Immunoediting and Angiogenesis in Ovarian Cancer

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For my beautiful wife, Melanie

Abstract

Advances in the treatment of ovarian cancer have had a limited impact on prognosis over recent decades. Alternatives to the traditional surgical and chemotherapeutic approach are being sought. Many novel therapies relate to a greater understanding of the molecular changes which occur during carcinogenesis and the development of targeted therapies to exploit these abnormalities. The aim of this thesis was to investigate the prognostic significance of markers relating to tumour immunology, angiogenesis and apoptosis, through the use of Tissue Microarray Technology.

339 cases of ovarian cancers diagnosed between 1982 and 1997 were assessed. Tumours were analysed immunohistochemically for expression of components of the IFNγ (IFNGR1, STAT1, p27, caspase 1), TRAIL (DR4 and DR5) and angiogenic (VEGF) pathways.

Loss of expression of IFNGR1 was an independent predictor of poor prognosis, although STAT 1 was not. High levels of cytoplasmic and nuclear p27 expression were associated with a reduced survival; cytoplasmic was independently prognostic. Tumours with reduced levels of caspase 1 had improved survival. These results suggest that only patients expressing IFNGR1 may benefit from IFNγ therapy and provides evidence of immunoediting in ovarian cancer.

DR4 and DR5 did not predict prognosis suggesting that the TRAIL pathway may not be significant in ovarian cancer apoptosis with implications for TRAIL-related therapy.

High levels of VEGF occurred infrequently, being an independent marker of poor prognosis. This may identify a group of patients who may preferentially benefit from anti-angiogenic therapy.

The thesis illustrates that ovarian cancers are heterogeneous and variations in expression of protein markers can predict tumour behaviour and stratify for therapy. Future targeted therapies may be selected on the basis of an immunohistochemical profile which predicts the pathways that are still functioning. New therapies as they arise should be trialed and targeted to tumours expressing the appropriate molecular markers.

Publications resulting from this thesis

Peer reviewed publications

Duncan, T. J., Rolland, P. *et al.* (2007). Loss of IFN gamma receptor is an independent prognostic factor in ovarian cancer. Clin Cancer Res 13(14): 4139-45.

Duncan, T.J., Watson, N.F.S. *et al.* (2007) The role of MUC1 and MUC3 in the biology and prognosis of colorectal cancer. World Journal of Surgical Oncology (5): 31

Duncan, T. J., Al-Attar, A. *et al.* (2008). Vascular endothelial growth factor expression in ovarian cancer: a model for targeted use of novel therapies? Clin Cancer Res 14(10): 3030-5.

Duncan, T.J., Al-Attar, A. *et al.* (2010) Subcellular localization of p27kip1 expression independently predicts poor prognosis in ovarian cancer. Int J Gyn Pathology 29(1): 8-18.

Poster presentations

Durrant, L.G., **Duncan, T.J.** *el al.* Novel biomarkers in Ovarian Cancer
Presented at the RCOG 1st Ovarian Cancer International Conference, March 2008

Duncan, T.J., Al-Attar, A. *et al.* Prognostic significance of VEGF expression in ovarian cancer
Presented at the British Gynaecological Cancer Society Meeting, November 2007

Duncan, T.J., Rolland, P. *el al.* The Evidence for Immunoediting in Ovarian Cancer
Presented at the British Gynaecological Cancer Society Meeting, November 2006

List of Abbreviations

AA	Ahmad Al-Attar
ABC	(strept)avidin-biotin-enzyme complex
ACTION	Adjuvant Chemotherapy Trial in Ovarian Neoplasia
APC	Antigen presenting cell
Bax	Bcl-2 associated X protein
BCR	B- cell receptor
C	Complement
Ca	Cancer antigen
CAP	Cyclophosphamide, adriamycin and cisplatin
CD	Cluster of differentiation
CDK	Cyclin dependent kinase
CEA	Carcinoembryonic antigen
CHORUS	CHemotherapy OR Upfront Surgery
CI	Confidence intervals
CK	Cytokeratin
CT	Computed tomography
CTLs	Cytotoxic T lymphocytes
CXCL	Chemokine ligand
DAB	3,3'-diaminobenzidine
DCs	Dendritic cells
DCGH	Derby City General Hospital
DD	Death domain
DFS	Disease free survival
DNA	Deoxyribonucleic acid
DSS	Disease specific survival
DPX	Distyrene, Plasticiser and Xylene
DR	Death receptor

EDTA	Ethylene diamine tetracetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EORTC	European Organisation for Research & Treatment of Cancer
ER	Oestrogen receptor
Erb	Endoplasmic reticulum binding
Fab	Fragment antigen binding
FADD	Fas associated protein with death domain
Fc	Fragment crystalline
FGF	Fibroblast growth factors
FFPE	Formaldehyde fixed paraffin embedded
FIGO	International Federation Gynaecologists & Obstetricians
FOXP3	Forkhead box protein 3
G1	Grade 1 - well differentiated
G2	Grade 2 - moderately differentiated
G3	Grade 3 - poorly differentiated
GF	Growth factor
GOG	Gynecologic Oncology Group
H&E	Haematoxylin and eosin
HER 2	Human epidermal growth factor receptor 2
HIF1α	Hypoxic inducible factor 1α
HLA	Human leukocyte antigen
HRT	Hormone replacement therapy
HNPCC	Hereditary nonpolyposis colorectal cancer syndrome
IDO	Indoleamine 2,3-dioxygenase
HR	Hazard ratio
HRP	Horse radish peroxidase

HRT	Hormone replacement therapy
ICAM	Intercellular adhesion molecule
ICE	IL1β converting enzyme
ICON	International Collaborative Ovarian Neoplasm
IFNGR	IFNγ receptor
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
IP	Intra-peritoneal
IRF	Interferon regulatory factor
IS	Intensity score
Jak	Janus tyrosine kinase
mAb	Monoclonal antibodies
MCA	Methylcholanthrene
MDT	Multi-disciplinary team
MHC	Major histocompatibility complex
MICA/B	Major histocompatibility complex class I polypeptide-related sequence A/B
MIG	Monokine induced by gamma interferon
MMP	Matrix metalloproteinases
MMR	Mismatch repair system
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
MVD	Microvessel density
NBF	Neutral buffered formalin
NHS	National Health Service

NK	Natural killer cells
NSS	Normal swine serum
OCS	Ovarian cancer screening
OCP	Oral contractive pill
OS	Overall survival
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCOS	Polycystic ovarian syndrome
PDGF	Platelet derived growth factor
PET	Positron emission tomography
PFS	Progression free survival
Pgp	Permeability glycoprotein
PID	Pelvic inflammatory disease
PKB	Protien kinase B
pRb	Retinoblastoma protein
PhR	Phil Rolland
PR	Progesterone receptor
PRR	Pattern recognition receptors
pTEN	Phosphatase and tensin homolog
RAG	Recombinase activating gene
RCT	Randomised controlled trial
RNA	Ribonucleic acid
RMI	Risk of malignancy index
RR	Relative risk
SCOTROC	Scottish Randomised TRial in Ovarian Cancer
SEER	Surveillance, epidemiology and end results
SOCS1	Suppressor of cytokine signalling 1
STAT1	Signal transducer and activator of transcription 1

TAA	Tumour associated antigen
TALs	Tumour associated lymphocytes
TAMs	Tumour associated macrophages
TAP	Transport protein
TBS	Tris buffered saline
TCR	T cell receptor
TD	Tim Duncan
TGFβ	Transforming growth factor β
Th	Helper T cell
TILs	Tumour infiltrating lymphocytes
TNF	Tumour necrosis factor
TMA	Tissue micro array
TRAIL	TNF related apoptosis inducing ligand
Treg	Regulatory T cell
TVS	Trans-vaginal ultrasound scan
UK	United Kingdom
UKCTOCS	United Kingdom Trial Of Ovarian Cancer Screening
UK-FOCSS	UK Familial Ovarian Cancer Screening Study
US	United States
VEGF	Vascular endothelial growth factor
vHL	Von Hippel-Lindau
WHI	Women's Health Initiative
WHO	World Health Organisation
WT-1	Wilms tumour 1 protein
XIAP	X-linked inhibitor of apoptosis protein

Acknowledgments

In line with the recommendations from the Intellectual Property Office of the UK, all figures included in this thesis, which have been adapted from previous works, have been fully referenced.

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1 Introduction

1.1 Ovarian Carcinoma

1.1.1 Epidemiology

Ovarian cancer is an uncommon disease which, unfortunately, is fatal in the majority of cases. The lifetime risk of ovarian cancer is approximately 2% and it is the fourth most common cause of cancer-related death in women within the UK, although its incidence worldwide is slightly less [1]. In 2005 almost 7000 cases of ovarian cancer were diagnosed, and until recently it was the most common gynaecological malignancy. Current data illustrates that it is now the second commonest after uterine cancer. The incidence of ovarian cancer has remained constant, but there has been an exponential rise in the rates of endometrial cancer [2]. The incidence of ovarian cancer appears to be slowly increasing with rates of 15 and 17 per 100,000 women in 1975 and 2005 respectively, which may simply reflect the proportionally older population in the UK compared with 30 years ago [2]. Ovarian cancer is predominately a disease of older, postmenopausal women. The incidence increases rapidly after the age of 50, with over 85% of ovarian cancers occurring above that age [2]. Less than 1% of epithelial ovarian cancers occur below the age of 20, with two thirds of the ovarian malignancies in these young patients being germ cell tumours [3]. Since germ cell tumours represent less than 5% of ovarian cancers and tend to present at an earlier stage, they make minimal impact on incidence and mortality rates [4].

The impact of age in ovarian cancer is not limited to incidence alone, increasing age is coupled to decreasing performance status which may limit the extent to which the most aggressive treatments can be instituted or sustained. Hence, increasing age and reduced performance status have been linked with poor prognosis [5], which is independent of other prognostic factors [6].

Worldwide there are almost 200,000 new cases of ovarian cancer annually. Incidence rates vary considerably with highest rates in Northern Europe and the USA and lowest rates in Africa and Asia. This may reflect the protective effect of higher parity in these areas [7]. There is conflicting data from migrant studies pertaining to the impact on risk of women moving from areas of contrasting incidence of ovarian cancer. A study of immigrants to Israel, from countries with a higher incidence of ovarian cancer, demonstrated that the risks were not significantly different to the background population [8], although other authors did not find this risk adjustment to occur [9]. These conflicting results illustrate the complexity of risk, which includes interactions between racial, cultural and socio-economic factors. The Surveillance, Epidemiology and End Results (SEER) programme registration data for cases diagnosed in the US over a 7 year period recorded an incidence of 17.2 and 11.8 per 100,000 women in Caucasian and non-Caucasian women respectively [10].

1.1.2 The Hallmarks of Cancer

Dysregulation of a number of pathways, *the hallmarks of cancer*, are necessary for oncogenesis. These include self sufficient growth signalling, insensitivity to inhibitory growth signals, evasion of apoptosis, limitless replication potential, sustained angiogenesis, tissue invasion and metastasis of tumour (Figure 1.1) [11].

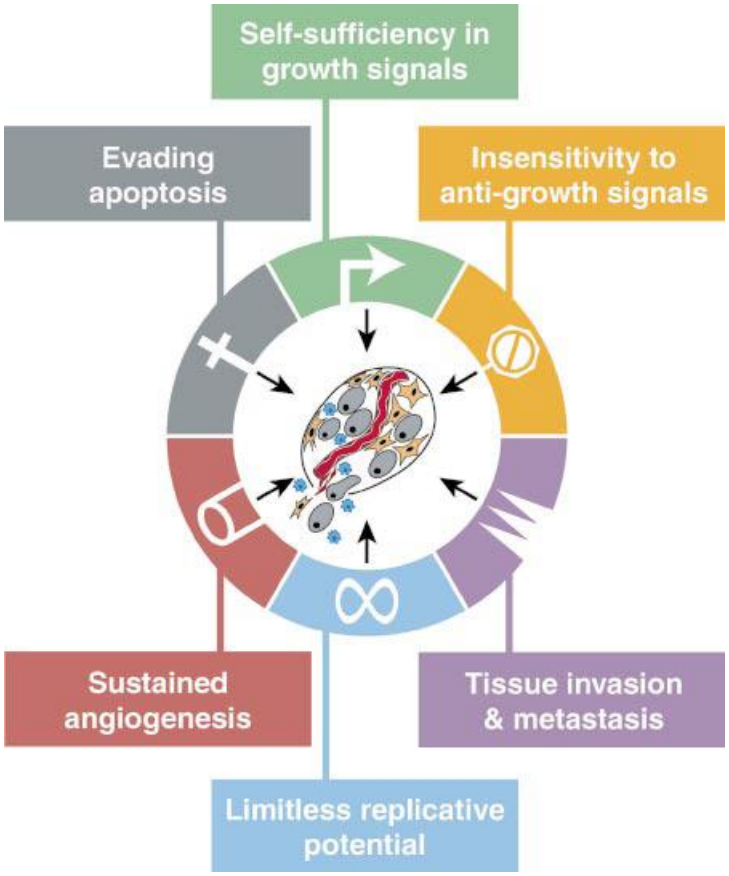


Figure 1.1 Acquired capabilities of cancer. Adapted from Hanahan *et al*. [11].

1.1.2.1 Self sufficiency in growth factor signals

One of the most obvious properties of cancer cells is the capacity for uncontrolled growth. Tumour cells' independence from normal growth factor (GF) control is in part due to the generation of its own GFs. This is in contrast to normal cells which only respond to exogenous GFs [11]. There are three molecular strategies by which tumours achieve growth autonomy, including alteration in extra cellular growth signals, receptor transducers of these signals and alteration of intracellular circuits which translate these signals into actions. Two illustrations of tumours secreting and responding to their own GFs in a positive feedback loop involve platelet derived growth factor (PDGF) and tumour growth factor α [11]. Receptor over-expression may enable tumour cells to be hyper-responsive to ambient levels of GF. For example, epidermal growth factor 1 (EGFR1) is over-expressed in ovarian cancer, with a subsequent negative impact on prognosis [12].

Downstream cytoplasmic circuitry can be altered, resulting in abnormally prolonged stimulation of cells following receptor signalling which is often related to the ras pathway. The ras pathway involves numerous proteins involved with cell proliferation [11].

1.1.2.2 Insensitivity to anti-growth signals

Within normal tissue, multiple anti-proliferative signals operate to maintain cellular quiescence. The retinoblastoma protein (pRb) acts as a central coordinator of anti-proliferative signals. pRb blocks proliferation by sequestering and inactivating transcription factors from the E2F group of genes which are involved in cell cycle regulation. These control the expression of genes essential for progression from G1 into S phase [13]. Disruption of the pRb pathway liberates E2Fs, thus allowing cellular proliferation and rendering cells unresponsive to anti-proliferative signals. TGFβ represents a widely studied anti-proliferative factor, which prevents phosphorylation and subsequent inactivation of pRb. TGFβ blocks phosphorylation of pRb by stimulation of $p15^{INK4B}$ and p21, which block cyclin:CDK complexes which are responsible for pRb phosphorylation [14]. Loss of TGFβ responsiveness has been demonstrated in tumours via down-regulation of TGFβ receptors [11].

1.1.2.3 Evading apoptosis

Apoptotic machinery is present in all cells and is divided into sensors and effectors. The sensors include cell surface receptors that bind to survival (e.g. IGF-1, IGF-2 and IL-3) and death factors (Fas ligand, TNFα and TRAIL) [11]. Many apoptotic signals converge on the mitochondria which produces cytochrome c, a potent catalyst of apoptosis. The release of cytochrome c is controlled through the Bcl-2 family of proteins which have either pro-apoptotic (Bax) or anti-apoptotic effects (Bcl-2 and Bcl-XL) [11]. The ultimate effectors of apoptosis are the caspases which can be stimulated through cytochrome c or death receptors.

Tumour cells all develop strategies to avoid apoptosis. One of the most common involves mutations and ultimately inactivation of p53, which is seen in over 50% of human cancers. Physiologically, p53 is a potent coordinator of apoptosis resultant from DNA damage, hypoxia and oncogenes hyper-expression [15]. Defects in the TRAIL pathway, the major p53 independent pathway for apoptosis, can also occur with disruptions to the TRAIL receptor and downstream signalling pathways [16]. In addition, over-activity of anti-apoptotic pathways can inhibit cell death. For example, the PI3kinase-AKT/PKB pathway can be hyper-stimulated through extra-cellular factors such as IGF1/2 and IL-3, intracellular factors, for example ras and via loss of pTEN (a tumour suppressor gene) [11]. A mechanism that inhibits cell death through fas, involving upregulation of decoy death receptors, has been demonstrated in tumour cells [17].

1.1.2.4 Limitless replicative potential

Normal cells have a limited number of possible cell divisions before they enter a state of senescence. Tumour cells have been shown *in vitro* to be immortalised with limitless replicative potential. This is thought to be in part due to defects in the pRb and p53 tumour suppressor systems [18], although effects on telomere maintenance may play a more significant role. Telomeres represent the ends of chromosomes, they act as the counting device for cell division. Each cell division leads to progressive loss of base pairs from the telomere and this erosion eventually leads to loss of protection of the chromosomal DNA. This in turn leads to fusion of chromosomal ends producing severe karyotype abnormalities resulting in cell death [19]. Telomere maintenance is seen in most cancer cells, with the upregulation of telomerase enzymes which add base pairs to the telomere. This preservation of the telomere effectively inactivates the cells usual counting mechanism for cell division [20].

1.1.2.5 Sustained angiogenesis

The oxygen and nutrients supplied by a tissue's vasculature are crucial for cell function and survival, necessitating that all cells are within 100μm of a blood capillary. The process by which new vessels form to ensure adequate blood supply to developing tissue, angiogenesis, is controlled by stimulatory signals such as vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGF).

The rapid growth of tumours requires a high level of angiogenesis. This has been demonstrated to be strongly associated with VEGF, since neutralising antibodies to VEGF inhibited tumour angiogenesis in mice models [21]. Tumours appear to activate the angiogenic switch through increased expression of VEGF and FGF with reduced expression of angiogenesis inhibitors such as thrombospondin-1 [22]. Different tumour types may use different strategies to activate the angiogenic switch, which makes the development of drugs to inhibit this process potentially challenging.

1.1.2.6 Tissue invasion and metastasis

The characteristic behaviour of tumour cells, which differs from normal cells, is the ability to invade tissue stroma and metastasise. Tumours utilise extra-cellular proteases and changes to the physical coupling of cells to achieve this.

1.1.3 Aetiology

The precise cause of epithelial ovarian cancer remains unclear; however, several factors are thought to play a role in its aetiology.

1.1.3.1 Environmental Factors

The fact that the incidence of ovarian cancer is highest in industrialised regions, such as Northern Europe and North America, points to an environmental link to the disease [7].

The exception to this rule is Japan, which in spite of being heavily industrialised has a low incidence of ovarian cancer, approximately 3 per 100,000 women. However, a higher rate of ovarian cancer is observed in Japanese immigrants in the USA, this risk eventually increases to equal that of their white counterparts within 2-3 generations [23]. This suggests that the causative carcinogen is likely to be within the immediate local environment, such as food.

Some have suggested that exposure to a chemical carcinogen might occur via transmission through the vagina, uterus and fallopian tubes to reach the ovaries. This potential route is feasible following studies by Venter et al using radio-nucleotides [24]. Whilst a variety of chemicals come into contact with the female genital tract, talc exposure is the most extensively studied. Some authors have described an increased risk with talc exposure, although they fail to produce a sound rationale for these findings [25]. In addition, other workers failed to find a similar association [26].

1.1.3.2 Dietary Factors

One of the major dietary differences among people in industrialised compared with non-industrialised countries is the high intake of meat and animal fat, which has been reported to be associated with an increase of ovarian cancer [27]. However, another study failed to show any significant effect of fat, protein, fibre or vitamins [28]. Cramer et al suggested that galactose may be associated with increased ovarian cancer risk, by inducing ovarian damage [29]. Others authors have failed to substantiate these conclusions, finding no increased risk with lactose, galactose or lactose intolerance [30]. Occasional reports have linked caffeine [31], alcohol [32] and tobacco [33] with an increased risk of ovarian cancer, but none have been confirmed by other investigators.

1.1.3.3 Reproductive factors

Parity

The protective effect of parity on ovarian cancer risk is well-documented. A European based study suggested a reduced risk in parous women compared with nulliparous, with a relative risk (RR) of 0.7. The relative risk was as low as 0.6 in grand multips [34]. A large study in the US estimated that the risk may be reduced by as much as 50% in parous women. This study also calculated that a 13-19% reduction in ovarian cancer risk occurred with each pregnancy [35].

Lactation

A number of studies suggest a modest protective effect from breast feeding. Whittemore et al demonstrated a long term reduction of 19% in women who breast fed. This remained significant when controlling for the effects of parity [35]. An Italian study suggested the risk reduction may be as high as 50% in women who have breast fed for over 12 months [36].

Infertility

The effect of infertility on ovarian cancer incidence is a particularly complicated relationship to investigate. This is primarily due to a number of confounding factors known to exert their own effects on ovarian cancer risk. For example, high parity and prolonged use of the oral contractive pill (OCP) are both protective against ovarian cancer, although for obvious reasons tend to occur less frequently in women with infertility. In addition, the use of medication to treat infertility further clouds the picture. When one considers the ovulation and gonadotrophin hypotheses relating to ovarian cancer (discussed later), it is clear that ovulation-induction agents which increase levels of gonadotrophin and rates of ovulation may increase the risk of ovarian cancer. Therefore, it is not surprising that case control studies demonstrated increased rates of ovarian cancer in women with infertility [35]. This risk is seen to be especially high with prolonged, multiple fertility treatments [37]. In view of the confounding factors mentioned, no convincing data exists to suggest that infertility per se is an independent risk factor for ovarian cancer.

1.1.3.4 Hormonal factors

A few case control studies have postulated that a late menopause is associated with an increase risk of ovarian cancer [38]. However, this data was not supported by a large cohort study [39], or a meta-analysis of six similar case control studies [40].

Early meta-analysis studies presented contradictory findings with regard to the impact of hormone replacement therapy (HRT). Some suggested that risk was increased [41], whilst others argued to the contrary [42]. Data from a large prospective cohort study has provided more robust evidence, and suggests that a slight increase in the incidence of ovarian cancer is seen with HRT (RR 1.2). This risk was substantially greater if HRT was used for over 10 years (RR 2.2). The risk was thought to be lower if combined preparations with progesterone were used [43]. However, the results of such studies may be academic following the Women's Health Initiative (WHI) study which concluded that a significant increase in the risk of breast cancer, pulmonary emboli, stroke and ischaemic heart disease existed with HRT. In the light of this report the use of HRT has reduced dramatically, especially long term use. Therefore, any potential effects of HRT on ovarian cancer incidence are likely to be minimal [44].

A large number of studies have illustrated the protective effects of the OCP on ovarian cancer risk. In the Oxford Family Planning Association study, the use of the OCP was associated with a 60% reduction in risk [45]. There was a trend towards increased protection the longer the OCP was used, and this has been supported by findings from a similar study [46].

1.1.3.5 Gynaecological Surgery

Hysterectomy with ovarian preservation is associated with up to a 33% reduction in rates of ovarian cancer [35]. In addition, data suggests tubal ligation can produce similar protective effects with reduced incidence [47] and mortality [48] from ovarian cancer.

The mechanism by which these surgeries reduce ovarian cancer risk is unclear. It has been suggested that this may reflect a degree of ovarian screening which occurs prior to

such operations. Others argue that tubal ligation and hysterectomy both prevent carcinogens such as talc from reaching the ovaries, although the potential impact of either of these mechanisms is unlikely to produce such large effects. Thirdly, it is possible that both procedures could affect ovarian function and ovulation through disruption of the ovarian blood supply. These theories remain to be substantiated.

1.1.3.6 Genetic factors

Between 5-10% of all ovarian cancer are thought to be hereditary; the result of mutations in a cancer predisposition gene. BRCA1 and BRCA2 gene mutations are the most commonly associated with familial ovarian and breast cancer, and are inherited in an autosomal dominant pattern. Mutations in the BRCA1 gene, located in chromosome 17, carry a 40-60% and 90% lifetime risk of ovarian and breast cancer respectively. In addition these women carry an increased risk of fallopian tube (RR 50-120) and primary peritoneal cancer (RR 50) [49]. BRCA2 mutations carry a lower risk of ovarian cancer in the order of 20-30% lifetime risk [50].

Ovarian cancer also forms part of the spectrum of cancers seen with hereditary nonpolyposis colorectal cancer syndrome (HNPCC). This is the result of mutation in the genes responsible for DNA mismatch repair and is expressed in an autosomal dominant pattern. Typically HNPCC is associated with early onset colorectal cancer, with the lifetime risk of ovarian cancer being approximately 12%. HNPCC is also associated with other extra-colonic cancers such as endometrial, upper gastrointestinal and renal [51].

Ovarian cancer is related to other familial cancer syndromes such as Peutz-Jegher and Multiple Endocrine Neoplasia type I [52]. These syndromes only account for those in which a genetic mutation has been identified; it is likely that there are others which remain to be discovered.

1.1.4 Mechanisms of ovarian carcinogenesis

There are four theories of causation for ovarian cancer, which attempt to explain the risk factors discussed previously.

1.1.4.1 Incessant Ovulation Hypothesis

This hypothesis suggests that the process of ovulation damages the ovarian epithelium. This can lead to erroneous DNA repair, especially if there is a short interval between individual ovulations. The progressive damage to the DNA from inaccurate repair could lead to the development of cancer [53]. This hypothesis would explain the protective effects seen with the OCP and pregnancy which both inhibit ovulation. A study supporting this theory suggested that each year of ovulation equated to a 20% increase in lifetime risk of ovarian cancer in women under thirty [54]. The protective effects seen with the progesterone only pill which does not inhibit ovulation cannot be accounted for in this model [30].

1.1.4.2 Gonadotrophin hypothesis

This hypothesis originates from both animal and epidemiological studies. Mice exposed to high levels of gonadotrophin are shown to have an increased risk of ovarian cancer [55]. Cramer et al suggest that the raised level of gonadotrophin does not cause ovarian cancer per se, but induces ovarian secretion of oestrogen which induces proliferation and transformation of ovarian epithelium. They suggest that this hypothesis would explain the reduced risk seen in parous women and those using the OCP, both of whom have low levels of gonadotrophin. In addition, it explains the increased incidence seen in postmenopausal women and those with Polycystic Ovarian Syndrome (PCOS), where gonadotrophin levels are significantly elevated [27].

1.1.4.3 Androgen hypothesis

Risch et al suggest that androgens may play a role in ovarian carcinogenesis, since the ovary is known to contain androgen receptors, and androgens have been shown to stimulate growth in ovarian cancer cell lines [56, 57]. Epidemiologically the theory is supported by the increased risk seen with PCOS when levels of androgens are increased [58]. In addition, the OCP is known to reduce androgen levels, which could potentially explain its protective effects [59].

1.1.4.4 Progesterone hypothesis

Progesterone is an anti-mitotic, which has been shown to increase wild type p53 tumour suppressor gene and induce apoptosis in ovarian cancer cell lines [57]. The rate of ovarian cancer in hens has been reduced by administration of progesterone [60]. Epidemiological data supports a role for progesterone through the protective effects of pregnancy, OCP and the progesterone only pill, all of which increase circulating levels of progesterone [61].

1.1.5 Pathology

1.1.5.1 Histological Types of Epithelial Ovarian Cancer

Primary ovarian neoplasia can arise from a large variety of cell types and can behave in a benign, malignant or borderline (low malignant potential) manner. The diversity of disease is highlighted by the classification of over 120 subtypes, with over 30 being malignant [62]. Ovarian epithelium is derived embryologically from the celomic epithelium, which gives rise to the Mullerian ducts from which the epithelium of the fallopian tube, endocervix and endometrium are derived. Hence, differentiation of ovarian neoplastic cells can produce various histological types. Tumours differentiating along the fallopian tube pathway

produce serous subtype, endocervical produce mucinous subtype, and cells from the endometrial pathway produce endometrioid and clear cell subtypes.

Serous cystadenocarcinoma

The serous subtype of ovarian cancer is the most common, accounting for 40-50% of cases [5, 10]. They are associated with a poor prognosis compared with other subtypes. However, this is likely to be due to their relatively late presentation. When stage is corrected for, there is no difference in prognosis [63].

Endometrioid adenocarcinoma

Endometrioid tumours of the ovary are malignant in 80% of cases, and represent 25% of all ovarian cancers. They have a similar appearance to colonic metastases which are known to spread trans-celomically to the ovaries [64]. Ovarian endometrioid tumours have a strong association with both pelvic and ovarian endometriosis with the suggestion that these tumours may in fact represent malignant transformation in endometriosis [65]. A concomitant endometrial carcinoma is present in up to 20% of cases, often raising a dilemma as to whether these tumours are synchronous separate primaries or metastases from one organ to another. If the tumours are confined to each organ then the prognosis is good, favouring the independent primary hypothesis [65].

Mucinous cystadenocarcinoma

Traditionally mucinous cancers accounted for approximately 10% of all ovarian cancers, although this rate is now thought to be lower. Overall they have a relatively favourable prognosis; this is probably due to the preponderance of early stage at presentation [5, 10]. Typically, they can present as huge pelvic masses, and tend to be resistant to platinum chemotherapy. Those that do present late, however, tend to have a poorer prognosis in comparison to other subtypes [66].

Clear Cell adenocarcinoma

Clear cell cancers represent approximately 5% of ovarian cancers [5, 10]. They are strongly associated with endometriosis, even more so than endometrioid subtypes [67]. In comparison with serous cancers they tend to present earlier; however, they are more

likely to display platinum insensitivity. In consequence, when correcting for stage, their prognosis is poor when compared with other subtypes [5, 68].

Undifferentiated carcinomas

Undifferentiated ovarian cancer incidence varies considerably between studies and accounts for between 5-25% of all ovarian cancers They present at a late stage, are chemoresistant and as such carry a very poor prognosis [5, 10].

The subtypes described above reflect the genuine heterogeneity which occurs in ovarian cancer. The influence of the histological type on prognosis is difficult to predict since strong independent factors such as stage and degree of cytoreducibility at surgery contribute greatly to prognosis and compound any potential effects exerted by the subtype of cancer. This heterogeneity also makes ovarian cancer studies more challenging, especially as some suggest that each subtype is a separate disease entity and should be investigated as such.

1.1.5.2 Histological Grade

The histological grade of a tumour refers to a system used to quantify the microscopic appearance of the cells into well (G1), moderate (G2) and poorly differentiated (G3). Numerous systems exist to produce this score and include consideration of nuclear atypia, number of mitoses and architectural appearance of the cells. The grading systems aim to provide prognostic information regarding the tumour; however, due to the heterogeneity of ovarian cancers the same system does not appear applicable to all subtypes. For example, only nuclear atypia provides prognostic information in clear cell tumours, whereas all three components are useful in serous, endometrioid and mucinous subtypes [69]. Overall grading is not considered to be a useful independent prognostic factor, although it can be useful on a sub-stage level, especially in early stage disease [5].

Immunohistochemical characteristics

Many metastatic adenocarcinomas can involve the ovary such as colorectal, gastric, breast, pancreas, gall bladder, renal and other gynaecological malignancies. Morphologically, these tumours may appear very similar and as such a potential metastatic origin should always be considered when assessing an ovarian tumour. This is especially true of mucinous ovarian tumours; a recent study demonstrated less than 3% of true ovarian cancers were mucinous. This figure is much lower than in earlier literature, suggesting that metastatic mucinous tumours have previously been incorrectly deemed ovarian in origin [70]. Since such similarities exist in tumour morphology light microscopy can be unreliable. Immunohistochemistry provides crucial additional information on a tumour's characteristics and likely origin, which will clearly influence management and subsequent prognosis [71].

The cornerstones of ovarian immunohistochemistry are antibodies specific to cytokeratin 7 and 20, Carcinoembryonic Antigen (CEA) and Cancer Antigen 125 (Ca125). Mucinous ovarian cancers tend to be CK7+, Ca125+ and CEA-, CK20-, although there are always exceptions. This most commonly occurs in mucinous tumours in which CK7 and CK20 can be positive in both colorectal and ovarian cancer [71]. However, ovarian tumours tend to be diffusely positive for CK7 and only focally positive for CK20, with the opposite occurring in colorectal tumours [72].

Extra-colonic ovarian metastases can be more challenging to distinguish immunohistochemically since most are CK7+ and CK20-. The use of additional markers such as oestrogen and progesterone receptor (ER and PR) as well as gross cystic disease fluid protein-15 can be used to distinguish a breast primary, in which these markers are usually positive [73]. With pancreatic cancers, Wilms Tumour 1 is usually negative, with the majority of ovarian cancers being positive [74]. Renal clear cell cancers can mimic ovarian cancers, when the use of CD10 a renal cell carcinoma antigen can be useful [75].

Practically, the determination of the site of tumour origin does not rely solely on histological morphology and immunohistochemical profiles but on the combination of these features coupled with clinical and radiological information. The importance of a cohesive and thorough assessment of all these components highlights one of the major roles of an oncology multi-disciplinary team (MDT) meeting.

1.1.5.3 Stage

Staging in ovarian cancer is traditionally surgical i.e. based on the operative findings at the commencement of the procedure. This is typically performed through a longitudinal midline abdominal incision, although more recently laparoscopic staging has been performed [76]. With the increasing role of neoadjuvant chemotherapy (discussed later), there is a trend towards image guided staging, typically with computed tomography (CT) and integrated positron emission tomography and CT (PET/CT) [77]. However, it should be noted that currently the gold standard for staging of ovarian cancer is a laparotomy.

Ovarian cancer most commonly spreads trans-peritoneally, although lymphatic spread is also seen. Therefore, close inspection and biopsies of the peritoneal surfaces including the diaphragm, para-colic gutters and omentum are required to detect microscopic metastases. In addition, ascites or peritoneal washings are assessed cytologically. The role of both pelvic and para-aortic lymphadenectomy in ovarian cancer staging remains controversial, particularly in advanced disease. In presumed early stage disease the risk of occult metastases in lymph nodes is approximately 10-20% [78, 79]. Therefore, without lymph node sampling many patients would be under-staged. This is of particular importance when determining adjuvant treatment following surgery and has been implicated in producing contradictory results between clinical trials in which thorough staging was inconsistently performed [80].

The major challenge to treating ovarian cancer effectively relates to the vast majority of patients presenting with advanced stage disease. Over the period in which the cohort of

patients in this study were diagnosed, a number of ovarian cancer series were reported in which over half the cases presented at stage III-IV [3, 10, 81-83].

There exists a number of staging systems for ovarian cancer, although the International Federation of Gynaecologists and Obstetricians (FIGO) system is the most widely used. This was adapted into its current format in 1987 [84].

In essence, stage I disease is limited to the ovaries, stage II is limited to the pelvis, stage III extends outside the pelvis and stage IV has distant metastases usually in the chest or liver parenchyma (Figure 1.2 and Figure 1.3 and appendix 1). Clinical staging is vitally important for planning of treatment and represents the strongest independent predictor of prognosis.

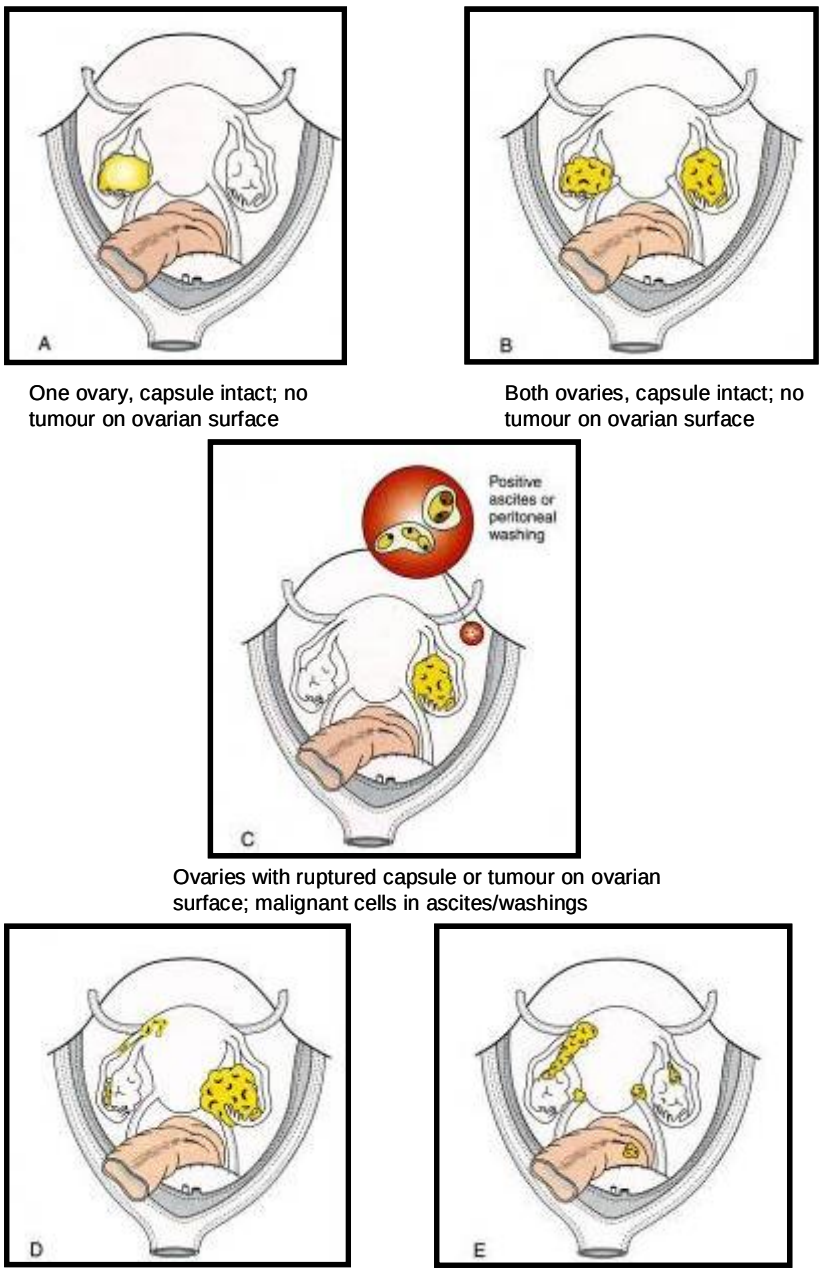
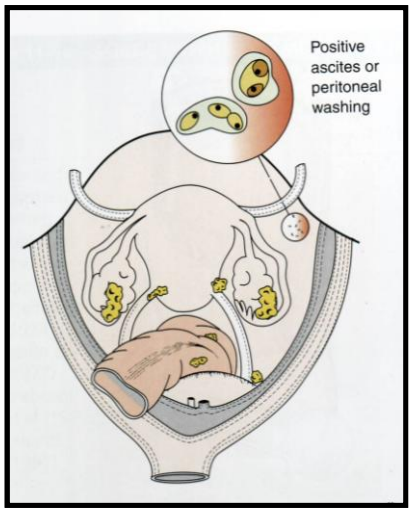
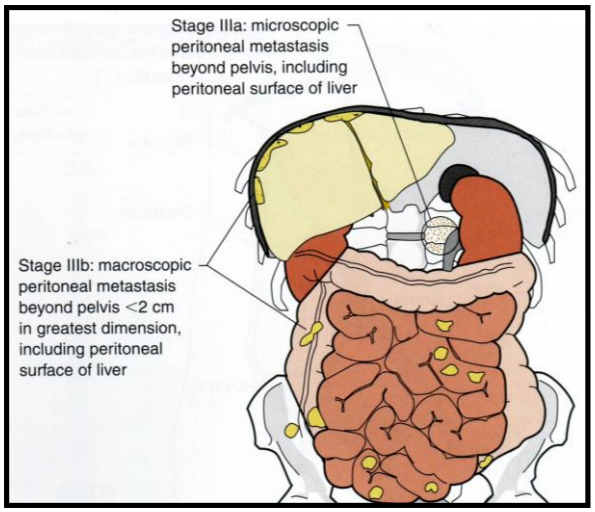


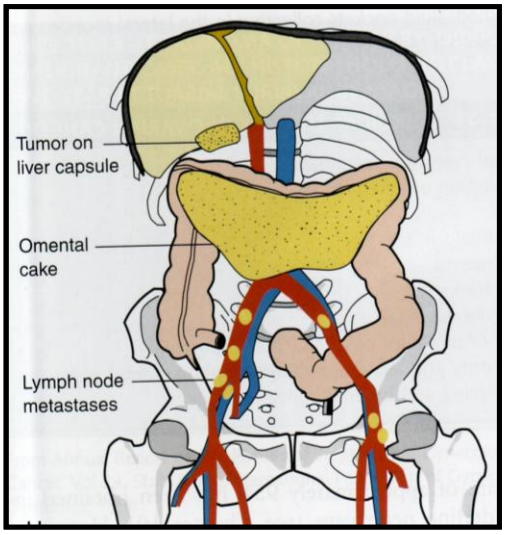
Figure 1.2 Ovarian Carcinoma Staging. A, Stage Ia. B, Stage Ib, C. Stage Ic. D, Stage IIa. E, Stage IIb. Illustration adapted from DiSaia et al. Clinical Gynecologic Oncology [85].



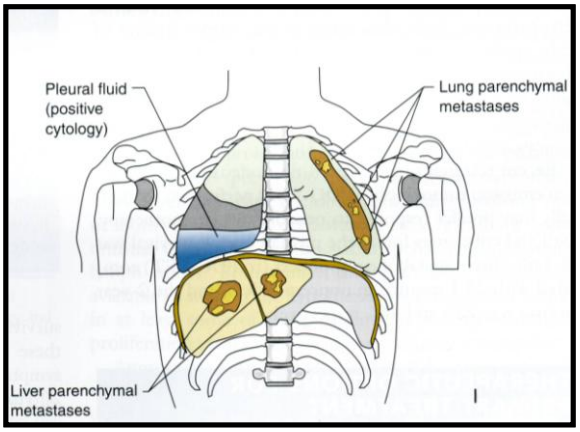
A: Implants in other pelvic tissues with malignant cells in ascites/washings



B: Microscopic or <2cm deposits beyond pelvis including peritoneal surface of liver



C: Peritoneal deposits beyond pelvis >2cm or regional lymph nodes metastasis



D: Liver parenchymal metastases, lung involvement or distant metastases

Figure 1.3 Ovarian cancer staging A: Stage IIc, B: Stages IIIa and IIIb, C: Stage IIIc, D: Stage IV. Illustration from adapted from DiSaia et al. Clinical Gynecologic Oncology [85].

1.1.6 Diagnosis

1.1.6.1 Symptoms and signs

Symptoms of ovarian cancer are typically non-specific and vague leading to late presentation and ultimately poor prognosis. This has led to ovarian cancer being named

“the silent killer”, although this definition is not strictly true since 95% of patients have symptoms prior to diagnosis [86]. The vagueness of these symptoms does, in the majority of cases, lead to them being ignored or discounted by both the patient and the General Practitioner.

In the early stage of disease, premenopausal women may develop irregular menses. If a pelvic mass is compressing the bladder or rectum then urinary frequency or constipation may develop. Acute symptoms such as pain secondary to ovarian rupture or torsion are rare.

In the advanced stages, symptoms are usually related to ascites and omental and bowel metastases. These symptoms include abdominal distension, bloating, nausea and constipation. Not surprisingly, these symptoms are often attributed to irritable bowel syndrome until the clinical signs become more obvious. In a survey of almost 2000 women with ovarian cancer, 70% had gastrointestinal symptoms, 58% pain, 34% urinary symptoms and 26% pelvic discomfort [86].

Classical signs which highlight the possibility of ovarian cancer in postmenopausal women are the detection of a pelvic mass, often fixed, in association with ascites (shifting dullness) and a palpable mass in the upper abdomen (omental cake). However, only 3% of palpable masses of less than 5cm are malignant in this population. The risk is clearly increased with the detection of ascites and an omental cake [87].

1.1.6.2 Investigation

The gold-standard investigation and staging of suspected ovarian cancer is via a laparotomy; however, this carries significant morbidity especially when a number of suspected cancers prove to be benign. Further investigations are divided into radiological, haematological and histopathological.

Serum tumour markers are often used when a pelvic mass is detected. Ca-125 is an antigen derived from celomic epithelium and is the classical serum marker for ovarian cancer. Unfortunately, it is relatively non-specific with a number of benign conditions

causing elevated levels, including endometriosis, pelvic inflammatory disease, menstruation, pregnancy, and rheumatoid arthritis. In addition, various other malignancies are able to produce an elevated Ca-125 such as colorectal and breast cancers [88]. Bast *et al* demonstrated that elevations in serum Ca-125 levels were seen more frequently in women with ovarian carcinoma (82%) compared to women in the general population (1%), women with benign disease (6%) or women with non-gynaecological cancers (28%) [89]. Ca-125 is also useful as a guide to chemotherapy response and is discussed later.

Other tumour markers can be useful in highlighting whether a pelvic mass could potentially be a metastasis from another primary site. For example, CEA (colorectal) [90], Ca-153 (breast) [90], and Ca-19.9 (pancreatic) [91].

The first line imaging modality for a pelvic mass is ultrasound. This can usually determine the origin of the mass, although in difficult cases pelvic Magnetic Resonance Imaging (MRI) can be useful [92]. In those patients with an ovarian mass, various features can increase the suspicion of ovarian cancer (see RMI below). Although laparotomy is the gold-standard for staging of ovarian cancer, cross sectional imaging can be useful preoperatively in treatment planning. For example, the site of surgery (Cancer Unit or Centre) and who will be operating (sub-specialty trained Gynaecological Oncologist or General Gynaecologist). In addition, with increasing use of neoadjuvant chemotherapy image guided staging is utilised. There appears to be no difference in the accuracy of CT and MRI for ovarian cancer staging [93].

Image guided biopsies are increasingly being used to confirm the diagnosis of ovarian cancer, either through ultrasound guided aspiration of ascitic fluid providing cytology, or a CT guided biopsy of an omental cake or pelvic mass to provide tissue for histological analysis. In general, a tissue diagnosis is preferred, however, adenocarcinoma cells in ascites cytology coupled with an elevated Ca-125 would be deemed adequate for the diagnosis of ovarian cancer prior to neoadjuvant chemotherapy.

1.1.6.3 Risk of Malignancy Index

An elevated Ca-125 level, as previously mentioned, is associated with ovarian cancer. In addition there are various features on an ultrasound scan which raise the suspicion that an ovarian mass may be malignant. The Risk of Malignancy Index (RMI) represents an algorithm which incorporates the ultrasound features of a pelvic mass with the Ca-125 level and the patent's menopausal status to produce a quantitative assessment of the risk of an individual mass being malignant [94]. The RMI has been shown to give a sensitivity of 85% and specificity of 97% for predicting ovarian cancer if the score is greater than 200 [94]. This has been tested on retrospective and prospective data with encouraging results [94, 95]. The model has been subject to some adaptation into the most widely used format at present (Table 1.1) [96]. The advantage of such a predictive model is that it allows effective planning of when, where and who performs a patient's surgery. This is particularly pertinent as observational data suggests that there is an improved outcome from ovarian cancer when initial surgery is carried out by a Gynaecological Oncologist in a Cancer Centre [97]. This model has been successfully used to triage patients in peripheral units for referral into Cancer Centres in the UK [98].

Table 1.1 The modified risk of malignancy index developed by Tingulstad et al [96].

Parameter	Score
Ultrasound appearances	
Multilocular cyst	None or one features = 1 Two or more features = 3
Solid areas	
Bilateral lesions	
Ascites	
Intra-abdominal metastases	
Menopausal status	
Post-menopausal	3
Pre-menopausal	1
Ca-125 level	U/ml

RMI= ultrasound score x menopausal score x Ca-125

1.1.7 Screening

Ovarian cancer typically has a late presentation which is thought to account for the high case: fatality ratio, since early stage disease is associated with an 85% 5-year survival. Ovarian cancer screening (OCS) aims to detect cases early, with subsequent benefits from improved survival rates. As with any screening test there are a number of hurdles including adequate sensitivity, specificity and acceptability of the test. Cost benefit considerations and ultimately the effectiveness at reducing mortality rates need to be assessed. At present there is no conclusive evidence to suggest that OCS is effective. Preliminary data exists from a randomised controlled trial (RCT) of 22 000 women. Out of those women within the trial who developed ovarian cancer those that participated in screening had an improved median survival (72 months) compared with controls (42 months) [99]. Additional evidence from a Japanese OCS study showed an increased proportion of stage I cancers in screened women compared with unscreened women (59% vs. 30%) [100].

OCS consists of two components, firstly a serum Ca-125 measurement and secondly a trans-vaginal ultrasound scan (TVS). These have been used individually and in combination. Significant improvements have been made to these tests. For example, using serial Ca-125 measurements, as opposed to an isolated test, allows trends in the level to be detected. This is thought to improve specificity, since only levels which continue to rise are indicative of ovarian cancer. Many elevated levels which then subsequently fall are the result of a benign process [101]. Use of abnormal ovarian morphology is more predictive of ovarian cancer than ovarian volume [102]. Thirdly, screening only postmenopausal women who have a proportionally higher incidence of ovarian cancer would make such screening more specific. In addition, conditions which can lead to a misleading abnormality on TVS, or a benign Ca-125 elevation such as endometriosis, fibroids, pelvic infection (PID), pregnancy and menstruation do not commonly occur in this age group [102].

There are two populations to consider for screening, the general population and those with a strong family history of ovarian and/ or breast cancer. The aim is to develop a strategy that has greater than 90% sensitivity for detecting ovarian cancer in asymptomatic women, with an acceptable false positive rate. This rate is often set at a maximum of ten operations in women without ovarian cancer for every case of ovarian cancer detected [102]. Currently, there are two large RCTs in the general population to assess the impact of OCS on ovarian cancer mortality. The Prostate, Lung, Colorectal and Ovarian Cancer screening study is a US-based study of over 74 000 women. The women being screened have an annual Ca-125 and TVS, and follow up will continue for at least 13 years following randomisation [103]. The United Kingdom Trial Of Ovarian Cancer Screening (UKCTOCS) is a similar study with 200 000 women; results are expected by 2012 [102].

In the high risk population with a strong family history, there is again sparse data to support screening at present. The multi-centre UK Familial Ovarian Cancer Screening Study (UK-FOCSS) is underway to assess potential benefits in this challenging patient

group. The additional problem posed in this population is the relatively high proportion of primary peritoneal cancers (a subgroup of ovarian cancer) occurring. These are not reliably detected by Ca-125 and TVS [104].

1.1.8 Surgery

Surgery in ovarian cancer varies according to the stage of disease. In presumed early stage disease it is vital to perform thorough surgical staging, whereas in advanced disease optimal debulking of the tumour represents the main surgical goal.

