therefore enabling tumour expansion. Although it may seem counter intuitive that expression of an anti-apoptotic protein would result in a favourable prognosis, it has been postulated that Bcl-2, by virtue of inhibiting apoptosis, retards the rate of proliferation in solid tumours (i.e. cells do not die, but they do not divide either) and hence results in slow growing tumours with a favourable outcome. [129] Whilst in advanced stage ovarian cancer, Bcl-2 has shown a tendency to decline with increasing tumour aggressiveness, this did not reach statistical significance, [112] and more studies have agreed with the present findings that Bcl-2 expression does not have any correlation with survival in epithelial ovarian carcinoma. [99,110,115,122,130] (See Table 4f: Prior Study Data: Expression of p53 & Bcl-2 in relation to overall survival in epithelial ovarian cancer).

It has been suggested that an inverse relationship exists between Bcl-2 and p53 expression in many cancers, with the most favourable survival rate being when p53 expression is negative and Bcl-2 positive. [116] When positive expression of p53 exists with non-expression of Bcl-2 then a significantly worse survival is suggested, [107] with death due to disease being most frequent in this group. [116] Conversely, as in this study, other studies have found no relationship between p53 and Bcl-2 expression. [99,112,130] We must consider however that in this study very few tumours were p53 negative thus evaluation of differences between groups may not be particularly informative in our series.

Mutated p53 over-expression has been found to be associated with a higher risk of relapse [107] and both excessive [109,123] and negative [123] p53 expression to be associated with poor DFS. This study found both percent expression of p53 ($p=0.011$) and p53 grade

(p=0.04) to be significant predictors of DFS with worsening levels corresponding to shorter DFS times. Of the apoptotic markers, percent expressivity of p53 (p=0.013) was found to be the strongest predictor of DFS, but like other studies [119] failed to retain independent prognostic significance when compared with clinicopathologic parameters.

Research on cancer treatments indicate that cell death induced by both radiotherapy and chemotherapy is predominantly due to apoptosis. [107,116,132] P53 has been shown to play a role in apoptosis in response to chemotherapy-induced DNA damage, [125] and evidence exists that wild type p53 product is involved in the cellular response to a number of cytotoxic agents. [132] Cells with p53 gene transduction have shown higher sensitivity to cisplatin, and p53 gene transduction to enhance cisplatin-induced apoptosis. [103] Chemotherapeutic drug resistance may be a resistance to apoptosis. [116] Mutation of the p53 gene has been associated with a lack of response to platinum agents. [132] Inactivation of p53 could confer resistance to cisplatin and other DNA damaging agents [132] and progressive accumulation of p53 has been observed during the development of drug resistance. [113]

Bcl-2 inhibits apoptosis and has been shown to exert anti-apoptotic activity in ovarian cancer cells responding to chemotherapy, [110] and hence should be correlated with chemoresistance. [112] Progressive Bcl-2 accumulation has been observed during the development of drug resistance, and over-expression of Bcl-2 has been shown to confer resistance to cisplatin. [113] Bcl-2 is also an additional intracellular target of taxanes and its down-regulation is also involved in taxane resistance. [134]

Expression of p53 and prediction of chemotherapy response has been found significant in prior studies [113,132] with p53 expression associated with platinum resistance. [108,113] Conversely, however, like our study no data has been found to support the view that

p53 [124] or Bcl-2 expression is associated with response to chemotherapy.

Although it is well accepted that there is a concordance between genetic methods that detect p53 gene mutations, and IHC methods that measure mutant p53 protein accumulation, [135-8] variations in study findings on p53 may relate to the use of differing types of p53 antibodies or indeed marked variation in the interpretation of nuclear staining. Differing proportions of early stage tumours may also contribute to varying findings between studies and we must consider that this study includes a large proportion of advanced stage tumours. On the whole this study has found Bcl-2 of no value as a prognostic marker in this group of serous ovarian tumours, and p53 of limited value only, being largely overshadowed by the prognostic capability of FIGO stage and extent of residual disease.

If mutation of p53 and its consequent over-expression is an early event in ovarian tumorigenesis then p53 assessment is more likely to be beneficial in the assessment of either borderline [130] ovarian tumour as a possible indicator for malignant progression, or in early stage [140] ovarian tumours as a marker of tumour aggression or likelihood of recurrence. P53 analysis in a large group of stage I ovarian tumours may also, theoretically be able to identify those early stage tumours that may benefit from chemotherapeutic intervention.

Table 4f: Prior Study Data: Expression of p53 & Bcl-2 in relation to overall survival in epithelial ovarian cancer

Study Reference (Year)	Study Details						p53 over expression & Overall Survival		Bcl-2 & Overall Survival	
	n	Study Type	FIGO Stage	Histological Sub-Type	Assessment Method	Folllow Up (months)	Univariate Analysis	Multivariate Analysis	Univariate Analysis	Multivariate Analysis
99 (1996)	148	retrospective	I-IV	s,m,e,cc,u	positivity	120	0.0001	X	Not sig	Not sig
104 (1994)	55	prospective	I-IV	s,m,e,cc,mi	positivity	38 (5-60)	0.002	X	X	X
106 (1996)	221	retrospective	I-IV	s,,m,e,cc,tcc,B	graded	84	0.049	Not sig	X	X
107 (2001)	106	retrospective	Ia-IIc	s,m,e,cc	positivity	87 (57-125)	0.046	Not sig	Not sig	Not sig
108 (2001)	108	retrospective	I-IV	s,m,e,cc,B,u,mi ,un	positivity	31 (1.5-12yrs)	0.056	Not sig	X	X
109 (1993)	83	retrospective	I-IV	s,m,e,cc,u	graded	2-120	0.0025	X	X	X
110 (2002)	90	retrospective	I-IV	s,m,e,cc,tcc,u, mi	positivity	N/S	Not sig	Not sig	Not sig	Not sig
111 (1995)	95	retrospective	I-IV	s,m,e,cc,u,un	positivity	22.2 (1-55)	0.06	0.72	X	X
115 (1996)	70	retrospective	II-IV	s,m,e,cc,mi	graded	N/S	Not sig	Not sig	Not sig	Not sig
116 (2002)	109	retrospective	I-II	s,m,e,cc,a	positivity	48 (15-80)	0.007	0.020	Not sig	Not sig
117 (2003)	229	retrospective	Iib-IV	s,e,cc,u,o	positivity & graded	41 (7-146.7)	Not sig	Not sig	Not sig	Not sig
118 (1997)	112	retrospective	I-IV	s,m,e,cc,mi	graded	46 (2-148)	0.0004	x	Not sig	Not sig
119 (1999)	316	retrospective	I-IV	s,m,e,cc,m	positivity	n/s	<0.0001	0.026	X	X
121 (1995)	136	retrospective	I-IV	s,m,e,cc,a	positivity	120	0.002	0.008	X	X
122 (2000)	103	retrospective	I-IV	s,m,e,cc,tcc,u	percent expression	60	0.006	0.0032	Not sig	Not sig
124 (2001)	81	retrospective	I-IV	s,m,e,cc,m,mi	graded	N/S	0.45	N/S	X	X
125 (2003)	111	retrospective	I-IV	n/s	graded	N/S	Not sig	X	X	X
126 (2002)	50	retrospective	I-IV	s,m,e	positivity	45 (10-93)	Not sig	Not sig	X	X
123 (2004)	226	retrospective	Ia-IIc	n/s	graded	115-125	0.002	0.007	X	X

Key to histological sub-type: s=serous, m=mucinous, e=endometrioid, mi=mixed, cc=clear cell, tcc = transitional cell, B=Brenner, a=anaplastic, u =undifferentiated, un=unclassified, o=other

Chapter 5. Mitotic Activity Index

5.1. Introduction

5.1.1 Mitotic Counting

Mitotic counts (i.e. proportion of cells in mitosis) have been used to assess the growth fraction, or proliferation index, of a tumour. Mendelsohn 1962 [141] defined the growth fraction (GF) as the relation between the number of proliferating cells (P) and the total number of cells, including quiescent cells (Q), which are out of the mitotic cycle (GF=P/P + Q). The proliferation Index (PI) is defined as the ratio of nuclear area stained to the total nuclear area. [142] In theory, the greater the mitotic activity, or the greater the number of proliferating cells, the greater a tumour's potential to invade and metastasise. The rate at which tumour cells proliferate has long been considered to bear a relationship to the clinical course. [143] Counting techniques are used to deduce the number of mitotic figures per high power field, or as an expression of the percentage of mitotic figures to the total number of cells per high power field. Mitotic counting must be done reproducibly and rigorously to be useful [144] and has indeed shown excellent intra- and interobserver reproducibility. [145-6]

5.1.2 Ki67

Light microscopic methods of estimating mitotic index only identify cells in metaphase and telophase, [142] so detect only cells in those parts of the cell cycle, and are not able to distinguish non-cycling cells (G0) from cells in other phases of the cycle. The application of immunohistochemical (IHC) methods is therefore popular in assessing the state of cellular proliferation in histological material, [147] because they can be used to detect cells in all phases of the cycle.

Ki67 is commonly used as a marker for cell proliferation. It is an IgG murine

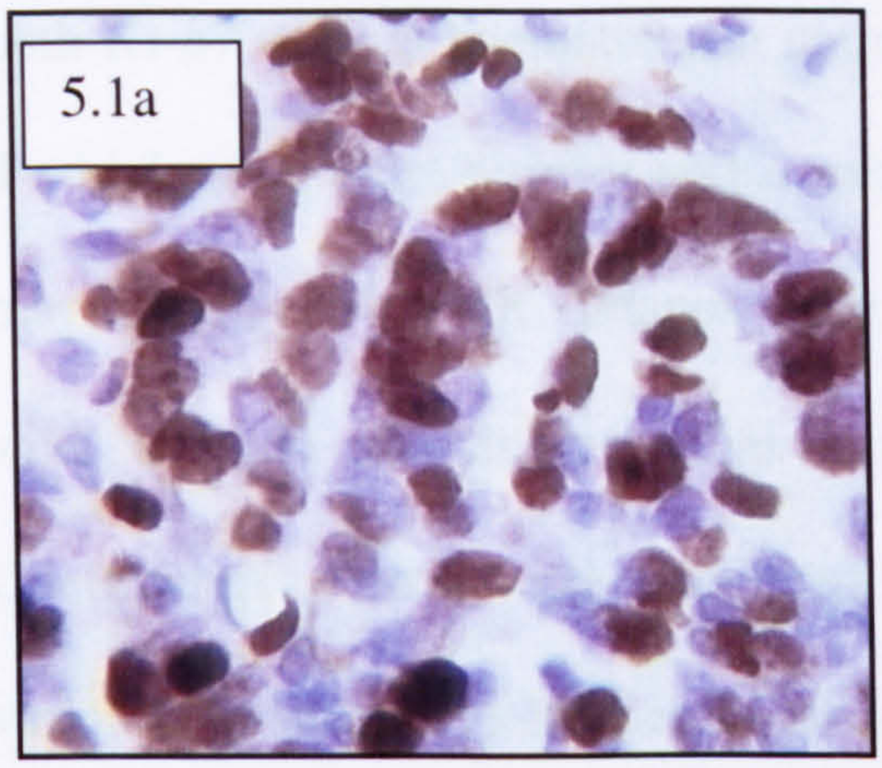
monoclonal antibody used to assess the growth fraction of a tumour. [7,8] Its gene is located at chromosome 10q25. Ki67 is present in cycling cells (all phases) but not non-cycling (G0) cells, [7,9] and reacts with a nuclear non-histone protein of 395 and 343 kDa. [10,11] In general Ki67 expression is an excellent indicator of proliferation in histological material, [147] but expression seems to be influenced by cell nutritional supply, so tissue taken from a central tumour area may give an erroneously low value for the growth fraction. [148] Tissue taken from a small biopsy may also not reflect the predominant proliferation rate of a tumour and, although Ki67 reflects the number of proliferating cells, [151] it only provides information about whether a cell is in cycle or not and does not tell us about cell cycle length. [148] This study utilizes Ki67 immunostaining to assess MAI.

5.2 Methods

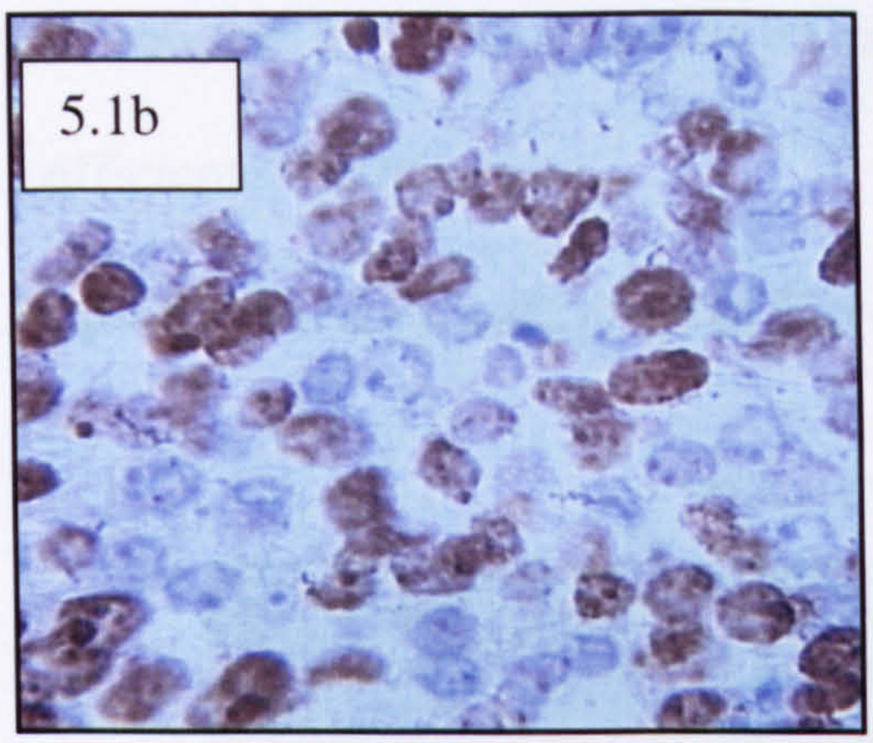
5.2.1 Immunostaining Technique

IHC studies were performed on 4-6μm formalin-fixed, paraffin-embedded tissue using the peroxidase-anti peroxidase (PAP) complex technique. Areas representative of the invasive component of the tumour were selected from archived sections stained with H&E. Two sections containing areas of worst atypia were selected and immunostained from each case. Sections were immunostained using the monoclonal antibody Ki-67, (DAKO Monoclonal Mouse Anti-Human Ki-67 Antigen Clone Ki-S5, Code No M 7187). A positive and negative control slide was included in each staining run. Haematoxylin was used as a counter-stain for Ki-67. Fig. 5.1 shows examples of Ki67 staining of carcinomas from three different women showing the range of staining found.

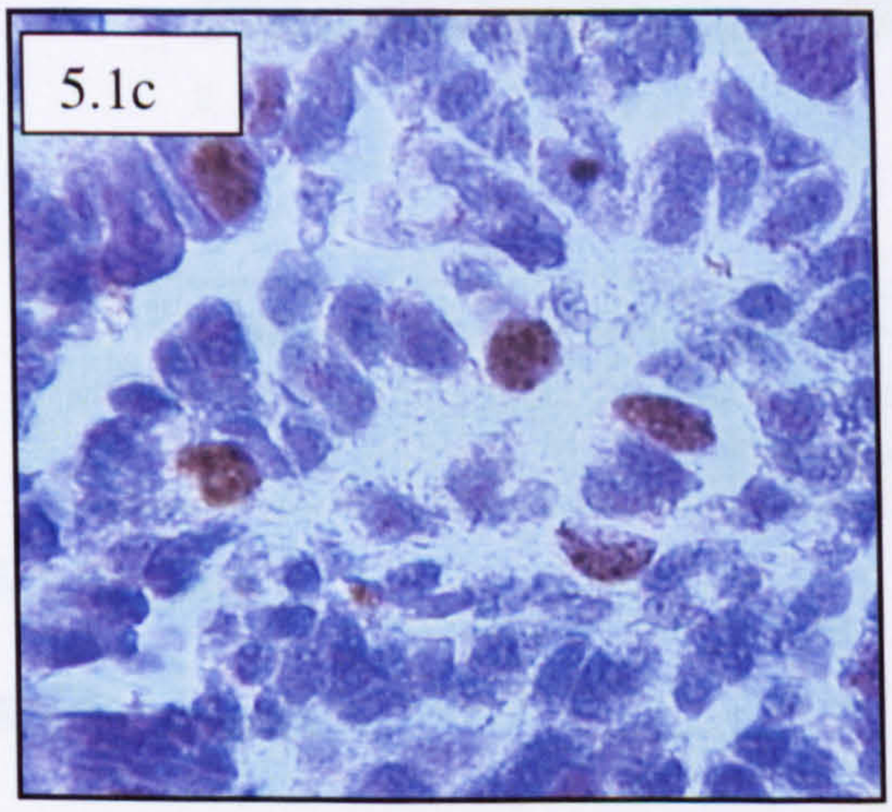
Fig 5.1: Ki67 staining and related survival in Serous Ovarian Tumours



Panel a: frequent Ki67 staining in a patient who survived one month



Panel b: rather less frequent staining, patient survived 26 months

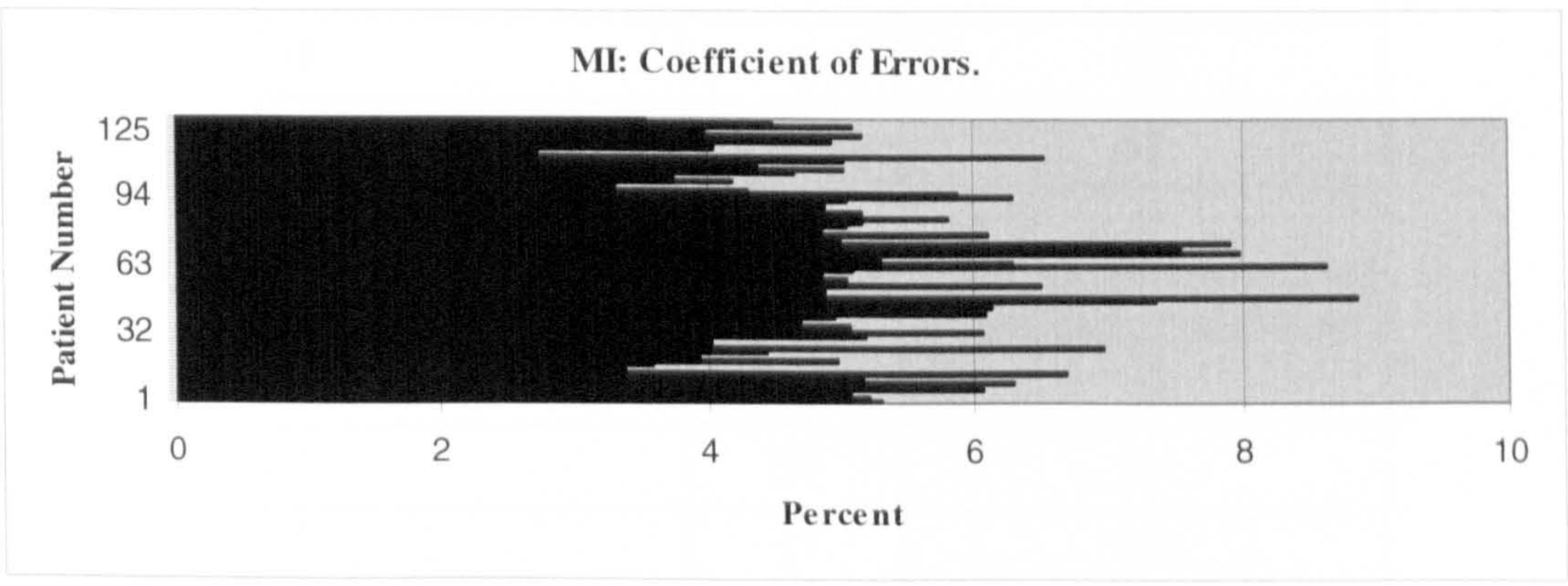


Panel c: sparse staining, patient still alive 60 months after resection

5.2.2 Mitotic Counting

Sections containing areas of worst nuclear atypia were initially re-cut and stained with Ki-67 using a haematoxylin counter-stain. Sections were scanned for areas including the highest mitotic activity and a mitotic count was performed on these areas at a final magnification of 400X. Images were captured onto the screen and Ki67 positive nuclei were counted and expressed as a percentage of total nuclei in the field of vision. Coefficients of error were calculated to ensure adequate sample sizes were attained (<5% variability) and up to 20 fields, randomly selected within the larger most atypical areas, were counted producing a count of greater than 1000 nuclei per case (See Graph 5a: MAI Coefficient of Error).

Graph 5a: MAI - Coefficient of Error



Coefficients of error were calculated to ensure adequate sample sizes were attained. Up to 20 visual fields were counted, encompassing a count of greater than 1000 nuclei per case

5.3 Results

5.3.1 Patients

Of a total 132 cases, 2 were excluded from MAI assessment as they were referral cases and original tissue blocks were not available for further assessment. Of those analysed 16% were Stage I, 15% stage II, 62% stage III and 7% stage IV as determined by FIGO. 17% were grade one, 39% grade two and 44% grade three. Thirty four percent had

an estimated disease residuum of <2cm and 66% > 2cm as defined by the operator. Mean age was 61.4 years (range 26-82 years). Cause of death was determined from patient records or via the West Midlands Cancer Registry. Overall 5year survival was 26%. (See Table 5a: Summary of clinicopathologic data)

Analysis was performed using mean values for each measurement parameter (see Table 5b: MAI mean values and clinicopathologic data). P values <0.05 were regarded as significant.

Table 5a: Summary of Clinicopathologic Data

Parameter	n	%
Mean Age	130	61.4yrs (26-82yrs)
FIGO Stage		
I	21	16
II	19	15
III	81	62
IV	9	7
Grade		
1	22	17
2	51	39
3	57	44
Residual Disease		
<2cm	44	34
>2cm	86	66

Table 5b: MAI Mean Values and Clinicopathologic Data

Clinical Parameters	MAI (range)
FIGO Stage	
I	20.329 (7.889-45.011)
II	35.432 (10.27-70.087)
III	36.755 (10.814-70.544)
IV	41.81 (8.36-61.139)
Tumour Grade	
1	20.766 (10.02-48.349)
2	35.676 (7.889-70.544)
3	38.837 (8.36-70.087)
Residual Disease	
<2cm	28.581 (7.889-60.415)
>2cm	37.599 (8.36-70.544)

5.3.2 Reproducibility

Intraobserver reproducibility was performed at a 6-month interval. Both intra- and interobserver reproducibility were performed on 10 cases. Intra- and interobserver reproducibility was excellent for MAI. (See Table 5c: Reproducibility MAI).

Table 5c: Reproducibility of MAI

Parameter		r	r^2	p	Spearman	2- tailed p
MAI	Intraobserver Error	0.996	0.9914	<0.0001	0.9879	<0.0001
	Interobserver Error	0.9910	0.9821	<0.0001	0.9879	<0.0001

Table 5c illustrates reproducibility of mitotic counts. Ten cases were counted at a 6month interval. Reproducibility was found to be excellent at both intra- and interobserver level.

5.3.3 Correlations

On linear regression analysis MAI was found to correlate strongly with extent of residual disease, tumour grade, presence of ascites and FIGO stage. (See Table 5d: MAI – correlation with clinicopathologic data).

Table 5d: MAI Correlation with Clinicopathologic Data.

Parameter	r	r^2	p	Significant
Age	0.05568	0.003100	0.5292	No
Residual Disease	0.3352	0.1123	<0.0001	Yes
Grade	0.3789	0.1436	<0.0001	Yes
Ascites	0.3244	0.1052	0.0002	Yes
Ca125 pre-op	0.1526	0.02328	0.1488	No
FIGO stage	0.3585	0.1285	<0.0001	Yes

Table 5d illustrates correlations between MAI and clinicopathologic parameters. MAI was found, on linear regression analysis, to strongly correlate with FIGO stage, tumour grade, ascites status and residual disease status

5.3.4 Survival Analysis

OS data was available for all 130 cases and DFS data was available for 94 cases. P values below 0.05 were regarded as significant.

Clinicopathologic variables and MAI were analysed using the univariate Cox model. Of the clinicopathologic data, FIGO stage (p=<0.01), tumour grade (p=0.002), pre-operative Ca125 levels (p=0.016), presence of ascites (p=<0.01) and extent of disease residuum (p=<0.01) were found to be significant predictors of OS and DFS. Age (p=0.065) was found to be insignificant. MAI was also found to be both a significant predictor of OS and DFS. For results of univariate survival analysis see table 5e.

Table 5e: Univariate Analysis: OS & DFS – Clinicopathologic and MAI Data

Parameter	n	Mean Survival Time	p	n	Mean Time to Relapse	p
FIGO Stage			**<.01**			**<.01**
Stage I	21	64.9	**<.01**	19	69.1	**0.037**
Stage II	19	49.53	**0.003**	15	40.73	0.417
Stage III	83	31.65	0.158	56	23.52	0.555
Stage IV	9	22.56		4	36.5	
Tumour Grade			**0.002**			**0.001**
Grade 1	22	56.55	**0.004**	20	60.5	**0.001**
Grade 2	53	34.26	0.846	33	33.76	0.384
Grade 3	57	34.72		41	25.93	
Age	132		0.065	94		0.722
Ascites	132		**<.01**	94		**<.01**
Pre-op Ca125	91		**0.016**	94		**0.004**
Residual Disease						
<2cm	46	28.29	**<.01**	40	51.63	**<.01**
>2cm	86	58.69		54	24.48	
MAI	130		**<.01**	94		**<.01**

Table 5e illustrates results of Cox univariate survival analysis for MAI and clinicopathologic parameters. MAI was found prognostically significant for both OS and DFS. (p values <0.05 were regarded as significant).

To estimate the simultaneous influence of prognostic factors all variables were entered into Cox regression analysis. This analysis revealed MAI (p=0.003) and residual disease (p=<0.01) to retain independent prognostic significance for OS. FIGO stage (p=<0.01) and MAI (p=0.011) retained independent prognostic significance for DFS. The other factors were not of additional prognostic relevance. (See table 5f Multivariate analysis: survival – MAI & clinicopathologic data).

Table 5f: Multivariate Analysis: OS & DFS – MAI & Clinicopathologic Data

Parameter	Overall Survival (OS)					Disease Free Survival (DFS)				
	df	Sig.	Exp (B)	95% CI Lower	95% CI Upper	Parameter	Sig.	Exp (B)	95% CI Lower	95% CI Upper
Residual Disease	1	**<.01**	.290	.209	.576	**MAI**	**.01**	1.02	1.005	1.038
MAI	1	**.003**	1.019	1.006	1.033	**Stage**	**<.01**			
Age	1	.326				**Stage(1)**	.172	.369	.088	1.544
Grade	2	.090				**Stage(2)**	.653	.747	.209	2.667
Grade(1)	1	.761				**Stage(3)**	.308	1.85	.567	6.034
Grade(2)	1	.045				**Age**	.174			
Stage	3	.150				**Grade**	.438			
Stage(1)	1	.352				**Grade(1)**	.278			
Stage(2)	1	.077				**Grade(2)**	.285			
Stage(3)	1	.052				**Ascites**	.610			
Ascites	1	.415				**Ca125 pre-op**	.357			
Ca125 pre-op	1	.348				**Residual Disease**	.506			

Table 5f illustrates results of Cox multivariate survival analysis for MAI and clinicopathologic parameters. MAI was found to retain independent prognostic significance for both OS and DFS.
(p values <0.05 were regarded as significant).

5.3.5 Prediction of Chemotherapy Response – Results

Univariate logistic regression analysis found tumour grade (p=0.02), FIGO stage (p=0.004), extent of residual disease (p=<0.01) and presence of ascites (p=0.004) to be

significant predictors of chemotherapy response, with correct classification to chemotherapy response group being 70.3%, 68.8%, 68.8% and 67.2% respectively. MAI was not found to be a significant predictor of chemotherapy response.

Multivariate logistic regression analysis found extent of residual disease (p=0.009) an independent predictor of chemotherapy response with the remaining factors not retaining independent significance. (See Table 5g for analysis of chemotherapy response data)

Table 5g: Logistic Regression Analysis: MAI and Clinicopathologic Data & Chemotherapy Response

Parameter	df	Sig. (p)	Correct Classification	95% CI Lower	95% CI Upper
Univariate Analysis					
Clinicopathologic Variables					
Grade	2	**.002**	**68.8%**		
Grade(1)				2.412	67.918
Grade (2)				.728	7.917
Stage	3	**.004**	**68.8%**		
Stage(1)				1.028	560.178
Stage(2)				.385	78.573
Stage (3)				.121	17.913
Residual Disease	1	**<.01**	**70.3%**	2.420	29.383
Presence of Ascites	1	**.004**	**67.2%**	1.566	13.822
Pre-operative Ca125	1	.093			
Age	1	.554			
MAI					
MAI	1	.352			
Multivariate Analysis					
MAI & Clinicopathologic Data					
Residual Disease	1	**.005**	**66.1%**	1.524	19.849

Table 5g illustrates univariate and multivariate logistic regression analysis results for MAI & clinicopathologic parameters and prediction of chemotherapy response. MAI was not found to be of significance.

5.4 Discussion

For prognostic factors to be reliable they must be reproducible, and MAI has shown excellent intra- and interobserver reproducibility. [145-6]

Higher MAI estimates were found to strongly correlate with increasing tumour grade, worsening FIGO stage, greater extent of residual disease and the presence of ascites. In prior study data on ovarian tumours, increasing levels of Ki67 have been found with increasing grade, [149,152-4] with the highest Ki67 values being found in grade 2 and 3 as compared to grade 1 tumours. [155] This suggests a possible correlation of high proliferative activity with mainly poorly differentiated tumours. Although high Ki67 values have been correlated with advanced stage, [153] numerous other studies dispute this finding. [142,149,152,154-6] Theoretically, if greater mitotic activity correlates to a tumours potential to invade and metastasise then correlation to advanced tumour stage, worsening tumour grade, the presence of ascites, and increasing amounts of residual disease is unsurprising.

A study in borderline ovarian tumours showed high MAI to be a strong negative prognosticator for survival [157] and generally low values of MAI seem to have good prognosis concerning survival. [158]

In early and late stage ovarian tumours results have been conflicting. Mitotic activity has shown varying importance as a prognostic factor, and in advanced stage disease, in some studies, has seemed to be prognostically less important than other factors for survival. [6,159] Nonetheless in other studies, both univariate and multivariate analyses have shown high Ki67 scores to be significantly associated with decreased survival, both in early [160] and advanced stage tumours. [149,152-3,158,161] In early stage tumours high Ki67 scores have correctly predicted cancer recurrence in 84% of cases [160] and, in general, significant disease free survival correlation has been shown for patients with low

proliferation indices. [155,160] This study clearly agrees with these findings, showing MAI to be a strong independent prognosticator not only for OS (0.003), but also for DFS (p=0.01) with higher MAI values conferring a worse prognosis.

Although there seems relatively little data, in ovarian carcinoma, regarding the value of MAI as a prognosticator for chemotherapy response, prior multivariate analysis has shown MAI able to independently differentiate patients on a basis of responders and non-responders to chemotherapeutic agents. [162] In another study however, MAI has also been found to be insignificant with regard to prediction of chemotherapy outcome, [158] which agrees with our findings. Discrepancies may exist as these studies have considered mixed histological sub-types, advanced stage (FIGO III & IV) disease only, and have comprised relatively small study populations. Due to the recruitment dates of these studies, varying chemotherapeutic regimes have also been employed in patient treatment, whereas our study has considered a well-selected group of serous ovarian tumours treated with primary carboplatin or carboplatin / paclitaxel regimes.

Overall MAI was found a very simple and highly reproducible investigation to perform, capable of providing independent prognostic information for survival. Results suggest that MAI certainly surpasses tumour grade in both its reproducibility, and value, as a prognostic factor in this group of patients and may have a significant role to play in future therapeutic decision-making. Larger studies of stage I & II ovarian tumours, would further clarify the role of MAI in earlier stage disease.

Chapter 6.Volume Percentage Epithelium.

6.1 Introduction

6.1.1 VPE Introduction

The estimation of the percentage epithelial area of a tumour or volume percentage of epithelium (VPE) has been used as a prognostic factor for survival in epithelial ovarian cancer. Estimation has been calculated by both manual and semi-automated techniques. Increased epithelial percentage denotes a more solid tumour with reduced areas of stroma, i.e. tightly packed sheets of cells, which are purportedly correlated to the invasive and metastatic potential of a tumour. In ovarian cancer neoplasms with relatively high VPE counts tend to be associated with extra-ovarian spread. [157]

Epithelial percentage has traditionally been estimated manually, by point counting, i.e. the number of points in a grid falling onto epithelium denotes the point fraction of the epithelium. The point fraction is an estimate of the area fraction, which is an estimate of the volume fraction where:-

$$\text{Volume fraction of epithelium} = \frac{\text{Volume occupied by epithelium}}{\text{Total volume of tissue.}}$$ [163]

Manual estimation of epithelial percentage, or point counting technique, is a rather tedious and time-consuming method with some degree of subjectivity. [164] It has however shown good overall intra- and interobserver reproducibility. [145,165] Any lack of reproducibility may be due to differences in sampling of visual fields between observers, or due to an insufficient number of points being measured per tumour section. [166]

Due to the time consuming and tedious approach of manual point counting, semi-automated techniques have been developed for the determination of VPE. Good correlation has been found between manual and semi-automated techniques [165,1677] with measurements being reproducible, cheap and easy to perform. [167]

6.1.2 Epithelial Markers

6.1.2.1 Cytokeratin

Cytokeratins (CK) occur in most normal epithelial tissues and antibodies against them have been used to characterise a wide variety of epithelial tumours. [168] The cytokeratins belong to the intermediate filaments, which create a cytoskeleton in almost all eukaryotic cells. They are made up of a highly complex multigene family of polypeptides with molecular masses ranging from 40 to 68 kDa. [169] Anti-human cytokeratin (Clone MNF 116) is a broad spectrum anti-keratin reagent reacting with intermediate and low-molecular-weight keratins. [169]

6.1.2.2 Epithelial Membrane Antigen

Epithelial Membrane Antigen (EMA) belongs to a heterogeneous group of heavily glycosylated proteins with a molecular mass range of 250-400kDa known as human milk fat globule (HMFG) membrane proteins. [170] These proteins are present in a variety of epithelia in both normal and neoplastic tissues. EMA is a useful tool for the identification of neoplastic epithelia, labelling epithelial cells in a wide variety of tissues. [171] Anti-human EMA (Clone E29) labels bands of 265-400 kDa.

The detection of cytokeratin intermediate filaments is widely used to identify tumours of epithelial origin with CK and EMA often used together in a diagnostic panel. [168] As CK and EMA are the most widely and successfully used epithelial markers, certainly in prior ovarian cancer studies, this study utilises both CK and EMA for VPE estimation purposes.

6.2 Method

6.2.1 Immunostaining Technique

IHC studies were performed on 4-6μm formalin-fixed, paraffin-embedded tissue

using the peroxidase-anti peroxidase (PAP) complex technique. Areas representative of the invasive component of the tumour were selected from archival sections stained with H&E. Two sections containing areas of worst atypia were selected and immunostained from each case. Sections were immunostained using CK (Monoclonal Mouse Anti-Human Cytokeratin, Clone MNF116, Code No M 0821) and EMA (Monoclonal Mouse Anti-Human Epithelial Membrane Antigen, Clone E29, Code No M0613). A positive and negative control slide was included in each staining run. No counter-stain was used for CK and EMA so as to optimise image analysis. (Fig 6.1 illustrates CK staining and related survival).

6.2.2 Epithelial Measurements – Manual Point Counting

Epithelial area was assessed in 30 cases by a standard manual point counting method. Using the archival H&E sections, random selection of the most epithelium rich area of tumour was performed. A Chalkey graticule (36 point grid) was placed into the microscope eyepiece and fields selected randomly, within the epithelial-rich areas, using a meandering technique. Measurements were performed at a final magnification of 400X and point counting was performed over 20 visual fields providing a maximum of 720 points per case.

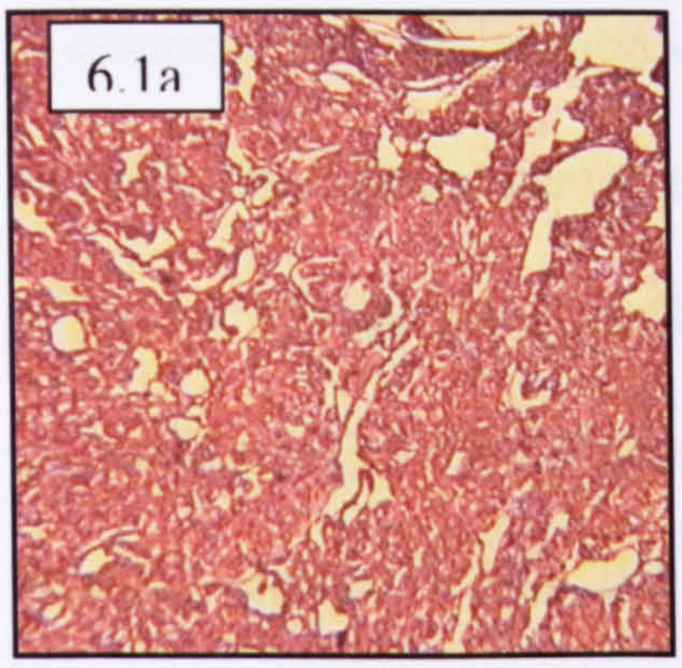
6.2.3 Epithelial Measurements – Automated

For automated epithelial assessments, sections containing the worst areas of atypia were initially re-cut and stained using combined CK and EMA. EMA proved to give more background staining than CK making automated analysis problematic. Therefore sections were stained with CK alone to perform VPE estimates in all cases.

