The Expression of HLA Class I Molecules and Complement Regulatory Proteins in Ovarian Cancer

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For Ida, of course.

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Abstract

Over recent decades, translational ovarian cancer research has been impeded by its underappreciated molecular heterogeneity and five-year survival has remained poor. One strategy for addressing this problem is to search for molecular biomarkers that can better inform the development and targeting of novel treatments. The aim of this thesis was to construct and validate a tissue microarray of ovarian cancer cases and to survey the expression and prognostic capabilities of immunological molecular markers: specifically HLA class I and the membrane bound complement regulatory proteins CD46, CD55 and CD59. These are central to the efficacy of certain immunotherapies and while they have been shown to have prognostic power in breast and colorectal cancer, they have been investigated less in ovarian cancer.

Five copies of a tissue microarray representing 339 cases of ovarian cancer which presented to Derby City General Hospital between 1982 and 1997 were made. The array was stained for CK7, CK20, CA125, CEA, p53 and Bcl-2 following a standard immunohistochemical protocol. A linked clinical database was adapted and assessed for data consistency and subsequently used to analyse the prognostic and clinicopathological associations of the expression data. The array was then stained for HLA class I, $\beta_2$microglobulin and CD59 using commercial antibodies and for CD55 and CD46 using in-house antibodies. Retained expression of HLA class I molecules independently predicted improved prognosis. High expression of CD55 and CD59 were associated with worse prognosis, though not independently of other factors. CD55 expression was more widespread than previously appreciated.

This thesis describes the discovery of a new independent marker of prognosis which suggests that immunoediting occurs in ovarian cancer, describes the distribution of markers known to have a negative impact on immunotherapy in ovarian cancer in a large series for the first time and documents the production of a valuable resource for future studies.

Abbreviations

ACS	American Cancer Society
ADCC	Antibody Dependent Cell Cytotoxicity
AJCC	American Joint Committee on Cancer
ANO	Array Number
APC	Antigen Presenting Cell
APES	3-aminoPropyltriEthoxySilane
AUC	Area Under Curve
β2m	β2microglobulin
Bcl-2	B Cell Lymphoma 2
BCR	B cell receptor
BEP	Bleomycin Etoposide Platinum
BRCA	BReast CAncer gene
BSA	Bovine Serum Albumin
C	Complement
CA125	Cancer Antigen 125
CAP	Cyclophosphamide Adriamycin cisPlatin
CCND1	Cyclin D
CD	Cluster Differentiation
CDC	Complement Dependent Cytotoxicity
CDCC	Complement Dependent Cellular Cytotoxicity
CDX2	Caudal related homeobox transcription factor 2
CEA	Carcino Embryonic Antigen
CHORUS	CHemotherapy OR Upfront Surgery
CI	Confidence Intervals
CIN	Cervical Intraepithelial Neoplasia
Cisplatin	CIS-diamminedichloridoPLATINum (II)
CK20	Cytokeratin 20
CK7	Cytokeratin 7
COCP	Combined Oral Contraceptive Pill
COREC	Central Office for Research Ethics Committees
CR	Complete Response
CRP	C Reactive Protein
CSF	Colony Stimulating Factor
CT	Cancer Testis
CT	Computerised Tomography
CTL	Cytotoxic T Lymphocytes
DAB	3, 3'-DiAminoBenzidine
DAF	Decay Accelerating Factor
DC	Dendritic Cell
DCGH	Derby City General Hospital
dH_2O	deionised H_2O
DNA	DeoxyriboNucleic Acid