1.1.8.1 Early stage disease

Ovarian cancer can only be termed early stage if a thorough staging laparotomy has been performed. A series by Young *et al* highlighted the importance of staging in patents with disease which appears to be confined to the ovaries. Those patients undergoing suboptimal staging had a 5 year survival of only 60% compared with survival of over 90% in those patients in whom thorough staging had been performed. This difference is likely to be due to inclusion of some patients with more advanced disease in the group with inadequate staging, where occults metastases could be missed. Clearly, inclusion of these cases would lead to a lower than expected survival rate [105].

In a patient where an ovarian cancer is suspected, a frozen section of the mass enables an intra-operative diagnosis to be made, with subsequent staging if necessary. There are limitations to frozen section with sensitivity being reported to be as low as 65% [106]. Optimal staging, if an ovarian cancer is confirmed, consists of peritoneal fluid for cytology, biopsies of suspicious areas on the peritoneal surfaces, including the diaphragm, omentectomy, pelvic and para-aortic lymph node dissections. Lymph node dissections are particularly important since up to 15% of apparent stage I cancers have nodal metastases [107]. Finally, a total abdominal hysterectomy and removal of the remaining ovary is performed. In carefully selected cases, where the woman wishes to retain her fertility, a unilateral oophorectomy can be performed with preservation of the uterus and

contralateral ovary. Careful follow up is required with Ca-125 and TVS. Completion surgery is usually recommended after child bearing.

1.1.8.2 Advanced disease

In patients with advanced ovarian cancer the surgical goal remains the removal of as much disease as possible at primary laparotomy. This approach is supported by numerous studies suggesting the degree of debulking surgery is an independent prognostic factor (Figure 1.4) [108]. Each 10% increase in maximum cytoreduction was associated with a 5.5% increase in median survival time. The definition of maximal cytoreduction varies from tumour nodules of <2cm through to no visible macroscopic disease [109-111]. The measurement of residual disease is inaccurate and frequently underestimated [112]. It may also be more relevant to measure total residual tumour volume as opposed to the maximum diameter of the residual disease nodules [108].

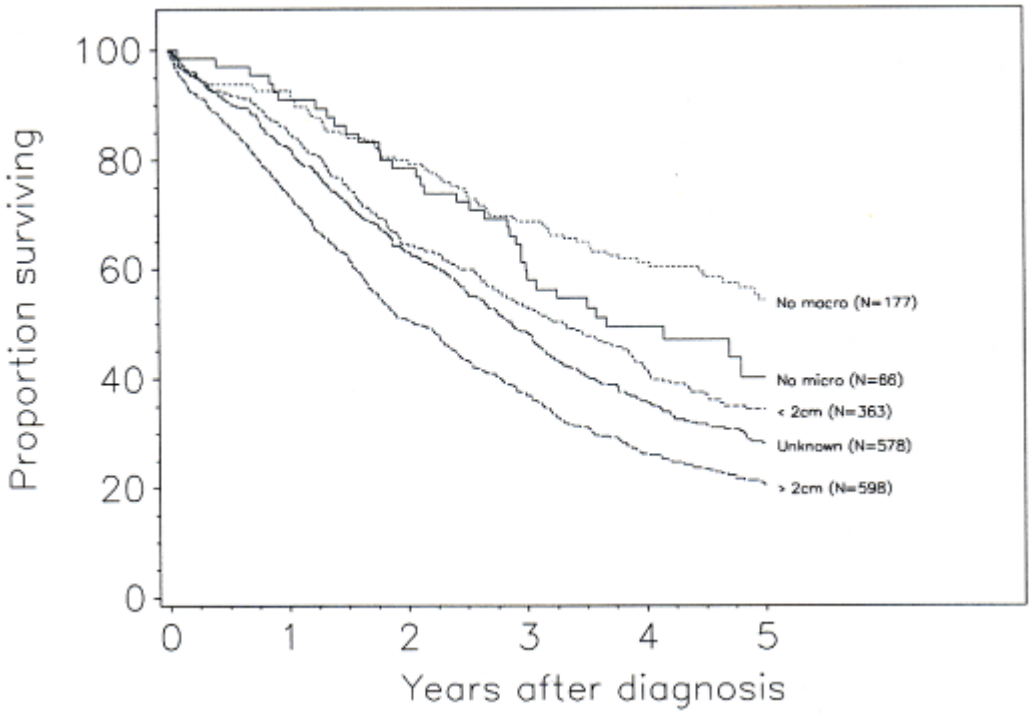


Figure 1.4 Survival of patients with stage IIIC epithelial ovarian cancer based on the maximum size of residual tumour after laparotomy and tumour resection. Adapted from Heintz *et al* (2003) [113].

The rationale for cytoreductive surgery relates to three theoretical considerations - physiological benefits, improved tissue perfusion and growth fraction, and enhanced immunological competence of the patient.

Often patients' symptoms improve after a large volume of tumour and ascites is removed, alleviating nausea, discomfort and satiety. The reduction in intestinal symptoms may improve the nutritional status of the patient prior to further adjuvant treatment.

Large bulky tumours tend to contain areas which are necrotic and poorly vascularised, which will be exposed to suboptimal concentrations of chemotherapeutic agents. In addition large tumour masses tend to contain a high proportion of non-dividing cells which will be resistant to the mechanisms employed by most chemotherapy. Debulking surgery produces smaller volumes of disease with improved blood supply and high growth fraction therefore allowing greater exposure of potentially more sensitive tumour cells to chemotherapy.

The fractional kill hypothesis of Skipper [114] postulates that a constant proportion of cells are killed with each cycle of treatment. Therefore the smaller the initial tumour burden at the start of treatment, the less cycles of treatment will be required to eradicate all the tumour cells. Hence, if a tumour burden is reduced prior to chemotherapy i.e. debulked, fewer cycles of chemotherapy will be needed to eradicate disease. The less treatment exposure needed reduces the potential for drug resistance to occur. However, large tumours have a greater chance of having inherent resistance prior to treatment, potentially negating this benefit. Another benefit of cytoreduction is related to enhanced immunological responses, since large tumours exert a greater non-specific immunosuppressive effect. In addition, recognition of tumour-associated antigens may be overwhelmed by large numbers of tumour cells [115].

In spite of the evidence suggesting a role and survival advantage from maximal surgical effort and optimal cytoreduction some still question its role in advanced ovarian cancer [116]. There is no prospective data supporting its role, and the ability to optimally debulk a tumour may be a reflection of the biological aggressiveness of the tumour as opposed to

the skill of the surgeon. The morbidity and impact on quality of life may be unacceptability high after radical surgery, especially when newer chemotherapy agents have a greater effect on “log kill” than surgery. Overall, the evidence regarding the optimum surgical approach to ovarian cancer remains controversial, with evidence and biological hypothesises promoting both radical and conservative approaches.

1.1.8.3 Gynaecological oncology sub-specialisation

There is strong evidence to suggest that the training and experience of the surgeon treating patients with ovarian cancer influences survival. Patients are more likely to be optimally staged, achieve optimal cytoreduction and ultimately survive longer if treated by a gynaecological oncologist as opposed to a general surgeon or general gynaecologist [97, 117]. This forms the basis for sub-specialisation of gynaecological cancer care in the UK with centralisation and coordination of care within recognised Cancer Centres.

1.1.9 Chemotherapy

1.1.9.1 Early stage disease

Adjuvant treatment in early stage ovarian cancer is determined by the grade and sub-stage of disease. Guthrie *et al* established the same prognosis with and without adjuvant chemotherapy in stages 1A and 1B (grade 1 and 2), with both groups having a 5 year survival of over 95%. In those patients with poorly differentiated tumours (grade 3), or stage 1C-IV, further treatment was deemed necessary [118].

The evidence for adjuvant treatment of early stage ovarian cancer is derived mainly from two parallel RCTs, the International Collaborative Ovarian Neoplasm Trial 1 (ICON1) and the Adjuvant Chemotherapy Trial in Ovarian Neoplasia (ACTION) [80]. In the ICON1 trial stage I and IIA disease was included, however, optimal surgical staging was not required to enter the trial. Hence, an unquantifiable number of patients would have had undetected occult metastases i.e. stage III disease. Patients were randomised to platinum based chemotherapy or observation. There was an improved 5 year survival in the treatment arm

of 73% compared with 63% in the control arm. In the ACTION trial about a third had optimal staging, and randomisation was to either platinum chemotherapy or observation, similar to ICON1. There was a survival advantage in the optimally staged patients in the observation arm compared to those without optimal staging. In the non-optimally staged patients there was a survival advantage in the chemotherapy, compared to the control arm, in optimally staged patients there was no difference seen. The differences in survival seen in the ACTION study might be explained by a number of occult stage III tumours being included with the suboptimally staged patients. If this is the case then benefit is likely to be achieved with chemotherapy. In those optimally staged patients i.e. true stage I disease, this survival advantage with chemotherapy may not be present. The conclusion from these studies is that chemotherapy is warranted in non-optimally staged early ovarian cancer but this benefit may not be as relevant in those who remain early stage after thorough staging [80]. The optimum type and duration of chemotherapy is yet to be confirmed, but is likely to be either three or six cycles of either single agent carboplatin or carboplatin in combination with taxol. The results of GOG 175 will hopefully provide these answers.

1.1.9.2 Advanced disease

Systemic chemotherapy has formed a major component of treatment in advanced ovarian cancer for many years. The platinum based compounds were introduced in the 1970s, initially cisplatin followed by carboplatin. The anticancer activity of platinum drugs results from their ability to link themselves between strands of DNA. This inhibits DNA replication, DNA repair and induces apoptosis. Paclitaxel was incorporated into platinum regimes in the 1990s. Paclitaxel is derived from the bark of the Pacific Yew Tree. Taxanes bind to tubulin preventing disassembly of microtubules and this inhibits mitosis during the M phase of the cell cycle.

Cisplatin replaced cyclophosphamide as a single agent showing improved survival and reduced toxicity rates [119]. The introduction of a second generation platinum analogue,

carboplatin was then introduced. This was superior to cisplatin with reduced gastrointestinal side effects, especially nausea and vomiting. Less nephrotoxicity, neurotoxicity and ototoxicity was noted, although carboplatin was seen to be more myelosuppressive [120, 121]. An RCT (ICON 2) compared carboplatin with cyclophosphamide, adriamycin and cisplatin (CAP) - the established standard regime at the time. This study confirmed that single agent carboplatin was as effective as CAP with less grade 3 and 4 toxicities [122].

Following the establishment of platinum agents as the primary form of chemotherapy in advanced ovarian cancer, paclitaxel was introduced as a potential adjunct. Initial studies compared combinations of paclitaxel with either cisplatin or carboplatin, two studies both demonstrated similar DFS and OS with the two regimes but the toxicity profile was improved in the carboplatin/ paclitaxel arm [123, 124]. In addition a semi-synthetic mechanism has produced a second generation taxane called docetaxel. This has been shown to reduce neurotoxicity and haematological suppression compared to paclitaxel when used in combination with carboplatin [125].

There are four main studies which assessed the use of platinum agents in conjunction with paclitaxel [126-129]. These trials were all asking the same basic question - is combined paclitaxel/ platinum superior to platinum based controls? The GOG 111 was the first to report and compared cisplatin/ paclitaxel with cyclophosphamide/ cisplatin and found improved OS in the paclitaxel arm [127]. The OV10, a European study with similar study arms, confirmed these findings [129]. However, these studies were contradicted in the GOG132 in which cisplatin/ paclitaxel produced no survival advantage compared with single agent cisplatin [128]. The most recent study to report, ICON3, compared carboplatin/ paclitaxel with either single agent carboplatin or CAP (this study was commenced before the findings of ICON 2 were known i.e. that carboplatin is superior to CAP). ICON3 demonstrated no difference in OS between the two study arms [126]. There are a number of explanations for differences in the findings of these large RCTs. Firstly, differing platinum compounds were used, either carboplatin or cisplatin. Carboplatin is

now known to be superior. There was also significant cross over to paclitaxel in the non-paclitaxel arm when disease progression or recurrence occurred. Finally, there was a suggestion that the control arms in the OV10 and GOG111 trials had suboptimal doses of platinum in the control arm [130]. Overall, the evidence has produced confusion and ultimately variation in clinical practice. Often, the combined approach of carboplatin/ paclitaxel is being favoured in the younger, fitter patients who are more able to tolerate the increased risk of toxicity. The UK national guidelines suggest that carboplatin can be used as a single agent or in combination with paclitaxel [131].

1.1.10 Treatment of recurrent disease

First line chemotherapy in advanced ovarian cancer, with platinum and paclitaxel, yield response rates of 80% with 40-60% complete response. In spite of this initial response the majority relapse, with median PFS of 18 months [132]. Recurrent ovarian cancer is incurable; however, use of chemotherapy may significantly prolong survival. The duration of remission is crucial in deciding which form of chemotherapy to use. Disease which recurs after more than 12 months of platinum treatment are deemed platinum sensitive, since it is likely that the tumour will respond to repeat administration of platinum agents. There is also evidence that addition of paclitaxel may improve OS, even if this was used in prior regimes [133]. This is supported by the results of ICON4 which demonstrated improved OS with carboplatin/ paclitaxel over single agent chemotherapy in relapsed disease [134].

The use of single agent paclitaxel and liposomal doxorubicin are recommended for those tumours which are platinum resistant (recurring within six months of last platinum chemotherapy), since the likelihood of response to further platinum is 10-20% [135]. Topotecan is usually reserved as a third line option [136]. The failure of current cytotoxics to cure patients with chemosensitive disease at presentation, and the progressive development of drug resistance at relapse, is responsible for the poor overall 5 year survival. This provides the impetus to pursue novel therapeutic strategies.

1.1.11 Novel chemotherapy regimes

1.1.11.1 Dose dense, maintenance and novel combination chemotherapy

There is some evidence that escalating the dose of chemotherapy produces increased survival. A study by Kaye *et al* demonstrated an improved OS with double the typical dose of cisplatin, although this difference disappeared on long term follow up [137]. A similar RCT (SCOTROC4), which is yet to report, is comparing a similar dose-escalating schedule with carboplatin. A weekly, as opposed to three weekly, administration of paclitaxel may provide advantages in reducing toxicity and improving response rates, even in platinum refractory disease [138]. Extended courses of cisplatin have not been shown to alter PFS or OS [139], however, there is early evidence that prolonged treatment with paclitaxel improves PFS and reduces platinum resistance [140].

There are numerous studies attempting to alter combinations of chemotherapy regimes and include new agents, an example is ICON5, a five arm study including carboplatin, paclitaxel, liposomal doxorubicin and gemcitabine. This study assessed the role of adding a third cytotoxic agent (liposomal doxorubicin or gemcitabine) to carboplatin and paclitaxel. The data is still maturing, although there does not appear to be any improvement in PFS with any of the aforementioned combinations [141].

1.1.11.2 Intra-peritoneal administration

Intra-peritoneal (IP) chemotherapy has the theoretical advantage that it acts directly on the tumour cells. There is evidence that there may be a survival advantage with IP delivery of platinum agents [139, 142]. There are limitations in that there will only be benefit if adequate peritoneal circulation of the chemotherapy is possible, which is unlikely in cases of advanced suboptimally debulked tumours. There are also catheter-related problems and potentially increased toxicity. The results of the recent GOG172 study comparing IP carboplatin and paclitaxel with conventional intravenous administration showed a marginal improvement in OS but with significant increase in toxicity. This

treatment route is yet to become established [143], although is the focus of a Cancer Research UK study which is randomising patients with advanced ovarian cancer to either standard chemotherapy (carboplatin and paclitaxel) or standard chemotherapy with IP carboplatin [144].

1.1.11.3 Neoadjuvant chemotherapy

Various studies have investigated the role of neoadjuvant chemotherapy, principally in tumours which on pre-operative assessment were deemed unlikely to be resectable. Essentially neoadjuvant chemotherapy refers to the administration of chemotherapy prior to surgery. This approach does not appear to impact on survival rates and may improve the resectability of the tumour [145, 146]. Currently, there are two main trials looking at this approach in advanced ovarian cancer, the CHORUS trial (CHemotherapy OR Upfront Surgery) and the EORTC 55971 trial. Whilst CHORUS is still recruiting, provisional reports from the EORTC trial have suggested that there is no improvement in OS with neoadjuvant chemotherapy, but the rate of optimal debulking is increased and surgical complication rates are reduced. Once data from these trials has matured it is likely that a move towards neoadjuvant chemotherapy will occur.

1.1.11.4 Agents to overcome chemoresistance

Cisplatin binds to DNA which results in erroneous DNA replication and subsequent cell death through the DNA mismatch repair system (MMR). During development of platinum resistance components of the MMR system can be inactivated such as the gene hMLH1 (through methylation). Therefore, drugs such as decitabine, a demethylation agent, may reactivate hMLH1 and restore platinum sensitivity [147]. Other methods of platinum resistance include mechanisms which reduce the intracellular concentration of platinum through reduced uptake or increased efflux. Permeability glycoprotein (Pgp) is over expressed in platinum resistant cells, resulting in increased efflux of platinum from the cell. Trials using Pgp inhibitors have only had limited success [148].

1.1.12 Novel targeted therapies

The increased understanding of tumour biology provides a number of potential therapeutic targets. These include the use of growth factor inhibitors, anti-angiogenic therapy, gene therapy and immunotherapy.

1.1.12.1 Growth factor inhibitors

Epidermal growth factor receptor (EGFR) is one of the most extensively studied growth factors in ovarian cancer. Cefitinib and Erlotinib are tyrosine kinase inhibitors which block signal transduction from EGFR receptors. These drugs have provided a good response in lung cancer but the effects in ovarian cancer have been disappointing [149]. A similar tyrosine kinase inhibitor, Lapatinib, which acts on both EGFR and Her2 receptors, is being assessed in advanced ovarian cancer by the GOG.

Raf-1 kinase inhibitor has been shown to have some inhibitory action in ovarian cancer. This occurs through the prevention of signal transduction in the Raf pathway, which is responsible for cell growth and proliferation [149]. The P13K/Akt pathway also stimulates cell growth and proliferation and is amplified in 30% of ovarian cancers. Inhibitors of this pathway are in development. MTOR is a downstream target of the P13K/Akt pathway, for which inhibitors are at a phase I stage of development [149].

1.1.12.2 Apoptosis inducers

Phenoxodiol is a topoisomerase II inhibitor, which induces apoptosis and may have a role in re-sensitising paclitaxel resistant ovarian cancer [149]. This is achieved by inhibiting the X-linked inhibitor of apoptosis protein (XIAP); XIAP is shown to active on paclitaxel resistant tumours [150]. Monoclonal antibodies which stimulate TRAIL pathway apoptosis are discussed in a later chapter.

1.1.12.3 Anti-angiogenic therapy

Bevacizumab is a mAb which binds to and inactivates all isoforms of VEGF-A leading to inhibition of angiogenesis. Phase II studies demonstrated a 17% response rate amongst patients with recurrent ovarian cancer the median time to recurrence being ten months [151]. VEGF receptor tyrosine kinase inhibitors are also being developed. A large multinational RCT comparing standard chemotherapy with carboplatin and paclitaxel, with standard treatment plus Bevacizumab is ongoing (ICON7).

Thalidomide is known to produce anti-angiogenic effects and has been shown to have some modest effects in stabilising recurrent ovarian cancer [149]. Carboxyamidotriazole is a calcium channel blocker with anti-angiogenic properties which has some activity in ovarian cancer. In addition PDGF receptor inhibitors are also showing some promise in arresting angiogenesis and tumour growth in ovarian cancer [149].

1.2 The Immune System

The immune system is comprised of two separate but complimentary systems; the innate system provides the first line of defence against infectious agents, whilst the adaptive system provides a more versatile and targeted immune response. The two systems work synergistically and rarely in isolation. The cells of the innate system provide a crucial role in initiating and directing adaptive immune responses and also participate in the removal of pathogens targeted by this system.

1.2.1 Innate immune system

1.2.1.1 Complement

The complement system consists of a large number of plasma proteins which react with one another to opsinise pathogens and induce inflammatory responses. Complement factors are distributed as inactive precursors before the triggering of the complement cascade triggers sequential activation of individual factors resulting in a large rapid

amplification of the complement response. The complement cascade can be triggered by three distinct pathways, the classical, alternative and MB lectin, all of which converge to generate the same effector molecules and hence produce the same effects.

The classical pathway can be triggered by direct interaction with pathogens (innate response) or through binding to antibody:antigen complexes (adaptive response). The MB lectin pathway is initiated by binding of mannan-binding lectin, a serum protein, to factors on the surface of the pathogen. The alternative pathway is triggered by stabilisation of C3b on the surface of pathogens, which under normal circumstances would be inactivated to iC3b. The activation of the complement cascade, via any of the three pathways, produces three effects, recruitment of inflammatory cells, opsonisation of pathogens and killing of pathogens. Killing of pathogens is achieved through the formation of the Membrane Attack Complex which creates pores in the cell membrane leading to cell death. C3b also covalently binds to the bacterial cell membrane (opsonisation) enabling phagocytes to internalise them. Thirdly C3a, C4a and C5a mediate an acute inflammatory response by stimulating mast cells to release histamine, chemokines, interleukins, leukotrienes, prostaglandins and thromboxanes, resulting in increased vascular permeability and recruitment of appropriate inflammatory cells.

1.2.1.2 Phagocytes

Phagocytes are the principal cells involved in ingesting and destroying foreign material, the predominant types are macrophages and neutrophils. These cells recognise foreign material by the expression of Pathogen Associated Molecular Patterns (PAMPs) via Pattern Recognition Receptors (PRR). The interaction between PAMPs and PRRs triggers a number of events including phagocytosis and release of cytokines with subsequent initiation of the acute inflammatory response [152].

1.2.1.3 Natural killer (NK) cells

NK cells are a type of lymphocyte defined by the expression of CD56 and the absence of CD3. NK cells play an important role in innate immunity by mediating direct cytotoxicity and secreting cytokines such as IFNγ, IL-10, IL-13 and TNFα [153]. NK cell activity is dependent on a complex balance between inhibitory and stimulatory receptors which interact through MHC class molecules on potential target cells. Ultimately the NK cell will be triggered or inhibited depending on the balance of these signals. The loss or absence of HLA class I molecules on the surface of the target cell tends to produce triggering of the NK cell [154]. Once activated the NK cells trigger apoptosis in the target cell through initiation of the caspase cascade. Apoptosis may also be induced through interaction of fasL and TNF Related Apoptosis Inducing Ligand (TRAIL) with the respective death receptors [155].

1.2.1.4 Gammadelta ($\gamma\delta$) T cells

Most T cell receptors consist of two polypeptide chains (α and β) similar to the Fab fragments of an immunoglobulin molecule. There is a small subgroup of T cells which have different polypeptide chains (γ and δ) making up the receptor [152]. These $\gamma\delta$ T cells are distinct as they are able to respond to antigens without the need for presentation through HLA molecules. The TCR in this subclass is able to recognize a range of epitopes, reducing the need for clonal expansion. These cells are thought to remove damaged and stressed cells [156]. Mice deficient in this cell type are predisposed to tumour development [157, 158].

1.2.1.5 Dendritic cells (DCs)

DCs along with macrophages and B cells are known as professional APCs, as they present antigen via HLA class I and II molecules, to cytotoxic and helper T cells (signal). Immature DCs originate in the bone marrow and migrate to peripheral tissues, where they

recognise and ingest foreign material. In this immature form they lack the ability to stimulate T cells. Following exposure to a pathogen or stressed cells the DC is activated and migrates to regional lymph nodes where maturation and morphological changes occur. These provide the vital second signal for activation of naïve T cells. These cells therefore represent an important interface between the innate and adaptive immune systems [152].

1.2.2 The adaptive immune system

1.2.2.1 B-cells

B-cells originate from the bone marrow. During maturation these cells undergo rearrangements in the genes which encode the variable regions on their surface immunoglobin, the B-cell receptor (BCR). This produces a population of cells with highly diverse, specific receptors able to respond to a wide range of antigenic stimuli. Once a B-cell encounters the specific antigen for which its BCR is coded, it binds and internalises the antigen. Subsequent processing finally occurs, resulting in the antigen being expressed on the cell surface combined with a HLA class II molecule; this enables interaction with other immune cells. B-cells may develop into plasma cells, which following clonal expansion, are capable of producing large qualities of identical immunoglobulin with a specific antigenic target [152, 159, 160].

1.2.2.2 Antibodies

Antibodies, or immunoglobulins, are produced by activated B-cells. They bind to unique antigenic sequences on foreign organisms which initiate a specific, targeted immune attack. The structure of the antibody incorporates two identical light and heavy protein chains which schematically are illustrated by a “Y” shaped molecule (Figure 1.5). This molecule possesses a stem, the Fragment crystalline (Fc), and two arms, the Fragment antigen binding unit (Fab). The Fc unit binds to the effector cells while the Fab units, which contain a highly variable region, bind to specific antigenic sequences on the

pathogen. There are a number of Fc subtypes which determine the class of antibody and include IgG, IgE, IgA and IgD. Different effector cells have Fc receptors for a particular subclass of antibody and hence each antibody type triggers different effector mechanisms [152].

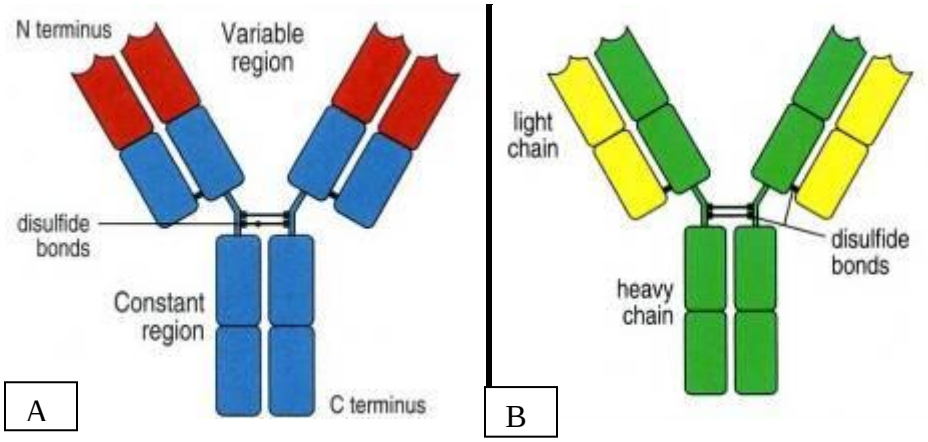


Figure 1.5 The antibody structure. A simplified schematic representation of an antibody molecule showing (A) the variable (red) and constant regions (blue), and (B) the heavy (green) and light chains (yellow). Figure adapted from Immunobiology. Chapter 3. Janeway *et al* [152].

1.2.2.3 T-cells

T-cells can be divided into two main groups; those expressing CD8 molecules, cytotoxic T lymphocytes (CTLs), and those expressing CD4 molecules, helper T cells (Th). These are co-receptors for HLA class I and II respectively.

Immature T cells lacking both CD4 and CD8 are produced in the bone marrow, prior to migration and maturation occurring in the thymus. In the thymus both CD molecules are initially expressed, one type is then removed and it is this which determines the ultimate cellular phenotype. As everyone is born with the same T-cell repertoire it is essential to select the cells that are necessary for each individual. The process governing this maturation of T-cells in the thymus eventually results in the destruction of the majority. Only approximately 2% become viable mature T-cells. T-cells recognising the individual's inherited HLA are selected (positive selection), and those capable of responding too

strongly to self antigens are removed (negative selection) in order to prevent auto-immunity. Ultimately T-cells will be produced which express either CD4 or CD8 co-receptors.

CD8+ cells

These cytotoxic T-cells have TCR which bind to HLA class I molecules on the surface of cells displaying peptides which are typically from viral infection or abnormal cellular functioning. The mechanism of cell killing is similar to NK cells.

CD 4+ cells

Naïve CD4 cells can differentiate upon activation into different subtypes, which vary in the cytokines they produce and hence in their function. The determining factors involved in this process are unclear. Some cytokines relate to cell-mediated immunity through the activation of macrophages and NK cells, others produce a humoral response with stimulation of B-cells to produce vast quantities of immunoglobulin. The nature of the antigenic stimulus dictates the proportion of each cell's type and hence the nature of the immune response, i.e. cell mediated or humoral.

1.2.2.4 Human leukocyte antigen (HLA)

HLA is the human form of Major Histocompatibility Complex (MHC), whose function is to bind peptide fragments from pathogens and display them on the cell surface for recognition by the appropriate T-cells. The result of the interaction between MHC and T-cells is varied and dependent on the specific cell type which binds. There are two main subtypes of HLA (class I and II). In addition, there are several different genes encoding HLA of each subtype and these are also highly polymorphic. This in essence means that each individual carries a large number of different HLA molecules and the variation in types between individuals is also high.

HLA class I is a heavy chain polypeptide which is expressed on the cell surface and is associated with a light chain (β2microglobulin). HLA class I, which is expressed on virtually all human cells, presents antigens which occur during abnormal cellular

conditions. The antigen is processed within the cell by the proteosome and transported to the endoplasmic reticulum and Golgi apparatus and finally to the cell surface by transporter proteins (TAP 1 and 2). During processing the peptide is bound to β2microglobulin before finally combining at the cell surface with the heavy chain to produce the HLA-peptide complex, which becomes a target for cytotoxic T-cells [152].

HLA class II molecules are expressed by a more limited population of cells. Expression is limited to B-cells, DCs, macrophages, endothelial and stressed epithelial cells. The primary role of HLA class II molecules is to present antigens to CD4 cells [152].

1.2.2.5 Regulatory T-cells

Regulatory T-Cells (Treg) or suppressor T-cells are a subpopulation of CD4+ T-cells which also express high levels of IL-2 receptor (CD25) and transcription factor Forkhead box p3 (Foxp3) [161]. Treg are primarily produced in the thymus, although a small proportion develops peripherally. Treg produce their effects through a number of cytokines including IL-10 and transforming growth factor β (TGFβ) [162]. Physiologically, Tregs prevent unwanted autoimmune reactions and curtail the immune response once the original stimulus is neutralised [163]. The suppressive effects of Tregs may be harnessed by pathogens and cancers to evade immune response [164].

1.2.2.6 Cytokines

Cytokines are low molecular weight proteins which are secreted by cells to produce various responses. They consist of a number of types including interleukins, colony stimulating factors, TNFs and interferons (IFNs). The functions of the various cytokines are diverse, reflecting the array and complexity of the cytokine groups. In broad terms these functions include control of cellular differentiation, upregulation of receptors, the initiation of the inflammatory response, anti-tumour and anti-viral activity, direct cytotoxicity and priming of the adaptive immune response.

1.3 Tumour Immunology

Tumour immunology represents an area of rapidly developing cancer research. This consists of exploration of the mechanisms which underlie immunological recognition and elimination of cancer and the mechanisms through which tumours evade such attack. However, the theory behind the immune system being able to recognise and eliminate tumours was first postulated 100 years ago [165]. This concept was later termed cancer immunosurveillance, although technological limitations at that time prevented significant development of the theory. With the emergence of transgenic mouse technology, coupled with the production of monoclonal antibodies to specific immune factors, the immunosurveillance hypothesis has attracted renewed attention. Modern molecular techniques allow robust, specific investigation of this model. As our knowledge of the concept expands it appears that not only does the immune system exert a suppressive effect on tumour cells, it also acts as a Darwinian type model to select and promote tumour cells with lower immunogenicity - this concept is termed immunoediting [166, 167].

1.3.1 Cancer immunosurveillance in mice

Initial interest in the concept of immunosurveillance was developed in the 1950s, however, this hypothesis lost favour over the subsequent 20 years. This was primarily due to results from experiments on nude mice, thought to be immunodeficient, which failed to show the predicted increase in methylcholanthrene (MCA) induced and spontaneous tumours compared with wild type controls [168]. With hindsight these seemingly negative findings can be explained by the demonstration that nude mice are not as immunodeficient as first assumed. They have a low but detectable level of αβ T-cells [169] and normal levels of NK cells; the existence and role of which was not known at that time [170]. In addition these mice had a fully functioning innate system, whose influence on the adaptive immune system was not appreciated [171]. Hence, the nude mouse is an imperfect model because

low levels of cancer immunosurveillance probably occur, explaining the negative findings of such studies [168, 172].

Some of the most convincing data relating to the theory of immunosurveillance comes from work done using transplantation techniques between immunocompetent and immunodeficient mice, which highlight the specific influence of lymphocytes. Immunodeficient mice lacked the recombinase activating gene-2 (RAG-2). RAG-2 is required to rearrange lymphocyte antigen receptors; hence, mice lacking RAG-2 will lack functional T, B and NK cells. Following exposure to MCA, 129/SvEv RAG-2-/- mice developed sarcomas more rapidly than wild type controls [167]. Similar effects were observed with spontaneous tumours. When tumours from both types of mice were transplanted into an immunodeficient host tumour growth was observed. However, when tumours from both groups were transplanted into immunocompetent hosts 40% of those arising in immunodeficient donors were rejected. Hence, tumours which developed in immunodeficient environments were significantly more immunogenic than those which developed in wild type controls. This gives some convincing evidence for the sculpting effect of the immune system on tumour characteristics i.e. immunoediting [167].

1.3.2 IFNγ in cancer immunosurveillance

Interest in immunosurveillance was rekindled following a series of studies involving IFNγ. Injection of a neutralising antibody for IFNγ resulted in the blocking of tumour rejection in mice transplanted with established tumours [173]. Similar effects were seen with the increased incidence of MCA-induced tumours in IFNγ insensitive mice compared to wild type controls. Mice lacking either one of the subunits of the IFNγ receptor (IFNGR1) or STAT1, a crucial transcription factor in the pathway, led to a 10 - 20 fold increase in tumour formation compared to wild type mice [174]. These findings have been reproduced by others [175]. In addition, the tumours produced in IFNGR1 deficient mice grew aggressively when transplanted into immunocompetent hosts. Therefore, tumours lacking IFNGR1 had their immunogenicity blocked because of an inability to respond to IFNγ.

The relationship between IFNγ and lymphocytes on tumour suppression was investigated using mice lacking either a functioning IFNγ pathway (IFNGR1 or STAT1 deficient), functioning lymphocytes (RAG-2-/-) or both. As expected, MCA-induced tumours occurred more frequently than in wild type controls, however, there was no synergistic effect seen within the mice lacking both functional lymphocyte and IFNγ pathways [167]. This suggests, not surprisingly, that there is significant overlap in the anti-tumour effects of IFNγ and lymphocytes. This is supported by the finding that mice lacking both IFNGR1 and perforin (a prime mediator of cytotoxic T lymphocyte killing), were not at significantly increased risk of tumour formation compared with those with only one defective pathway [175].

The source of IFNγ utilised during cancer elimination appears to originate primarily from γδ T-cells. Gao *et al* demonstrated an increased incidence of tumours in mice lacking γδ T-cells which was equivalent to that of IFNγ deficient mice [176]. The production of IFNγ in this pathway is thought to be mediated by IL-12. IL-12 is a potent cytokine produced by macrophages which stimulate release of IFNγ. IL-12 dependent rejection of sarcomas was blocked by IFNγ neutralising antibody [177].

Although evidence suggests that IFNγ is critical in immunosurveillance, it was not clear whether the tumour cell itself was a direct target for IFNγ activity. Using tumour cell transplantation models, MCA-induced tumour cells which were engineered to be unresponsive to IFNγ, (by over expression of a dominant negative mutant for IFNGR1), were transplanted along with controls into wild type mice. The tumour cells rendered unresponsive to IFNγ grew more aggressively than the controls, suggesting that tumour cells are a physiological target of IFNγ in the tumour rejection process [173].

1.3.3 Specific effects of IFNγ on tumour cells

IFNγ has been shown to upregulate components of the MHC class I pathway, resulting in increased immunogenicity and ultimately tumour rejection [178]. IFNγ has marked anti-

proliferative effects on tumour cells through the induction of cell cycle inhibitors such as p21 [179] and p27 [180]. Pro-apoptotic pathways can also be induced through IFNγ promotion of genes producing caspase-1, Fas and Fas ligand, which can trigger programmed cell death [181-183]. Release of potent chemokines from tumour cells can be induced by IFNγ, CXCL-9 and CXCL-10 (IP-10) and act as powerful chemo-attractants for specific leukocytes as well as inhibiting angiogenesis [184, 185]. Although the aforementioned effects of IFNγ on tumour cells have been clearly demonstrated in isolation, the relative contribution of each to the process of immunosurveillance is unclear.

1.3.4 Immunosurveillance in humans

Having established evidence of immunosurveillance using mouse models, can these findings be extrapolated into humans? The evidence for immunosurveillance in humans is much less developed. However, there are three main areas of evidence. One obvious cohort, in whom the potential effects of the immune system on tumour formation might be observed, is in those individuals with immune deficiency. Immunodeficiency can be both congenital, acquired or through the effects of immunosuppressive drugs. Unfortunately, these patients are also more prone to viral infections, and in consequence, to virally induced tumours such as Kaposi's sarcoma and cervical cancer which are caused by Human Herpes Virus 8 and Human Papilloma Virus respectively [186]. Hence any effect of immune suppression on non-virally induced tumour, which is likely to be more subtle, may be clouded by such observations. There are three lines of enquiry pointing to a potential role for immunosurveillance in humans. Evidence from immunosuppressed transplant patients, immune responses to established cancers and the prognostic significance of lymphocytes within tumours.

1.3.4.1 Tumour formation in transplant patients

Evidence for an increased relative risk of many non-virally associated tumours have been documented in renal [187] and cardiac [188] transplant patients. These included colon,

lung, bladder and renal cancers. There is the possibility that a hitherto unknown viral cause exists, but with current understanding it appears that drug-induced immune suppression results in increased risk of tumour formation. This may result from *de novo* tumours or through the outgrowth of occult tumours previously suppressed by the functioning immune system.

1.3.4.2 Immune responses to tumours in humans

Clearly the transplantation techniques used in the mouse model would not be applicable to humans. Therefore *in vitro* approaches have been adopted to demonstrate immune responses to tumour antigens. Autologous typing is an example of such a technique. Tumour cell lines were established from patients with melanoma and then used to assess humoral and cellular responses. This system identified T-cells (both CD4 and CD8) with antibodies specific to tumour cell surface antigens [189]. NY-ESO-1, a tumour specific antigen, provides an excellent illustration of current understanding and evidence surrounding specific immune cellular responses to tumours. NY-ESO-1 has been identified in some melanoma, bladder and lung cancers [190]. The antibody to this has only been detected in tumours expressing the antigen and not in NY-ESO-1 negative tumours or normal controls [191]. The antibody is further linked to the presence of the NY-ESO-1 positive tumour since surgical removal leads to disappearance of the antibody [192]. There is some evidence that suggests that patients with spontaneous immunity to NY-ESO-1 carry a more favourable prognosis. This is yet to be established.

The innate immune system also appears to play a role in immunosurveillance. MICA and MICB (MHC class 1 glycoprotiens) act as tumour cell surface ligands for interaction with NK cells and γδ T-cells. They are non-classical MHC proteins which do not associate with β macroglobulin or require TAP for expression [193]. MICA/B is only found in the large and small intestine of normal tissues, but is seen in lung, breast, kidney, ovarian and colon cancers [194]. Interaction of NK c and γδ T-cells with MICA/B is via NKG2D

receptors, with inhibition of cell lysis produced by pre-treatment with inhibitory monoclonal antibody to NKG2D [195].

MICA/B has been linked with immunosurveillance following the correlation, *in vivo*, of MICA/B expression in tumours with tumour infiltration by γδ T-cells [194]. Secondly, soluble MICA shed by tumour cells causes downregulation of NKG2D receptors on T-cells leading to a reduced responsiveness of tumour antigen specific T-cells. This may be a method by which a tumour evades immune attack [196].

1.3.4.3 Tumour infiltrating lymphocytes

Over recent years the presence of tumour infiltrating lymphocytes (TILs) has been shown to exert some influence on tumour behaviour. In particular Zhang *et al* studied 174 advanced ovarian carcinomas and demonstrated that a favourable clinical outcome could be predicted by the presence of intratumoral TILs [197]. Similar studies in colorectal cancer demonstrated the same prognostic influence [198]. These studies as well as others involving lung, gastric and oesophageal cancer [166], all suggest a profound influence of the immune system on the behaviour and control of established tumours. This influence appears to be exerted on established cancers, i.e. those which have escaped immunosurveillance. However, it is likely that similar effects are occurring at a sub-clinical level to eliminate or suppress occult tumours.

Tregs are CD4+ T-cells which release cytokines such as IL-10 [199] and TGF-β [200] to inhibit the function of other T-cells. Physiologically, Tregs prevent unwanted autoimmune reactions [163]. Tumours, however, may highjack these cells to prevent effective immune response to tumour antigens [164, 201]. This was illustrated in a murine model with transplanted tumours, which were rejected when the recipient mice were pre-depleted of Tregs [202]. γδ T-cells represent a further subset of T- cells which have inhibitory properties with exposure to TGF-β promoting an inhibitory role. However, they also have stimulatory roles dependent on the cytokine milieu [203]. Tumour-associated γδ T-cells

have been associated with inhibition of anti-tumour immune responses through the inhibition of NK cells [204].

1.3.5 Cancer immunoediting

In spite of the numerous studies pointing to a protective effect from the immune system against tumour development, tumours are seen occurring in patients with intact immune systems. Hence, if the immune system is able to detect and destroy tumour cells then tumour cells must develop methods of evading such attack. This concept is central to tumour biology, namely that they escape the mechanisms that operate to suppress them.

Shankaran *et al* demonstrated in a mouse model that tumours which develop in the absence of an intact immune system appear to be more immunogenic [167]. Takeda *at el* [205], whose work focuses on TRAIL-mediated cytotoxicity in tumours, assessed the influence on characteristics of tumours which developed in the presence or absence of a functioning TRAIL pathway. Tumours developed in mice with the TRAIL blocking antibody, produced tumours that were sensitive to TRAIL-mediated cytotoxicity. However, tumours established with a functioning TRAIL pathway, produced tumours that were resistant to TRAIL killing. This suggests that tumours exposed to the TRAIL pathway need to develop phenotypes which could evade such attacks. Whereas those grown in a “TRAIL-free” environment did not require this evasion to develop and hence were sensitive to TRAIL-mediated attack when exposed [205].

This series of experiments shows that tumours are imprinted by the immunological environment in which they form. Tumours developing within an environment containing an intact immune system produce cells which are rendered resistant to immune attack and/ or recognition. This phenomenon is likely to occur from the selection pressures exerted by the immune system. Specifically, tumour cells which develop mutations allowing evasion of the immune system will have a survival advantage and hence be selectively favoured as the tumour grows. The selective pressure of the immune system is highlighted by the sensitivity to immune attack of tumours developed in the absence of a functioning immune

system. Mutations producing the ability to evade the immune system do not provide a survival advantage in an immunodeficient environment and therefore are not positively selected. This leads to a tumour which remains sensitive to immune-mediated cytotoxicity. During tumour development a series of selected mutations will occur leading to sculpting of the tumour. This happens at an early, sub-clinical stage, and hence clinically apparent tumours will already have been attenuated by the sculpting effects of the immune system.

1.3.5.1 Refining the immunosurveillance theory

The original concept of immunosurveillance suggested that cells from the adaptive immune system produced a protective role against potential cancers developing by recognition and elimination of such cells. It now appears that the innate as well as the adaptive system is involved. The influence of the immune system is not limited to merely preventing tumour development, but also imposes an influence on the immunogenicity of tumours which do develop. This sculpting, or editing, effect occurs through Darwinian-style selection pressures exerted on the tumour, in which tumour cells able to evade immune attack are positively selected. This process has been termed immunoediting [206]. A potential model for how this process occurs involves “the three Es ”- elimination, equilibrium and escape (Figure 1.6).

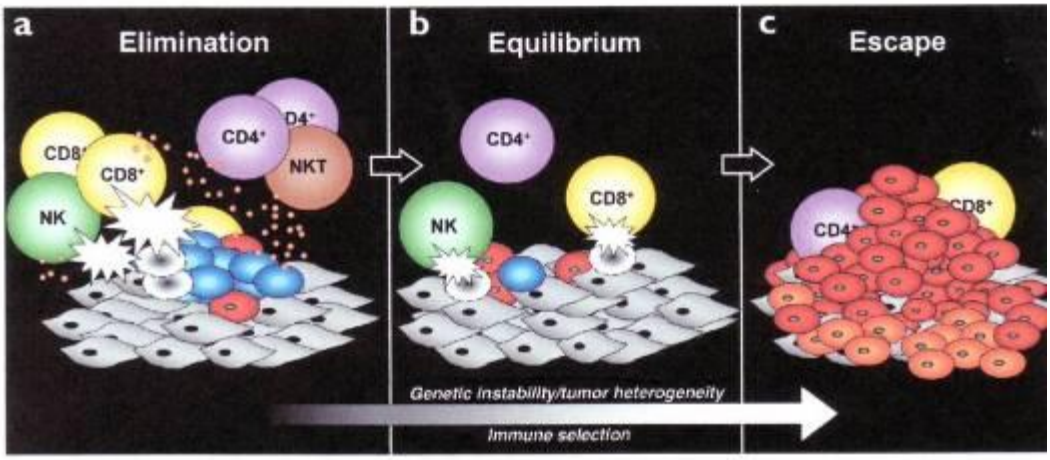


Figure 1.6 The three Es of cancer immunoediting. (a) Elimination corresponds to immunosurveillance. (b) Equilibrium represents the process by which the immune system selects and/ or promotes tumour cell variants with increased capacity to survive immune attack. (c) Escape is the process wherein the immunologically sculpted tumour expands in an uncontrolled manner in the immunocompetent host. Developing tumour cells (blue), tumour cell variants (red), additional tumour cell variants (orange), stroma (grey) and white flashes represent lymphocytic cytotoxic activity against tumour cells. Diagram adapted from Dunn *et al* [166].

Elimination

This phase represents the initial concept of immunosurveillance - i.e. the killing of tumour cells by the immune system. Immunological recognition and elimination of tumour cells at this stage is similar to that seen with anti-microbial effects. The initiation of the anti–tumour effects occurs from the stromal disruption resulting from the invasive tumour growth pattern and angiogenesis. This releases inflammatory chemokines that summon cells of the innate system (NK, γδ T-cells and macrophages) to the source of danger [207]. These cells recognise tumour cells via NKG2D or TCR receptors on the tumour surface [208]. The end result of these early interactions is the production of IFNγ. A positive feedback loop involving various chemokines, including IL12, produces a rapidly increasing level of IFNγ, leading to a number of IFNγ-dependent processes including anti-proliferative [180], pro-apoptotic [209] and angiostatic [184] effects, which lead to tumour killing. In addition, IFNγ activates macrophages and NK cells which produce further cell death through the TRAIL [205] and caspase pathways [210]. As a result of these

processes dead tumour cells are made available for recruitment by the adaptive immune system in to the process.

The following step relates to the development of tumour-specific adaptive immune responses. Immature DCs that have been recruited to the tumour site are activated by cytokines which are either released by the innate system in response to the tumour, or via direct interaction with NK cells [211]. The DCs ingest tumour antigens from the cell debris produced from the killed cells, following which they migrate to regional lymph nodes [212]. In the lymph node DCs activate naive tumour-specific Th1 CD4+ T-cells which in turn facilitate the development of tumour specific CD8+ CTLs [213]. The specific CD4+ and CD8+ T-cells then migrate to the tumour site where they proceed to kill antigen positive tumour cells. In addition to direct killing these T-cells also produce vast amounts of IFNγ which stimulates further IFNγ specific anti-tumour responses.

This process must be repeated for each new cancer which develops, since each will be antigenically unique and hence require specific adaptive immune responses. This constant need for ongoing immunosurveillance might in part explain the increased incidence of cancers in the elderly population where it is recognised that immune function deteriorates.

Equilibrium

Any tumour cells that have the ability to avoid being killed enter into a phase of equilibrium, during which time the immune system has the ability to contain, but not fully eliminate, the tumour cells. This is similar to the old concept of tumour dormancy [214]. This cell population is genetically unstable with microsatellite and chromosomal instability which leads to a constantly evolving heterogeneous group of cells with frequent production of new clones. Tumour variants with increased ability to avoid immune attack will be positively selected in a Darwinian-style model. Hence, the tumour becomes increasing resistant to immune attack until it is able to escape and grow unhindered by the inhibitions of the immune system. This period of equilibrium is the longest of the three

phases and may occur over many years. For example, there can be a 20 year interval between initial carcinogen exposure and clinical detection of a tumour [215].

Further evidence is offered from case reports of renal transplant patients with donor-derived melanoma even when the donor has no history of malignancy. It is suggested that the immune suppression required following transplantation allowed the escape of occult tumour which was being held in equilibrium by the donor's intact immune system [216].

Escape

Multiple tumour sculpting events, in the equilibrium phase, leads to an immunogenic phenotype capable of growing in the presence of a functioning immune system. This requires evasion of both the adaptive and innate systems. There is evidence that tumours can exert inhibitory effects through the release of immunosuppressive cytokines such as TGF-β and IL-10, or via mechanisms involving T-cells with immunosuppressive properties (Treg) [199, 200].

Changes in the tumour at a cellular level can also produce alterations in tumour recognition and the effectiveness of immune attack. Loss of MHC [217], shedding of NKG2D ligands (MICA/B) [196] and development of IFNγ insensitivity [174] produce reduced immunogenicity. Kaplan *et al* demonstrated that 25% of lung adenocarcinoma cell lines were unresponsive to IFNγ due to absence or dysfunction of components in the IFNγ pathway [174]. Cellular changes can also prevent immune-mediated cellular destruction, for example, through defects in the TRAIL pathway [205].

Improved understanding of immune elimination of tumour cells may enable augmentation of a patient's natural immune response to stimulate tumour rejection. Further understanding of the escape phase, in terms of the specific mechanisms tumours employ to evade the immune system, will allow strategies to be developed which unmask tumour immunogenicity and thereby re-sensitise tumours to immune attack.

1.3.5.2 Immunoediting in ovarian cancer

The vast amount of evidence pertaining to immunoediting originates from mouse models. However, evidence does exist to illustrate the effects of the immune system on humans and particularly in ovarian cancer. NK cells are sparsely seen in ovarian cancers, with activity been significantly reduced in comparison to normal ovaries [218, 219]. A reduction in NK activity in peripheral blood was seen to correlate with disease progression, [218] and reduced function of NK cells has been demonstrated in tumour-associated lymphocytes (TALs) obtained from the ascitic fluid of patients with advanced ovarian cancer [220]. These NK cells had reduced IL2 induced proliferation and reduced secretion of IFNγ [220].

Tumour associated macrophages (TAMs) are found in abundance in ovarian cancers with some evidence suggesting they may actually promote tumour growth [221]. This may be due to the release of cytokines by TAMs which result in inhibition of tumour-specific effector T-cells [222], although this has not been associated with prognosis [223].