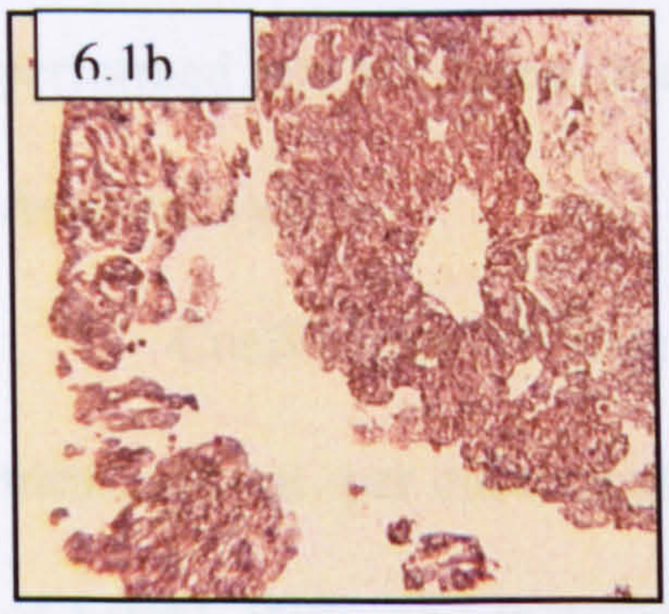
The live image was initially set up with regards to white balance to attain even illumination. The stained section was then randomly scanned using a set of up to 20 fields

at a final on screen magnification of 25X (1 pixel = 0.00323 microns). Once live image acquisition was attained the image was converted to binary enabling further image

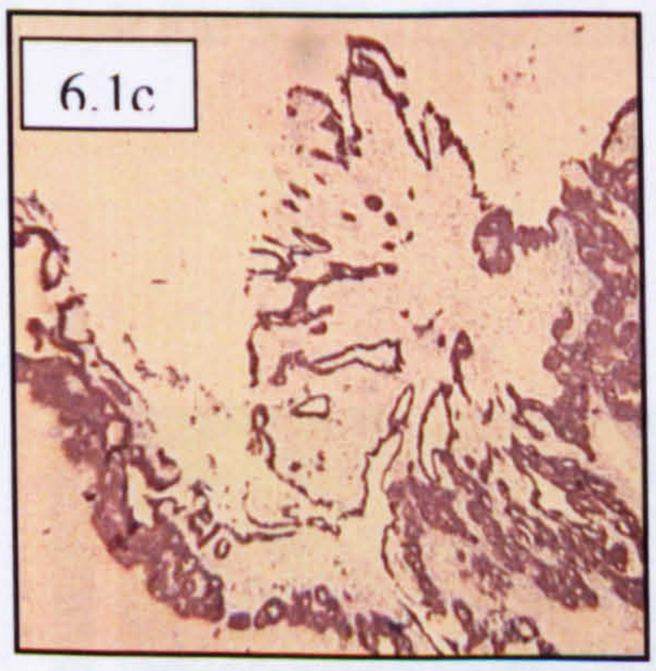
Fig 6.1: Shows CK staining and related survival in Serous Ovarian Tumours



Panel a. Cytokeratin staining in a patient surviving 1 month.



Panel b. Cytokeratin staining in a patient surviving 26 months.

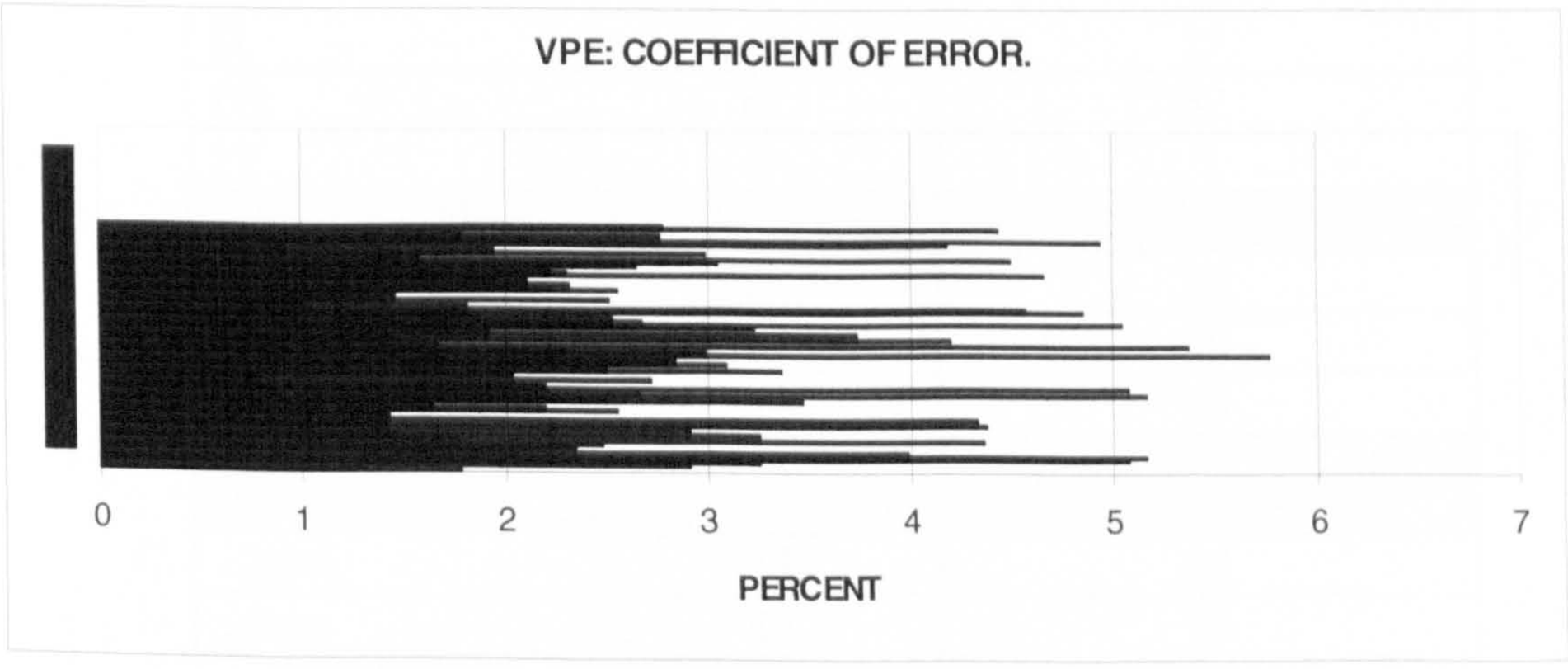


Panel c. Cytokeratin staining in a patient alive at study closure.

processing prior to measurement. A grey level detection was adjusted to correspond well to CK positive areas and negate any further unwanted regions. The binary images were amended permitting the image to be modified in such a manner as to obliterate the holes caused by nuclei present in the CK positive epithelium by means of dilatation. (Dilatation is essentially the expansion of image detail by moving boundaries outwards by a uniform amount. Dilatations were therefore performed upon each image in order to close the holes produced by epithelial nuclei with each dilatation constituting the size of 1 pixel in circumference). A Field measurement was attained (representing a summed value for all selected regions within the measurement frame) to in turn attain the area fraction. As manual and automated epithelial measurements were found to highly correlate (r=0.99), manual counts were only performed upon the first 30 cases and automated counts performed on all cases. Measurements were made using the LEICA Q500MC Image Analysis System.

Coefficients of error were used to calculate the number of fields required for measurement, per case, to attain adequate sample size. (See Graph 7a: VPE – Coefficient of Error). Intra- and interobserver error was calculated by repeating measurements for ten cases at a 6-month interval

Graph 6a: VPE - Coefficient of Error



CE ≤5% Indicates a high level of measurement precision

6.3 Results

6.3.1 Patients

Of the 132 cases, 2 were excluded from VPE assessment as they were referral cases and original tissue blocks were not available for further assessment. Of those analysed 16% were stage I, 15% stage II, 62% Stage III and 7% Stage IV as determined by FIGO. 17% were grade 1, 39% grade 2 and 44% grade 3. Thirty four percent had an estimated disease residuum of <2cm and 66% > 2cm as defined by the operator. Mean age was 61.4 years (range 26-82 years). Cause of death was determined from patient records or via the West Midlands Cancer Registry. Overall 5year survival was 26%. (See Table 6a: Summary of clinicopathologic data)

Analysis was performed using mean values for each measurement parameter (see Table 6b: VPE mean values and clinicopathologic data). P values <0.05 were regarded as significant.

Table 6a: Summary of Clinicopathologic Data

Parameter	n	%
Mean Age	130	61.4yrs (26-82yrs)
FIGO Stage		
I	21	16
II	19	15
III	81	62
IV	9	7
Grade		
1	22	17
2	51	39
3	57	44
Residual Disease		
<2cm	44	34
>2cm	86	66

Table 6b: VPE Mean Values and Clinicopathological Data

Clinical Parameters	VPE (%) (range)
FIGO Stage	
I	45.183 (15.059-69.14)
II	59.824 (27.015-83.463)
III	58.866 (21.143-80.275)
IV	62.629 (15.83-84.02)
Tumour Grade	
1	42.135 (15.059-73.74)
2	59.165 (28.06-72.8)
3	61.253 (12.646-84.027)
Residual Disease	
<2cm	52.404 (26.544-72.8)
>2cm	62.093 (12.646-90.859)

6.3.2 Reproducibility

Intra- ($p=<0.0001$) and inter-observer ($p=<0.0001$) reproducibility was excellent for VPE parameters. (See Table 6c: Reproducibility of VPE parameters).

Table 6c: Reproducibility of VPE Parameters

Parameter VPE		r	r^2	p	Spearman	2- tailed p
Manual vs. Automated	**Intra-Error**	**0.990**	**0.9805**	**<0.0001**	**0.9831**	**<0.0001**
Automated Technique		**0.992**	**0.9844**	**<0.0001**	**0.9636**	**0.0001**
Automated Technique	**Inter-Error**	**0.9866**	**0.9734**	**<0.0001**	**0.9515**	**<0.0001**

Table 6c illustrates reproducibility of VPE.
Manual assessment was found to strongly correlate with the semi-automated technique.Ten cases were counted at a 6month interval. Reproducibility was found to be excellent at both intra- and interobserver level.

6.3.3 Correlation between parameters

On linear regression analysis VPE was found to correlate with extent of residual disease, tumour grade, presence of ascites, FIGO stage, patient age and pre-operative Ca125 levels. (See Table 6d: VPE correlation with clinicopathologic data).

Table 6d: VPE Correlation with Clinicopathologic Data

VPE	r	r^2	p	Significant
Age	0.2010	0.04038	**0.0219**	Yes
Residual Disease	0.3345	0.1119	**0.0001**	Yes
Grade	0.3335	0.1112	**0.0001**	Yes
Ascites	0.2778	0.07715	**0.0014**	Yes
Ca125 pre-op	0.2812	0.07905	**0.0080**	Yes
FIGO Stage	0.3241	0.1051	**0.0002**	Yes

Table 6d illustrates correlations found between VPE and clincopathologic parameters on linear regression analysis. VPE estimates were found to strongly correlate with FIGO stage, tumour grade, pre-operative Ca125 levels, ascites status, residual disease status a, and patient age at presentation.

6.3.4 Survival Analysis

OS data was available for all 130 cases and DFS data available for 94 cases. P values below 0.05 were regarded as significant.

Clinicopathologic variables and VPE were analysed using the univariate Cox model. Of the clinicopatholgic data, FIGO stage, tumour grade, pre-operative Ca125 levels, presence of ascites and extent of disease residuum were found to be significant predictors of OS and DFS. Age was found to be insignificant. VPE was also found to be a significant predictor for both OS and DFS. (See table 6e for results of univariate survival analysis).

Table 6e: Univariate Analysis Survival –VPE & Clinicopathologic Data

	Overall Survival (OS)			Disease Free Survival (DFS)		
Parameter	**n**	**Mean Survival Time (months)**	**p**	**n**	**Mean Relapse Time (months)**	**p**
FIGO Stage			**<.01**			**<.01**
Stage I	21	64.9 (8-123)	**<.01**	19	69.1 (19-123)	**0.037**
Stage II	19	49.53 (7-96)	**0.003**	15	40.73 (8-96)	0.417
Stage III	81	31.65 (1-106)	0.158	56	23.52 (1-100)	0.555
Stage IV	9	22.56 (1-104)		4	36.5 (9-104)	
Grade			**0.002**			**0.001**
Grade 1	22	56.55 (6-123)	**0.004**	20	60.5 (9-123)	**0.001**
Grade 2	51	34.26 (1-119)	0.846	33	33.76 (1-119)	0.384
Grade 3	57	34.72 (1-106)		41	25.93 (4-96)	
Age	130		0.065	94		0.722
Ascites	130		**<.01**	94		**<.01**
Pre-op Ca125	91		**0.016**	94		**0.004**
Residual Disease						
<2cm	44	28.29 (1-104)	**<.01**	40	51.63 (1-123)	**<.01**
>2cm	86	58.69 (1-123)		54	24.48 (2-104)	
VPE	130		**<.01**	94		**0.013**

Table 6e illustrates results of Cox univariate survival analysis for VPE and clinicopathologic parameters. VPE was found a significant prognosticator for both OS and DFS.
p values <0.05 were regarded as significant.

To estimate the simultaneous influence of prognostic factors, VPE and all clinicopathologic variables were entered into Cox regression analysis. Both VPE (p=0.03) and extent of residual disease (p=<0.01) achieved independent prognostic significance for prediction of OS whereas FIGO stage alone retained independent prognostic significance for prediction of DFS. (See table 6f for multivariate survival analysis).

Table 6f: Multivariate Analysis: Survival – VPE & Clinicopathologic Data

Overall Survival						Disease Free Survival				
Parameter	**df**	**Sig.**	**Exp (B)**	**95% CI Lower**	**95% CI Upper**	**Parameter**	**Sig.**	**Exp (B)**	**95% CI Lower**	**95% CI Upper**
Residual Tumour	1	**<.01**	.331	.178	.615	**Stage**	**<.01**			
VPE	1	**.03**	1.02	1.002	1.038	**Stage(1)**	**.001**	.090	.021	.391
Age	1	.319				**Stage(2)**	**.019**	.198	.051	.767
Grade	2	.297				**Stage(3)**	.348	.566	.173	1.859
Grade(1)	1	.527				**Age**	.059			
Grade(2)	1	.123				**Grade**	.145			
Stage	3	.150				**Grade (1)**	.05			
Stage(1)	1	.174				**Grade (2)**	.374			
Stage(2)	1	.235				**Ascites cat**	.195			
Stage(3)	1	.098				**Ca125 cat**	.516			
Ascites cat	1	.225				**Residual Tumour**	.183			
Ca125 cat	1	.442				**VPE**	.095			

Table 6f illustrates results of Cox multivariate survival analysis for VPE and clinicopathologic parameters. VPE was found to be of independent prognostic significance for OS, but not for DFS, being superseded by FIGO stage. p values <0.05 were regarded as significant.

6.3.5 Prediction of Chemotherapy Response

Univariate logistic regression analysis found tumour grade FIGO stage, extent of residual disease and presence of ascites to be significant predictors of chemotherapy response, with correct classification to chemotherapy response group being 70.3%, 68.8%, 68.8% and 67.2% respectively. VPE was also found to be significant (p=0.002) for prediction of chemotherapy response with an overall correct classification to chemotherapy response group of 76.3%.

Multivariate logistic regression analysis found extent of residual disease (p=0.042) and VPE (p=0.003) to be independent predictors for chemotherapy response with a correct overall classification to chemotherapy response group of 75%. (See table 6g for logistic regression analysis and prediction of chemotherapy response.

Table 6g: Logistic Regression Analysis: VPE and Clinicopathologic Data & Prediction of Chemotherapy Response

Parameter	df	Sig. (p)	Correct Classification	95% CI Lower	95% CI Upper
Univariate Analysis					
Clinicopathologic Variables					
Grade	2	**.002**	**68.8%**		
Grade(1)				2.412	67.918
Grade (2)				.728	7.917
Stage	3	**.004**	**68.8%**		
Stage(1)				1.028	560.178
Stage(2)				.385	78.573
Stage (3)				.121	17.913
Residual Tumour	1	**<.01**	**70.3%**	2.420	29.383
Presence of Ascites	1	**.004**	**67.2%**	1.566	13.822
Pre-operative Ca125	1	.093			
Age	1	.554			
VPE					
VPE	1	**<.01**	**76.3%**	0.899	0.976
Multivariate Analysis					
VPE & Clinicopathologic Data					
VPE	1	**.003**	**Group 1=63.2%** **Group 2=81.1%** **Overall = 75.0%**	.892	.987
Residual Tumour	1	**.042**		1.009	15.840

Table 6g illustrates results of univariate and multivariate logistic regression analysis for VPE & clinicopathologic parameters, and prediction of chemotherapy response. VPE was found of independent prognostic significance.
(Group 1 = no / partial response: Group 2 = Complete Remission)

6.4 Discussion

Like other studies, [145,165,167] this study, on a well-selected group of serous ovarian tumours, has shown VPE assessment techniques to have both excellent intra and inter observer reproducibility and good correlation between manual and semi-automated techniques, with semi-automated measurements not only being highly reproducible but also faster, easier and cheap to perform.

Higher VPE estimates were found to strongly correlate with increasing tumour grade, worsening FIGO stage, greater extent of residual disease and the presence of ascites.

Prior study data on ovarian tumours has shown a tendency for VPE to increase with tumour grade, [157] but considerable variability has been found. Strong linear correlation however has been found between VPE and FIGO stage. [172] Prior studies have found VPE to both exceed the prognostic value of histological sub-type and extent of residual disease in borderline tumours of the ovary, [157] and to be a valuable prognosticator for survival in both borderline and invasive carcinomas of the ovary.

Although VPE estimation in FIGO stage I epithelial ovarian cancer has not shown prognostic value in all studies, [173] other studies have shown VPE scores of < or > 65% to be strong positive or negative predictors of survival respectively, especially when combined with mitotic activity indices (MAI). [174] VPE in advanced stage epithelial ovarian carcinoma has shown strong associations with survival, [172] with VPE scores >70% and 76% respectively being associated with poor prognosis in terms of patient survival. [157,172] The epithelial percentage, as established with digital image processing, also appears to be a strong prognosticator for survival. [172] This study found VPE, as determined by semi-automated analysis, a strong prognosticator, on univariate analysis, for both OS ($p<0.01$) and DFS ($p=0.013$) with higher VPE estimates conferring poorer prognosis. VPE estimates also retained independent prognostic value on multivariate analysis for prediction of OS ($p=0.030$).

In borderline, [157] early, [174] and advanced stage [158] epithelial ovarian tumours the combination of VPE and MAI has been used as a successful prognosticator for survival with lower values related to enhanced survival and low values of mitoses seeming to confer better prognosis when related to cisplatin chemosensitivity. [175] In this study, when using a scoring system for MAI and VPE (Group A – MAI <30, VPE <65; Group B – MAI <30, VPE > 65; Group C – MAI >30) as per Baak et al, [157] mean survival per group was

found to be 57 months, 39 months and 30 months respectively. This MAI/VPE scoring system showed prognostic significance on univariate analysis for OS (p=<0.01) and DFS (p=<0.01), but grouped combinations of VPE and MAI however, failed to achieve independent prognostic significance on multivariate analysis thus offering little benefit over analysing VPE and MAI as prognostic factors in their own right.

When considering the value of VPE as a prognostic factor to predict chemotherapy response, there appears to be a paucity of literature. This study finds VPE estimation not only to be an independent predictor of chemotherapy response, but also to attain a relatively high degree of, and to surpass the predictive capabilities of traditional clinicopathologic parameters such as extent of disease residuum.

This study found automated VPE estimates, not only extremely easy to perform and highly reproducible, but also to have independent prognostic ability for not only survival prediction, but also for prediction of chemotherapy response, which certainly seems an area worthy of further investigation.

Chapter 7. Nuclear Morphometry

7.1 Introduction

7.1.1 Morphometry Background

Morphometry, literally meaning the measurement of form or 'the quantitative description of a structure' [176] is generally used to denote measurement of geometric cell and tissue features, where morphometric measurements essentially aim at 2d (planimetric) or 3d (stereologic) descriptions of tissues. [163]

Quantitative techniques can improve assessment and provide highly reproducible and objective measurements, [173] hence morphometric approaches to evaluating changes in tissue are likely to be more reproducible than ordinal grading because they are quantitative. Morphometry may provide an exact and objective system of malignancy grading [6] and is likely to decrease variability in quantitating features of cells and tissues. It may provide a numerical, reproducible scale of quantitative features and increase sensitivity in detecting minimal change. [177] It is possible that morphometric analysis of tumours may enhance the observation and interpretation of morphologic features and thus lead to greater accuracy and precision of diagnosis. [178]

Accurate evaluation of the primary tumour is important to determine prognosis and treatment strategies in any cancer. Prognostic tools should ideally allow an individualised therapy that minimises the risk of severe side effects, [3] whilst maximising survival. As overall survival in epithelial ovarian carcinoma is poor and therapeutic strategies such as platinum chemotherapy agents have considerable side effects (see appendix 1), morphometry used as a prognostic tool may enable accurate patient selection, and select only those patients who will benefit from therapy. [4] Patients with advanced ovarian cancer may have only a very short- term survival (FIGO stage IV <5% 5 year survival) thus less

toxic palliative therapy may be favourably selected. Conversely 20%-40% of FIGO stage I patients, who are traditionally not felt to benefit from adjuvant therapy, will die from recurrent disease [5] and these patients may potentially be identified as benefiting from more aggressive therapy. Current therapeutic results in ovarian cancer are reached at the expense of severe side effects with most patients undergoing radical treatment. Knowledge of predictive factors could lead to a better selection of patients who will benefit from aggressive therapy. [6] Morphometric data may not only be used to correlate with traditional tumour classification, but it has been argued that it could also be used as a prognostic parameter in survival and chemotherapy response. [158]

7.1.2 Nuclear Morphometry

Neoplasms differ histologically from their corresponding normal tissue by various features including loss of differentiation, loss of nuclear cohesion, nuclear enlargement and increased mitotic activity, and therefore may exhibit nuclear pleomorphism (greater variety in size, shape and polarity), hyperchromatism (denser, coarser chromatin staining) and increased mitotic index etc. Cancer consists of cells with uncontrolled growth, resulting in cell nuclei that deviate from the size and shape of a normal nucleus, these differences can be studied and quantified by morphometric image analysis. [179] Although nuclear size may alter with respect to cell cycle position, nuclear size as measured through the entire cell cycle has been shown as an independent prognostic indicator of survival in epithelial ovarian cancer. [179]

Prior studies on nuclear measurements have concentrated upon nuclear area, diameter, perimeter and form factors. These quantitative features may be correlated to more traditional qualitative features of proliferation, abnormal differentiation or invasion and metastatic potential. High nuclear area measurements (mean nuclear area and standard

deviation of nuclear area) correlate with enlarged, pleomorphic nuclei, abnormal differentiation and increased metastatic potential as do other shape parameters such as nuclear diameter, shortest nuclear axis and longest nuclear axis. (See Table 7a: Qualitative and Quantitative Pathological Features) [180]

Table 7a: Qualitative and Quantitative Pathological Features. [180]

Qualitative Feature	Quantitative Feature
Proliferation	
Mitosis numerous and often abnormal	↑ MAI, %Ki-67 positive cells.
Nuclei enlarged and pleomorphic	Higher values of MNA, sdNA, longest and shortest axes ↑, Other shape factors changed.
Nucleoli usually large	↑Mean nucleolar area
Coarse and clumped chromatin	Quantitative chromatin features and/or ploidy abnormal
Cytoplasmic changes	↑N:C ratio
Haemorrhage and Necrosis	Volume percentage of red blood cells or necrosis ↑
Abnormal Differentiation	
Little microscopic resemblance to normal tissue	MNA, sdNA and other nuclear and nucleolar features changed. Volume percentage of glands ↑, shape factor of glands changed
Function may be retained, lost or abnormal	Oestrogen and/or progesterone receptor content different. Abnormal immunophenotype. Quantitative sub-cellular changes.
Invasion and metastatic Potential	
Capsule intact or not: local invasion frequent Metastases present	Surface/volume ratio of epithelial fields in tumour, Epithelial percentage, large MNA, irregular cell and nuclear shape

Previous studies suggest a potentially important role for morphometry in ovarian cancer but there have been few confirmatory studies utilising well-selected, and staged tumours with a single epithelial type, and a fully platinum-based treatment regime. This study therefore aims to assess the prognostic and predictive value of nuclear morphometric features in well-staged, platinum treated, serous ovarian tumours.

7.2 Methodology

7.2.1 Tissue Processing

Nuclear morphometry was performed on archived, paraffin-embedded, H&E stained, sections. Morphometric measurements were made using the LEICA Q500MC Image Analysis System.

7.2.2 Nuclear Measurements

H&E stained archival specimens were selected for sections representing the most atypical areas of tumour. Randomly selected fields were focused using the microscope, then interactively captured onto the computer screen avoiding areas of inflammation, necrosis and calcification. Prior to acquisition, the live image views were white balanced ensuring even image illumination and optimal light intensity. Images had been calibrated from pixels to micrometers allowing lens selection and hence the associated calibration value (using Mag. 63X, 10X objective – 1 pixel = 0.00725μm). The selected image was then acquired enabling the image to be frozen and stored as a single frame ready for measuring.

Nuclei were measured using the edit mode of the image program with the mouse being used to trace the perimeter of each nucleus. Nuclei were then systematically measured within the captured field avoiding overlapping nuclei and concentrating upon nuclei with clearly defined edges to improve accuracy of measurement.

Defined nuclei were measured using the feature measurement tool of the image analysis program using the pre-determined measurement parameters of mean nuclear area (MNA) (the total number of detected pixels within the feature), nuclear breadth (NB) (the breadth of the longest feret), equivalent circle diameter (ED) (i.e. the diameter of a circle having the same area as the feature), fullness ratio (FR) (a shape factor, equal to the square

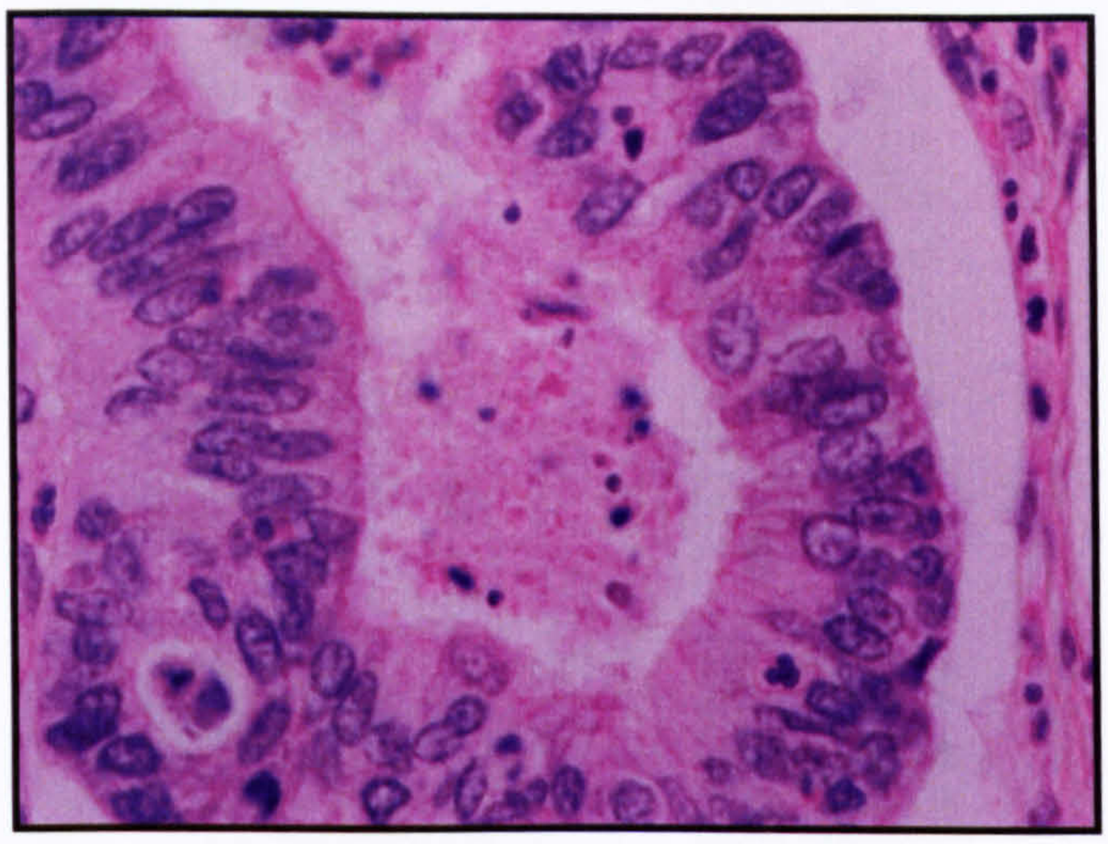
root of the ratio of area to circumscribed area), nuclear length (NL) (the length of the longest feret), orthogonal feret (the length of the feret that is at right angles to the longest feret), nuclear perimeter (NP) (the total length of the boundary of the feature), nuclear roundness (NR) (a shape factor which gives a minimum value of unity for a circle calculated from the ratio of perimeter squared to area), XFCP (the X co-ordinate of the feature count point), and YFCP (the Y co-ordinate of the feature count point). (See appendix 2: Nuclear Morphometry Parameter Definitions).

Nuclei were initially measured at an on screen magnification of 1200X (40X Objective). This resulted in a single intra-observer correlation of 93.68% when used to measure 50 consecutive nuclei. As a mouse is used to trace the nuclear outline, a hand tremor factor was evident and measurement at the higher on screen magnification of 1900X (63X objective) was found to give an improved correlation of 97.56%. 100 nuclei per case were measured at a final on screen magnification of 1900X. (See Fig 7.1 for morphometry methodology

Coefficients of error were used to calculate the number of nuclei and fields required for measurement per case to attain adequate sample size. When measurement's remained stable over several fields (<5% variation), this was accepted as a representative sample. (See fig 7.2: Morphometric Parameters - Coefficients of Error)

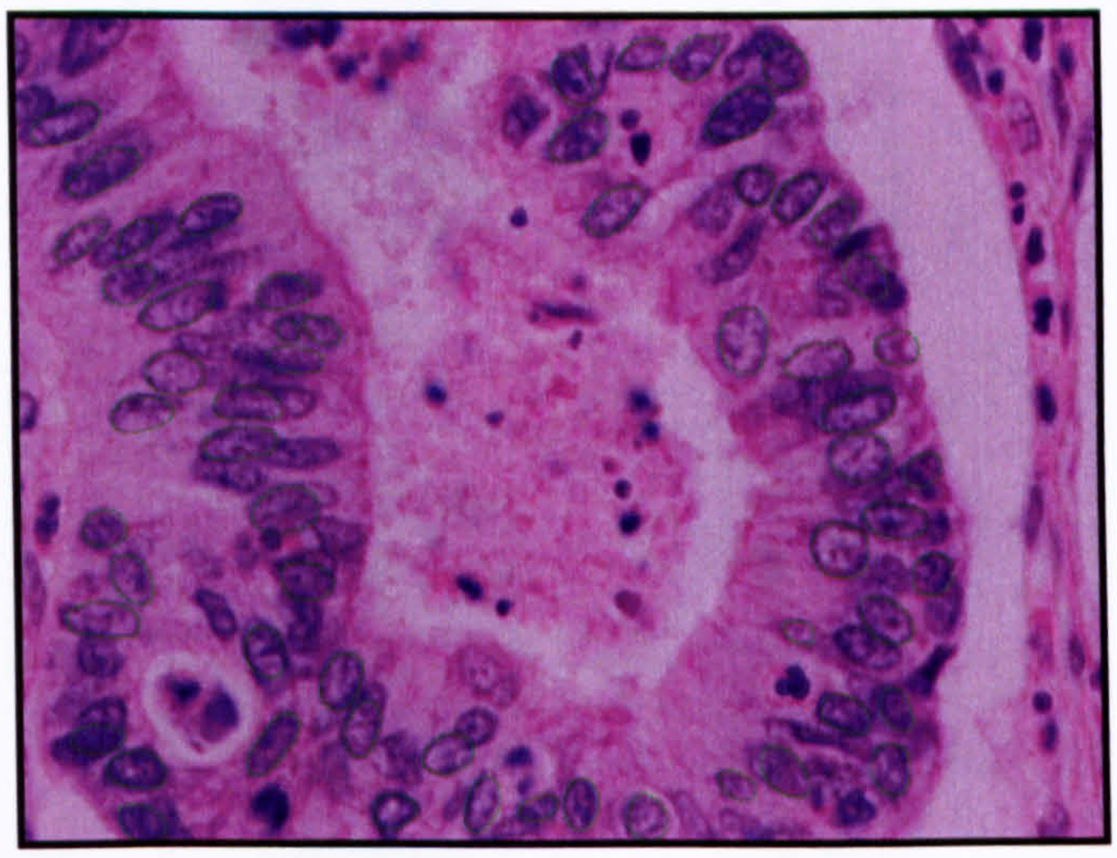
Intra-observer error was calculated by repeating measurements for ten cases at a 6 - month interval. Inter-observer error was calculated over ten cases for each measurement variable. Two observers without prior knowledge of patient characteristic's or outcome made assessments.

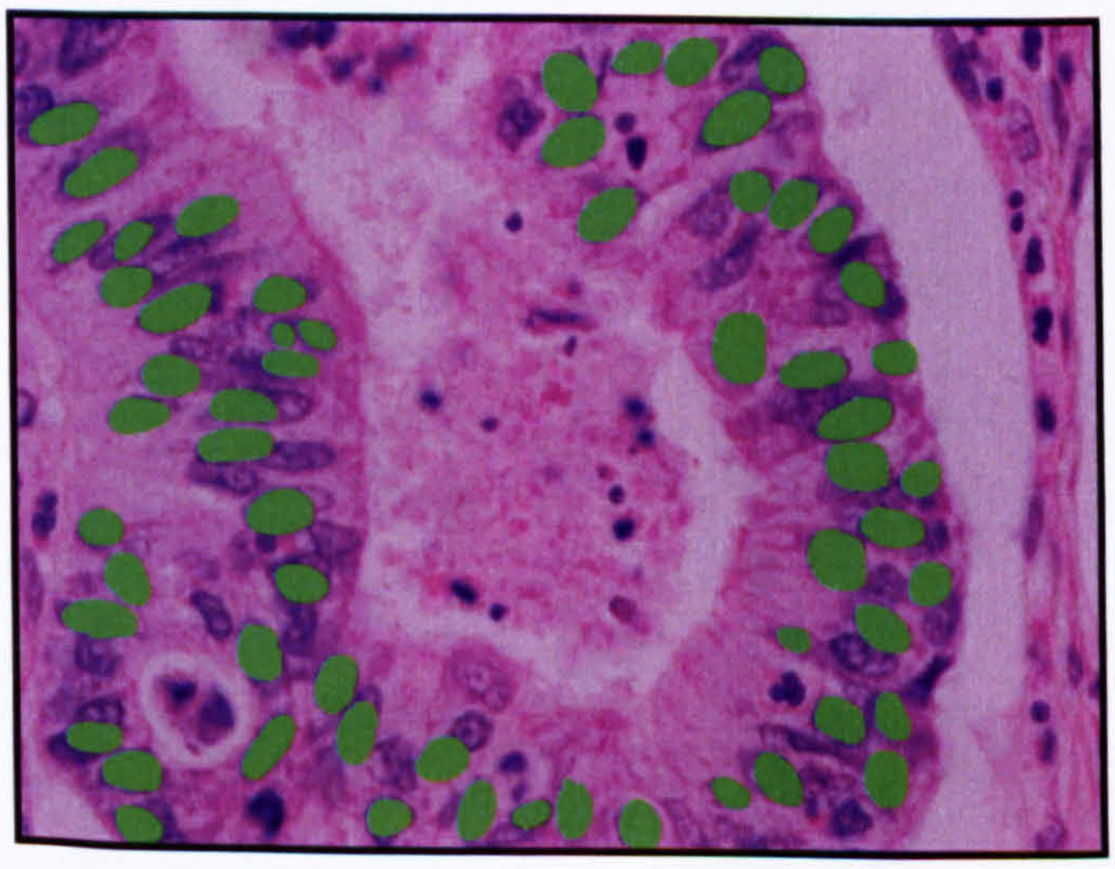
Fig 7.1: Morphometry Methodology



Haematoxylin and Eosin stained archived specimens were selected for areas representing the most atypical area of tumour.

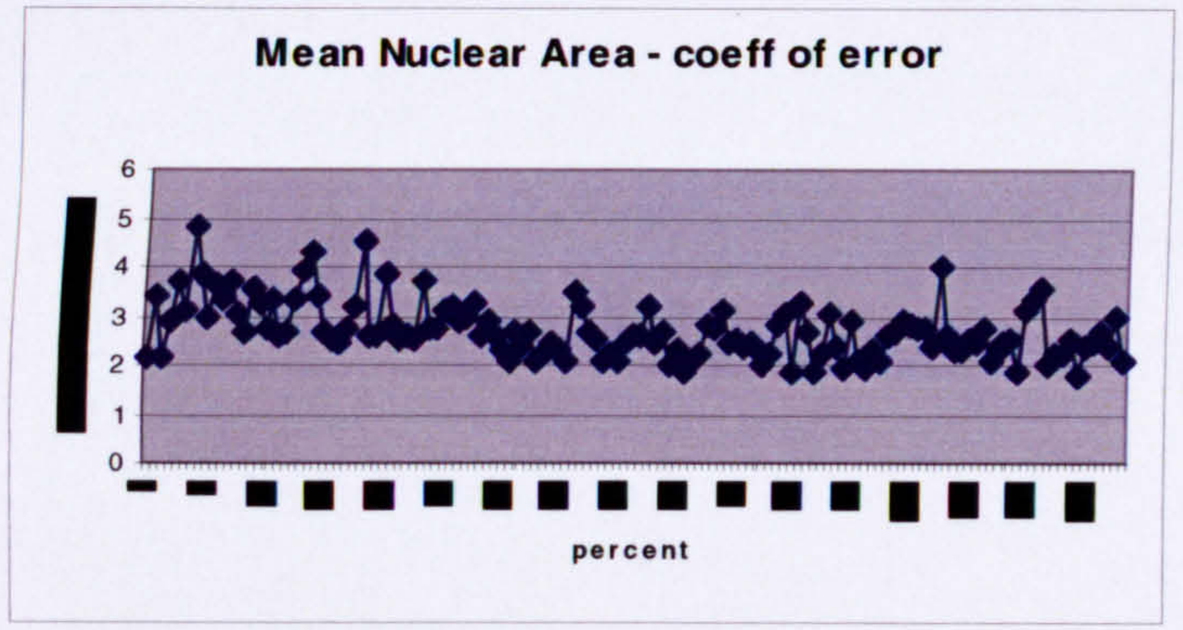
Nuclei were measured using the edit mode of the image program with the mouse being used to trace the perimeter of each nucleus. Nuclei were then systematically measured within the captured field avoiding overlapping nuclei and concentrating upon nuclei with clearly defined edges to improve accuracy of measurement.