DPC4	Deleted in Pancreatic Cancer 4
DPCL	DNA Platinum Protein Complexes
DPX	Distyrene, Plasticiser and Xylene
DSS	Disease Specific Survival
EABA	Endogenous Avidin Binding Activity
EDTA	Ethylene Diamine Tetracetic Acid
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked ImmunoSorbent Assay
EPCam	EPithelial Cell adhesion molecule
ER	Oestrogen Receptor
ERCC1	Excision Repair Cross-Complementation group 1
ErbB	Erythroblastic leukemia viral oncogene homolog
ESR	European Standarad Rate
Fab	Fragment Antigen Binding
fasL	fasLigand
Fc	Fragment crystalline
FFPE	Formalin Fixed Parafin Embedded tissue
FIGO	International Federation of Gynaecology and Obstetrics
Foxp3	Forkhead bOX p3
FSH	Follicle Stimulating Hormone
G0	Cell cycle resting phase
G1	Grade 1 - well differentiated
G2	Grade 2 - moderately differentiated
G3	Grade 3 - poorly differentiated
GCDFP	Gross Cyst Disease Fluid Protein
GCIG	Gynaecologic Cancer Inter Group
GI	GastoIntestinal
GMCSF	Granulocyte Macrophage Colony Stimulating Factor
GOG	Gynaecology Oncology Group
GPI	Glycosyl Phosphatidyl Inositol
GSTpi	Glutathione S Transferase pi
GX	Grade unassesable
H&E	Haemotoxylin and Eosin
H_2O	Water
HAMA	Human Anti Mouse Antibodies
HAT	Hypoxanthine Aminopterinein Thiamine
HcG	Human chorionic Gonadotrophin
HCl	Hydro Chloric acid
HLA	Hyman Leukocyte Antigen
HMFG	Human Milk Fat Globule
HNPCC	Hereditary Non Polyposis Colorectal Cancer
HOX	HomeobOX
HR	Hazard Ratio
HRP	Horse Radish Peroxidase

HSP	Heat Shock Protein
HT	Human Tissue act
HTA	Human Tissue Authority
HUS	Haemolytic Uraemic Syndrome
I.S	Ian Spendlove
I.Sc	Ian Scott
iC3b	Inactive C3b
ICON	International Collaboration in Ovarian Neoplasia
IFN	InterFeroN
INFɣ	Inter FeroN gamma
Ig	Immunoglobulins
IHC	Immuno Histo Chemistry
IL	Interleukin
ILT	Ig Like Transcript
IP	Intra Peritoneal
IRAS	Integrated Research Application System
IS	Intensity Score
KIR	Killer cell Immunoglobulin like Receptor
KLH	Keyhole Limpet Haemocyanin
KM	Kaplan-Meier curve
LH	Lutenising Hormone
LPS	LipoPolySaccharide
LREC	Local Regional Ethics Committee
mAb	monoclonal Antibody
MAC	Membrane Attack Complex
MASP	Mannose binding lectin Associated Serine Protease
MBL	Mannose Binding Lectin
MBS	Maleimidobenzoyl-N-hydroxysulfosuccinimide
MCP	Membrane Cofactor Protein
mCRP	membrane Complement Regulatory Protein
mDC	myeloid DC
MDR	Multi Drug Resistance
MDT	Multi Disciplinary Team
MHC	Major Histocompatability Complex
MICA/B	MHC Class I polypeptide-related sequence A/B
MLH1	Mut L Homolog 1
MMP4	MatrixMetalloProteinase4
MMR	Miss Match Repair gene family
MR	Magnetic Resonance
MRC	Medical Research Council
MREC	Multi Regional Ethics Committee
MS	Mass Spectrometry
MTTB	Multi Tissue Tumour Block
MUC4	Mucin 4

NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NBF	Neutral Buffered Formalin
NCH	Nottingham City Hospital
NCI	National Cancer Institute
NCR	Natural Cytotoxicity Receptor
NER	Nucleotide Excision Repair
NHS	National Health Service
NICE	National Institute of Clinical Excellence
NIH	National Institute for Health
NK cell	Natural Killer cell
NKCC	Natural Killer Cell Cytotoxicity
NKR	NK Receptor
NKT	Natural Killer T cell
NPV	Negative Predictive Value
NSS	Normal Swine Serum
OS	Overall Survival
OVCA	OVarian CAncer
P.R	Philip Rolland
p53	protein 53
PAMP	Pathogen Associated Molecular Patterns
PCOS	PolyCystic Ovarian Syndrome
PCR	Polymerase Chain Reaction
PD	Progressive Disease
pDC	plasmacytoid DC
PFS	Progression Free Survival
PgP	P glycol Protein
PNH	Paroxysmal Nocturnal Haemoglobinuria
POP	Progesterone Only Pill
PPV	Positive Predictive Value
PR	Progesterone Receptor
PR	Partial Response
pRb	Retinoblastoma protein
PRR	Pattern Recognition Receptors
PSA	Prostate Specific Antigen
PTEN	Phosphatase and TENsin homolog
QMC	Queens Medical Centre
R&D	Research and Development
RCA	Regulators of Complement Activation
RECIST	Response Evaluation Criteria In Solid Tumours
RGF	Research Governance Framework
RMI	Risk of Malignancy Index
RNA	Ribose Nucleic Acid
RR	Relative Risk