With regard to the adaptive immune system, Zhang *et al* studied 186 advanced ovarian carcinomas and demonstrated a favourable clinical outcome with the presence of CD3+ TILs [197]. TILs were present in significant numbers in 55% of tumours, with associated elevated levels of IFNγ and IL2 (a T-cell growth factor), suggesting a functionally active infiltration. Patients with TILs had a 38% 5 year survival compared to only 4% in those without, this was independently prognostic [197]. There was an association between presence of TILs and the likelihood of optimal surgical debulking, suggesting that spontaneous anti-tumour immune responses may restrict infiltrative tumour growth [197]. These findings were recently supported by Tomsova *et al*, however, they distinguished between stromal and epithelial TILs and found that only intraepithelial TILs were an independent predictor of survival [224]. In addition, an inverse relationship has been demonstrated between γδ T-cells and prognosis in 95 advanced ovarian cancers, thereby supporting the immunosuppressive effects of these cells [158].

In late stage ovarian cancer Woo *et al* demonstrated an increased percentage of Tregs. This was associated with low levels of IL-2, IFNγ and TNFβ [225]. Other workers have illustrated an inverse correlation between tumour infiltrating Tregs and overall survival independent of stage or extent of debulking surgery [226]. Wolf *et al*, using FOXP3 as a marker of Treg expression, also found them to be a negative prognostic factor [227]. Sato *et al* examined the relationship between levels of Tregs and cytotoxic (CD8+) cells. A high CD8+:Treg ratio was associated with a 70% reduction in mortality, suggesting a close relationship between immune cytotoxic activity and Tregs in ovarian cancer [228].

When considering these data, and the aforementioned evidence from mouse models, it appears that ovarian cancer is immunogenic mediating both cellular and humoral responses from the adaptive and innate systems. The influence of these cells, and associated cytokines, on prognosis and tumour behaviour suggests that the interaction and manipulation of the immune system by ovarian cancer, i.e. immunoediting, appears to be a genuine phenomenon.

1.3.6 Immunotherapy in ovarian cancer

The origin of cancer immunotherapy can be traced back to William Coley who, in the 1890s, observed that bacterial infection adjacent to a partly resected tumour could induce an effective anti-tumour response [229].

Cancer immunotherapies, or cancer vaccines, are terms used for harnessing the immune system in the targeting of cancer cells for antigen specific elimination. This encompasses a variety of strategies from prophylactic to therapeutic vaccines with active and passive immune responses. Passive immunotherapy is based on transfer of *ex-vivo* activated immune cells, immuno-modulators (e.g. IFNγ) or monoclonal antibodies. These strategies do not generate an immunological memory and hence the duration of their effect is limited. Active immunotherapy is aimed at activating the patient's own immune system against tumour antigens, which has the advantage of producing immunological memory

with potentially longer lasting effects. The majority of trials have so far centred on passive techniques and in particular the use of monoclonal antibodies [230].

The major challenge in developing successful therapeutic vaccines includes the following: (i) the identification of suitable tumour-associated antigens (TAAs) with tissue-specific expression, (ii) the development of strategies to induce an immunological response sufficient to eliminate the tumour cells and (iii) the development of techniques to overcome the immuno-evasive mechanisms inherent in tumour biology.

1.3.6.1 Monoclonal antibodies

Monoclonal antibodies (mAb) are increasingly used to target various pathways involved in oncogenesis including growth factor inhibitors and anti-angiogenic factors. Whist some mAb such a herceptin (trastuzumab) have become established treatments in breast cancer, the role of mAb in ovarian cancer is less clear [231].

In ovarian cancer the most extensively studied mAb relating to growth factor pathways are Erb1 (EGFR) and Erb2 (Her2). Since 20 - 30% of ovarian cancers are reported to overexpress Her2, trastuzumab, a mAb against Her2, was evaluated. This had a very limited clinical response rate of 7%. This was especially disappointing as only tumours with moderate to high immunohistochemical expression of Her2 were included, which accounted for less than 10% of all tumours assessed [232]. There are other Her2 mAb trials ongoing using different drugs, the results of which are hoped to provide improved efficacy.

TRM-1 is a TRAIL-R1 agonist mAb which is able to activate apoptosis through the TRAIL pathway. It appears to be active as a single agent in addition to potentiating the effects of established chemotherapy agents such as cisplatin and paclitaxel. The studies, however, are all at phase I stage at present [233].

1.3.6.2 Adoptive T-cell transfer

Adoptive cellular therapy is a novel approach to passive immunotherapy through the use of gene-modified autologous T-cells. Kershaw *et al*, in a recent phase I study, used modified T-cells, with reactivity against the alpha-folate receptor, a TAA of ovarian cancer. The results were disappointing with no effect on tumour load and a rapid decline in the T-cell population after 48 hours [234]. Further studies involving T cells with reactivity to WT-1, another ovarian TAA, are ongoing. There remains much interest in the use of adoptive T-cells although strategies need to incorporate techniques to prolong T-cell persistence.

1.3.6.3 Immuno-modulators

Various immunological cytokines have been investigated in the treatment of ovarian cancer. IFNγ is the most extensively researched. The potent anti-tumour effects seen *in vitro* led to its introduction into a phase II trial with the then standard chemotherapeutic regime of cisplatin and cyclophosphamide. Patients with advanced ovarian cancer, demonstrated improved PFS [235, 236]. However, when repeating the trial with modern chemotherapeutic agents (carboplatin and taxol) OS was reduced in the IFNγ arm and the trial was abandoned [237]. Potential explanations include a reduction in the dose of standard chemotherapeutic agents in the IFNγ arm due to treatment toxicities, or potentially the role of longer term IFNγ administration on the proliferation of Tregs with subsequent immunosuppressive effects [238]. Therefore the role of IFNγ in ovarian cancer is yet to be established.

1.3.6.4 Active vaccination

Active vaccines can include peptides, whole proteins or tumour cell lysates, with the principle of these strategies being to challenge the immune system with TAAs processed by APCs. The processed antigens are then presented to CD8+/CD4+ TCRs in conjunction with HLA class I/II to mediate CD8+ lysis. The TAAs used in vaccines are classified

according to the mechanism of action, for example mutational antigens refer to altered forms of a protein occurring in cancer cells, e.g. caspase-8 [239] and p53 [240]; amplification antigens refer to altered levels of a protein occurring, e.g. Her2/neu [241].

An example of a current vaccination strategy used in ovarian cancer is Oregovomab. This is a murine monoclonal antibody which binds with high affinity to circulating Ca-125. The Ca-125:antibody complex is processed by APCs, such as macrophages and DCs, which produce an adaptive immune response with production of antigen-specific antibodies. This is an example of an "antibody as antigen" strategy [242]. Oregovomab has been associated with improved overall survival in phase II trials in recurrent ovarian cancer [243, 244]. In a placebo controlled RCT of patients in clinical remission from advanced ovarian cancer, Oregovomab was used as a maintenance treatment. Overall, this failed to demonstrate prolonged remission [245]. Subgroup analysis, however, revealed that patients with optimal surgical resection, prior to maintenance with Oregovomab, did show improved OS. This is the basis for a subsequent trial investigating this subgroup of patients (IMPACT) [245].

1.3.6.5 Subverting immune escape mechanisms

In spite of developing activated tumour specific T-cells and immuno-modulators, these techniques may fail to exert their effector function due to an immunosuppressive tumour microenvironment [230]. Several approaches are being investigated to modify this environment through the use of immuno-stimulatory compounds. Indoleamine 2,3-dioxygenase (IDO) is an important enzyme which can contribute to tumour tolerance via the inactivation of T-cell effector functions and the development of Tregs [246]. IDO has been shown to be produced by tumour cells [247]. Concomitant inhibition of IDO during vaccination may therefore improve T-cell activation. 1-methyl-tryptophan inhibits IDO and has shown some benefits when used with chemotherapeutic agents that have an immune cell activating function such as paclitaxel [248]. Similar inhibitors to PGE2, VEGF and

TGF-β, all of whom have immunosuppressive effects in the tumour microenvironment, are being investigated.

A second axis to reduce immunosuppression in tumours is to eliminate intratumoural Tregs. This may be possible through monoclonal antibodies to CD25, which are predominantly expressed in Tregs [249].

A combined approach to vaccination with standard chemotherapeutic agents may have some merit, since chemotherapy produces tissue necrosis with release of immune activators which facilitate tumour antigen presentation to cancer vaccines. In addition, chemotherapy has been shown to preferentially eliminate and inactivate Tregs. This has been demonstrated with cyclophosphamide, producing immuno-stimulation which could be coupled effectively with a cancer vaccine [250].

Low dose radiation has been shown to induce upregulation of MHC class I expression and may improve tumour antigen presentation [251]. Therefore coupling of immunotherapy with radiation and/ or chemotherapy may be beneficial.

1.3.7 Prognosis

The prognosis of patients with ovarian cancer is related to a number of established clinical variables. In addition there are novel molecular, histological and serum parameters presented as useful makers of tumour behaviour which can assist in our understanding of carcinogenesis and may be of use clinically in the planning and development of future treatments.

1.3.7.1 Established prognostic parameters

Age, as with most cancers, predicts survival, with worsening prognosis with increasing age. Even adjusting for stage, patients younger than 50 years have a 5 year survival of 40% compared with about 15% for those older than 50 years [5]. This is likely to reflect numerous factors which are coupled to age such as co-morbidities and general health.

Stage represents the strongest prognostic indictor in ovarian cancer (Figure 1.7). Typically, 5 year survival for fully staged patients with stage I disease is 76 - 93%, depending on tumour grade. For stage II 5 year survival is 60 - 74%, for stage IIIA 41%, for stage IIIB and C 25%, and for stage IV 11% (Figure 1.8) [5].

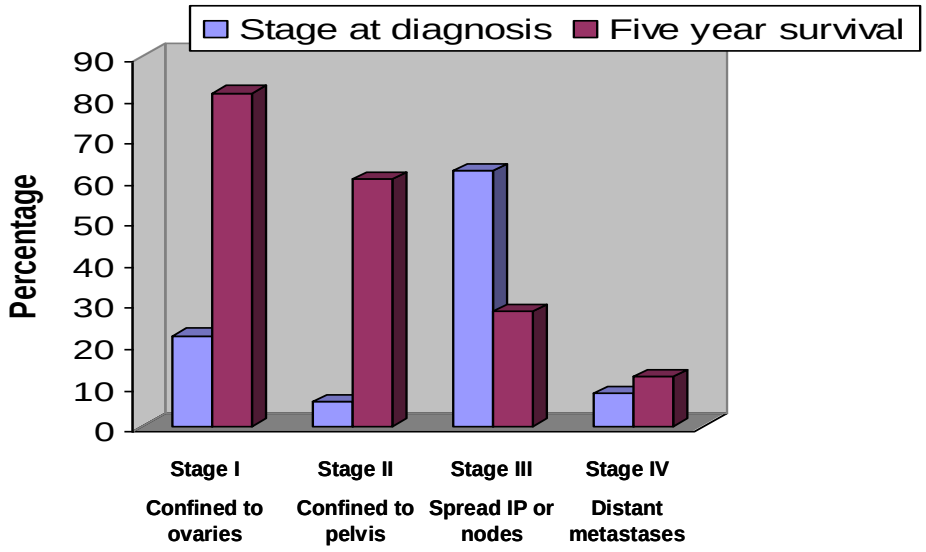


Figure 1.7 Survival of patients with epithelial ovarian cancer by stage [252].

The grade of a tumour correlates with survival especially in early stage where grade I tumours have a 5 year survival of 91% compared to approximately 75% for grades II and III. Similar differences occur in more advanced disease but to a lesser degree [5].

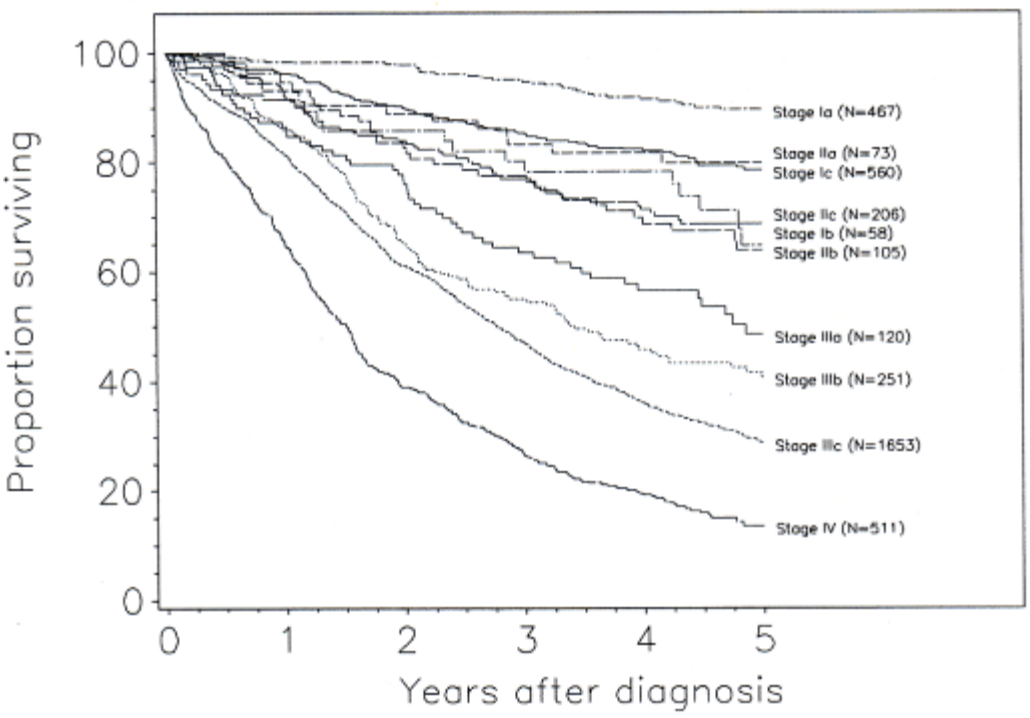


Figure 1.8 Survival of patients with epithelial ovarian cancer by substage. Adapted from Heintz *et al* [113].

As previously discussed the amount of residual tumour following surgery represents a significant independent prognostic variable. This has produced fervent debate with regard to surgical approaches for ovarian cancer.

There has been a slight improvement in prognosis in recent years, which is thought to reflect the introduction of platinum-based chemotherapy. Overall the 5 year survival for women with ovarian cancer remains poor at approximately 30% [113].

1.3.7.2 Novel prognostic markers

Ovarian cancer is a heterogeneous disease and therefore the use of various clinical and pathological markers which can predict the behaviour of an individual tumour is very useful. Biomarkers can be either prognostic (relating to survival) or predictive (relating to tumour behaviour other than survival, e.g. response to chemotherapy). They allow a clinician to provide patients with information on the likely outcome of their disease. This

enables patients and relatives to prepare psychologically, financially and practically for the future. It also allows planning of treatment resources and enables meaningful comparisons to be made between different groups of patients after correcting for confounding prognostic variables. Furthermore, applying equal stratification of known prognostic factors within experimental arms of a clinical trial allows the effect of the intervention to be independently assessed.

Since there are clear advantages to accurately predicting prognosis, novel markers are constantly being sought. New independent markers are those which predict outcome and which are not dependent on an association with an established prognostic factor. As these factors add prognostic information, and are not surrogates for known factors, they also imply causation. Discovering a molecular marker with independent prognostic or predictive power may therefore reveal the importance of a particular intracellular process and identify a target to which novel therapy can be developed. For a prognostic marker to become clinically useful it must be reproducible, add additional value to current markers and have biological plausibility with regard to carcinogenesis [11]. Most biomarkers are derived from analysis of serum or tissue and are related to one of the aspects key hallmarks of carcinogenesis [253].

1.3.7.3 Serum biomarkers

Ca-125 is the most studied tumour marker in ovarian cancer; however, its usefulness is controversial. Some studies suggest that elevated preoperative Ca-125 in stage I disease gives a relative risk of dying of disease of 6.37 [254]. Low postoperative Ca-125 following optimal debulking may be independently predictive of prognosis. In addition, variations in the kinetics of the Ca-125 response during chemotherapy may also be prognostic.

Low preoperative haemoglobin and high platelet count are associated with poor prognosis. These parameters have been successfully used in a trial setting to produce a normogram which accurately predicts both PFS and OS [255].

Tumour cells release pro-coagulant and fibrinolytic factors which result in fibrin split products (D Dimers). There is some evidence that raised D Dimers may predict poor prognosis, although associations with occult thromboembolism may play a role [12].

1.3.7.4 Tissue biomarkers

Cell cycle and apoptosis regulatory proteins, which play an obvious role in tumour biology, have been highlighted as potential biomarkers including p27, p21, p53, cyclin D and E which are discussed elsewhere.

Elevated expression of pro-apoptotic protein bax has been demonstrated to be independently predictive of chemosensitivity and prolonged survival in advanced ovarian cancer. In contrast Bcl-XL, an anti-apoptotic protein, is related to chemoresistance and poor prognosis, although is not independent [12].

Growth factor receptors, such as EGFR and Her-2/neu, have also been shown to be overexpressed in ovarian cancer. Various studies have related overexpression with poor prognosis [256].

Tissue matrix metalloproteinases (MMP) represent a large group of zinc- and calcium-dependent proteolytic enzymes which are capable of degrading most constituents of the extracellular matrix. As such, they are associated with metastatic potential in tumours. Various studies illustrate a negative effect on survival with high expression of MMPs [12].

1.3.8 Exploring novel prognostic marker and tissue microarrays

Molecular markers may be found in bodily fluids (e.g. urine and serum), cell lines or cancer tissue. Cancer induces molecular change in genes which can be detected at through alterations in DNA, RNA or protein expression.

The classical starting point for detection of aberrant pathways in carcinogenesis is at the genome level. However, not all genetic defects result in changes at a protein level, the level which is required to exert a biological effect. Therefore, it is often more practical to detect abnormal protein expression, from which genetic abnormalities can be sought.

Immunohistochemistry (IHC) is a commonly used tool for assessing protein expression. Over recent years a high throughput platform has been developed to enable rapid and consistent analysis of tissue immunohistochemically. This technique is called Tissue Micro Array (TMA) [257].

Traditionally IHC studies relied on staining whole sections of tissue, which was labour intensive and time consuming when analysing large numbers of samples. In addition, since only a limited number of slides could be processed at any one time, multiple IHC runs were needed. This could produce considerable variation in the experimental conditions, and may introduce inconsistencies in analysis. Studies in which large numbers of samples were used proved inefficient with high reagent cost and the consumption of large amounts of archived tissue.

To address these drawbacks the concept of transferring small samples of many tissue blocks into a single block was explored, and a number of techniques have been attempted. The most recent technique was developed by Konnen *et al* who described the use of a custom built instrument, called a Tissue Microarrayer, which produced 0.6mm wide cylindrical tissue cores from archived blocks which could be transferred to holes precisely punched out in a recipient paraffin block [257].

TMA methods reduce reagent costs, variability of experimental conditions and is more time efficient [257]. The use of TMAs in ovarian cancer was validated by Rosen who stained six copies of a TMA containing 45 ovarian cancers for ER, Ki-67 and mutant p53. There was a high concordance of protein expression between the whole tissue sections (used as the gold standard) and the TMA cores [258].

1.4 Aims

There is intense interest in defining prognostic markers which would provide both the physician and the patient with more accurate assessments of likely tumour behaviour. In addition, new prognostic markers can be signposts towards potential targets for molecular therapies, as well as allowing tailoring of established treatments based on the likelihood of effect. This thesis is based on defining the potential effects of immune and angiogenic pathways on ovarian cancer through assessment of the relationship between specific biomarkers and ovarian cancer behaviour. The prognostic significance of these biomarkers will hopefully allow greater selectivity of treatment and indicate abnormalities within tumour metabolism which could be targets for novel therapeutic agents.

Immunohistochemistry is a commonly used tool for assessing protein expression, and in particular prognostic markers. This thesis utilised advances in the technology surrounding IHC by using the TMA. The TMA represents a high throughput platform for IHC which reduces reagent costs, amount of tissue required, variability of experimental conditions and is more time efficient when compared with traditional techniques [257]. The TMA with coupled database had been previously validated [259].

There is a large weight of evidence illustrating the considerable influence of the immune system on tumour development and progression, especially the need for tumours to develop mechanisms to evade immune attack. These mechanisms are not clear, hence, studying the expression of elements within the IFNγ pathway (the pathway which is predominantly involved in the anti-tumour activity of the immune system), may provide an insight into immune evasion in ovarian cancer. By assessing immunohistochemically the expression of IFNGR1, STAT1, p27 and caspase 1 in relation to clinicopathological features, particularly prognosis, has allowed us to test the hypothesis that defects within

this pathway will lead to a poorer prognosis. Patients expressing an intact IFNγ pathway should benefit from IFNγ therapy.

The TNFα super-family represents an apoptotic pathway related to tumour cell killing; TRAIL is one of the ligands in the pathway [260]. The evidence of dysregulation of TRAIL both in tumour development and in tumour resistance to chemotherapy, as well as in its potential therapeutic role, provides an interesting avenue of investigation using the ovarian cancer TMA. The hypothesis follows that tumours with low expression of DR4 and DR5 (TRAIL receptors) would be less prone to TRAIL mediated-attack and therefore apoptosis. This would be reflected in reduced patient survival. If this proved correct these patients would be ideal for TRAIL ligand or anti-TRAIL mab therapy.

Angiogenesis has been established as a vital component among the mechanisms involved in tumour growth and metastasis [261, 262]. VEGF-A is the most extensively researched angiogenic protein. Previous research has produced inconsistent evidence with regard to the importance of VEGF in ovarian cancer and its relation to prognosis. The hypothesis was that tumours with high expression of VEGF would have extensive angiogenesis and a poor prognosis. If this proved correct they would be ideal candidates for bevacizumab therapy.

2 Materials and Methods

2.1 Ovarian Cancer Tissue Microarray

2.1.1 Tissue microarray construction

An overview of the steps involved in producing a TMA is outlined in Figure 2.1. Firstly, potential cases were identified from a prospectively maintained clinical database (Figure 2.2). The original pathology reports from potential cases were reviewed, following which archival tissue for each case was then assessed contemporaneously by a gynaecological pathologist. This enabled identification of representative tissue biopsy sites as well as exclusion of inappropriate cases, for example, metastatic disease from other sites (Figure 2.3). Tissue cores were then physically extracted from the appropriate blocks at sites determined from the previously stained haematoxylin and eosin (H & E) stained sections. This was performed using the Beecher Instruments Manual Tissue Microarrayer. The donor cores were inserted into the recipient tissue array blocks in a grid pattern (Figure 2.4 and Figure 2.5). A meticulous record of the positioning of the tissue from each tumour was produced (Figure 2.6). The final TMA blocks were then sectioned (5μm) and mounted on microscope slides in preparation for immunohistochemical analysis (Figure 2.7).

The cases included were recruited from those patients diagnosed with ovarian cancer between 1st January 1982 and 31st December 1997. The cohort of cases considered for inclusion numbered 424 after initial exclusions had been made. It was possible to find pathology reports for 403. Following contemporaneous review further cases were excluded due to lack of availability of suitable tissue, or the cases not representing ovarian cancer e.g. borderline tumours. Ultimately, tissue cores from a total of 339 surgically treated primary ovarian cancers were represented in four TMA blocks with two representative cores from each case being used to construct two copies of the TMA (Figure 2.2).

Initial validation and assessment of the TMA has been performed by Rolland and Deen [259]. The use of CK7, CK20, CEA and Ca-125 were used to confirm that expression of

these proteins was consistent with established patterns seen in previous studies. This work confirmed that the TMA was capable of being used for immunohistochemical analysis and that the pattern of expression of these commonly studied proteins was consistent with established parameters [259].

Five copies of the TMA were available for analysis; however, only two copies were used for immunohistochemical assessment of the various markers. Previous work has identified that the accuracy of assessment of a biomarker is high when assessing two copies of a TMA, i.e. two samples from each tumour. The assessment of more copies failed to improve accuracy significantly [258]. In addition, the cost of reagents and limiting tissue wastage had to be considered. Conversely, the loss of tumour cores needed to be accounted for, which tended to occur during the antigen retrieval process. Previous work using the current TMA suggested that the rate of loss was 8 - 10%. This compares to rates of 2 - 25% in other studies [258]. Overall, using two copies of the TMA allowed efficient use of tissue and reagents, whilst maintaining accurate assessment of protein expression.

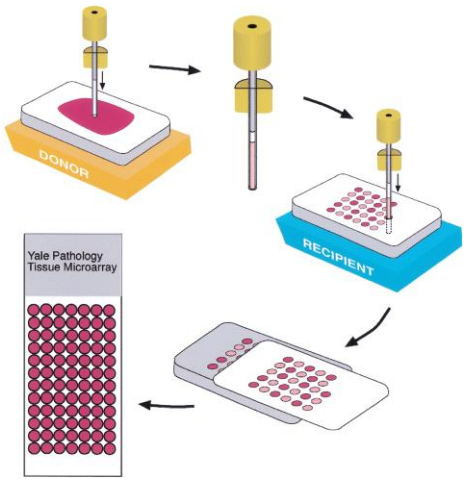


Figure 2.1 A schematic of the current mechanism of tissue microarray construction. An arraying device is used to obtain a core biopsy from a tissue block at a location of interest defined by previous examination of a stained section. The core is then transferred from the donor block to the recipient block, where it is placed, by gentle pressure on the stylus, into a pre-made hole at a specific location. Once the recipient block is completely filled (as determined by the design of the individual array), it is sectioned to reveal 0.6 mm diameter circles of tissue from each case. The array sections may be visualised by all methods used for conventional formalin-fixed, paraffin-embedded tissue sections. Figure adapted from Rimm *et al* [263].

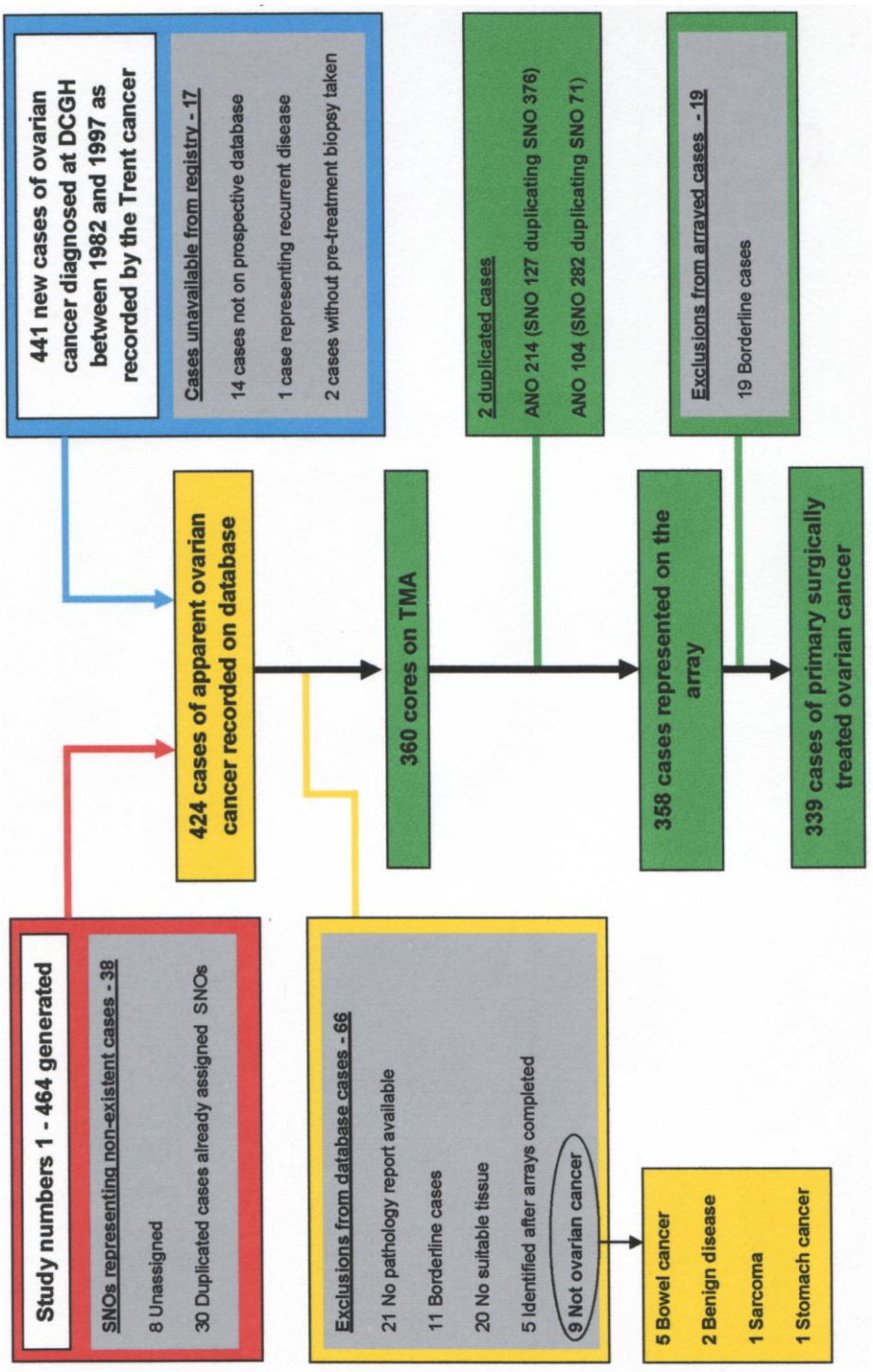


Figure 2.2 A schematic displaying the excluded cases. The study numbers were allocated before the database had been audited for duplicate cases and too many were issued, hence 30 duplicate cases. Borderline tumours were erroneously included on the first array block but subsequently excluded. There were other reasons why cases weren't included in the final array following their histopathological review. Two cases were accidentally arrayed twice. Despite the complex logistics, 339 individual cases of primary ovarian cancer treated surgically were represented on the four final array blocks from the investigated cohort of 441 presenting between 1982 and 1997 at DCGH. Diagram from previous thesis, Rolland [259].

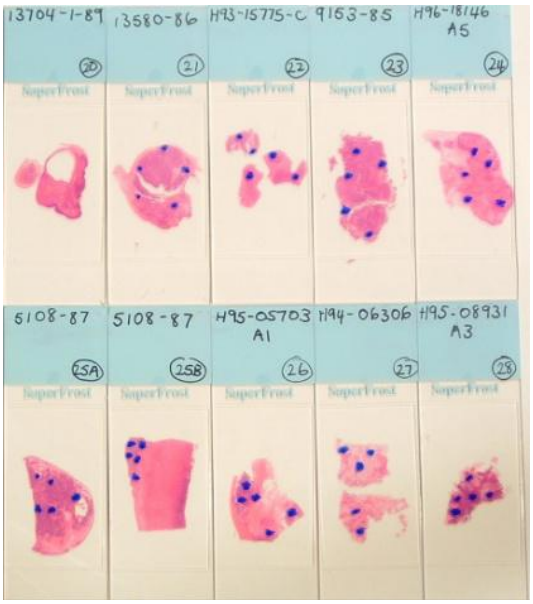
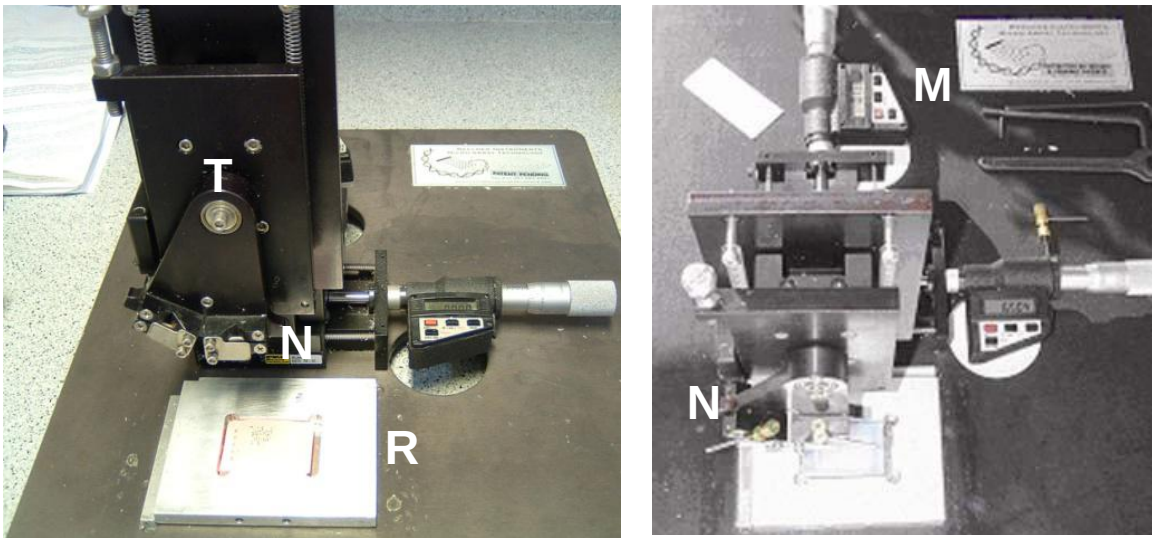


Figure 2.3 Identification of representative areas to include on the array. H&E sections. Sections of these blocks were reviewed, typed and graded by a pathologist with extensive experience in gynaecological oncology. The most representative areas on the H&E slides were marked [259].

Figure 2.4 Identification of representative areas to sample. Marking the blocks. Using the H&E slides, the corresponding representative blocks identified were selected. The marker dots from the H&E slides were transferred onto the blocks to mark the area for extraction by the microarrayer [259].



(A) Frontal view **(B) Seen from above**

Figure 2.5 The Beecher tissue microarrayer [259].
(A) Positioning of a recipient block beneath the tissue microarrayer turret. A tissue core is accurately removed at the position highlighted by the marker points precisely sampled and then transferred using a needle punch (N) mounted on a swinging turret (T) from the donor to the recipient block (R). The turret moves upwards and downwards, allowing the needles maneuverability in the Z axis, ensuring that a correct depth of sample is transferred.
(B) The perpendicular orientation of the micrometers is illustrated (M). These alter the alignment of the turret and needle punch in precise increments. This enables each sample to be accurately placed close to adjacent tumour cores in the X and Y axes to produce rows and columns of tumours which are readily identified using a grid.

J	K								
I									
H	147	148	149	150	151	152			
G	137	138	139	140	141	142	143	144	145	146
F	127	128	129	130	131	132	133	134	135	136
E	117	118	119	120	121	122	123	124	125	126
D	107	108	109	110	111	112	113	114	115	116
C	97	98	99	100	101	102	103	104	105	106
B	87	88	89	90	91	92	93	94	95	96
A	77	78	79	80	81	82	83	84	85	86
	1	2	3	4	5	6	7	8	9	10
	K									

Figure 2.6 An example of a TMA grid used for scoring of tumour cores. The numbers correspond to an individual tumour. K represents the position of a section of kidney tissue, which was used to aid orientation of the microscope slide with the TMA grid.

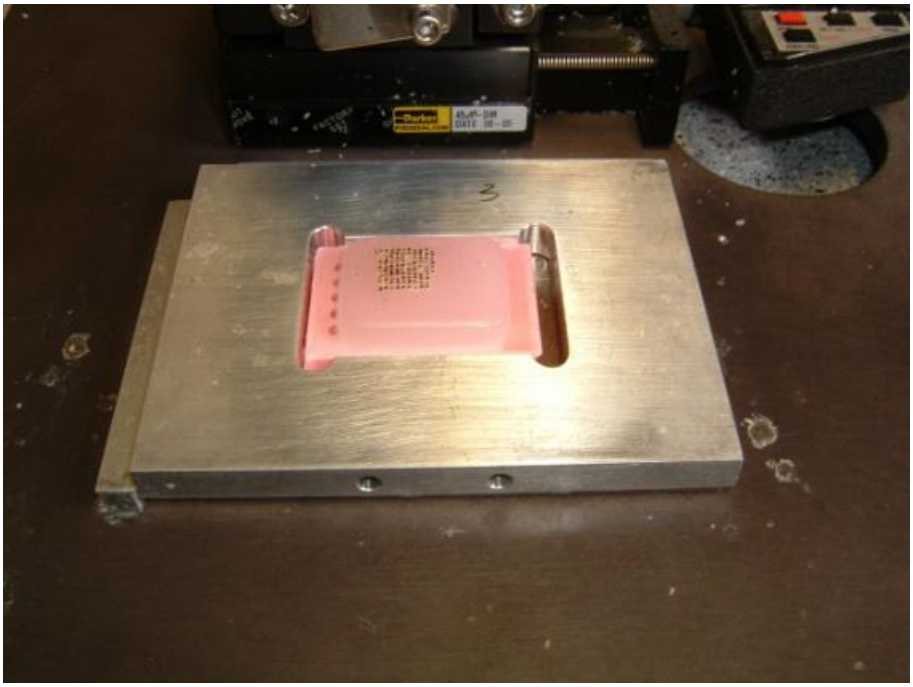


Figure 2.7 A final recipient TMA block. **Once the all the donor cores were inserted, the block was heated for 10-15 minutes at 37°C and the surface leveled using a glass microscope slide. The TMA block was then ready to be sectioned using a standard microtome [259].**

2.1.2 Clinicopathological aspects of the tissue microarray

2.1.2.1 Age

The age of the patients at diagnosis was available for all but one patient with a range of 24 - 90 years. The age distribution was compared with data produced from a large epidemiological study by SEER [10] (Table 2.1). This illustrates that the age distribution of patients within the TMA dataset was comparable with a typical ovarian cancer population. The age of a patient is known to be a prognostic factor in a number of diseases, and this appears to be the case with the patients included in the TMA. On testing the data in SEER categories, increasing age was seen to be associated with reduced survival although in this form it was not an independent prognostic factor (Table 2.2) (Figure 2.8).This is consistent with another group's findings [5]. However, when age was assessed in its continuous form, age did exert independent prognostic power (Table 2.3). This finding is

again consistent with the work of others and further supports the validity of the dataset contained within the TMA [6]. Since there was an apparent independent contribution of age (as a continuous variable) with prognosis, it was included in the Cox model for assessment of the various molecular markers in the thesis.

Table 2.1 The age distribution of patients in the ovarian TMA database when categorised and compared according to the Surveillance, Epidemiology and End Results (SEER) programme of the National Cancer Institute.

Age Categories Years	Cases in SEER series (n)	Cases in SEER Series (%)	Cases in TMA database (n)	Cases TMA database (%)
≤ 30	783	4	1	<1
30-59	9690	43	135	40
≥ 60	11905	53	202	60
Total	22378	100	338	100

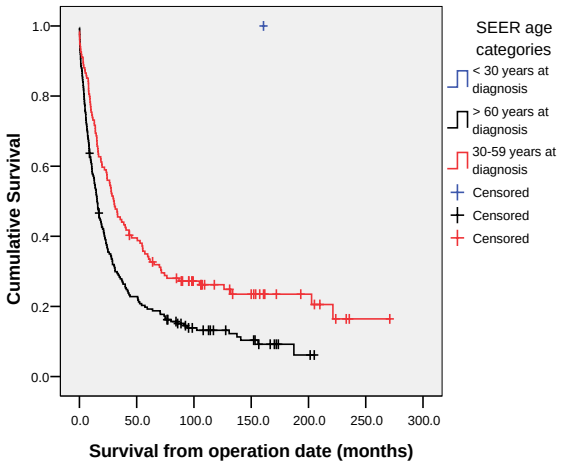


Figure 2.8 A Kaplan Meier plot of age within the Ovarian TMA database when reclassified into the categories used in reporting the SEER data. A statistically significant worsening of survival is seen with advancing age. Log rank= 15.85, p<0.001. Note that age <30 years at diagnosis only has one data point and is therefore represented by a single point.

Table 2.2 Cox multivariate analysis of independent prognostic factors and age data divided into SEER categories. In this model age was not an independent factor.

Variable	Hazard Ratios (95% Confidence intervals)	Significance (p)
FIGO Stage		
I	1	
II	2.424 (1.426-4.121)	
III	4.621 (2.864-7.456)	
IV	5.901 (3.394-10.261)	**<0.001**
Optimal Debulking		
Yes	1	
No	2.238 (1.600-3.131)	**<0.001**
Patient received Chemotherapy		
No	1	
Yes	0.441 (0.315-0.617)	**<0.001**
Age at diagnosis (years) SEER categories		
≤ 30	1	
30-59	2.070 (0.286-14.992)	0.471
≥ 60	3.242 (0.449-23.417)	0.244

Table 2.3 Cox multivariate analysis of independently prognostic factors showing that age data as a continuous variable was an independent prognostic factor.

Variable	Hazard Ratios (95% Confidence intervals)	Significance (p)
FIGO Stage		
I	1	
II	2.567 (1.503-4.383)	
III	4.719 (2.914-7.643)	
IV	6.562 (3.737-11.523)	**<0.001**
Optimal Debulking		
Yes	1	
No	2.131 (1.519-2.991)	**<0.001**
Patient received Chemotherapy		
No	1	
Yes	0.437 (0.312-0.613)	**<0.001**
Age at diagnosis (years) Continuous data	1.024 (1.014-1.035)	**<0.001**

2.1.2.2 Pathology

The 339 cases included in the TMA were mostly epithelial ovarian cancer (98.5%), which is above the 90% normally seen in unselected series [264]. This probably reflects the referral of the rarer subtypes from DCGH to a supra-regional centre. With the exception of the low rates of non-epithelial cancers, the breakdown of histological type was otherwise typical (Table 2.4).

In line with the published frequencies, represented in parenthesises, 52% of the cases were of the serous subtype (40-50%), 12% were endometrioid (12-18%), 10% were mucinous (9-15%), 7% were clear cell (4-6%), and 16% were undifferentiated (5-25%) [5, 10]. Borderline ovarian cancers made up 8% of all cases reviewed (up to 15%) [84].

This series was more likely to have cases with higher grade disease (66%) than generally reported (47%) [5]. This may be a true difference but is more likely to reflect the well documented difficulty in objectively reproducing the commonly used grading systems for all histological types between observers [265]. There is also variation in how ovarian cancers are graded by individual pathologists. Some workers alter their grading system depending on the histological type [266], others grade all subtypes with standard grading systems such as the WHO classification [62] or FIGO [264]. Therefore, due to the inconsistencies in grading of ovarian cancers, it is difficult to draw any meaningful conclusions from comparisons with other studies.

Tumour grade did appear to have prognostic significance on univariate analysis (Figure 2.9). However, this was not maintained on multivariate analysis (Table 2.5). This suggests that the impact of tumour grade on prognosis is dependent on other markers of tumour biology including stage and response to chemotherapy. Since tumour grade was not an independent prognostic marker, it was not included in the Cox model for assessing new markers.

Table 2.4 The distribution of histological subtypes and grades of tumours within the TMA.

Histological type	Number	% of series (n=339)
Epithelial	334	99
Serous	178	53
Serous papillary	21	6
Endometrioid	42	12
Mucinous	35	10
Undifferentiated	54	16
Clear cell	25	7
Non-epithelial	5	1
Yolk Sac	1	
Teratoma	1	
Granulosa	1	
Mixed Mullarian	2	
Borderline cancers*	30/369	8
Grade		
Un-gradable**	2	1
Well differentiated (G1)	39	11
Moderately differentiated (G2)	73	22
Poorly differentiated (G3)	225	66

* 30 borderline cases were excluded during the creation of the 339 sized series
** 1 yolk sac tumour and 1 clear cell tumour were considered un-gradable

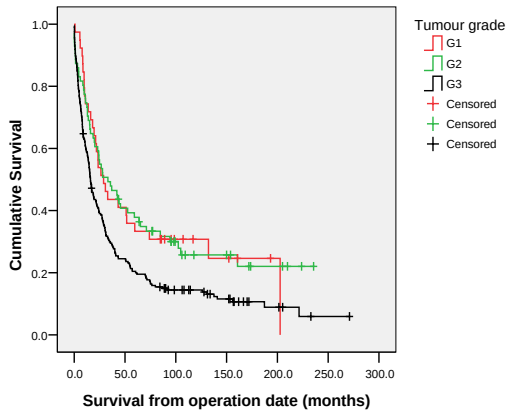


Figure 2.9 A Kaplan Meier plot of grade within the Ovarian TMA database. A statistically significant worsening of survival is seen with poorly differentiated (G3) compared to moderate and well differentiated (G1 and G2) tumours. Log rank= 13.23, p<0.001.

Table 2.5 Cox multivariate analysis of independently prognostic factors showing that tumour grade was not an independent prognostic factor.

Variable	Hazard Ratios (95% Confidence intervals)	Significance (p)
Age at diagnosis (years) Continuous data	1.026 (1.015-1.038)	**<0.001**
FIGO Stage		
I	1	
II	2.434 (1.410-4.201)	
III	4.662 (2.857-7.606)	
IV	6.167 (3.486-10.910)	**<0.001**
Optimal Debulking		
Yes	1	
No	2.031 (1.442-2.861)	**<0.001**
Patient received Chemotherapy		
No	1	
Yes	0.440 (0.313-0.618)	**<0.001**
Histological grade		
G1	1	0.258
G2	1.327 (0.820-2.149)	0.250
G3	1.425 (0.931-2.176)	0.101

2.1.2.3 Stage

Data was available for 330 of the 339 cases, with the classical distribution of cases according to stage being seen. The majority of cases presented at an advanced stage with 63% being stage III or IV (Figure 2.10) (Table 2.6). These frequencies are consistent with those of other contemporaneous cohorts [3, 5, 10, 81-83]. Using the χ^2 test no association between stage and age was revealed (in SEER categories), although worsening grade was associated with a more advanced tumour stage (13.55, p= 0.035).

Not surprisingly there was a significant association between stage and survival both in univariate and multivariate analysis (Table 2.7 and Figure 2.11). This aids to validate both the survival and stage data [5]. Since stage was clearly an independent prognostic factor it was included in Cox's models for assessment of other markers.

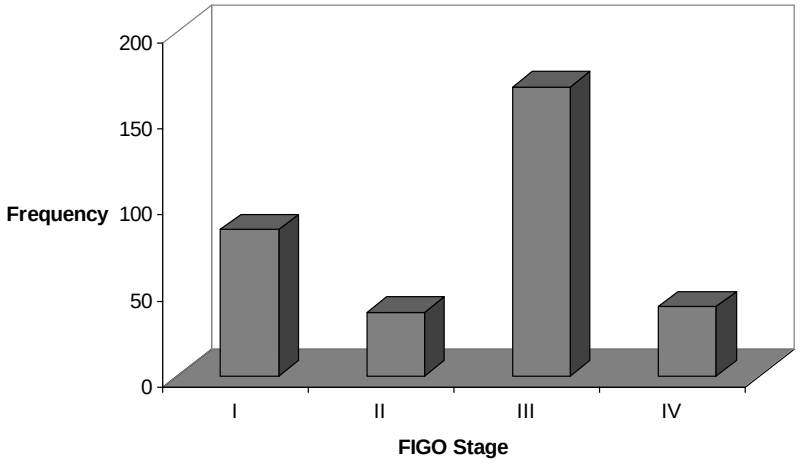


Figure 2.10 A histogram demonstrating the distribution of cases within the FIGO stage groupings.

Table 2.6 The distribution and survival of cases divided according to FIGO stage.

FIGO stage	Cases- n (%)	Mean survival (months)	Median survival (months)	5-year overall survival (%)
I	85 (26)	129.4	137.6	60
II	37 (11)	79.3	40.7	38
III	168 (51)	26.3	13.0	10
IV	40 (12)	15.4	7.3	5
Missing data	9			
Total	330			

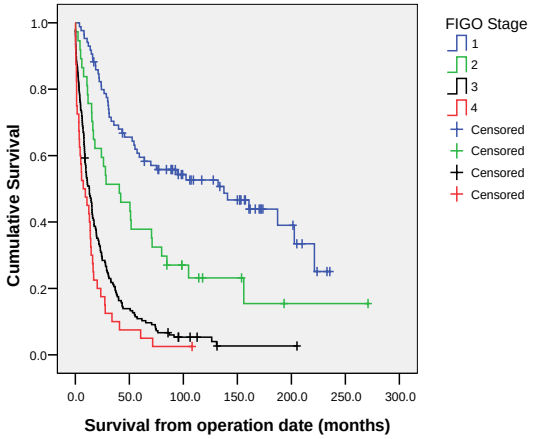


Figure 2.11 A Kaplan Meier plot of stage within the Ovarian TMA database. A statistically significant worsening of survival is seen with increasing stage. Log rank= 114.24, p<0.0001.

Table 2.7 Cox multivariate analysis of independently prognostic factors showing that tumour stage was an independent prognostic factor.

Variable	Hazard Ratios (95% Confidence intervals)	Significance (p)
Age at diagnosis (years) Continuous data	1.026 (1.013-1.036)	**<0.001**
Optimal Debulking		
Yes	1	
No	1.996 (1.419-2.807)	**<0.001**
Patient received Chemotherapy		
No	1	
Yes	0.437 (0.311-0.614)	**<0.001**
FIGO Stage		
I	1	**<0.001**
II	2.463 (1.428-4.247)	**0.001**
III	4.868 (2.979-7.956)	**<0.001**
IV	6.545 (3.706-11.559)	**<0.001**

2.1.2.4 Surgery

There were 13 cases in which information regarding cytoreduction was unavailable. Of those remaining, 130 (40%) had no macroscopic disease after surgery (optimally debulked), 89 (27%) had tumour deposits of less than 2cm and 107 (33%) had deposits of greater than 2cm. Overall survival was better in patents who were optimally debulked compared with the two other groups (Figure 2.12). This relationship was still apparent when cases with any macroscopic disease were compared with no macroscopic disease (Figure 2.12). These relationships were seen to be independently prognostic in the Cox model (Table 2.8).

The proportion of cases debulked to <2cm in the literature range from 36 - 87% [5, 63, 97] and those with no visible macroscopic disease from 15 – 85% [5, 109, 110]. This series data were well within these ranges, therefore further supporting its consistency. Optimal debulking by either standard is an accepted independent prognostic factor, which was found to be the case in this series, again validating the dataset. Most recent literature

defines optimal debulking as no macroscopic disease. Therefore, this simpler definition of optimal debulking was used when including debulking status in subsequent survival analyses.

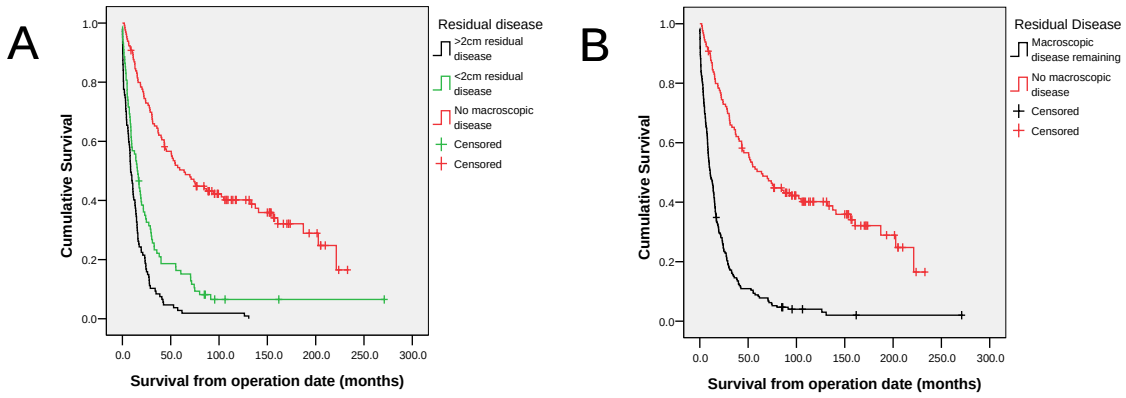


Figure 2.12 A Kaplan Meier plot of the degree of surgical debulking with survival in the Ovarian TMA database. (A) Illustrates survival when categorised into three groups. This demonstrated significant reduction in survival with increasing volumes of residual disease. Log rank= 130.11, p<0.0001. (B) Categorised into two groups. This illustrates statistically significant worsening of survival with any macroscopic disease. Log rank= 107.41, p<0.0001.