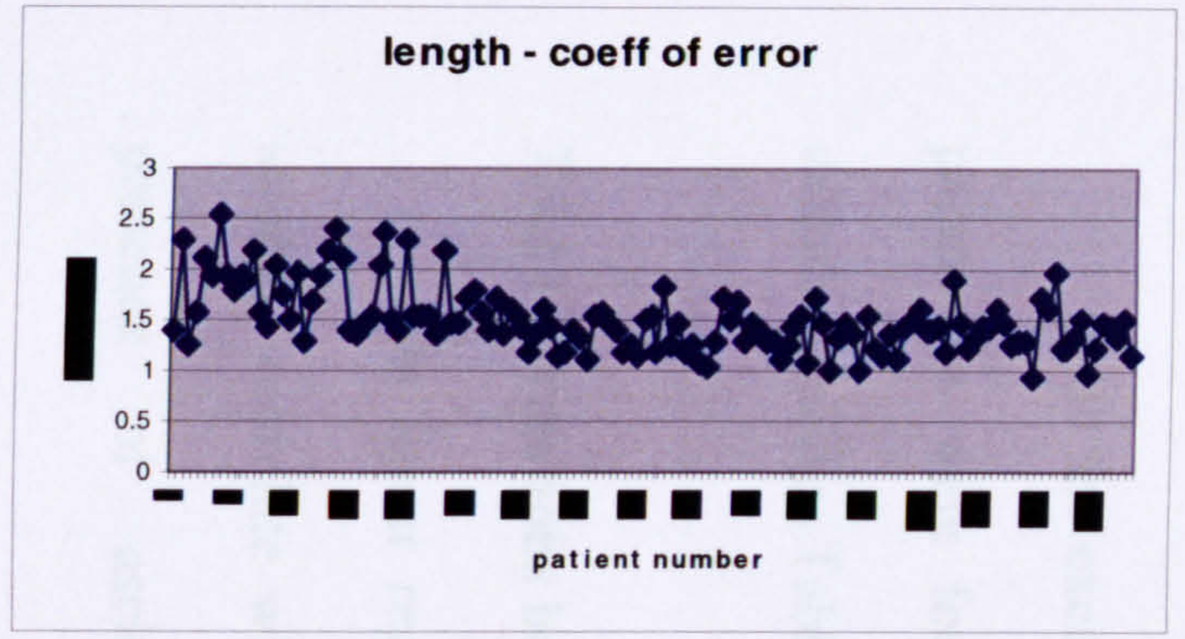


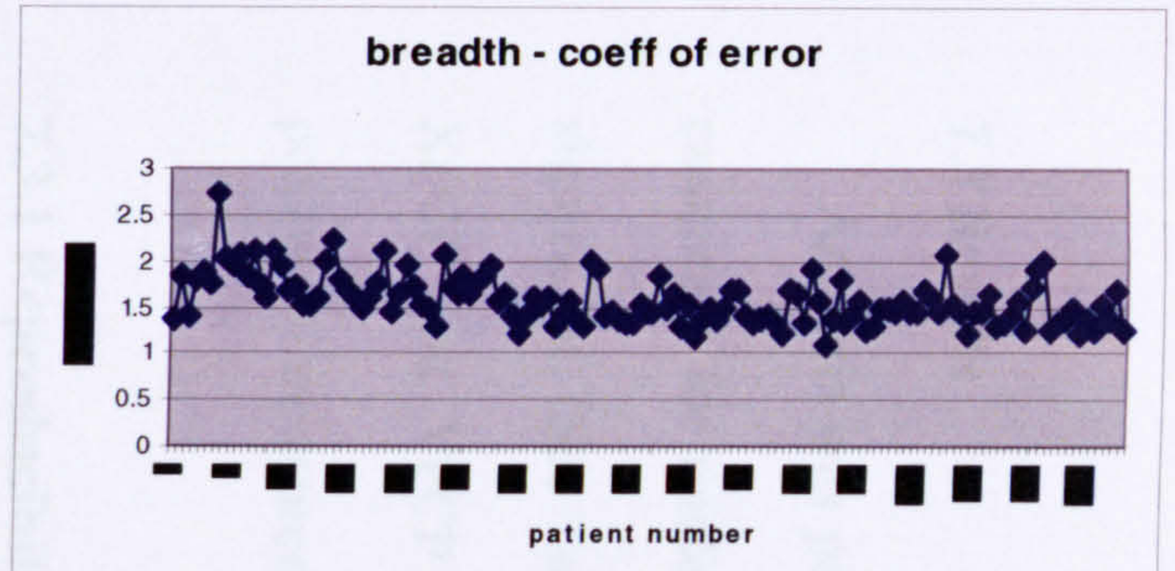


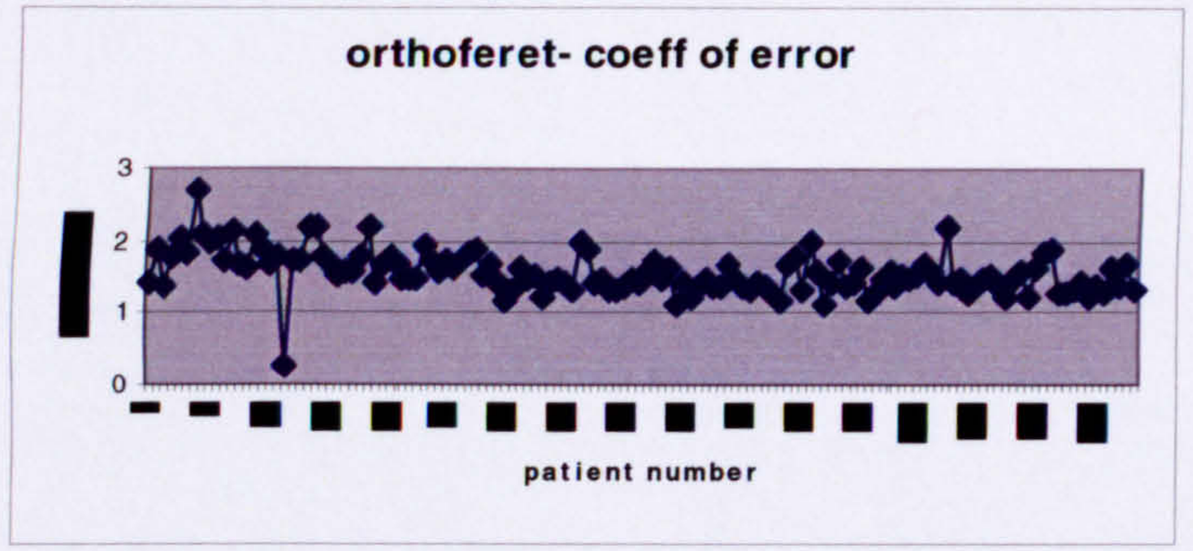
Defined nuclei were measured using the feature measurement tool of the image analysis program using the pre-determined measurement parameters of area, breadth, equivalent circle diameter, fullness ratio, length, orthogonal feret, perimeter, roundness.

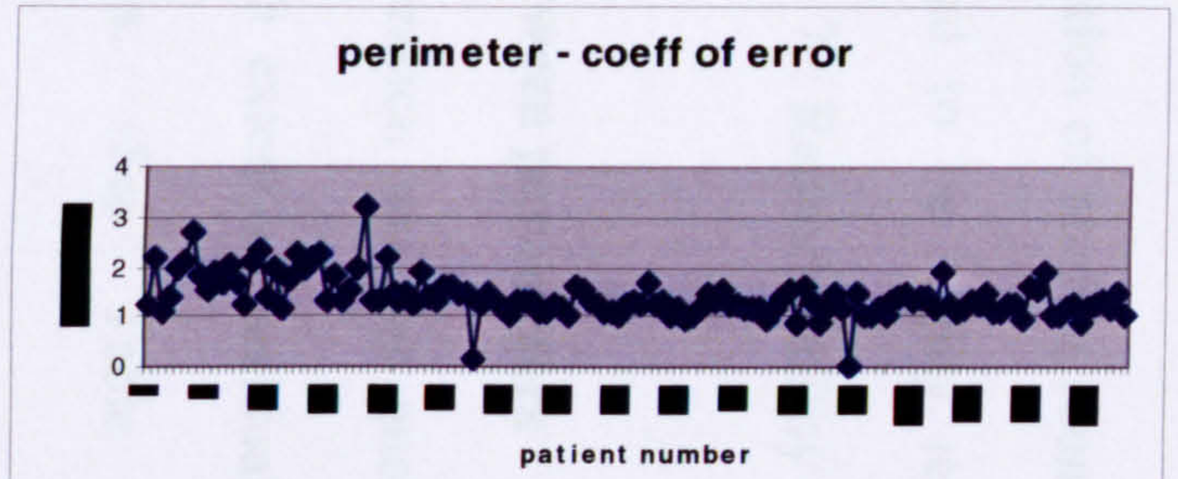
Fig. 7.2: Morphometric Parameters - Coefficients of Error

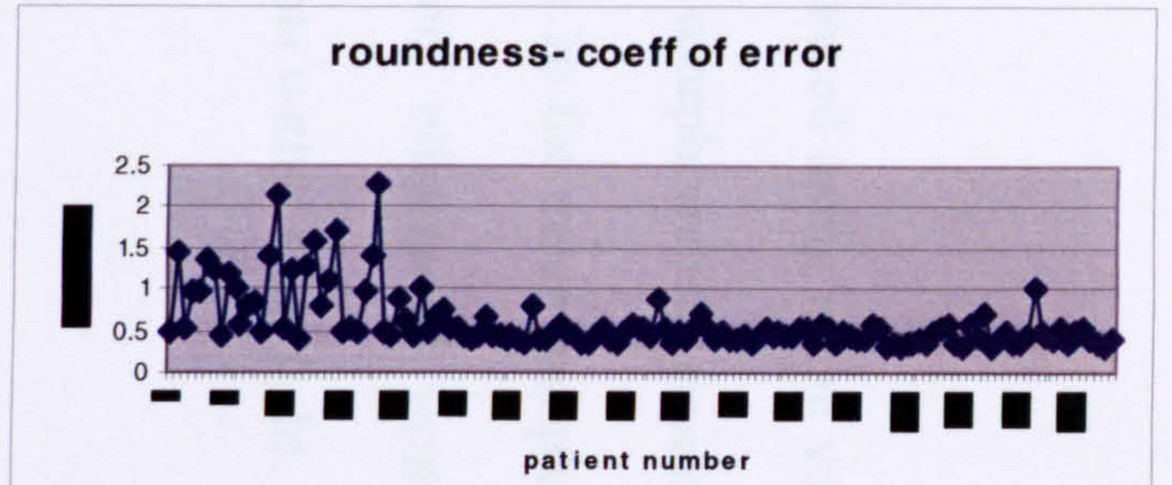


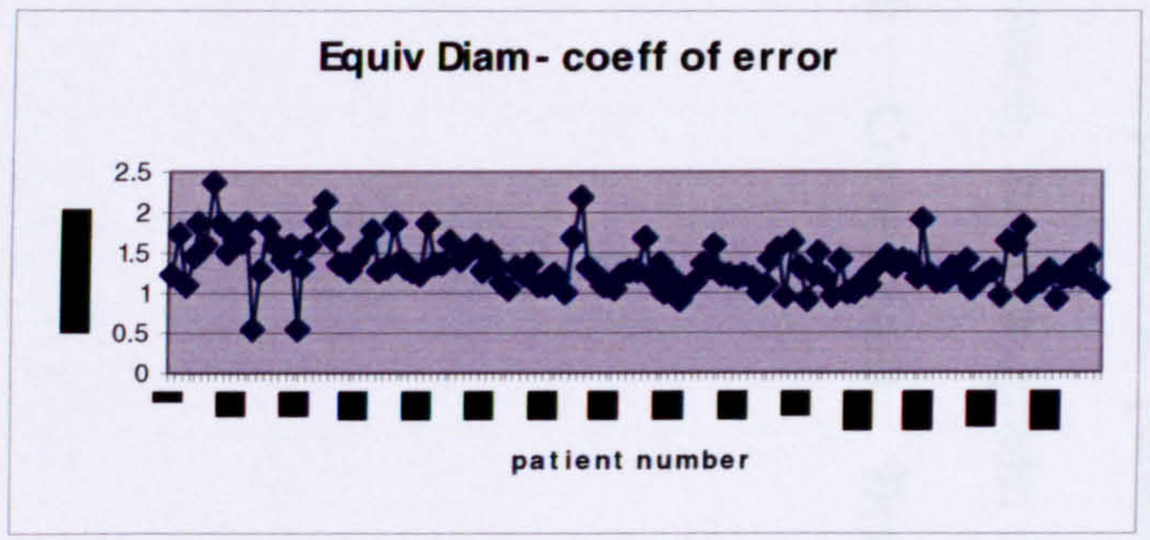












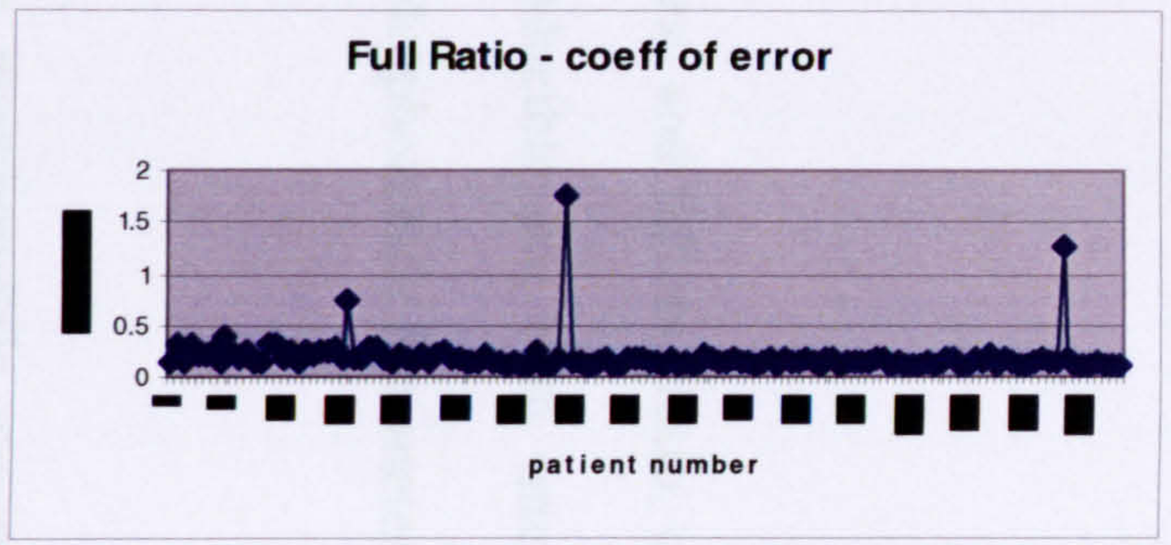


Fig 7.2 illustrates coefficient of error graphs for nuclear morphometry features. 100 nuclei were measured per case. A coefficient of error ≤5% Indicates a high level of measurement precision

7.3 Results

Analysis was performed using mean values for each measurement. All 132 cases were examined for nuclear morphometric parameters. P values <0.05 were regarded as being significant. (See Table 7b for mean morphometric parameters and clinicopathologic data). XFCP and YFCP were eliminated from analysis as these simply represent nuclear positional measurements within each field.

7.3.1 Reproducibility

With the exception of nuclear roundness and fullness ratio, nuclear morphometric parameters were found to be highly reproducible for both inter and intra-observer calculations (see Table 7c: Reproducibility – Morphometric Parameters).

7.3.2 Correlations between parameters

On linear regression analysis nuclear morphometric parameters were found to strongly correlate with extent of residual disease, tumour grade, FIGO stage and the presence of ascites. (See Table 7d: Correlations Between Parameters).

Table 7b: Mean Morphometric Parameters and Clinicopathologic Data

Clinical Parameters	MNA (μ)	sd NA (μ)	Nuclear Perimeter (NP) (μ)	Nuclear Roundness (NR)	Fullness Ratio	Nuclear Length (NL) (μ)	Nuclear Breadth (NB) (μ)	Orthoferet	Equivalent Diameter
FIGO Stage I	0.099	0.030	1.187	1.111	0.967	0.417	0.298	0.314	0.348
II	0.199	0.038	1.440	1.097	0.970	0.507	0.364	0.379	0.451
III	0.152	0.040	1.463	1.103	0.970	0.514	0.374	0.388	0.445
IV	0.175	0.049	1.585	1.099	0.969	0.558	0.402	0.419	0.467
Grade 1	0.111	0.031	1.253	1.108	0.968	0.441	0.315	0.331	0.395
2	0.142	0.038	1.422	1.102	0.969	0.501	0.363	0.375	0.418
3	0.180	0.043	1.492	1.102	0.971	0.523	0.379	0.396	0.459
Residual Disease <2cm	0.126	0.034	1.332	1.100	0.969	0.468	0.337	0.353	0.405
>2cm	0.168	0.042	1.473	1.105	0.969	0.518	0.375	0.389	0.446

Table 7c: Reproducibility - Morphometric Parameters

Nuclear Parameter	Intra-observer Error	r	r^2	p	Spearman	2- tailed p	Inter-observer Error	r	r^2		Spearman	2-tailed p
MNA		0.995	0.9890	<0.0001	0.9758	<0.0001		0.9648	0.9308	<0.0001	0.9758	<0.0001
sdNA		0.993	0.9867	<0.0001	0.9879	<0.0001		0.7707	0.5939	0.0091	0.8303	0.0047
Nuclear Length		0.994	0.9872	<0.0001	0.9758	<0.0001		0.9606	0.9227	<0.0001	0.9636	<0.0001
Nuclear Breadth		0.994	0.9888	<0.0001	0.9758	0.0016		0.9504	0.9033	<0.0001	0.9758	0.0016
Orthoferet		0.993	0.9865	<0.0001	0.9879	<0.0001		0.9501	0.9028	<0.0001	0.9273	<0.0001
Nuclear Perimeter		0.994	0.9881	<0.0001	0.9879	<0.0001		0.9649	0.9311	<0.0001	0.9758	<0.0001
Nuclear Roundness		0.895	0.8003	0.0005	0.8545	0.0029		0.2223	0.04941	0.5371	0.1879	0.6073
Equivalent Diameter		0.995	0.9896	<0.0001	0.9758	<0.0001		0.9620	0.9254	<0.0001	0.9515	<0.0001
Fullness Ratio		0.898	0.8069	0.0004	0.9030	0.0008		0.2629	0.06913	0.4630	0.3333	0.3487

Table 7c illustrates reproducibility of nuclear morphometry parameters. Ten cases were measured at a six month interval. With the exception of nuclear roundness and fullness ratio, nuclear morphometric parameters were found to be highly reproducible for both inter and intra-observer calculations.

Table 7d: Correlations Between Parameters

Nuclear Parameter	Age			Grade			Stage			Ca125 pre-op			Ascites			Residual Disease		
	r	r^2	p	r	r^2	p	r	r^2	p	r	r^2	p	r	r^2	p	r	r^2	p
MNA	0.065	0.0042	0.46	0.22	0.48	0.01	0.109	0.012	0.22	-0.02	0.003	0.88	0.18	0.03	0.04	0.17	0.03	0.05
SdNA	0.096	0.009	0.27	0.30	0.09	0.0005	0.31	0.098	0.0002	0.22	0.05	0.04	0.29	0.084	0.0007	0.27	0.07	0.002
Nuclear Length	0.07	0.005	0.42	0.35	0.12	<0.0001	0.45	0.21	<0.0001	0.14	0.02	0.19	0.3	0.11	0.0001	0.31	0.094	0.0003
Nuclear Breadth	0.054	0.003	0.558	0.35	0.12	<0.0001	0.452	0.204	<0.0001	0.127	0.016	0.23	0.32	0.1	0.0002	0.29	0.089	0.0005
Orthoferet	0.04	0.002	0.66	0.37	0.14	<0.0001	0.45	0.20	<0.0001	0.13	0.016	0.23	0.31	0.09	0.0003	0.29	0.083	0.0008
Nuclear Perimeter	0.065	0.005	0.46	0.36	0.13	<0.0001	0.045	0.21	<0.0001	0.14	0.02	0.19	0.32	0.11	0.0001	0.3	0.09	0.0004
Nuclear Roundness	0.051	0.003	0.56	-0.05	0.002	0.6	-0.06	0.004	0.49	0.07	0.005	0.496	0.02	0.0004	0.812	0.045	0.002	0.61
Equivaent Diameter	0.12	0.015	0.169	0.24	0.055	0.0065	0.29	0.087	0.0006	0.079	0.007	0.45	0.19	0.04	0.022	0.19	0.035	0.033
Fullness Ratio	-0.182	0.033	0.037	0.20	0.041	0.02	0.13	0.016	0.151	-0.07	0.004	0.54	0.03	0.001	0.69	-0.015	0.0002	0.867

Table 7d illustrates correlations between nuclear morphometry features and clinicopathologic parameters. morphometric parameters were found to strongly correlate with residual disease status, tumour grade, FIGO stage and the ascites status.
P values <0.05 were regarded as significant.

7.3.3 Survival Analysis

OS data was available for all 132 patients and DFS data for 94 patients. P values below 0.05 were regarded as significant.

Morphometric and clinicopathologic variables were analysed using the univariate Cox model. Of the clinicopatholgic data, FIGO stage (p=<0.01), tumour grade (p=0.002), pre-operative Ca125 levels (p=0.016), presence of ascites (p=<0.01) and extent of disease residuum (p=<0.01) were found to be significant predictors of OS. FIGO stage (p=<0.01), tumour grade (p=0.001), pre-operative Ca125 levels (p=0.004), presence of ascites, (p=<0.01) and extent of disease residuum (p=<0.01) were found to be significant predictors of DFS. For morphometric variables sdNA (p=0.001), NP (p=0.001), sdNP (p=0.038), NL (p=0.001), NB (p=0.001), orthoferet (p=0.003), and ED (p=<0.01) were found to be significant predictors of OS, with higher values of these being prognostically less favourable. sdNA (p=0.018), NP (p=0.003), NL (p=0.003), NB (p=0.003), orthoferet (p=0.003) and ED (p=0.015) were also found to be significant predictors of DFS, again with higher values of these being prognostically less favourable

Using the Cox multivariate model for morphometric parameters alone, ED showed to be a significant predictor of OS (p=0.001) and orthoferet a significant predictor of DFS (p=0.003).

To estimate the simultaneous influence of prognostic factors all clinicopathologic and morphometric variables were entered into Cox regression analysis. This analysis revealed extent of disease residuum (p=<0.01) and ED (p=0.002) to be significant predictors for OS in this group of patients. FIGO stage (p=<0.01) and ED (=0.039) were found to be a significant predictor for DFS. The other factors were not of additional prognostic relevance. (See table 7e for univariate and multivariate survival analysis).

Table 7e: Positive Findings – Univariate and Multivariate Survival Analysis

Parameter	df	Sig. (p)	Exp (B)	95% CI Lower	95% CI Upper	Parameter	Sig. (p)	Exp (B)	95% Lower CI	95% Upper CI
Univariate Analysis										
Overall Survival (OS)						**Disease Free Survival (DFS)**				
Clinicopathologic Variables						**Clinicopathologic Variables**				
Grade	2	**.008**				**Grade**	**.004**			
Grade(1)	1	**.004**	.344	.168	.705	**Grade(1)**	**.001**	.288	.138	.603
	1					**Grade(2)**	**.384**	.798	.479	1.327
Stage	3	**<.01**				**Stage**	**<.01**			
Stage(1)	1	**<.01**	.151	.056	.403	**Stage(1)**	**.037**	.228	.057	.913
Stage(2)	1	**.003**	.256	.103	.639	**Stage(2)**	**.417**	.592	.167	2.101
						Stage(3)	**.555**	1.421	.442	4.568
Residual Disease	1	**<.01**	.341	.204	.568	**Residual Disease**	**<.01**	.341	.204	.568
Ascites	1	**<.01**	.377	.237	.599	**Ascites**	**<.01**	.358	.212	.605
Pre-operative Ca125	1	**.016**	1.000	1.000	1.000	**Pre-operative Ca125**	**.001**	1.000	1.000	1.000
Morphometric Variables										
MNA	1	.071	2.799	0.916	8.550	**MNA**	**.104**	2.657	0.817	8.647
sdNA	1	**.001**	21457 92807. 524	5434.46	8E+014	**sdNA**	**.016**	37524 95240. 3	63.515	2E+017
Nuclear Length	1	**.001**	78.318	5.662	1083.29	**Nuclear Length**	**.004**	93.287	4.266	2039.772
Nuclear Breadth	1	**.001**	266.78 4	9.342	7618.82	**Nuclear Breadth**	**.003**	380.06	7.511	19232.1
Nuclear Perimeter	1	**.001**	4.420	1.773	11.022	**Nuclear Perimeter**	**.004**	4.928	1.680	14.457
sd perimeter	1	**.038**	16.662	1.174	236.571	**sd perimeter**	.852	1.416	0.036	55.243
Nuclear Roundness	1	.926	.841	0.021	33.254	**Nuclear Roundness**	.316	.045	0.000	19.573
sd roundness	1	.673	.411	0.007	25.43	**sd roundness**	.164	.014	0.000	5.785
orthoferet	1	.003	355.71	7.423	3938.88	**orthoferet**	**.003**	355.72	7.423	17046.04
Fullness Ratio	1	.627	.001	.000	1E+009	**Fullness Ratio**	.102	31285 41574 39642 6.0	0.001	1.2E+03 4
Equivalent diameter	1	**<.01**	22.403	4.791	104.768	**Equivalent diameter**	**.006**	9.932	1.955	50.46
Multivariate Analysis										
Morphometric Data										
Equiv Diameter	1	**.000**	22.40 3	4.791	104.76	**Orthoferet**	**.003**	355.7 2	7.423	17046.0 47
Morphometric & Clinicopathologic Data										
Residual Disease	1	**<.01**	.337	.209	.542	**Equivalent Diameter**	**.039**	9.409	1.114	79.478
Equivalent Diameter	1	**.002**	16.60	2.783	99.088	**Stage**	**<.01**			
						Stage(1)	.089	.090	.072	1.205
						Stage(2)	.359	.198	.154	1.972
						Stage(3)	.454	.566	.485	5.042

Table 7e illustrates univariate and multivariate Cox survival analysis results for nuclear morphometry features and clinicopathologic parameters. At multivariate level, equivalent nuclear diameter was found of independent prognostic significance for both OS and DFS
p values <0.05 were regarded as significant.

7.3.4 Response to Chemotherapy

Of the clinicopathologic data, tumour grade (p=0.02), FIGO stage (p=0.004), extent of residual disease (p=<0.01) and presence of ascites (p=0.004) were found significant predictors of chemotherapy response with overall correct classification to chemotherapy response group being 70.3%, 68.8%, 68.8% and 67.2% respectively. For the morphometric data, sdNA (p=0.017), NP (p=0.004), NL (p=0.005), NB (p=0.005) and orthoferet (p=0.005) were found to be significant predictors of chemotherapy response with correct overall classification to chemotherapy response group being 64.1% for sdNA, NP and NL, 65.6% for NB and 65.6% for orthoferet diameter.

Multivariate logistic regression analysis of morphometric parameters alone revealed NP to be a significant predictor of survival (p=0.009) with a correct overall classification rate of 64.1%.

To estimate the simultaneous influence of prognostic factors all clinicopathologic and morphometric variables were entered into multivariate logistic regression analysis. This analysis showed extent of residual disease (p=0.003) and NL (p=0.041) to be the strongest predictors of chemotherapy response with correct classification rates of 61.5% to group 1 and 73.7% to group 2, and an overall correct classification rate to chemotherapy response group of 68.8%. (See Table 7f Logistic Regression Analysis: Morphometric and Clinicopathologic Data & Chemotherapy Response).

Table 7f: Logistic Regression Analysis: Morphometric and Clinicopathologic Data & Chemotherapy Response

Parameter	df	Sig. (p)	Correct Classification	95% CI Lower	95% CI Upper
Univariate Analysis					
Clinicopathologic Variables					
Grade	2	.002	68.8%		
Grade(1)				2.412	67.918
Grade (2)				.728	7.917
Stage	3	.004	68.8%		
Stage(1)				1.028	560.178
Stage(2)				.385	78.573
Stage (3)				.121	17.913
Residual Disease	1	<.01	70.3%	2.420	29.383
Presence of Ascites	1	.004	67.2%	1.566	13.822
Pre-operative Ca125	1	.093			
Age	1	.554			
Morphometric Variables					
sdNA	1	.017	64.1%	.000	.001
Nuclear Length	1	.005	64.1%	.000	.076
Nuclear Breadth	1	.005	65.6%	.000	.044
Nuclear Perimeter	1	.004	64.1%	.002	.407
orthoferet	1	.005	65.6%	.000	.042
MNA	1	.060			
sd perimeter	1	.290			
Nuclear Roundness	1	.523			
sd roundness	1	.305			
Fullness Ratio	1	.414			
Equivalent diameter	1	.076			
Multivariate Analysis					
Morphometric Data					
Nuclear Perimeter	1	.004	64.1%	.002	.407
Morphometric & Clinicopathologic Data					
Nuclear Length	1	.041	Group 1 = 61.5% Group 2 = 73.7% Overall = 68.8%	.000	.691
Residual Disease	1	.003		1.960	25.738

Table 7f illustrates univariate and multivariate logistic regression analysis results for nuclear morphometry features, clinicopathologic parameters and prediction of chemotherapy response. Numerous morphometry parameters were found significant at univariate level. Nuclear length was found to retain independent prognostic significance, and, in combination with residual disease status, provide an overall 69% correct classification to chemotherapy response group.

(Group 1 = no / partial response: Group 2 = Complete Remission)

7.4 Discussion

Morphometric analysis has been applied to various tumours (eg breast, prostate, colon) and used to predict outcome. Like this study nuclear morphometric measurements have, on the whole, shown good intra- and interobserver reproducibility [4,146,181] and mean nuclear area has shown to be more reproducible than grade and to give more accurate prediction of survival. [173]

Correlation of nuclear morphometric variables with clinicopathologic data has been less widely studied. In general smaller nuclei have been found in lower stage and grade tumours, and mean nuclear volume (MNV) has also been found as significantly lower in lower grade tumours. [3] This study has found strong correlations between morphometric parameters and tumour grade, FIGO stage, the presence of ascites and extent of residual disease, showing higher nuclear morphometric measurements to be associated with poorer clinicopathologic variables.

Nuclear morphometry has successfully been used to differentiate benign, borderline and malignant ovarian serous tumours with significant differences in nuclear size and shape being found. [181] Higher Mean Nuclear Area (MNA) has been found to correlate with shorter survival times and rapid progression and to be of prognostic significance between lower (I) and higher (II & III) tumour grades. [179] In borderline tumours morphometric techniques were also found capable of predicting, with a high degree of accuracy, the outcome of patients. [182]

Studies in FIGO Stage I patients have found MNA and Mean Nuclear Volume (MNV) to be objective, and accurate predictors of survival, with MNA and MNV being the strongest single prognostic factors for overall survival. [173] In Stage I clear cell ovarian carcinoma the presence of giant nuclear cells or nuclear irregularity has been found

positively associated with tumour prognosis. [183]

In advanced stage (FIGO III – IV) epithelial ovarian cancer MNA has shown to be a most important prognostic factor for survival in both univariate and multivariate analysis. [6,158-9,175] Standard deviation of the nuclear area (sdNA), [158,159,162,175] nuclear perimeter (NP), [158,162] largest perpendicular axis, [158,162] longest axis, [162] and shortest axis [158] have also shown strong associations with survival. In advanced stage, significantly different morphometric features have been found between survivors and non-survivors, [158] and morphometric data has appeared to increase the prognostic power compared to traditional morphology.

A study comparing survival in advanced stage patients having second look laparotomy showed variations of nuclear size as significant predictive variables for survival with sdNA, MNP, largest perpendicular axis and largest axis all significantly correlating with survival. [162]

Nuclear morphometry has also been used to predict chemotherapeutic response. In advanced stage disease nuclear size has been noted as an important predictor of the sensitivity of tumour cells to cisplatin, [184] with MNA and sdNA having significant correlation, and MNA the strongest prognostic factor in multivariate analysis. [175] Morphometric features have also been found to be predictors of survival in advanced ovarian cancer after cisplatin treatment, especially when combined with FIGO stage and preoperative tumour load. [159] For prediction of chemosensitivity, morphometric data seems a stronger discriminator than subjective histological grading and staging [184]

For prediction of OS and DFS this study on serous ovarian tumours has shown several nuclear morphometric parameters of significance on both univariate and multivariate analysis with high levels of measurement reproducibility

Prior studies have largely been performed with patients recruited from the late 1970's to 1980's [6,145,158,159,162,173,175,183] using relatively small population sizes, [6,145,158,159,162,175] advanced stage disease (III-IV) only, [6,145,158,159,162,175] and have incorporated mitotic activity index [6,145,158,159,162,175] and epithelial area estimates [145,158,175] into their analysis and not concentrated purely on nuclear morphometry. Numerous studies have also been performed incorporating mixed histological sub-types. [6,158,173,159,162,175] Comparisons between studies are therefore fraught with difficulties and this may be reflected in morphometry and outcome. See table 7 for a summary of prior morphometric studies.

There is increasing evidence suggesting that the molecular pathogenesis of the various histological sub-types of ovarian cancer may be distinct. A study performed by Miller 1991 [184] showed mucinous ovarian tumours to significantly differ in MNA estimations as compared to endometrioid and serous type tumours. A previous study on a pure serous group of ovarian tumours failed to show nuclear morphometry as an independent prognosticator for survival when results were corrected for clinical variables [145] This suggests that perhaps histological sub-types should be considered as separate entities, certainly when considering nuclear morphometric analysis.

Few prior studies have investigated the value of nuclear morphometry to predict chemotherapy response. [158,162] Again these prior studies have been small, considered advanced stage disease only, with different histological sub-types, and, due to the recruitment dates of these studies, varying chemotherapeutic regimes have been employed in patient treatment. This is one of the few studies to consider patients treated with primary carboplatin or carboplatin / paclitaxel regimes. For prediction of chemotherapy response nuclear morphometric measurements were shown to achieve independent prognostic

significance.

This study revealed extent of disease residuum to be an important factor for both survival and chemotherapy response prediction. Although the volume of residual tumour after debulking surgery appears to relate well to the clinical outcome there is no accurate measure and the critical volume for long-term survival remains unknown i.e. the precise definition of 'what is optimal surgery' has not been established. Present methods of quantifying residual tumour are imprecise [26] and the maximal diameter of residual disease is a very crude estimate. [32] This study regarded the extent of residual disease on the basis of < or >2cm total residuum. As variation exists in the meaning of optimal cytoreduction and estimation of extent of disease residuum caution must be taken in its interpretation with regards to survival benefit.

Objective methods of tumour assessment are required as accepted prognostic factors should be reproducible. This study found nuclear morphometric measurements not only to be fast, easy to perform and highly reproducible, but results to also suggest that nuclear morphometry can provide significant information to predict both survival and chemotherapy response in platinum treated serous ovarian cancer. Interactions between various morphological and clinical variables are clearly complex and merit studies on larger patient groups and more stage I tumours, but our results suggest that morphometry may have a significant role to play in future therapeutic decision-making.