S.D	Suha Deen
SCR	Short Consensus Repeat
SD	Stable Disease
SDAH	Southern Derbyshire Acute Hospitals
SDS-PAGE	Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis
SEER	Surveillance, Epidemiology, and End Results
SNO	Study Number
STP	Serine Threonine Proline rich region
T	Thymic
TAA	Tissue Associated Antigen
TAG72	Tumour Associated Glycoprotein 72
TAP	Transporters associated with Antigen Processing
TBS	Tris Buffered Saline
Tc	cytotoxic T cell
TCM	Central Memory T cell
TCR	T Cell Receptor
TEM	Effector Memory T cell
TGF β	Transforming Growth Factor β
Th	T helper cell
TIL	Tumour Infiltrating lymphocyte
TLR	Toll Like Receptor
TMA	Tissue Micro Array
TNF	Tumour Necrosis Factor
TNM	Tumour Nodes Metastasis
TRAIL	TNF Related Apoptosis Inducing Ligand
Treg	T regulatory cell
TRIS	TRIShydroxymethylaminomethane
TTF1	Thyroid Transcription Factor 1
TVS	Trans Vaginal Scan (Ultrasound)
UICC	Union Internationale Contre le Cancer
UKCTOCS	United Kingdom Collaborative Trial of Ovarian Cancer Screening
UKFOCS	United Kingdom Familial Ovarian Cancer Screening
UoN	University of Nottingham
USS	Ultra Sound Scanning
VEGF	Vascular Endothelial Growth Factor
VGEFR	Vascular Endothelial Growth Factor Receptor
w/v	weight per volume
WHO	World Health Organisation
WT1	Wilms Tumour 1
WTp53	Wild Type p53

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Specific contributions of the author and other workers

The body of work contained within this thesis is solely that of the author, supported as outlined below by those individuals named in the acknowledgements.

Dr Ian Spendlove conceived the project and submitted the initial application for ethical approval along with Mr Ian Scott. The author was responsible for organising the tailoring of this agreement as the project progressed and for obtaining R+D approval. Dr Spendlove checked the final manuscripts for the submitted papers written by the author which comprise the latter chapters of the thesis. Sam Crockett was responsible for the production of the initial clinical database which was audited in the first instance by Sheila O'Malley. The author was responsible for subsequently transforming this clinical database into a final anonymised, validated, consistent and more substantially audited analysis database which could be used for the statistical analyses.

Mr Ian Scott prospectively identified the cohort of patients with ovarian cancer studied within this work. The author used this information to physically identify and extract the appropriate tissue blocks from the pathology archives. The author then organised and participated in the production of new slides representing these cases, subsequently coordinating and recording their review by the histopathologist, Dr Suha Deen.

Claire Paisch physically arrayed tissue from the first 76 cases on the first block of the first copy of the tissue microarray as proof of principal. She was also responsible for taking sections from the final array blocks. The author used the manual tissue arrayer to produce the subsequent three blocks of the first copy of the array and the remaining 16 blocks of the other four copies - recording their production in the form of array grids. The author was also responsible for cutting sections of control tissue to be used in the staining runs.

One copy of the array was stained for CK7, CK20, CEA and CA125 by the staff of the histopathology department at Derby City General Hospital with the remainder of the staining work being performed by the author alone at the Department of Academic and Clinical Oncology at the University of Nottingham. The scoring of the staining of the tissue cores was performed in conjunction with the histopathologist Dr Deen with the results being recorded and statistically analysed by the author alone. Drs Lewis and McKeever periodically gave statistical advice and were responsible for performing the power calculations. The work behind the in-house production and validation of the anti-human CD55 and CD46 antibodies was performed by Dr Maryam Madjd alone as detailed in her publications and thesis – extracts of which are included within this thesis for the convenience of the reader. Otherwise the text of the thesis is entirely the work of the author alone.

Publications resulting from this thesis

Peer reviewed publications

Rolland, P., I. Spendlove, et al. (2007). "The p53 positive Bcl-2 negative phenotype is an independent marker of prognosis in breast cancer." Int J Cancer 120(6): 1311-7.

Rolland, P., Z. Madjd, et al. (2007). "The ubiquitin-binding protein p62 is expressed in breast cancers showing features of aggressive disease." Endocr Relat Cancer 14(1): 73-80.