Table 2.8 Cox multivariate analysis of independently prognostic factors showing that residual disease after surgery when separated into two or three groups was an independent prognostic factor.

Variable	Hazard Ratios (95% Confidence intervals)	Significance (p)
Age at diagnosis (years) Continuous data	1.023 (1.012-1.034)	**<0.001**
FIGO Stage		
I	1	
II	2.579 (1.489-4.465)	
III	4.795 (2.913-7.892)	
IV	6.210 (3.480-11.079)	**<0.001**
Patient received Chemotherapy		
No	1	
Yes	0.426 (0.303-0.601)	**<0.001**
Residual disease (3 groups)		
No macroscopic disease	1	
Macroscopic disease (<2cm)	1.675 (1.154-2.432)	**0.007**
Macroscopic disease (>2cm)	2.555 (1.743-3.748)	**<0.001**
Residual disease (2 groups)		
No macroscopic disease	1	
Macroscopic disease	1.996 (1.419-2.807)	**<0.001**

2.1.2.5 Survival

All survival rates were calculated from the date of surgery to the date of death, and these were compared to histology reports to ensure accuracy. Data were 100% complete. Patients were followed up according to a local protocol, three monthly for two years, 6 monthly for 5 years and thereafter annually and indefinitely. The date of latest follow up was recorded in the database to facilitate calculation of latest known survival date. Data were available for 336 cases (99% complete). Data concerning date of death were also available for 99% of cases. Using this data, patient survival status was coded as 0 (alive) or 1 (dead). When the database was locked on 31st November 2005, 57 (17%) of patients were still alive. The precise cause of death was not specifically recorded but anecdotally the majority died of related causes with only four women known to have died from causes not attributable to ovarian cancer.

The data concerning date of diagnosis, latest follow-up, date of death and survival status were used to generate crude overall survival (OS) data with 1 year and 5 year survival rates. There were three instances of missing data (99% complete). 24 patients (7%) died within a 30 day period following surgery. OS ranged from 0 – 271 months with a mean OS of 60.5 months (95% CI 50.6 – 70.44) and median OS of 19.5 months (95% CI 15.5 – 23.5). The crude one year OS was 63.7% and the corresponding five year measurement 25% (Figure 2.13). Mean, median and crude five year OS decreased in a stepwise manner within the FIGO stage categorisation with stage I cancer having a median OS of 137.6 months and a five year OS of 60% whereas stage IV cancers had a median OS of 7.3 months and a five year OS of 5% (Table 2.6).

During the follow up period, which ranged up to 270 months, 83% of women died. The majority of these women are likely to have died from ovarian cancer, which was the anecdotal observation. However, the case fatality rate appears higher than expected given the established fatality rate of approximately 65% [267]. In spite of the higher than expected fatality rate, known associations with clinicopathological factors with accepted independent prognostic value was consistently reproduced in this series. Therefore, it was decided that although the fatality rate was high, the otherwise expected correlations with previous studies of ovarian cancer survival, allows for its valid use in assessing novel prognostic marker expression. In addition, as the markers assessed compared differences in survival within the tumour population, any confounding effects on survival would be equally attributable to all patients within the cohort.

Reasons for this worse survival might include patient factors (older age group, worse performance status), tumour factors (above average number of high grade cases) and treatment factors (inadequate staging procedure, inadequate treatment, delays in diagnosis). Similar observations, suggesting that survival rates in the UK for gynaecological cancer were poor in comparison with other developed countries, has led to implementation of changes to the framework of cancer services. These improvements

have been coordinated through initiatives such as The NHS Cancer Plan [268] and The Cancer Reform Strategy [269].

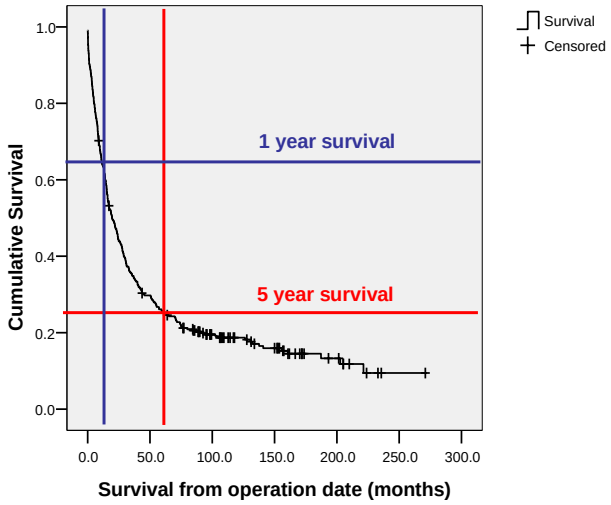


Figure 2.13 A Kaplan Meier plot of overall survival in the Ovarian TMA database. Illustrating both one and five year survival of 65% and 25% respectively.

2.2 Immunohistochemistry

The principle method of investigation used during this work consisted of immunohistochemistry (IHC); a technique which utilises the specific interaction between antigens and antibodies to produce an accurate representation of the expression of a particular protein within tissue. This technique can be used on freshly frozen tissue or Formalin Fixed Paraffin Embedded (FFPE) tissue, which may have been stored for a number of years. FFPE tissue is known to produce stable conditions, which maintains tissue morphology for many years [270]. All the tissue used during this work was provided from archived FFPE. This study related to comparative differences in protein marker expression between tumours and how this altered prognosis. Therefore, as the aim was not to determine variations in relation to non-cancerous tissue, marker expression was not investigated in normal ovaries.

2.2.1 Processing of samples

Following surgical removal, the tissue was usually fixed for 24 hours but longer for larger tissue specimens, in 10% NBF. The samples that were used originated from cases between 1982 and 1997, and therefore had been archived for between 6 and 23 years.

Chemical fixation enables the preservation of tissue. However, due to cross linking of molecules within DNA, RNA and protein, alternations in molecular structure can occur. These bonds can only be disrupted by hydrolysis [271]. Cross linking of proteins has the advantage of stabilizing the antigens involved in IHC, however, these antigens may be hidden and result in reduced immunoreactivity [272].

The immunoreactivity of some tissue is seen to reduce with time. This is related to the techniques used in fixation and the tissue type. NBF was the fixation method employed on the tissue within this study, which has been shown to preserve antigenicity well [273].

All the tissue used during the experiments including the TMA, whole sections of ovarian cancer and positive control tissue were cut into 4 μm thick sections. These were mounted onto slides which were positively charged; this improved the adherence and reduced the risk of tissue loss during processing. The control tissue used was normally tonsil, which was known to express all the markers under investigation in the study. For assessment of each marker two copies of the TMA were used. This allowed efficient use of the study tissue whilst still maintaining accuracy approaching that of whole sections [258]. Whole sections of ovarian cancer from the study cohort we used to assess the pattern of staining of each marker prior to assessment of expression on the TMA.

The tissue was embedded in paraffin wax which required removal prior to tissue processing. The slides were heated at 60ºC in an oven for 10 minutes to soften the paraffin. The slides were then placed in a xylene bath (stored in a fume cupboard) for 20 minutes to dissolve the wax. Rehydratrion of the tissue was then achieved by immersing the slides in graded ethanol baths (100, 95 and 75%) for 10 seconds in each.

2.2.2 Antigen retrieval

For tissue embedding the chemical fixation and heating of the tissue at 60^{O}C leads to denaturing of the antigenic epitopes. Formalin fixation can lead to cross linking of unrelated proteins to the target antigen resulting in masking and ultimately reducing immunogenicity. To improve immunogenicity of the tissue, treatment with antigen retrieval solutions can be used, prior to application of the primary antibody [274]. Two antigen retrieval solutions were employed during this work, Citrate buffer at pH 6.0 or Ethylene Diami Tetracetic Acid (EDTA) at pH 8.0 - 9.0 (Table 2.9). An 800W microwave was used to heat the retrieval solution.

2.2.3 Preventing non-specific binding

IHC relies on primary antibodies binding to specific amino acid sequences to accurately locate a particular protein. Unfortunately, it is possible for antibodies to bind to unrelated proteins through hydrophobic interactions. This will lead to non-specific staining. Normal swine serum (NSS) was used to prevent this type of interaction occurring. NSS will mask the hydrophobic sites on the tissue, responsible for non specific binding, which would otherwise interfere with the specific interaction of the antibody and epitope.

2.2.4 Primary antibody

2.2.4.1 Selection

The primary antibodies selected for IHC on FFPE tissue should be related to epitopes which are known to be active following the fixing and embedding process. Fortunately, in this work there were commercially available antibodies for all of the proteins studied. Commercially developed antibodies have a number of advantages; they have proven specificity, are available in sufficient quantities, and in general have been used in previously published work.

2.2.4.2 Titration

The optimal working dilution for a specific antibody equates to the concentration at which consistent specific staining is produced with no or minimal background staining. The optimum antibody concentration will be affected by a number of variables including the fixation technique and storage method used on the tissue. The time elapsed between sectioning and staining the tissue along with the antigen retrieval methods used will also influence the affinity of the antibody to the epitope. The age and storage conditions of the primary antibody and whether an avidin-biotin blocking step is performed may alter its activity [275].

Clearly, due to the numerous factors which could potentially affect the concentration of primary antibody required to produce optimum staining, titration of the antibody is required. The antibody concentration suggested by the manufacturer was used as a guide, and serial dilutions around this concentration were used to determine the optimum conditions. Occasionally, variation in the application time of the antibody provided improvements in the quality of staining, however, the majority of antibodies required 60 minutes application.

2.2.4.3 Negative control

The negative control had all of the reagents applied to it with the exception of the primary antibody. This ensured that all staining of experimental tissue was produced by the specific interaction between the antibody and epitope. In place of the primary antibody, either NSS or an antibody of the same subclass and coding for non-human epitopes were used.

2.2.5 Washing

The slides were mounted onto Shandon cover plates and placed into Shandon Sequenza™ slide racks. This system utilises a capillary gap produced by holding the slide

and cover plate upright and apart by a fixed distance with reagents applied via a small reservoir above. This ensures that consistent volumes of reagents are incubated with each slide (Figure 2.14).

Thorough washing of the sections between steps with Tris Buffered Saline (TBS) was essential. This prevented both drying of the tissue sections and non-specific staining produced from interaction of residual reagents which had not bound to the target protein.

Figure 2.14 The Shandon cover plate Sequenza™ system

2.2.6 Signal amplification

The technique of IHC relies firstly on the ability of an antibody produced in another species (e.g. mouse, goat, rabbit) to recognise a human antigen. Secondly, the reaction of an oxidizing agent, such as HorseRadish Peroxidase (HRP), with a chemical which produces a colour change (chromogen) e.g. 3, 3'-DiAminoBenzidine (DAB).

Initially a direct method of antigen staining was used. This involved an enzyme labelled 'primary antibody' which recognised the specific antigen within the tissue. The application of a substrate chromogen (i.e. a substrate which produces a coloured end product) reacts with the primary antibody to produce staining at sites on the tissue where the antibody has bound (Figure 2.15) [275].

The direct method has been widely replaced by a two step, or indirect method, which allows amplification of the signal, produced, and was used in this work. This technique involved binding of the primary antibody to the tissue antigen as in the direct method, however, following this an enzyme labelled 'secondary antibody' is added. This secondary

antibody binds to the primary antibody (now the antigen). This can be at multiple sites and thus numerous secondary antibodies can bind to a single primary antibody producing an amplification effect (Figure 2.15) [275].

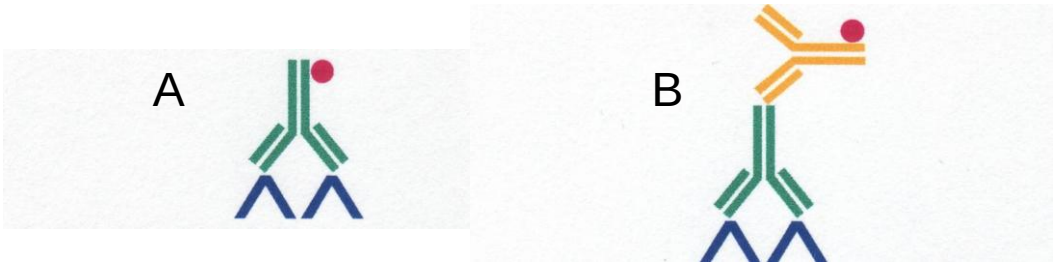


Figure 2.15 Direct method: Enzyme labelled 'primary antibody' reacts with tissue antigen (A). Two step indirect method: Enzyme labelled 'secondary antibody' reacts with 'primary antibody' bound to tissue antigen (B). Figures adapted from Immunohistochemical Staining methods 3rd Ed. DAKO [275].

Following the amplification of the secondary antibody, a further amplification in signal is achieved when applying the enzyme component. This consists of a (strept)avidin-biotin-enzyme complex (ABC), and exploits a phenomenon whereby one molecule of streptavidin, a protein produced in bacterium Streptomyces, can bind four molecules of biotin (vitamin B7). This enables streptavidin to bind to biotinylated antibodies, as well as biotin complexed with an enzyme, to react with a chromogen (Figure 2.16). HRP was the enzyme linked to the avidin-biotin complex, and when exposed to the chromogen, 3,3'-diaminobenzidine (DAB), oxidises DAB to produce a brown substrate which can be seen during light microscopy [275].

The secondary antibody used is biotinylated; it is engineered to express multiple molecules of biotin, each able to bind avidin once. The following two reagents are then mixed and given at least 30 minutes to associate; they are "A" streptavidin and "B" biotin complexed with HRP. When associated into "AB", each streptavidin molecule binds several biotin-HRP complexes. After addition to the tissue each multi-HRP expressing

complex can bind to each biotin site on the secondary antibody, therefore multiplying the sites available for chromogen binding many times in comparison with the direct method (Figure 2.16) [275].

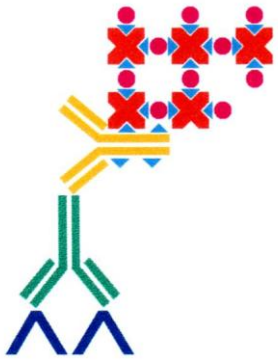


Figure 2.16 In the ABC method, the streptavidin-biotin-enzyme complex reacts with the biotinylated secondary antibody. Figure adapted from Immunohistochemical Staining Methods 3rd Ed. DAKO [275].

A commercially produced kit was used for this work:

(StreptABCComplex/ HRP Duet, Mouse/ Rabbit - K0492; Dako, Ely, UK).

A- streptavidin in 0.01 mol/L PBS, 15mmol/L NaN3; pH 7.2 diluted to 1:100

B- biotinylated horseradish peroxidase in 0.01 mol/L PBS, 15mmol NaN3; pH 7.2 diluted to 1:100

C- biotinylated goat anti-mouse/rabbit secondary antibody diluted to 1:100.

Avidin binding capacity can be seen in normal tissues. This could potentially lead to binding of “AB” to sites on the tissue unrelated to the antibody complex, resulting in non-specific staining. To eliminate this problem incubation of the tissue with avidin and biotin prior to the application of the “AB” solution will saturate these sites and prevent binding of “AB” to anything other than the secondary antibody [276].

A commercial blocking kit was used to perform this: (Avidin-Biotin blocking kit – SP-2001, Vector Laboratories, Burlingame, US).

2.2.7 Visualisation of the primary antibody

The chromogen DAB was used to highlight the location of the antibody complex. This complex consisted of the primary and secondary antibody to which the streptavidin-biotin proteins with HRP are bound. The classical brown colour is produced by oxidisation of DAB by HRP. The brown substrate is clearly seen on light microscopy, and even by the naked eye if protein expression is strong.

Some tissues contain endogenous peroxidase activity which can produce nonspecific staining by oxidizing DAB in areas which do not contain the primary antibody complex. This can be prevented by blocking any endogenous peroxidase activity before the antigen retrieval step. This is achieved using 0.3% hydrogen peroxide in methanol, produced by adding 4mls 30% hydrogen peroxide to 400mls methanol.

The staining was of DAB was intensified by the application of 0.5% copper sulphate in 0.8% sodium chloride. Haematoxylin then was used to counter-stain the background blue.

The tissue slides were dehydrated in the graded alcohols, xylene was used to remove the ethanol, before allowing the slides to dry in a fume cupboard. The cover slips were mounted in the slides using Distyrene, Plasticiser and Xylene (DPX) in preparation for light microscopy.

Table 2.9 The 'in-house' preparation of solutions most commonly used in immunohistochemistry.

Solution	Materials	Company	Quantity	Method
Citrate buffer concentrate	Citric Acid	Sigma-Aldrich, Gillingham, UK	21g	Working dilution- Add 450mls dH2O to 50mls citric acid concentrate. Adjust the pH to 6.0 with 5 mmol HCl or NaOH.
	NaOH pellets	Sigma-Aldrich, Gillingham, UK	10g	
	dH2O	In-house	500mls	
EDTA concentrate	TRIS	Sigma-Aldrich, Gillingham, UK	12.1g	Working dilution- Add 450mls dH2O to 50mls EDTA concentrate. Adjust the pH to 8.0 with 5 mmol HCl or NaOH.
	EDTA	Sigma-Aldrich, Gillingham, UK	374g	
	dH2O	In-house	500mls	
TBS	Tris Base	Sigma-Aldrich, Gillingham, UK	6.06g	For 1000mls of 0.05m Tris, 0.15 NaCl TBS- Dissolve Tris base in 90ml dH2O and adjust ph with 10mmol HCl to 7.6. Dissolve NaCl in 100mls dH2O. Add remaining dH2O.
	NaCl	Sigma-Aldrich, Gillingham, UK	8.76g	
	dH2O	In-house	1000mls	
0.5% Copper Sulphate solution	CuSO4	Sigma-Aldrich, Gillingham, UK	2.5g	For 500mls 0.5% CuSO4 solution- Add 2.5g CuSO4 to 500mls dH2O and add 4.0g NaCl.
	NaCl	Sigma-Aldrich, Gillingham, UK	4.0g	
	dH2O	In-house	500mls	

dH_2O: Deionised Water **NaOH: Sodium Hydroxide**

TBS: TRIS Buffered Saline **NaCl: Sodium Chloride**

$CuSO_4$: Copper Sulphate **EDTA: Ethylene Diamine Tetracetic Acid**

Tris: TRIS(hydroxymethyl)aminomethane

2.2.8 IHC protocol

The following protocol summarises the methods above and was used as the template for each experiment.

1. Place TMA slides into a slide basket and then heat in an oven at 60º for 10 minutes.

2. Immerse the slide basket in a xylene bath for 10 minutes. Repeat this step using an additional xylene bath.

3. Immerse the slide basket in graded ethanol baths from 100% to 95% to 75% for 10 seconds in each.

4. Immerse the slide basket into a hydrogen peroxide (0.3%) and methanol bath for 15 minutes.

Steps 2-4 should be performed in a fume cupboard.

5. Wash the slides in a container of tap water with constant irrigation of fresh tap water. Avoid direct water current over side baskets.

6. Immerse the slide basket in 500mls of the specific antigen retrieval solution made up to the required dilution and pH placed in a microwavable plastic container. Place the container in the microwave (800W) and heat for 10 minutes on high power and then 10 minutes on low power.

7. Wash the slides in a container of tap water with constant irrigation of fresh tap water. Avoid direct water current over side baskets.

8. Using Sequenza cover plates mount the slides and place them in a Sequenza rack™.

9. Wash the sections with TBS.

10. Apply 100µl of NSS diluted 1:5 in TBS to each slide for 15 minutes.

11. Apply 100µl of avidin D solution to each slide for 15 minutes and before rinsing three times with TBS. Next apply 100µl of biotin solution to each slide for 15 minutes.

12. Rinse the slides with TBS three times, for 5 minutes each time.

13. Apply 100µl of optimally diluted primary antibody to each slide, with the exception of the negative control. Leave for 60 minutes.

14. For the negative control apply 100µl of an appropriate negative control immunoglobulin, or TBS if such an antibody is not available. Leave for 60 minutes.

15. Wash the sections three times, for 5 minutes each time, with TBS.

16. Apply 100µl of the secondary antibody (reagent “C”) diluted to 1:100 in TBS, to each slide. Leave for 30 minutes.

17. Wash the sections three times, for 5 minutes each time, with TBS.

18. Prepare the “AB” solution, this must be at done at least 30 minutes before the “AB” can be used. The “AB” solution is produced by combing and diluting reagent “A” and “B” to 1:100, by adding equal quantities to the appropriate volume of TBS.

19. Apply 100µl of the “AB” complex to each section. Leave for 60 minutes.

20. Wash the sections three times, for 5 minutes, with TBS.

21. Dilute DAB to 1:50 in its substrate buffer. Apply 100µl of DAB to each slide. Leave for 5 minutes. Repeat this step 3.

22. Wash the sections three times, for 5 minutes each time, with TBS.

23. Apply 100µl of 0.5% copper sulphate solution to each slide. Leave for 5 minutes. Repeat this step.

24. Wash the sections three times, for 5 minutes each time, with TBS.

25. Apply 100µl of haematoxylin to each slide. Leave for 5 minutes. Repeat this step.

26. Dismount the slides from the Sequenza rack™ and cover plates. Wash the slides in a container of tap water with constant irrigation of fresh tap water. Avoid direct water current over side baskets.

27. Remount the slides in the slide basket and dehydrate them by rotating them through the graded alcohol baths in reverse order to the step, spending 10 seconds in each.

28. Place the sections into a xylene bath for 10 seconds and repeat this step in a further xylene bath.

29. Mount slide covers onto the slides using DPX and leave the slides overnight.

Steps 27-29 should be performed in a fume cupboard.

2.2.9 Specific immunohistochemical protocols for individual markers

2.2.9.1 IFNGR1 protocol

The standard methodology was used as described in the general methods section. 500mls of pH 6.0 citrate buffer was used for antigen retrieval. Test sections were incubated with 100µl of mouse anti-human Interferon-gamma receptor 1 (IFNGR1) monoclonal antibody (MMHGR-1, Tebu-Bio©) which was found to be optimally diluted at 1/50 (v/v) in TBS for 60 minutes at room temperature. This commercial antibody had been previously validated [277]. Positive control tissue comprised whole sections of colorectal

cancer. The primary antibody was omitted from the negative control, which was left incubating in NSS.

2.2.9.2 Evaluation of IFNGR1 staining

The tumour cores were assessed by two experienced observers (TD and PhR). Two different copies of the TMA were used which provided two samples from different areas of the tumour in each case. The cores were scored independently by the observers with a consensus being reached in difficult cases and those were scoring differed by more than 5%. There was high concordance between observers with less than 10% of cores requiring reassessment. This concordance is in line with previous authors findings [278]. Following initial review of the staining characteristics a semi-quantitative system, using both intensity and percentage of cells staining, was adopted as the most accurate method of demonstrating marker expression. The scoring was performed in a coded manner with observers blinded to the clinical and pathological parameters of the case. To utilise both the staining intensity (graded as: 0 - absent, 1 - weak, 2 - moderate, 3 - strong) and distribution (% of cells staining) in the assessment of each core, an intensity score was calculated (% cells staining × intensity of staining). To produce a single value of marker expression for each tumour a mean value was calculated for the IS of the two cores from each tumour. To determine low and high expression the median intensity score for the cohort was used, with an intensity score of greater than 90 defining high IFNGR1 expression.

2.2.9.3 STAT1 protocol

Standard protocol described in the general methods section was used. 500mls of pH 6.0 citrate buffer was used for antigen retrieval. Test sections were incubated with 100µl of mouse anti-human STAT1 monoclonal antibody (ST1-3d4, Zymed©) which was found to be optimally diluted at 1/50 (v/v) in TBS for 60 minutes at room temperature. This was a commercial antibody which has previously been used in analysis of STAT1 expression in

breast cancer [279]. Positive control tissue comprised whole sections of normal tonsil. The primary antibody was omitted from the negative control, which was left incubating in NSS.

2.2.9.4 Evaluation of STAT1 staining

The expression of STAT1 was seen in two main sub-cellular locations, namely the cytoplasm and the nucleus. As such, the analysis consisted of scoring each tumour separately for these two locations.

There were a number of scoring systems for cytoplasmic expression of STAT1. We opted to use that of Chen *et al* [280] in which tumours were deemed positive if greater than 5% of tumour cells expressed STAT1. As for nuclear STAT1, no such previous studies were found and therefore any nuclear STAT1 was taken as positive. These scoring systems were decided prior to any interpretation of the cores. All tumour cores were scored independently by two scorers, both with extensive experience of TMA work (TD & AA). Those cores in which there was discrepancy were reassessed and agreement reached.

2.2.9.5 p27 protocol

The standard methodology was used as described in the general methods section. 500mls of pH 9.0 EDTA buffer was used for antigen retrieval. Test sections were incubated with 100µl of mouse anti-human p27kip1 monoclonal antibody (M7203, DAKO) which was found to be optimally diluted at 1/50 (v/v) in TBS for 60 minutes at room temperature. This commercial antibody has been previously validated and used on colorectal and breast carcinomas [281]. Positive control tissue comprised whole sections of tonsil. The primary antibody was omitted from the negative control, and was replaced with mouse IgG_1 to Aspergillus niger glucose oxidase, an enzyme not present in human tissue and hence a suitable negative control (X0931, DAKO).

2.2.9.6 Evaluation of p27 staining

The expression of p27 was seen in two main sub-cellular locations, namely the cytoplasm and the nucleus. As such the analysis consisted of scoring each tumour separately for these two locations. Numerous methods exist for evaluation of immunohistochemical p27 expression, with most studies designing novel scoring systems. In consequence, adoption of the established system of intensity scoring (see IFNGR1 methods) was deemed an appropriate system [282]. This system incorporates both the intensity and proportion of tumour cells staining. This scoring system was decided prior to any interpretation of the cores. All tumour cores were scored independently by two scorers, both with extensive experience of TMA work (TD & AA). Those cores in which there was discrepancy were reassessed and agreement reached. To determine low and high expression the median intensity score was used, with an intensity score for cytoplasmic and nuclear p27 of greater than 40 and 25 respectively defining high expression.

2.2.9.7 Caspase 1 protocol

The standard methodology was used as described in the general methods section. 500mls of pH 6.0 citrate buffer was used for antigen retrieval. Test sections were incubated with 100µl of rabbit anti-human caspase 1 polyclonal antibody (06-503 Upstate Biotechnology) which was found to be optimally diluted at 1/40 (v/v) in TBS for 60 minutes at room temperature. This was a commercially developed antibody which has been used previously in immunohistochemical analysis of pancreatic carcinoma and detects expression of the pro-enzyme (p45) and active p20 subunit [283]. Positive control tissue comprised whole sections of tonsil. The primary antibody was omitted from the negative control, which was left incubating in NSS.

2.2.9.8 Evaluation of caspase 1 staining

The expression of caspase 1 was seen almost exclusively in the cytoplasm. Yang *et al* used a semi-quantitative analysis which used intensity and proportion of tumour cells displaying immunoreactivity, therefore, a similar system was adopted for analysis of the TMA [283]. This scoring system was decided prior to any interpretation of the cores. All tumour cores were scored independently by two scorers, both with extensive experience of TMA work (TD & AA) Those cores in which there was discrepancy were reassessed and agreement reached. To determine low and high expression the median intensity score was used, with an intensity score of greater than 150 defined as high expression.

2.2.9.9 VEGF protocol

The standard methodology was used as described in the general methods section. 500mls of pH 6.0 citrate buffer was used for antigen retrieval. A pre-diluted rabbit-anti-human VEGF antibody was used (SP28, Abcam, Cambridge, UK). Optimisation of the staining was performed on ovarian cancer whole section mounts, using a range of incubation times. Two hour incubation was chosen to stain the arrays as it showed the best results with minimal background staining. Positive control tissue comprised whole sections of tonsil. The primary antibody was omitted from the negative control, which was left incubating in NSS.

2.2.9.10 Evaluation of VEGF staining

When staining was positive, it was primarily of cytoplasmic location and its pattern was uniform among the cancer cells within each core. The staining ranged in intensity from mild to strong and was initially recorded as such. However, to interpret the expression, and for the purposes of analysis, a scoring system was designed to identify potentially sensitive tumours to anti-VEGF therapies. As such cases were categorised as either

"high" expressors (represented by the strongly-stained group) or "low expressors (comprised of the negative, weak and moderate groups). This scoring system was decided prior to any interpretation of the cores. All tumour cores were scored independently by two scorers, both with extensive experience of TMA work (TD & AA). Those cores in which there was discrepancy were reassessed and agreement reached.

2.2.9.11 DR4 and DR5 protocol

The standard methodology was used as described in the general methods section. 500mls of pH 6.0 citrate buffer was used for antigen retrieval. Test sections for DR4 and DR5 were incubated with 100µl of rabbit anti-human DR4 polyclonal antibody (H-130, Santa Cruz) and goat anti-human DR5 polyclonal antibody (616380, Calbiochem) respectively. The antibody to DR4 was found to be optimally diluted at 1/30 (v/v) and DR5 at 1/50 (v/v) in TBS for 60 minutes at room temperature. These were commercial antibodies which had been previously validated [284, 285]. Positive control tissue comprised whole sections of tonsil. The primary antibody was omitted from the negative control, which was left incubating in NSS.

2.2.9.12 Evaluation of DR4 and DR5

Expression of DR4 and DR5 was mainly in the cytoplasm, with occasional nuclear staining with DR5. The expression of both proteins was homogenous with all cells in the cores being either positive or negative. Scoring was initially recorded as intensity and the percentage of cells staining. For the purposes of analysis, and in line with previous authors' work [286], cores were deemed positive if there was any immunoreactivity. This scoring system was decided prior to any interpretation of the cores. All tumour cores were scored independently by two scorers, both with extensive experience of TMA work (TD & AA) Those cores in which there was discrepancy were reassessed and agreement reached.

Table 2.10 Summary of immunohistochemical protocols and antibodies used in individual marker studies.

Antigen	Antibody Type	Company	Clone Code	Dilution	Antigen retrieval solution	Positive Control Tissue	Negative Control
IFNγ receptor 1	IgG_1 Monoclonal Mouse	Tebu-Bio, Peterbrough, UK	MMHGR-1	1:50	Citrate pH 6.0	Colorectal cancer	Omission of primary antibody NSS
STAT1	IgG_1-Kappa Monoclonal Mouse	Zymed, S. Francisco, US	ST1-3D4	1:50	Citrate pH 6.0	Tonsil	Omission of primary antibody NSS
P27	IgG_1-Kappa Monoclonal Mouse	DAKO, Ely, UK	SX53G8	1:50	EDTA pH 9.0	Tonsil	Mouse IgG_1 X0931, DAKO
Caspase 1	IgG Polyclonal Rabbit	Upstate Biotech, Herts, UK	06-503	1:40	Citrate pH 6.0	Tonsil	Omission of primary antibody NSS
VEGF	IgG_1 Monoclonal Rabbit	Abcam, Cambridge, UK	SP-28	Pre-diluted	Citrate pH 6.0	Tonsil	Omission of primary antibody NSS
DR4	IgG Polyclonal Rabbit	Santa Cruz, CA, US	H-130	1:30	Citrate pH 6.0	Tonsil	Omission of primary antibody NSS
DR5	IgG Polyclonal Goat	Calbiochem, Darmstadt, Germany	616380	1:50	Citrate pH 6.0	Tonsil	Omission of primary antibody NSS

2.3 Statistical tests

The statistical analyses were all performed using the computer package SPSS versions 11.0 & 13.0.

2.3.1 Null and alternative hypothesis and p values

Many statistical tests assessing differences between groups use null and alternative hypotheses. If the null hypothesis is correctly accepted, there is no difference between groups. Accepting the alternative hypothesis indicates that there is significant difference between the groups. A false positive result is that which occurs when the null hypothesis is incorrectly rejected. Conversely a false negative occurs when the alternative hypothesis is falsely rejected.

A p value gives the probability that the null hypothesis has been incorrectly rejected. This is expressed as a value between zero and one. p values close to one suggest that the observed difference has occurred by chance, whereas those close to zero are more likely to represent genuine differences. If a p value is 0.05, this would indicate that there is a 5% chance the observed difference has occurred by chance i.e. a false positive. Typically a p value of less than 0.05 is used to indicate differences that are statistically significant.

2.3.2 Univariate analysis – Persons Chi squared (χ^2) test

Much of the data produced in this study was of a qualitative nature, this necessitated division of clinicopathological characteristics into groups. Since the data was qualitative comparisons between groups required the χ^2 test. This test compares the frequency of observed results (O) against expected frequencies (E) if there was no difference between the groups. A contingency table of the data is produced. The categories are cross tabulated against each other forming tables which range in size from 2 x 2 upwards,

dependent upon the subdivision of the categories. If positive and negative expression of a marker is being compared with grade 1, 2 or 3 disease a 2 x 3 table would be formed containing six individual cells. The difference between O and E is calculated for each variable, and to ensure all values are positive the square is used. For example:

$$\frac{[E-O]^2}{E}$$

The results of each of these calculations are added together to give the $\chi 2$ value:

$$\chi 2 = \Sigma \frac{[E-O]^2}{E}$$

Hence, the higher the value of χ^2 the greater the difference between the observed and expected values. The calculation of the p value incorporates the χ^2 value and the number of degrees of freedom, which can be gleaned from the table. In this work the value was calculated through statistical software. The degrees of freedom relates to the number of factors being compared. The degrees of freedom = (number of columns - 1) x (number of rows - 1).

The χ^2 test is a simplified version of Fisher's Exact test, the χ^2 test compares favorably if the cells within the table contain above the specified minimum number. No more than 20% of cells should contain less than five cases and no cells should be empty, the Fisher's Exact test was used if these parameters were not met. Yates correction was applied when the numbers in a fourfold table were small. The Yates correction reduced the χ^2 value by subtracting 0.5 from the difference between the observed and expected values. This reduces the risk of overestimating statistical significance if the data sets are small [287]. These methods are automatically applied by the statistical package SPSS.

2.3.3 Univariate analysis of associations with survival

2.3.3.1 Mean and median survival times

The simplest illustration of survival between groups is through the calculation of average survival time. This can be represented by either the mean or the median survival time. The mean is most appropriately used in normally distributed data and median in non-

parametric data sets. For the purposes of analysis both values were calculated, although as the data was non-parametric the median survival times were most relevant.

2.3.3.2 Kaplan-Meier plot

At any point in time patients entered into a study are either alive, dead or lost to follow up. To achieve the most accurate representation of survival no patients should be lost to follow up and analysis should be performed once all the patients have died. Realistically this is rarely possible and would take an unfeasible amount of time. A Kaplan-Meier calculation allows analysis of survival in incomplete data sets i.e. with patients lost to follow up and some still being alive.

To perform a Kaplan-Meier analysis a life table is constructed which consists of patients' survival time from shortest to longest. At regular time intervals the proportion of patients at risk of dying is calculated. As patients are lost to follow up they are censored i.e. removed from subsequent analyses. The resulting cumulative survival is plotted against time as a curve with censored cases being denoted by "+". When comparing groups of patients within a population a separate life table is constructed for each group with the resultant survival curve. These curves are plotted on the same axis to enable comparison.

For this analysis to be accurate various assumptions are made, firstly, censoring of patients is a random event and that these cases would behave no differently from the other cases in the group they originated from. An example where bias could be introduced at this point is that dead patients don't attend follow up for obvious reasons, and if the death is not known, the patient may be incorrectly censored, hence biasing the results. In addition, patients may be censored if death occurs from another cause; this is only statistically valid if the cause of death is completely unrelated to the risk of dying from the disease being studied. Kaplan-Meier analysis also assumes that patients entered into the study early have the same long term survival prospects as those entered later. Lastly, the survival data should be continuous and not grouped to the nearest follow up point [287].

2.3.3.3 Log rank statistic

A Kaplan-Meier plot displays the differences in survival between groups graphically. Whilst apparent differences may be observed between survival curves, the statistical significance can not be deduced from the plot. A log rank test can be applied to the data to determine whether any differences are statistically significant. As in Kaplan-Meier analyses, life tables are constructed and sequential calculations of observed and expected deaths occurring in each group is made each time a death occurs. This is performed using a 2 x 2 table of observed and expected deaths in each group with a χ^2 test applied. When data are censored, they are excluded from subsequent analyses. The end result is a number of χ^2 values representing each death day which is then used to generate the log rank statistic and the p value.

A log rank test allows interpretation of survival differences across the whole study period, as opposed to assessing survival rates at arbitrary time points. For the test to be accurate the same assumptions made in the Kaplan-Meier analysis must hold true. In addition the relative proportions of deaths occurring in each group should not change over the study period. Therefore, if the Kaplan-Meier survival curves cross, indicating variation in the relative proportions of deaths between the groups, then the log rank test should not be applied [287].

2.3.3.4 Multivariate analysis of associations with survival – Cox proportional hazards model

The log rank test applied in survival analysis provides an estimate of the likelihood that an individual factor affects prognosis; however, it does not allow for the potential influence of associated factors which could produce a confounding effect. In addition, it does not calculate the magnitude of the effect on survival. To address these issues a multivariate analysis was performed using the Cox regression model. This analysis measures

interrelationship between multiple variables and produces an estimate of the degree of effect produced called a hazard ratio (HR).

This model produces a baseline survival curve and calculates the HR for each variable by measuring the effect of adding and removing each variable from the model. This provides an estimate of the independence and size of effect for each variable on the overall hazard rate. The HR produced for each variable reflects the increased or decreased likelihood of death at any time point due to this factor. A HR of one indicates the factor has no influence on survival. A HR of greater than one means the factor is associated with increased likelihood of death, and less than one with a reduced likelihood of death. The further the HR is from one the greater the effect of the factor on prognosis. 95% confidence intervals (CI) are also generated. These delineate the range of values within which the true HR has a 95% chance of lying. For a factor to have a statistically independent effect on survival the CI range must lie either wholly above or below one. A factor with a 95% CI which straddles one could have either a negative or positive effect on survival and therefore is not a statistically significant independent prognostic factor.

Factors may appear statistically significant on univariate log rank testing but fail to be significant on Cox analysis, i.e. 95% CI straddles one. This suggests that the variable is confounded by other factors which are truly prognostic; this factor is simply acting as a surrogate. For example, if a particular factor simply reflects the stage of a cancer, then on univariate analysis it will appear significant since stage is strongly prognostic. However, when this factor is included in a Cox model with stage, the confounding effect of stage is accounted for and the factor will fail to be significant.

Similarly to Kaplan-Meier and log rank testing, the Cox model assumes that a factor's effect on prognosis remains constant throughout the study period. Therefore, factors with Kaplan-Meier curves that cross should not be included in this model because in that situation this assumption is not valid. Clearly factors which are not statistically significant prognostic factors on univariate analysis should not be included in a Cox model [288].

3 The Interferon Gamma Pathway

3.1 Introduction

3.1.1 Interferons

Interferons are a group of cytokines of which there are two types. Type I, which include interferon alpha (IFNα) and beta (IFNβ), are produced by most cells. Type II, of which interferon gamma (IFNγ) is the sole member, is only produced by specific immune cells. IFNγ is structurally different to those in group I, binds to a different receptor and is coded for on a different gene locus. IFNγ has a pivotal role in immune response through a number of mechanisms, including its direct anti-proliferative and pro-apoptotic effects on stressed cells and its indirect effects on immune cells such as macrophages. Macrophage stimulation by IFNγ produces direct anti-tumour and anti-microbial effects and also induces them to release chemokines to trigger immune infiltration [289].

3.1.2 Interferon gamma receptor

The functional IFNγ receptor is made up of four subunits, two ligand binding IFNGR1 chains (also known as α chains) and two signal transducing IFNGR2 chains (also known as β chains). The receptor belongs to the class II cytokine receptor family since it binds ligands in a 'V'-shape formed within the extracellular domain [290]. In lymphocytes, IFNGR2 is thought to be the rate limiting factor since IFNGR1 is usually found in surplus at the cell surface [291]. Both IFNGR chains lack intrinsic kinase/ phosphatase activity and hence must be associated with additional signalling machinery to achieve signal transduction [292, 293]. This is accomplished through binding of Janus tyrosine kinase (Jak) 1 and 2, and signal transducer and activator of transcription 1 (STAT1), to specific motifs in the intracellular domain of the IFNγ receptor [294] (Figure 3.1).

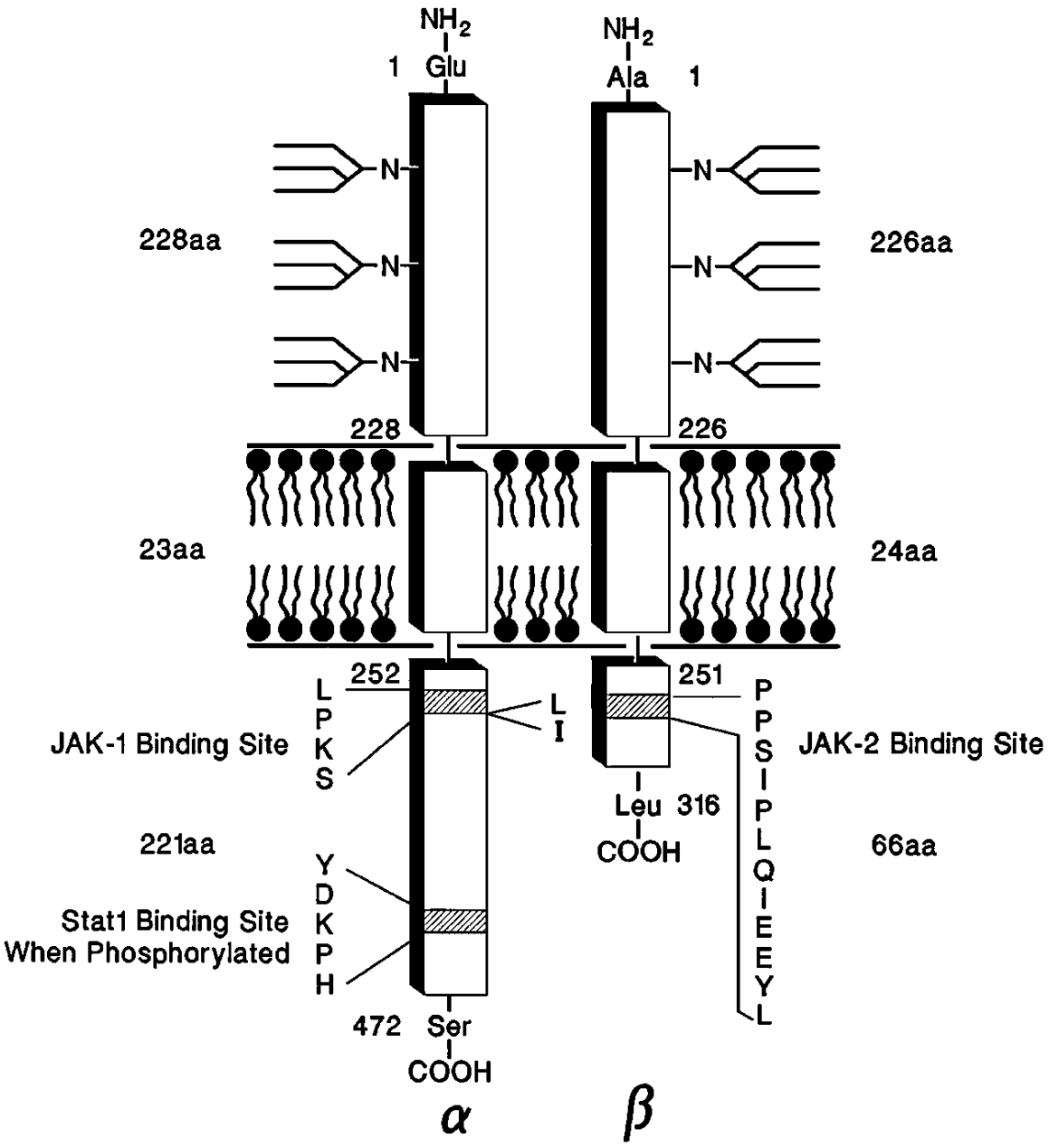


Figure 3.1 Polypeptide chain structure of the human IFN receptor. The IFN receptor consists of two species-matched polypeptides. The IFN receptor α chain is required for ligand binding and signalling. The IFN receptor β chain is required primarily for signalling and plays only a minor role in ligand binding. The intracellular domain of the receptor α chain contains two functionally important sequences: (1) an LPKS sequence required for α chain association with the tyrosine kinase Jak1, and (2) a YDKPH sequence that, when phosphorylated, forms the docking site for latent Stat1. The intracellular domain of the receptor β chain contains a functionally important box1/ box2 sequence required for Jak2 association. Figure adapted from Bach *et al* [295].

3.1.3 Interferon gamma signal transduction and STAT1

Ligand binding of two IFNγ molecules to the associated pair of IFNGR1 induces auto-phosphorylation and activation of Jak2; this in turn trans-phosphorylates Jak1. Activated Jak1 proceeds to phosphorylate a functionally critical tyrosine residue on each IFNGR1 chain, which leads to the formation of two adjacent docking sites for the SH2 domain of latent STAT1 [178]. The STAT1 pair recruited to the docking sites of the two IFNGR1 molecules is phosphorylated, probably by Jak2, before forming an activated STAT1 homodimer and dissociating from the receptor. The phosphorylation of the four critical tyrosines (Jak1, Jak2, IFNGR1 and STAT1) has been demonstrated to occur in less than one minute of treatment with IFNγ [292]. The activated STAT1 homodimer enters the nucleus and binds to DNA at promoter elements which initiate or suppress transcription of IFNγ related genes (Figure 3.2). Whilst STAT1 acts as a transcription factor for numerous pathways, it appears that it is the sole transcription factor for the IFNγ pathway. This was demonstrated in $STAT^{-/-}$ mice which displayed similar unresponsiveness to IFNγ as $IFNGR1^{-/-}$ mice [296].

In type 1 IFN pathways, STAT1 can bind with other molecules, such as other STAT and IRF molecules, to produce different signalling effects. This is known as heterodimerization, although the STAT1:STAT1 homodimer formed during IFNγ signalling remains the most common binding combination [297]. A number of different stimuli result in phosphorylation of STAT1, including type I (α and β) and type II (γ) IFN, lipopolysaccharide, IL-2, IL-12, TNFα and platelet derived growth factor [298]. The ability of STAT1 to activate or suppress gene transcription relies on the presence of other transcription factors at the promoter element, for example IRF-9, specificity protein-1 and heat shock factor-1 [299].

Although STAT1 homodimers are most closely related to IFNγ there are (as aforementioned) other pathways which utilise STAT1. This suggests that the functioning of STATs may be more refined than originally thought. There is some suggestion that the

concentration and rate of STAT activation may influence the type of signalling produced [296]. It is also recognised that STATs can form heterodimers, which will clearly affect their function. These factors coupled with the previously mentioned inhibitory factors associated with STAT1 result in a complex, dynamic signalling system which provides a number of functions, not simply the transmission of IFNγ signals.

There is some early evidence of a STAT1-independent signalling pathway for IFNγ. This may exert proliferative effects, as opposed to the anti-proliferative effects, of the STAT1:IFNγ pathway, possibly through an as yet unknown IFNγ receptor [300].

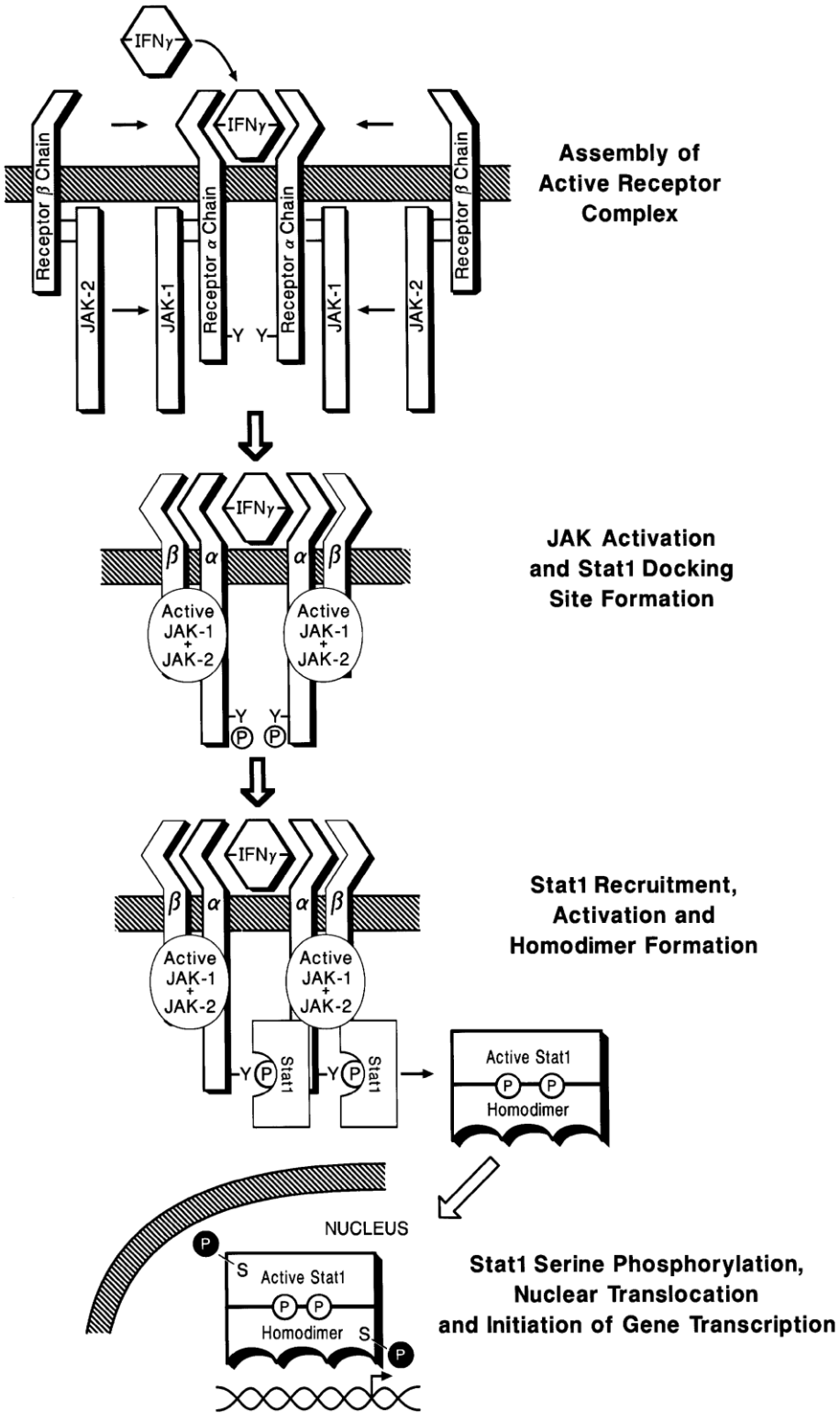


Figure 3.2 Proposed signalling mechanism for IFNγ pathway. Figure adapted from Bach *et al* [295].