Table 7g: Summary of Prior Morphometry Studies

Study [Ref]	Study Period	Sample Size	FIGO Stage	Histological Type	Follow up	Chemotherapy Type	Multivariate Statistical Methods	Clinicopath Data Included	VPE Included	MAI Included	Multivariate survival analysis Nuclear Morphometry significant	Mulitvariate analysis Chemo prediction Nuclear Morphometry significant
Baak [183] 1988	1980-84	73	III & IV	Epithelial Sub-type n/s	Med 44/12	cisplatin, doxorubicin and cyclophos	Cox proportional hazards	Yes	Yes	Yes	No	Not Investigated
Weger[162] 1989	1978-86	63	III & IV	Serous Mucinous Endometrioid Mixed Undifferentiated	n/s	mixed regimes:- 5-FU, Cyclophos, Doxorubicin, Cisplatin	Cox multivariate/ linear stepwise discrimanant	Yes	No	Yes	Nuclear Roundness p=0.01	sdNA nuclear roundness MNA 10th centile
Ludescher[6] 1990	1978-84	49	III & IV	Serous Mucinous, Mixed Undifferentiated	5yrs	5-FU + cyclophos or adriamycin, 5FU & cyclophos cisplatin patients excluded	Stepwise Cox regression	Yes	No	Yes	No	Not Investigated
Hogberg[159] 1992	1984-1987	65	IIIB-IV	Serous Endometrioid Clear Cell TC,M,U	n/s	Cisplatin & Doxorubicin	Stepwise Cox regression	Yes	No	Yes	sd largest nuclear axis p=0.0003	Not Investigated
VanDiest[175] 1994	1979-84	58	III & IV	Serous Mucinous Endometrioid Clear Cell	Med 40.3/12 (1-111 months)	CHAP-5	Cox multivariate model	Yes	Yes	Yes	MNA + FIGO stage + Pre-op tumour load P=0.0034	Not Investigated
Katsoulis[10] 1995	1982-88	60	III & IV	Serous Mucinous Endometrioid	5yrs / until death	cisplatin cyclophos	Cox proportional hazards	Yes	Yes	Yes	sdNA p=0.037 sd longest axis p=0.002	MNA p=0.0006 SdNA p=0.0019
Brinkhuis[145] 1995	1981-84	45	III & IV	Serous	median 37/12 (27-60/12)	cisplatin cyclophos +/- adriomycin	Cox multivariate model	Yes	Yes	Yes	No	Not Investigated
Brugghe[173] 1998	1979-91	102	Ia, Ib, Ic	Serous Mucinous Endometrioid Clear Cell	Med 48/12 12-155/12	Untreated	Cox multivariate model	Yes	No	No	MNA + FIGO Stage p=<0.0001	Not Investigated

Chapter 8: Syntactic Structure Analysis

8.1 Introduction

8.1.1 Background

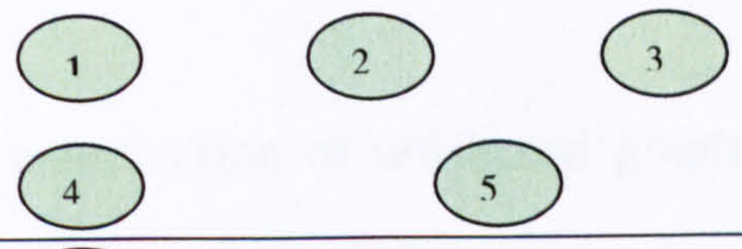
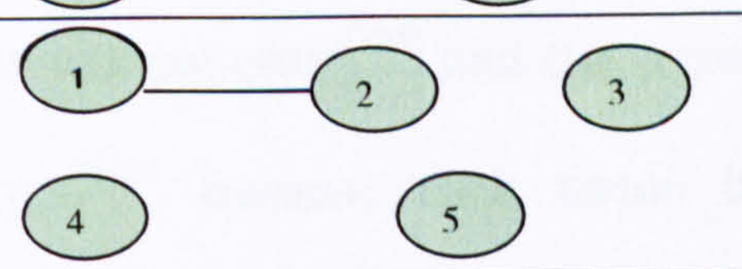
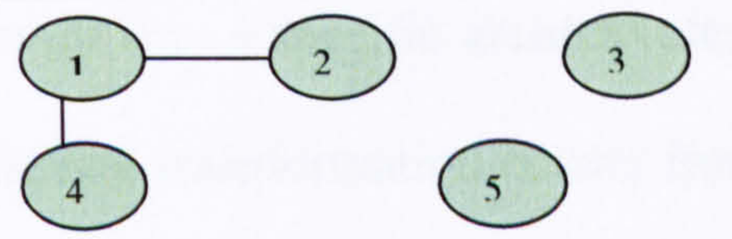
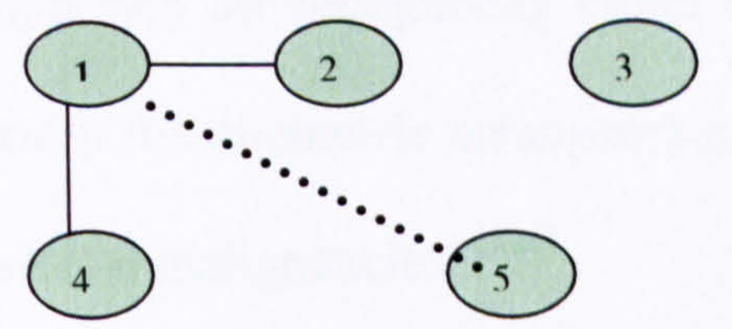
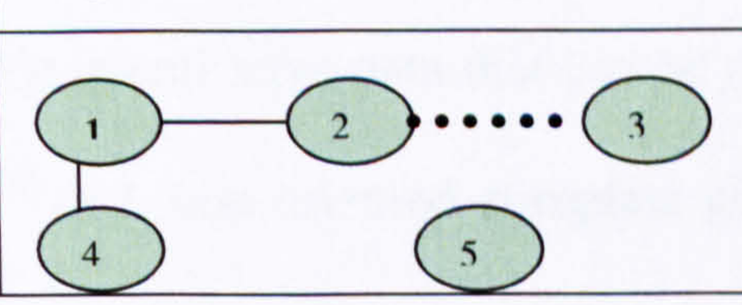
Based on the theory of attributed graphs, [185] syntactic structure analysis (SSA) deals with properties of cells or higher order structures. [186] It is a fast, interactive, quantitative microscope technique used for describing the organisation of histologic structures. [187] SSA reduces a structure to points and interconnecting lines according to a certain mathematical function, [187] and measures the arrangement (texture) of cells, independent of single cell properties. [185] By choosing certain reproducible basic units of major interest, a geometric network can be constructed by graph theory i.e. nuclei (vertices or points) connected by lines (edges or intercellular bridges). [188]

Malignant cells are identified primarily by their cellular atypia (abnormal size, shape etc) but, in cases with minimal atypia, disordered cell-to-cell arrangement may give a clue in the diagnosis of malignancy. [189] Carcinoma cells can be considered as single points (vertices) described in a 2d space. The geometric arrangement of carcinoma cells (nuclei) can be analysed by connecting the points (nuclei) with related features by construction of a network graph.

8.1.2 The Minimum Spanning Tree and Neighbourhood Conditions

The minimum spanning tree (MST) is a tree that connects all objects in a population in such a way that the sum of the length of the lines is minimal, [190] It is a graph in which every point is interconnected by a line to one or more neighbouring points, without forming loops (per definition – the sum of the length of all lines must be minimal). [187,191] Therefore cell nuclei represent the vertices and the MST connects the vertices by lines (i.e. inter-nuclear distance).

Fig 8.1. Schematic Diagram - Computation of Neighbouring Nuclei: (Adapted from Fig 1. Scheme of computation of neighbouring glands, Kayser K, Shaver M, Modlinger K et al 1986) [188]

Nuclei interactively marked and numbered	
Compute: nearest nuclei (nucleus 2) of nucleus 1: **1st neighbour**	
Compute: 2nd nearest nuclei (nucleus 4), not hidden beyond neighbours already computed (nucleus 2): **2nd Neighbour.**	
Compute: 3rd nearest nuclei (nucleus 5), situated between neighbours already computed (nucleus 2 and 4). Distance nuclei 2-4 < nuclei 1-5. Therefore nucleus 2 is a neighbour of nucleus 4: **no neighbour.**	
Compute: 4th nearest nuclei (nucleus 3), hidden beyond neighbour already computed (nucleus 2): **no neighbour.**	

To construct an MST, the centres of gravity of all nuclei are first marked and the distance between these vectors is calculated hence forming the first branch of the tree by connecting the 2 nuclei having the shortest distance apart. The nuclei closest to either one of these points are then selected forming the second tree branch. The procedure continues until all nuclei are connected and linked in a tree. Spanning tree analysis may be used to compute the number of nuclei, the MST and varying inter-nuclear features (length MST – total line length between nuclei; max. MST – the maximum distance between nuclei, min MST – the minimum distance between nuclei with standard deviations per parameter).

Another possibility in analysing relationships in the geometrical arrangement of cells is neighbourhood conditions. [188] In normal human tissue every cell has at least one neighbour, in solid structures the number of neighbours is ideally normally distributed, [192] with the presence of 3 to 5 neighbours being typical of solid tissue. [193] (See Fig 8.1: Computation of neighbouring nuclei). In cancer, structures become disordered and cell neighbourhoods are found to alter.

Increasing evidence exists that the construction of attributed graphs is a suitable technique for the analysis of microscopic growth patterns [194] and can provide a powerful tool for objectifying tumour morphology. [195] Because each tissue has a specific arrangement of cells with respect to each other (i.e. a specific architecture), SSA may be used to determine tissue origin. [196] As malignant transformation occurs from well to more anaplastic, SSA could be considered a potent tool for recognising either tumour type or grade, [196] and as SSA deals with properties of the geometric arrangement of cells it may well be used to reflect the biological behaviour of malignancies. [186]

Construction of the MST can provide quantitative data that can be used for tumour classification and prognosis prediction, [195] and, non-oriented complete graphs, based on neighbourhood condition have been found closely related to the histopathologic diagnosis. [197]

8.2 Methods

8.2.1 Tissue Processing: Immunostaining Technique

MST analysis was performed on archived, paraffin-embedded, H&E stained sections 4-6μm thick. Sections were chosen containing the worst areas of atypia, as confirmed by 2 pathologists.

8.2.2 Computer-Aided Image Analysis System

Computer aided image analysis was performed using a digital interactive video overlay

system (Qprodit, version 6.1; Leica, Cambridge, UK). MST measurements were performed in the department of pathology, Stavanger University Hospital, Stavanger, Norway under the supervision of Professor Jan Baak.

8.2.3 MST Measurement

To calculate the MST, archival H&E stained sections, 4-6microns thick, were used for each case. The MST was calculated from areas containing the worst atypia avoiding areas of necrosis, inflammation or calcification. Areas were selected, captured and frozen onto the screen and in each field of vision the centres of gravity for tumour cell nuclei were marked with an interactive graphic tablet. The minimal total line length then automatically connected these points without forming loops, thus forming the MST. MST measurement parameters included length (length MST), average length (mean MST), minimum length (min MST), maximum length (max MST), standard deviation (sdMST), and percentage connectivity to 1,2,3,4 and 5 nearest neighbours. (See Fig. 8.2 for MST Calculations).

MST was calculated at a final on screen magnification X1200 (objective X40). Intra-field reproducibility was correlated as 97.6% for each tumour grade thus deemed adequate as tumour cells and non-tumour cells could be easily differentiated at this level.

MST was calculated over an average of 10 fields of vision (marking an average of 870 points per case). The number of visual fields required to achieve adequate measurement precision were calculated by conducting running means and coefficients of error (CE). Measurements of MST parameters were conducted until a CE of <5% variability was achieved. (See Fig 8.3 for Coefficient of Error Graph's).

The systematic sample of, on average 87 points per field over an average of 10 fields was also used to cope with inter-field heterogeneity and to counteract boundary effects, [195] as, the size of the MST may be limited by the borders of the visual field.

Fig 8.2: Calculation of the minimum spanning tree

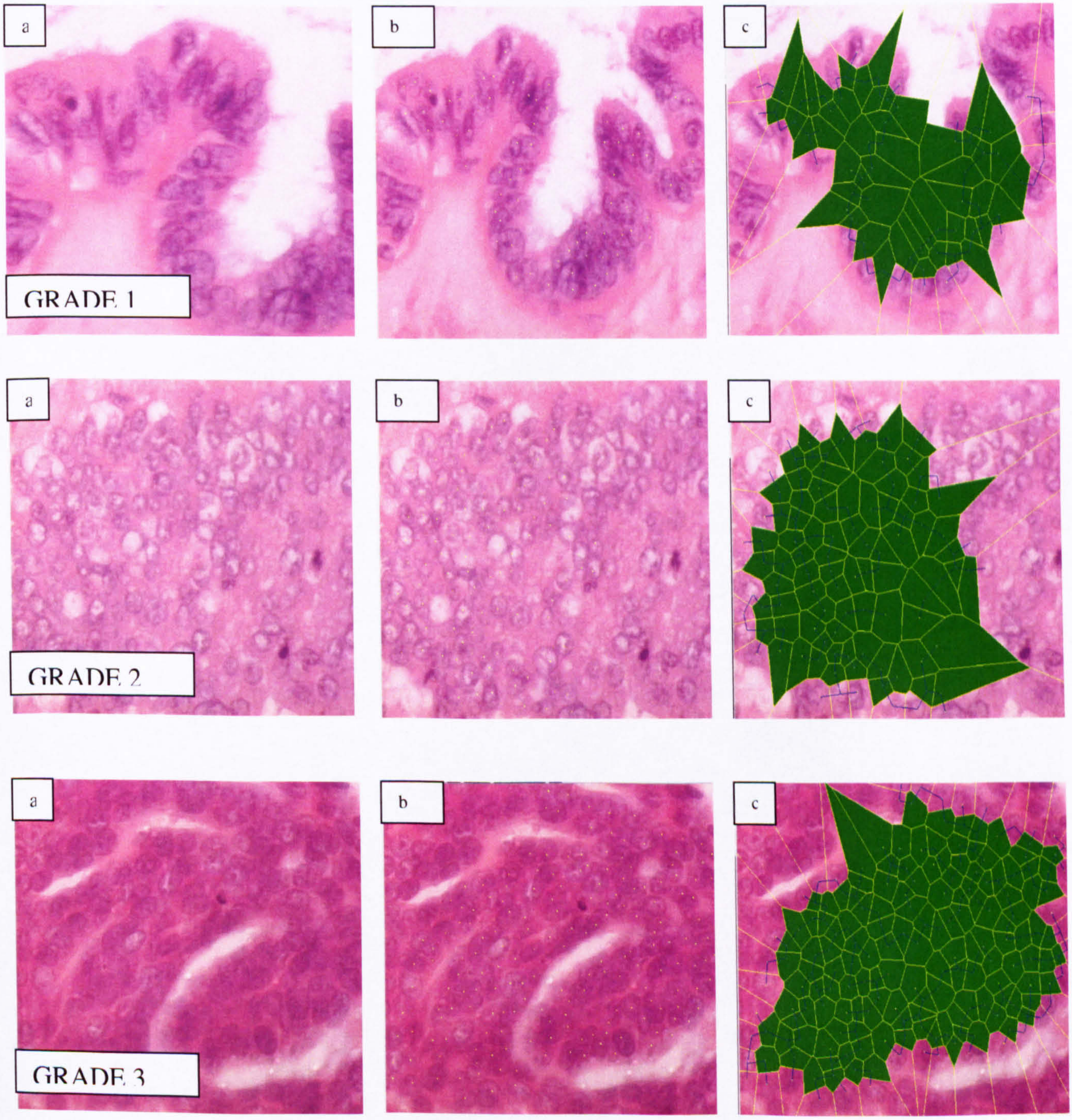
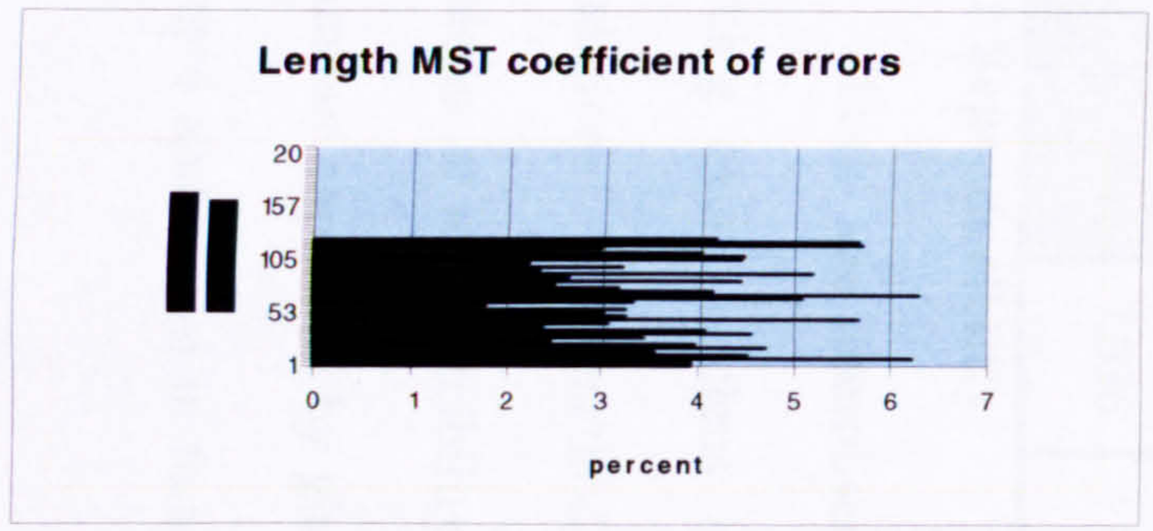
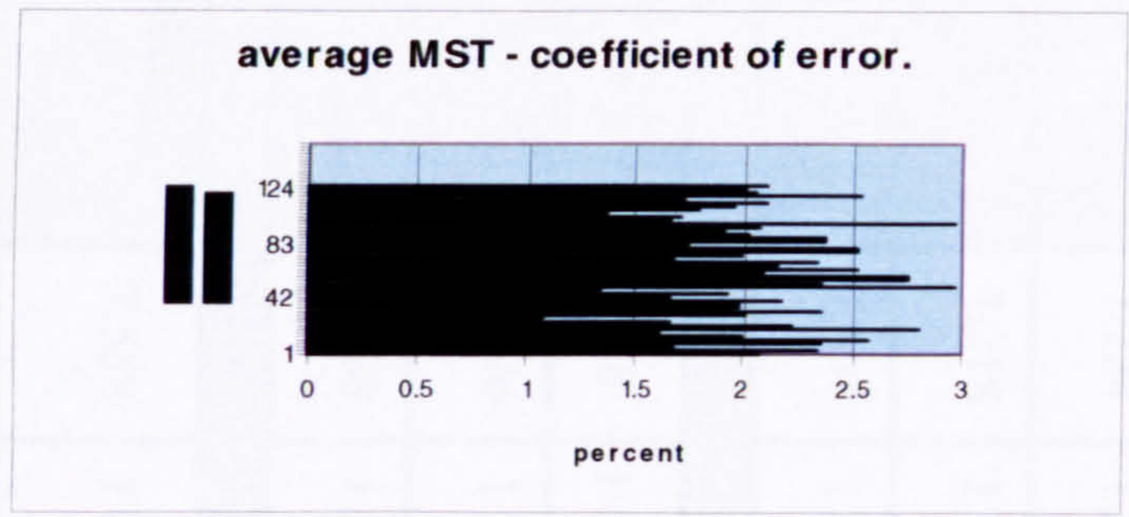
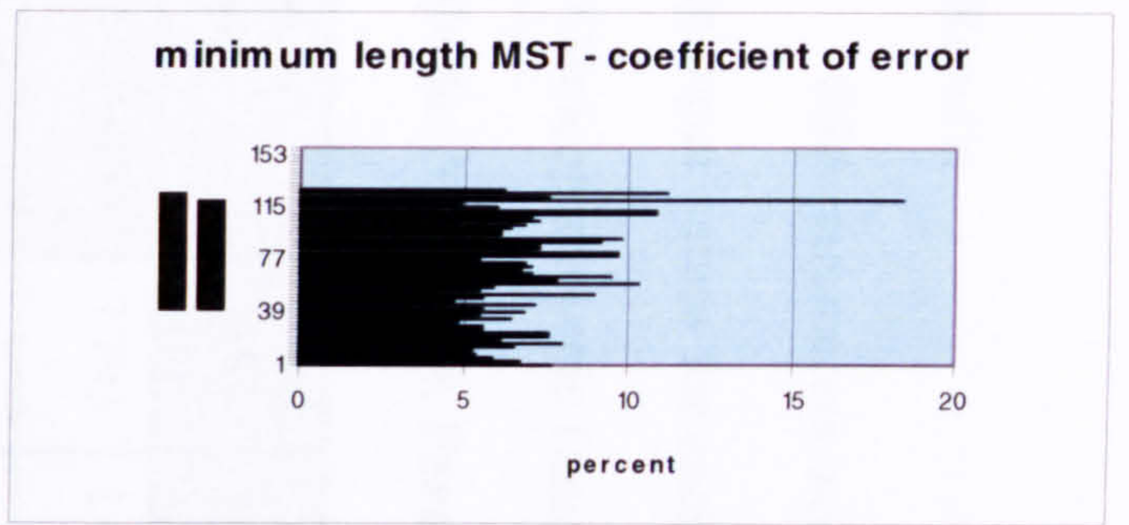


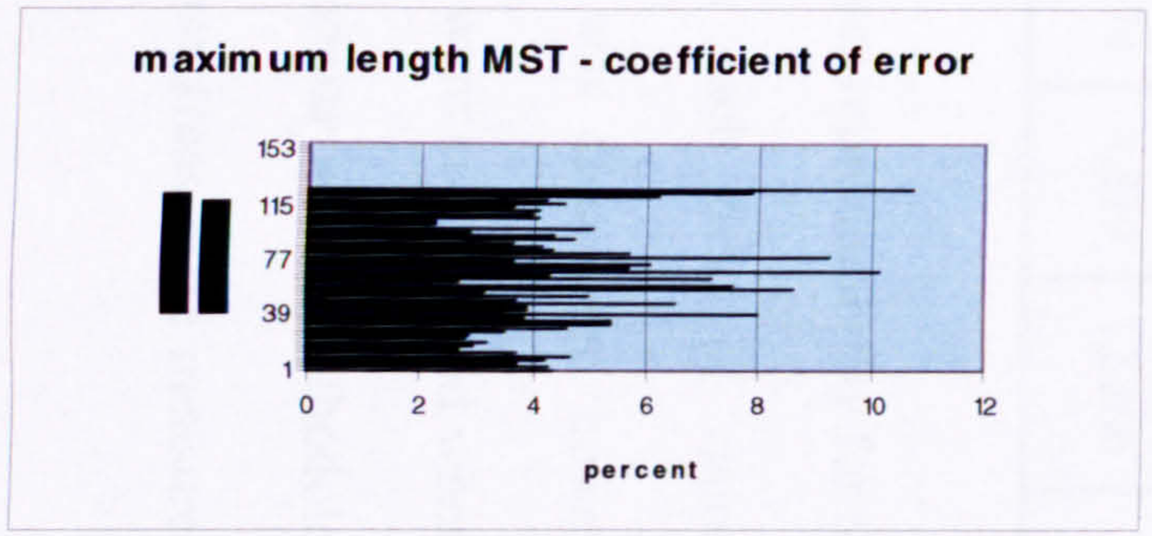
Fig 8.2 – Panel a. Shows initial tumour selection
- Panel b. marking of centres of gravity, and
- Panel c. MSTcomputation
in grades 1-3 serous ovarian tumours.

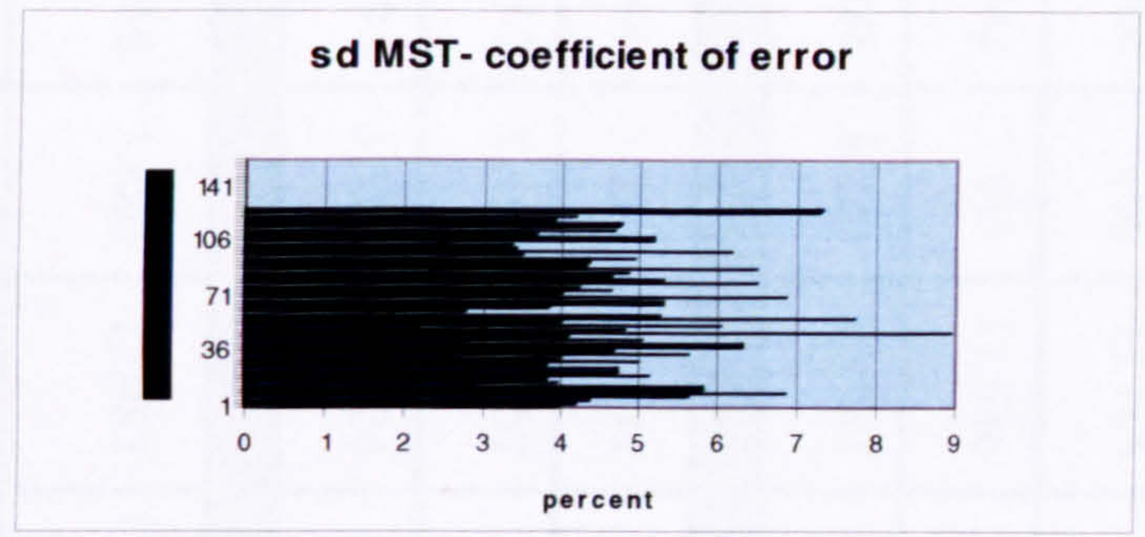
Fig 8.3: Syntactic Structure Analysis: Coefficient of Error Graphs

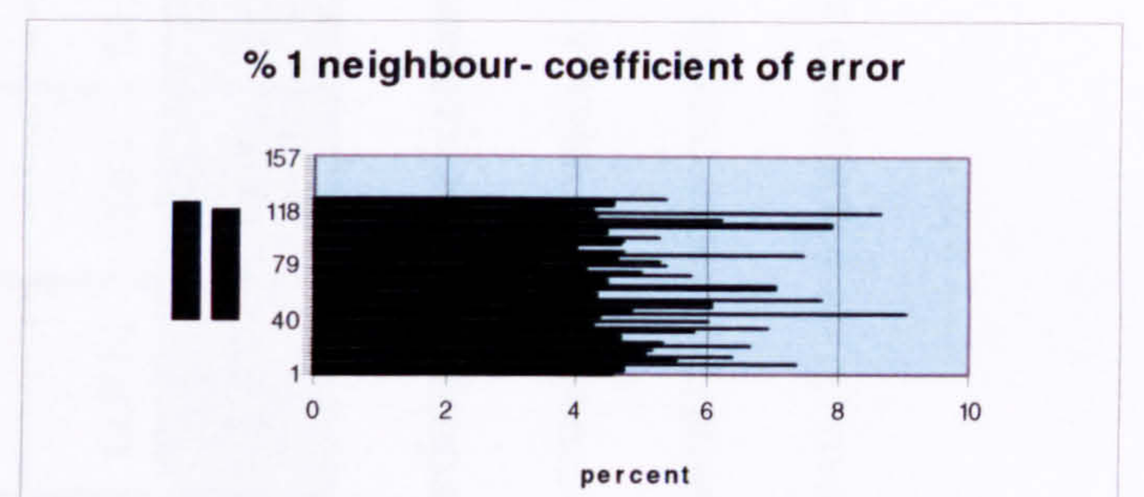


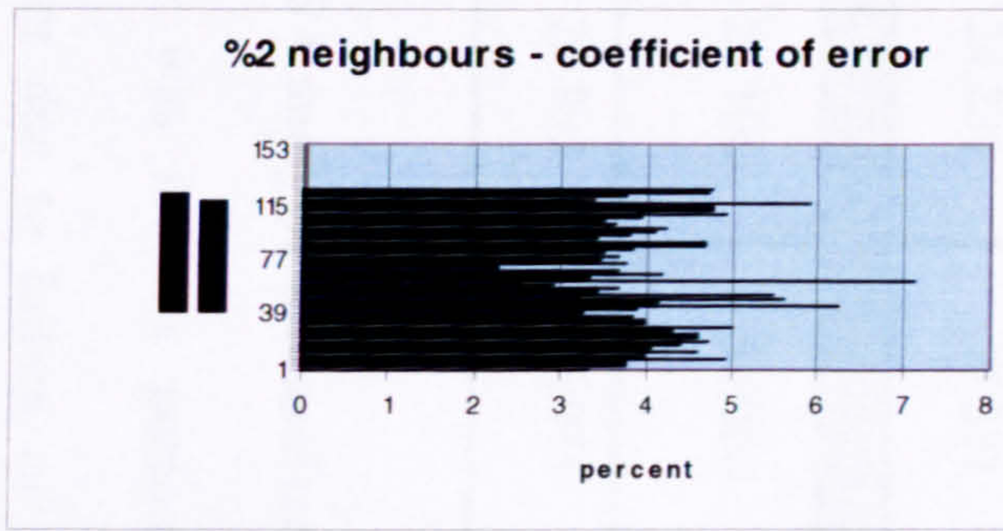












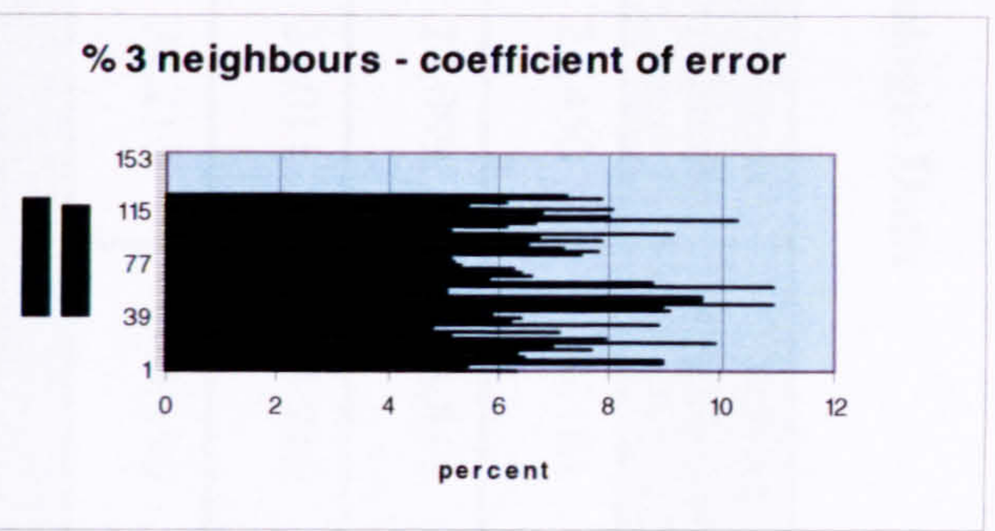


Figure 8.3 illustrates coefficient of error graphs for syntactic structure analysis.
MST was calculated over an average of 10 fields of vision (marking an average of 870 points per case).
CE ≤5% Indicates a high level of measurement precision

8.3 Results

SSA parameters were conducted on all available 132 cases. Analysis was performed using mean values for each measurement parameter (see Table 8a: Mean SSA Parameters and Clinicopathologic Data). P values <0.05 were regarded as being significant.

Table 8a: Mean SSA Parameters and Clinicopathologic Data

Clinical Parameters	Mean MST (μ)	sd MST (μ)	Min MST (μ)	Max MST (μ)	1 Nearest Neighbour (%)	2 Nearest Neighbours (%)	3 Nearest Neighbours (%)
FIGO Stage: I	7.793	1.919	3.932	13.477	22.450	59.164	17.365
II	7.724	1.866	3.686	13.145	24.099	78.819	41.748
III	7.218	1.742	3.483	11.946	24.011	55.916	18.714
IV	7.308	1.988	3.220	13.947	24.213	55.136	19.356
Grade: 1	7.48	1.868	3.733	12.974	22.554	59.442	17.684
2	7.276	1.762	3.514	12.207	23.782	56.382	18.599
3	7.388	1.798	3.513	12.474	24.231	55.331	19.016
Residual Disease: <2cm	7.558	1.865	3.680	12.987	23.106	58.060	18.199
>2cm	7.336	1.774	3.529	12.266	23.993	55.911	18.692

8.3.1 Reproducibility

Intra- and interobserver reproducibility for SSA analysis was assessed over 8 cases. Marking every nucleus in each field of vision was initially performed for MST calculations. This method was discounted however due to failure of reproducibility. Problems of reproducibility were encountered where fields of vision contained solid areas of tumour separated by glandular acini. Methodology for MST calculation was therefore changed and nuclei in tumour areas were measured without crossing lumina and a fixed

number of nuclei were marked for each field of vision (50-100 vertices). Intra observer reproducibility was excellent for mean MST (p=<0.0001), sd MST (p=<0.0001), max MST (p=<0.0001), min MST (p=0.002) and percent connectivity to 1 nearest neighbour (p=0.004). Inter observer reproducibility was excellent for mean and max MST only (p=0.02), but not significant for the remaining parameters. (See Table 8b: Reproducibility SSA).

8.3.2 Correlations

On linear regression analysis increase in mean, min, max MST and percent connectivity to 1 & 2 nearest neighbours were found to strongly correlate with FIGO stage. Percent Connectivity to 1 & 2 nearest neighbours were also found to strongly correlate with tumour grade, presence of ascites and the extent of residual disease. (See Table 8c: Correlations Between Parameters).

8.3.3 Survival analysis

OS data was available for all 132 cases. DFS data was available for 94 cases. P values below 0.05 were regarded as significant.

8.3.3.1 Significance of single parameters to predict survival / Univariate Analysis

SSA and Clinicopathologic variables were analysed using the univariate Cox model. Of the clinicopathologic data, FIGO stage (p=<0.01), tumour grade (p=0.002), pre-operative Ca125 levels (p=0.016), presence of ascites (p=<0.01) and extent of disease residuum (p=<0.01) were found to be significant predictors of OS. For SSA variables max MST (p=0.036), percent connectivity of 1 nearest neighbour (p=0.003) and 2 nearest neighbours (0.013) were also found to be significant predictors of survival with min MST being of borderline significance (p=0.053). The remaining variables were not significantly correlated.

Table 8b: Reproducibility SSA Data

	Mean MST		Sd MST		Max MST		Min MST		1 Nearest Neighbour (%)		2 Nearest Neighbours (%)		3 Nearest Neighbours (%)	
Observer	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Correlation	Intra	Inter	Intra	Inter	Intra	Inter	Intra	Inter	Intra	Inter	Intra	Inter	Intra	Inter
r	0.9832	0.9384	0.9856	0.4517	0.9905	0.8524	0.7885	0.4936	0.6405	0.6631	0.6158	0.2803	0.5039	0.1973
r2	0.9668	0.8806	0.9714	0.204	0.981	0.7266	0.6218	0.2437	0.4102	0.4397	0.3792	0.07855	0.2539	0.03894
P	<0.0001	0.0182	<0.0001	0.309	<0.0001	0.0148	0.0201	0.2603	0.0871	0.1044	0.1041	0.5427	0.2030	0.6715
Spearman	0.9286	0.9	0.9286	0.3929	1	0.6071	0.8095	0.2857	0.7619	0.3921	0.3792	0.07855	0.2539	0.03894
2 Tailed p	0.0022	0.0833	0.0022	0.3956	<0.0001	0.1667	0.0218	0.556	0.0368	0.3956	0.1041	0.5427	0.2030	0.6715

Table 8b illustrates reproducibility of SSA features.
Although line features (Mean, Max, Min MST) were generally found to be reproducible, neighbourhood features were not.

Table 8c: Correlation Between Parameters

	mean MST			min MST			max MST			1 Neighbour			2 Neighbours			3 Neighbours		
	r	r^2	p	r	r^2	p	r	r^2	p	r	r^2	p	r	r^2	p	r	r^2	p
Age	0.0516	0.0027	0.558	-0.016	0.0003	0.86	-0.003	8.23E-06	0.974	0.152	0.023	0.083	-0.116	0.013	0.189	0.11	0.012	0.208
Grade	0.012	0.00014	0.892	-0.04	0.0018	0.63	-0.045	0.002	0.609	0.275	0.076	0.0015	-0.391	0.153	<0.0001	0.23	0.054	0.0073
Stage	-0.18	0.0325	0.039	-0.23	0.051	0.009	-0.192	0.0368	0.028	0.3	0.092	0.0004	-0.35	0.123	<0.0001	0.2319	0.054	0.0077
Ca125 Pre op	0.069	0.0047	0.517	0.056	0.003	0.599	0.06	0.0036	0.57	0.047	0.0022	0.66	-0.058	0.0034	0.583	0.025	0.0006	0.81
Ascites	-0.09	0.008	0.306	0.1685	0.028	0.054	0.109	0.012	0.21	0.18	0.033	0.039	0.252	0.064	0.0037	0.128	0.016	0.14
Residual Disease	-0.09	0.008	0.3	-0.097	0.00	0.272	-0.14	0.019	0.116	0.28	0.081	0.001	-0.35	0.119	<0.0001	0.163	0.03	0.06

Table 8c illustrates correlations between SSA and clinicopathologic parameters.
Mean MST, min MST, max MST, and percent connectivity to 1 & 2 nearest neighbours were found to strongly correlate with FIGO stage.

For DFS, of the clinicopathologic data, FIGO stage (p=<0.01), tumour grade (p=0.001), pre-operative Ca125 levels (p=0.004), presence of ascites (p=<0.01) and extent of disease residuum (p=<0.01) were found to be significant predictors. For SSA variables min MST (p= 0.044), percent connectivity to 1 nearest neighbour (p=0.014) and 2 nearest neighbours (p=0.009) were also found to be significant predictors of DFS. Max MST reached borderline significance (p=0.057). The remaining variables were not found to be significant.

8.3.3.2 Multivariate Survival Analysis

SSA variables were analysed using the Cox multivariate model. Overall max MST (p=<0.01), sd MST (p=0.001), percent connectivity of 1 nearest neighbour (p=<0.01) and 2 nearest neighbours (p=0.021) showed to be a significant predictor's of OS, with the remaining SSA variables adding no additional prognostic relevance.

To estimate the simultaneous influence of all prognostic factors clinicopathologic and all SSA variables were entered into Cox regression analysis. This analysis showed overwhelmingly that extent of disease residuum was found to be a significant predictor for OS (p=<0.01) in this group of patients. The other factors did not achieve independent significance.

Min. MST (p=0.016) and percent connectivity to 2 nearest neighbour's (p=0.005) showed to be significant predictors of DFS, with the remaining SSA failing to achieve independent significance. Using Multivariate Cox models all clinicopathologic and SSA variables were considered. Overwhelmingly FIGO stage was found to be a significant predictor for DFS (p=<0.01) in this group of patients. The other factors did not achieve independent significance. (See Table 8d for positive findings on univariate and multivariate OS & DFS analysis.)

8.3.4 Prediction of Chemotherapy Response

Ca125 data was available for 69 cases, 5 of which did not receive primary chemotherapy so results of 64 patients were analysed. All patients were treated with primary carboplatin or carboplatin / paclitaxel regimes.