Rolland, P., S. Deen, et al. (2007). "Human leukocyte antigen class I antigen expression is an independent prognostic factor in ovarian cancer." Clin Cancer Res 13(12): 3591-6.

Duncan, T. J., P. Rolland, 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., A. Al-Attar, 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.

Oral Presentations

Rolland,P., S,Deen., I.Scott.,L.Durrant., I.Spendlove. “Loss of MHC Class I is an Independent Prognostic Factor in Ovarian Cancer.” British Gynaecological Cancer Society International Scientific Meeting, 30th November 2006, Manchester.

Oral Presentations continued

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Poster presentations

“The Multi-Functional Protein P62 is Over-expressed in Poorly Differentiated, Poor Prognosis Breast Cancers” NCRI Conference, Birmingham 2005.

“BCL-2 Expression and Improved Survival from Breast Cancer – The use of a Tissue Microarray to Study a Large Series” NCRI Conference, Birmingham, 2005.

Submitted

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Chapter 1: Introduction

Ovarian cancer has a poor five-year survival which has improved little over the last 40 years despite our knowledge of the basic science underlying cancer increasing substantially during this period. While such knowledge has led to palpable practical improvements at sites such as breast cancer, the same translation of basic science research has been less successful in ovarian cancer - mainly due to the relative complexity of its genetics and the heterogeneity of its key molecular pathways.

Ovarian cancer is characterised by the development of resistance to first line platinum based chemotherapies and subsequently death. A major translational challenge therefore continues to be the development of effective molecular methods for the targeting of alternative treatment modalities. This is particularly pertinent to the large number of cases which progress rapidly on first line treatment and miss the opportunity to trial novel therapies. The informed search for biomarkers whose presence correlates with clinical outcome and response to treatment attempts to address this by translating research from the laboratory to the clinic and back again through the identification of subgroups particularly amenable to novel treatment modalities at an early stage. The components of a small number of molecular processes (such as angiogenesis, cell adhesion, growth signalling and so on) are likely to represent the most promising candidate biomarkers and this thesis concerns the search for those ones with possible roles within the immune based processes affecting ovarian cancer behaviour. This work therefore represents an attempt to add knowledge which could help in the development and targeting of immunotherapies such as cytokines, vaccines and monoclonal antibodies – which appear theoretically particularly suited to use in ovarian cancer.

One important obstacle which has traditionally hampered such translational research in ovarian cancer has been its relatively low

frequency. This has frustrated the accrual of the large, homogenously processed cohorts of cases required for the study of a disease which displays divergent outcomes within seemingly identical groups of cases. Other barriers to the study of biomarkers in ovarian cancer using immunohistochemistry and whole sections have been its time consuming and expensive nature, a tendency towards under powering and the occurrence of intra- and inter- operator discrepancies when large numbers of cases are stained in batches. The aim of this work is to circumvent such traditional translational difficulties by taking advantage of a meticulously accrued clinicopathological cohort of several hundred cases of ovarian cancer and by the production and use of a tissue microarray – a validated technology allowing several hundred specimens to be simultaneously stained for a particular marker on a single slide. This was then stained for immunologically relevant candidate biomarkers in order ultimately to help inform the more global translation of laboratory based immunotherapies into the clinic setting. This introduction aims to provide a relevant background to ovarian cancer in general, biomarker research and tumour immunology, including the use immunotherapy in ovarian cancer.

1.1. Ovarian Cancer

1.1.1. Epidemiology

Ovarian cancer is an uncommon disease which is commonly fatal. The lifetime risk of ovarian cancer is approximately 2% and it is the sixth and fourth most common cancer in women globally and in the UK respectively (Quinn 2001). In the UK, the direct age-standardised incidence rate has varied over the last 40 years (in part due to differences between birth cohorts) from 14.5/100 000 in 1971 to a peak of 19/100 000 in 1997, falling back to 16.7/100 000 in 2005 when this equated to 5456 new cases (Statistics 2008). The incidence of ovarian cancer varies widely between geographical regions and in 2002 the World Standard Rate (per 100 000) of ovarian cancer was 6.6 world-wide, 11.3 in Western Europe, 10.6 in the US, 3.2 in China and 13.4/100 000 in the UK (GLOBOCAN 2004). Though migrants generally

adopt the background risk of their host nation, ethnic group and degree of social deprivation can remain linked to incidence through confounding, because racial, cultural and socioeconomic factors are often intertwined with the reproductive behaviour that determines the risk of developing ovarian cancer – those having higher numbers of children being relatively protected (Bartlett 2000). Indeed, the Surveillance, Epidemiology, and End Results (SEER) Programme registration data for cases diagnosed in the US between 1992 and 1999 recorded an incidence (age-adjusted to the standard million population/100 000) of 17.24 for white women and 11.28 for non-white women (Quirk and Natarajan 2005).