3.1.4 Interferon gamma regulated genes

IFNγ has diverse functions that include anti-viral, anti-proliferative, pro-apoptotic and immune modulation effects. The anti-proliferative effects of IFNγ are primarily translated through p21 and p27, which are produced from IFNγ dependent genes with IFNγ stimulation resulting in increased transcription of both. These molecules are cyclin dependent kinase (CDK) inhibitors. CDK is required during the cell cycle with inhibition causing arrest in the G1-S phase. IFNγ also downregulates c-myc which is responsible for activating cyclin:CDK complexes and inducing transcription of genes required for S phase [301] .

p27, also known as Kip 1, is expressed in most cells and, along with p21, is a member of the Cip/Kip family of cell cycle inhibitors [302]. The established role of p27 is to bind and inhibit cyclin-cyclin dependent kinase (CDK) complexes, thereby inhibiting cell cycle progression between G1 and S phase [303]. p27 binds to cyclin D and CDK4 in the cytoplasm which promotes translocation of the complex to the nucleus. In non-cycling cells, p27 binds to cyclinD-CDK4 complexes as well as cyclinE-CDK2. The p27-cyclinE-CDK2 complex results in inhibition of CDK2 resulting in cell cycle arrest. In proliferating cells levels of cyclinD-CDK4 increase and preferentially bind p27 resulting in degradation of p27, this produces release of cyclinE-CDK2 from p27. The liberation of CDK2 from p27 results in loss of inhibition, and subsequent stimulation of cell cycle progression [302], (Figure 3.3). This anti-proliferative effect gives p27 a significant role as a tumour suppressor gene. p27 is rarely mutated and is predominantly regulated at a post-transcriptional level by degradation in the ubiquitin-proteosome pathway [304]. As previously alluded to, some of the anti-proliferative effects of IFNγ are through p27, hence its inclusion in the current study.

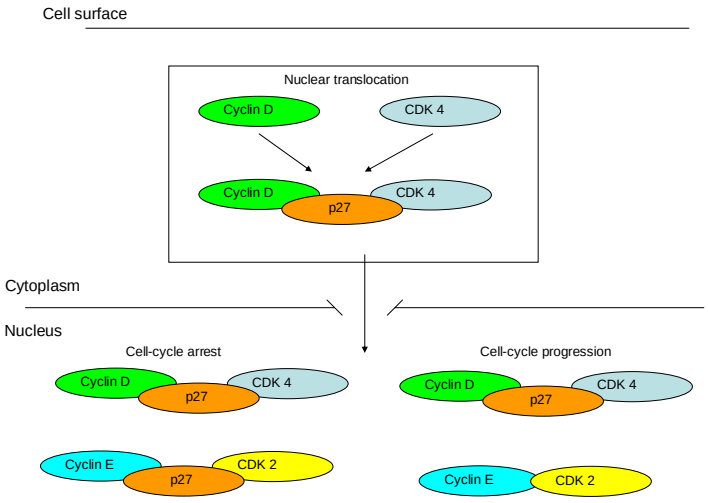


Figure 3.3 Cip/Kip protein (p27) regulates cyclin-CDK complexes in the cytoplasm. p27 acts as a bridge between cyclin D and cdk4 to promote their association. Following binding, p27 enhances the nuclear translocation of the complex. Once in the nucleus, nascent cyclin-D-CDK complexes titrate p27 from cdk2, thereby inducing cell cycle progression. Upon cell cycle arrest, the levels of p27 increase, saturate D type proteins and then bind to cyclin-E-cdk2 to block catalytic activity of the kinase.

With such a well documented tumour suppressor role, it is intriguing that such controversy exists as to the prognostic significance of p27, including its sub-cellular location. Low p27 expression has been associated with poor prognosis in a range of malignancies including breast, colorectal, ovary, prostate, bladder and pancreatic tumours [306-309]. Other studies contrast these findings with a loss of p27 producing a favourable prognostic outcome in pancreatic [310], colon, oesophageal and endometrial cancers [311]. Therefore the prognostic influence of p27 as part of the IFNγ pathway is clearly of interest, especially in view of the controversial findings of previous studies.

The pro-apoptotic effects of IFNγ are mediated through a number of pathways including Fas and TNFα. IRFs are a family of transcription factors induced by type I and II IFN pathways and as such are associated with a variety of immune, cell cycle regulation and apoptotic effects. Interferon regulatory factor 1 (IRF1), a tumour suppressor gene, is thought to play a central role in producing the apoptotic effects of IFNγ [312]. This is primarily through the activation of caspase 1 [181] and discussed later. Levels of IRF1 may be the determining factor as to whether IFNγ induces or protects against apoptosis.

Exposure of cells with high levels of functioning IFNγ receptor to IFNγ produces a rapid activation of STAT1, thereby inducing high levels of IRF1 which in turn trigger the apoptotic pathway. However, if cells have low levels of IFNγ receptor, then exposure to IFNγ produces lower levels of phosphorylated STAT1 and in consequence low levels of IRF1. These lower levels of IRF1 are not sufficient to trigger apoptosis [291]. This is illustrated in experiments in which over expression of IFNγ receptor in cells usually expressing low levels, changes the response to IFNγ exposure from an anti-apoptotic/ proliferative effect to a pro-apoptotic effect [291]. This may explain the differing responses of cell types to IFNγ. Another member of the IRF family, IRF2, acts as a suppressor of IRF1, by binding to the same gene promoter elements [313]. These are both produced at low levels in the non-stimulated cells; however IRF2 is more stable than IRF1 and so accumulates in the nucleus and represses the actions of IRF1 [314]. During IFNγ signalling the subsequent up-regulation of IRF1 allows competition with IRF2 and stimulates transcription of many IFNγ inducible genes [315].

Caspase 1 is a cysteine protease belonging to a family of caspases which represent the common final pathway for a number of apoptotic mechanisms. Caspases tend to act as the principle executors in these pathways and produce an amplification cascade between individual caspases. Caspases are expressed as inactive pro-enzymes prior to proteolytic activation via a number of pro-apoptotic stimuli [316]. Caspase 1 can be triggered by a number of mechanisms but is associated with producing IFNγ mediated apoptosis through IRF1 [181]. Hence, tumour cell evasion from immunosurveillance through defects in the IFNγ pathway may be through inhibition of the pro-apoptotic effects of IFNγ induced caspases. Therefore reduced expression of caspase 1 through a defective IFNγ-IRF1-caspase 1 pathway may result in a poorer prognosis.

3.1.5 Regulation of the IFNγ pathway

A number of mechanisms exist which can prevent overstimulation of a cell by IFNγ, which is illustrated by the inhibition of STAT1 activation within one hour of IFNγ treatment [298]. Following signal transduction the IFNGR1-IFNγ complex is internalised and enters the endosomal pathway. Once internalised the ligand is degraded and the receptor is usually returned to the cell surface, however, the receptor can also be degraded, thereby producing a negative feedback loop [317].

IRF2 inhibits IRF1, thereby influencing the degree of cell stimulation by IFNγ [314]. Some IFNγ inducible targets provide further negative feedback. SOCS1 (suppressor of cytokine signalling 1) represents one mechanism which controls IFNγ signalling, and this gene has binding sites on its promoter region for STAT1 [318]. SOCS1 binds to and inhibits Jak and hence produces a negative feedback loop [319], which occurs through high affinity binding to the SH2 region thus preventing STAT1-Jak interaction [320]. SOCS1 is strongly induced after IFNγ stimulation [321]. Over expression of SOCS1 inhibits IFNγ signalling through inhibition of Jak 1 and 2 [322]. SOCS1 deficient mice die soon after birth from excessive IFNγ dependent multi system inflammatory tissue destruction [323]; the effects of which are reversed by administration of anti-IFNγ antibodies [324].

There is a large weight of evidence illustrating that the immune system has considerable influence on tumour development and progression, especially the need for tumours to develop mechanisms to evade immune attack. These mechanisms are not clear, hence, studying the expression of elements within the IFNγ pathway (the pathway which is predominantly involved in the anti-tumour activity of the immune system), may provide an insight into immune evasion in ovarian cancer. Immunohistochemical assessment of the expression of IFNGR1, STAT1, p27 and caspase 1 in relation to clinicopathological features, and in particular prognosis, will allow us to test the hypothesis that defects or deficiencies within this pathway will lead to a poorer prognosis.

3.2 Results

3.2.1 IFNGR1

3.2.1.1 Clinicopathological characteristics

Of the 395 patients identified within the series, 339 had archived tissue available. These tumours were included in the tissue array. Five of the 339 tumour cores had no tissue available for analysis, however, due to its loss during immunohistochemical processing. This resulted in the availability of over 98% of cases for analysis, this core loss rate compares favourably with previous TMA studies. This subgroup of 334 patients was similar to that of the overall group which, as previously discussed, is representative of a typical ovarian cancer population (Table 3.1). Of particular importance is the comparability of five year survival, which is 24% in the whole series and 25% in those analysed.

3.2.1.2 IFNGR1 staining

Staining was seen predominantly within the cytoplasm and cell membrane. Complete lack of staining was observed in 22% of cores. There was an even distribution of staining intensity seen within the remainder with mild, moderate and strong staining occurring in 23%, 30% and 26% respectively (Table 3.2 and Table 3.3). In those tumours with IFNGR1 expression the staining tended to be of uniform intensity with the majority of tumour cells within the core being positive. Almost half of tumours (45%) displayed immunoreactivity in at least 75% of cells, and no staining was seen within the negative controls (Figure 3.4).
The use of an intensity score allowed the heterogeneity of IFNGR1 expression to be taken into account, with the mean intensity score being taken between two cores from each tumour. The median intensity score for the tumours was 90, this was used to divide the tumours into high and low expression of IFNGR1. Using this system there were 163 low intensity and 171 high intensity cores.

Table 3.1 Clinicopathological characteristics of the whole series and those analysed for IFNGR1 expression.

Variable	Whole series n=395 (%)	IFNGR1 analysed cases n=334 (%)
Age (years)	n=394	n=333
<30	1 (0.3)	1 (0.3)
30-59	167 (42.4)	133 (39.9)
≥60	226 (57.4)	199 (59.8)
FIGO Stage	n=375	n=326
I	99 (22.6)	83 (26.5)
II	46 (12.3)	37 (11.3)
III	188 (50.1)	167 (51.2)
IV	42 (11.2)	39 (12.0)
Optimal Debulking	n=376	n=322
Yes	157 (41.8)	129 (40.1)
No	219 (58.2)	193 (59.9)
Tumour Grade	n=376	n=332
1	50 (13.3)	38 (11.4)
2	93 (24.7)	72 (21.7)
3	233 (62)	222 (66.9)
Histological Type	n=395	n=334
Serous	203 (51.4)	177 (53.0)
Endometrioid	46 (11.7)	42 (12.6)
Mucinous	50 (12.7)	34 (10.2)
Undifferentiated	65 (16.5)	51 (15.3)
Clear Cell	26 (6.6)	25 (7.5)
Other	5 (1.3)	5 (1.5)
Chemotherapy	n=388	n=332
Platinum	196 (50.5)	166 (50.0)
Non-platinum	80 (20.6)	72 (21.7)
None	112 (28.9)	94 (28.3)
Randomised Treatment	69/395 (17.5)	57/334 (17.1)
Five Year Survival	n=385	n=329
Yes	91 (23.6)	82 (24.9)
No	294 (76.4)	247 (75.1)
Alive at time of data censoring	n=388	n=331
Yes	60 (15.5)	56 (16.9)
No	328 (84.5)	275 (83.1)

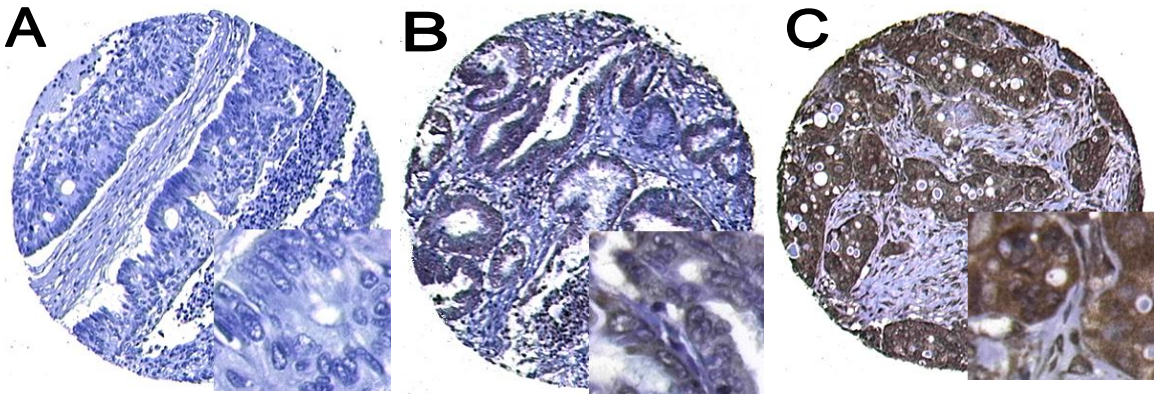


Figure 3.4 (A) Shows a core from tumour demonstrating negative IFNGR1 staining. B & C show cores of tumour demonstrating moderate (B) and strong (C) IFNGR1 staining at x100 magnification (inserts at x400 magnification).

Table 3.2 Intensity of cytoplasmic staining with IFNGR1.

Intensity of Staining	Number	Percentage
No staining	72	21.6
Mild	77	23.1
Moderate	97	29.6
Strong	87	26.1

Table 3.3 Proportion of viable tumour cells staining with IFNGR1.

Proportion of tumour cells staining	Number	Percentage
No cells	72	21.6
1 - 25%	18	5.4
26 - 50%	59	17.7
51 - 75%	35	10.5
76 - 100%	149	44.7

3.2.1.3 Comparison of IFNGR1 expression and patient tumour characteristics including survival

In a univariate analysis, using the χ^2 test, no significant relationship between IFNGR1 expression and the standard clinical and pathological variables was apparent. There did, however, appear to be a significant difference in the five year survival and the overall

survival of patients. Patients with tumours expressing high levels of IFNGR1 had a greater overall (p=0.034) and five year survival rate (p=0.006; Table 3.4). Patients in whom tumours expressed a high level of IFNGR1 had five year survival and overall survival rates of 31% and 21% respectively, compared to 18% and 12% in patients with low expression.

Correlations between IFNGR1 expression and patient survival were also assessed using Kaplan Meier survival curves and log-rank testing (Figure 3.5). There was a statistically significant difference in the Disease Specific Survival (DSS) between patients with high and low IFNGR1 expression, with high expression predicting an improved outcome (log rank= 5.710 (p=0.017)). The mean DSS was 72 months and 46 months for high and low receptor expression respectively, which represents a 25 month difference in survival between the two groups. The median DSS also supported the favourable survival outcome with high receptor expression (27 vs. 16 months; Table 3.5 and Figure 3.5).

Whilst initial classification into high and low expression using the median intensity score was used, a further sub-stratification was analysed in which tumours were divided into three groups: those with no expression of IFNGR1 (complete loss), those with low level expression and those with high level expression. This illustrated that much of the difference in survival rates between the patients appeared to be related to complete loss of IFNGR1 (Figure 3.6) (Table 3.6). This is clearly seen in the Kaplan Meier analysis in which the survival curves for tumours of high and low expression were very similar; tumours with no receptor expression appeared to have a comparatively poorer prognosis (p=0.043). Since the behaviour of the tumours appeared to correlate more closely with whether any receptor at all was detected, it seemed intuitive to assess the patients based on these criteria, hence, the groups were divided according to the loss or presence of IFNGR1 (Figure 3.6). This analysis showed that complete loss was associated with a poorer outcome (log rank=6.005, p=0.014); median survival with complete receptor loss was 15 months compared with 23 months in tumours expressing any receptor (Table 3.6).

Table 3.4 IFNGR1 expression in all of the cases included in the final analysis. This includes univariate analysis by $\chi 2$ of IFNGR1 status and clinicopathological criteria.

Variable	Analysed cases N=334 (%)	High IFNGR1 expression (%)	Low IFNGR1 expression (%)	$\chi 2$ (p value)
Age (years)	n=333	n=171	n=162	1.17 (0.557)
<30	1 (0.3)	0	1 (0.6)	
30-59	133 (39.9)	67 (39.2)	66 (40.7)	
≥60	199 (59.8)	104 (60.8)	95 (58.6)	
FIGO Stage	n=326	n=168	n=158	1.86 (0.601)
I	83 (26.5)	41 (24.4)	42 (26.6)	
II	37 (11.3)	18 (10.7)	19 (12.0)	
III	167 (51.2)	85 (50.6)	82 (51.9)	
IV	39 (12.0)	24 (14.3)	15 (9.5)	
Optimal Debulking	n=322	n=168	n=157	0.42 (0.838)
Yes	129 (40.1)	67 (40.6)	62 (39.5)	
No	193 (59.9)	98 (59.4)	95 (60.5)	
Tumour Grade	n=332	n=165	n=161	0.31 (0.856)
1	38 (11.4)	20 (11.7)	18 (11.2)	
2	72 (21.7)	35 (20.5)	37 (23.0)	
3	222 (66.9)	116 (67.8)	106 (65.8)	
Histological Type	n=334	n=171	n=163	14.53 (0.104)
Serous	177 (53.0)	92 (53.8)	85 (52.2)	
Endometrioid	42 (12.6)	22 (12.9)	20 (12.3)	
Mucinous	34 (10.2)	14 (8.2)	20 (12.3)	
Undifferentiated	51 (15.3)	33 (19.3)	18 (11.0)	
Clear Cell	25 (7.5)	8 (4.7)	17 (10.4)	
Other	5 (1.5)	2 (1.2)	3 (1.8)	
Chemotherapy	n=328	n=169	n=163	2.43 (0.487)
Platinum	164 (50.0)	88 (52.1)	76 (47.8)	
Non-platinum	93 (28.4)	46 (27.3)	43 (27.0)	
No chemotherapy	71 (21.6)	35 (20.7)	36 (22.6)	
Randomised Treatment	57/334 (17.1)	29/171 (17.0)	28/163 (17.1)	0.26 (0.873)
Five Year Survival	n=329	n=169	n=160	**7.69 (0.006)**
Yes	82 (24.9)	53 (31.4)	29 (18.1)	
No	247 (75.1)	116 (68.6)	131 (81.9)	
Alive at time of data censoring	n=331	n=170	n=161	**4.508 (0.034)**
Yes	56 (16.9)	36 (21.2)	20 (12.4)	
No	275 (83.1)	134 (78.8)	141 (87.6)	

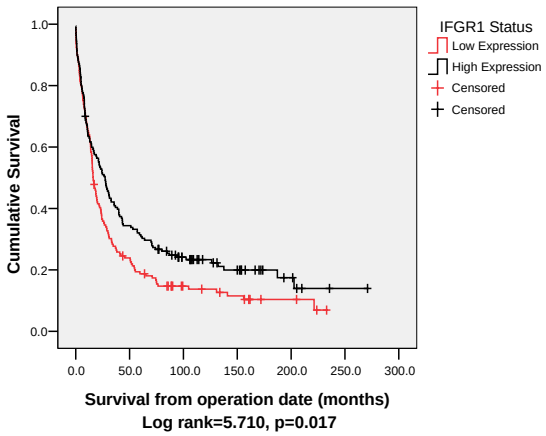


Figure 3.5 Kaplan-Meier plot for disease specific survival. High IFNGR1 expression tumours compared with low IFNGR1 expression tumours (n=334).

Table 3.5 Mean and median results for survival time in relation to high and low expression of IFNGR1, and Log Rank test for univariate survival analysis.

	Mean[(a)]				Median			
	Estimate (months)	Std. Error	95% Confidence Interval		Estimate (months)	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Low	45.9	5.6	34.9	56.9	16.1	1.3	13.6	18.7
High	71.9	7.8	56.6	87.1	27.3	3.4	20.6	34.0
Overall	60.2	5.1	50.2	70.2	19.5	2.1	15.5	23.6

(a) Estimation is limited to the largest survival time if it is censored.

	Chi-square	d.f.	p value
Log Rank (Mantel-Cox)	5.710	1	**0.017**

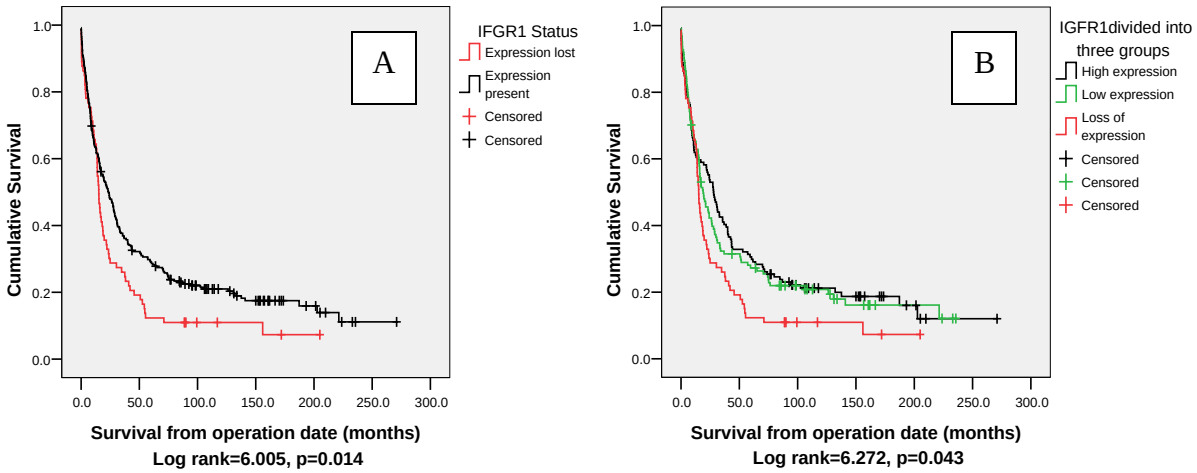


Figure 3.6 Kaplan-Meier plots for disease specific survival. IFNGR1 expression: (A) present vs. loss and (B) high vs. low vs. loss in tumours (n=334).

Table 3.6 Mean and median results for survival time in relation to complete loss of IFNGR1 with division into three groups. Log Rank test for univariate survival analysis.

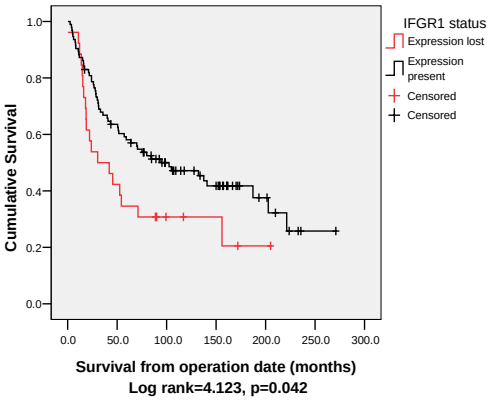
	Mean[(a)]				Median			
	Estimate (months)	Std. Error	95% Confidence Interval		Estimate (months)	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
IFNGR1 receptor lost or present								
Lost	36.7	6.6	23.6	49.8	15.2	1.1	13.0	17.3
Present	65.9	6.0	54.2	77.7	23.4	3.1	17.3	29.4
Overall	60.2	5.1	50.2	70.2	19.5	2.1	15.5	23.6
IFNGR1 receptor expression divided into three groups								
Lost	36.7	6.6	23.6	49.8	15.2	1.1	13.0	17.3
Low	60.1	7.6	45.1	75.1	19.0	3.0	13.1	24.8
High	68.3	8.3	52.0	84.7	27.8	3.3	21.3	34.3
Overall	60.2	5.1	50.2	70.2	19.5	2.1	15.5	23.6

(a) Estimation is limited to the largest survival time if it is censored.

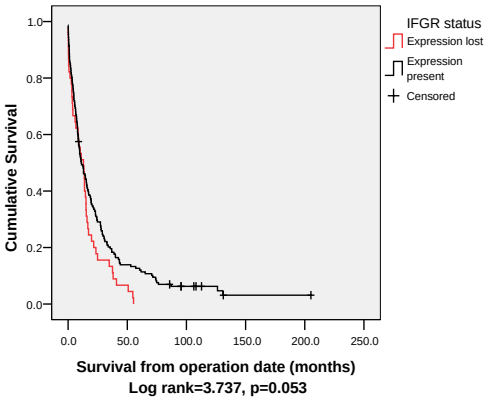
Log Rank (Mantel-Cox)	Chi-square	d.f.	p value
IFNGR1 receptor lost or present	6.005	1	**0.014**
IFNGR1 receptor expression (three groups)	6.272	2	**0.043**

Tumour stage predominantly dictates prognosis in ovarian cancer. Whilst the effects of IFNGR1 status on patient survival are independent of stage (see multivariate analysis), an analysis of early with late stage tumours was performed to assess if these effects were more marked in one subgroup. Tumours were assessed according to high/ low expression and loss/ presence of IFNGR1 following division of patients into those with early (stage I and II) and advanced disease (stage III and IV) (Figure 3.7). Both classifications of IFNGR1 status demonstrated that although the negative effects on patient survival were seen in both early and late stages, the effects may be more pronounced in early stage disease ($p=0.042$ vs. $p=0.053$ and $p=0.017$ vs. $p=0.045$). The effects were most marked when comparing receptor loss with receptor presence in early stage disease; median survival being 30 months and 102 months respectively (Table 3.7 and Table 3.8).

A

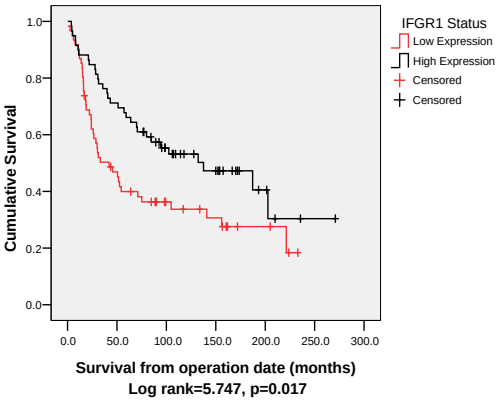


Early Stage tumours (Stage I-II) with complete loss of IFNGR1 expression

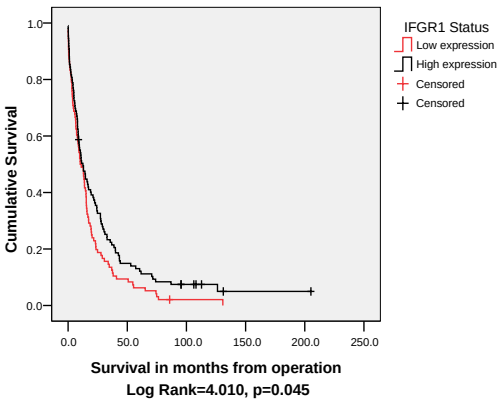


Late Stage tumours (Stage III-IV) with complete loss of IFNGR1 expression

B



Early Stage tumours (Stage I-II) with high and low IFNGR1 expression



Late Stage tumours (Stage III-IV) with high and low IFNGR1 expression

Figure 3.7 Kaplan-Meier plots for disease specific survival with early and late stage disease, using both loss vs. presence (A) and high vs. low (B) IFNGR1 expression (n=334).

Table 3.7 Mean and median results for survival time in relation to complete loss of IFNGR1 with early and late stage disease. Log Rank test for univariate survival analysis.

	Mean[(a)]				Median			
	Estimate (months)	Std. Error	95% Confidence Interval		Estimate (months)	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Early stage (I and II)								
IFNGR1 lost	76.5	15.5	46.1	106.9	30.3	14.9	1.05	59.6
IFNGR1 present	129.8	12.0	106.2	153.4	102.4	29.1	45.4	159.5
Overall	120.5	10.6	99.7	141.3	71.0	21.0	30.0	112.2
Late stage (III and IV)								
IFNGR1 lost	14.7	2.2	10.4	19.1	13.0	3.1	7.0	19.0
IFNGR1 present	27.0	3.4	20.2	33.7	11.0	2.0	7.2	14.9
Overall	24.3	2.8	18.9	30.0	11.3	1.5	8.5	14.2

(a) Estimation is limited to the largest survival time if it is censored.

Log Rank (Mantel-Cox)	Chi-square	d.f.	p value
Early stage (I and II)	4.123	1	**0.042**
Late stage (III and IV)	3.737	1	0.053

Table 3.8 Mean and median results for survival time in relation to high and low IFNGR1 expression with early and late stage disease. Log Rank test for univariate survival analysis.

	Mean[(a)]				Median			
	Estimate (months)	Std. Error	95% Confidence Interval		Estimate (months)	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Early stage (I and II)								
IFNGR1 low	91.7	12.2	67.8	115.6	42.0	11.8	19.0	65.0
IFNGR1 high	143.2	15.3	113.2	173.2	137.6	44.4	50.5	224.6
Overall	120.5	10.6	99.7	141.3	71.1	21.0	30.0	112.5
Late stage (III and IV)								
IFNGR1 low	18.0	2.4	13.2	22.8	10.2	2.2	6.0	14.4
IFNGR1 high	30.2	4.7	21.0	39.4	12.5	2.2	8.2	16.7
Overall	24.3	2.7	18.9	30.0	11.3	1.5	8.5	14.2

(a) Estimation is limited to the largest survival time if it is censored.

Log Rank (Mantel-Cox)	Chi-square	d.f.	p value
Early stage (I and II)	5.747	1	**0.017**
Late stage (III and IV)	4.010	1	**0.045**

3.2.1.4 Multivariate analysis

In order to assess whether IFNGR1 status was an independent marker of prognosis, the relative influence of IFNGR1 expression and other known standard clinicopathological prognostic variables were included in a multivariate analysis. Factors shown to predict prognosis independently of each other were age, FIGO stage, the absence of macroscopic disease after surgery and whether the patient received chemotherapy. These factors along with IFNGR1 status were included in the Cox regression analysis. IFNGR1 when divided into high and low expression was seen to retain its power to predict an improved prognosis in the study population, independent of other prognostic factors, HR 0.727 (95% CI 0.570-0.927), p=0.01. This was also seen when patients were divided according to complete loss of IFNGR1, HR 0.744 (95% CI 0.558-0.992), p=0.045 (Table 3.9).

Table 3.9 Multivariate analysis using Cox regression model demonstrating IFNGR1 expression (low vs. high or loss vs. present) predicts a positive survival advantage independently of other accepted independent prognostic factors.

Variable	Hazard Ratio (95% Confidence intervals)	Significance (p)
Age at diagnosis (years)	1.024 (1.013-1.036)	**<0.001**
FIGO Stage		
I	1	
II	2.542 (1.469-4.440)	
III	5.091 (3.109-8.330)	
IV	6.851 (3.854-12.180)	**<0.001**
Optimal Debulking		
Yes	1	
No	1.955 (1.389-2.720)	**<0.001**
Patient received Chemotherapy		
No	1	
Yes	0.438 (0.312-0.615)	**<0.001**
IFNGR1 expression		
Low	1	
High	0.727 (0.570-0.927)	**0.010**
Lost	1	
Present	0.744 (0.558-0.992)	**0.045**

3.2.2 STAT1

Two tumour samples were used from each patient and were represented by two cores on the TMA. There were 318 patients in whom the immunohistochemical processing had produced cores in which the staining and tissue preservation were possible to evaluate. This gave a core loss of 6.2%, which is within recognised standards for TMA work. Reasons for cores being lost to analysis included lack of viable tumour cells from extensive tumour necrosis and damage to the actual core itself during processing leading to detachment or folding of the core. Both of these reasons render it impossible to interpret the core.

3.2.2.1 Clinicopathological characteristics

When compared with the whole study series of 395 cases, the subgroup analysed were similar and consistent with recognised clinicopathological patterns from other studies [5, 10]. Of particular importance was the comparability of the five year survival rates, which were 24% in the whole series and 26% in those analysed for STAT1 expression (Table 3.10).

Table 3.10 Clinicopathological characteristics of the whole series and those analysed for STAT1 expression.

Variable	Whole series n=395 (%)	STAT1 analysed cases n=318 (%)
Age (years)	n=394	n=317
<30	1 (0.3)	1 (0.3)
30-59	167 (42.4)	126 (39.7)
≥60	226 (57.4)	190 (59.9)
FIGO Stage	n=375	n=310
I	99 (22.6)	80 (25.8)
II	46 (12.3)	34 (11.0)
III	188 (50.1)	160 (51.6)
IV	42 (11.2)	36 (11.6)
Optimal Debulking	n=376	n=318
Yes	157 (41.8)	124 (40.5)
No	219 (58.2)	182 (59.5)
Tumour Grade	n=376	n=318
1	50 (13.3)	38 (12.0)
2	93 (24.7)	72 (22.7)
3	233 (62)	207 (65.3)
Histological Type	n=395	n=318
Serous	203 (51.4)	169 (53.1)
Endometrioid	46 (11.7)	41 (12.9)
Mucinous	50 (12.7)	33 (10.4)
Undifferentiated	65 (16.5)	48 (15.1)
Clear Cell	26 (6.6)	24 (7.5)
Other	5 (1.3)	3 (1.0)
Chemotherapy	n=388	n=312
Platinum	196 (50.5)	159 (51.0)
Non-platinum	80 (20.6)	71 (22.7)
None	112 (28.9)	82 (26.3)
Randomised Treatment	69/395 (17.5)	55/312 (17.7)
Five Year Survival	n=385	n=318
Yes	91 (23.6)	83 (26.3)
No	294 (76.4)	232 (73.3)
Alive at time of data censoring	n=388	n=315
Yes	60 (15.5)	56 (17.8)
No	328 (84.5)	259 (82.2)

3.2.2.2 Immunohistochemical expression of STAT1 in ovarian cancer

As expected, STAT1 expression was seen in both the cytoplasm and nucleus. Expression in both these locations has been seen previously in ovarian cancer and normal ovarian tissue [280]. The antibody used could detect STAT1 in both its inactive and active (phosphorylated) form. It was thought that the STAT1 within the nucleus was most likely to represent the active form. Analysis of expression looked at both subcellular locations. Photomicrographs of the types of staining seen are illustrated (Figure 3.8). There was no staining seen within the negative controls.

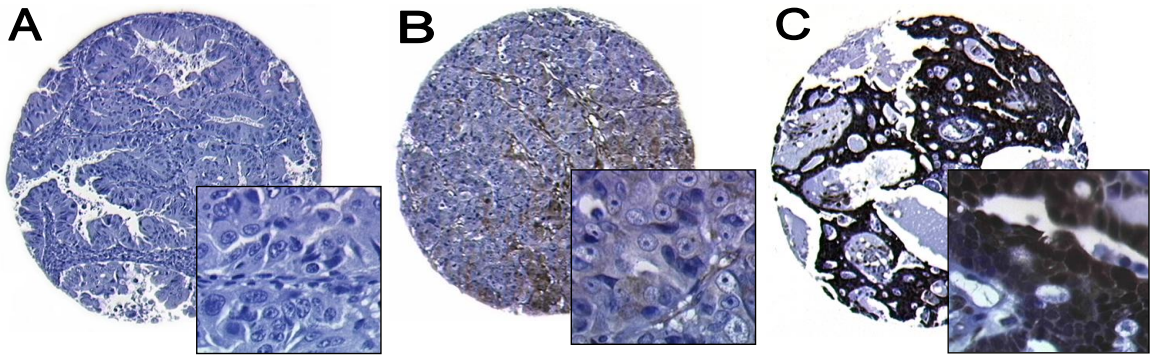


Figure 3.8 A shows a core from tumour demonstrating negative STAT1 staining. B shows a core of tumour demonstrating weak cytoplasmic and absent nuclear staining. C illustrates strong cytoplasmic and nuclear staining. Tumour cores at x100 magnification (insets at x400 magnification).

Less than half of tumours (45%) expressed STAT1 within the cytoplasm; the majority were of mild and moderate intensity, with only 22 (7%) of tumours having strong STAT1 cytoplasmic staining. The cytoplasmic staining was heterogeneous between the tumour cells, with the majority demonstrating positive staining in less than 50% of cells within each core (Table 3.11 and Table 3.12).

Nuclear STAT1 expression was less prevalent than cytoplasmic; only 20% expressed positive nuclear staining, and that tended to be of mild intensity (Table 3.11 and Table 3.12). In those tumour cores which were negative for any STAT1 staining it was

interesting to note that only 3% had evidence of STAT1 expression within the surrounding stroma (Table 3.13) implying lack of IFNγ within the tumour micro-environment.

A previously determined scoring system was used, in which tumours with greater than 5% of cells demonstrating cytoplasmic staining were deemed positive and any nuclear staining was seen as positive [280]. 39% of tumour cores demonstrated positive cytoplasmic staining compared to only 19% with nuclear staining (Table 3.14). As expected, there was strong correlation between the expression of nuclear and cytoplasmic STAT1 (χ^2 94.98, $p<0.00001$). Whilst 67 (54%) of tumours showing cytoplasmic expression had no nuclear expression, there were only four (6%) tumours with positive nuclear expression in the absence of cytoplasmic expression. This pattern could be predicted by the nature of STAT1 functioning within the cell, namely the production within the cytoplasm and translocation to the nucleus (Table 3.14).

Table 3.11 Intensity of cytoplasmic and nuclear staining with STAT-1.

Intensity of staining	Cytoplasmic		Nuclear	
	Number	Percentage	Number	Percentage
No staining	175	55.0	256	80.5
Mild	70	22.0	38	12.0
Moderate	51	16.0	11	3.5
Strong	22	7.0	13	4.0

Table 3.12 Proportion of viable tumour cells demonstrating cytoplasmic and nuclear staining with STAT-1.

Proportion of cells staining	Cytoplasmic		Nuclear	
	Number	Percentage	Number	Percentage
No staining	175	55.0	256	80.5
1 - 25%	79	24.8	42	13.2
26 - 50%	36	11.3	10	3.1
51 - 75%	14	4.4	5	1.6
76 - 100%	14	4.4	5	1.6

Table 3.13 Background STAT1 stromal staining in tumour cores with absent STAT1 cytoplasmic staining.

	Present (%)	Absent (%)
Stromal staining	4 (3)	171 (97)

Table 3.14 Expression and correlation of cytoplasmic and nuclear STAT1, using the aforementioned scoring system.

	Positive (%)		Negative (%)
Cytoplasmic STAT1	125 (39.3)		193 (60.7)
Nuclear STAT1	62 (19.5)		256 (80.5)
Cytoplasmic STAT1	**Nuclear STAT1 (%)**		**χ^2 (p value)**
	Positive	**Negative**	
Positive	58 (94)	67 (26)	**94.98 (p<0.0001)**
Negative	4 (6)	189 (74)	

3.2.2.3 Comparison of cytoplasmic STAT1 and clinicopathological variables

There was a correlation between cytoplasmic STAT1 expression and increasing stage and grade of tumour. A correlation also existed with having a lower likelihood of optimal tumour debulking, however, this may be due to the associated trend to higher stage at presentation. There was a higher proportion of serous tumours with STAT1 positivity and less endometriod and clear cell subtypes. Age, types of chemotherapy received and, most importantly, five year survival were not related to cytoplasmic STAT1 expression (Table 3.15).

3.2.2.4 Comparison of nuclear STAT1 and clinicopathological variables

STAT1 nuclear expression was related to a higher grade of tumour with almost all being moderately or poorly differentiated (98%). As with cytoplasmic STAT1 a similar trend towards higher rate of expression in serous subtype was seen. There was no correlation with any of the remaining clinicopathological variables, including five year survival (Table 3.15).

3.2.2.5 STAT1 expression and survival

Using Kaplan-Meier analysis there was no statistically significant difference in survival between STAT1 cytoplasmic or nuclear expression, although there was a trend towards reduced survival with high cytoplasmic STAT1 expression (Figure 3.9 and Figure 3.10). The mean survival time was 66.5 months for cytoplasmic STAT1 positive tumours and 52.8 months for negative, the log rank test not reaching significance (p=0.111)

Table 3.16). The mean survival time for nuclear STAT1 positive tumours was 67.2 months compared with 58.5 months in negative tumours. Although there appears to be a difference between these groups this was not significant on log rank testing (p=0.887). (Table 3.17).

Table 3.15 Expression of STAT1 divided according to sub cellular location. Univariate analysis by χ^2 of IFNGR1 status and clinicopathological criteria (>5% cytoplasmic staining = positive, any nucleus staining = positive).

Variable	STAT1 Analysed Cases (%)	Cytoplasmic STAT1 Present (%)	Cytoplasmic STAT1 Absent (%)	χ^2 (p value)	Nuclear STAT1 Present (%)	Nuclear STAT1 Absent (%)	χ^2 (p value)
Age (years)	n=317	n=125	n=192	2.20 (p=0.334)	n=62	n=255	4.16 (p=0.125)
<30	1 (0.3)	1 (0.8)	0		1 (1.6)	0	
30-59	126 (39.7)	46 (36.8)	80 (41.7)		25 (40.3)	101 (39.6)	
≥60	190 (59.9)	78 (62.4)	112 (58.3)		36 (58.1)	154 (60.4)	
FIGO Stage	n=310	n=123	n=187	**18.82 (p<0.0001)**	n=62	n=248	6.37 (p=0.095)
I	80 (25.8)	16 (13.0)	64 (34.2)		9 (14.5)	71 (28.6)	
II	34 (11.0)	13 (10.6)	21 (11.2)		9 (14.5)	25 (10.1)	
III	160 (51.6)	78 (63.4)	82 (43.9)		38 (61.3)	122 (49.2)	
IV	36 (11.6)	16 (13.0)	20 (10.7)		6 (9.7)	30 (12.1)	
Optimal debulking	n=318	n=121	n=185	**5.71 (p=0.017)**	n=60	n=246	0.01 (p=0.927)
Yes	124 (40.5)	39 (32.2)	85 (45.9)		24 (40.0)	100 (40.7)	
No	182 (59.5)	82 (67.8)	100 (54.1)		36 (60.0)	146 (59.3)	
Tumour Grade	n=318	n=125	n=192	**25.66 (p<0.0001)**	n=62	n=255	**9.53 (p=0.009)**
1	38 (12.0)	5 (4.0)	33 (17.2)		1 (1.6)	37 (14.5)	
2	72 (22.7)	18 (14.4)	54 (28.1)		12 (19.4)	60 (23.5)	
3	207 (65.3)	102 (81.6)	105 (54.7)		49 (79.0)	158 (62.0)	
Histological Type	n=318	n=125	n=193	**33.88 (p<0.0001)**	n=62	n=256	**13.60 (p=0.018)**
Serous	169 (53.1)	81 (64.8)	88 (45.6)		41 (66.1)	128 (50.0)	
Endometrioid	41 (12.9)	8 (6.4)	33 (17.1)		5 (8.1)	36 (14.1)	
Mucinous	33 (10.4)	3 (2.4)	30 (15.5)		0	33 (12.9)	
Undifferentiated	48 (15.1)	27 (21.6)	21 (10.9)		12 (19.4)	36 (14.1)	
Clear Cell	24 (7.5)	5 (4.0)	19 (9.8)		4 (6.5)	20 (7.8)	
Other	3 (1.0)	1 (0.8)	2 (1.0)		0	3 (1.2)	
Chemotherapy	n=312	n=123	n=189	5.00 (p=0.172)	n=62	n=250	2.92 (p=0.403)
Platinum	159 (51.0)	67 (54.5)	92 (48.7)		37 (59.7)	122 (48.8)	
Non-platinum	71 (22.7)	32 (26.0)	39 (20.6)		11 (17.7)	60 (24)	
None	82 (26.3)	24 (19.5)	58 (30.7)		14 (22.6)	68 (27.2)	
Randomised treatment	55/312 (17.7)	17/123 (13.8)	38/189 (19.1)	2.15 (p=0.172)	8/62 (12.9)	47/248 (19.0)	1.24 (p=0.265)
Five Year Survival	n=318	n=124	n=191	2.21 (p=0.138)	n=62	n=253	0.05 (p=0.831)
Yes	83 (26.3)	27 (21.8)	56 (29.3)		17 (27.4)	66 (26.1)	
No	232 (73.3)	97 (78.2)	135 (70.7)		45 (72.6)	187 (73.9)	
Alive at time of data censoring	n=315	n=124	n=191	3.32 (p=0.068)	n=62	n=253	0.13 (p=0.717)
Yes	56 (17.8)	16 (12.9)	40 (20.9)		12 (19.4)	44 (17.4)	
No	259 (82.2)	108 (87.1)	151 (79.1)		50 (80.6)	209 (82.6)	

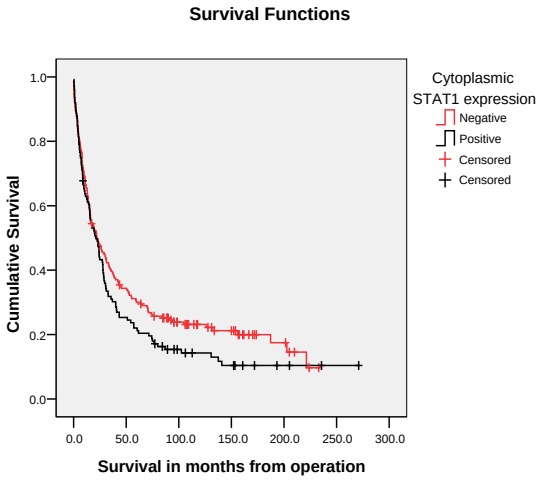


Figure 3.9 Kaplan Meier Survival Plots for disease specific survival with positive (>5% of tumour cells staining) and negative (<5%) cytoplasmic STAT1 expression. Log rank - 2.5, p=0.111.

Table 3.16 Mean and median results for survival time in relation to cytoplasmic STAT1 expression. Log Rank test for univariate survival analysis.

	Mean[(a)]				Median			
	Estimate (months)	Std. Error	95% Confidence Interval		Estimate (months)	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Negative	66.5	6.2	53.4	77.7	22.1	4.4	13.5	30.7
Positive	52.8	7.4	38.2	67.4	21.1	3.5	14.2	28.1
Overall	62.9	5.3	52.5	73.4	22.1	2.8	16.7	27.6

(a) Estimation is limited to the largest survival time if it is censored.

	Chi-square	d.f.	p value
Log Rank (Mantel-Cox)	2.5	1	0.111

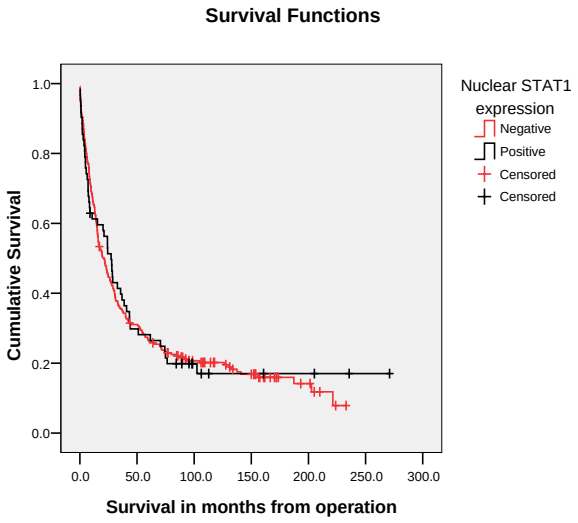


Figure 3.10 Kaplan-Meier Survival Plots for disease specific survival with positive (any nuclear staining in tumour cells) and negative (no nuclear staining) nuclear STAT1 expression. Log rank - 0.2, p=0.887.

Table 3.17 Mean and median results for survival time in relation to nuclear STAT1 expression. Log Rank test for univariate survival analysis.

	Mean[(a)]				Median			
	Estimate (months)	Std. Error	95% Confidence Interval		Estimate (months)	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Negative	58.5	5.0	48.6	68.3	21.1	2.5	16.2	26.0
Positive	67.2	12.6	42.6	91.2	27.3	4.0	19.4	35.2
Overall	62.9	5.3	52.5	73.4	22.1	2.8	16.6	27.6

(a) Estimation is limited to the largest survival time if it is censored.

	Chi-square	d.f.	p value
Log Rank (Mantel-Cox)	0.2	1	0.887

Further analysis of STAT1 functioning within the cell was performed by subgroup analysis, according to the relative distribution of STAT1 in the subcellular locations. Cells with no STAT1 expression were deemed to not be signalling at that point in time and those with STAT1 within the nucleus and cytoplasm, were deemed to be either signalling correctly. Both these groups are likely to have intact signalling STAT1 pathways. In contrast, those cells with high cytoplasmic expression in the absence of translocation and expression of STAT1 in the nucleus may have a defective IFNγ pathway. These groups were assessed using Kaplan-Meier analysis. There was a trend towards an improved survival with an intact compared with a defective STAT1 pathway, with mean survivals of 69 and 39 months respectively. This failed to reach statistical significance (p= 0.08). (Figure 3.11 and Table 3.18).

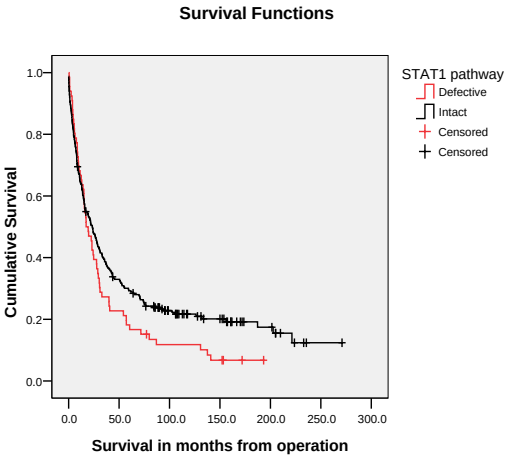


Figure 3.11 Kaplan-Meier Survival Plots for disease specific survival with an intact or defective STAT1 pathway (see text for definition). Log rank - 3.063, p=0.080.

Table 3.18 Mean and median results for survival time in relation to STAT1 pathway status. Log Rank test for univariate survival analysis.

	Mean[(a)]				Median			
	Estimate (months)	Std. Error	95% Confidence Interval		Estimate (months)	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Intact	68.8	6.3	54.4	81.2	23.5	3.5	16.7	30.3
Defective	39.5	6.4	26.9	52.1	17.3	3.4	10.7	24.0
Overall	62.9	5.3	52.5	73.4	22.1	2.8	16.6	27.6

(a) Estimation is limited to the largest survival time if it is censored.