Table 8d: Positive Findings – Univariate and Multivariate Survival Analysis SSA

'arameter	df	Sig. (p)	Exp (B)	95% CI Lower	95% CI Upper	Parameter	Sig. (p)	Exp (B)	95% Lower CI	95% Upper CI
Univariate Analysis										
Overall Survival						**Disease Free Survival**				
Clinicopathologic Variables						**Clinicopathologic Variables**				
Grade	2	**.008**				**Grade**	**.004**			
Grade(1)	1	**.004**	.344	.168	.705	**Grade(1)**	**.001**	.288	.138	.603
	1					**Grade(2)**	**.384**	.798	.479	1.327
Stage	3	**<.01**				**Stage**	**<.01**			
Stage(1)	1	**<.01**	.151	.056	.403	**Stage(1)**	**.037**	.228	.057	.913
Stage(2)	1	**.003**	.256	.103	.639	**Stage(2)**	**.417**	.592	.167	2.101
	1					**Stage(3)**	**.555**	1.421	.442	4.568
Residual Disease	1	**<.01**	.341	.204	.568	**Residual Disease**	**<.01**	.341	.204	.568
Presence of Ascites	1	**<.01**	.377	.237	.599	**Presence of Ascites**	**<.01**	.358	.212	.605
Pre-operative Ca125	1	**.016**	1.000	1.000	1.000	**Pre-operative Ca125**	**.001**	1.000	1.000	1.000
SSA Variables						**SSA Variables**				
Length MST	1	0.29				**Length MST**	0.407			
sdMean MST	1	0.48				**sdMean MST**	0.151			
Mean MST	1	**0.092**				**Mean MST**	0.093			
Min MST	1	0.053				**Min MST**	**0.044**	.704	.497	.996
Max MST	1	**0.036**	.925	.861	.995	**Max MST**	**0.057**			
sd MST	1	0.125				**sd MST**	0.136			
1 Nearest Neighbour	1	**0.003**	1.187	1.057	1.333	**1 Nearest Neighbour**	**0.014**	1.161	1.032	1.307
2 Nearest Neighbours	1	**0.017**	.928	.873	.987	**2 Nearest Neighbours**	**0.009**	.915	.854	.980
3 Nearest Neighbours	1	.189				**3 Nearest Neighbours**	0.130			
Multivariate Analysis										
SSA Data										
Max MST	1	**<.01**	.541	.391	.749	**Min MST**	**.016**	.647	.454	.923
sdMST	1	**.001**	28.54	3.848	211.638	**2 Nearest Neighbours**	**.005**	.901	.839	.968
1 nearest neighbour	1	**<.01**	1.48	1.241	1.764					
2 nearest neighbours	1	**.021**	.832	.712	.972					
SSA & Clinicopathologic Data										
Residual Disease	1	**<.01**	.272	.150	.494	**Stage**	**<.01**			
						Stage(1)	.001	.090	.021	.391
						Stage(2)	.019	.198	.051	.767
						Stage(3)	.348	.566	.173	1.859

Of the clinicopathologic data, tumour grade (p=0.02), FIGO stage (p=0.004), extent of residual disease (p=<0.01) and presence of ascites (p=0.004) were found significant predictors of chemotherapy response with an overall correct classification to chemotherapy response group being 70.3%, 68.8%, 68.8% and 67.2% respectively. For the spanning tree data 2 nearest neighbours (p=0.006) was found to be a significant predictor of chemotherapy response with an overall correct classification to chemotherapy response group of 53.1%. The remaining spanning tree parameters were insignificant.

To estimate the simultaneous influence of prognostic factors all clinicopathologic and SSA variables were entered into multivariate logistic regression analysis. This analysis showed extent of residual disease (p=0.005) to be the strongest predictor of chemotherapy response with correct overall classification rates of 66.1%. The remaining parameters did not retain significance. (See Table 8e Logistic Regression Analysis: SSA & Clinicopathologic Data and Chemotherapy Response).

8.4 Discussion

Variability of intercellular arrangement may be an important clue to the diagnosis of malignancy. In the differential diagnosis of lung tumours prior studies showed SSA to be a valuable tool in the classification of mesothelioma and a supplement to visually appraised diagnosis, [196] with malignant mesothelioma being readily distinguishable from adenocarcinoma by internuclear distances. [198] Studies have found MST able to easily distinguish small cell from non-small cell anaplastic carcinoma. [197] SSA was also shown to provide a powerful tool for specifically assessing, at least partially, some of the cytonuclear changes in dysplasia of colorectal adenomatous polyps. [187]

Prior SSA studies in lung carcinoma revealed patient survival to be strongly related to structural features, [194] and smaller inter-nuclear distances to have worse prognosis than

Table 8e: Table 8e Logistic Regression Analysis: SSA & Clinicopathologic Data and Chemotherapy Response

Parameter	df	Sig. (p)	Correct Classification	95% CI Lower	95% CI Upper
Univariate Analysis					
Clinicopathologic Variables					
Grade	2	.002	68.8%		
Grade(1)				2.412	67.918
Grade (2)				.728	7.917
Stage	3	.004	68.8%		
Stage(1)				1.028	560.178
Stage(2)				.385	78.573
Stage (3)				.121	17.913
Residual Disease	1	<.01	70.3%	2.420	29.383
Presence of Ascites	1	.004	67.2%	1.566	13.822
Pre-operative Ca125	1	.093			
Age	1	.554			
SSA Variables					
2 Nearest Neighbours	1	.006	53.1%	1.042	1.460
Length MST	1	.508			
Mean MST	1	.900			
sd Mean MST	1	.663			
sdMST	1	.848			
Max MST	1	.751			
Min MST	1	.797			
1 Nearest Neighbour	1	.059			
Multivariate Analysis SSA & Clinicopathologic Data					
Residual Disease	1	.005	66.1%	1.524	19.849

Table 8e illustrates univariate and multivariate logistic regression analysis of SSA features, clinicopathologic parameters and prediction of chemotherapy response. Although number of nuclei with 2 nearest neighbours showed significance at univariate level, independent prognostic significance was not achieved. (p values <0.05 were regarded as significant).

larger distances in small cell anaplastic carcinoma of the lung. [197] Mean and max MST have also exhibited significance for survival in breast carcinoma. [199] With regard to neighbourhood parameters the maximum number of nuclei with 2 or 3 neighbours, were

found to be the best prognosticators in breast carcinoma, [200] in uveal melanoma, [193] and in predicting the development of metachronous cancer in colorectal adenomas. [201]

The present study of serous ovarian tumours encountered reproducibility problems whilst computing both the MST and neighbourhood features. Although intra-observer reproducibility was generally excellent for MST parameters, interobserver reproducibility was less impressive. Neighbourhood features remained overall poorly reproducible. It thus may be better to replace interactive morphometric SSA and MST analysis, by automated digital image analysis.

In invasive breast cancer both MST and neighbourhood features have been reported as highly reproducible. [199-200] Some measurements however were repeated using the same static field of vision by both observers. In uveal melanomas, intra-observer reproducibility was found acceptable with a mean correlation coefficient of 0.70, yet no inter-observer correlations were reported. [193] In advanced ovarian cancer reproducibility has been quoted as high for intra-observer and as acceptable for inter-observer analysis with, as in this study, neighbourhood variables remaining less well reproducible. [190]

Use of image analysis should essentially offer reduced disagreement and a possibility to compare inter-laboratory data. [196] Computation of the MST may become difficult where nuclei overlap i.e. when no agreement is met as to whether overlapping nuclei should be counted as 1 or 2 discrete entities, and where visual fields include glandular acini. Serous ovarian tumours represent a markedly heterogeneous group with ovarian cancer certainly having more variation in nuclear spacing than for example melanomas or breast carcinomas. Such variations in tumour area, cyst size and e.g. papilla formation may reflect the finding of high inter-observer variation and hence limit the use of these methods when applied to serous ovarian tumours. Also for MST and neighbourhood analysis to offer the possibility to

compare study data, analysis should not be performed on identical static images, as in reality no pathologist would choose precisely the same region for analysis as another.

This study found MST and neighbourhood parameters to strongly correlate with FIGO stage, with nearest neighbour parameters also strongly correlating with tumour grade, extent of residual disease and the presence of ascites. It therefore appears that MST, and neighbourhood parameters, are associated with tumour aggression. Univariate analysis revealed several SSA parameters of significance with wider inter-nuclear distances being associated with improved OS and DFS, and able to predict response to chemotherapy. Multivariate combination of SSA parameters improved the prognostic capability of standard deviation MST suggesting that variability in nuclear arrangement may be a more important finding for the prediction of OS. Multivariate analysis combining SSA and clinicopathologic parameters however did not reveal SSA features as independent prognosticators for either survival or prediction of chemotherapy response.

Further SSA sub-group analysis in this tumour set of early (FIGO I & II) and late stage tumours (FIGO III & IV) (see appendix 3) also failed to achieve independent significance on multivariate analysis. This may reflect that this data set contained only 31% early stage tumours (16% were Stage I, 15% stage II) with the majority of cases being of more advanced stage (69%) and grade (83%). Clearly a large study of FIGO stage I tumours might be of particular use here in differentiating the real value of SSA parameters when freed from the effects of advanced disease.

Where marked tumour heterogeneity occurs, we must consider that the set of points representative of the biological sample is derived from a larger set of points, so has to be large enough to enable recognition of the biological sample. [201] In practice the size of the MST will always be limited by the borders of visual field, [195] be it from natural boundary

effects such as size of tumour area, cystic walls, the presence of papillae or from system-induced boundary effects. Magnification must be large enough to adequately differentiate the areas and points of interest from non-tumour areas but increased magnification in turn reduces the size of the visual field and hence the size of the MST. It is therefore not possible to eliminate all of the boundary effects of an MST. [202] A prior study had suggested that boundary effects were observed mainly when the number of points in the MST was smaller than 64. [195] Our study assessed an average of 870 points over 10 fields giving a mean count of 87 points per field. Sub-analysis of only those cases containing >65 points per field also failed to achieve independent prognostic significance for SSA features. As acceptable intraobserver reproducibility and significant results on univariate analysis were achieved with this method we feel that if such systematic error occurred then it is likely to be a limiting factor in the practical application of this technique.

Use of lower magnification, combination of adjacent visual fields, or larger field sizes may be the ultimate answer but this would reduce operator precision at accurately identifying points of interest and have obvious time implications. Again automated image analysis probably must be used for highly reproducible SSA and MST analysis.

Chapter 9. Angiogenesis

9.1 Introduction

9.1.1 Background

Angiogenesis is the formation of new blood vessels by proliferation of new capillaries from pre-existing vessels. [203] Angiogenesis is linked to physiological conditions such as ovarian and endometrial alterations during the menstrual cycle, wound healing and tumour growth. [204] When considering tumour proliferation, the 'pre-vascular' phase is associated with limited tumour growth with few or no metastases, the 'vascular' phase with increased tumour growth and metastatic spread. [203] Pre-vascular tumours may remain dormant in-situ for months or years. [205]

Tumour angiogenesis is thought initiated by an increase in the level of angiogenic stimuli and a concomitant decrease in the level of angiogenic inhibitors. [206] 'Once tumour has occurred, every increase in tumour cell population must be preceded by an increase in new capillaries that converge upon the tumour.' [206] The pre-vascular phase can maintain tumour only up to a tumour size of <2mm. [207] Beyond this critical volume the onset of angiogenesis permits rapid expansion of the tumour population, if the tumour cells are capable of rapid proliferation. [208] Micro vessels supplying tumours are strikingly hyper permeable to circulating plasma proteins [203] and neoplastic capillaries have fragmented, leaky basement membranes making them more accessible to tumour cell migration and facilitation of haematogenous metastasis. [209] Highly angiogenic tumours may therefore facilitate a rapidly increasing volume of tumour cells, provide access to the circulatory system and promote the development of lymphatic channels. [210]

There is mounting evidence that, in general, tumour progression and metastasis are angiogenesis dependent [211] with studies demonstrating direct correlation between the

degree of angiogenesis in tumours and more aggressive tumour biology. [210] It is possible therefore that the degree of angiogenesis may be a marker for the aggressiveness of ovarian cancers [210] and could be used as a prognostic factor for survival.

9.1.2 Assessment of Angiogenesis

Angiogenesis is assessed quantitatively by measuring intratumoral micro vessel density (IMVD or MVD). [212] IMVD is assumed to reflect the intensity of tumour angiogenesis. The most commonly used antibodies or endothelial cell markers, used to highlight blood vessels by IHC are anti-factor VIII antigen, CD31 and CD34, being assessed under high power microscopy. Gross examination, angiography, and vascular injection techniques could conceivably be used to evaluate the degree of tumour vascularity, [213] but practically, angiogenesis assessment is conducted by observing and quantifying vascularization histologically, to provide an estimate of the final product of a complex process. [214]

Several methods of IMVD measurement have been used including manual counting of micro vessels in tumour regions of greatest vascularity, estimation using point counting, [214] and multiparametric computer analysis systems. The most conventional method of IMVD estimation is by 'hot spot' counting as defined by Weidner and associates. [215] The heterogeneity of tumour vasculature is well recognised and most prognostic studies evaluate MVD in regions of high MVD or areas termed as vascular 'hot spots.' [216] Angiogenesis is assessed in areas of tumour stroma, with the highest angiogenic density in areas surrounded by solid tumour. [217] These areas of greatest MVD would theoretically be more likely to disseminate cells and produce rapidly growing, clinically detectable metastases. [218] Once regions with elevated vascular density, or 'hot spots', are identified then positive IHC staining is counted as a microvessel. Small vessels are counted including capillaries,

arterioles and venules. [217] Any highlighted endothelial or endothelial cell cluster, clearly separate from adjacent microvessels, tumour cells and other connective tissue elements is considered as a single countable vessel [212] (i.e. no lumen or red blood cell's required). Larger arteries with thick smooth muscle walls and widely distended venous sinuses should be excluded. [217]

Problems may arise in variations of vessel counting techniques. Visual counting at high magnification is tedious and may be subject to poor reproducibility. [219] Manual counting may certainly have limitations. Single tortuous vessels may be counted as several vessels, distinguishing individual vessels may not be possible in areas of tangled capillaries, [220] and no agreement may be met if only luminal structures are counted. Further limitations may result from observer error or sampling error as marked heterogeneity in MVD may be observed in different areas of the same tumour. [204] Since identification of the neovascular 'hot spot' relies upon the judgement of the 'pathologist' the relative skills and experience of different pathologists may be problematic, with the inexperienced pathologist having a tendency to overestimate the number of vessels. [221]

Image analysis techniques are used as an objective method of quantifying angiogenesis to overcome the potential subjectivity of manual counts. [220] Application of computer-aided image analysis may help to standardise microvessel counts and help eliminate observer variables such as inexperience and 'hot spot' selection bias, [222] but, image analysis may also result in multiple counts of the same vessel and background noise may read as falsely positive. [220] Based on the assumption that the maximally vascular area within a tumour determines metastatic potential, automated morphometric techniques that provide an average micro vessel count may give less prognostic information. [214] Standardisation of angiogenesis quantification is necessary to facilitate confirmation of the suggested

prognostic value of IMVD in different tumour types. [212]

9.1.3 Angiogenesis Markers

Anti Factor VIII antigen (von Willebrand Factor Antibody), CD31 (PECAM-1, platelet endothelial cell adhesion molecule-1) and CD34 are the most commonly used antibodies, or endothelial cell markers, used to highlight blood vessels by IHC. [223]

9.1.3.1 Anti Factor VIII Antigen

Factor VIII is a glycoprotein present in human plasma. Factor VIII is reported present in human endothelial cells, megakaryocytes and platelets. As Factor VIII related antigen is present in plasma and platelets, positive staining of intravascular plasma inevitably occurs in areas of haemorrhage, vascular damage and leakage of plasma. [224] In cases of ovarian cancer anti-factor VIII has occasionally been shown to stain lymphatics, [203] and has appeared to stain fewer microvessels. [216] Generalised background staining at the site of exudation, haemorrhage and necrosis has seemed to make some cases un-interpretable for vWF. [225] Therefore anti factor VIII is not used in this study.

9.1.3.2 Anti CD31

CD31, also known as PECAM-1 (platelet endothelial cell adhesion molecule 1), is a 130kDa integral membrane protein mediating cell-to-cell adhesion. CD31 is expressed on the surface of adult and embryonic endothelial cells and is weakly expressed on many peripheral leucocytes and platelets. It has also been detected on bone marrow-derived haematopoietic stem cells and embryonic stem cells. [226] In ovarian cancer CD31 has been found more sensitive for identification of vessels, but not specific i.e. can bind to granulocytes, monocytes, megakaryocytes, platelets and occasional plasma cells, in addition to endothelial cells. [210] CD31 has also been found to occasionally stain inflammatory cells. [203] Anti CD31 showed stronger staining in small vessels and capillaries than in large

vessels with no connective tissue elements positive. [225]

9.1.3.3 Anti CD34

CD34 antigen is a single chain trans-membrane glycoprotein, Mr 105 to 120 kDa. The CD34 antigen is present on immature haematopoietic precursor cells and all haematopoietic colony-forming cells in bone marrow and blood. [227] In ovarian cancer studies, anti CD34 has shown to react with endothelia of arteries, venules and capillaries and to be more intense in small vessels, especially capillaries. [225] Anti CD34 has shown positivity in some stromal elements, [203,216] but antibodies to CD31 and CD34 have been shown to highlight similar numbers of micro vessels. [216] In image analysis of epithelial ovarian carcinoma anti CD34 was found to have the highest specific staining and least background stain. [220] It has been implied that anti-CD34 may be a more reproducible and reliable antibody for routine studies. [212]

Increased IMVD appears to adversely effect prognosis suggesting that angiogenic properties are correlated with tumour aggressiveness. [205] Evidence of angiogenesis dependence in carcinoma of the ovary is apparent in peritoneal metastasis. Metastasis to the peritoneal membrane, as tiny avascular seeds, rarely grows beyond a limited size until after vascularisation. [208] The early and extensive metastatic dissemination of ovarian cancer suggests that angiogenesis may be an early and important event. [207] This could be of use in identifying those patients at increased risk of relapse or more distant dissemination. [207]

9.2 Methods

9.2.1 Tissue Processing: Immunostaining Technique

IHC studies were performed on 4-6μm formalin-fixed, paraffin-embedded tissue using the peroxidase-anti peroxidase (PAP) complex technique. Areas representative of the

invasive component of the tumour were selected from archived paraffin-embedded sections stained with Haematoxylin &Eosin (H&E). 2 sections containing areas of worst atypia were selected and immunostained from each case. Sections were immunostained, using antibodies to the endothelial cell marker CD34 (Mouse Anti-CD34 (Clone My10)(Beckton Dickinson, Cat#347660) and CD31 (Rat Anti-Mouse CD31 (PECAM-1) (Pharmingen, Cat#553370). A positive and negative control slide was included in each staining run. No counterstain was used. Sections were again washed then dehydrated, cleaned and mounted.

9.2.2 Manual Counting

An initial 10 cases were randomly selected from the study group for staining with both CD31 and CD34 with a methylene blue counter-stain. Counts were performed using both staining techniques. Anti CD34 was found to have the highest specific staining and least background staining compared to CD31. Therefore all cases were stained with CD34 without counter-stain to be further used for both manual counts and image analysis techniques.

9.2.3 Intratumoral Microvessel Density Determination

Sections were scanned under light microscopy for areas of most intense neovascularisation at low magnification (100X). Microvessel density for CD34 was heterogeneous throughout the tumour sections, but occurred most frequently at the periphery of the invasive tumour. Sections were scanned in parallel to the original H&E stained slides to assure analysis was restricted to invasive carcinoma.

Once neovascular 'hot spots' were identified, individual microvessels were counted in a 200X and 400X field. 'Any brown staining cluster that was clearly separate from adjacent microvessels, tumour cells and other connective tissue elements was considered as a single countable microvessel.' [14] Luminal structures or the presence of red blood cells

was not deemed necessary to define a micro vessel. Each count was expressed as the mean number of micro vessels per field (mean MVD) or the highest count (max MVD) identified within any X200 or X400 field. Two observers without prior knowledge of patient characteristic's or outcome made assessments.

9.2.4 Computer-Aided Image Analysis

9.2.4.1 Computer-Aided Image Analysis System

Computer aided image analysis was performed using a digital interactive video overlay system (LEICA Q500MC, Leica, Cambridge, UK) for image analysis microvessel counts and estimation of endothelial areas. A digital interactive video overlay system (Qprodit, version 6.1; Leica, Cambridge, UK) was used for angiogenesis spanning tree estimates.

9.2.4.2 Image Analysis Microvessel Determination

For each case, image analysis was performed on the same 'hot spot' area as manual micro vessel density determination, using a X25 objective. Each pixel was calibrated at 0.00323μ.The live image was initially set up with regards to white balance to attain even illumination. The selected area was then scanned using a set of initially 3 and up to 10 fields. A set of 10 fields was used to ensure maximum 'hot spot' areas were included and to reduce area selection bias. Once acquisition of the live image was attained the image was converted to binary to enable further image processing prior to measurement. Grey level detection was adjusted to corresponded well to CD34 positive areas and negate any background 'noise. A Field measurement was automatically attained (representing a summed value for all selected regions within the measurement frame) to attain the area fraction. This area occupied by endothelial cells, automatically detected by the image analysis system, expressed as a percentage of total examined area, equated the endothelial

area. Pixel counts were used for the endothelial area measurement.

Sections were scanned in parallel to the original H&E stained slides to ensure analysis was restricted to invasive carcinoma. 2 observers without prior knowledge of patient characteristic's or outcome made assessments.

9.2.4.3 Spanning Tree analysis

To calculate the MST and neighbourhood parameters anti CD34 stained sections were examined adjacent to original H&E sections to ensure analysis was restricted to invasive carcinoma. The MST was calculated from areas of highest microvessel density. Areas were selected, captured and frozen onto the screen. In each field of vision the centres of gravity for each microvessel were interactively marked with a graphic tablet and the area of interest chosen. Points were automatically connected by the minimal total line length, without forming loops, thus forming the MST. An average of 27 points per field were analysed over an average of 14 fields per case.

MST parameters measured included mean (mean MST) length (length MST), min length (min MST), max length (max MST) and standard deviation (sdMST) thus denoting mean, min and max intercapillary distances. Percent connectivity to 1,2,3 and 4 nearest neighbours was also calculated. Connectivity to 4 nearest neighbours was a rare event and thus excluded from further analysis.

MST was calculated at X100 final magnification. Intra-field reproducibility was calculated as >95% thus deemed adequate as microvessels could be easily identified at this level. The number of visual fields required to achieve adequate measurement precision were calculated by conducting running means and coefficients of error. Measurements of mean MST were conducted until a CE of <5% variability was achieved. The systematic sample of, on average, 14 fields of vision was also used to cope with inter-field

heterogeneity (i.e. confronts variation of microvessel density in different fields of vision) and to counteract boundary effects [195] as the borders of visual fields may limit the size of the MST.

9.3 Results

Analysis was performed on all available 132 cases using mean values for each measurement parameter (see Table 9a: Mean Microvessel Parameters and Clinicopathologic Data). P values <0.05 were regarded as significant. Figure 9.1 illustrates CD34 staining as related to patient survival.

Fig 9.1: CD34 Staining and Survival: shows CD34 staining as related to patient Survival

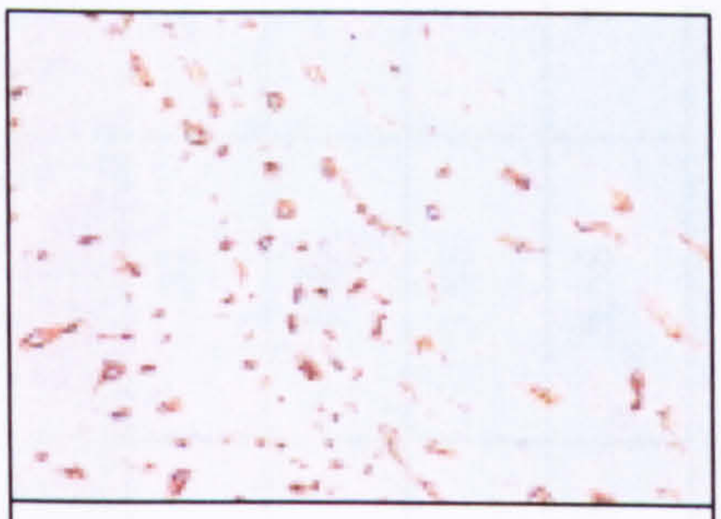


Fig 9.1 a

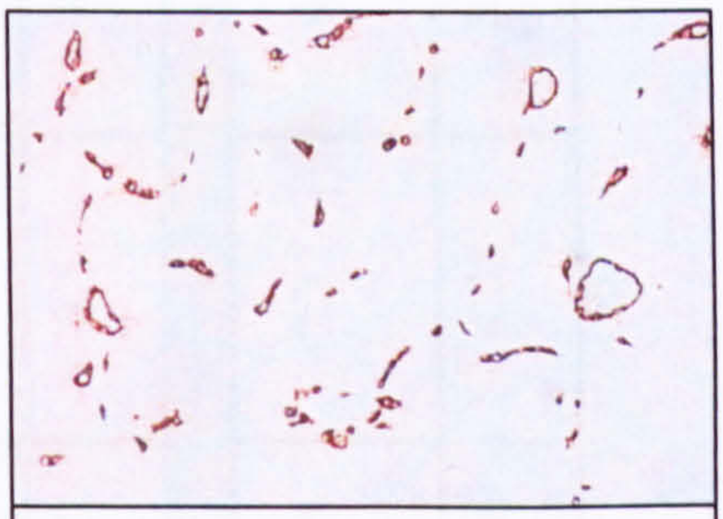


Fig 9.1 b

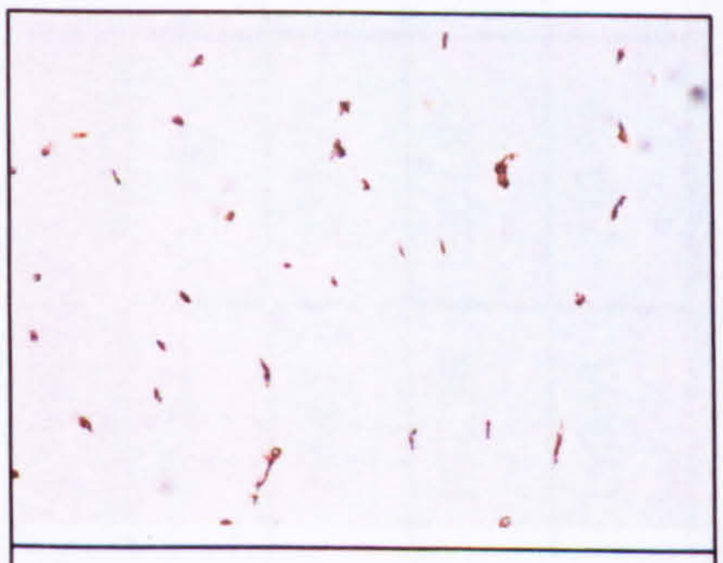


Fig 9.1 c

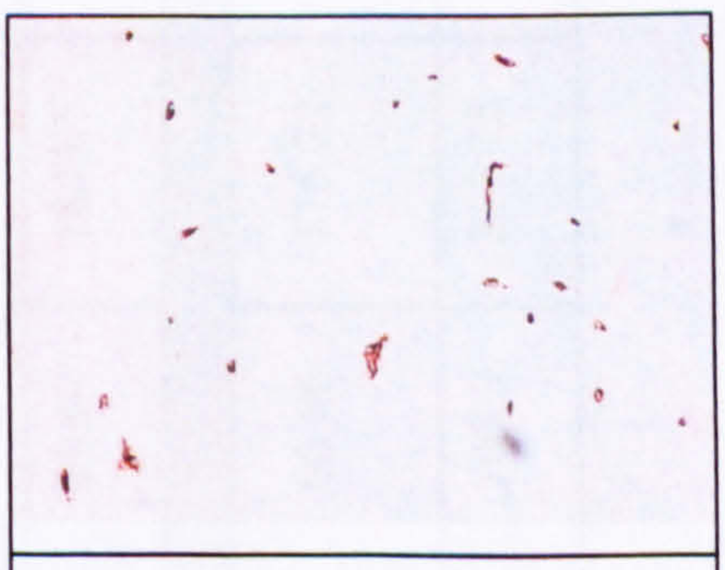


Fig 9.1 d

Fig.9.1

Panel a - Survival 6 months –FIGO III, Grade 1;

Panel b – Survival 21 months - FIGO III, Grade 3;

Panel c - Survival 72 months –FIGO IV, Grade 2;

Panel d – Alive at study closure– FIGO III, Grade 3

Table 9a: Mean Microvessel Parameters and Clinicopathologic Data

Clinical Parameters	Manual Angiogenesis Parameters				Image Analysis Angiogenesis Parameters				Spanning Tree Angiogenesis Parameters				
	X400 Mean MVD (%)	X400 Max MVD (%)	X200 Mean MVD (%)	X200 Max MVD (%)	Mean IA MVD Count (pixels)	Max IA MVD Count (pixels)	Mean Endothelial Area (μm)	Max Endothelial Area (μm)	Mean MST (μm)	Max MST (μm)	Min MST (μm)	sdMST (μm)	1 Nearest Neighbour (μm)
FIGO Stage I	19.76	23.38	48.56	55.62	127	170	2.31	3.446	23.13	40.16	10.4	9.14	32.37
II	26.31	31.11	59.86	68.63	151.77	536	3.07	4.21	21.79	38.93	9.36	8.84	31.29
III	38.15	43.3	90.36	101.36	201.87	261	3.88	5.54	17.11	30.74	6.97	6.61	27.95
IV	55.26	65.25	127.5	150.63	270.99	351.63	5.58	8.32	11.34	21.53	3.74	4.31	25.75
Tumour Grade 1	23.45	27.36	54.79	62.09	128.3	170.37	2.7	4.52	21.56	37.87	9.4	8.48	30.76
2	39.44	45.34	92.79	104.85	214.91	275.09	4.08	5.68	17.18	30.99	7.12	6.75	28.51
3	33.84	39.4	82.32	93.67	184.05	235.17	3.5	4.86	18.33	32.67	7.62	7.12	28.84
Residual Disease <2cm	22.61	26.7	53.44	60.86	175.22	131.39	2.72	4.32	22.66	39.27	10.01	8.92	31.89
>2cm	40.88	46.61	96.42	108.9	216.75	274.26	4.08	5.56	16.21	29.6	6.49	6.32	27.48

9.3.1 Reproducibility

9.3.1.2 Reproducibility CD31 and CD34

Ten cases were selected and stained for CD31 and CD34. Manual counts were performed and assessed for reproducibility of technique at 400X magnification. Both CD31 and CD34 showed good reproducibility for MVD determination (r=0.9957 and r=0.9996 respectively). High Correlation (r= 0.993) was also achieved between CD31 and CD34 counts (see table 9b: CD31 & CD34 Correlations). CD31 was found to be more difficult to count due to stain intensity and more prominent background staining. Further cases were therefore only stained with CD34 to enable use in both manual counting and image analysis techniques.

Table 9b: CD31 and CD34 Correlation

Patient number	CD34 Round 1	CD34 Round 2	CD31 Round 1	CD31 Round 2
5	18.75	19	17.25	16.75
6	34.5	35.75	30.25	31
13	40.75	40.25	48.25	48
15	40.75	41.5	30.5	31
17	18.5	18.25	18.75	18.25
18	40.5	40.75	25.5	25.75
19	67.5	66.75	65.5	66.25
21	15.25	16	14.75	14.75
22	30.5	30.5	28.75	28.75
28	10	10	10.75	10.75
Single Observer Reproducibility	r= 0.9996		r= 0.9957	
Correlation CD34 + CD31	r=0.993			

9.3.1.3 Reproducibility CD34 Manual Count

Ten cases were randomly selected as stained with CD34. Manual counting was performed at 200X and 400X and intra and interobserver reproducibility calculated for

both mean micro vessel density and highest count. All parameters were highly reproducible. (See table 9c: Intra- and Inter-observer Reproducibility CD34).

Table 9c: Intra- and Inter-observer Reproducibility CD34

	400X Mean MVD Count		400X Max MVD Count		200X Mean MVD Count		200X Max MVD Count	
Observer	1	2	1	2	1	2	1	2
Correlation	Intra	Inter	Intra	Inter	Intra	Inter	Intra	Inter
r	0.8097	0.868	0.8303	0.8595	0.9758	0.9771	0.989	0.978
r^2	0.6556	0.7534	0.6893	0.7388	0.9521	0.9457	0.978	0.9566
p	0.045	0.0011	0.029	0.0014	<0.0001	<0.0001	<0.0001	<0.0001
Spearman	0.679	0.679	0.8511	0.772	0.8024	0.8424	0.9362	0.8632
2 Tailed p	0.0306	0.0029	0.0126	0.0072	0.0037	0.0037	<0.0001	<0.0001

Table 9c illustrates reproducibility of manual MVD counts. All manual counts, irrespective of magnification (X200 or X400) were shown to be highly reproducible at intra- and interobserver level

9.3.1.4. Reproducibility: Image Analysis Microvessel Counts

Ten randomly selected cases stained with CD34 were counted and intra and interobserver reproducibility calculated for both mean and max image analysis micro vessel counts and mean and maximum endothelial areas. Intra and inter observer reproducibility for mean and max microvessel counts and mean endothelial area were highly reproducible $p<0.0001$. Intra observer ($p<0.0001$) and inter-observer ($p=0.0014$) counts for max endothelial area were also highly reproducible. (See table 9d Reproducibility Image Analysis Microvessel Counts).

Table 9d: Reproducibility Image Analysis Microvessel Counts

	Mean MVD Count image analysis		Max MVD Count image analysis		Mean Endothelial Area		Max Endothelial Area	
Observer	1	2	1	2	1	2	1	2
Correlations	Intra	Inter	Intra	Inter	Intra	Inter	Intra	Inter
r	0.9984	0.9908	0.9969	0.9948	0.9945	0.9231	0.9802	0.8598
r^2	0.9967	0.9817	0.9939	0.9895	0.989	0.8552	0.9608	0.7393
p	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0001	<0.0001	0.0014
Spearman	0.9879	0.9152	0.9879	0.8788	0.9758	0.8909	0.9636	0.903
2 Tailed p	<0.0001	0.0005	<0.0001	0.0016	<0.0001	0.0011	<0.0001	0.0008

Table 9d illustrates reproducibility of image analysis microvessel counts, and endothelial area estimates. All counts were shown to be highly reproducible at intra- and interobserver level.

9.3.1.5 Reproducibility: Spanning Tree Analysis Angiogenesis

Five randomly selected cases, stained with CD34, were measured for spanning tree and nearest neighbour parameters and intra and inter observer reproducibility calculated for mean MST, max MST, min MST, sdMST and 1, 2 and 3 nearest neighbours. Max MST, min MST, sdMST and 1 nearest neighbour showed to be highly reproducible (see table 9e: Angiogenesis MST - Reproducibility).

Table 9e: Angiogenesis SSA Reproducibility

	Mean MST		Max MST		Min MST		sd MST		1 nearest neighbour		2 nearest neighbours		3 nearest neighbours	
Observer	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Correlation	Intra	Inter	Intra	Inter	Intra	Inter	Intra	Inter	Intra	Inter	Intra	Inter	Intra	Inter
r	0.9819	0.9774	0.9995	0.9973	0.9452	0.8939	0.9958	0.9975	0.9694	0.9656	0.6439	0.4087	0.6057	0.7583
r^2	0.9642	0.9553	0.999	0.9945	0.8931	0.799	0.9917	0.9951	0.9397	0.9325	0.4146	0.167	0.3669	0.575
p	0.0029	0.0041	<0.0001	0.0002	0.0153	0.0408	0.0003	0.0001	0.0064	0.0076	0.241	0.4945	0.279	0.1374
Spearman	0	1	1	1	0	0.9	0	1	0	0.9	0.4	0.2	0.4	0.6
2Tailed p	0.0167	0.0167	0.0167	0.0167	0.0167	0.0833	0.0167	0.0167	0.0167	0.0833	0.5176	0.7833	0.45	0.35

Table 9d illustrates reproducibility of syntactic structure analysis of microvessel architecture. Line features (mean, max, min, sdMST), were found to be highly reproducible at intra- and interobserver level, neighbourhood features were less impressive.

9.3.2 Correlations between Parameters

On linear regression analysis the manual angiogenesis parameters, of increasing mean and maximum counts at 200X and 400X, were found to strongly correlate with increasing automated microvessel counts, endothelial area estimates and angiogenesis spanning tree factors. Correlation with the extent of residual tumour was also highly significant ($p<0.0001$) as was correlations with FIGO stage and presence of ascites. Manual angiogenesis parameters were also found to correlate with pre-operative Ca125 levels. Neither age, nor tumour grade correlated with any of the angiogenesis parameters. For all angiogenesis correlations see table 9f and 9g.

9.3.3 Survival Analysis

Survival data was available for all 132 cases. P values below 0.05 were regarded as significant.

9.3.3.1 Significance of single parameters to predict survival / Univariate Analysis

Angiogenesis and clinicopathologic variables were analysed using the univariate Cox model. Of the clinicopathologic data, FIGO stage, tumour grade, pre-operative Ca125 levels, presence of ascites and extent of disease residuum were found to be significant predictors of OS and DFS. All angiogenesis parameters were found to be highly significant predictors for OS and all except number of capillaries with 3 nearest neighbours were found to be highly significant predictors for DFS.