	Chi-square	d.f.	p value
Log Rank (Mantel-Cox)	3.062	1	0.080

3.2.2.6 STAT1 correlation with IFNGR1 status

As STAT1 is an integral component of the IFNγ pathway, a correlation between IFNGR1 status and STAT1 expression was performed using the χ^2 test. Both high/ low and present/ absent IFNGR1 expression were compared to cytoplasmic and nuclear STAT1. There was a significant association between IFNGR1 status and the expression of both nuclear and cytoplasmic STAT1 (Table 3.19). 68% of nuclear (i.e. active) STAT1 occurred in high expressers of IFNγ receptors, increasing to 93% in those tumours expressing any receptors.

Table 3.19 Correlation of IFNGR1 expression with cytoplasmic and nuclear STAT1.

IFNGR1 expression	Cytoplasmic STAT1 (%)		χ^2 (p value)	Nuclear STAT1 (%)		χ^2 (p value)
	Present	Absent		Present	Absent	
High	85 (69)	86 (45)	**16.4 (p<0.001)**	42 (68)	129 (51)	**5.50 (p=0.019)**
Low	39 (31)	104 (55)		20 (32)	123 (49)	
Total	124	190		62	252	
Present	115 (93)	146 (77)	**13.5 (p<0.001)**	58 (93)	203 (80)	**5.99 (p=0.014)**
Absent	9 (7)	44 (23)		4 (7)	49 (20)	
Total	124	190		62	252	

3.2.3 p27

3.2.3.1 Clinicopathological characteristics

Of the 339 tumours that were included in the array, 305 were suitable for the assessment of the level of p27 expression. This relatively high loss of tumour cores during immunohistochemical processing was due to the buffer required for antigen retrieval. This required the use of EDTA buffer which tends to damage the tumour cores. This subgroup of 305 patients was similar to that of the overall group, which as previously discussed is representative of a typical ovarian cancer population (Table 3.20). Of particular importance was the comparability of the five year survival rate, which was 24% in the whole series and 26% in those analysed for p27 expression.

3.2.3.2 p27 staining

In keeping with previous studies, p27 expression was seen in both the cytoplasm and the nucleus [325, 326]. This staining was heterogeneous with regard to the number and intensity of staining of the cells, in both the nuclear and cytoplasmic sub-cellular locations, and as such both aspects were scored in the initial analysis. There was strong p27 expression in the positive control (tonsil) and no immunoreactivity in the negative control section where the primary antibody was omitted (Figure 3.12).

3.2.3.3 Cytoplasmic staining of p27

There was an even distribution of intensity of p27 immunoreactivity within the cytoplasm with 22% having no expression, and 25%, 28% and 26% displaying mild, moderate and strong staining respectively. Cytoplasmic p27 expression was not uniform throughout the tumour cores, with only 24% having positive staining of the majority of cells (Table 3.21 and Table 3.22).

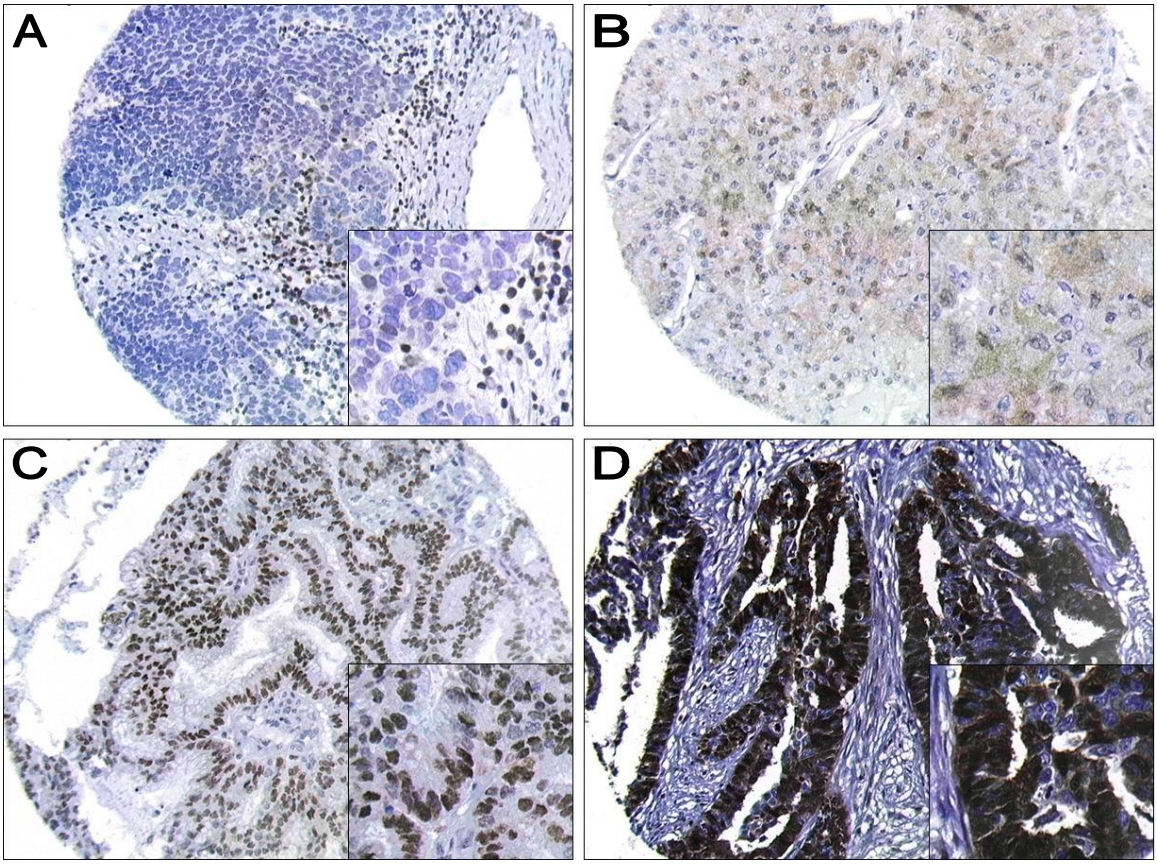


Figure 3.12 Photomicrographs of p27 immunohistochemical staining of ovarian TMA cores using the Avidin-biotin complex protocol. Panel A shows a negative tumour core, with positive stromal cells (internal positive controls). Panels B-D show weak, moderate, and strong staining respectively. Cores are shown at x100 magnification; insets are x200.

3.2.3.4 Nuclear staining of p27

Nuclear p27 expression was seen in less tumour cores than cytoplasmic, 65% and 78% respectively. The tumour cores demonstrating nuclear p27 expression had less variation in the intensity of staining when compared to cytoplasmic expression. The majority of tumour cells displaying immunoreactivity within the nucleus had moderate or strong staining; only 16% of tumours had mild nuclear p27 expression. As was seen with the cytoplasmic expression, nuclear p27 expression was not uniform within all the cells in an individual core, with the majority of cores expressing immunoreactivity in less than 50% of cells (Table 3.21 and Table 3.22).

The use of an intensity score allowed the heterogeneity of both cytoplasmic and nuclear p27 expression to be taken into account. The mean intensity score was taken between the two cores from each tumour. The intensity of staining within the tumour cores varied from mild to strong (Table 3.21), as did the proportion of cells staining within each core (Table 3.22). However, the intensity of staining within the cells of an individual core was relatively uniform; therefore, an intensity score provided an accurate representation of the immunoreactivity. If the intensity of staining within individual cells had varied significantly within an individual core, the "H scoring system" could have been utilised which allows for scoring of each intensity of staining separately within the core, providing a more accurate representation of heterogeneous staining. The median intensity score for the cytoplasmic p27 expression in tumours overall was 40 and for nuclear p27 was 25. The median IS was used to divide the tumours into high and low expression for cytoplasmic p27. The scoring system for nuclear expression was simplified to presence (>5% of cells) or absence (<5% of cells) of immunoreactivity as described by previous authors [326].

Table 3.20 Clinicopathological characteristics of whole series and those analysed for p27 expression.

Variable	Whole series n=395 (%)	p27 analysed cases n=305 (%)
Age (years)	n=394	n= 304
<30	1 (0.3)	1 (0.3)
30-59	167 (42.4)	120 (39.5)
≥60	226 (57.4)	183 (60.2)
FIGO Stage	n=375	n= 297
I	99 (22.6)	74 (24.9)
II	46 (12.3)	33 (11.1)
III	188 (50.1)	156 (52.5)
IV	42 (11.2)	34 (11.4)
Optimal Debulking	n=376	n= 293
Yes	157 (41.8)	116 (39.6)
No	219 (58.2)	177 (60.4)
Tumour Grade	n=376	n= 304
1	50 (13.3)	35 (11.5)
2	93 (24.7)	67 (22.0)
3	233 (62)	202 (66.4)
Histological Type	n=395	n= 305
Serous	203 (51.4)	161 (52.8)
Endometrioid	46 (11.7)	39 (12.8)
Mucinous	50 (12.7)	30 (9.8)
Undifferentiated	65 (16.5)	48 (15.7)
Clear Cell	26 (6.6)	24 (7.9)
Other	5 (1.3)	3 (1)
Chemotherapy	n=388	n= 299
Platinum	196 (50.5)	152 (50.8)
Non-platinum	80 (20.6)	68 (22.7)
None	112 (28.9)	79 (26.4)
Randomised treatment	69/395 (17.5)	52/299
Five Year Survival	n=385	n= 300
Yes	91 (23.6)	78 (26.0)
No	294 (76.4)	222 (74)
Alive at time of data censoring	n=388	n= 302
Yes	60 (15.5)	54 (17.9)
No	328 (84.5)	248 (82.1)

Table 3.21 Intensity of cytoplasmic and nuclear staining with p27.

Intensity of Staining	Cytoplasmic		Nuclear	
	Number	Percentage	Number	Percentage
No staining	67	22.0	106	34.8
Mild	73	24.0	50	16.4
Moderate	87	28.5	81	26.6
Strong	78	25.6	68	22.2

Table 3.22 Proportion of viable tumour cells with cytoplasmic and nuclear p27 expression.

Proportion of tumour cells staining	Cytoplasmic		Nuclear	
	Number	Percentage	Number	Percentage
No cells	67	22.0	106	34.8
1-25%	87	28.5	69	22.6
26-50%	77	25.2	66	21.6
51-75%	37	12.1	32	10.5
76-100%	37	12.1	32	10.5

3.2.3.5 Comparison of cytoplasmic p27 expression and patient tumour characteristics

Dividing the cores into high and low expression of cytoplasmic p27 produced 147 low, and 157 high, expression tumours. In a univariate analysis, using the χ^2 test, no significant relationship between cytoplasmic p27 expression and age, rates of tumour debulking, tumour grade or types of chemotherapy received were apparent. However, there was a trend towards earlier stage in tumours with low cytoplasmic p27 expression. 43% of tumours with low cytoplasmic p27 expression were stage I and II, compared with 29% of those with high p27 expression, although this was not statistically significant (p=0.068). A greater proportion of tumours with high p27 expression were of serous subtype and a lower proportion were mucinous compared with tumours of low p27 expression (Table 3.23). There was a clear difference in the prognosis of the two groups with regard to five

year survival rates, with high and low cytoplasmic p27 expression being 19% and 35% respectively (p=0.002). This is illustrated further in the Kaplan-Meier graphs below.

3.2.3.6 Comparison of nuclear p27 expression and patient tumour characteristics

The tumours cores were divided according to whether or not there was any expression of nuclear p27. 199 (65%) of tumour cores expressed some degree of nuclear p27 expression. As with cytoplasmic p27 there was no significant relationship between nuclear p27 positivity and age, rates of tumour debulking, tumour grade or types of chemotherapy received. There was a trend towards a higher proportion of early stage tumours (stage I and II) in those with absent, compared to those with nuclear p27 expression; 43% and 32% respectively. This trend was not as clear as with cytoplasmic p27 and was also not statistically significant (p=0.261) (Table 3.23).

As was seen with cytoplasmic expression, there were higher rates of serous and lower rates of mucinous subtypes in tumours expressing nuclear p27. There was a clear difference in the prognosis of the two groups in terms of five year survival rates, with positive and negative nuclear p27 expression being 21% and 36% respectively (p=0.005). This is illustrated further in the Kaplan-Meier graphs below.

Table 3.23 Expression of p27 divided according to sub cellular location. Univariate analysis by χ^2 p27 status and clinicopathological criteria.

Variable	p27 analysed cases n=305(%)	High Cytoplasmic p27 expression (%)	Low Cytoplasmic p27 expression (%)	χ^2 (p value)	Nuclear p27 present (%)	Nuclear p27 absent (%)	χ^2 (p value)
Age (years)	n= 304	n= 157	n= 147	3.912 (p= 0.141)	n= 199	n= 105	2.10 (p=0.350)
<30	1 (0.3)	0	1 (0.7)		0	1 (1)	
30-59	120 (39.5)	55 (35.0)	65 (44.2)		77 (38.7)	43 (41.0)	
≥60	183 (60.2)	102 (65.0)	81 (55.1)		122 (61.3)	61 (58.0)	
FIGO Stage	n= 297	n= 156	n= 141	7.12 (p=0.068)	n= 198	n= 99	4.00 (p=0.261)
I	74 (24.9)	33 (21.2)	41 (29.1)		43 (21.7)	31 (31.3)	
II	33 (11.1)	13 (8.3)	20 (14.2)		21 (10.6)	12 (12.1)	
III	156 (52.5)	88 (56.4)	68 (48.2)		111 (56.1)	45 (45.5)	
IV	34 (11.4)	22 (14.1)	12 (8.5)		23 (11.6)	11 (11.1)	
Optimal Debulking	n= 293	n= 156	n= 137	1.90 (p= 0.168)	n= 195	n= 98	1.13 (p= 0.287)
Yes	116 (39.6)	56 (35.9)	60 (43.8)		73 (37.4)	43 (43.9)	
No	177 (60.4)	100 (64.1)	77 (56.2)		122 (62.6)	55 (56.1)	
Tumour Grade	n= 304	n= 157	n= 147	3.43 (p= 0.180)	n= 198	n= 106	4.85 (p= 0.088)
1	35 (11.5)	13 (8.3)	22 (15.0)		17 (8.6)	18 (17.0)	
2	67 (22.0)	37 (23.6)	30 (20.4)		46 (23.2)	21 (19.8)	
3	202 (66.4)	107 (68.2)	95 (64.6)		135(68.2)	67(63.2)	
Histological Type	n= 305	n= 157	n= 148	**13.71 (p=0.018)**	n= 199	n= 106	**16.8 (p= 0.005)**
Serous	161 (52.8)	94 (59.9)	67 (45.3)		114(57.3)	47(44.3)	
Endometrioid	39 (12.8)	21 (13.4)	18 (12.2)		25 (12.6)	14(13.2)	
Mucinous	30 (9.8)	7 (4.5)	23 (15.5)		10 (5.0)	20(18.9)	
Undifferentiated	48 (15.7)	22 (14.0)	26 (17.6)		30 (15.1)	18(17.0)	
Clear Cell	24 (7.9)	12 (7.6)	12 (8.1)		18 (9.0)	6 (5.7)	
Other	3 (1)	1 (0.6)	2 (1.4)		2 (1.0)	1 (0.9)	
Chemotherapy	n= 299	n= 157	n= 142	2.19 (p= 0.534)	n= 199	n= 100	5.85 (p= 0.199)
Platinum	152 (50.8)	81 (51.6)	71 (50.0)		107(53.8)	45(45.0)	
Non-platinum	68 (22.7)	39 (24.8)	29 (20.4)		48 (24.1)	20(20.0)	
None	79 (26.4)	37 (23.6)	42 (29.6)		44 (22.1)	35(35.0)	
Randomised treatment	52/299	28/157	24/141	0.034 (p= 0.854)	33/199	19/100	0.251 (p= 0.616)
Five Year Survival	n= 300	n=156	n= 144	**9.276 (p= 0.002)**	n= 197	n= 103	**8.026 (p= 0.005)**
Yes	78 (26.0)	29 (18.6)	49 (34.0)		41 (20.8)	37 (35.9)	
No	222 (74)	127 (81.4)	95 (66.0)		156 (79.2)	66 (64.1)	
Alive at time of data censoring	n= 302	n= 157	n= 147	**5.888 (p= 0.015)**	n= 198	n= 104	**5.475 (p= 0.019)**
Yes	54 (17.9)	20 (12.7)	34 (23.4)		28 (14.1)	26 (25.0)	
No	248 (82.1)	137 (87.3)	111 (76.6)		170 (85.9)	78 (75.0)	

3.2.3.7 Cytoplasmic p27 expression and survival

Correlation between cytoplasmic p27 expression and patient survival was assessed using a Kaplan-Meier survival curve and log-rank testing (Figure 3.13). There was a statistically significant difference in the DSS between patients with high and low p27 expression; with low expression predicting an improved outcome (log rank= 7.849 (p=0.005)). The mean DSS was 73 months and 51 months for low and high cytoplasmic p27 expression respectively. This relates to a 22 month difference in survival between the two groups. The median DSS also supported the favourable survival outcome with low p27 expression (28 vs. 17 months) (Table 3.24).

In an attempt to describe the relationship between cytoplasmic p27 expression and prognosis, expression was sub-stratified into three groups; those with absent, low (less than median IS) and high (greater than median IS) expression (Figure 3.13). This figure illustrates that there is a progressively worsening prognosis with increasing p27 expression. The mean DSS with absent, low and high expression was 76, 68 and 50 months respectively (p=0.015) (Table 3.24).

The Kaplan-Meier survival curve demonstrated that as a group those tumours with high cytoplasmic p27 expression behaved particularly poorly. Therefore, the groups were reanalysed according to whether there was high expression in comparison with the remainder of the tumours (Figure 3.14). This illustrated the profound effect of high p27 expression on prognosis, with the mean DSS being 50 months in comparison with 72 months for the remainder of the patients ((p=0.004) (Table 3.25).

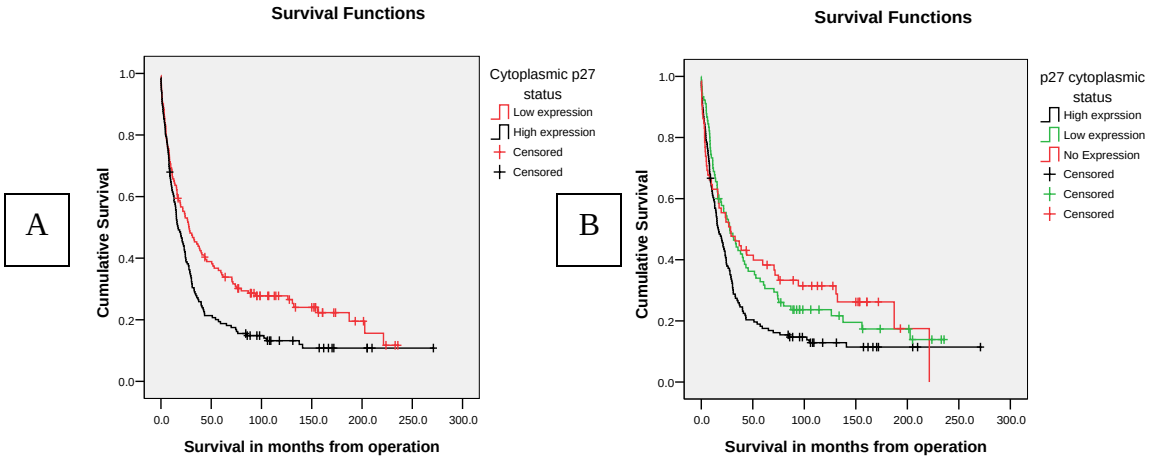


Figure 3.13 Kaplan-Meier plots for disease-specific survival. A: High cytoplasmic p27 expression compared with low and (B) high cytoplasmic p27 expression compared with low and absent expression in tumours (n=305).

Table 3.24 Mean and median survival time in relation to high and low cytoplasmic p27 expression. Division into three groups and Log Rank test for univariate survival analysis.

	Mean(a)				Median			
	Estimate (months)	Std. Error	95% Confidence Interval		Estimate (months)	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Cytoplasmic p27 expression								
Low	73.3	7.5	58.5	88.1	28.0	5.5	17.2	38.7
High	50.6	6.7	37.4	63.8	16.9	2.6	11.8	22.1
Overall	63.2	5.5	52.3	73.9	21.9	2.8	16.4	27.3
Cytoplasmic p27 expression in 3 groups								
Nil	76.0	11.3	53.9	98.1	28.4	10.1	8.6	45.2
Low	67.8	8.9	50.3	85.2	27.6	5.7	16.5	38.8
High	50.5	7.1	36.5	64.4	16.4	2.7	11.1	21.8
Overall	63.1	5.5	52.3	73.9	21.9	2.8	16.4	27.3

(a) Estimation is limited to the largest survival time if it is censored.

Log Rank (Mantel-Cox)	Chi-square	d.f.	p value
Cytoplasmic p27 high or low expression	7.849	1	**0.005**
Cytoplasmic p27 expression (3 groups)	8.386	2	**0.015**

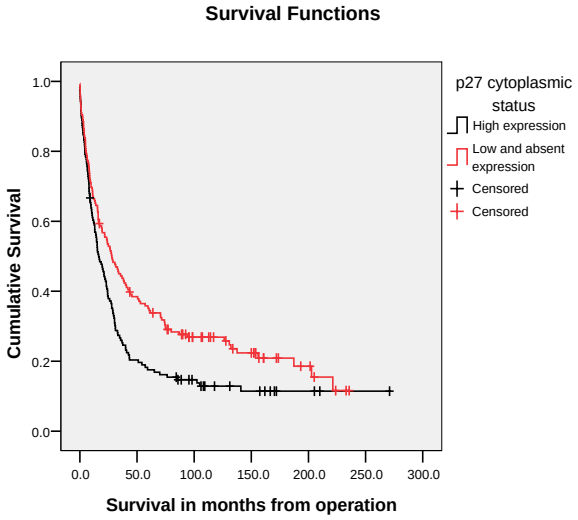


Figure 3.14 Kaplan-Meier plots for disease specific survival. High cytoplasmic p27 expression compared with low and absent expression in tumours (n=305).

Table 3.25 Mean and median survival time in relation to high, low and absent expression of cytoplasmic p27. Log Rank test for univariate survival analysis.

	Mean(a)				Median			
	Estimate (months)	Std. Error	95% Confidence Interval		Estimate (months)	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Low/Absent	71.9	7.1	57.9	85.8	28.0	5.0	18.1	37.8
High	50.5	7.1	36.5	64.4	16.4	2.7	11.1	21.8
Overall	63.1	5.5	52.3	73.9	21.9	2.8	16.4	27.3

(a) Estimation is limited to the largest survival time if it is censored.

	Chi-square	d.f.	p value
Log Rank (Mantel-Cox)	8.304	1	**0.004**

3.2.3.8 Nuclear p27 expression and survival

The initial analysis of nuclear p27 expression included division of tumours into high and low expressers according to whether the IS of the tumour was less than or greater than the median IS of 25. The results were similar to that of cytoplasmic expression. High expression of nuclear p27 correlated with a poorer prognosis, having a mean DSS of 53 months compared with 72 months for those with low expression (p=0.018) (Figure 3.15) (Table 3.26). The patients were then divided into three groups, those with high, low and

absent expression of nuclear p27, as was performed for cytoplasmic p27. Kaplan-Meier analysis revealed two distinct groups in terms of prognosis. Those patients with low and high nuclear p27 expression were seen as having a very similar prognostic outlook, with mean DDS of 54 and 53 months respectively, whereas the DSS for those patients with absent nuclear p27 expression was 79 months (Figure 3.15) (Table 3.26). Intuitively, reanalysis of the groups according to presence (high and low expression) or absence of nuclear p27 seemed reasonable (Figure 3.16). This clearly demonstrated that expression of nuclear p27 is a poor prognostic marker with a difference in the mean DSS between the two groups of 25 months (p=0.0014) (Table 3.27).

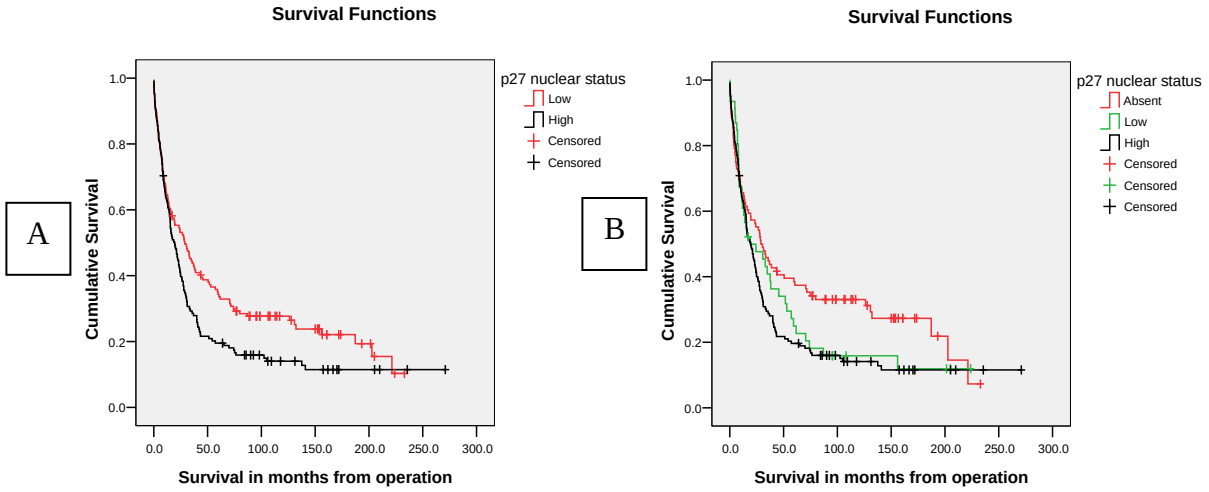


Figure 3.15 Kaplan-Meier plots for disease-specific survival. A: High nuclear p27 expression compared with low and B: high nuclear p27 expression compared with low and absent expression in tumours (n=305).

Table 3.26 Mean and median survival time in relation to high and low nuclear p27 expression, with division into three groups. Log Rank test for univariate survival analysis.

	Mean(a)				Median			
	Estimate (months)	Std. Error	95% Confidence Interval		Estimate (months)	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Nuclear p27 expression								
Low	72.1	7.5	57.4	86.7	28.9	6.0	17.2	40.6
High	52.6	7.2	38.6	66.7	19.5	2.8	13.9	25.0
Overall	64.3	5.7	53.2	75.4	22.4	3.0	16.5	28.4
Nuclear p27 expression in 3 groups								
Nil	78.7	9.4	60.1	97.1	28.9	5.6	17.8	40.0
Low	54.3	10.7	33.4	75.3	19.0	10.4	0	39.6
High	53.0	7.2	38.9	67.1	19.4	2.8	13.9	39.6
Overall	64.3	5.7	53.2	75.4	22.4	3.0	16.5	28.4

(a) Estimation is limited to the largest survival time if it is censored.

Log Rank (Mantel-Cox)	Chi-square	d.f.	p value
Nuclear p27 high or low expression	5.637	1	**0.018**
Nuclear p27 expression (3 groups)	6.626	2	**0.036**

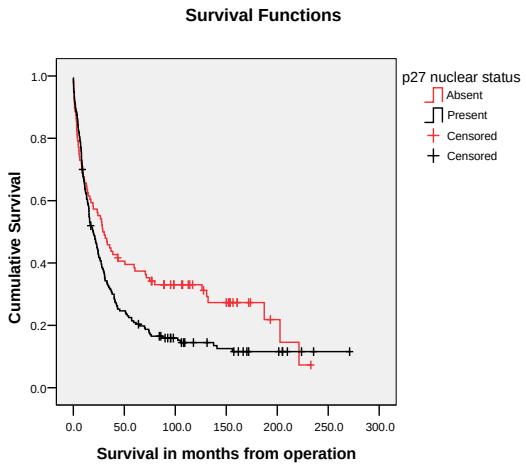


Figure 3.16 Kaplan-Meier plots for disease-specific survival. Present nuclear p27 expression in comparison with absent expression in tumours (n=305).

Table 3.27 Mean and median survival time in relation to present and absent expression of nuclear p27. Log Rank test for univariate survival analysis.

	Mean(a)				Median			
	Estimate (months)	Std. Error	95% Confidence Interval		Estimate (months)	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Absent	78.9	9.4	60.3	97.1	28.9	5.6	17.8	40.0
Present	54.5	6.3	42.2	66.8	19.4	2.8	14.0	24.8
Overall	64.3	5.7	53.2	75.4	22.4	3.0	16.5	28.4

(a) Estimation is limited to the largest survival time if it is censored.

	Chi-square	d.f.	p value
Log Rank (Mantel-Cox)	6.080	1	**0.014**

3.2.3.9 Multivariate analysis of cytoplasmic and nuclear p27 expression and survival

In order to assess whether cytoplasmic p27 status was an independent marker of prognosis, the relative influence of cytoplasmic p27 expression and other known standard clinicopathological prognostic variables were included in a multivariate analysis. Factors shown to predict prognosis independently of each other were age, FIGO stage, the absence of macroscopic disease after surgery and whether the patient received chemotherapy.

These factors along with p27 status were included in the Cox regression analysis. Cytoplasmic p27 expression when divided into high, low and absent expression was seen to retain its power to predict a poorer prognosis in the study population. This was independent of other prognostic factors (HR 1.313; p=0.042) (Table 3.28).

Nuclear p27 expression when divided into present compared with absent expression did not retain its power to predict a poorer prognosis in the study population (HR 1.304; p=0.079) (Table 3.29).

Table 3.28 Multivariate analysis using Cox regression model demonstrating that high cytoplasmic p27 expression predicts worse survival independently of other accepted prognostic factors.

Variable	Hazard Ratios (95% Confidence intervals)	Significance (p)
Age at diagnosis (years)	1.023 (1.012-1.038)	**<0.001**
FIGO Stage		
I	1	
II	2.859 (1.569-5.210)	
III	5.587 (3.238-9.641)	
IV	6.347 (3.382-11.911)	**<0.001**
Optimal Debulking		
Yes	1	
No	2.019 (1.404-2.904)	**<0.001**
Patient received Chemotherapy		
No	1	
Yes	0.424 (0.290-0.622)	**<0.001**
Cytoplasmic p27 expression		
Low/ absent	1	
High	1.313 (1.010-1.707)	**0.042**

Table 3.29 Multivariate analysis using Cox regression model demonstrating that high nuclear p27 expression fails to predict improved survival independently of other accepted prognostic factors.

Variable	Hazard Ratios (95% Confidence intervals)	Significance (p)
Age at diagnosis (years)	1.025 (1.013-1.037)	**<0.001**
FIGO Stage		
I	1	
II	2.865 (1.557-5.273)	
III	5.203 (2.987-9.064)	
IV	6.278 (3.315-11.891)	**<0.001**
Optimal Debulking		
Yes	1	
No	2.003 (1.382-2.902)	**<0.001**
Patient received Chemotherapy		
No	1	
Yes	0.455 (0.308-0.671)	**<0.001**
Nuclear p27 expression		
Absent	1	
Present	1.304 (0.970- 1.753)	0.079

3.2.3.10 Correlation between nuclear and cytoplasmic p27 expression

The survival analysis for both cytoplasmic and nuclear p27 demonstrated similar findings, with expression of both appearing to confer a survival disadvantage. However, this was only independent with cytoplasmic sub-cellular location. As might be predicted there appears to be a strong correlation between expression of nuclear and cytoplasmic p27. 95% of tumours with positive cytoplasmic p27 also had positive nuclear p27, with only seven tumours having positive cytoplasmic p27 in the absence of nuclear expression. This correlation was also seen in those tumours with negative cytoplasmic p27 expression, although not as strongly, as 67% of tumours with negative cytoplasmic expression also having negative nuclear expression (Table 3.30).

Table 3.30 Expression and correlation of cytoplasmic and nuclear p27, using described scoring system.

	Positive (%)		**Negative (%)**
Cytoplasmic p27 (high/low)	157 (51.5)		148 (48.5)
Nuclear p27 (present/absent)	199 (65.2)		106 (34.8)
Cytoplasmic p27	**Nuclear p27 (%)**		**χ2 (p value)**
	positive	**negative**	
Positive	150 (95.5)	7 (4.5)	**130.959**
Negative	49 (33.1)	99 (66.9)	**(p< 0.0001)**

3.2.3.11 Relationship of p27 expression with IFNGR1 and STAT1 expression

Considering that p27 represents one of the factors induced by IFNγ stimulation there did not appear to be a correlation between the presence or absence of either cytoplasmic or nuclear p27 expression and IFNγ receptor status (Table 3.31). This included classifying receptor positivity according to high/ low expression and presence/ absence of the receptor.

However, expression of STAT1, the IFNγ transcription factor within the cytoplasm, did appear to be related to expression of both cytoplasmic and nuclear p27. Tumours which expressed cytoplasmic STAT1 had a high proportion of cytoplasmic p27 positivity (60% vs. 44%, p= 0.004). A similar effect was demonstrated, but to a greater extent, with nuclear p27 with 75% of tumours being p27-positive in the presence of cytoplasmic STAT1 staining compared with only 57% in STAT1-negative tumours (p=0.001). Interestingly, a similar relationship was not displayed when comparing nuclear STAT1 with p27 expression (Table 3.32).

Table 3.31 Correlation of IFNGR1 with cytoplasmic and nuclear p27, including high vs. low and present vs. absent expression.

IFNGR1 expression	Cytoplasmic p27 (%)		χ2 (p value)	Nuclear p27 (%)		χ2 (p value)
	High	Low		Present	Absent	
IFNGR1 High	88 (51.8)	82 (48.2)	0.002 (p= 0.962)	109 (64.1)	61 (35.9)	0.308 (p= 0.579)
IFNGR1 Low	69 (51.5)	65 (48.5)		90 (67.2)	44 (32.8)	
IFNGR1 Present	132 (51.0)	127 (39.4)	0.323 (p= 0.570)	167 (64.1)	92 (35.5)	0.746 (p= 0.388)
IFNGR1 Absent	25 (55.6)	20 (44.4)		32 (71)	13 (28.9)	

Table 3.32 Correlation of nuclear and cytoplasmic STAT1 expression with cytoplasmic and nuclear p27.

STAT1 expression	Cytoplasmic p27 (%)		χ2 (p value)	Nuclear p27 (%)		χ2 (p value)
	High	Low		Present	Absent	
Cytoplasmic STAT1 Present	86 (60.6)	56 (39.4)	**8.192 (p= 0.004)**	106 (74.6)	36 (25.4)	**10.210 (p= 0.001)**
Cytoplasmic STAT1 Absent	71 (44.1)	90 (55.9)		92 (57.1)	69 (42.9)	
Nuclear STAT1 Present	35 (56.5)	27 (43.5)	0.671 (p= 0.413)	44 (71.0)	18 (29.0)	1.088 (p= 0.297)
Nuclear STAT1 Absent	122 (50.6)	119 (49.4)		44 (63.9)	87 (36.1)	

3.2.4 Caspase1

3.2.4.1 Clinicopathological characteristics

Of 395 tumours within the study, 301 cases were suitable for immunohistochemical analysis for caspase 1 expression. 38 cores were lost during the antigen retrieval process, an 11% core loss rate. There was no difference in the clinical or pathological features of the tumours assessed when compared with the whole series. Most importantly, survival rates were similar, with five year survival in the whole series and in the assessed cores being 24% and 25% respectively (Table 3.33).

3.2.4.2 Caspase 1 staining

Caspase 1 expression was mainly cytoplasmic, with very little expression within the nucleus or cell membrane (Figure 3.17). Those tumour cores displaying caspase 1 immunoreactivity tended to display this in the majority of the tumour cells, with 75% of tumours cores expressing caspase 1 in at least half of cells. Caspase 1 expression was either moderate or strong (Table 3.34 and Table 3.35). There was strong caspase 1 expression in the positive control and no immunoreactivity in the negative control. Tonsillar tissue was used for both controls, with the negative control having the antibody omitted and the positive control exposed to the caspase 1 antibody as per the TMA slides. As with previous markers an intensity score which comprised both intensity and proportion of tumour cells staining was used, with the median IS dividing tumours into high and low expression. The median IS of 150 was relatively high compared with previous markers, reflecting the high levels of expression in both intensity and proportion of cells staining. Tumours were also assessed according to presence of any caspase 1 expression compared with a complete lack of expression, 32 tumours (10%) demonstrated a complete lack of caspase 1 expression.

Table 3.33 Clinicopathological characteristics of whole series and of those analysed for caspase 1 expression.

Variable	Whole series n=395 (%)	Caspase 1 analysed cases n= 301 (%)
Age (years)	n=394	n=300
<30	1 (0.3)	1 (0.3)
30-59	167 (42.4)	117 (39.0)
≥60	226 (57.4)	182 (60.7)
FIGO Stage	n=375	n= 294
I	99 (22.6)	75 (25.5)
II	46 (12.3)	30 (10.2)
III	188 (50.1)	155 (52.7)
IV	42 (11.2)	34 (11.6)
Optimal Debulking	n=376	n= 290
Yes	157 (41.8)	119 (41.0)
No	219 (58.2)	171 (59.0)
Tumour Grade	n=376	n= 301
1	50 (13.3)	36 (11.9)
2	93 (24.7)	69 (22.9)
3	233 (62)	196 (65.1)
Histological Type	n=395	n= 301
Serous	203 (51.4)	159 (52.8)
Endometrioid	46 (11.7)	39 (13.0)
Mucinous	50 (12.7)	30 (10.0)
Undifferentiated	65 (16.5)	47 (15.6)
Clear Cell	26 (6.6)	23 (7.6)
Other	5 (1.3)	3 (1)
Chemotherapy	n=388	n= 296
Platinum	196 (50.5)	150 (50.7)
Non-platinum	80 (20.6)	67 (22.6)
None	112 (28.9)	79 (26.7)
Five Year Survival	n=385	n= 299
Yes	91 (23.6)	75 (25.1)
No	294 (76.4)	224 (74.9)
Alive at time of data censoring	n=388	n= 299
Yes	60 (15.5)	51 (17.1)
No	328 (84.5)	248 (82.9)

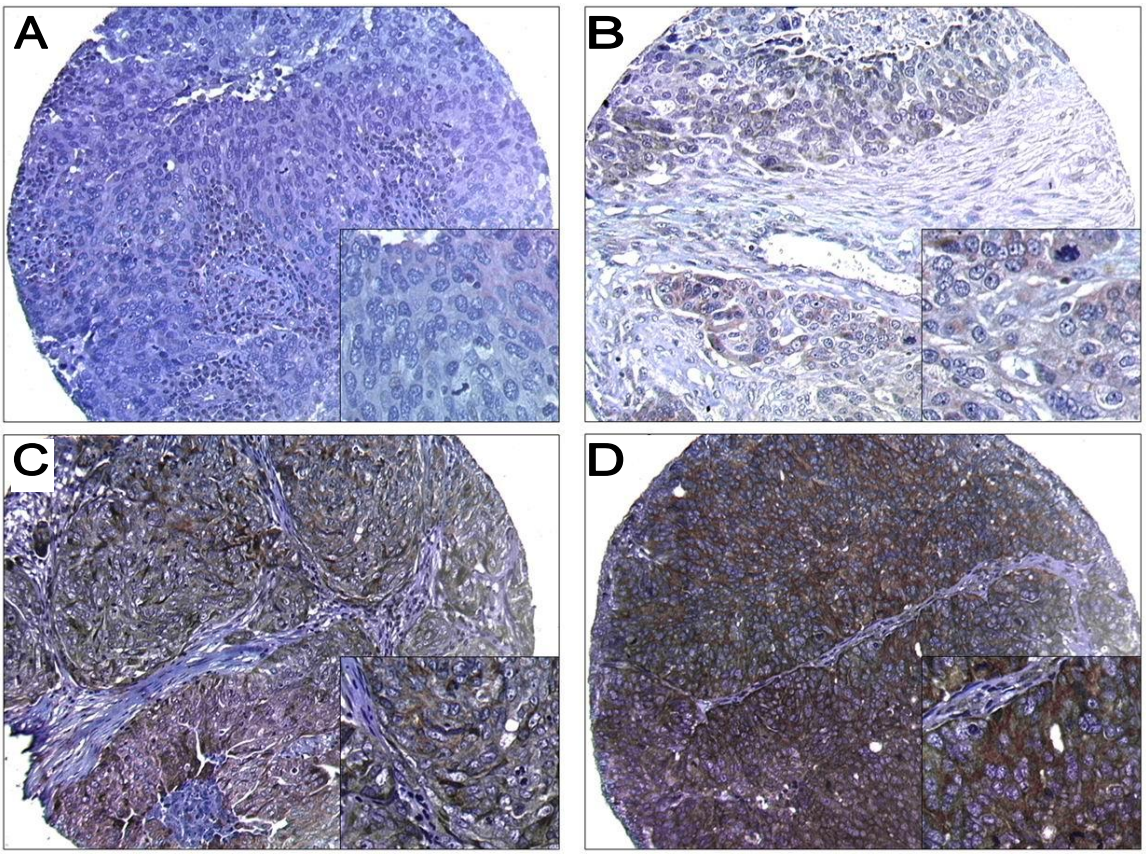


Figure 3.17 Photomicrographs of caspase 1 immunohistochemical staining of ovarian TMA cores using the Avidin-biotin complex protocol. Panel A shows a negative tumour core. Panels B - D show weak, moderate, and strong staining respectively. Cores are shown at x100 magnification; insets are x200.

Table 3.34 Intensity of cytoplasmic staining with caspase1.

Intensity of Staining	Number	Percentage
No staining	32	10.6
Mild	57	18.8
Moderate	120	40.0
Strong	92	30.6

Table 3.35 Proportion of viable tumour cells staining with caspase1.

Proportion of tumour cells staining	Number	Percentage
No cells	32	10.6
1-25%	14	4.7
26-50%	57	18.9
51-75%	37	12.3
76-100%	161	53.5

3.2.4.3 Comparison of cytoplasmic caspase 1 expression and patient tumour characteristics

Expression of caspase 1 according to both high vs. low and also presence vs. absence, was compared to the established clinicopathological features (Table 3.36). When dividing the cores according to an absence of staining only 32 (10%) tumours had no caspase 1 expression, hence comparisons with other factors should be viewed with caution.

There was preponderance of undifferentiated and endometrioid subtypes and fewer clear cell subtypes, in tumours with high expression of caspase 1. Low caspase 1 expression appeared to signify a significant survival advantage, with a 30% five year survival compared with only 20% in those with high expression (p= 0.036). This relationship is further illustrated with Kaplan-Meier survivals curves (Figure 3.18 and Figure 3.19). There were no other associations with other clinical parameters.

Table 3.36 Expression of caspase 1 divided according to high/ low and presence/ absence. Univariate analysis by χ2 caspase 1 status and clinicopathological criteria.

Variable	Caspase 1 Analysed Cases n=301 (%)	Caspase 1 high expression (%)	Caspase 1 low expression (%)	X^2 (p value)	Caspase 1 present (%)	Caspase 1 absent (%)	X^2 (p value)
Age (years)	n=300	n=147	n= 153	2.11 (p=0.348)	n= 268	n= 32	**9.85 (p=0.007)**
<30	1 (0.3)	0	1 (0.7)		0	1 (3.1)	
30-59	117 (39.0)	53 (36.1)	64 (41.8)		108 (40.3)	9 (28.1)	
≥60	182 (60.7)	94 (63.9)	88 (57.5)		160 (59.7)	22 (68.8)	
FIGO Stage	n= 294	n= 144	n= 150	5.64 (p= 0.130)	n= 262	n= 32	1.37 (p=0.714)
I	75 (25.5)	33 (22.9)	42 (28.0)		65 (24.8)	10 (31.3)	
II	30 (10.2)	14 (9.7)	16 (10.7)		27 (10.3)	3 (9.4)	
III	155 (52.7)	74 (51.4)	81 (54.0)		138 (52.7)	17 (53.1)	
IV	34 (11.6)	23 (16.0)	11 (7.3)		32 (12.2)	2 (6.3)	
Optimal Debulking	n= 290	n= 143	n= 147	2.54 (p= 0.11)	n= 259	n= 31	0.78 (p= 0.379)
Yes	119 (41.0)	52 (36.4)	67 (45.6)		104 (40.2)	15 (48.4)	
No	171 (59.0)	91 (63.3)	80 (54.4)		155 (59.8)	16 (51.6)	
Tumour Grade	n= 301	n= 148	n= 153	1.65 (p= 0.649)	n= 269	n= 32	**14.38 (p=0.002)**
1	36 (11.9)	15 (10.1)	21 (13.8)		34 (12.6)	2 (6.2)	
2	69 (22.9)	35 (23.6)	34 (22.2)		57 (21.2)	12 (37.5)	
3	196 (65.1)	98 (66.2)	98 (64.1)		178 (66.2)	18 (56.3)	
Histological Type	n=301	n=148	n= 153	**11.85 (p=0.037)**	n= 269	n= 32	**19.50 (p=0.002)**
Serous	159 (52.8)	75 (47.2)	84 (54.9)		142 (52.8)	17 (53.1)	
Endometrioid	39 (13.0)	21 (14.2)	18 (11.8)		37 (13.8)	2 (6.3)	
Mucinous	30 (10.0)	18 (12.2)	12 (7.8)		28 (10.4)	2 (6.3)	
Undifferentiated	47 (15.6)	28 (18.9)	19 (12.4)		45 (16.7)	2 (6.3)	
Clear Cell	23 (7.6)	6 (4.1)	17 (11.1)		15 (5.6)	8 (25.0)	
Other	3 (1)	0	3 (2.0)		2 (0.7)	1 (3.1)	
Chemotherapy	n= 296	n= 145	n= 151	3.05 (p=0.383)	n= 264	n= 32	1.87 (p=0.601)
Platinum	150 (50.7)	73 (50.3)	77 (51.0)		133 (50.4)	17 (53.1)	
Non-platinum	67 (22.6)	33 (22.8)	34 (22.5)		61 (23.0)	6 (18.8)	
None	79 (26.7)	39 (26.9)	40 (26.5)		70 (26.6)	9 (28.1)	
Five Year Survival	n= 299	n= 147	n= 152	**4.14 (p=0.036)**	n= 267	n= 32	0.20 (p= 0.658)
Yes	75 (25.1)	29 (19.7)	46 (30.3)		68 (25.5)	7 (21.9)	
No	224 (74.9)	118 (80.3)	106 (69.7)		199 (74.5)	25 (78.1)	
Alive at time of data censoring	n= 299	n= 147	n= 152	1.57 (p= 0.21)	n= 267	n= 32	0.05 (p=0.820)
Yes	51 (17.1)	21 (14.3)	30 (19.7)		46 (17.2)	5 (15.6)	
No	248 (82.9)	126 (85.7)	122 (80.3)		221 (82.8)	27 (84.4)	

3.2.4.4 Caspase 1 and survival

Kaplan-Meier survival curves were used to assess the prognostic significance of caspase 1 expression, initially using high and low expression according to median IS. This demonstrated improved survival with low caspase 1 expression with a mean DSS of 67 months compared to 46 months with high expression (p=0.025) (Figure 3.18) (Table 3.37). The relationship between caspase 1 expression and survival was further described by dividing tumours into presence or complete absence of caspase 1 (Figure 3.19) (Table 3.38). This illustrated very similar survival curves, with no significant difference in DSS (p= 0.15). Dividing the tumours into three groups (absent, low and high expression) failed to clarify the relationship. This is because both loss and high expression of caspase 1 appeared to predict poor prognosis, with mean DSS of 51 and 46 months respectively, whereas tumours with low expression had improved prognosis with a mean DSS of 70 months.

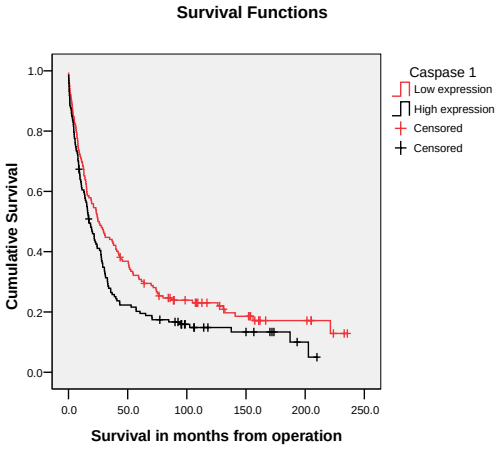


Figure 3.18 Kaplan-Meier survival plots for Caspase 1 expression. High expression refers to an IS greater than median, whereas low expression is an IS less than median (n=301).

Table 3.37 Mean and median survival time in relation to high and low Caspase 1 expression. Log Rank test for univariate survival analysis.

	Mean(a)				Median			
	Estimate (months)	Std. Error	95% Confidence Interval		Estimate (months)	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Low	66.7	6.9	53.2	80.3	24.9	3.9	17.3	32.5
High	46.0	5.6	35.1	56.9	17.3	2.4	12.7	22.0
Overall	57.4	4.6	48.3	66.5	21.7	2.7	16.4	27.1

(a) Estimation is limited to the largest survival time if it is censored.

	Chi-square	d.f.	p value
Log Rank (Mantel-Cox)	5.0	1	**0.025**

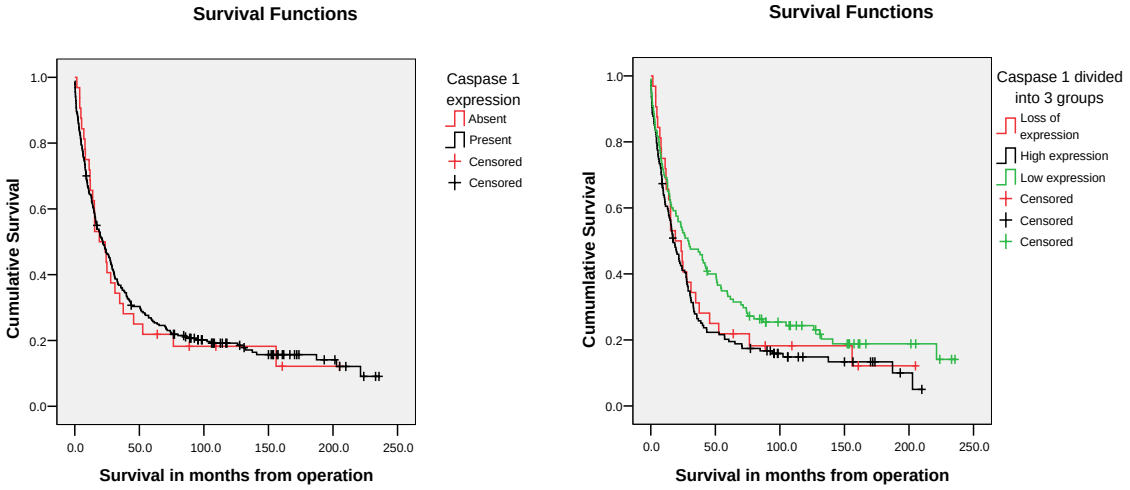


Figure 3.19 Kaplan-Meier survival plots for Caspase 1 expression. A: Presence of caspase I expression compared with absence, and B: high caspase 1 expression compared with low and absence, in tumours (n=301)

Table 3.38 Mean and median survival time in relation to present and absent Caspase 1 expression. Log Rank test for univariate survival analysis.