9.3.3.2 Multivariate Analysis: Manual Counts and Survival

To estimate the simultaneous influence of prognostic factors all clinicopathologic and manual count angiogenesis variables were entered into Cox regression analysis. This analysis showed mean count at X400 magnification $p=<0.01$) and mean count at X200

Table 9f: Correlations Between Angiogenesis Measurement Parameters

Parameter	X200 Mean MVD	X200 Max MVD	X400 Mean MVD	X400 Max MVD	Mean image analysis MVD Count	Max image analysis MVD Count	Mean Endothelial Area	Max Endothelial Area	Mean MST	sdMST	Max MST	Min MST	1NN	2NN	3NN
	r														
X200 Mean MVD Count		0.9932	0.9434	0.9329	0.9237	0.8804	0.7235	0.5838	-0.724	-0.713	-0.756	-0.665	-0.576	0.3177	0.3646
X200 Max MVD Count	0.9932		0.9398	0.9392	0.9176	0.8788	0.7247	0.5933	-0.736	-0.723	-0.767	-0.673	-0.58	0.3185	0.3728
X400 Mean MVD Count	0.9434	0.9398		0.9883	0.9059	0.8707	0.7050	0.5873	-0.696	-0.674	-0.722	-0.633	-0.52	0.28	0.354
X400 Max MVD Count	0.9329	0.9392	0.9883		0.8949	0.8623	0.6763	0.5681	-0.708	-0.681	-0.731	-0.643	-0.53	0.2817	0.3485
Mean MVD Count image analysis	0.9237	0.9176	0.9059	0.8949		0.9796	0.7799	0.6338	-0.727	-0.707	-0.747	-0.666	-0.572	0.29	0.377
Max MVD Count image analysis	0.8804	0.8788	0.8707	0.8623	0.9796		0.789	0.681	-0.714	-0.677	-0.724	-0.656	-0.579	0.336	0.316
Mean Endothelial Area	0.7235	0.7247	0.7050	0.6763	0.7799	0.789		0.9266	-0.649	-0.590	-0.646	-0.598	-0.536	0.345	0.248
Max Endothelial Area	0.5838	0.5933	0.5873	0.5681	0.6338	0.681	0.9266		-0.584	-0.5301	-0.588	-0.535	-0.482	0.3255	0.2042
Mean MST	-0.724	-0.736	-0.696	-0.708	-0.727	-0.714	-0.649	-0.584		0.9239	0.9659	0.9513	0.8437	-0.515	-0.475
sdMST	-0.713	-0.723	-0.674	-0.681	-0.707	-0.677	-0.590	-0.5301	0.9239		0.9724	0.8048	0.778	-0.394	-0.564
Max MST	-0.756	-0.767	-0.722	-0.731	-0.747	-0.724	-0.646	-0.588	0.9659	0.9724		0.8781	0.785	-0.445	-0.496
Min MST	-0.665	-0.673	-0.633	-0.643	-0.666	-0.656	-0.598	-0.535	0.9513	0.8048	0.8781		0.8311	-0.521	-0.441
1 Nearest Neighbour	-0.576	-0.58	-0.524	-0.53	-0.572	-0.579	-0.536	-0.482	0.8437	0.778	0.785	0.8311		-0.824	-0.212
2 Nearest Neighbours	0.3177	0.3185	0.28	0.2817	0.29	0.336	0.345	0.3255	-0.515	-0.394	-0.445	-0.521	-0.824		-0.364
3 Nearest Neighbours	0.3646	0.3728	0.354	0.3485	0.377	0.316	0.248	0.2042	-0.475	-0.564	-0.496	-0.441	-0.212	-0.364	

Table 9f illustrates correlations between varying angiogenesis measurement techniques. mean and maximum counts at 200X and 400X, were found to strongly correlate with increasing automated microvessel counts, endothelial area estimates, and angiogenesis spanning tree factors.

Table 9g: Correlations: Clinicopathologic and Angiogenesis Variables

	Age			Grade			Stage			Residual Disease			Ca125 Pre op			Ascites		
Parameter	**r**	**r^2**	**p**	**r**	**r^2**	**p**	**r**	**r^2**	**p**	**r**	**r^2**	**p**	**r**	**r^2**	**p**	**r**	**r^2**	**p**
X200 Mean MVD Count	0.102	0.104	0.25	0.144	0.021	0.1	0.42	0.18	<0.0001	0.42	0.18	<0.0001	0.29	0.088	0.0045	0.29	0.088	0.0007
X20 Max MVD Count	0.104	0.011	0.24	0.15	0.023	0.089	0.43	0.19	<0.0001	0.43	0.18	<0.0001	0.29	0.085	0.0052	0.29	0.089	0.0006
X40 Mean MVD Count	0.101	0.01	0.26	0.099	0.0097	0.27	0.38	0.14	<0.0001	0.37	0.14	<0.0001	0.26	0.069	0.0127	0.26	0.067	0.0031
X40 Max MVD Count	0.108	0.012	0.23	0.11	0.013	0.21	0.39	0.15	<0.0001	0.37	0.13	<0.0001	0.25	0.064	0.0161	0.27	0.07	0.0022
Mean Endothelial Area	0.114	0.013	0.22	0.084	0.007	0.73	0.34	0.14	0.0002	0.28	0.08	0.0020	0.093	0.009	0.4156	0.22	0.05	0.0161
Max Endothelial Area	0.108	0.012	0.25	0.023	0.0005	0.80	0.32	0.1	0.0005	0.18	0.034	0.0488	0.079	0.0062	0.4905	0.18	0.032	0.0560
Mean MVD Count image analysis	0.083	0.0069	0.39	0.11	0.013	0.24	0.35	0.12	0.0003	0.37	0.134	0.0001	0.16	0.027	0.1492	0.21	0.046	0.0274
Max MVD Count image analysis	0.087	0.0075	0.38	0.103	0.011	0.29	0.34	0.12	0.0003	0.36	0.13	0.0002	0.17	0.027	0.1445	0.20	0.04	0.0398
Mean MST	-0.14	0.019	0.13	-0.12	0.014	0.19	-0.44	0.19	<0.0001	-0.45	0.20	<0.0001	-0.17	0.029	0.1102	-0.38	0.143	<0.0001
sdMST	-0.07	0.004	0.469	-0.12	0.015	0.17	-0.44	0.19	<0.0001	-0.43	0.18	<0.0001	-0.19	0.036	0.0745	-0.36	0.13	<0.0001
Min MST	-0.13	0.019	0.12	-0.13	0.016	0.16	-0.47	0.22	<0.0001	-0.44	0.198	<0.0001	-0.15	0.023	0.1598	-0.37	0.14	<0.0001
Max MST	-0.12	0.0140	0.19	-0.13	0.016	0.17	-0.45	0.20	<0.0001	-0.43	0.19	<0.0001	-0.19	0.036	0.0725	-0.4	0.16	<0.0001
1 Nearest Neighbour	-0.097	0.0093	0.29	-0.15	0.021	0.1	-0.48	0.23	<0.0001	-0.52	0.27	<0.0001	-0.20	0.04	0.0611	-0.33	0.1	0.0002
2 Nearest Neighbours	0.163	0.027	0.07	0.096	0.009	0.29	0.3	0.09	0.0007	0.39	0.15	<0.0001	0.139	0.019	0.1946	0.22	0.05	0.0156
3 Nearest Neighbours	-0.13	0.016	0.158	0.05	0.003	0.57	0.21	0.043	0.0215	0.15	0.02	0.1057	0.049	0.0024	0.6526	0.12	0.015	0.1697

Table 9g illustrates correlations between varying angiogenesis measurement techniques and clinicopathologic parameters. Correlation with residual disease status was highly significant as were correlations with FIGO stage and ascites status. Neither age, nor tumour grade correlated with any angiogenesis parameters.

magnification (p=<0.01), and FIGO stage (p=0.002) to be significant predictors for DFS. The other factors were not of additional prognostic relevance.

9.3.3.3 Multivariate Analysis: Angiogenesis Image Analysis and Survival

To estimate the simultaneous influence of prognostic factors all clinicopathologic and angiogenesis image analysis variables were entered into Cox regression analysis. This analysis showed mean endothelial area (p=<0.01) and extent of disease residuum (p=0.001) to be significant predictors for OS and mean endothelial area (p=<0.01) and stage (p=0.001) to be significant predictors for DFS. The other factors were not of additional prognostic relevance.

9.3.3.4 Multivariate Analysis: Angiogenesis Spanning Tree Analysis and Survival

To estimate the simultaneous influence of prognostic factors all clinicopathologic and angiogenesis spanning tree variables were entered into Cox regression analysis. This analysis showed max MST (p=<0.01) and number of capillaries with 2 Nearest Neighbours (p=0. <0.01) to be significant predictors for OS and max MST (p=<0.01), number of capillaries with 3 Nearest Neighbours (p=0.032) and FIGO stage (p=<0.01) to be significant predictors of DFS. The other factors were not of additional prognostic relevance.

9.3.3.5 Multivariate Analysis: All Angiogenesis Variables and Survival

To estimate the simultaneous influence of prognostic factors all clinicopathologic and all angiogenesis variables were entered into Cox regression analysis This analysis showed Max MST (p=0.009), Length MST (p=0.005), 1 Nearest Neighbour (p=<0.01) and X400 Mean count (o=0.0001) to be significant predictors for OS and mean endothelial area (p=<0.01) and FIGO stage (p=0.001) to be significant predictors for DFS. The other factors were not of additional prognostic relevance. Table 9h illustrates positive results for univariate and multivariate analysis.

Table 9h: Positive Findings – Univariate and Multivariate Analysis
Clinicopathologic & Angiogenesis Parameters

	df	Sig.	Exp (B)	95% CI Lower	95% CI Upper	Parameter	Sig.	Exp (B)	95% Lower CI	95% Upper CI
Univariate Analysis										
Overall Survival						**Disease Free Survival**				
Clinicopathologic Variables						**Clinicopathologic Variables**				
Grade	2	**.008**				**Grade**	**.004**			
Grade (1)	1	**.004**	.344	.168	.705	**Grade (1)**	**.001**	.288	.138	.603
	1					**Grade (2)**	**.384**	.798	.479	1.327
Stage	3	**<. 01**				**Stage**	**<. 01**			
Stage (1)	1	**<. 01**	.151	.056	.403	**Stage (1)**	**.037**	.228	.057	.913
Stage (2)	1	**.003**	.256	.103	.639	**Stage (2)**	**.417**	.592	.167	2.101
	1					**Stage (3)**	**.555**	1.421	.442	4.568
Residual Disease	1	**<. 01**	.341	.204	.568	**Residual Disease**	**<. 01**	.341	.204	.568
Presence of Ascites	1	**<. 01**	.377	.237	.599	**Presence of Ascites**	**<.01**	.358	.212	.605
Pre-operative Ca125	1	**.016**	1.000	1.000	1.000	**Pre-operative Ca125**	**.001**	1.000	1.000	1.000
Angiogenesis Variables						**Angiogenesis Variables**				
X200 mean MVD Count	1	**<.01**	1.021	1.017	1.025	**X200 mean MVD**	**<.01**	1.013	1.008	1.018
X200 max MVD Count	1	**<.01**	1.018	1.015	1.022	**X200 max MVD**	**<.01**	1.011	1.007	1.015
X400 mean MVD Count	1	**<.01**	1.041	1.033	1.049	**X400 mean MVD**	**<.01**	1.031	1.020	1.042
X400 max MVD Count	1	**<.01**	1.036	1.029	1.044	**X400 max MVD**	**<.01**	1.023	1.014	1.033
Mean Endothelial Area	1	**<.01**	1.451	1.329	1.584	**Mean Endo Area**	**<.01**	1.635	1.334	2.004
Max Endothelial Area	1	**<.01**	1.234	1.165	1.307	**Max Endo Area**	**<.01**	1.226	1.119	1.343
Mean MVD count image analysis	1	**<.01**	1.008	1.006	1.009	**Mean image analysis count**	**<.01**	1.005	1.002	1.008
Max image analysis count	1	**<.01**	1.006	1.005	1.008	**Max image analysis count**	**<.01**	1.226	1.119	1.343
Mean MST	1	**<.01**	.790	.750	.832	**Mean MST**	**<.01**	.901	.857	.947
sdMST	1	**<.01**	.636	.570	.710	**sdMST**	**<.01**	.805	.715	.907
Max MST	1	**<.01**	.866	.840	.892	**Max MST**	**<.01**	.929	.900	.959
Min MST	1	**<.01**	.719	.660	.782	**Min MST**	**<.01**	.867	.800	.939
1 nearest neighbour	1	**<.01**	.756	.698	.819	**1 nearest neighbour**	**.001**	.886	.825	.951
2 nearest neighbours	1	**<.01**	1.137	1.079	1.197	**2 nearest neighbours**	**.019**	1.074	1.012	1.141
3 nearest neighbours	1	**.037**	1.089	1.005	1.181					
Multivariate Analysis										
Manual Angiogenesis & Clinicopathologic Variables										
Residual Disease	1	**.002**	.379	.203	.707	**X200 mean MVD Count**	**<.01**	1.010	1.005	1.016
X400 Mean MVD Count	1	**<.01**	1.038	1.027	1.050	**Stage**	**.002**			
						Stage(1)	**.016**	.129	.024	.687
Image Analysis Angiogenesis & Clinicopathologic Variables										
Residual Disease	1	**.001**	.347	.185	.653	**X200 mean MVD Count**	**<.01**	1.010	1.005	1.016
Mean Endothelial Area	1	**<.01**	1.434	1.270	1.619	**Stage**	**.002**			
						Stage(1)	**.016**	.129	.024	.687
Spanning Tree Angiogenesis & Clinicopathologic Variables										
Max MST	1	**<.01**	.873	.839	.908	**Stage**	**<.01**			
2 nearest neighbours	1	**<.01**	1.144	1.064	1.230	**Max MST**	**<.01**	.924	.887	.963
						3 nearest neighbours	**.032**	.875	.775	.988
All Angiogenesis & Clinicopathologic Variables										
Max MST	1	**.009**	.934	.887	.983	**Stage**	**.001**			
Length MST	1	**.005**	.995	.991	.998	**Stage(1)**	**.092**	.231	.042	1.272
1 nearest neighbour	1	**<.01**	.770	.675	.877	**Mean Endothelial Area**	**<.01**	1.627	1.277	2.073
X400 Mean MVD Count	1	**.001**	1.028	1.012	1.044					

9.3.4 Prediction of Chemotherapy Response - Results

Of the clinicopathologic data tumour grade (p=0.02), FIGO stage (p=0.004), extent of residual disease (p=<0.01) and presence of ascites (p=0.004) were found significant predictors of chemotherapy response with overall correct classification to chemotherapy response group being 70.3%, 68.8%, 68.8% and 67.2% respectively. For the angiogenesis data all measurement parameters except percent connectivity to 3 nearest neighbours were significant. See table 9i for univariate analysis of angiogenesis parameters and chemotherapy response.

Multivariate logistic regression analysis of clinicopathologic and manual angiogenesis parameters revealed max count at 200X magnification (p=0.004) and the presence of ascites (p=0.046) to be of independent prognostic significance with an overall 76.4% correct classification rate to chemotherapy response group. Analysis of clinicopathologic and image analysis angiogenesis parameters revealed max image analysis vessel count (p=0.018) and presence of ascites p=0.021) to retain significance with an overall 80.9% correct classification rate to chemotherapy response group. Analysis of clinicopathologic and MST angiogenesis parameters revealed sdMST (p=<0.002) and tumour grade (p=<0.041) to retain significance with an overall correct classification to chemotherapy response group of 88.5% respectively.

Multivariate logistic regression analysis of all clinicopathologic and angiogenesis parameters revealed tumour grade (p=<0.057) and sdMST (p=<0.003) of independent prognostic significance with an overall 82.2% correct classification to chemotherapy response group. (See table 9j for multivariate analysis of clinicopathologic & all angiogenesis parameters and prediction of chemotherapy response).

Table 9i: Angiogenesis Parameters and Prediction of Chemotherapy Response: Univariate Analysis

Parameter	df	Sig. (p)	Correct Classification	95% CI Lower	95% CI Upper
Univariate Analysis					
Clinicopathologic Variables					
Grade	2	.002	68.8%		
Grade(1)				2.412	67.918
Grade (2)				.728	7.917
Stage	3	.004	68.8%		
Stage(1)				1.028	560.178
Stage(2)				.385	78.573
Stage (3)				.121	17.913
Residual Disease	1	<.01	70.3%	2.420	29.383
Presence of Ascites	1	.004	67.2%	1.566	13.822
Pre-operative Ca125	1	.093			
Age	1	.554			
Angiogenesis Variables					
X200 Mean MVD Count	1	<.01	71.0%	.926	.982
X200 Max MVD Count	1	<.01	71.0%	.932	.984
X400 Mean MVD Count	1	<.01	74.2%	.819	.954
X400 Max MVD Count	1	<.01	71.0%	.847	.960
Mean MVD Count Image Analysis	1	.027	63.0%	.402	1.006
Max MVD Count Image Analysis	1	<.01	68.5%	.978	.997
Mean Endothelial Area	1	.027	63.0%	.402	1.006
Max Endothelial Area	1	.033	63.0%	.599	1.031
Mean MST	1	<.01	75.8%	1.094	1.436
Sd MST	1	<.01	79.0%	1.230	2.347
Max MST	1	<.01	79.0%	1.057	1.270
Min MST	1	<.01	71.7%	1.145	1.781
1 nearest neighbour	1	<.01	74.6%	1.156	1.775
2 nearest neighbours	1	.013	64.4%	.734	.976

Table 9i illustrates univariate logistic regression analysis of angiogenesis & clinicopathologic parameters and prediction of chemotherapy response.
For the angiogenesis data all measurement parameters, except percent connectivity to 3 nearest neighbours, were found to be significant.
p values <0.05 were regarded as significant.

Table 9j: Multivariate Analysis - clinicopathologic & all angiogenesis parameters and prediction of chemotherapy response

Parameter	B	S.E.	Wald	df	Sig.	Exp(B)	95.0% C.I .for EXP(B)		Correct Classification
							Lower	Upper	
	Clinicopathologic &Manual Angiogenesis Parameters								
X200 Max MVD Count	-.046	.016	8.414	1	**.004**	.955	.926	.985	Group 1 = 52.6 % Group 2 = 88.9 % Overall = 76.4 %
Ascites	1.434	.717	3.996	1	**.046**	4.195	1.028	17.112	
Constant	3.252	1.176	7.655	1	**.006**	25.853			
	Clinicopathologic & Image Analysis Angiogenesis Parameters								
Max MVD Count Image analysis	-.013	.005	5.558	1	**.018**	.987	.977	.998	Group 1 = 55.6 % Group 2 = 96.6 % Overall = 80.9 %
Ascites	1.757	.760	5.344	1	**.021**	5.798	1.307	25.728	
Constant	2.052	.990	4.293	1	**.038**	7.784			
	Clinicopathologic & MST Angiogenesis Parameters								
Sd MST	.869	.277	9.837	1	**.002**	2.385	1.385	4.105	Group 1 = 70.6 % Group 2 = 97.1 % Overall = 88.5 %
Grade			6.413	2	**.041**				
Grade(1)	2.967	1.172	6.411	1	**.011**	19.439	1.955	193.286	
Grade(2)	1.229	.955	1.657	1	.198	3.418	.526	22.215	
Constant	-7.53	2.504	9.060	1	**.003**	.001			
	Clinicopathologic & All Angiogenesis Parameters								
Grade			5.713	2	**.057**				Group 1 = 70.6% Group 2 = 89.3 % Overall = 82.2 %
Grade(1)	2.794	1.172	5.686	1	**.017**	16.349	1.645	162.545	
Grade(2)	1.081	1.028	1.105	1	.293	2.948	.393	22.120	
sdMST	0.917	.310	8.749	1	**.003**	2.501	1.362	4.590	
Constant	6.640	2.276	8.512	1	**.004**	764.894			

Table 9j illustrates multivariate logistic regression analysis of angiogenesis & clinicopathologic parameters and prediction of chemotherapy response.
Angiogenesis parameters retained significance at multivariate level, and, for all combinations, angiogenesis parameters remained the strongest predictiors of chemotherapy response.
(Group 1 = no / partial response: Group 2 = Complete Remission)

9.4 Discussion

Statistically significant differences in MVD counts have previously been reported between benign, borderline and malignant tumours of the ovary, [202] with analysis of MVD perhaps being helpful in the distinction of these tumours. However, in ovarian carcinoma, prior studies of angiogenesis in relation to clinicopathologic criteria have largely reported contradictory results. Increased MVD has been found by some to correlate with increasing age, [228-30] FIGO stage, [204, 207, 229,230-31] and worsening tumour grade. [204,230-31] Other

studies have, however, shown no correlation to age, [205,210-11,217,232] stage, [205,209-11,217,232] or grade. [207,209-11,232] With regard to histological type, increased MVD counts have been reported more frequently in mucinous as compared to serous and endometrioid tumours, [211,229,232] serous as compared to other types, [204,231] and some have reported no correlation. [205,207,209,232] When considering residual disease, increased MVD counts have also shown contradictory findings. Theoretically, less vascular tumours should have a lesser tendency to metastasise and therefore be easier to surgically resect. Increased MVD has been correlated with both high [205,229] and low amounts of residual tumour. [205,217,232]

Turning to survival analysis, increased MVD counts have been associated with a worse prognosis for survival and disease-free survival in both univariate [209,211,229,231,233] and multivariate analysis. [204,207,209-10,221,228,234] By image analysis techniques, increased mean vessel area / mean endothelial area per mm^3 has also shown worsening prognosis for survival. [220-221] Micro vessel density has shown to correlate well to metastasis with significant correlation of MVD to size of metastasis, [205] and, micro vessel counts in metastases significantly predictive of survival. [217] Numerous other studies have however reported no correlation of MVD counts with survival. [205,216-7,232,235] Heterogeneity of ovarian tumours may decrease the prognostic value of angiogenesis in epithelial ovarian cancer [232] and differences in measurement techniques may have affected results. The lack of standardisation of methodology, patient selection and variation in treatment and outcome data make these studies very difficult to compare.

The area of drug sensitivity and angiogenesis is a particularly interesting one. Anti cancer drugs gain access to solid tumours via the blood supply and must penetrate through the extra vascular space to reach cancer cells in sufficient concentration to cause lethal toxicity. [236] Essentially while increased IMVD may promote tumour growth and spread, it might also be expected to facilitate the delivery of cytotoxic chemotherapy to the tumour

site. [230] Neovascularisation however has been suggested counter-intuitively to reduce tumour accessibility to chemotherapeutic drugs as compared to normal tissues. Tumours may not outgrow their blood supply, but instead compress it, i.e. vascular compression causing eventual central necrosis. [237] Blood flow in tumours is often irregular and the intercapillary distance in neovascularised areas may be relatively large compared to normal tissue. Penetration of some chemotherapeutic drugs, through such tissue, may be slow and slow tissue penetration may contribute to the development of clinical resistance. [234]

It is perhaps not surprising, in view of this balance of potential effects, that studies on IMVD and chemotherapeutic response have shown conflicting results. Studies have found increased IMVD to be a significant predictor of improved chemotherapeutic response and complete response. [211,228] Conversely increased IMVD has also been reported as a predictor for unfavourable chemotherapeutic response. [234]

It may be that intrinsic cell properties are more important with regard to survival in ovarian cancer [235] and the confounded literature reflects the fact that angiogenesis may not prove a practically useful technique in outcome prediction in ovarian cancer. This study however, in a well-selected patient group with robust follow-up and outcome data has shown angiogenesis variables to be highly reproducible with strong correlations between techniques used.

Increasing angiogenesis parameters were all found to strongly correlate with increased extent of residual disease, worsening FIGO stage and presence of ascites, and on univariate analysis all angiogenesis parameters strongly correlated with OS with increased counts reflecting worsening survival. Angiogenesis parameters also strongly correlated with DFS where increased counts again reflected shorter times to relapse. For each set of angiogenesis variables, whether determined manually, by semi-automated technique or by spanning tree methods, multivariate analysis showed them to be by far the strongest

prognosticators for OS and stronger than FIGO stage for relapse prediction.

This study has also found angiogenesis parameters, irrespective of measurement techniques applied, to be strong predictive factors for chemotherapy response on univariate analysis. When combined with clinicopathologic data, multivariate analysis has also shown angiogenesis parameters to retain independent significance, to supersede the predictive ability of such traditional pathologic markers as tumour grade, stage and extent of disease residuum, and to identify those patients likely to achieve complete remission, with a high degree of accuracy.

This study has used a well-selected group of single histological sub-type, platinum-treated serous ovarian tumours with varied angiogenesis measurement techniques applied to the same tumour sections. Irrespective of measurement technique applied, angiogenesis parameters were found to be strong predictive factors for chemotherapeutic outcome in this group of tumours. This would certainly seem to be an area worthy of further study.

We therefore feel that such measurements should be further investigated in carefully chosen prospective series of ovarian carcinomas of single histological type, preferably in a clinical trial setting where prospective clinical data collection is likely to be more precise. We would suggest that such selection problems and inconsistencies in clinical data might well have confounded previous studies.

Chapter 10. Final Analysis

10.1 Reproducibility of Parameters

Intraobserver analysis revealed semi-automated parameters to be highly reproducible. Interobserver reproducibility was also impressive on the whole, yet analysis of nuclear spanning tree parameters showed to be less reliable. Table 10a illustrates overall reproducibility of all measurement parameters.

Table 10a: Reproducibility of Parameters

Parameter	Intra-observer Reproducibility			Inter-observer Reproducibility		
	r	r^2	p	r	r^2	p
p53						
P53 percent expression	0.9983	0.9966	<0.0001	0.9985	0.9772	<0.0001
Mitotic Activity Index						
Mitotic Activity Index	0.996	0.9914	<0.0001	0.9910	0.9821	<0.0001
Morphometry Parameters						
VPE	0.992	0.9844	<0.0001	0.9866	0.9734	<0.0001
MNA	0.995	0.9890	<0.0001	0.9648	0.9308	<0.0001
SdNA	0.993	0.9867	<0.0001	0.7707	0.5939	0.0091
Nuclear Length	0.994	0.9872	<0.0001	0.9606	0.9227	<0.0001
Nuclear Breadth	0.994	0.9888	<0.0001	0.9504	0.9033	<0.0001
Orthoferet	0.993	0.9865	<0.0001	0.9501	0.9028	<0.0001
Nuclear Perimeter	0.994	0.9881	<0.0001	0.9649	0.9311	<0.0001
Nuclear Roundness	0.895	0.8003	0.0005	0.2223	0.04941	0.5371
Equivalent Diameter	0.995	0.9896	<0.0001	0.9620	0.9254	<0.0001
Fullness Ratio	0.898	0.8069	0.0004	0.2629	0.06913	0.4630
Spanning Tree Parameters						
Mean MST	0.9832	0.9668	<0.0001	0.9384	0.8806	0.0182
sdMST	0.9856	0.9714	<0.0001	0.4517	0.204	0.309
MaxMST	0.9905	0.981	<0.0001	0.8524	0.7266	0.0148
Min MST	0.7855	0.6218	0.0201	0.4936	0.2437	0.2603
1 Nearest Neighbour	0.6405	0.4102	0.0871	0.6631	0.4397	0.1044
2 Nearest Neighbours	0.6158	0.3792	0.1042	0.2803	0.07855	0.5427
3 Nearest Neighbours	0.5039	0.2539	0.2030	0.1973	0.03894	0.6715
Manual Angiogenesis Parameters						
200X Mean MVD	0.9758	0.9521	<0.0001	0.9771	0.9457	<0.0001
200X max MVD	0.989	0.978	<0.0001	0.978	0.9566	<0.0001
400X Mean MVD	0.8097	0.6556	0.045	0.868	0.7534	0.0011
400X Max MVD	0.8303	0.6893	0.029	0.8595	0.7388	0.0014
Angiogenesis Image Analysis Parameters						
Mean MVD Count	0.9984	0.9967	<0.0001	0.9908	0.9817	<0.0001
Max MVD Count	0.9969	0.9939	<0.0001	0.9948	0.9895	<0.0001
Mean Endothelial Area	0.9945	0.989	<0.0001	0.9231	0.8552	0.0001
Max Endothelial area	0.9802	0.9608	<0.0001	0.8598	0.7393	0.0014
Angiogenesis Spanning Tree Parameters						
Mean MST	0.9819	0.9642	0.0029	0.9774	0.9553	0.0041
sdMST	9958	9917	0.0003	9975	9991	0.0001
MaxMST	0.9995	0.999	<0.0001	0.9973	0.9945	0.0002
Min MST	0.9432	0.8931	0.0153	0.8939	0.799	0.0408
1 Nearest Neighbour	0.9694	0.9397	0.0064	0.9656	0.9325	0.0076
2 Nearest Neighbours	0.6439	0.4146	0.241	0.4087	0.167	0.4945
3 Nearest Neighbours	0.6057	0.3669	0.279	0.7583	0.575	0.1374

10.2 Correlations between parameters

Percent expression of p53 and p53 staining grade were found to strongly correlate with MAI, and numerous nuclear morphometric parameters, yet not with angiogenesis parameters. MAI was also found to strongly correlate with numerous nuclear morphometric and angiogenesis parameters. Correlations between measurement parameters are illustrated in appendix 5.

10.3 Survival Analysis

Multivariate Cox logistic regression analysis was performed on all parameters showing positive associations on univariate analysis for OS and DFS (see table 10b for univariate OS analysis results).

10.3.1 Multivariate Overall Survival Analysis

Multivariate analysis revealed MAI (p=<0.01), and the angiogenesis parameters of X400 mean MVD (p=0.029), mean endothelial area (p=0.035), and number of microvessels with one nearest neighbour (p=<0.01) to retain independent prognostic ability. The remaining parameters were insignificant. Table 10c illustrates multivariate results for OS analysis.

10.3.2 Multivariate Disease Free Survival Analysis

Multivariate analysis revealed FIGO stage (p=0.02), MAI (p=0.046) and mean endothelial area (p=<0.01) to retain independent prognostic ability. The remaining parameters were not significant. Table 10c Illustrates, multivariate results for DFS analysis.

Table 10b: Univariate Analysis: OS and DFS - All Parameters

Parameter	df	Sig. (p)	Exp (B)	95% CI Lower	95% CI Upper	Sig. (p)	Exp (B)	95% Lower CI	95% Upper CI
	Overall Survival					Disease Free Survival			
Grade	2	.008				.004			
Grade(1)	1	.004	.344	.168	.705	.001	.288	.138	.603
	1					.384	.798	.479	1.327
Stage	3	<.01				<.01			
Stage(1)	1	<.01	.151	.056	.403	.037	.228	.057	.913
Stage(2)	1	.003	.256	.103	.639	.417	.592	.167	2.101
	1					.555	1.421	.442	4.568
Residual Disease	1	<.01	.341	.204	.568	<.01	.341	.204	.568
Ascites	1	<.01	.377	.237	.599	<.01	.358	.212	.605
Pre-op Ca125	1	.016	1.000	1.000	1.000	.001	1.000	1.000	1.000
Age	1	0.065				0.722			
p53 Percent Expression	1	0.74				0.011	1.013	1.003	1.024
p53 Grade	1	0.28				0.040	1.046	1.011	1.082
p53 Positive	1	0.91				0.216			
bcl-2 Grade	1	0.88				0.257			
bcl-2 Positive	1	0.98				0.658			
p53/bcl-2 combined	1	0.94				0.607			
MAI		<.01				<.01			
VPE		<.01				0.013			
MNA	1	.071	2.799	0.916	8.550	.104	2.657	0.817	8.647
sdNA	1	.001	2145792807.524	5434.469	8E+014	.016	3752495240.3	63.515	2E+017
Nuclear Length	1	.001	78.318	5.662	1083.29	.004	93.287	4.266	2039.772
Nuclear Breadth	1	.001	266.784	9.342	7618.82	.003	380.06	7.511	19232.1
Nuclear Perimeter	1	.001	4.420	1.773	11.022	.004	4.928	1.680	14.457
sd perimeter	1	.038	16.662	1.174	236.571	.852	1.416	0.036	55.243
Nuclear Roundness	1	.926	.841	0.021	33.254	.316	.045	0.000	19.573
sd roundness	1	.673	.411	0.007	25.43	.164	.014	0.000	5.785
orthoferet	1	.003	355.719	7.423	3938.88	.003	355.72	7.423	17046.047
Fullness ratio	1	.627				.102	3128541574396426.0	0.001	1.2E+034
Equivalent diameter	1	<.01	22.403	4.791	104.768	.006	9.932	1.955	50.46
Length MST	1	0.29				0.407			
sdMean MST	1	0.48				0.151			
Mean MST	1	0.092				0.093			
Min MST	1	0.053				0.044	.704	.497	.996
Max MST	1	0.036	.925	.861	.995	0.057			
sd MST	1	0.125				0.136			
1 Nearest Neighbour	1	0.003	1.187	1.057	1.333	0.014	1.161	1.032	1.307
2 Nearest Neighbours	1	0.017	.928	.873	.987	0.009	.915	.854	.980
3 Nearest Neighbours	1	.189				0.130			
Angiogenesis Parameters									
X200 mean MVD	1	<.01	1.021	1.017	1.025	<.01	1.013	1.008	1.018
X200 max MVD	1	<.01	1.018	1.015	1.022	<.01	1.011	1.007	1.015
X400 mean MVD	1	<.01	1.041	1.033	1.049	<.01	1.031	1.020	1.042
X400 max MVD	1	<.01	1.036	1.029	1.044	<.01	1.023	1.014	1.033
Mean Endothelial Area	1	<.01	1.451	1.329	1.584	<.01	1.635	1.334	2.004
Max Endothelial Area	1	<.01	1.234	1.165	1.307	<.01	1.226	1.119	1.343
Mean MVD image analysis count	1	<.01	1.008	1.006	1.009	<.01	1.005	1.002	1.008
Max MVD image analysis count	1	<.01	1.006	1.005	1.008	<.01	1.226	1.119	1.343
Mean MST	1	<.01	.790	.750	.832	<.01	.901	.857	.947
sdmst_	1	<.01	.636	.570	.710	<.01	.805	.715	.907
Max MST	1	<.01	.866	.840	.892	<.01	.929	.900	.959
Min MST	1	<.01	.719	.660	.782	<.01	.867	.800	.939
1 Neighbour	1	<.01	.756	.698	.819	.001	.886	.825	.951
2 Neighbours	1	<.01	1.137	1.079	1.197	.019	1.074	1.012	1.141
3 Neighbours	1	.037	1.089	1.005	1.181				

Table 10c: Multivariate Analysis: OS and DFS - All Parameters

Parameter	df	Sig. (p)	Exp (B)	95% CI Lower	95% CI Upper	Parameter	Sig. (p)	Exp (B)	95% CI Lower	95% CI Upper
Multivariate Analysis										
Overall Survival						**Disease Free Survival**				
Mitotic Activity Index	1	<.01	1.037	1.017	1.058	Stage	.002			
X400 mean MVD	1	.029	1.018	1.002	1.033	Stage(1)	.148	.282	.051	1.567
Mean endothelial area	1	.035	1.210	1.014	1.444	Stage(2)	.148	.275	.048	1.582
1 nearest neighbour angiogenesis	1	<.01	.739	.652	.836	Stage(3)	.698	1.331	.314	5.642
						Mitotic Activity Index	.046	1.022	1.000	1.045
						Mean endothelial area	<.01	1.579	1.226	2.034

Table 10c illustrates Cox multivariate survival analysis results for all parameters. Overall the angiogenesis parameters, as determined by image analysis, were the strongest independent prognosticators for both OS and DFS. p values <0.05 were regarded as significant.

10.4 Response to Chemotherapy

Multivariate logistic regression analysis was performed on all parameters showing positive associations on univariate analysis for prediction of chemotherapy response. (See table 10d for overall univariate results).

10.4.1 Multivariate analysis: prediction of chemotherapy response

Multivariate logistic regression analysis revealed tumour grade, VPE (p=0.013), nuclear length (p=0.009) and number of microvessels with one nearest neighbour (p=0.005) to retain independent predictive significance with overall correct classification to chemotherapy response group of 84.6%. The remaining parameters were not significant.

(See table 10e for multivariate analysis of prediction of chemotherapy response).

Table 10d: Univariate Prediction of Chemotherapy Response: All Parameters

Parameter	df	p	95.0% C.I .for EXP(B)		Correct Classification
			Lower	Upper	
Grade	2	.010			68.8%
Grade(1)	1	.003	2.412	67.918	
Grade(2)	1	.151	.728	7.917	
FIGO Stage	3	.025			68.8%
Stage(1)	1	.048	1.028	560.178	
Stage(2)	1	.209	.385	78.573	
Stage(3)	1	.761	.121	17.913	
Residual Disease	1	.001	2.420	29.383	70.3%
Ascites	1	.006	1.566	13.822	67.2%
Age	1	.554			
Pre-op Ca125	1	.093			
p53 Grade	1	.217			
p53 Positive	1	.235			
p53 Percent Expression	1	.663			
bcl-2 Positive	1	.771			
bcl-2 Grade	1	.718			
Mitotic Activity Index	1	.352			
VPE	1	<.01	0.899	0.976	76.3%
MNA	1	.060			
sdNA	1	.017	.000	.001	64.1%
Nuclear Length	1	.005	.000	.076	64.1%
Nuclear Breadth	1	.005	.000	.044	65.6%
Nuclear Perimeter	1	.004	.002	.407	64.1%
sd nuclear perimeter	1	.290			
Nuclear Roundness	1	.523			
sd nuclear roundness	1	.305			
Orthoferet	1	.005	.000	.042	65.6%
Fullness Ratio	1	.414			
Equivalent diameter	1	.076			
Length MST nuclear	1	.508			
sd Mean MST nuclear	1	.663			
Mean MST nuclear	1	.900			
Min MST nuclear	1	.797			
Max MST nuclear	1	.751			
sd MST nuclear	1	.848			
1 Nearest Neighbour nuclear	1	.059			
2 Nearest Neighbours nuclear	1	.006	1.042	1.460	53.1%
X200 mean MVD	1	<.01	.926	.982	71.0%
X200 max MVD	1	<.01	.932	.984	71.0%
X400 mean MVD	1	<.01	.819	.954	74.2%
X400 max MVD	1	<.01	.847	.960	71.0%
Mean Endothelial Area	1	.027	.402	1.006	63.0%
Max Endothelial Area	1	.033	.599	1.031	63.0%
Mean MVD image analysis count	1	.027	.402	1.006	63.0%
Max MVD image analysis count	1	<.01	.978	.997	68.5%
Mean MST angiogenesis	1	<.01	1.094	1.436	75.8%
sd MST angiogenesis	1	<.01	1.230	2.347	79.0%
Max MST angiogenesis	1	<.01	1.057	1.270	79.0%
Min MST angiogenesis	1	<.01	1.145	1.781	71.7%
1 nearest neighbour angiogenesis	1	<.01	1.156	1.775	74.6%
2 nearest neighbours angiogenesis	1	.013	.734	.976	64.4%

Table 10d illustrates univariate logistic regression analysis results for all parameters and prediction of chemotherapy response . Significant parameters were used in multivariate analysis p values <0.05 were regarded as significant.