	Mean(a)				Median			
	Estimate (months)	Std. Error	95% Confidence Interval		Estimate (months)	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Caspase 1 present or absent								
Absent	51.5	12.1	27.7	75.3	19.0	6.3	6.5	31.5
Present	57.8	4.9	48.1	67.4	21.7	3.0	15.9	27.5
Overall	57.4	4.6	48.3	66.5	21.7	2.8	16.4	27.1
Caspase 1 expression divided into 3 groups								
Absent	51.5	12.1	27.7	75.3	19.0	6.4	6.5	31.5
Low	70.2	7.9	54.7	85.8	28.7	7.1	14.8	42.6
High	46.0	5.6	35.1	56.9	17.3	2.4	12.7	22.0
Overall	57.4	4.6	48.3	66.5	21.7	2.7	16.4	27.1

(a) Estimation is limited to the largest survival time if it is censored.

Log Rank (Mantel-Cox)	Chi-square	d.f.	p value
Caspase 1 present or absent	0.15	1	0.904
Caspase 1 expression (3 groups)	5.8	2	0.056

3.2.4.5 Multivariate analysis of caspase 1 expression and survival

In order to assess whether caspase 1 status was an independent marker of prognosis, the relative influence of caspase 1 expression and the other previously mentioned clinicopathological prognostic variables were included in a multivariate analysis. Caspase 1 when divided into high compared with low expression was seen to lose its power to predict prognosis, suggesting dependence on the other prognostic factors (HR 1.180; p=0.214) (Table 3.39).

Table 3.39 Multivariate analysis using Cox regression model demonstrating that low compared with high caspase 1 expression does not predict a survival advantage independently of other accepted prognostic factors.

Variable	Hazard Ratios (95% Confidence intervals)	Significance (p)
Age at diagnosis (years)	1.020 (1.008-1.032)	**<0.001**
FIGO Stage		
I	1	
II	3.551 (1.901-6.631)	
III	5.594 (3.285-9.528)	
IV	6.257 (3.373-11.608)	**<0.001**
Optimal Debulking		
Yes	1	
No	2.261 (1.561-3.277)	**<0.001**
Patient received Chemotherapy		
No	1	
Yes	0.384 (0.262-0.564)	**<0.001**
Caspase 1 expression		
Low	1	
High	1.180 (0.909-1.533)	0.214

3.2.4.6 Relationship of caspase 1 expression with IFNGR1 and STAT1 expression

Caspase 1 expression (high vs. low) was seen to correlate with IFNGR1 status. Presence of IFNGR1 expression was associated with a higher proportion of caspase 1 positivity compared with absent expression; 51% and 34% respectively (p= 0.026). A similar relationship was seen with cytoplasmic STAT1, with the presence of STAT1 being associated with high caspase 1 expression (58% vs. 42%, p= 0.006). Nuclear STAT1 expression was also related to high caspase 1 expression, although this did not reach statistical significance (Table 3.40).

Table 3.40 Correlation of IFNGR1 and STAT1 expression with caspase 1.

	Caspase 1 (%)		χ2 (p value)
	High	Low	
IFNGR1 Present	131 (51.8)	122 (48.2)	**4.989 (p= 0.026)**
IFNGR1 Absent	16 (34.0)	31 (66.0)	
Cytoplasmic STAT1 Present	80 (58.0)	58 (42.0)	**7.680 (p= 0.006)**
Cytoplasmic STAT1 Absent	67 (41.9)	93 (58.1)	
Nuclear STAT1 Present	36 (60.0)	24 (40.0)	3.423 (p= 0.064)
Nuclear STAT1 Absent	111 (46.6)	127 (53.4)	

3.3 Discussion

3.3.1 Interferon gamma receptor

Previous authors have established a protective role for IFNγ in ovarian cancer biology (reviewed in [206]), although the mechanism through which ovarian cancer is able to evade such action is unclear. In this chapter we investigated a potential route by which the anti-tumour effects of IFNγ may be avoided, by the down-regulation of IFNγ receptor on the tumour cell surface, reduced signalling through STAT1, and effects on two products of IFNγ stimulation: caspase 1 and p27.

The value of TMA technology is highlighted in the analysis of 339 ovarian cancers used in the study. With the use of a comprehensive database of clinicopathological variables, this provided a powerful tool to examine the components of the IFNγ pathway in an unselected cohort of ovarian cancer patients, including the influence IFNγ exerted on survival. As with all TMA work, some loss of tissue cores during antigen retrieval occurred. However, this amounted to between two and 11%, and so was therefore unlikely to affect the results, especially as the core loss, which mainly occurred during antigen retrieval, was random. The initial study population included 395 patients; however, archived histopathological material was only available for analysis from 334. This, in addition to the aforementioned

core loss experienced during each experiment, resulted in less tumours being analysed for each marker than the original cohort. To ensure the sample analysed for each marker was representative, a chi-squared analysis of the original cohort and the group analysed for each specific marker were performed. This illustrated that the group analysed was representative for each of the markers (Table 3.1, 3.10, 3.20 and 3.33).

The IFNγ receptor is made up of two subunits, the first IFNGR1 is responsible for ligand binding and signal transduction, and the second IFNGR2 is mainly involved with signal transduction. Whilst there is some evidence that IFNGR2 is found in lower concentrations and may act as a rate-limiting step during signal transduction in lymphocytes [291], the majority of studies relating to receptor function indicate that it is IFNGR1 that is relevant in carcinogenesis. Overexpression of defective IFNGR1 in tumours leads to a lack of response to IFNγ [173]. In addition, IFNGR1 knockout mice were found to develop tumours more frequently when exposed to the chemical carcinogen, methylcholanthrene (MCA), than wild type. These tumours when transplanted into naive mice continued to grow; however, restoring IFNGR1 expression in these tumours resulted in rapid rejection [295]. We decided to look at expression of IFNGR1 as it is most actively involved in IFNγ binding and has the most evidence for a role in tumour immunology.

There was variation in both the intensity of staining and the proportion of tumour cells displaying immunoreactivity to IFNGR1 antibody. Using an IS it was possible to incorporate both factors when calculating the degree of receptor expression. The median IS enabled a statistically valid division into high and low expression of IFNGR1. This illustrated a clear relationship between low IFNGR1 expression and reduced survival, a difference of 24 months mean survival time ($p=0.017$) (Figure 3.5). To explore this relationship further, the tumours were subdivided into absent, low and high receptor expression. This indicated that tumours with any receptor present, regardless of the concentration, behaved in a similar manner having very similar survival curves (Figure 3.6). This might be explained by the fact that IFNGR1 tends to be more abundant in the cell membrane than IFNGR2 [291], therefore, it may be that even low levels of IFNGR1

are sufficient to combine with the limited supply of IFNGR2 to produce physiologically normal levels of the combined receptor. Consequently, the true effects of reduced IFNGR1 may only be seen when complete loss occurs. In addition, most of the experimental data to date has assessed the potential effects of IFNGR1 loss, as opposed to high and low expression, on tumour growth [173, 174]. As such, a reanalysis was performed separating those tumours with complete receptor loss from those with any receptor expression. This demonstrated that much of the effect on survival was due to loss, rather than simple reduction, of IFNGR1 expression (p=0.014). Using complete loss of IFNGR1, as opposed to variations in degrees of receptor expression, may be more biologically valid and can perhaps more reliably predict which tumours have functioning IFNγ pathways. This apparent complete loss of IFNGR1 contrasts with the findings of Burke *et al* who demonstrated 100% expression in ovarian cancers. This was a small set of 12 patients, however, and employed RT-PCR to detect mRNA, and is therefore not directly comparable with immunohistochemical methods of detection [327].

The improved DSS in tumours maintaining IFNGR1 suggests that interruption of the IFNγ pathway, and hence evasion of subsequent anti-tumour effects, leads to a tumour being more aggressive with a poorer prognosis for the patient. This uncoupling from the immune system provides support to the theory of immunoediting in ovarian cancer.

The lack of correlation with any of the histopathological variables suggests that the effects of IFNGR1 on survival are independent of any associated confounding factors. There have not been previous studies of IFNGR1 expression in ovarian cancers with which to compare these findings.

In view of the absence of correlation between IFNGR1 and any confounding variables it was not surprising to demonstrate that loss of IFNGR1 was an independent prognostic marker. This is illustrated by multivariate analysis in which the prognostic value of IFNGR1 expression maintained significance when all other prognostic markers (tumour stage, degree of cytoreductive surgery and chemotherapy) are included (Table 3.9).

Interestingly, although the effects of IFNGR1 expression on survival were seen at all tumour stages, they may be more pronounced in early stage disease (Figure 3.7). This would support the hypothesis that loss of IFNGR1 is an early phenomenon in ovarian cancer development, with these effects being diluted by subsequent cellular mutations which occur as the tumour escapes from immunological control.

The immunoediting theory would suggest that loss of IFNGR1 should confer a survival advantage to those cells, which would then become the predominant phenotype leading to loss of IFNγ responsiveness. This hypothesis has been supported by animal studies. Overexpression of a dominant negative mutant of IFNGR1 produced enhanced tumourogenicity and reduced immunogenicity in a mouse model [173]. Furthermore, mice lacking IFNGR1 or STAT1 were 10 - 20 times more likely to develop tumours in response to the carcinogen (MCA) than their wild type counterparts [174]. To assess if the effects of IFNγ insensitivity were related to the lack of expression in the tumour cells or immune effector cells, tumours derived from mice lacking IFNGR1 were seen to grow rapidly when transplanted into wild type hosts. This suggested that the effects were related to a lack of IFNγ function within the tumour cells. Using cell lines from these tumours, IFNGR1 was transfected into the cells thereby renewing the cells' IFNγ sensitivity. These tumours were highly immunogenic and were rejected in a CD4+ and CD8+ T cell dependent manner. These findings confirm a key role for IFNγ and IFNGR1 in promoting tumour immunogenicity [174] and were independently supported by similar results using C57BL/6 strain mice lacking the gene for IFNγ itself [328]. These data suggest that the host immune system alters the phenotype of the tumours and selects for more aggressive tumour variants with less immunogenicity.

IFNγ upregulates the quantity, quality and diversity of MHC class I. Upregulation of MHC class I is important in the host immune response to intracellular pathogens and acts by displaying antigens on the cell surface for interaction with CD8+ T cells. Diversity and quality of MHC class I products is enhanced by IFNγ inducing changes in the subunits within the MHC proteosome. IFNγ also upregulates TAP1 and TAP2, which are the key

transport molecules involved in movement of peptides within the MHC pathway. Production of MHC class I heavy chain and β2 macroglobulin, the constituent parts of MHC class I, are increased in response to IFNγ [289]. The increased expression of MHC class I has been shown to be important in the immune response to tumours, and in the context of tumours evading the immune system. Loss of MHC has been implicated in this process [329], thereby preventing activation of CD8+ cytotoxic T cells and subsequent apoptotic pathways [330]. HLA class I has been documented to be reduced in a variety of tumours including ovarian cancer [331, 332]. Loss of MHC class I has also been shown to have a direct effect on tumour behaviour with lack of expression being an independently prognostic factor in breast, colorectal and ovarian cancers [333-335]. Clearly, any effects on the functioning of the IFNγ pathway may result in disruption of the MHC system with potential effects on prognosis.

Tumour transformation results in an upregulation of stress molecules that are recognised by γδ T cells and NK cells with the subsequent release of high levels of IFNγ. Although there is little NK infiltration in ovarian cancer, suggesting that these cells play a limited role in immunosurveillance of this cancer, mice lacking γδ T cells have high levels of spontaneous tumours [176]. IFNγ orchestrates the trafficking of specific immune cells to sites of inflammation and stress, through various chemokines and adhesion molecules including MIG [336] and ICAM1 [337]. Therefore, disruption in the functioning of the IFNγ pathway may result in alterations in the presence of lymphocytes within the tumour milieu. There is evidence from a number of cancers, and in particular advanced ovarian cancer, that the presence of tumour infiltrating T lymphocytes (TILs) produces an improved clinical outcome in terms of disease free and overall survival. The presence of TILs gives a five year survival of 38% compared with 4% in those without. There were 39% of tumours with no detectable TILs. Interestingly, these findings were even more pronounced in patients with complete response to initial first line treatment (surgery and chemotherapy), where those with TILs had a 10 fold increase in survival [197]. Since stimulation of cells with IFNγ is required to produce signalling for recruitment of further lymphocytes, the loss of the

IFNγ receptor demonstrated in our study group may account for the absence of TILs witnessed by Zhang *et al* [197]. There is contrasting data relating to the presence of TILs which suggests that excessive accumulation is associated with increased tumour size. This may represent immunosuppressive effects from myeloid suppressor cells which inhibit the function of the accumulated T cells, and again it is defects in the IFNγ receptor that have been implicated in this process [338]. Unfortunately, due to the size of cores used in the TMA it was not possible to examine for the presence of TILs within the tumour, although this would provide a fascinating avenue for future investigation.

Evidence from sarcoma cell lines demonstrated reduced levels of IFNγ receptor in lung metastases relative to non-metastatic tumours, and this was reflected in reduced levels of expression of IFNγ-related genes, in particular, caspase 1 and Fas [339]. This provides another potential avenue through which the observed prognostic impact of receptor loss demonstrated in our study might be produced, that is through the reduction in apoptotic pathways such as Fas and caspase 1.

In addition to the aforementioned influence IFNγ exerts on stimulating immune effector cells, it is also known to exert a number of direct anti-tumour effects in ovarian cancer. A number of studies have shown IFNγ to produce negative effects on proliferation [327, 340]. The anti-proliferative effects are thought to be via the actions of p21 [327, 341] and p27 that inhibit cyclin-dependent kinases [341] (see later discussion). In ovarian cancer cell lines, IFNγ has been shown to downregulate HER-2/neu, a proto-oncogene responsible for inducing proliferation and being a marker of poor prognosis, producing a reduction in proliferation rates [342]. Similarly IFNγ was seen to upregulate H-REV 107-1, a growth inhibitory gene resulting in apoptosis [343].

IFNγ has also been implicated in TRAIL-dependent apoptotic pathways, with IFNγ inducing transcription of mRNA for KILLER/DR5 TRAIL receptors mediating p53 independent apoptosis [344].

Whilst the prognostic influence of IFNGR1 has not previously been documented, Marth *et al* did demonstrate, using RT-PCR, that increased expression of IFNγ in ovarian cancer

was an independent prognostic factor. This was associated with transcription of known IFNγ-associated genes (SOCS-1 and IRF-1) and reduced expression of HER2/neu [345].

There are now a number of studies supporting a clinical role for IFNγ. The anti-proliferative effects of IFNγ were seen to act synergistically when combined with cisplatin in ovarian cancer cell lines [340]. Pro-apoptotic effects of IFNγ have been demonstrated via caspase pathways in ovarian cancer cells retrieved from ascitic fluid. Yet again, synergistic effects were seen with the addition of cisplatin [235], as well as inhibitory effects on anti-apoptotic molecules Bcl-2 and Bcl-XL [346]. Wall *et al* combined *in vivo* and *in vitro* methods to demonstrate the anti-proliferative effects in eight ovarian cancer cells lines. However, only two of six patients with advanced ovarian cancer demonstrated a clinical improvement following administration of intra-peritoneal IFNγ, with reduced ascites formation [347].

A number of clinical trials have been conducted in an attempt to utilise the potential anti-tumour effects of IFNγ, in particular, the synergistic effects seen *in vitro* with cisplatin. Early evidence of a potential clinical role came in 1988 when Welander *et al* [348] demonstrated some response to IFNγ in four of 14 patients with relapsed ovarian cancer. The intra-peritoneal approach, with the potential advantages of bioavailability via this route, was investigated and shown to produce a 31% response rate at second look laparotomy in patients with residual disease [349].

In a randomised phase III study, 148 women with stage Ic-III ovarian cancer were treated with cisplatin and cyclophosphamide alone versus chemotherapy plus subcutaneous IFNγ. Unfortunately, the trial was terminated early due to changes in the recommended standard first line chemotherapy for ovarian cancer. At that point, a significantly improved PFS, from 38% in the control group to 51% in the IFNγ treatment group, was seen (RR of progression = 0.48, 95% CI 0.28 – 0.82). Three year overall survival was also improved, with 58% and 74% respectively, although this was not statistically significant, possibly due to the premature termination of the trial [236]. Recent chemotherapy regimes which tend to include paclitaxel appear to be safe with regards to toxicity when combined with IFNγ

[237]. In light of this data, a recent large multi-centre phase III trial was conducted similar to the Windbichler *et al* study [236] but using carboplatin and taxol as the standard chemotherapeutic regime. This study was stopped early due to a pre-designated stopping boundary being reached during the second interim analysis, revealing significantly shorter OS time in patients receiving the IFNγ in addition to the standard chemotherapy. 40% of patients in the IFNγ group had died in comparison to 30% in the control arm. Higher adverse events were also seen in the IFNγ group, primarily due to serious haematological toxicities [350]. Explanations postulated as to reasons for treatment failure included direct toxicity from IFNγ and alterations in dose regime of chemotherapy, due to toxicity and activation of Tregs, resulting in immune suppression. The timing of the deaths tended to be after the completion of treatment making direct toxic effects from IFNγ unlikely. There was significantly greater incidence of grade 3 (severe) and 4 (life threatening) events with IFNγ, especially related to neutropenia. The increase in such toxicities in the IFNγ arm led to less patients completing the six cycles of carboplatin/ taxol or there was a requirement for dose reductions and delays. This is most likely to account for the reduced survival demonstrated, since the administration of sub-therapeutic chemotherapy as a result of toxicity occurred in 18% of the IFNγ group compared with only 9% of those receiving standard treatment. The third possibility is that IFNγ may have induced Treg cells, which have been shown to repress the cancer immune response [249]. The activity of Tregs has been linked to reduced survival in ovarian cancer [227, 249]. Hence, there is a possibility that exogenous IFNγ may paradoxically suppress the cancer immune response through the inhibitory effects of Tregs. Our data illustrates that IFNγ receptor loss occurs in 22% of tumours. Hence, a significant proportion of the patients in the study by Albert *et al* were unlikely to have benefited from what was clearly a toxic cocktail of agents, due to a defective IFNγ pathway. Therefore, these patients could potentially be receiving the additional toxicity from IFNγ without the potential beneficial anti-tumour effects. This might partly explain the negative impact IFNγ had on overall survival in this unselected

population. Assessment of the IFNGR status of the tumours prior to inclusion in such a study would highlight those tumours with an intact IFNγ signalling pathway which may benefit from IFNγ treatment. Equally it would exclude those in which a disrupted pathway would render the patient insensitive to such treatment.

In summary, the expression of IFNGR1 in a typical ovarian cancer population is variable, with 22% showing complete loss of the receptor. Reduced receptor expression appears to have a negative effect on survival and is unrelated to other clinicopathological variables. The low expression of IFNGR1 is an independent prognostic marker in ovarian cancer. These data suggest that evasion of the IFNγ effects, through reduced receptor expression, results in more aggressive tumours, and supports the theory of immunoediting. Future work focusing on the targeting of susceptible tumours (those with functioning receptors) to IFNγ therapy may provide improved outcomes.

3.3.2 STAT1

The antibody used in this work detects STAT1 in both its inactive and active (phosphorylated) form. The process of activation of STAT1 via phosphorylation with subsequent translocation to the nucleus is well established [351]. Therefore, STAT1 within the nucleus is most likely to represent the active form with STAT1 in the cytoplasm being a combination of both forms. Similarly, deactivation and subsequent removal of STAT1 from the nucleus through the action of nuclear tyrosine phosphatases has also been demonstrated [352]. Therefore, the assumption that the appearance of STAT1 within the nucleus represents active STAT1 seems reasonable.

The distribution of STAT1 expression within the nucleus and cytoplasm (Figure 3.1), has previously been described [280]. The nuclear STAT1 is likely to represent the functioning component, however, high levels of phospho-STAT1 (i.e. activated) have been shown to predominantly reside within the cytoplasm, with weaker nuclear expression [280]. In addition, Meyer *et al* have demonstrated some level of functioning of unphosphorylated STAT1 within the nucleus, independent of the IFNγ activation [353]. This further

complicates the analysis of STAT1 expression as a component of the IFNγ pathway. We found a similar pattern of STAT1 expression to Chen *et al* [280] with cytoplasmic and nuclear immunoreactivity of 45% and 20% respectively (Table 3.11). There are a number of potential explanations for higher cytoplasmic, compared with nuclear, expression. Firstly, as was previously alluded to, the antibody which we used targeted both phosphorylated and non-phosphorylated STAT1. Therefore, we may be demonstrating a predominantly inactive reserve of STAT1 which does not correlate with cell signalling. However, as Chen *et al* [280] described similar findings, when only detecting activated STAT1, this seems unlikely. The use of an antibody for phospo-STAT1 may have removed this potential dilemma, but when this was used on the current TMA it produced non-specific staining and therefore the current antibody was preferred. Secondly, it has been suggested that a defective STAT1 transport system could occur in cancer cells, resulting in an accumulation of STAT1 within the cytoplasm [353].

When assessing the distribution of STAT1 expression, it was interesting to note that in the majority (97%) of cores in which there was an absence of STAT1 within the tumour cells, there was also loss of expression within the stromal cells. This suggests a generalised lack of IFNγ production within the tumour milieu (Table 3.13)

There was a strong correlation between the expression of cytoplasmic and nuclear STAT1 (Table 3.14). The vast majority (94%) of nuclear STAT1 was seen in tumours expressing cytoplasmic STAT1, which is to be expected since STAT1 translocates from the cytoplasm to the nucleus [351]. Interestingly, just fewer than half of the tumours expressing cytoplasmic STAT1 also displayed nuclear STAT1 expression .

A correlation was seen between nuclear and cytoplasmic STAT1 and increasing tumour grade (Table 3.15). This had been previously demonstrated [354] and is thought to reflect an increasing stress response of the cell to dedifferentiation. A similar explanation may attribute the association with advanced stage and cytoplasmic STAT1 status, although this was not seen in nuclear STAT1.

Since STAT1 is the key signal transducer for IFNγ, it would be expected that IFNGR1 status and STAT1 expression would be closely correlated, and this is indeed what is seen. In those tumours expressing high levels of STAT1 (both nuclear and cytoplasmic) the majority (93%) had expression of the IFNγ receptor ($p<0.001$) (Table 3.19). This relationship suggests that most of the demonstrated STAT1 expression is in the activated form.

The survival analysis did not demonstrate a significant correlation between STAT1 expression and prognosis, although there was a trend towards reduced survival with positive cytoplasmic STAT1 (Figure 3.9 and Figure 3.10). This suggests the possibility of an accumulation of STAT1 in the cytoplasm, resulting from defects in the upstream signalling pathway or defective translocation of STAT1 to the nucleus. To further investigate this possibility the tumours were regrouped according to this hypothesis, that is tumours in which there was positive cytoplasmic and negative nuclear STAT1 expression were grouped together and were deemed to have a potentially defective pathway. These were assessed against the remainder of the group (Figure 3.11). This demonstrated reduced survival in tumours with the "defective STAT1 pathway", although this failed to reach statistical significance. Clearly, this hypothesis can not be proven by immunohistochemical methods, and would require other techniques to investigate it further. There is early evidence that mutations in the nuclear import protein importin β p97, normally responsible for translocation of activated STAT1, could result in inhibition of this function with accumulation of STAT1 within the cytoplasm and a lack STAT1 within the nucleus [353]. This effective inhibition of IFNγ signalling might explain the trend towards reduced survival with high cytoplasmic and low nuclear STAT1.

There is little data regarding the prognostic significance of STAT1 in ovarian cancer; however, contrasting findings have been illustrated in breast cancer where a positive correlation with STAT1 expression and prognosis was seen by one group [279], although others were unable to reproduce these findings [355]. In addition, activation of STAT1 in melanoma patients was seen to be a predictor of poor prognosis [356].

Interestingly, although there was a correlation between IFNGR1 status and STAT1 expression, STAT1 failed to produce similar prognostic features seen with the IFNGR1. There are a number of explanations for this; firstly, the IFNγ receptor represents a more stable protein. Only small fluctuations are produced with the ubiquitination and loss of some of the receptor molecules following signalling, the majority returning to the cell membrane [317]. Conversely, STAT1 is more unstable, with rapid deactivation through dephosphorylation and ubiquitin proteosome degradation pathways. This is possibly via the STAT-interacting ubiquitin ligase enzyme [357] and so may be producing a snapshot of the cells' activity, which may not be entirely representative. Secondly, whilst STAT1 represents the main signal transducer for IFNγ, it can also be associated with other signalling pathways, for example, type I IFN. Heterodimers and heterotrimers are produced with other STATs and IRFs, such as the STAT1:STAT2:IRF9 complex, which produces differing cellular effects [294]. Therefore, STAT1 expression will not exclusively represent IFNγ signalling, which may distort the perceived correlation of STAT1 with the IFNγ signalling pathway.

3.3.3 p27

p27 represents a prognostic marker in ovarian cancer which has sparked much controversy and debate. Various studies conducted on breast [358], gastric [359], colorectal [307], bladder [360], endometrial [311] and ovarian cancers [308, 361-365] have demonstrated seemingly contrasting results regarding the influence of p27 on tumour behaviour.

Many studies in ovarian cancer have focused on the expression of nuclear p27, since this is its site of action as a CDKI [362, 366]. Some authors have demonstrated expression of p27 in both cytoplasmic and nuclear locations [325, 326, 367]. In the current study we support these findings with clear expression of nuclear and cytoplasmic p27 (Figure 3.12). The distribution of p27 expression was patchy in both sub-cellular locations with less then 25% of tumour cores displaying positivity in the majority of the cells (Table 3.21). This

correlates with previous work in which the variation in nuclear staining was between eight and 99% of tumour cells [366]. This heterogeneity of staining is not ideal in TMA analysis, since only a relatively small area of the tumour is represented; the staining pattern could result in inaccurate assessment of protein expression if a non-representative area has been sampled. It was for this reason that two cores were assessed from each tumour, in an attempt to improve the likelihood of samples being representative. However, it is clear that this could not be as robust as using whole sections of tissue. This is one of the sacrifices made in the use of such high throughput immunohistochemical techniques.

There has been huge variation as to whether nuclear or cytoplasmic p27 expression is relevant, with some authors suggesting the importance of nuclear [366] and others cytoplasmic [325, 367], whilst some do not distinguish [362]. To fully assess the potential influence of p27, we felt it necessary to assess both sub-cellular locations.

With cytoplasmic p27 expression there was very limited data regarding a scoring system. Some studies showing limited cytoplasmic expression, which was not recorded or analysed [362], whilst others documented any immunoreactivity as positive [326]. Using an IS provided the maximum semi-quantitative assessment, using a well-recognised scoring system [368].

Many studies have failed to describe any association between p27 and clinicopathological parameters [325, 362, 367]. We have demonstrated a trend towards early stage, with low compared to high cytoplasmic p27 expression, with 42% and 29% being early stage respectively ($p=0.068$). This relationship has previously been described [326]. Both high nuclear and cytoplasmic p27 expression was seen more predominantly in serous subtypes and less frequently in mucinous, which has also been reported previously [367]. High tumour grade has been associated with low p27 expression [366, 369, 370], but this was not seen in our study (Table 3.23).

The initial survival analysis for cytoplasmic p27 showed a clear association between high expression and reduced survival (Figure 3.13), which supports the findings of Rosen *et al* [326]. The current analysis elaborates on this relationship, when the group is sub-

stratified. It appears that there is a progressive impact on prognosis from no expression through to high expression, with increasing levels of cytoplasmic p27 resulting in increasingly poorer prognosis (Figure 3.13). Comparing those tumours with high expression with the remainder highlights these tumours as a particularly poor prognostic group (Figure 3.14).

The analysis of nuclear p27 expression was also initially recorded as an IS. This demonstrated a reduced survival with high expression, and the subgroup analysis revealed that tumours with absent expression (<5% of cells positive), appeared to behave differently to those with low or high expression (Figure 3.15). Since most previous studies have divided tumours according to <5% as being negative, this method was favoured (Figure 3.15) [326, 366]. This clearly demonstrated that tumours with nuclear p27 expression had a poorer prognosis (Figure 3.16). Using the previously mentioned established prognostic factors in a Cox regression model demonstrated that high cytoplasmic p27 expression was an independent prognostic factor (p=0.042), but nuclear expression was not (p=0.079) (Table 3.28 and Table 3.29).

The nucleus is clearly the primary site of p27 action, however, a number of studies have demonstrated high levels of cytoplasmic expression in colorectal [371] and ovarian cancers [326]. This sequestering of p27 to the cytoplasm has been suggested as a mechanism for inactivation, by removal from its site of action [372]. This process is thought to occur as a result of PKB/Akt phosphorylation of p27, which itself may be related to the hyperactivation of Ras signalling [373]. Hence, activation of the Ras pathway results in the sequestration and inactivation of p27, which leads to increased levels of function CDK2 with subsequent triggering of the G1 phase of the cell cycle.

There is increasing evidence that cytoplasmic expression of p27 does not simply reflect a method of inactivation, and that it has specific functions within the cytoplasm. p27 is associated with the formation of cyclin D and E-CDK complexes within the cytoplasm and the subsequent translocation to the nucleus [374]. Hence, high levels of cytoplasmic p27 may result in increased formation and translocation of cyclin-CDK complexes to the

nucleus with subsequent stimulation of cell proliferation. Cytoplasmic p27 may have an anti-apoptotic effect through inhibition of cytochrome c release [375] and regulation of expression of the anti-apoptotic protein mcl-1 [375, 376]. The stimulation of cyclin-CDK complexes and the anti-apoptotic effects of p27 within the cytoplasm may explain the negative prognostic influence of high cytoplasmic p27 demonstrated in our study population. Recent evidence suggests that cytoplasmic p27 may have an influence on cellular motility as it has been shown to bind to and inhibit RhoA. The increased motility of tumour cells may effect their ability to invade and metastasise, which intuitively may influence tumour behaviour and hence prognosis [377]. The prognostic significance of the subcellular location of p27 again provides contrasting results with Watson *et al* demonstrating improved survival with high expression of cytoplasmic p27 in colorectal cancers [378]. Rosen *et al,* however, suggest the opposite effect in a series of ovarian cancers [326].

The novel role suggested for cytoplasmic p27 fails to explain our findings that increased nuclear p27 also predicts an unfavourable prognosis. This result seems paradoxical when considering that the role of p27 within the nucleus as a cell cycle inhibitor is well established [303]. However, whilst a number of studies have shown the expected effect of a correlation between reduced nuclear p27 and poor survival in ovarian cancer [363, 379], overexpression of nuclear p27 has also been proposed as a mechanism for inactivation. Two other studies failed to demonstrate these effects [361, 380], whilst the findings of Psryrri *et al* concurred with our results [325].

Nuclear p27 overexpression has been demonstrated in a number of tumours, including endometrial [311, 381], pancreatic [310] and breast [382]. Paradoxically, increased nuclear p27 has been linked to an increased cellular proliferation rate in endometrial [311], colorectal [383] and lung cancer [384]. This upregulation of p27 has been proposed to induce, as opposed to inhibit, cyclinE-CDK2 signalling and hence promote cell division [374, 383].

Since mutation in the p27 gene is very rare [385], the explanation of the seeming dysregulation of p27 in malignant tissue relates to post-translational effects. The tumour cells may become resistant to inhibition by p27, resulting in its amplification of p27 in an attempt to overcome this resistance [386]. Secondly, alterations in the level of associated factors such as cyclin D and E may influence p27 effects [281]. Thirdly, there may be alterations in the ubiquitin-related p27 degradation pathway, leading to elevation of p27 levels within the nucleus resulting in disruption of normal functioning [387].

3.3.4 Caspase 1

Caspase 1 is a protease, which cleaves inactive pro-interleukin-1β (IL1β) to produce the active 17 kDa mature form, which is a key mediator of inflammation [388, 389]. Hence, the alternative name for caspase 1, IL1β converting enzyme (ICE). In addition to its role in triggering inflammation, caspase 1 is also associated with apoptosis [390]. The inactive precursor of caspase 1 is the 45 kDa protein, p45. Activation occurs through the cleaving of the terminal subunit to create two active components p20 and p10. The active caspase 1 complex consists of two p10 and two p20 monomers [391, 392]. The caspase 1 antibody used in this study (06-503 Upstate Biotechnology) recognises both the inactive pro-caspase (p45) and one of the active subunits (p20), hence any immunoreactivity demonstrated may have represented either inactive or active caspase 1.

In normal cells caspase 1 predominantly exists within the cytoplasm as the p45 proform [393] which is converted via upstream initiator caspases such as caspase 8 and 9 [316]. Yang *et al*, working with the same antibody used in our study showed immunoreactivity in 71% of tumour cells compared to 90% in our population [283] (

Table 3.35), and demonstrated overexpression of caspase 1 in pancreatic carcinomas and in chronic pancreatitis. Feng *et al* reported opposite effects, with reduced levels of caspase 1 in ovarian cancer cell lines compared with surface epithelium of normal ovaries. This was associated with reduced caspase 1 mRNA, suggesting reduced production as opposed to increased utilisation of the enzyme, which was also linked to reduced apoptosis [394]. Caspase 1 has a predominantly cytoplasmic location [393], and this was clearly seen in the pattern of staining within the TMA (Figure 3.17).The preponderance to high caspase 1 expression in undifferentiated and endometrioid cell types has not previously been reported (Table 3.36).

An alternative route of activation of caspase 1 is via IRF1 to produce IFNγ-mediated apoptosis [181]. This process was illustrated in the loss of IFNγ-induced caspase 1 apoptosis in IRF1-deficient mice [395]. Induced caspase 1 activates downstream caspases such as caspase 3, a highly active apoptotic target which destroys the homeostatic functions within a cell, to produce a final common caspase mediated apoptotic pathway [316]. Therefore, one of the principle effects of IFNγ on tumour cells, apoptosis, is thought to be via activation of IRF1 with subsequent triggering of the caspase cascade via caspase 1. This pathway has been illustrated using pancreatic [182] and ovarian cell lines [396]. Consequently, tumour cell evasion from immunosurveillance through defects in the IFNγ pathway may be through inhibition of the pro-apoptotic effects of IFNγ induced caspases. Therefore, reduced expression of active caspase 1 through a defective IFNγ-IRF1-caspase 1 pathway may result in a poorer prognosis.

In our population, tumours with reduced caspase 1 expression had significantly improved overall survival, with mean survival time 20 months greater than tumours with high caspase 1 expression (p=0.025) (Figure 3.18) (Table 3.37) although this was not independently significant (Table 3.39). This effect was not reproduced when looking at absence vs. presence of caspase 1, which may be due to the small number of patients within the absent caspase 1 group (10% of total). This would require a bigger difference in survival to produce a significant association with survival (Figure 3.19) (Table 3.38).

Since the antibody recognises both active and inactive caspase 1, the interpretation of these results is difficult. Caspase 1 is known to exist mainly in the inactive form within cells. A cell which is actively converting pro-caspase would therefore produce a reduction in the total amount of caspase 1 within the cell. Hence, the low expression of total caspase 1 within tumour cells may represent a cell with increased utilisation of the caspase 1 pathway, which in turn triggers higher levels of apoptosis. This theory could explain why reduced levels of caspase 1, as a marker of increased apoptosis, would produce a more favourable prognosis since greater tumour killing is occurring.

Whilst caspase 1 has a clear role in apoptosis, overexpression has been associated with elevated levels of cyclin D1 [283], which has been demonstrated to lead to increased cell proliferation and contribute to aggressive tumour behaviour in lung [397] and ovarian cancers [341]. Overexpression of caspase 1 has also been linked to increased levels of epidermal growth factor (EGF) and epidermal growth factor receptor (EGFR) [283] which have also been linked to poor prognosis in ovarian cancer [256].

Interestingly, of the six ovarian cancer cell lines used by Feng *et al,* one had very high levels of caspase 1 expression in the absence of any caspase-dependent apoptosis [394]. This suggests a defect in the downstream caspase pathway which could result in increasing levels of caspase 1 without subsequent apoptosis. This may be an alternative explanation for the association between poor prognosis and the high levels of caspase 1 in the current study.

The positive correlation displayed between high caspase 1 expression, IFNGR1 and STAT1 status (Table 3.40) suggests that the signalling pathway in tumours with a functioning IFNγ pathway, that is STAT1 and IFNGR1 positive, produces elevated levels of caspase 1. The correlation between STAT1 activation, through IFNγ stimulation, and subsequent caspase 1-induced apoptosis has been demonstrated in cancer cell lines [181]. This association would contradict the hypothesis that low caspase 1 levels were a result of excessive signalling leading to reduced reserves of pro-caspase 1. This association would instead suggest that the elevated caspase 1 expression seen was a

result of increased levels of the activated isoforms. This could possibly lead to increased levels of EFG, EGFR and cyclin D1, or reflect a defective downstream pathway, both of which would produce the observed reduction in survival seen. Further work investigating the influence of these factors and IRF1 may elucidate the relationship between high caspase 1 expression and reduced survival.

3.4 Conclusion

Loss of IFNGR1 was demonstrated to be an independent marker of poor prognosis. This effect was seen in both early and late stage tumours, suggesting that this was an early phenomenon in tumourigenesis. The loss of IFNGR1 in a significant number of tumours might explain the negative findings of previous clinical trials using IFNγ [350].

STAT1, the main intracellular signalling molecule for the IFNγ pathway, failed to show the same strong correlation with prognosis as IFNGR1. There was a non-significant association between a defective STAT1 pathway and prognosis.

p27 showed a clear association between high cytoplasmic and nuclear p27 expression and reduced survival; however, only cytoplasmic expression was an independent prognostic factor. This result seems paradoxical when considering that the role of p27 within the nucleus as a cell cycle inhibitor is well established, although increased nuclear p27 has been linked paradoxically to an increased cellular proliferation rate [311, 374, 384]. This upregulation has been proposed to induce, as opposed to inhibit, cyclinE-CDK2 signalling and hence promote cell division [374, 383].

Tumours with reduced caspase 1 expression demonstrated significantly improved overall survival, although this was not independent of other prognostic factors. Reduced levels of caspase 1, as a marker of increased apoptosis, would produce a more favourable prognosis since greater tumour killing is occurring. Alternatively, overexpression of caspase 1 has been associated with elevated levels of cyclin D1 [283], with increased cell proliferation and aggressive tumour behaviour [341].

4 TRAIL Pathway

4.1 Introduction

Dysregulated apoptosis plays a key role in carcinogenesis producing an increased tumour cell life span as well as contributing to the development of chemoresistance. The tumour necrosis factor α (TNFα) super-family represents a significant apoptotic pathway. The death receptor ligands for this pathway include Fas ligand, TNFα, TL1A and TNF- related apoptosis inducing ligand (TRAIL). These ligands can signal following release into the extracellular matrix or whilst bound to the cell membrane during cell-cell contact [260].

TRAIL has attracted recent attention as a potential anticancer agent due to its apoptotic activity against cancer cells *in vitro* [398]. Unlike other TNF family members, soluble TRAIL seems inactive against normal cells [399].

Thus far, there have been five TRAIL receptors identified, two agonistic receptors DR4 (or TRAIL-R1) and DR5 (TRAIL-R2), and three antagonistic TRAIL-R3, TRAIL-R4 and osteoprotegerin [16]. For the reminder of this work DR4 and DR5 will be used as opposed to TRAIL-R1/-R2, although these terms are interchangeable.

Both DR4 and DR5 are transmembrane proteins which contain an extracellular receptor with an intracellular tail containing a death domain (DD), which engages downstream apoptotic pathways after ligand binding [398]. TRAIL-R4 has a non-functioning cytosolic tail and TRAIL-R3 lacks a cytosolic tail entirely, hence they fail to transmit ligand signalling. Since all the TRAIL receptors have similar extracellular domains they all bind to the same ligand, therefore TRAIL-3 and -4 have the effect of acting as decoy receptors [16].

TRAIL forms homotrimers that bind three receptor molecules resulting in trimerisation of receptors with clustering of the intracellular DDs. The clustering of the DDs leads to the recruitment of the adaptor molecule Fas-associated protein with death domain (FADD), which in turn activates caspase 8 and caspase 10. The activation of caspase 8 and 10 leads to direct and indirect (through mitochondrial cytochrome c) conversion of pro-

caspase 3 and 7 to the active forms, both of which lead to cellular disassembly (Figure 4.1) [16].

The evidence of dysregulation of TRAIL in the development of tumours and their resistance to chemotherapy, as well as its potential therapeutic role, provides an interesting avenue of investigation using the ovarian cancer TMA. The hypothesis follows that tumours with low expression of DR4 and DR5 would be less prone to TRAIL-mediated attack and therefore apoptosis. This would be reflected in reduced patient survival.

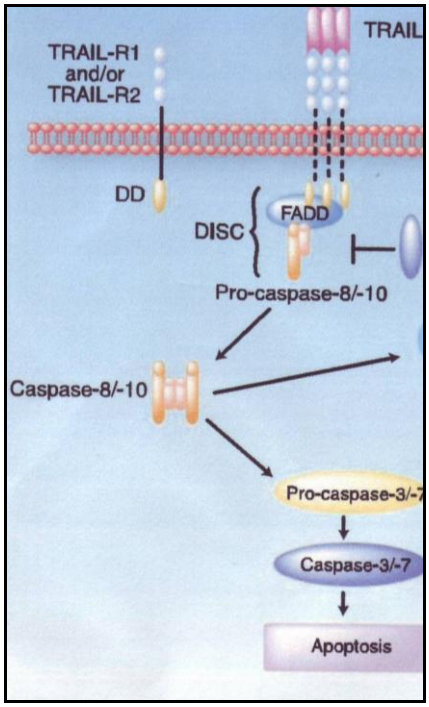


Figure 4.1 TRAIL apoptosis pathway. Trimerisation of death receptors (R1 and R2) by TRAIL triggers activation of caspase 8 and caspase 10. This subsequently activates caspase 3 and caspase 7 leading to apoptosis. Adapted from Carlo-Stella *et al* [16].

4.2 Results

4.2.1 Clinicopathological characteristics

Of the 339 tumour cores on the TMA, 40 were lost with DR4 and 39 with DR5 immunohistochemical processing. This left approximately 88% of cases available for analysis. The subgroups analysed were similar to that of the overall group, which as previously discussed, is representative of a typical ovarian cancer population (Table 4.1). Of particular importance was the comparability of five year survival, being 24% in the whole series and 25% in those analysed for DR4 and DR5 expression.

4.2.2 DR4 and DR5 staining

The subcellular distribution of both DR4 and DR5 expression was mainly seen within the cytoplasm. There was very occasional membrane staining and some nuclear staining seen with DR4 and DR5, although this was most prominent with DR4 (Figure 4.2 and Figure 4.3). In line with previous studies, expression within the cytoplasm was assessed [286, 400-402]. DR4 expression, when present, was of predominantly mild to moderate intensity (67%), with only 14% of cores showing strong staining and 19% showing no immunoreactivity (Table 4.2). DR4 expression tended to be homogeneous within the positive cores and was demonstrated in at least 75% of cells (Table 4.3).

DR5 expression was also mainly mild to moderate in intensity, although a greater proportion of cores were negative (36%) compared with DR4 (Table 4.2). The staining was more heterogeneous than DR4, with less than 50% of cells demonstrating immunoreactivity within each positive core cells (Table 4.3). For the purposes of analysis, and in line with previous work [286], a core was deemed positive if there was the presence of staining and negative if there was a complete absence of immunoreactivity. This system was decided upon prior to analysis in order to avoid potential bias. When classifying tumour cores, according to these criteria, 81% and 65% were positive for DR4 and DR5 respectively.

Table 4.1 Clinicopathological characteristics of whole series and those analysed from the TMA.

Variable	Whole series n=395 (%)	DR4 Analysed Cases n=299 (%)	DR5 Analysed Cases n=300 (%)
Age (years)	n=394	n=298	n=299
<30	1 (0.3)	1 (0.3)	1 (0.3)
30-59	167 (42.4)	118 (39.6)	117 (39.1)
≥60	226 (57.4)	179 (60.1)	181 (60.5)
FIGO Stage	n=375	n=292	n=292
I	99 (22.6)	74 (25.3)	74 (25.3)
II	46 (12.3)	32 (11.0)	31 (10.6)
III	188 (50.1)	153 (54.4)	153 (52.4)
IV	42 (11.2)	33 (11.3)	34 (11.6)
Optimal Debulking	n=376	n= 288	n=289
Yes	157 (41.8)	117 (40.6)	118 (40.8)
No	219 (58.2)	135 (59.4)	171 (59.2)
Tumour Grade	n=376	n=298	n=299
1	50 (13.3)	34 (11.4)	33 (11.0)
2	93 (24.7)	67 (22.5)	67 (22.4)
3	233 (62)	197 (66.1)	199 (66.6)
Histological Type	n=395	n=299	n=300
Serous	203 (51.4)	160 (53.5)	160 (53.3)
Endometrioid	46 (11.7)	39 (13.0)	40 (13.3)
Mucinous	50 (12.7)	30 (10.0)	28 (9.3)
Undifferentiated	65 (16.5)	14 (15.7)	46 (15.3)
Clear Cell	26 (6.6)	20 (6.7)	23 (7.7)
Other	5 (1.3)	3 (1.0)	3 (1.0)
Chemotherapy	n=388	n=294	n=294
Platinum	196 (50.5)	150 (51.0)	150 (51.0)
Non-platinum	80 (20.6)	69 (23.5)	70 (23.8)
None	112 (28.9)	75 (25.5)	74 (25.2)
Randomised treatment	69/395 (17.5)	53/299 (17.7)	52/300 (17.3)
Five Year Survival	n=385	n=294	n=295
Yes	91 (23.6)	74 (25.2)	76 (25.8)
No	294 (76.4)	226 (74.8)	219 (74.2)
Alive at time of censoring	n=388	n=296	n=297
Yes	60 (15.5)	50 (16.9)	52 (17.5)
No	328 (84.5)	246 (83.1)	245 (82.5)

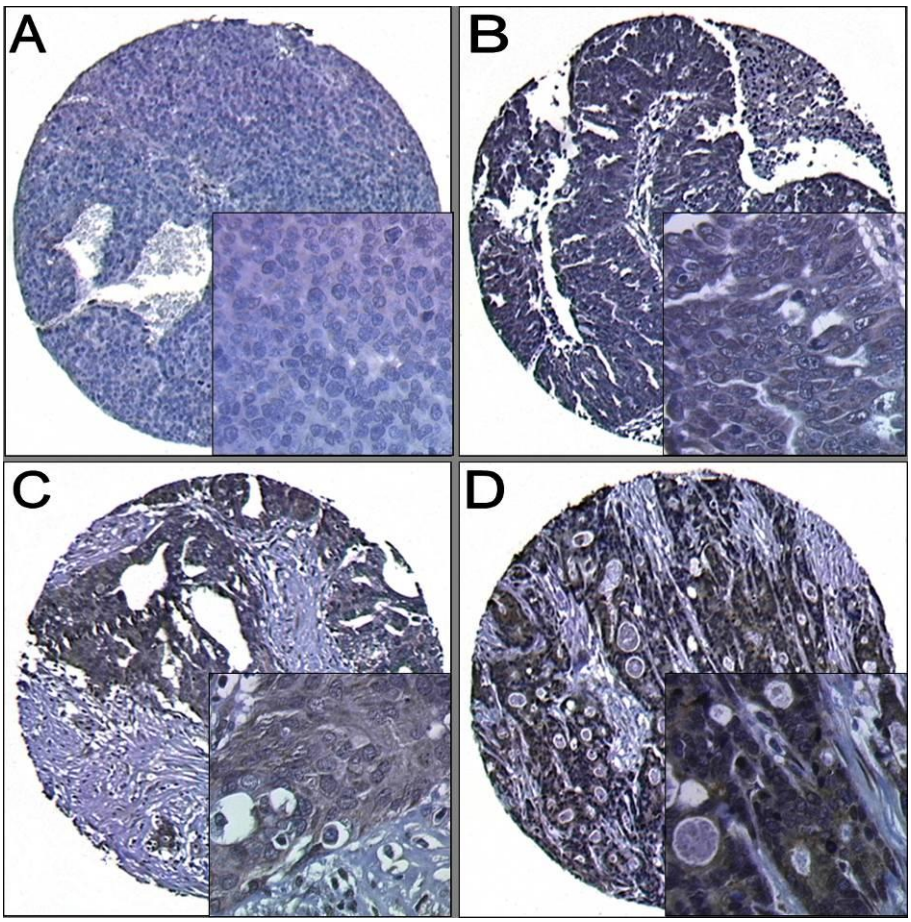


Figure 4.2 Photomicrographs of ovarian TMA cores immunohistochemically stained for DR4. The level of expression ranged from none (A), weak (B), moderate (C) through strong (D). Expression was mainly cytoplasmic but nuclear staining was also seen. Magnification: x100, inset x400.

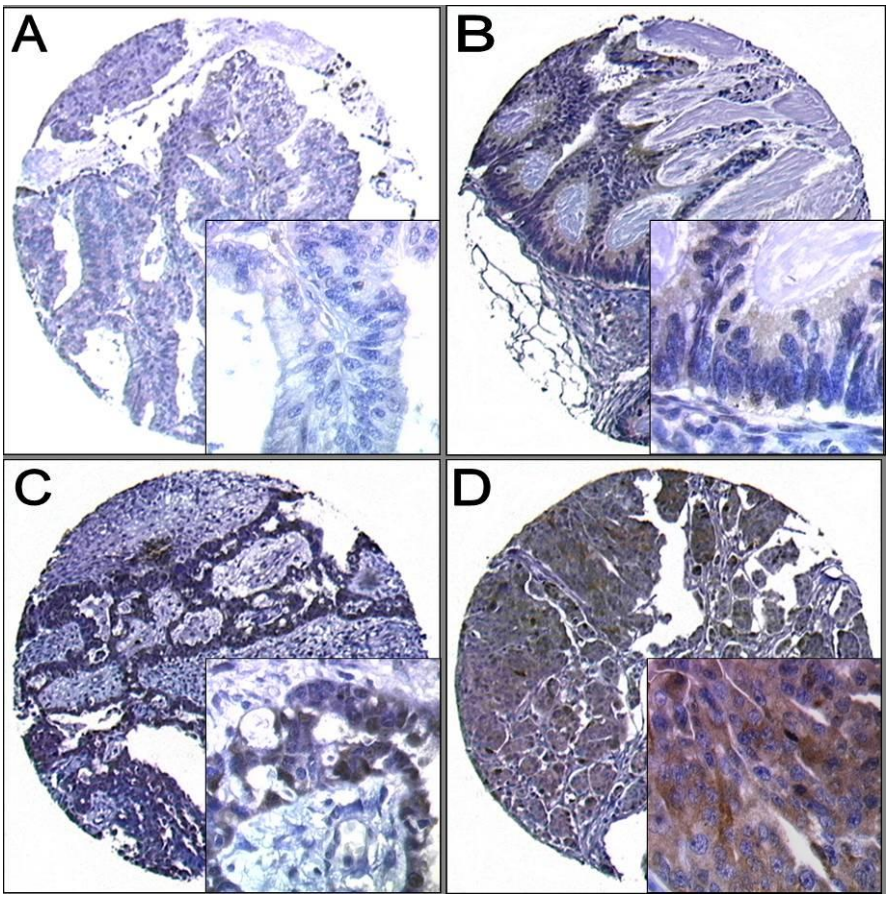


Figure 4.3 Photomicrographs of ovarian TMA cores immunohistochemically stained for DR5. The level of expression ranged from none (A), weak (B), moderate (C) through strong (D). Expression is mainly cytoplasmic, although occasional nuclear staining is seen. Magnification: x100, inset x400.

Table 4.2 Intensity of cytoplasmic staining with DR4 and DR5.

Intensity of Staining	DR4		DR5	
	Number	Percentage	Number	Percentage
No staining	56	18.7	106	35.5
Mild	91	30.4	72	24.0
Moderate	101	37.2	100	33.3
Strong	41	13.7	22	7.4

Table 4.3 Proportion of viable tumour cells staining with DR4 and DR5.

Proportion of tumour cells staining	DR4		DR5	
	Number	Percentage	Number	Percentage
No cells	56	18.7	106	35.5
1 - 25%	13	4.3	25	8.3
26 - 50%	39	13.0	57	19.0
51 - 75%	30	10.0	29	9.7
76 - 100%	161	54.0	83	27.7

4.2.3 Comparison of DR4 and DR5 expression clinicopathological characteristics including survival

In a univariate analysis, using the χ^2 test, no significant relationship between DR4 expression and the standard clinical and pathological variables was apparent. This included five year survival and overall patient survival. There was a higher proportion of endometrioid and mucinous subtypes with DR4 positive tumours, although this was not statistically significant.

There was a trend towards improved prognosis with positive DR4 expression, with 27% vs. 18%, five year survival. However, this also failed to reach statistical significance (p=0.107), which may be due to the relatively small proportion of tumours with negative DR4 status (Table 4.4).

Further analysis of the prognostic significance of DR4 status is provided by Kaplan-Meier survival curves and log rank testing (Figure 4.4). There is a suggestion that loss of DR4 expression leads to a poorer prognosis, with mean and median survival times of 39 and 16 months respectively. This prognosis compares unfavourably with tumours which express DR4, as these have mean and median survival times of 64 and 23 months respectively. This difference between the two groups failed to reach statistical significance (p=0.153), and is possibly due to the small group of patients with no DR4 expression, leading to wide confidence intervals (Table 4.6).

Table 4.4 DR4 expression in all cases included in final analysis including univariate analysis by χ^2 of DR4 status and clinicopathological criteria.

Variable	Analysed DR4 cases n= 299	Positive DR4 expression	Negative DR4 expression	$\chi 2$ (p value)
Age (years)	n=298	n=193	n=56	4.65 (0.098)
<30	1 (0.3)	0	1 (1.8)	
30-59	118 (39.6)	98 (40.5)	20 (35.7)	
≥60	179 (60.1)	144 (59.5)	35 (62.5)	
FIGO Stage	n=292	n=237	n=55	1.93 (0.588)
I	74 (25.3)	64 (27.0)	10 (18.2)	
II	32 (11.0)	26 (11.0)	6 (10.9)	
III	153 (54.4)	121 (51.1)	32 (58.2)	
IV	33 (11.3)	26 (10.9)	7 (12.7)	
Optimal Debulking	n= 288	n=233	n=55	1.04 (0.307)
Yes	117 (40.6)	98 (42.1)	19 (34.5)	
No	135 (59.4)	135 (57.9)	36 (65.5)	
Tumour Grade	n=298	n=242	n=56	4.20 (0.122)
1	34 (11.4)	32 (13.2)	2 (3.6)	
2	67 (22.5)	53 (21.9)	14 (25.0)	
3	197 (66.1)	157 (64.9)	40 (71.4)	
Histological Type	n=299	n=243	n=56	9.25 (0.099)
Serous	160 (53.5)	127 (52.3)	33 (58.9)	
Endometrioid	39 (13.0)	35 (14.4)	4 (7.1)	
Mucinous	30 (10.0)	29 (11.9)	1 (1.8)	
Undifferentiated	14 (15.7)	35 (14.4)	12 (21.4)	
Clear Cell	20 (6.7)	15 (6.2)	5 (8.9)	
Other	3 (1.0)	2 (0.8)	1 (1.8)	
Chemotherapy	n=294	n=238	n=56	2.09 (0.554)
Platinum	150 (51.0)	125 (52.5)	16 (28.6)	
Non-platinum	69 (23.5)	54 (22.7)	25 (44.6)	
None	75 (25.5)	59 (24.8)	15 (26.8)	
Randomised treatment	53/299 (17.7)	40/171 (17.6)	10/56 (17.8)	0.26 (0.87)
Five Year Survival	n=294	n=238	n=56	1.96 (0.107)
Yes	74 (25.2)	64 (26.9)	10 (17.9)	
No	226 (74.8)	174 (73.1)	46 (82.1)	
Alive at time of censoring	n=296	n=240	n=56	0.33 (0.563)
Yes	50 (16.9)	42 (17.5)	8 (14.3)	
No	246 (83.1)	198 (82.5)	48 (85.7)	

DR5 expression did demonstrate some associations with a number of clinicopathological features. There did appear to be an increased likelihood of optimal debulking surgery in DR5 negative tumours (49% vs. 37%, p=0.047). There was also a propensity for clear cell tumours to have a DR5 negative status. In addition, more patients with DR5 positive tumours received platinum-based chemotherapy (55% vs. 43%, p=0.039). In spite of these differences, however, this failed to translate into an alteration in prognosis, with DR5 positive patients having a 27% five year and a 19% overall survival respectively, compared with 23% and 15% in DR5 negative patients (p=0.487 and p=0.415) (Table 4.5). Unsurprisingly, using a Kaplan-Meier survival curve and log rank testing confirmed that there was no prognostic value to DR5 status (p=0.538) (Figure 4.4 and Table 4.6).

Table 4.5 DR5 expression in all cases included in final analysis including univariate analysis by χ^2 of DR5 status and clinicopathological criteria.

Variable	Analysed DR5 cases	Positive DR5 expression	Negative DR5 expression	χ2 (p value)
Age (years)	n=299	n=193	N=106	2.30 (0.317)
<30	1 (0.3)	0	1 (0.9)	
30-59	117 (39.1)	73 (37.8)	44 (41.5)	
≥60	181 (60.5)	120 (62.2)	61 (57.6)	
FIGO Stage	n=292	n=189	n=103	1.58 (0.663)
I	74 (25.3)	44 (23.3)	30 (29.1)	
II	31 (10.6)	22 (11.6)	9 (8.7)	
III	153 (52.4)	100 (52.9)	53 (51.5)	
IV	34 (11.6)	23 (12.2)	11 (10.7)	
Optimal Debulking	n=289	n=186	n=103	**3.94 (0.047)**
Yes	118 (40.8)	68 (36.6)	50 (48.5)	
No	171 (59.2)	118 (63.4)	53 (51.5)	
Tumour Grade	n=299	n=194	n=105	3.64 (0.161)
1	33 (11.0)	18 (9.3)	15 (14.3)	
2	67 (22.4)	49 (25.3)	18 (17.1)	
3	199 (66.6)	127 (65.5)	72 (86.6)	
Histological Type	n=300	n=194	n=106	**13.32 (0.021)**
Serous	160 (53.3)	108 (55.7)	52 (49.1)	
Endometrioid	40 (13.3)	27 (13.9)	13 (12.3)	
Mucinous	28 (9.3)	20 (10.3)	8 (7.3)	
Undifferentiated	46 (15.3)	30 (15.5)	16 (15.1)	
Clear Cell	23 (7.7)	9 (4.6)	14 (13.2)	
Other	3 (1.0)	0	3 (2.8)	
Chemotherapy	n=294	n=190	n=104	**8.34 (0.039)**
Platinum	150 (51.0)	105 (55.3)	45 (43.3)	
Non-platinum	70 (23.8)	36 (19.0)	34 (32.7)	
Other	74 (25.2)	49 (25.8)	25 (24.0)	
Randomised treatment	52/300 (17.3)	34/194 (17.5)	18/106 (17.0)	0.26 (0.87)
Five Year Survival	n=295	n=190	n=105	0.720 (0.487)
Yes	76 (25.8)	52 (27.4)	24 (22.9)	
No	219 (74.2)	138 (72.6)	81 (77.1)	
Alive at time of censoring	n=297	n=191	n=106	0.665 (0.415)
Yes	52 (17.5)	36 (18.8)	16 (15.1)	
No	245 (82.5)	155 (81.2)	90 (84.9)	

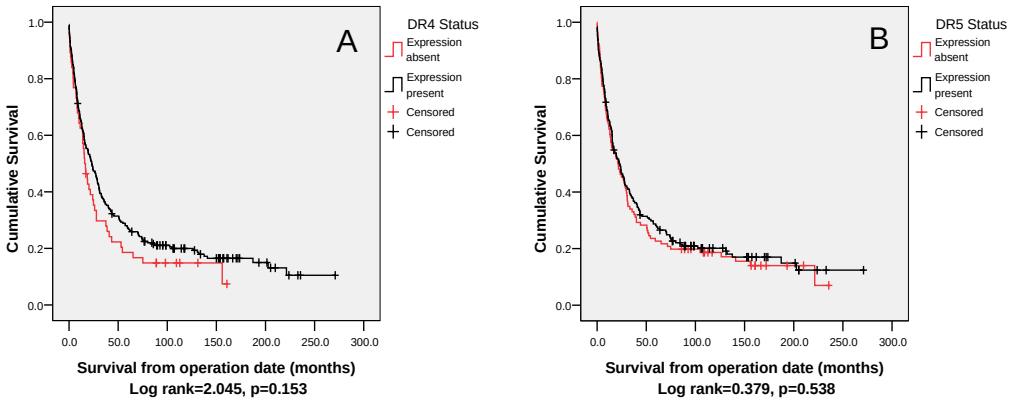


Figure 4.4 Kaplan-Meier plot for disease-specific survival. A: Absent compared with present DR4 expression in tumours (n=299). No association between DR4 status and survival shown. Log rank= 2.045, p=0.153. B: Absent compared with present DR5 expression in tumours (n=300). No association between DR5 status and survival shown. Log rank= 0.379, p=0.538.

Table 4.6 Mean and median survival time in relation to DR4 and DR5 expression. Log Rank test for univariate survival analysis.

	Mean(a)				Median			
	Estimate (months)	Std. Error	95% Confidence Interval		Estimate (months)	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
DR4								
DR4 lost	39.2	7.1	25.3	53.1	15.9	2.5	11.0	20.7
DR4 present	63.8	6.0	52.0	75.5	23.1	3.3	16.9	29.5
Overall	60.6	5.3	50.2	71.1	21.6	2.6	16.4	26.8
DR5								
DR5 lost	55.3	7.6	40.5	70.2	21.1	5.1	11.2	31.1
DR5 present	65.0	7.0	51.3	78.7	23.1	3.7	15.8	30.4
Overall	62.3	5.4	51.6	73.0	22.1	2.9	16.4	27.8

(a) Estimation is limited to the largest survival time if it is censored.

Log Rank (Mantel-Cox)	Chi-square	d.f.	p value
DR4	2.0451	1	0.153
DR5	0.379	1	0.538

Since DR4 and DR5 have similar functions (they both act as agonist receptors for TRAIL) an analysis of the combined receptor status was performed (Table 4.7 and Figure 4.5). As illustrated in the Kaplan-Meier curves there was no clear relationship when assessing receptor status in combination. The absence or presence of both receptors resulted in similar median survival times of 22 and 24 months respectively. Prognosis did appear to

be worse in those patients expressing only a single receptor, but overall the relationship failed to reach significance (p=0.078).

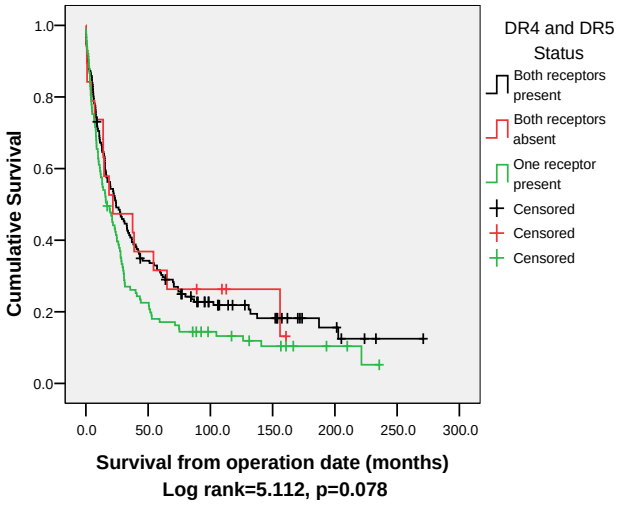


Figure 4.5 Kaplan-Meier plot for disease-specific survival according to combined DR4 and DR5 receptor status (n=299)

Table 4.7 Mean and median survival time in relation to combined DR4 and DR5 expression. Log Rank test for univariate survival analysis.

	Mean(a)				Median			
	Estimate (months)	Std. Error	95% Confidence Interval		Estimate (months)	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Both DR4 and DR5 absent	57.0	14.4	28.7	85.3	21.6	16.6	0.0	54.2
One receptor lost	44.8	6.6	31.9	57.6	16.9	3.5	10.0	23.9
Both DR4 and DR5 present	68.8	7.8	53.4	84.1	24.2	4.2	16.0	32.6
Overall	60.2	5.3	49.7	70.7	21.7	2.9	16.1	27.3

(a) Estimation is limited to the largest survival time if it is censored.

	Chi-square	d.f.	p value
Log Rank (Mantel-Cox)	5.112	2	0.078

4.3 Discussion

This chapter focused on the theoretical possibility that changes in the expression of TRAIL receptors DR4 and DR5 may occur in ovarian cancer cells, and that these differences might produce differences in tumour behaviour. The loss of death receptor expression, thereby enabling evasion of immune attack, would be in keeping with the theory of immunoediting [206].

The expression of DR4 and DR5 within the tumour cores was mainly seen in the cytoplasm with occasional nuclear and membrane staining. These patterns are similar to those of a previous study of ovarian cancers [286], suggesting that the staining distribution was representative. Previous work has demonstrated that loss of DR4 and DR5 expression in ovarian cancer is associated with increasing tumour grade and stage [286]. This was not seen in the current cohort, although other workers also failed to demonstrate this association [403].

Since TRAIL is an important pathway in apoptosis, intuitively a role in tumour behaviour seems likely. Evidence exists for a role in tumourogenesis and immune response to tumours. In a mouse model, it was shown that TRAIL is important in the surveillance of tumour metastases by NK cells [404].

There are a number of studies suggesting that dysregulation of the TRAIL pathway, through alteration in the expression of receptors, leads to effects on patient survival [286, 400-402]. The results are varied and may be dependent on the type of cancer. Renal cell cancers were seen to have poorer prognosis with high DR5 and low TRAIL-R4 (a decoy receptor) expression, which seems counterintuitive [401]. However, this has been reproduced in breast [402] and lung cancers [405]. The opposite finding of an improved prognosis with high expression of DR4 and DR5 has been seen in colorectal and glioblastomas respectively [406, 407]. It has been postulated that the association between high death receptor expression and poor prognosis is due to a proliferative response to TRAIL from DR4 and DR5 receptors in cells with TRAIL resistance. This mechanism has

also been suggested to promote invasion and metastasis in some tumour types [401]. In ovarian cancer there has been an association between high DR5 expression and poor survival; however, as was found with the current cohort no association was seen with DR4 [286].

Most chemotherapy and radiotherapy produce cellular damage which triggers apoptosis through the tumour suppressor p53 pathway. Due to the survival advantage conferred, continued treatment eventually results in selection of tumour cells with inactivated p53 pathways. This renders the tumours resistant to treatment. Activation of the TRAIL death receptors represents an extrinsic, p53-independent, pathway for apoptosis. This mechanism may potentially resensitise tumours to chemotherapy through the use of TRAIL in combination with conventional treatment [16]. There is also evidence to suggest that TRAIL-related apoptosis may be highly selective for tumour cells, since low levels of death receptors are seen in normal cells, and there seems to be a limited effect on normal cells from exposure to TRAIL [399].

A number of TRAIL-related complexes have been developed including recombinant TRAIL and agonist monoclonal antibodies to DR4 and DR5. These monoclonal antibodies have the theoretical advantage of only stimulating the death receptors and not the decoys [16]. These therapies are currently at the phase I stage in solid tumours in conjunction with carboplatin and paclitaxel chemotherapy [408]. These DNA-damaging agents may act synergistically with TRAIL by upregulating the death receptors [409]. There is some evidence that mutations and defects in the receptors (DR4 and DR5) and downstream components may lead to resistance to TRAIL-mediated therapy [410]. TRAIL targeting therapeutics are highly specific for TRAIL death receptors. Therefore, the level of receptor expression should be examined as a prerequisite for patients' inclusion in clinical studies of such agents. In the current cohort of ovarian cancers contained within the TMA it seems that both agonist receptors are expressed in the majority of cases (72% DR4, 65% DR5). Hence, most patients would be eligible for such studies. However, the influence of

prior exposure of tumours to chemotherapy to the levels of death receptor expression is not yet known.

This current work does not appear to demonstrate any prognostic significance from either DR4 or DR5, but since both receptors can respond independently to TRAIL, a combined analysis was performed. This failed to show any effect from either complete loss, or presence, of both receptors. There are a number of reasons why no prognostic significance was seen in the ovarian TMA compared with previous studies [286, 400-402]. Firstly, there do appear to be varying effects dependent on tumour type. Therefore, ovarian cancer may not have a prominent TRAIL-dependent component of apoptosis relative to other tumour sites. In addition to alterations in the levels of expression of the receptor, there may also be changes in the downstream pathway, such as the caspase cascade, which would alter the responsiveness to TRAIL. This was not assessed in the current work, but could be a focus of future work. DR4 and DR5 are both agonist receptors whose effects are balanced by decoy receptors (TRAIL R3, R4 and OPG); alterations in their expression may contribute to the response of the cell to TRAIL. Previous work has suggested that high levels of decoy receptors may produce resistance to TRAIL-mediated apoptosis [411], although other authors suggest that the ratio of death receptors and decoys does not correlate with TRAIL resistance [412]. Finally, as with all immunohistochemical studies there may also be variations in antibody and antigen preparation and inter-observer variations between the current work and previous studies.

4.4 Conclusion

Of the patients included in the ovarian TMA neither DR4 nor DR5 expression was prognostic. This may not reflect the cells' responsiveness to TRAIL, since analysis of decoy receptors was not included and could be the focus of future work. The death receptors are expressed in the majority of tumours, making ovarian cancer an ideal candidate for clinical studies of TRAIL and TRAIL monoclonal antibodies.

5 Vascular Endothelial Growth Factor

5.1 Introduction

Angiogenesis has been established as a vital component among the mechanisms involved in tumour growth and metastasis [261, 262]. The angiogenic potential of tumours can be assessed by measuring microvessel density (MVD). Earlier studies illustrated the importance of angiogenesis in tumour development, with MVD directly correlating with a poor prognosis in ovarian [413] and other tumours [414, 415]. The mounting evidence of a pivotal role for neovascularisation in carcinogenesis has lead to the development of drugs targeting these mechanisms and hence the degree of vascular activity within a specific tumour may be vital when planning treatment.

Angiogenesis is the process of new vessel formation, which can be either physiological or pathological. For the purposes of clarification angiogenesis refers to the formation of small vessels, as opposed to arteriogenesis and lymphangiogenesis which are the development of arteries and lymphatics respectively. There are some common agents, however, which are involved in all these processes, an example being VEGF [416].

Physiological angiogenesis occurs in the foetus and developing adult, as well as during wound healing, leading to a well ordered hierarchy of vessels from arterioles to capillaries and finally post-capillary venules and veins. In contrast, pathological angiogenesis which occurs in tumours leads to a disordered, chaotic vascular pattern with irregular branches and arterio-venous shunts [417]. These vessels are functionally heterogeneous with variable fenestrations which are often highly permeable to plasma and plasma proteins. This is of significance when considering the potential for metastasis in tumour-associated vessels.

The VEGF group of proteins are multifunctional cytokines that stimulate angiogenesis and increase microvascular permeability through binding to specific receptors expressed on vascular endothelial cells [418, 419]. Although VEGF is produced by a number of tumours and hypoxic tissues [420] its receptors are expressed primarily by endothelial cells. VEGF

has been shown to have a crucial role in neovascular formation in tumours, providing nourishment for the highly metabolic tumour cells as well as providing access to the host vasculature [317].

The VEGF family of proteins is thought to represent the most important of all angiogenic factors. Of these, VEGF-A is the most extensively researched and seemingly the most important. VEGF-B is linked with coronary artery development, and VEGF-C and -D are primarily associated with lymphangiogenesis [421, 422]. There are numerous other factors and cytokines which have angiogenic activity, for example, fibroblast growth factors 1 and 2, platelet derived growth factor (PDGF), tumour necrosis factor alpha (TNFα) and angiopoietins 1 and 2. These factors are of secondary importance when considering angiogenesis, and indeed many exert their effects through modification of VEGF expression.

VEGF-A is a glycoprotein which is encoded for on a single gene. Although alternative splicing leads to a number of isoforms, these are thought to have similar but not identical activities. VEGF-A is produced by most tissues, often during physiological angiogenesis; however, the pathway of recent interest is that of tumour-associated VEGF production. Expression of VEGF-A is influenced by a number of cytokines, as well as hypoxic inducible factor 1α (HIF1α) which is expressed in hypoxic environments [423]. Hypoxia promoting VEGF production, via HIF1α, is thought to be crucial in solid tumour angiogenesis since such tumours have a high metabolic turnover and rapidly expanding mass and frequently become hypoxic because of their increasing distance from the nearest blood supply. Various oncogenes (src, ras) and tumour suppressor genes (p53, p73 and von Hippel-Laudau (vHL)) also influence VEGF-A expression. For example, vHL gene product forms part of the protein complex which targets HIF1α for proteolysis. Hence, if vHL is inactivated during tumour development this leads to an accumulation of HIF1α which subsequently leads to upregulation of VEGF-A even in normoxic conditions [424].

The VEGF family members bind to three high affinity tyrosine kinase receptors (VEGFR1-3). VEGFR1 and 2 are expressed on normal vascular endothelium and are regulated mainly by interaction with VEGF-A [425]. VEGF-A is thought to exert most if its actions via VEGFR2. Upon binding of VEGF-A, receptor dimerisation and autophosphorylation occurs with subsequent activation of downstream targets such as nitric oxide synthase leading to increased vascular permeability [416]. This increase in vascular permeability causes extravasation of fibrinogen and other clotting proteins to produce a fibrin gel. This increase in vascular permeability is also thought to lead to ascites formation in ovarian cancer [426]. Following the formation of the pro-angiogenic gel, VEGF-A stimulates endothelial cell division, migration and prolonged cell survival. This leads to new vessel buds forming and it is thought that VEGF also provides guidance for the direction of endothelial cell migration and hence the direction of subsequent new vessel growth.

Studies have suggested a specific role for VEGF in various phases of ovarian carcinogenesis, with effects on tumour growth and neovascularisation seen in animal models and in humans [427, 428]. Higher levels of VEGF are demonstrated in ovarian carcinomas when compared to normal ovaries [429, 430]. Recent interest has focused on the use of anti-angiogenic drugs in an attempt to inhibit the pro-tumour effects of VEGF and other such cytokines. These studies have demonstrated some anti-tumour effects but have shown significant side effects. The future role of such therapies is yet to be established [431-433].

Previous research has produced inconsistent evidence regarding the importance of VEGF in ovarian cancer and its relation to prognosis. These studies often suffered from low patient numbers and the use of subset analysis[434-436]. The aim of our work was two fold. Firstly, to determine the distribution of VEGF expression and its relation to prognosis, and secondly, to elucidate the prognostic role of VEGF in patients with ovarian cancer. This might enable the development of more individualised adjuvant treatments using emergent novel therapies to inhibit tumour angiogenesis.

5.2 Results

5.2.1 Clinicopathological characteristics

Of the 395 patients identified with ovarian cancer, 339 had archived tissue available, and these tumours were included in the tissue array. Nineteen of the 339 tumour cores had no tissue available for analysis due to loss during immunohistochemical processing, which left 94% of cases available for analysis. This core loss rate compares favourably with previous TMA studies. This subgroup of 320 patients was similar to that of the overall group, which as previously discussed is representative of a typical ovarian cancer population (Table 5.1). Of particular importance was the comparability of five year survival, which was 24% in the whole series and 27% in those analysed for VEGF expression.

5.2.2 VEGF staining

The intensity of the staining was estimated on a 4-tiered scale; encoded as 0 (absent), 1 (weak), 2 (moderate), and 3 (strong). When staining was positive, it was primarily of cytoplasmic location and its pattern was uniform among the cancer cells within each core. Only 22 tumours (6.9%) were strongly positive, whilst the remaining tumours (298/ 320) exhibited either weak (42.2%) or moderate (39.4%) staining. Thirty seven tumour cores (11.6%) failed to show any noteworthy staining. A summary of the results is shown in Table 5.2, with photomicrographs of each category in Figure 5.1. For survival analysis purposes, cases were categorised as either “high” expressers (represented by the strongly-stained group) or “low” expressers (comprised of the negative, weak and moderate groups).

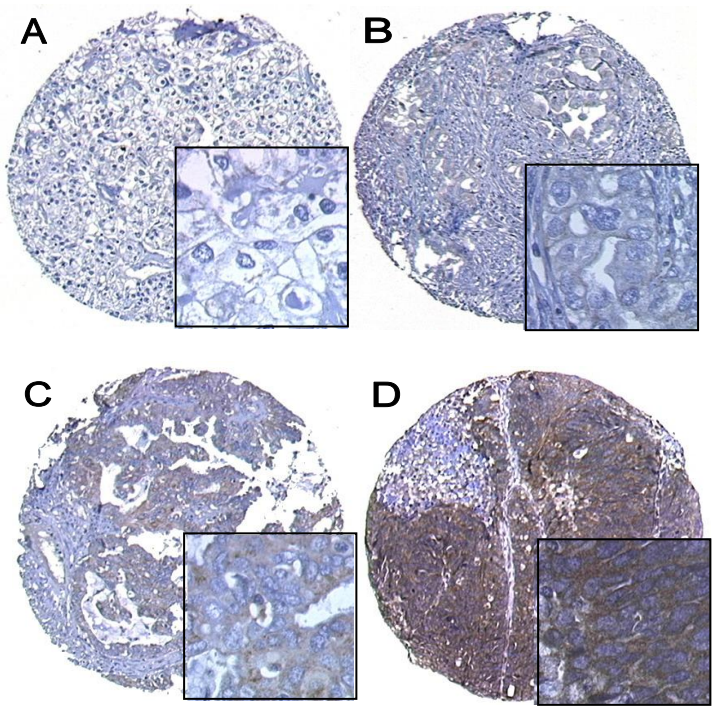


Figure 5.1 Photomicrographs of ovarian TMA cores immunohistochemically stained for VEGF. The level of expression ranged from none (A), weak (B), moderate (C) through strong (D). Magnification: x100, inset x400.

Table 5.1 Clinicopathological characteristics of whole series and those analysed for VEGF expression.

Variable	Whole series n=395 (%)	VEGF Analysed Cases n=320 (%)
Age (years)	n=394	n=319
<30	1 (0.3)	1 (0.3)
30-59	167 (42.4)	126 (39.7)
≥60	226 (57.4)	192 (60.2)
FIGO Stage	n=375	n=312
I	99 (22.6)	84 (26.9)
II	46 (12.3)	32 (10.3)
III	188 (50.1)	162 (51.9)
IV	42 (11.2)	34 (10.9)
Optimal Debulking	n=376	n=308
Yes	157 (41.8)	132 (42.9)
No	219 (58.2)	176 (57.1)
Tumour Grade	n=376	n=302
1	50 (13.3)	36 (11.8)
2	93 (24.7)	69 (22.7)
3	233 (62)	199 (65.5)
Histological Type	n=395	n=320
Serous	203 (51.4)	168 (52.5)
Endometrioid	46 (11.7)	42 (13.1)
Mucinous	50 (12.7)	33 (10.3)
Undifferentiated	65 (16.5)	49 (15.3)
Clear Cell	26 (6.6)	25 (7.8)
Other	5 (1.3)	3 (1.0)
Chemotherapy	n=388	n=314
Platinum	196 (50.5)	156 (49.7)
Non-platinum	80 (20.6)	75 (23.9)
None	112 (28.9)	83 (26.4)
Randomised treatment	69/395 (17.5)	54/320 (16.8)
Five Year Survival	n=385	n=315
Yes	91 (23.6)	87 (27.6)
No	294 (76.4)	228 (72.4)
Alive at time of data censoring	n=388	n=317
Yes	60 (15.5)	58 (18.3)
No	328 (84.5)	259 (81.7)

Table 5.2 Intensity of cytoplasmic staining with VEGF.

Intensity of Staining	Number	Percentage
No staining	37	11.6
Mild	135	42.2
Moderate	126	39.4
Strong	22	6.9

5.2.3 Comparison of VEGF expression and patient tumour characteristics including survival

Using Chi-squared test, VEGF expression did not correlate with the age of the patient, tumour grade, stage, or histological type, nor was it associated with the presence or absence of residual disease or with the administration of adjuvant chemotherapy (Table 5.3).

Survival analysis revealed that patients who had tumours with low levels of VEGF had a median survival time of 24 months while that of high VEGF expression was 14 months (Table 5.4). The difference in mean survival time was 40 months (67 and 27 months for low VEGF and high VEGF groups, respectively). This difference in survival was shown to be statistically significant with the Log rank test (p value= 0.04) (Table 5.4). A Kaplan-Meier survival plot is shown in Figure 5.2.

Table 5.3 VEGF expression in all cases included in the final analysis including univariate analysis by $\chi 2$ of VEGF status and clinicopathological criteria.

Variable	Analysed Cases n=320 (%)	High VEGF expression (%)	Low VEGF expression (%)	$\chi 2$ (p value)
Age (years)	n=319	n=22	n=297	0.24 (0.886)
<30	1 (0.3)	0	1 (0.3)	
30-59	126 (39.7)	8 (36.4)	118 (39.8)	
≥60	192 (60.2)	14 (63.6)	178 (59.9)	
FIGO Stage	n=312	n=20	n=292	0.76 (0.860)
I	84 (26.9)	6 (30)	78 (26.7)	
II	32 (10.3)	3 (15)	29 (9.9)	
III	162 (51.9)	9 (45)	153 (52.4)	
IV	34 (10.9)	2 (10)	32 (11.0)	
Optimal Debulking	n=308	n=18	n=290	0.40 (0.528)
Yes	132 (42.9)	9 (50)	123 (42.4)	
No	176 (57.1)	9 (50)	167 (57.6)	
Tumour Grade	n=302	n=22	n=282	5.77 (0.056)
1	36 (11.8)	6 (27.3)	30 (10.6)	
2	69 (22.7)	3 (13.6)	66 (23.4)	
3	199 (65.5)	13 (59.1)	186 (66.0)	
Histological Type	n=320	n=22	n=298	9.20 (0.162)
Serous	168 (52.5)	10 (45.5)	158 (53.0)	
Endometrioid	42 (13.1)	7 (31.8)	35 (11.7)	
Mucinous	33 (10.3)	2 (9.1)	31 (10.4)	
Undifferentiated	49 (15.3)	2 (9.1)	47 (15.8)	
Clear Cell	25 (7.8)	1 (4.5)	24 (8.0)	
Other	3 (1.0)	0	3 (1.0)	
Chemotherapy	n=314	n=21	n=293	2.07 (0.559)
Platinum	156 (49.7)	8 (38.1)	148 (50.5)	
Non-platinum	75 (23.9)	5 (23.8)	70 (23.9)	
None	83 (26.4)	8 (38.1)	75 (25.6)	
Randomised treatment	54/320 (16.8)	3/21 (14.3)	49/293 (16.7)	0.26 (0.873)
Five Year Survival	n=315	n=22	n=293	**4.06 (0.031)**
Yes	87 (27.6)	2 (9.1)	85 (29.0)	
No	228 (72.4)	20 (90.9)	208 (71.0)	
Alive at time of data censoring	n=317	n=22	n=295	2.99 (0.063)
Yes	58 (18.3)	1 (4.5)	57 (19.3)	
No	259 (81.7)	21 (95.5)	238 (80.7)	

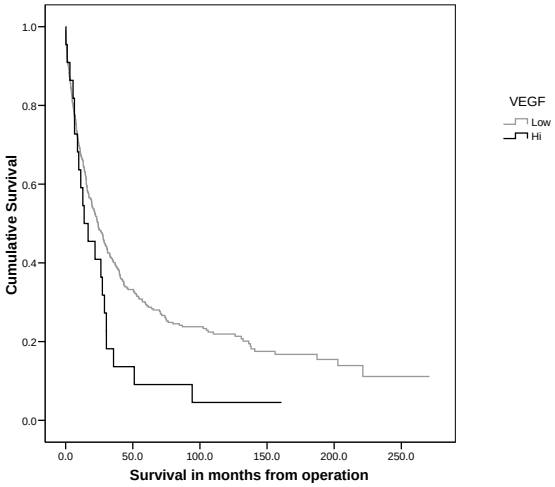


Figure 5.2 Kaplan-Meier plot for disease-specific survival, High compared with low VEGF expression tumours (n=319).

Table 5.4 Mean and median survival time in relation to VEGF expression. Log Rank test for univariate survival analysis.

	Mean[(a)]				Median			
	Estimate (months)	Std. Error	95% Confidence Interval		Estimate (months)	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Low	67.4	5.6	56.3	78.3	24.1	2.7	18.9	29.4
High	27.4	7.6	12.5	42.2	13.8	6.2	1.5	26.0
Overall	64.8	5.3	54.4	75.3	23.5	2.4	18.7	28.3

(a) Estimation is limited to the largest survival time if it is censored

	Chi square	d.f.	p value
Log Rank (Mantel-Cox)	4.2	1	**0.04**

5.2.4 Multivariate analysis

VEGF did not correlate with any of the established clinicopathological prognostic parameters, for example, age, stage or grade. This limited the possibility of any "confounder" effect on survival predictions. However, to ensure the findings were truly independent, a multi-variant Cox proportional hazards model was constructed incorporating age, residual disease, grade and the receipt of adjuvant therapy. A hazard ratio for high VEGF expression was obtained (HR = 1.75, p value= 0.02) (Table 5.5).

Table 5.5 Multivariate analysis using Cox regression model demonstrating that high VEGF expression predicts a poor prognosis independently of other accepted independent prognostic factors.

Variable	Hazard Ratios (95% Confidence intervals)	Significance (p)
Age at diagnosis (years)	1.025 (1.013-1.036)	**<0.001**
FIGO Stage		**<0.001**
I	1	
II	1.779 (1.041-3.040)	
III	3.081 (1.997-4.753)	
IV	4.233 (2.431-7.371)	
Optimal Debulking		**<0.001**
Yes	1	
No	2.173	
Patient received Chemotherapy		**<0.001**
No	1	
Yes	0.462 (0.320-0.666)	
VEGF expression		**0.02**
Low	1	
High	1.752 (1.062-2.899)	

5.3 Discussion

In spite of the assumed importance of VEGF in the progression of cancer, relatively few studies of the expression of this protein in ovarian cancer have been linked to comprehensive clinical data, and in particular data on survival. In addition, the low numbers in previous studies may have contributed to inconsistency in findings. Using TMA technology allowed 320 primary ovarian cancers to be immunohistochemically assessed under identical experimental conditions, and this coupled with the robust clinicopathological database allowed for a more confident assessment of VEGF expression and its role as a prognostic marker.

The expression of VEGF in patients exhibited a similar pattern to that of Goodheart *et al* [434] with a strong expression seen in 6.9% of the tumours. Lesser expression ranged from moderate (39.4%) through weak (42.2%) to no expression in 11.6% of the patients (Table 5.2). Other groups reported varying levels of VEGF. They used different antibodies to stain the tissue, different scoring systems and different cut-off points. Although this highlights the need for inter-laboratory standardisation of immunohistochemical techniques, it does not detract from the results as the differences observed in each study represent comparisons among different tumours treated uniformly following a standard protocol.

Our results indicate that patients with tumours which express high levels of VEGF have worse survival rates compared with those with medium, low or no VEGF expression. A median survival advantage of almost ten months is seen among the latter group when compared to the former. This was confirmed statistically using the log rank test (p= 0.04) and shown graphically using Kaplan-Meier curves (Table 5.4 and Figure 5.2). This result is further substantiated when a multivariate Cox regression model was constructed in which factors such as stage, microscopic residual disease, tumour grade, and the use of adjuvant therapy were accounted for. VEGF maintained statistical significance with regard to patient survival (p=0.02) (Table 5.5). The HR indicates that patients with high VEGF have an approximately 75% higher risk of dying than their low VEGF counterparts.

The role of angiogenesis in ovarian carcinoma development remains unclear, with contradictory studies existing regarding the influence of MVD in ovarian cancer prognosis [413, 437, 438]. Immunohistochemical assessment of VEGF within a tumour offers further information regarding the potential for angiogenic activity and its effects on tumour behaviour and subsequent prognosis for the patient. The literature has been equally controversial concerning VEGF expression within ovarian tumours; some authors demonstrate no independent relationship with prognosis[439, 440] whereas others have shown a significant independent prognostic influence. Patients with early stage disease (FIGO I and II) had a poorer prognosis with increased VEGF expression within the tumour

[441]. Shen *et al* demonstrated elevated expression of VEGF (using mRNA) to be predictive of a poor prognosis [430]. Interestingly, there was no correlation with MVD which is in contradiction to previous work [439]. Raspollini *et al* illustrated that VEGF and MVD were both independent predictors of survival in advanced disease (FIGO III) and this also correlated with the likelihood of response to chemotherapy [436]. Similar findings were also seen when including early stage disease [435]. Since we have such a large number of cases in our study we can clearly demonstrate that the prognostic effects of VEGF are seen at all disease stages and that these effects are independent of confounding variables such as stage, grade and residual disease.

The results illustrate no clear association between VEGF and any of the clinicopathological variables, including stage and grade of tumour. This is in agreement with some studies, most of which had a typical distribution of disease according to stage [413, 435, 439, 442, 443]. Some studies have suggested that stage and grade are associated with VEGF, although these studies tended to have an unusually high proportion of early stage disease (up to 58%) [430, 444, 445].

Studies investigating serum VEGF levels in ovarian cancer offer further evidence of a role for VEGF, with elevated levels being independently predictive of a poor prognosis [446, 447]. Rudlowski *et al* demonstrated similar elevations in VEGF level in ascites from malignant tumours compared with borderline and benign disease, which correlated with advanced stage and patient survival. Interestingly, there was no difference in serum VEGF levels between benign and malignant tumours in this study [445].

Zhang *et al* in a study of infiltrating T cells in ovarian cancer showed that the absence of intratumoral T cells was associated with higher levels of VEGF [197]. This group of patients had early recurrence rates and short survival. It is therefore thought that VEGF further affects the behaviour of ovarian tumours by reducing the number of T cells in the tumour milieu, suppressing the immune system's defences against ovarian cancer. The potential mechanism through which VEFG might inhibit migration of T cells into tumours is

not clear; especially considering the increase in vascular permeability associated with VEFG which promotes extravasation of intravascular contents.

There is early evidence that VEGF-mediated angiogenesis may be utilised as a novel pathway in the treatment of ovarian cancer as has been witnessed in colon [448], breast [449] and lung [450] carcinomas. Monk *et al* have demonstrated some clinical benefit from using a monoclonal antibody against VEGF (bevacizumab) in recurrent ovarian cancer [432]. This has also been used successfully in the palliative treatment of ascites in refractory disease [451]. The Gynecologic Oncology Group are completing a phase III trial (GOG 218), using bevacizumab in combination with standard chemotherapeutic agents, the results of which are eagerly awaited. Another strategy devised to suppress angiogenesis in ovarian cancer is the interception of VEGF with receptor decoys, such as VEGF-Trap, which has shown encouraging results in early phase trials. Studies performed on mouse cancer models have demonstrated that, combined with paclitaxel, VEGF-Trap inhibits tumour growth and prolongs survival [431]. Early results from a randomised, multi-centre, double-blind, phase 2 study suggest that VEGF-Trap may represent an important new option for patients who have received three or four prior chemotherapy regimens and have become resistant to platinum-based agents [452].

It should be noted that the subgroup of high VEGF expressing tumours accounts for less than 10% of patients and hence we are identifying a small group who appear to have a much worse prognosis. The small proportion of tumours expressing high levels of VEGF may explain why smaller studies failed to find significant prognostic associations [439, 440]. The clinical value of identifying these patients and adapting treatment will therefore have a limited effect on overall population survival; however, we may have identified a specific group of patients who are highly sensitive to anti-angiogenic drugs. VEGF status is independent of stage in chemotherapy-naïve patients suggesting that there may be a role for bevacizumab as first line treatment in addition to standard chemotherapy in selected patients. This would represent a significant new role for such agents, as most studies have looked at use in recurrent platinum-resistant disease [433, 453]. As

illustrated in this set of experiments, VEGF expression in tumours can be demonstrated using standard immunohistochemical techniques and therefore could be undertaken in most histopathology departments to aid the clinician in tailoring treatment to an individual patient. For example, the use of bevacizumab alone in those tumours expressing high levels of VEGF.

5.4 Conclusion

The expression of VEGF is an independent prognostic indicator in a large series of patients with early and late stage ovarian cancer. High VEGF expression only occurs in a small proportion of ovarian cancers but may denote a specific group in which anti-angiogenic therapy is more effective.

6 General Discussion

Ovarian cancer is an uncommon disease, however, due to the vagueness of symptoms patients often present at an advanced stage. This results in ovarian cancer being the fourth most common cause of cancer-related death in women in the UK [1]. Over the last 30 years there have been improvements in the surgical and postoperative care received by women with ovarian cancer. In addition, refinements in chemotherapeutic agents have been introduced, in particular the use of platinum-based compounds and more recently paclitaxel. Unfortunately, these changes have had little, if any, impact on prognosis with the typical five year survival rate of 30% [113]. Novel targeted therapy combined with a better prognostic index, which stratifies patients for the optimal therapy to cure their cancer, based upon its molecular background could significantly change the outcome from this disease as has been proved for breast cancer.

Established prognostic markers in ovarian cancer include age, stage of disease and extent of residual disease following surgery [5]. Patients with a curative resection that has not metastasised (stage Ia) can be considered cured and will not require adjuvant therapy but will be closely monitored for recurrent disease. All other patients will be considered for further therapy. The current work demonstrated that tumours which expressed high levels of VEGF had worse survival rates compared with those with medium, low or no VEGF expression. The literature is controversial with regard to VEGF expression and ovarian cancer prognosis. Some authors demonstrate no independent relationship with prognosis whereas other studies have shown a significant independent prognostic influence; however, the number of cases in these studies was often limited [439, 440]. These smaller studies may prove less robust when it is considered that less than 10% of tumours in the current series had high VEGF. Due to the large number of cases analysed in this thesis, we can clearly demonstrate that the prognostic effects of VEGF are seen at all stages of disease. These effects are independent of confounding variables such as stage, grade and residual disease.

The clinical value of identifying these patients and adapting treatment will therefore have a limited effect on overall population survival; however, we may have identified a specific group of patients who are highly sensitive to anti-angiogenic drugs. The prognostic influence of VEGF status is independent of stage in chemotherapy-naïve patients. Therefore, there may be a role for bevacizumab as first line treatment in addition to standard chemotherapy in selected patients. This would represent a significant new role for such agents, as most studies have looked at use in recurrent platinum-resistant disease [433, 453]. The prognostic value of high VEGF-A expression in these and future trials involving anti-VEGF therapy must be considered.

There is increasing evidence that the immune system plays a pivotal role in ovarian cancer, controlling its growth until a phenotypic variant that is resistant to immune attack arises [158, 197, 224]. Animal studies have identified IFNγ as a critical cytokine involved in recognition of stressed or transformed cells [167, 173-175]. In this thesis we provide the first evidence of immunoediting of the IFNγ pathway in ovarian cancer. Loss of IFNGR1 was demonstrated to be an independent marker of poor prognosis. This effect was seen in both early and late stage tumours, suggesting that this was an early phenomenon in tumourogenesis. Interestingly, later molecules involved in the IFNγ signalling pathway (STAT-1, p27, caspase 1) did not have such strong prognostic value. This may be because these other molecules can also be associated with other signalling pathways [294]. Tumours lacking the IFNGR1 receptor are unlikely to respond to IFNγ therapy. This may explain the lack of response to this cytokine in a recent phase III clinical trial [350]. If 30% of patients could not respond to the therapy, but had to have a reduction in the dose of platinum due to increased toxicity, then these patients would do worse and thus confound any positive benefit in the responsive group. This trial should be reassessed for response based upon expression of the IFNGR1. If this proves to be of prognostic value then there may be a role for IFNγ as first line treatment in addition to standard chemotherapy in selected patients. It follows that those patients with tumours that have low VEGF-A expression and absent IFNGR1 should avoid anti-angiogenic and IFNγ

therapies and be treated with standard chemotherapy alone or be considered for trials involving other targeted treatments [454-458].

The evidence of dysregulation of TRAIL in the development of tumours and their resistance to chemotherapy, as well as its potential therapeutic role, provides an interesting avenue of investigation. The hypothesis followed that tumours with low expression of DR4 and DR5 (TRAIL receptors) would be less prone to TRAIL-mediated attack and therefore apoptosis. This would be reflected in reduced patient survival. This current work does not appear to demonstrate any prognostic significance from either DR4 or DR5. However, there was high expression of both receptors, hence patients may benefit from TRAIL ligand or anti-TRAIL mab therapy [408].

Our studies would suggest that patients with stage II - IV disease who express high levels of VEGF-A should be stratified to first line therapy of bevacizumab plus chemotherapy. In contrast, stage II - IV patients with low levels of VEGF-A, but who still express IFNGR, should receive first line IFNγ plus chemotherapy. Only patients who have low VEGF-A and IFNGR negative expression should receive chemotherapy alone (Figure 6.1).

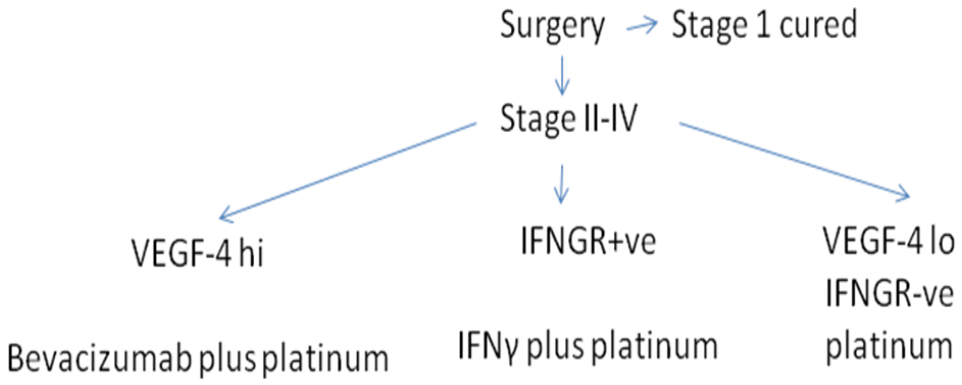


Figure 6.1 Novel treatment strategy for ovarian carcinoma reflecting the tumour's molecular profile.

The need for an extensive array of targeted therapies is highlighted by the poor prognosis seen in patients whose tumours are platinum-resistant VEGF-A low expressers and IFNGR1 negative. New therapies as they arise should be trialed and targeted to tumours expressing the appropriate molecular marker. These include PDGF receptor inhibitor

(imatinib) [456], PDGF tyrosine kinase inhibitor (gefitinib) [457], alpha folate receptor inhibitors (farletuzumab) [454], DNA repair inhibitors (PARP) [458] and P13K-Akt inhibitors [455]. All these novel treatments have pathways in which components can be identified through IHC techniques and hence a prediction of response is possible.

This thesis addresses three areas of tumour biology: immunoediting, angiogenesis and apoptosis, through the use of TMA immunohistochemical techniques. The work illustrates that ovarian cancers can be extremely heterogeneous and that variations in expression of protein markers between individual tumours can be used to predict tumour behavior. Due to the heterogeneous nature of ovarian cancer it is not surprising that a “one size fits all” approach to treatment with standard chemotherapeutic agents has met with limited success. Future ovarian cancer treatment will be individualised and based on the molecular characteristics of the tumour assessed through immunohistochemistry and genomic hybridisation [458]. This assessment will need to be performed at each relapse as potential therapeutic pathways may change. Of equal importance is the design of clinical trials of novel treatments. These will need to include predictive biomarkers of the particular pathway being targeted. This will ensure that only patients likely to benefit from a particular treatment will be exposed to the potentially toxic compound.

The markers used in this thesis have direct links to potential targeted therapies, and variations in the functionality of these pathways may exist between individual tumours. In the future targeted therapies may be selected on the basis of an IHC panel of markers which predict which pathways are still functioning and hence which therapy should be employed for each individual tumour.

7 Appendices

7.1 Appendix 1

Table 7.1 Staging of ovarian cancer by the FIGO classification [84].

Stage 0	**No evidence of primary tumour**	
Stage I	**Tumour confined to the ovaries**	
	IA	Tumour limited to one ovary, capsule intact No tumour on ovarian surface No malignant cells in ascites or peritoneal washings
	IIB	Tumour limited to both ovaries, capsules intact No tumour on ovarian surface No malignant cells in ascites or peritoneal washings
	IC	Tumour limited to one or both ovaries With any of the following:- Capsule ruptured, tumour on ovarian surface, positive malignant cells in the ascites or positive peritoneal washings
Stage II	**Tumour involves one or both ovaries with pelvic extension**	
	IIA	Extension and/ or implants in uterus and/ or tubes No malignant cells in the ascites or peritoneal washings
	IIB	Extension to other pelvic organs No malignant cells in the ascites or peritoneal washings
	IIC	IIA/B with positive malignant cells in the ascites or positive peritoneal washings
Stage III	**Tumour involves one or both ovaries with microscopically confirmed peritoneal metastasis outside the pelvis and/ or regional lymph node metastasis**	
	IIIA	Microscopic peritoneal metastasis beyond the pelvis
	IIIB	Macroscopic peritoneal metastasis beyond the pelvis 2cm or less in dimension
	IIIC	Peritoneal metastases beyond the pelvis more than 2cm in greatest dimension and/ or regional lymph node metastasis
Stage IV	**Distant metastasis beyond the peritoneal cavity (including parenchymal liver metastasis and pleural effusion with positive cytology)**	

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