Table 10e: Multivariate Analysis: Prediction of Chemotherapy Response – All Parameters

Parameter	df	Sig. (p)	Correct Classification	95% CI Lower	95% CI Upper
Grade	2	.100	Group 1 = 82.6% Group 2 = 86.2% Overall = 84.6%		
Grade(1)	1	.043		1.119	1757.396
Grade(2)	1	.651		.081	4.796
VPE	1	.013		.673	.955
Nuclear length	1	.009		.000	.000
1 nearest neighbour angiogenesis	1	.005		1.314	4.679
Constant	1	.322			

Table 10e illustrates multivariate logistic regression analysis results for all parameters and prediction of chemotherapy response.
Overall tumour grade, VPE, nuclear length, and number of microvessels with one nearest neighbour (p=0.005) were found to retain independent predictive significance with overall correct classification to chemotherapy response group of 84.6%.
p values <0.05 were regarded as significant.
(Group 1 = no / partial response: Group 2 = Complete Remission)

10.5 Discussion

The application of quantitative morphometric assessments aims to reduce variability in quantifying features of cells and tissues, with an overall aim to reduce intra- and interobserver variability, and to provide a numerical, reproducible scale of quantitative features. [17] As previously discussed, studies on traditional, qualitative, clinicopathologic parameters, such as tumour grade, have shown considerable intra and interobserver variability, [87,83,84,85,86,98] and present methods of quantifying residual tumour are imprecise [26] with definitions of optimal cytoreductive surgery markedly varying. [41] This study, with the exception of nuclear spanning tree parameters, found semi-automated image analysis techniques to be not only highly reproducible, but also to surpass the variation found in grading techniques. The analyses were also relatively fast and inexpensive, and did not require a learned pathologist to perform them.

This study found strong correlations between percent expression of p53 (p=0.0032), p53 staining grade (p=0.0002) and MAI. The correlation of p53 parameters and MAI is not surprising. A major feature of cancer is the inappropriate division of cells.

P53 plays a pivotal role in inducing apoptosis and loss of p53 suppressor function renders cells susceptible to uncontrolled growth, with mutation or deletion of p53 believed to result in uncontrolled cell proliferation, and failure of apoptosis. Prior IHC based studies have found Ki67 negative tumours also negative for p53 staining, [104] and positive correlations found between p53 accumulation and MIB 1 LI (MIB 1 labelling Index), with MIB 1 being a monoclonal antibody directed against Ki67 antigen. [119] The combination of p53 (nuclear accumulation in >10% neoplastic cells) and MIB 1 counts (MIB1 LI >30%) has been found strongly predictive of the highest risk ratio of death and the most powerful prognostic indicator of reduced survival. [119]

This study did not to show any correlations between p53 expression and angiogenesis parameters. Prior studies of p53 gene mutation in ovarian carcinoma have found a significant trend for the MVD count to increase as the p53 status of the cancer progressed from wild type to missense to null. [231] Mutation of the Tp53 tumour suppressor gene is involved in the down-regulation of angiogenesis inhibitors such as thrombospondin-1 (TSP-1) and in the up-regulation of angiogenic factors such as vascular endothelial growth factor (VEGF). [210] Both VEGF and TSP-1 contain p53 response elements and are involved in the formation and inhibition of new blood vessels respectively. [238-240] TSP-1 is a potent modulator of angiogenesis which has been shown to have inhibitory effects on the process of neovascularisation, so in cell culture, loss of p53 wild-type allele is associated with decreased levels of TSP-1; shifting the balance towards angiogenesis. [240] Despite it being well accepted that there is a concordance between genetic methods that detect p53 gene mutations, and IHC methods that measure mutant p53 protein accumulation, [37,38,39,40] like this study, IHC determination of p53 over-expression has previously not been found to correlate with tumour microvessel density assessment. [238.241]

The finding that p53 expression and numerous nuclear morphometric parameters showed significant correlations in this study is also not surprising. Higher values of MNA, sdNA, nuclear length and breadth for example relate to enlarged, pleomorphic nuclei, which, like MAI are regarded as markers of cell proliferation. As 96% of tumours in this study showed positive staining for p53, this explains the lack of correlation between p53 positivity and other measurement parameters.

This study found MAI to correlate with numerous nuclear morphometric and angiogenesis parameters. Explanation for the correlation of MAI and nuclear morphometric parameters is that cycling cells are likely to be larger than non-cycling. A prior study in ovarian cancer however had found no association between mitotic counts and nuclear features, but this was a relatively small study (n=49). [235]

The association between tumour cell proliferation and IMVD in endothelial cells has been previously recognised in studies of colorectal [242] and lung [243] carcinomas, yet no such correlation was found in a study on ovarian carcinoma, although this again was of a relatively small sample group (n=31). [244] This association between MAI and angiogenesis parameters appears to suggest that tumour progression and metastasis are angiogenesis dependent.

The combination of MAI, and the angiogenesis parameters of mean MVD at X400 magnification, mean endothelial area, and one nearest neighbour were independent prognosticators for OS. Mean endothelial area remained the strongest independent prognosticator for DFS. Multivariate analysis for prediction of chemotherapy response also revealed the angiogenesis parameter of number of capillaries with one nearest neighbour to be the strongest independent prognosticator. Overall, therefore angiogenesis determinants, particularly those determined by semi-automated image analysis, were found to be the strongest prognosticators in this group of serous ovarian carcinomas.

Chapter 11. Summary, Final Conclusion, and Future Perspectives.

11.1 Introduction

Due to the heterogeneous nature of ovarian tumours, it is probable that no single biological parameter will give accurate prognostic information in all ovarian cancer patients. Identification, however, of prognostic factors in ovarian cancer, enables an improved understanding of the natural history of the disease, provides clinicians with guidelines for decisions on treatment strategies, and adjusts for imbalances in comparing therapeutic regimens. [245] Identification of predictive factors, useful in selection of patients likely to benefit from a specific treatment such as targeted systemic therapy, has been an important goal to optimise therapy. Patients could be more appropriately stratified within treatment protocols if good and poor prognosis patient groups could be identified at initial surgery

This thesis has investigated the reproducibility, prognostic, and predictive value of qualitative clinicopathological, and quantitative pathological variables in a well-selected group of serous ovarian carcinomas, in an aim to determine whether basic morphometric data can predict outcome and chemotherapeutic response in ovarian serous carcinoma, to determine whether newer semi-automated methods of tumour morphometry provides similar results to older methods, and to determine whether advanced image analysis methods can offer further tumour outcome data etc in serous ovarian carcinoma.

11.2 Summary

In chapter 1 a review is given of clinicopathologic factors in ovarian carcinoma. Borderline tumours were not always excluded in the studies examined and histological sub-types were, most often, not considered as singular entities. The most important clinicopathologic prognostic factors in ovarian carcinoma appear to be residual disease

status, FIGO stage, and tumour grade, which are all fraught with difficulties in reproducibility and classification. The general conclusion, for most of these clinicopathologic prognostic factors however, was that conflicting results were obtained from different studies. **Chapter 3** therefore investigated the prognostic and predictive ability of clinicopathologic parameters, in a well–selected group of serous ovarian carcinomas, to ensure that this study's findings were comparable to others. Multivariate analysis revealed extent of residual disease to be the strongest prognostic factor for overall survival (OS) and strongest predictive factor for chemotherapy response, and FIGO stage the strongest prognosticator for disease free survival (DFS), which was unsurprising. As problems with reproducibility are well documented in tumour grade, and definitions of residual disease variable, then close consideration was taken as to reproducibility of all further parameters investigated.

Chapter 4 considered the prognostic and predictive value of p53 and Bcl-2 as determined by immunohistochemical techniques. Prior study data again showed conflicting results. Bcl-2 was found neither a valuable prognostic nor predictive factor in this group of patients. P53 percent expression was found to be highly reproducible. Although p53 percent expression and p53 grade were found significant predictors of DFS, they failed to retain independent prognostic significance, being superseded by FIGO stage. P53 was not found to be significant for prediction of chemotherapy response. From these findings it may be concluded that p53 is only of limited prognostic value in serous ovarian carcinomas.

Evidence has accumulated for the strong additional prognostic value of quantitative pathologic techniques. Such techniques allow greater objectivity than histological grading, typing, estimation of residual tumour etc. and have been shown in ovarian tumours to have considerable prognostic importance. **Chapters 5 and 6** investigated the value of mitotic

activity index (MAI) and volume percentage epithelium (VPE) estimates. Traditional point counting techniques for assessment of epithelial area were highly correlated to semi-automated image analysis techniques. For both parameters, assessment techniques were found to be highly reproducible at intra- and interobserver level. In agreement with prior study findings both MAI and VPE were found to be strong independent prognosticators for both OS and DFS. Although MAI was not significant, VPE was found to retain independent significance for prediction of chemotherapy response, superseding the prognostic ability of residual disease status. From these findings it may be concluded that MAI and VPE are of important value in prognostic determination in serous ovarian carcinomas.

The ability of nuclear morphometric features to predict outcome in serous ovarian carcinomas was investigated in **chapter 7**. Again this quantitative technique was found to be highly reproducible. For prediction of survival (OS & DFS), and chemotherapy response, multivariate analysis revealed equivalent diameter and nuclear length, respectively, to retain independent prognostic significance. It is therefore concluded that nuclear morphometric variables have important value in predicting prognosis in serous ovarian carcinoma and add to the classical prognostic parameters of residual disease status and FIGO stage.

Minimum spanning tree analysis (MST) is a method to analyse the arrangement of nuclei in tissue. In **Chapter 8** the reproducibility and prognostic ability of MST variables in serous ovarian carcinoma were investigated. Despite amendments in methodology, MST variables were not reproducible at interobserver level, and, despite attaining prognostic value on univariate analysis, MST variables failed to retain significance on multivariate analysis for prediction of OS, DFS and chemotherapy response. In conclusion, MST is of limited prognostic value in serous ovarian carcinomas, and the marked heterogeneity

observed in ovarian tumours is likely be a limiting factor in the practical application of this technique.

Angiogenesis is the formation of new blood vessels by proliferation of new capillaries from pre-existing vessels. **Chapter 9** investigated the prognostic and predictive ability of angiogenesis parameters in serous ovarian carcinoma. Parameters were determined by traditional light microscopic counting techniques, semi-automated image analysis techniques and by application of syntactic structure analysis techniques. Methods were found to be highly reproducible and to highly correlate with one another. Multivariate analysis found angiogenesis parameters to exceed the prognostic ability of traditional clinicopathologic parameters for the prediction of OS, DFS and chemotherapy response. In conclusion angiogenesis parameters have important value in predicting prognosis in serous ovarian carcinoma, with particular importance shown by those parameters determined by semi-automated image analysis techniques.

The overall prognostic value of clinical, qualitative, and quantitative pathological features were evaluated in **chapter 10**, in this study of a well-selected group of serous ovarian carcinoma patients, treated with primary debulking surgery, and platinum-based combination chemotherapy, with long-term follow-up. The determination of quantitative features was not only fast and easy to perform, but also relatively inexpensive. Reproducibility of quantitative parameters was excellent, and certainly exceeded the reproducibility of tumour grade, as reported in prior studies. Multivariate analysis revealed the combination of angiogenesis parameters (X400 mean MVD, mean endothelial area and percent connectivity of capillaries with one nearest neighbour) and MAI to be independent prognostic factors for OS, and the combination of FIGO stage, MAI and mean endothelial area to be independent prognostic factors for DFS. For the prediction of chemotherapy response, multivariate analysis revealed tumour grade, VPE, nuclear length and percent

connectivity of capillaries with one nearest neighbour to be independent prognostic factors. At multivariate level angiogenesis parameters, as determined by semi-automated image analysis techniques, were found overall to be the strongest prognosticators.

11.3 Final conclusion

From these findings it may be concluded that basic morphometric data can predict outcome and chemotherapeutic response in ovarian serous carcinoma, that newer semi-automated methods of tumour morphometry provides similar results to older methods and that advanced image analysis methods can offer further tumour outcome data in ovarian carcimoma. The rationale for the application of semi-automated and automated detection and outlining of abnormal regions of a tumour is that it may provide an unbiased sampling of a lesion and possibly a more representative estimate of areas that a human expert might label. Such determined, quantitative pathological findings were found to have important value in predicting prognosis in ovarian carcinoma and, if not to supersede, certainly to add to the classical prognostic factors of FIGO stage, tumour grade and residual disease status.

11.4 Future Perspectives

Prior studies have suffered from lack of multivariate analysis, use of mixed histological sub-types, small population sizes, and outcome analysis performed on patients treated with varying chemotherapeutic regimes. Future studies should be performed using multivariate analysis, as ideal prognostic classification should be derived from prognostic factors, which are identified by multivariate statistical analyses. [246] This thesis has attempted to achieve this. Use of different histological sub-types may have confounded prior study findings due to their distinct molecular pathogenesis and biological background. This thesis considers serous carcinomas only, and application to singular non-serous histological sub-types would be of interest to ensure that the data holds throughout the whole spectrum of epithelial ovarian tumours. This study group also contains a

relatively lower number of stage I tumours. Careful analysis and development of more accurate risk factors is important to indicate the 'high risk' early stage patients hence further analysis in a large group of stage I tumours is recommended to ensure these findings hold. Angiogenesis determinants were found to be the strongest prognosticators for survival and prediction of chemotherapy response. It would be particularly interesting to see whether they do so in larger studies and in particular early stage tumours.

The way forward probably lies in conducting large, well defined retrospective and prospective studies of single tumour types, using precisely conducted mitotic counts, well-selected nuclear measurements, VPE estimates, and angiogenesis determinants, all under careful statistical control.

This thesis illustrates that there may be no singular parameter uniformally capable of predicting patient outcome. However this data suggests that with further refinement and control, measurement of a range of parameters may contribute more than current subjective measurements.

Appendix 1: Side Effects of Cytotoxic Drugs:

General:

Extravasation of intravenous drugs.
Oral mucositis
Hyperuricaemia
Nausea & Vomiting
Bone Marrow suppression
Alopecia
Reproductive Function.

Cisplatin: Nephrotoxicity,
Ototoxicity
Peripheral Neuropathy
Hypomagnesemia,
Myelosuppression.

Carboplatin As above but generally milder
More myelosuppressive than cisplatin

Taxol Hypersensitivity reactions
(bradycardia or asymptomatic arrhythmia)
Myelosuppression
Peripheral neuropathy
Cardiac conduction defects with arrhythmia.
Alopecia
Muscle pain.

Topotecan Dose-limiting myelosuppression
GI effects (delayed diarrhoea)
Asthenia
Alopecia
Anorexia

Appendix 2: Glossary of Measurement Parameters:

1.Feature Measurement Parameters

Feature: Features are distinct areas within an image having a single continuous boundary. Image analysers recognise features rather than objects.

Area: Feature Area – The total number of detected pixels within the feature.

Equivalent Diameter: Equivalent Circle Diameter – i.e. the diameter of a circle having the same area as the feature.

Fullness Ratio: The Fullness Ratio – this is a shape factor, equal to the square root of the ratio of area to circumscribed area.

$$\text{Fullness Ratio} = \text{sqrt}\left(\frac{\text{Area}}{\text{Convex Area}}\right)$$

Length The length of the longest feret.

Feret Diameters

Sometimes known as calliper diameters, may be known as the orthogonal distance between a pair of parallel tangents to the feature at the specified angle to the scan. eg feret 0 corresponds to the feature width and feret 90 corresponds to the feature height.

Orthoferet Orthogonal feret – the length of the feret that is at right angles to the longest feret.

Perimeter The total length of the boundary of the feature. This is calculated from the horizontal and vertical projections, with an allowance for the number of corners.

Roundness A shape factor which gives a minimum value of unity for a circle. This is calculated from the ratio of perimeter squared to area.

Feature Count Point

Defines the X and Y co-ordinate for the feature. The Feature Count Point is the last (rightmost) pixel of the last (bottom) line contained in the object. An object whose FCP is out of the measurement frame will not be measured by a feature measurement, and will not be counted by a field or a feature measurement. Feature measurements produce results relating to a wide range of parameters for each isolated feature.

X FCP The X co-ordinate of the Feature Count Point.

Y FCP The Y co-ordinate of the Feature Count Point.

Appendix 3: Sub-group analysis of MST Parameters- Grouped FIGO stage I & II and III & IV.

Parameter	df	Sig. (p)	Exp (B)	95% CI Lower	95% CI Upper	Parameter	Sig. (p)	Exp (B)	95% Lower CI	95% Upper CI
Univariate Analysis FIGO ! & II										
Overall Survival						**Disease Free Survival**				
Clinicopathologic Variables						**Clinicopathologic Variables**				
Max MST	1	**.031**	.780	.622	.977	**Length MST**	1	.154		
Length MST	1	.385				**Mean MST**	1	.434		
Mean MST	1	.108				**Min MST**	1	.166		
Min MST	1	.169				**Max MST**	1	.568		
sd MST	1	.107				**sd MST**	1	.512		
1 Nearest Neighbour	1	.161				**1 Nearest Neighbour**	**.025**	1.229	1.026	1.473
2 Nearest Neighbours	1	.549				**2 Nearest Neighbours**	**.038**	.872	.766	.992
3 Nearest Neighbours	1	.163				**3 Nearest Neighbours**	**.015**	1.287	1.050	1.577
Univariate Analysis FIGO III & IV										
Length MST	1	.724				**Length MST**	.943			
Max MST	1	.686				**Max MST**	.816			
Mean MST	1	.842				**Mean MST**	.976			
Min MST	1	.748				**Min MST**	.751			
sd MST	1	.756				**sd MST**	.966			
1 Nearest Neighbour	1	.162				**1 Nearest Neighbour**	.453			
2 Nearest Neighbours	1	.295				**2 Nearest Neighbours**	.546			
3 Nearest Neighbours	1	.394				**3 Nearest Neighbours**	.454			

Appendix 4: Sub-group analysis spanning tree parameters – field count >65 points per field

Parameter	df	Sig. (p)	Exp (B)	95% CI Lower	95% CI Upper	Parameter	Sig. (p)	Exp (B)	95% Lower CI	95% Upper CI
Univariate Analysis										
Overall Survival						**Disease Free Survival**				
Clinicopathologic Variables						**Clinicopathologic Variables**				
Mean MST	1	**.038**	.821	.682	.989	**Mean MST**	.106			
Min MST	1	**.014**	.638	.447	.912	**Min MST**	.083			
Max MST	1	**.019**	.904	.831	.983	**Max MST**	.071			
sd MST	1	.070				**sdMST**	.133			
1 Nearest Neighbour	1	.057				**1 Nearest Neighbour**	.396			
2 Nearest Neighbours	1	.154				**2 Nearest Neighbours**	.397			
3 Nearest Neighbours	1	.714				**3 Nearest Neighbours**	.715			
Length MST	1	.137				**Length MST**	.146			

Appendix 5: Correlations Between Parameters

P53 Grade	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.2574	0.06623	0.0032	Yes
Morphometry Parameters				
VPE	0.1311	0.01718	0.1387	No
MNA	0.1571	0.02467	0.0755	No
sdNA	0.2482	0.06162	0.0046	Yes
Nuclear Perimeter	0.2266	0.05134	0.0098	Yes
Nuclear Roundness	-0.07219	0.005212	0.4162	No
Fullness Ratio	-0.1023	0.01046	0.2487	No
Nuclear Length	0.2322	0.05392	0.0081	Yes
Nuclear Breadth	0.1777	0.03158	0.0439	Yes
Orthoferet	0.2096	0.04393	0.0171	Yes
Equiv. Diameter	0.09122	0.008322	0.3039	No
bcl-2 Parameters				
Bcl-2 grade	0.01955	0.0003824	0.8259	No
Bcl-2 positive	0.002477	6.134E-06	0.9778	No
Spanning Tree Parameters				
Length MST	-0.03357	0.001127	0.7068	No
Sd average MST	-0.07905	0.006248	0.3751	No
Mean length MST	-0.1397	0.01953	0.1157	No
Min length MST	-0.1352	0.01828	0.1281	No
Max length MST	-0.1365	0.01864	0.1244	No
Sd MST	-0.1253	0.01570	0.1587	No
1 nearest neighbour	0.05207	0.002711	0.5594	No
2 nearest neighbour	-0.07690	0.005914	0.3883	No
3 nearest neighbours	0.07124	0.005075	0.4242	No

P53 Percent Expression	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.2574	0.06623	0.0032	Yes
Morphometry Parameters				
VPE	0.1311	0.01718	0.1387	No
MNA	0.1571	0.02467	0.0755	No
sdNA	0.2482	0.06162	0.0046	Yes
Nuclear Perimeter	0.2266	0.05134	0.0098	Yes
Nuclear Roundness	-0.07219	0.005212	0.4162	No
Fullness Ratio	-0.1023	0.01046	0.2487	No
Nuclear Length	0.2322	0.05392	0.0081	Yes
Nuclear Breadth	0.1777	0.03158	0.0439	Yes
Orthoferet	0.2096	0.04393	0.0171	Yes
Equiv. Diameter	0.09122	0.008322	0.3039	No
bcl-2 Parameters				
Bcl-2 grade	0.01955	0.0003824	0.8259	No
Bcl-2 positive	0.002477	6.134E-06	0.9778	No
Spanning Tree Parameters				
Length MST	-0.03357	0.001127	0.7068	No
Sd average MST	-0.07905	0.006248	0.3751	No
Mean length MST	-0.1397	0.01953	0.1157	No
Min length MST	-0.1352	0.01828	0.1281	No
Max length MST	-0.1365	0.01864	0.1244	No
Sd MST	-0.1253	0.01570	0.1587	No
1 nearest neighbour	0.05207	0.002711	0.5594	No
2 nearest neighbour	-0.07690	0.005914	0.3883	No
3 nearest neighbours	0.07124	0.005075	0.4242	No

Appendix 5: Correlations Between Parameters - Continued

P53 Positive	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.2574	0.06623	0.0032	Yes
Morphometry Parameters				
VPE	0.1311	0.01718	0.1387	No
MNA	0.1571	0.02467	0.0755	No
sdNA	0.2482	0.06162	0.0046	Yes
Nuclear Perimeter	0.2266	0.05134	0.0098	Yes
Nuclear Roundness	-0.07219	0.005212	0.4162	No
Fullness Ratio	-0.1023	0.01046	0.2487	No
Nuclear Length	0.2322	0.05392	0.0081	Yes
Nuclear Breadth	0.1777	0.03158	0.0439	Yes
Orthoferet	0.2096	0.04393	0.0171	Yes
Equiv. Diameter	0.09122	0.008322	0.3039	No
bcl-2 Parameters				
Bcl-2 grade	0.01955	0.0003824	0.8259	No
Bcl-2 positive	0.002477	6.134E-06	0.9778	No
Spanning Tree Parameters				
Length MST	-0.03357	0.001127	0.7068	No
Sd average MST	-0.07905	0.006248	0.3751	No
Mean length MST	-0.1397	0.01953	0.1157	No
Min length MST	-0.1352	0.01828	0.1281	No
Max length MST	-0.1365	0.01864	0.1244	No
Sd MST	-0.1253	0.01570	0.1587	No
1 nearest neighbour	0.05207	0.002711	0.5594	No
2 nearest neighbour	-0.07690	0.005914	0.3883	No
3 nearest neighbours	0.07124	0.005075	0.4242	No

Bcl-2 positive	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.1951	0.03807	0.0261	Yes
Morphometry Parameters				
VPE	-0.08969	0.008045	0.3102	No
MNA	-0.05161	0.002264	0.5598	No
sdNA	0.02485	0.0006173	0.7790	No
Nuclear Perimeter	0.02407	0.0005794	0.7858	No
Nuclear Roundness	0.03471	0.001205	0.6950	No
Fullness Ratio	0.1206	0.01454	0.1718	No
Nuclear Length	0.01466	0.0002091	0.8703	No
Nuclear Breadth	0.06617	0.004378	0.4545	No
Orthoferet	0.04318	0.001864	0.6257	No
Equiv. Diameter	0.002791	7.787E-06	0.9749	No
p53 Parameters				
P53 percent expression	-0.07713	0.005950	0.3849	No
P53 Grade	0.002477	6.134E-06	0.9778	No
P53 positive	-0.02784	0.0007753	0.7551	No
Spanning Tree Parameters				
Length MST	0.04960	0.002460	0.5767	No
Sd average MST	0.002008	4.033E-06	0.9820	No
Mean length MST	-0.05390	0.002905	0.5441	No
Min length MST	-0.07872	0.006196	0.3752	No
Max length MST	-0.006670	4.448E-05	0.9402	No
Sd MST	-0.01666	0.0002774	0.8514	No
1 nearest neighbour	0.04059	0.001648	0.6479	No
2 nearest neighbour	-0.09628	0.009270	0.2777	No
3 nearest neighbours	0.03189	0.001017	0.7198	No

Appendix 5: Correlations Between Parameters - Continued

Bcl-2 Grade	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.1529	0.02337	0.0825	No
Morphometry Parameters				
VPE	-0.01474	0.0002173	0.8678	No
MNA	-0.009056	8.202E-05	0.9185	No
sdNA	0.04299	0.001848	0.6272	No
Nuclear Perimeter	0.1285	0.0165	0.1451	No
Nuclear Roundness	-0.08656	0.007493	0.3274	No
Fullness Ratio	0.1595	0.02544	0.0699	No
Nuclear Length	0.1220	0.01490	0.1666	No
Nuclear Breadth	0.1852	0.03428	0.0349	Yes
Orthoferet	0.1433	0.02054	0.1038	No
Equiv. Diameter	0.05222	0.002727	0.5352	No
p53 Parameters				
P53 percent expression	-0.04297	0.001847	0.6287	No
P53 Grade	0.01955	0.0003824	0.8259	No
P53 positive	-0.06822	0.004653	0.4442	No
Spanning Tree Parameters				
Length MST	0.09110	0.008299	0.3045	No
Sd average MST	0.06078	0.003694	0.4938	No
Mean length MST	-0.02721	0.0007402	0.7596	No
Min length MST	-0.09067	0.008221	0.3068	No
Max length MST	0.02067	0.0004272	0.8161	No
Sd MST	0.02175	0.0004733	0.8067	No
1 nearest neighbour	0.05130	0.00263	0.5637	No
2 nearest neighbour	-0.1044	0.01089	0.2391	No
3 nearest neighbours	0.07086	0.005021	0.4249	No

MAI	r	r^2	p	Significant
Morphometry Parameters				
VPE	0.1616	0.02613	0.0662	No
MNA	0.06891	0.004749	0.4350	No
sdNA	0.2633	0.06933	0.0025	Yes
Nuclear Perimeter	0.2967	0.08802	0.0006	Yes
Nuclear Roundness	-0.1360	0.01850	0.1228	No
Fullness Ratio	0.09280	0.008612	0.2936	No
Nuclear Length	0.2966	0.08796	0.0006	Yes
Nuclear Breadth	0.3475	0.1208	<0.0001	Yes
Orthoferet	0.2927	0.08565	0.0007	Yes
Equiv. Diameter	0.08829	0.007795	0.3179	No
p53 & bcl-2				
P53 percent expression	0.3228	0.1042	0.0002	Yes
P53 Grade	0.2574	0.06623	0.0032	Yes
P53 positive	0.02632	0.0006929	0.7680	No
Bcl-2 grade	0.1529	0.02337	0.0825	No
Bcl-2 positive	0.1951	0.03807	0.0261	Yes
Spanning Tree Parameters				
Length MST	0.1444	0.02084	0.1027	No
Sd average MST	-0.1170	0.01368	0.1868	No
Mean length MST	-0.1285	0.01651	0.1467	No
Min length MST	-0.1860	0.03461	0.0348	Yes
Max length MST	-0.1220	0.01487	0.1685	No
Sd MST	-0.09330	0.008705	0.2930	No
1 nearest neighbour	0.2330	0.05429	0.0079	Yes
2 nearest neighbour	-0.3233	0.1045	0.0002	Yes
3 nearest neighbours	0.2843	0.08082	0.0011	Yes

Appendix 5: Correlations Between Parameters - Continued

VPE	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.1616	0.02613	0.0662	No
Morphometry Parameters				
MNA	0.2078	0.04317	0.0177	Yes
sdNA	0.3049	0.09296	0.0004	Yes
Nuclear Perimeter	0.3931	0.1545	<0.0001	Yes
Nuclear Roundness	0.05942	0.003530	0.5019	No
Fullness Ratio	0.4236	0.001795	0.6322	No
Nuclear Length	0.4059	0.1648	<0.0001	Yes
Nuclear Breadth	0.3094	0.09570	0.0003	Yes
Orthoferet	0.3579	0.1281	<0.0001	Yes
Equiv. Diameter	0.1917	0.03674	0.0289	Yes
p53 & bcl-2				
P53 percent expression	0.1749	0.03060	0.0474	Yes
P53 Grade	0.1311	0.01718	0.1387	No
P53 positive	0.03175	0.001008	0.7220	No
Bcl-2 grade	-0.01474	0.0002173	0.8678	No
Bcl-2 positive	-0.08969	0.008045	0.3102	No
Spanning Tree Parameters				
Length MST	-0.03922	0.001539	0.6590	No
Sd average MST	0.01552	0.002409	0.8614	No
Mean length MST	0.02333	0.0005442	0.7930	No
Min length MST	-0.02139	0.0004573	0.8099	No
Max length MST	-0.002377	5.65E-06	0.9787	No
Sd MST	0.03754	0.001409	0.6727	No
1 nearest neighbour	0.2738	0.07496	0.0017	Yes
2 nearest neighbour	-0.2721	0.07405	0.0018	Yes
3 nearest neighbours	0.3352	0.1124	0.0001	Yes

MNA	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.06891	0.004749	0.4350	No
VPE				
VPE	0.2078	0.04317	0.0177	Yes
p53 & bcl-2				
P53 percent expression	0.1745	0.03046	0.0479	Yes
P53 Grade	0.1571	0.02467	0.0755	No
P53 positive	0.02270	0.0005155	0.7992	No
Bcl-2 grade	-0.009056	8.202E-05	0.9185	No
Bcl-2 positive	-0.05161	0.002264	0.5598	No
Spanning Tree Parameters				
Length MST	0.02871	0.0008245	0.7448	No
Sd average MST	0.06499	0.004224	0.4608	No
Mean length MST	0.07641	0.005839	0.3857	No
Min length MST	-0.01191	0.0001420	0.7532	No
Max length MST	0.08346	0.006966	0.3432	No
Sd MST	0.1201	0.01443	0.1718	No
1 nearest neighbour	0.1503	0.02260	0.0865	No
2 nearest neighbour	-0.1708	0.02917	0.0511	No
3 nearest neighbours	0.1321	0.01745	0.1326	No

Appendix 5: Correlations Between Parameters - Continued

sdNA	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.2633	0.06933	0.0025	Yes
VPE				
VPE	0.3049	0.09296	0.0004	Yes
p53 & bcl-2				
P53 percent expression	0.2728	0.07443	0.0018	Yes
P53 Grade	0.2482	0.06162	0.0046	Yes
P53 positive	0.06613	0.004373	0.4853	No
Bcl-2 grade	0.04299	0.001848	0.6272	No
Bcl-2 positive	0.02485	0.0006173	0.7790	No
Spanning Tree Parameters				
Length MST	0.7905	0.006249	0.3694	No
Sd average MST	0.1568	0.02457	0.0738	No
Mean length MST	0.2121	0.04500	0.0150	Yes
Min length MST	0.1717	0.02950	0.0498	Yes
Max length MST	0.1500	0.02249	0.0874	No
Sd MST	0.1731	0.02998	0.0480	Yes
1 nearest neighbour	0.3303	0.1091	0.0001	Yes
2 nearest neighbour	0.3364	0.1131	<0.0001	Yes
3 nearest neighbours	0.2376	0.05645	0.0063	Yes

Nuclear Length	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.2966	0.08796	0.0006	Yes
VPE				
VPE	0.4059	0.1648	<0.0001	Yes
p53 & bcl-2				
P53 percent expression	0.2441	0.05958	0.0053	Yes
P53 Grade	0.2322	0.05392	0.0081	Yes
P53 positive	0.008810	7.762E-05	0.9214	No
Bcl-2 grade	0.1220	0.01490	0.1666	No
Bcl-2 positive	0.01466	0.0002091	0.8703	No
Spanning Tree Parameters				
Length MST	0.1204	0.01449	0.1708	No
Sd average MST	0.1979	0.03916	0.0235	Yes
Mean length MST	0.2039	0.04157	0.0195	Yes
Min length MST	0.1169	0.01366	0.1837	No
Max length MST	0.1624	0.02639	0.0638	No
Sd MST	0.1867	0.03486	0.0327	Yes
1 nearest neighbour	0.3109	0.09663	0.0003	Yes
2 nearest neighbour	-0.3493	0.1220	<0.0001	Yes
3 nearest neighbours	0.2526	0.06382	0.0036	Yes

Appendix 5: Correlations Between Parameters - Continued

Nuclear Breadth	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.3475	0.1208	<0.0001	Yes
VPE				
VPE	0.3094	0.09570	0.0003	Yes
p53 & bcl-2				
P53 percent expression	0.1992	0.03969	0.0236	Yes
P53 Grade	0.1777	0.03158	0.0439	Yes
P53 positive	-0.08452	0.007144	0.3428	No
Bcl-2 grade	0.1852	0.03428	0.0349	Yes
Bcl-2 positive	0.06617	0.004378	0.4545	No
Spanning Tree Parameters				
Length MST	0.2576	0.06636	0.0030	Yes
Sd average MST	0.1906	0.03632	0.0292	Yes
Mean length MST	0.1965	0.03863	0.0245	Yes
Min length MST	0.08788	0.007722	0.3182	No
Max length MST	0.1761	0.03099	0.0443	Yes
Sd MST	0.1887	0.03559	0.0309	Yes
1 nearest neighbour	0.3044	0.09267	0.0004	Yes
2 nearest neighbour	-0.3578	0.1280	<0.0001	Yes
3 nearest neighbours	0.2742	0.07518	0.0015	Yes

Nuclear Perimeter	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.2967	0.08802	0.0006	Yes
VPE				
VPE	0.3931	0.1545	<0.0001	Yes
p53 & bcl-2				
P53 percent expression	0.2421	0.05863	0.0057	Yes
P53 Grade	0.2266	0.05134	0.0098	Yes
P53 positive	0.01600	0.002559	0.8578	No
Bcl-2 grade	0.1285	0.0165	0.1451	No
Bcl-2 positive	0.02407	0.0005794	0.7858	No
Spanning Tree Parameters				
Length MST	0.1312	0.01721	0.1353	No
Sd average MST	0.2039	0.04156	0.0195	Yes
Mean length MST	0.2176	0.04733	0.0126	Yes
Min length MST	0.1280	0.01639	0.1450	No
Max length MST	0.1754	0.03077	0.0451	Yes
Sd MST	0.1972	0.03888	0.0240	Yes
1 nearest neighbour	0.3197	0.1022	0.0002	Yes
2 nearest neighbour	-0.3601	0.1297	<0.0001	Yes
3 nearest neighbours	0.2616	0.06842	0.0025	Yes

Appendix 5: Correlations Between Parameters - Continued

Nuclear Roundness	r	r^2	p	Significant
Mitotic Activity Index				
MAI	-0.1360	0.01850	0.1228	No
VPE				
VPE	0.05942	0.003530	0.5019	No
p53 & bcl-2				
P53 percent expression	-0.06970	0.004859	0.4325	No
P53 Grade	-0.07219	0.005212	0.4162	No
P53 positive	0.003324	1.105E-05	0.9703	No
Bcl-2 grade	-0.08656	0.007493	0.3274	No
Bcl-2 positive	0.03471	0.001205	0.6950	No
Spanning Tree Parameters				
Length MST	-0.9760	0.009526	0.2674	No
Sd average MST	0.01034	0.0001069	0.9067	No
Mean length MST	-0.07410	0.005491	0.4002	No
Min length MST	-0.02212	0.004893	0.8020	No
Max length MST	-0.1100	0.01210	0.2110	No
Sd MST	-0.09138	0.008350	0.2993	No
1 nearest neighbour	0.005255	2.762E-05	0.9525	No
2 nearest neighbour	-0.008390	7.039E-05	0.9242	No
3 nearest neighbours	0.001439	2.070E-06	0.9870	No

Equivalent Diameter	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.08829	0.007795	0.3179	No
VPE				
VPE	0.1917	0.03674	0.0289	Yes
p53 & bcl-2 Parameters				
P53 percent expression	0.1291	0.01666	0.1449	No
P53 Grade	0.09122	0.008322	0.3039	No
P53 positive	0.03009	0.0009056	0.7360	No
Bcl-2 grade	0.05222	0.002727	0.5352	No
Bcl-2 positive	0.002791	7.787E-06	0.9749	No
Spanning Tree Parameters				
Length MST	0.1369	0.01874	0.1190	No
Sd average MST	0.05220	0.002725	0.5538	No
Mean length MST	0.05168	0.002671	0.5577	No
Min length MST	-0.02179	0.0004747	0.8049	No
Max length MST	0.06144	0.003775	0.4857	No
Sd MST	0.07489	0.005608	0.3953	No
1 nearest neighbour	0.3682	0.1356	<0.0001	Yes
2 nearest neighbour	-0.1579	0.02493	0.0717	No
3 nearest neighbours	0.4205	0.001768	0.6334	No

Appendix 5: Correlations Between Parameters – Continued

Orthoferet	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.2927	0.08565	0.0007	Yes
VPE				
VPE	0.3579	0.1281	<0.0001	Yes
p53 & bcl-2 Parameters				
P53 percent expression	0.2305	0.05315	0.0086	Yes
P53 Grade	0.2096	0.04393	0.0171	Yes
P53 positive	0.03019	0.0009115	0.7351	No
Bcl-2 grade	0.1433	0.02054	0.1038	No
Bcl-2 positive	0.04318	0.001864	0.6257	No
Spanning Tree Parameters				
Length MST	0.1440	0.02074	0.1008	No
Sd average MST	0.2054	0.04220	0.0186	Yes
Mean length MST	0.2325	0.05406	0.0075	Yes
Min length MST	0.1400	0.01961	0.1106	No
Max length MST	0.1937	0.03750	0.0267	Yes
Sd MST	0.2093	0.04379	0.0165	Yes
1 nearest neighbour	0.3324	0.1105	0.0001	Yes
2 nearest neighbour	-0.3738	0.1397	<0.0001	Yes
3 nearest neighbours	0.2777	0.07709	0.0013	Yes

Fullness Ratio	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.09280	0.008612	0.2936	No
VPE				
VPE	0.4236	0.001795	0.6322	No
p53 & bcl-2				
P53 percent expression	-0.07340	0.005387	0.4084	No
P53 Grade	-0.1023	0.01046	0.2487	No
P53 positive	0.01273	0.0001621	0.8866	No
Bcl-2 grade	0.1595	0.02544	0.0699	No
Bcl-2 positive	0.1206	0.01454	0.1718	No
Spanning Tree Parameters				
Length MST	0.1494	0.02233	0.0885	No
Sd average MST	-0.02256	0.0005088	0.7982	No
Mean length MST	0.09626	0.009267	0.2741	No
Min length MST	0.03085	0.0009519	0.7265	No
Max length MST	0.1297	0.01682	0.1398	No
Sd MST	0.1040	0.01082	0.2372	No
1 nearest neighbour	0.08872	0.007871	0.3136	No
2 nearest neighbour	-0.1032	0.01065	0.2408	No
3 nearest neighbours	0.1186	0.01405	0.1775	No

Appendix 5: Correlations Between Parameters - Continued

Length MST	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.1444	0.02084	0.1027	No
Morphometry Parameters				
VPE	-0.03922	0.001539	0.6590	No
MNA	0.02871	0.0008245	0.7448	No
sdNA	0.7905	0.006249	0.3694	No
Nuclear Perimeter	0.1312	0.01721	0.1353	No
Nuclear Roundness	-0.9760	0.009526	0.2674	No
Fullness Ratio	0.1494	0.02233	0.0885	No
Nuclear Length	0.1204	0.01449	0.1708	No
Nuclear Breadth	0.2576	0.06636	0.0030	Yes
Orthoferet	0.1440	0.02074	0.1008	No
Equiv. Diameter	0.1369	0.01874	0.1190	No
p53 & Bcl-2				
P53 percent expression	-0.1381	0.01907	0.1201	No
P53 Grade	-0.03357	0.001127	0.7068	No
P53 positive	-0.1287	0.01656	0.1493	No
Bcl-2 grade	0.09110	0.008299	0.3045	No
Bcl-2 positive	0.04960	0.002460	0.5767	No

Mean MST	r	r^2	p	Significant
Mitotic Activity Index				
MAI	-0.1285	0.01651	0.1467	No
Morphometry Parameters				
VPE	0.02333	0.0005442	0.7930	No
MNA	0.07641	0.005839	0.3857	No
sdNA	0.2121	0.04500	0.0150	Yes
Nuclear Perimeter	0.2176	0.04733	0.0126	Yes
Nuclear Roundness	-0.07410	0.005491	0.4002	No
Fullness Ratio	0.09626	0.009267	0.2741	No
Nuclear Length	0.2039	0.04157	0.0195	Yes
Nuclear Breadth	0.1965	0.03863	0.0245	Yes
Orthoferet	0.2325	0.05406	0.0075	Yes
Equiv. Diameter	0.05168	0.002671	0.5577	No
p53 & bcl-2				
P53 percent expression	-0.05822	0.003389	0.5139	No
P53 Grade	-0.1397	0.01953	0.1157	No
P53 positive	0.05621	0.003160	0.5302	No
Bcl-2 grade	-0.02721	0.0007402	0.7596	No
Bcl-2 positive	-0.05390	0.002905	0.5441	No

Appendix 5: Correlations Between Parameters - Continued

Sd MST	r	r^2	p	Significant
Mitotic Activity Index				
MAI	-0.09330	0.008705	0.2930	No
Morphometry Parameters				
VPE	0.03754	0.001409	0.6727	No
MNA	0.1201	0.01443	0.1718	No
sdNA	0.1731	0.02998	0.0480	Yes
Perimeter	0.1972	0.03888	0.0240	Yes
Roundness	-0.09138	0.008350	0.2993	No
Fullness Ratio	0.1040	0.01082	0.2372	No
Length	0.1867	0.03486	0.0327	Yes
Breadth	0.1887	0.03559	0.0309	Yes
Orthoferet	0.2093	0.04379	0.0165	Yes
Equiv. Diameter	0.07489	0.005608	0.3953	No
p53 & bcl-2				
P53 percent expression	-0.1201	0.01442	0.1770	No
P53 Grade	-0.1253	0.01570	0.1587	No
P53 positive	-0.06465	0.004180	0.4702	No
Bcl-2 grade	0.02175	0.0004733	0.8067	No
Bcl-2 positive	-0.01666	0.0002774	0.8514	No

Max MST	r	r^2	p	Significant
Mitotic Activity Index				
MAI	-0.1220	0.01487	0.1685	No
Morphometry Parameters				
VPE	-0.002377	5.65E-06	0.9787	No
MNA	0.08346	0.006966	0.3432	No
sdNA	0.1500	0.02249	0.0874	No
Nuclear Perimeter	0.1754	0.03077	0.0451	Yes
Nuclear Roundness	-0.1100	0.01210	0.2110	No
Fullness Ratio	0.1297	0.01682	0.1398	No
Nuclear Length	0.1624	0.02639	0.0638	No
Nuclear Breadth	0.1761	0.03099	0.0443	Yes
Orthoferet	0.1400	0.01961	0.1106	No
Equiv. Diameter	0.06144	0.003775	0.4857	No
p53 & bcl-2				
P53 percent expression	-0.1541	0.02375	0.0824	No
P53 Grade	-0.1365	0.01864	0.1244	No
P53 positive	-0.09828	0.009658	0.2717	No
Bcl-2 grade	0.02067	0.0004272	0.8161	No
Bcl-2 positive	-0.006670	4.448E-05	0.9402	No

Appendix 5: Correlations Between Parameters - Continued

Min MST	r	r^2	p	Significant
Mitotic Activity Index				
MAI	-0.1860	0.03461	0.0348	Yes
Morphometry Parameters				
VPE	-0.02139	0.0004573	0.8099	No
MNA	-0.01191	0.0001420	0.7532	No
sdNA	0.1717	0.02950	0.0498	Yes
Nuclear Perimeter	0.1280	0.01639	0.1450	No
Nuclear Roundness	-0.02212	0.004893	0.8020	No
Fullness Ratio	0.03085	0.0009519	0.7265	No
Nuclear Length	0.1169	0.01366	0.1837	No
Nuclear Breadth	0.08788	0.007722	0.3182	No
Orthoferet	-0.02179	0.0004747	0.8049	No
Equiv. Diameter	-0.02179	0.0004747	0.8049	No
p53 & bcl-2				
P53 percent expression	-0.02766	0.0007650	0.7566	No
P53 Grade	-0.1352	0.01828	0.1281	No
P53 positive	0.07391	0.005463	0.4089	No
Bcl-2 grade	-0.09067	0.008221	0.3068	No
Bcl-2 positive	-0.07872	0.006196	0.3752	No

1 Nearest Neighbour	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.2330	0.05429	0.0079	Yes
Morphometry Parameters				
VPE	0.2738	0.07496	0.0017	Yes
MNA	0.1503	0.02260	0.0865	No
sdNA	0.3303	0.1091	0.0001	Yes
Nuclear Perimeter	0.3197	0.1022	0.0002	Yes
Nuclear Roundness	0.005255	2.762E-05	0.9525	No
Fullness Ratio	0.08872	0.007871	0.3136	No
Nuclear Length	0.3109	0.09663	0.0003	Yes
Nuclear Breadth	0.3044	0.09267	0.0004	Yes
Orthoferet	0.3324	0.1105	0.0001	Yes
Equiv. Diameter	0.3682	0.1356	<0.0001	Yes
p53 & bcl-2				
P53 percent expression	0.2913	0.08488	0.0008	Yes
P53 Grade	0.05207	0.002711	0.5594	No
P53 positive	0.1716	0.02964	0.0537	No
Bcl-2 grade	0.05130	0.00263	0.5637	No
Bcl-2 positive	0.04059	0.001648	0.6479	No

Appendix 5: Correlations Between Parameters - Continued

2 Nearest Neighbours	r	r^2	p	Significant
Mitotic Activity Index				
MAI	-0.3233	0.1045	0.0002	Yes
Morphometry Parameters				
VPE	-0.2721	0.07405	0.0018	Yes
MNA	-0.1708	0.02917	0.0511	No
sdNA	0.3364	0.1131	<0.0001	Yes
Nuclear Perimeter	-0.3601	0.1297	<0.0001	Yes
Nuclear Roundness	-0.008390	7.039E-05	0.9242	No
Fullness Ratio	-0.1032	0.01065	0.2408	No
Nuclear Length	-0.3493	0.1220	<0.0001	Yes
Nuclear Breadth	-0.3578	0.1280	<0.0001	Yes
Orthoferet	-0.3738	0.1397	<0.0001	Yes
Equiv. Diameter	-0.1579	0.02493	0.0717	No
p53 & bcl-2				
P53 percent expression	-0.3035	0.09213	0.0005	Yes
P53 Grade	-0.07690	0.005914	0.3883	No
P53 positive	-0.1444	0.02085	0.1053	No
Bcl-2 grade	-0.1044	0.01089	0.2391	No
Bcl-2 positive	-0.09628	0.009270	0.2777	No

3 Nearest Neighbours	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.2843	0.08082	0.0011	Yes
Morphometry Parameters				
VPE	0.3352	0.1124	0.0001	Yes
MNA	0.1321	0.01745	0.1326	No
sdNA	0.2376	0.05645	0.0063	Yes
Nuclear Perimeter	0.2616	0.06842	0.0025	Yes
Nuclear Roundness	0.001439	2.070E-06	0.9870	No
Fullness Ratio	0.1186	0.01405	0.1775	No
Nuclear Length	0.2526	0.06382	0.0036	Yes
Nuclear Breadth	0.2742	0.07518	0.0015	Yes
Orthoferet	0.2777	0.07709	0.0013	Yes
Equiv. Diameter	0.4205	0.001768	0.6334	No
p53 & bcl-2				
P53 percent expression	-0.2216	0.04909	0.0120	Yes
P53 Grade	0.07124	0.005075	0.4242	No
P53 positive	0.07088	0.005024	0.4284	No
Bcl-2 grade	0.07086	0.005021	0.4249	No
Bcl-2 positive	0.03189	0.001017	0.7198	No

Appendix 5: Correlations Between Parameters - Continued

X200 Mean MVD	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.2814	0.07917	0.0013	Yes
Morphometry Parameters				
VPE	0.3263	0.1065	0.0002	Yes
MNA	0.07925	0.006280	0.3739	No
sdNA	0.2239	0.05011	0.0111	Yes
Nuclear Perimeter	0.2330	0.05430	0.0081	Yes
Nuclear Roundness	-0.01741	0.0003031	0.8453	No
Fullness Ratio	0.09575	0.009168	0.2823	No
Nuclear Length	0.2426	0.05887	0.0058	Yes
Nuclear Breadth	0.2869	0.08231	0.0010	Yes
Orthoferet	0.2079	0.04324	0.0184	Yes
Equiv. Diameter	0.2110	0.04451	0.0168	Yes
p53 & bcl-2				
P53 percent expression	0.1493	0.02228	0.0940	No
P53 Grade	0.1035	0.01072	0.2467	No
P53 positive	0.1153	0.01330	0.1986	No
Bcl-2 grade	0.005194	2.698E-05	0.9536	No
Bcl-2 positive	-0.06136	0.003765	0.4914	No
Spanning Tree Parameters				
Length MST	-0.06655	0.004429	0.4572	No
Sd average MST	-0.09007	0.08113	0.3139	No
Mean length MST	-0.2631	0.06922	0.0028	Yes
Min length MST	-0.2662	0.07084	0.0025	Yes
Max length MST	-0.2691	0.07239	0.0022	Yes
Sd MST	-0.2249	0.05056	0.0110	Yes
1 nearest neighbour	0.1552	0.02409	0.0815	No
2 nearest neighbour	-0.1381	0.01907	0.1215	No
3 nearest neighbours	0.1211	0.01466	0.1751	No

X200 Max MVD	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.2932	0.08596	0.0008	Yes
Morphometry Parameters				
VPE	0.3311	0.1096	0.0001	Yes
MNA	0.07715	0.005952	0.03867	no
sdNA	0.2253	0.05077	0.0106	Yes
Nuclear Perimeter	0.2329	0.05422	0.0082	Yes
Nuclear Roundness	-0.01848	0.0003414	0.8360	No
Fullness Ratio	0.09687	0.009384	0.2767	No
Nuclear Length	0.2427	0.05890	0.0058	Yes
Nuclear Breadth	0.2859	0.08174	0.0011	yes
Orthoferet	0.2079	0.04322	0.0185	Yes
Equiv. Diameter	0.2158	0.04656	0.0144	Yes
p53 & bcl-2				
P53 percent expression	0.1485	0.02206	0.0956	No
P53 Grade	0.09838	0.009680	0.2711	No
P53 positive	-0.1061	0.01125	0.2371	No
Bcl-2 grade	-0.0005382	2.896E-07	0.9952	No
Bcl-2 positive	-0.06852	0.004695	0.4422	No
Spanning Tree Parameters				
Length MST	-0.006617	0.004379	0.4598	No
Sd average MST	-0.09878	0.009758	0.2692	No
Mean length MST	-0.2642	0.06982	0.0027	Yes
Min length MST	-0.2667	0.07112	0.0024	Yes
Max length MST	-0.2700	0.07291	0.0021	Yes
Sd MST	-0.2242	0.0502	0.0113	Yes
1 nearest neighbour	0.1552	0.02409	0.0815	No
2 nearest neighbour	-0.1323	0.01750	0.1381	No
3 nearest neighbours	0.1169	0.01368	0.1904	No

Appendix 5: Correlations Between Parameters - Continued

X400 Mean MVD	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.3021	0.09127	0.0005	Yes
Morphometry Parameters				
VPE	0.2519	0.06343	0.0041	Yes
MNA	0.04788	0.002293	0.5915	No
sdNA	0.1836	0.03372	0.0380	Yes
Nuclear Perimeter	0.1957	0.03829	0.0269	Yes
Nuclear Roundness	0.002928	8.571E-06	0.9738	No
Fullness Ratio	0.09521	0.009065	0.2851	No
Nuclear Length	0.2050	0.04203	0.0203	Yes
Nuclear Breadth	0.2867	0.08218	0.0010	Yes
Orthoferet	0.1723	0.02968	0.0518	No
Equiv. Diameter	0.1795	0.03222	0.0426	Yes
p53 & bcl-2				
P53 percent expression	0.09079	0.008243	0.3100	No
P53 Grade	0.06103	0.003725	0.4955	No
P53 positive	-0.1515	0.02295	0.0904	No
Bcl-2 grade	-0.02874	0.0008257	0.7475	No
Bcl-2 positive	-0.08811	0.007764	0.3227	No
Spanning Tree Parameters				
Length MST	0.009950	9.899E-05	0.9116	No
Sd average MST	-0.09088	0.008260	0.3095	No
Mean length MST	-0.2604	0.06779	0.0031	Yes
Min length MST	-0.28590	0.08177	0.0011	Yes
Max length MST	-0.2430	0.05905	0.0059	Yes
Sd MST	-0.2087	0.04357	0.0185	Yes
1 nearest neighbour	0.1428	0.02039	0.1092	No
2 nearest neighbour	-0.1330	0.01769	0.1361	No
3 nearest neighbours	0.1144	0.01309	0.2003	No

X400 Max MVD	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.3075	0.09458	0.0004	Yes
Morphometry Parameters				
VPE	0.2666	0.07107	0.0024	Yes
MNA	0.07194	0.005176	0.4197	No
sdNA	0.1917	0.03677	0.0301	Yes
Nuclear Perimeter	0.2038	0.04152	0.0211	Yes
Nuclear Roundness	0.01011	0.0001021	0.9099	No
Fullness Ratio	0.1051	0.01105	0.2377	No
Nuclear Length	0.2143	0.04593	0.0151	Yes
Nuclear Breadth	0.2881	0.08302	0.0010	Yes
Orthoferet	0.1772	0.03140	0.0454	Yes
Equiv. Diameter	0.1764	0.03111	0.0464	Yes
p53 & bcl-2				
P53 percent expression	0.08221	0.006758	0.3582	No
P53 Grade	0.04969	0.002469	0.5790	No
P53 positive	-0.1693	0.02876	0.0580	No
Bcl-2 grade	-0.01529	0.0002339	0.8640	No
Bcl-2 positive				
Spanning Tree Parameters				
Length MST	0.01283	0.0001647	0.8861	No
Sd average MST	0.1021	0.01042	0.2534	No
Mean length MST	-0.2465	0.06075	0.0052	Yes
Min length MST	-0.2777	0.07712	0.0016	Yes
Max length MST	-0.2329	0.05422	0.0084	Yes
Sd MST	-0.1942	0.03771	0.0287	Yes
1 nearest neighbour	0.1372	0.01881	0.1241	No
2 nearest neighbour	-0.1324	0.01754	0.1378	No
3 nearest neighbours	0.1182	0.01397	0.1857	No

Appendix 5: Correlations Between Parameters - Continued

Max Endothelial Area	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.1896	0.03392	0.0488	Yes
Morphometry Parameters				
VPE	0.2531	0.06406	0.0064	Yes
MNA	0.04583	0.002100	0.6267	No
sdNA	0.1319	0.01739	0.1601	No
Nuclear Perimeter	0.1678	0.02817	0.0730	No
Nuclear Roundness	0.05081	0.002582	0.5897	No
Fullness Ratio	-0.1588	0.02523	0.0900	No
Nuclear Length	0.1773	0.03142	0.0581	No
Nuclear Breadth	0.1438	0.02067	0.1253	No
Orthoferet	0.1498	0.02245	0.1100	No
Equiv. Diameter	0.1979	0.03916	0.0340	Yes
p53 & bcl-2				
P53 percent expression	-0.09517	0.009057	0.3138	No
P53 Grade	-0.1465	0.02146	0.1183	No
P53 positive	-0.1164	0.01356	0.2173	No
Bcl-2 grade	-0.1220	0.01488	0.1941	No
Bcl-2 positive	-0.1553	0.02411	0.0975	No
Spanning Tree Parameters				
Length MST	-0.1835	0.03369	0.0506	No
Sd average MST	-0.04763	0.002269	0.6148	No
Mean length MST	-0.2694	0.07257	0.0038	Yes
Min length MST	-0.2452	0.06011	0.0086	Yes
Max length MST	-0.2386	0.05692	0.0106	Yes
Sd MST	-0.2076	0.04308	0.0267	Yes
1 nearest neighbour	0.1200	0.01441	0.2033	No
2 nearest neighbour	-0.1334	0.01780	0.1571	No
3 nearest neighbours	0.1197	0.01434	0.2045	No

Mean Endothelial Area	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.2711	0.07349	0.0034	Yes
Morphometry Parameters				
VPE	0.2615	0.06837	0.0048	Yes
MNA	0.3461	0.001198	0.7135	No
sdNA	0.1354	0.01833	0.1491	No
Nuclear Perimeter	0.1692	0.02862	0.0707	No
Nuclear Roundness	0.04258	0.001813	0.6514	No
Fullness Ratio	-0.1405	0.01975	0.1342	No
Nuclear Length	0.1822	0.03321	0.0512	No
Nuclear Breadth	0.1449	0.02101	0.1222	No
Orthoferet	0.1432	0.02050	0.1269	No
Equiv. Diameter	0.1587	0.02519	0.0902	No
p53 & bcl-2				
P53 percent expression	-0.001991	3.964E-06	0.9832	No
P53 Grade	-0.07154	0.005117	0.4474	No
P53 positive	-0.09916	0.009833	0.2939	No
Bcl-2 grade	-0.08029	0.006446	0.3937	No
Bcl-2 positive	-0.1201	0.01443	0.2009	No
Spanning Tree Parameters				
Length MST	-0.1939	0.03759	0.0387	Yes
Sd average MST	-0.05899	0.003480	0.5330	No
Mean length MST	-0.2989	0.08932	0.0012	Yes
Min length MST	-0.2666	0.07109	0.0041	Yes
Max length MST	-0.2798	0.07828	0.0026	Yes
Sd MST	-0.2441	0.05958	0.0089	Yes
1 nearest neighbour	0.1315	0.01728	0.1632	No
2 nearest neighbour	-0.1346	0.01812	0.1533	No
3 nearest neighbours	0.1133	0.01284	0.2300	No

Appendix 5: Correlations Between Parameters - Continued

Max MVD Count Image Analysis	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.2389	0.05706	0.0137	Yes
Morphometry Parameters				
VPE	0.1877	0.03524	0.0540	No
MNA	0.04094	0.001676	0.6769	No
sdNA	0.2083	0.04337	0.0322	Yes
Nuclear Perimeter	0.1661	0.02759	0.0888	No
Nuclear Roundness	0.07155	0.005119	0.4661	No
Fullness Ratio	-0.1431	0.02047	0.1434	No
Nuclear Length	0.1731	0.02997	0.0760	No
Nuclear Breadth	0.2164	0.04684	0.0259	Yes
Orthoferet	0.1412	0.01995	0.1487	No
Equiv. Diameter	0.1383	0.01912	0.1575	No
p53 & bcl-2				
P53 percent expression	0.04679	0.002190	0.6355	No
P53 Grade	-0.01178	0.0001388	0.9046	No
P53 positive	-0.09496	0.009018	0.3352	No
Bcl-2 grade	-0.07450	0.005550	0.4479	No
Bcl-2 positive	-0.1201	0.01443	0.2200	No
Spanning Tree Parameters				
Length MST	-0.08753	0.007662	0.3746	No
Sd average MST	-0.05123	0.002624	0.6038	No
Mean length MST	-0.2067	0.04274	0.0343	Yes
Min length MST	-0.2054	0.04218	0.0356	Yes
Max length MST	-0.1955	0.03821	0.0457	Yes
Sd MST	-0.1531	0.02343	0.1190	No
1 nearest neighbour	0.1698	0.02884	0.0833	No
2 nearest neighbour	-0.1649	0.02718	0.0928	No
3 nearest neighbours	0.08845	0.007823	0.3696	No

Mean MVD Count Image Analysis	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.2623	0.06879	0.0066	Yes
Morphometry Parameters				
VPE	0.1846	0.03409	0.0581	No
MNA	0.03844	0.001478	0.6956	No
sdNA	0.1916	0.03670	0.0492	Yes
Nuclear Perimeter	0.1603	0.02569	0.1007	No
Nuclear Roundness	0.03784	0.001432	0.7001	No
Fullness Ratio	-0.1275	0.01626	0.1928	No
Nuclear Length	0.1691	0.02859	0.0831	No
Nuclear Breadth	0.2146	0.04604	0.0272	Yes
Orthoferet	0.1349	0.01819	0.1680	No
Equiv. Diameter	0.1401	0.01961	0.1522	No
p53 & bcl-2				
P53 percent expression	0.09788	0.009580	0.3205	No
P53 Grade	0.03996	0.001596	0.6843	No
P53 positive	-0.1154	0.01331	0.2412	No
Bcl-2 grade	-0.06509	0.004237	0.5074	No
Bcl-2 positive	-0.1272	0.016177	0.1939	No
Spanning Tree Parameters				
Length MST	-0.08394	0.007047	0.3946	No
Sd average MST	-0.06278	0.003941	0.5246	No
Mean length MST	-0.2278	0.05190	0.0194	Yes
Min length MST	-0.2279	0.05196	0.0194	Yes
Max length MST	-0.2237	0.05003	0.0218	Yes
Sd MST	-0.1787	0.03192	0.0682	No
1 nearest neighbour	0.1388	0.01927	0.1579	No
2 nearest neighbour	-0.1326	0.01758	0.1776	No
3 nearest neighbours	0.07595	0.005769	0.4412	No

Appendix 5: Correlations Between Parameters - Continued

Angiogenesis Mean MST	r	r^2	p	Significant
Mitotic Activity Index				
MAI	-0.2547	0.06035	0.0058	Yes
Morphometry Parameters				
VPE	-0.3101	0.09617	0.0004	Yes
MNA	-0.04090	0.001673	0.6507	No
sdNA	-0.1192	0.01420	0.1857	No
Nuclear Perimeter	-0.1378	0.01899	0.1254	No
Nuclear Roundness	-0.1145	0.01311	0.2036	No
Fullness Ratio	0.2092	0.04375	0.0192	Yes
Nuclear Length	-0.1574	0.02478	0.0796	No
Nuclear Breadth	-0.1401	0.01962	0.1192	No
Orthoferet	-0.09780	0.009564	0.2779	No
Equiv. Diameter	-0.1700	0.02891	0.0580	No
p53 & bcl-2				
P53 percent expression	-0.09213	0.008488	0.3088	No
P53 Grade	-0.07987	0.006379	0.3760	No
P53 positive	0.09980	0.009959	0.2701	No
Bcl-2 grade	0.01596	0.0002547	0.8598	No
Bcl-2 positive	0.04846	0.002348	0.5915	No
Spanning Tree Parameters				
Length MST	0.1383	0.01913	0.1255	No
Sd average MST	0.06125	0.003752	0.4992	No
Mean length MST	0.1924	0.03702	0.0323	Yes
Min length MST	0.1763	0.03108	0.0502	No
Max length MST	0.2671	0.07132	0.0027	Yes
Sd MST	0.2049	0.04197	0.0225	Yes
1 nearest neighbour	-0.1517	0.02300	0.0927	No
2 nearest neighbour	0.1296	0.01680	0.1514	No
3 nearest neighbours	-0.1046	0.01095	0.2475	No

Angiogenesis sd MST	r	r^2	p	Significant
Mitotic Activity Index				
MAI	-0.2605	0.06785	0.0033	Yes
Morphometry Parameters				
VPE	-0.2657	0.07062	0.0027	Yes
MNA	-0.08127	0.006605	0.3676	No
sdNA	-0.1422	0.02021	0.1138	No
Nuclear Perimeter	-0.1831	0.03354	0.0409	Yes
Nuclear Roundness	-0.01815	0.0003296	0.8407	No
Fullness Ratio	0.1355	0.01837	0.1318	No
Nuclear Length	-0.1983	0.03934	0.0266	Yes
Nuclear Breadth	-0.1974	0.03896	0.0274	Yes
Orthoferet	-0.1550	0.02404	0.0843	No
Equiv. Diameter	-0.1811	0.03279	0.0433	Yes
p53 & bcl-2				
P53 percent expression	-0.1020	0.01039	0.2599	No
P53 Grade	-0.09703	0.009414	0.2817	No
P53 positive	0.1548	0.02398	0.0859	No
Bcl-2 grade	-0.04192	0.001757	0.6426	No
Bcl-2 positive	0.03336	0.001113	0.7119	No
Spanning Tree Parameters				
Length MST	0.09495	0.009015	0.2942	No
Sd average MST	0.07106	0.005050	0.4329	No
Mean length MST	0.2140	0.04580	0.0170	Yes
Min length MST	0.2229	0.04969	0.0128	Yes
Max length MST	0.2446	0.05981	0.0062	Yes
Sd MST	0.1854	0.03437	0.0393	Yes
1 nearest neighbour	-0.1080	0.01166	0.2325	No
2 nearest neighbour	0.1215	0.01476	0.1789	No
3 nearest neighbours	-0.09907	0.009815	0.2736	No

Appendix 5: Correlations Between Parameters - Continued

Angiogenesis: Min MST	r	r^2	p	Significant
Mitotic Activity Index				
MAI	-0.1977	0.03907	0.0271	Yes
Morphometry Parameters				
VPE	-0.3014	0.09082	0.0006	Yes
MNA	-0.008958	8.025E-05	0.9210	No
sdNA	-0.09854	0.009711	0.2742	No
Nuclear Perimeter	-0.1162	0.01351	0.1968	No
Nuclear Roundness	-0.1365	0.01864	0.1290	No
Fullness Ratio	0.1909	0.03646	0.0329	Yes
Nuclear Length	-0.1354	0.01833	0.1322	No
Nuclear Breadth	-0.1087	0.01181	0.2276	No
Orthoferet	-0.07844	0.006153	0.3846	No
Equiv. Diameter	-0.1497	0.02242	0.0956	No
p53 & bcl-2				
P53 percent expression	-0.09682	0.009374	0.2847	No
P53 Grade	-0.07899	0.006240	0.3812	No
P53 positive	0.02285	0.0005222	0.8011	No
Bcl-2 grade	0.03610	0.001303	0.6894	No
Bcl-2 positive	0.06210	0.003856	0.4915	No
Spanning Tree Parameters				
Length MST	0.1511	0.02283	0.0939	No
Sd average MST	0.03966	0.001597	0.6595	No
Mean length MST	0.1459	0.02129	0.1058	No
Min length MST	0.1114	0.01241	0.2180	No
Max length MST	0.2605	0.06787	0.0035	Yes
Sd MST	0.2011	0.04042	0.0252	Yes
1 nearest neighbour	-0.1967	0.03870	0.0285	Yes
2 nearest neighbour	0.1608	0.02585	0.0745	No
3 nearest neighbours	-0.1321	0.01745	0.1436	No

Angiogenesis: Max MST	r	r^2	p	Significant
Mitotic Activity Index				
MAI	-0.2801	0.07844	0.0016	Yes
Morphometry Parameters				
VPE	-0.2999	0.08995	0.0007	Yes
MNA	-0.06105	0.003727	0.4988	No
sdNA	-0.1607	0.02583	0.0734	No
Nuclear Perimeter	-0.1835	0.03367	0.0405	Yes
Nuclear Roundness	-0.06431	0.004135	0.4762	No
Fullness Ratio	0.1799	0.03238	0.0446	Yes
Nuclear Length	-0.2001	0.04004	0.0253	Yes
Nuclear Breadth	-0.1971	0.03886	0.0276	Yes
Orthoferet	-0.1488	0.02213	0.0977	No
Equiv. Diameter	-0.1736	0.03014	0.0529	No
p53 & bcl-2				
P53 percent expression	-0.1073	0.01150	0.2357	No
P53 Grade	-0.1009	0.01019	0.2627	No
P53 positive	0.1318	0.01736	0.1447	No
Bcl-2 grade	0.006808	4.366E-05	0.9417	No
Bcl-2 positive	0.05036	0.002536	0.5770	No
Spanning Tree Parameters				
Length MST	0.1167	0.01361	0.1969	No
Sd average MST	0.07090	0.005026	0.4339	No
Mean length MST	0.2231	0.04979	0.0127	Yes
Min length MST	0.2201	0.04846	0.0140	Yes
Max length MST	0.2696	0.07268	0.0025	Yes
Sd MST	0.2064	0.04262	0.0214	Yes
1 nearest neighbour	-0.1291	0.01666	0.1530	No
2 nearest neighbour	0.1489	0.02217	0.0989	No
3 nearest neighbours	-0.1050	0.01102	0.2459	No

Appendix 5: Correlations Between Parameters - Continued

Angiogenesis: 1 nearest neighbour	r	r^2	p	Significant
Mitotic Activity Index				
MAI	-0.2242	0.05027	0.0123	Yes
Morphometry Parameters				
VPE	-0.3114	0.09696	0.0004	Yes
MNA	-0.1458	0.02125	0.1062	No
sdNA	-0.07149	0.005110	0.4301	No
Nuclear Perimeter	-0.1435	0.02060	0.1117	No
Nuclear Roundness	-0.09392	0.008821	0.2995	No
Fullness Ratio	0.1045	0.01093	0.2478	No
Nuclear Length	-0.1561	0.02437	0.0834	No
Nuclear Breadth	-0.1396	0.01948	0.1221	No
Orthoferet	-0.1193	0.01423	0.1869	No
Equiv. Diameter	-0.1950	0.03804	0.0299	Yes
p53 & bcl-2 Parameters				
P53 percent expression	-0.04926	0.002426	0.5885	No
P53 Grade	-0.03098	0.0009598	0.7327	No
P53 positive	-0.02463	0.0006065	0.7869	No
Bcl-2 grade	0.01080	0.0001166	0.9052	No
Bcl-2 positive	0.09377	0.008792	0.3003	No
Spanning Tree Parameters				
Length MST	0.08259	0.006821	0.3638	No
Sd average MST	0.06399	0.004095	0.4819	No
Mean length MST	0.09991	0.009982	0.2716	No
Min length MST	0.1149	0.01321	0.2055	No
Max length MST	0.1976	0.03904	0.0285	Yes
Sd MST	0.1355	0.01837	0.1350	No
1 nearest neighbour	-0.2319	0.05378	0.0099	Yes
2 nearest neighbour	0.1715	0.02943	0.0578	No
3 nearest neighbours	-0.1661	0.02579	0.0663	No

Angiogenesis : 2 Nearest Neighbours	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.1626	0.02645	0.0711	No
Morphometry Parameters				
VPE	0.2613	0.06828	0.0034	Yes
MNA	0.1921	0.03689	0.0326	Yes
sdNA	0.05756	0.003313	0.5254	No
Nuclear Perimeter	0.09293	0.008637	0.3046	No
Nuclear Roundness	0.1113	0.01239	0.2184	No
Fullness Ratio	-0.07724	0.005966	0.3938	No
Nuclear Length	0.09766	0.009537	0.2806	No
Nuclear Breadth	0.08334	0.006946	0.3574	No
Orthoferet	0.07770	0.006038	0.3910	No
Equiv. Diameter	0.1476	0.02178	0.1019	No
p53 & bcl-2				
P53 percent expression	-0.06624	0.004388	0.4666	No
P53 Grade	-0.07170	0.005140	0.4288	No
P53 positive	0.1549	0.02400	0.0871	No
Bcl-2 grade	-0.05722	0.003274	0.5279	No
Bcl-2 positive	-0.08192	0.006791	0.3657	No
Spanning Tree Parameters				
Length MST	0.02870	0.008240	0.7526	No
Sd average MST	-0.06055	0.003667	0.5058	No
Mean length MST	0.003537	1.251E-05	0.9690	No
Min length MST	-0.05400	0.002916	0.5531	No
Max length MST	-0.06712	0.004505	0.4607	No
Sd MST	-0.02240	0.0005016	0.8058	No
1 nearest neighbour	0.2309	0.05332	0.0102	Yes
2 nearest neighbour	-0.1817	0.03303	0.0442	Yes
3 nearest neighbours	0.1839	0.03381	0.0418	Yes

Appendix 5: Correlations Between Parameters - Continued

Angiogenesis 3 Nearest Neighbours.	r	r^2	p	Significant
Mitotic Activity Index				
MAI	0.08265	0.006831	0.3615	No
Morphometry Parameters				
VPE	0.04205	0.001769	0.6428	No
MNA	-0.1198	0.01434	0.1852	No
sdNA	0.005636	3.177E-05	0.9505	No
Nuclear Perimeter	0.03877	0.001511	0.6682	No
Nuclear Roundness	-0.005302	2.811E-05	0.9534	No
Fullness Ratio	-0.09926	0.009852	0.2727	No
Nuclear Length	0.05576	0.003109	0.5385	No
Nuclear Breadth	0.03791	0.001437	0.6760	No
Orthoferet	0.01688	0.002850	0.8524	No
Equiv. Diameter	0.03996	0.001597	0.6594	No
p53 & bcl-2				
P53 percent expression	0.1955	0.03822	0.0302	Yes
P53 Grade	0.1759	0.03095	0.0507	No
P53 positive	-0.2126	0.04520	0.0182	Yes
Bcl-2 grade	0.03635	0.001322	0.6885	No
Bcl-2 positive	-0.04674	0.002185	0.6062	No
Spanning Tree Parameters				
Length MST	-0.2141	0.04583	0.0174	Yes
Sd average MST	-0.002979	8.877E-06	0.9739	No
Mean length MST	-0.1767	0.03121	0.0506	No
Min length MST	-0.07687	0.005909	0.3981	No
Max length MST	-0.2271	0.05156	0.0115	Yes
Sd MST	-0.2027	0.04110	0.0245	Yes
1 nearest neighbour	-0.03642	0.001327	0.6892	No
2 nearest neighbour	0.06230	0.003881	0.4936	No
3 nearest neighbours	-0.08340	0.006955	0.3591	No

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Submitted Papers Under Consideration

1. Prognostic value of measurements of angiogenesis in serous carcinoma of the ovary.

Palmer JE, Sant Cassia LJ, Irwin C, Morris AG[2], Rollason TP. – Submitted to Int J Gynecol Pathol

2.The prognostic value of nuclear morphometric analysis in serous ovarian carcinoma.

Palmer JE, Sant Cassia LJ, Irwin CJ, Morris AG, Rollason TP. – Submitted to Int J Gynecol Pathol

3. p53 & bcl-2 Assessment in Serous Ovarian Carcinoma. Palmer Julia E, Sant Cassia Louis J, Irwin Clive J, Morris Alan G, Rollason Terence P. – Submitted to Int J Gynecol Cancer.

4. The prognostic and predictive value of syntactic structure analysis in serous carcinoma of the ovary. Palmer JE, Sant Cassia LJ, Irwin C, Morris AG, Janssen EAM, Baak JP, Rollason TP. - Submitted to J Clin Pathol

5. Manual and computer-aided image analysis measurements of angiogenesis and their ability to predict chemotherapy response in serous carcinoma of the ovary. Palmer Julia E, Sant Cassia Louis J, Irwin Clive J, Morris Alan G, Rollason Terry P. – Submitted to J Clin Pathol.

6. The prognostic and predictive value of mitotic activity index and volume percentage epithelial estimates in serous ovarian carcinoma. Palmer JE, Sant Cassia LJ, Irwin CJ, Morris AG, Rollason TP. – Submitted to Int J Gynecol Cancer.