Ovarian cancer is largely a disease of postmenopausal women with only 10% of cases occurring in women under the age of 45. As age advances, the incidence of ovarian cancer increases sharply – stabilising between the ages of 70 and 85 (at approximately 75/100 000 population at risk) and falling thereafter (Quinn 2001). The majority of ovarian cancer cases present in the 50-59 or 60-69 age groups, with the median age of diagnosis being between 60-63 years (Yancik 1993; Heintz, Odicino et al. 2001; Quirk and Natarajan 2005). Advancing age is linked to worsening performance status and affects the clinical management, as weaker patients are unlikely to withstand maximal therapy and may also be excluded from clinical trials (Gospodarowicz 2001). The effect of increasing age/decreasing performance status is therefore accepted to have a negative impact on prognosis (DiSilvestro, Peipert et al. 1997; Heintz, Odicino et al. 2001) and this prognostic power has occasionally been shown to be independent of other factors (Thigpen, Brady et al. 1993; Crijns, Boezen et al. 2003). In reporting age, the data have rarely been grouped in a standard way, having variously been analysed as a continuous variable, subdivided on the basis of the median into two groups, subdivided into two groups on the basis of arbitrary ages (such as 60, 55 or 50) or grouped into more complex categories (Heintz, Odicino et al. 2001; Crijns, Boezen et al. 2003; Quirk and Natarajan 2005; Beral, Doll et al. 2008).

1.1.2. Aetiology

1.1.2.1. Normal ovarian biology

The ovaries are the primary sex organs in the female and are a pair of oval shaped organs approximately 3-5cm in length which lie in the pelvis between the utero-ovarian and infundibulo-pelvic ligaments within a so-called ovarian fossa. The purpose of the ovaries is the secretion of sex hormones and the ovulation of oocytes. Under the influence of Follicle Stimulating Hormone (FSH) and Lutenising Hormone (LH), a Graffian follicle develops from within a pool of primordial follicles. This dominant follicle contains the mature pre-ovulatory oocyte and is the main source of cyclical oestrogens. Under the influence of a surge in LH, the oocyte is ejected and ruptures the ovarian capsule. Should fertilisation of the oocyte not occur, the redundant follicle undergoes fibro-hyaline degeneration to form the scar tissue of a corpus albicans. This cycle repeats itself until the pool of primordial follicles from which Graffian follicles develop falls to a critically low level, after which ovulation ceases and the menopause ensues. Ovulation may be suspended during pregnancy, lactation and the use of the combined oral contraceptive pill (McGee and Hsueh 2000).

1.1.2.2. Risk factors

Familial

Only a minority (5-10%) of ovarian cancer cases are associated with a familial tendency but for those individuals with cases in their immediate family this is their single most important risk factor. Where there is no specific genetic basis found the risk is approximately 9% but can vary depending on the number of first degree relatives affected and their age of onset of the disease (Holschneider and Berek 2000). Ovarian cancers with a known genetic basis often present at an earlier age than their sporadic equivalents and are associated with the development of cancer at other sites. Mutations within the BReast CAncer 1 and 2 genes (BRCA1/2) are associated with a 30-40% and 27% risk of developing ovarian cancer respectively and account for most familial

cases of ovarian cancer, occurring in 40% and 25% of ovarian cancer families and 0.06% of the general population (Ford, Easton et al. 1994; Ford, Easton et al. 1995; Szabo and King 1997; Whittemore, Gong et al. 1997). These mutations have a variable penetrance which is influenced by factors such as ethnicity and most women will not go on to have ovarian cancer. However, the risk of breast cancer is higher and 87% and 84% of BRCA1 and BRCA2 mutation carriers develop it by the age of 70. This risk is reduced by 50% following prophylactic oophrectomy and, though this also reduces the risk of developing ovarian cancer, it is not completely prevented within embryological remnants (Ford, Easton et al. 1994; Miracle-McMahill, Calle et al. 1997; Kauff, Satagopan et al. 2002). Hereditary NonPolyposis Colorectal Cancer (HNPCC) predisposes to the development of right-sided colonic cancers in young adults and to the development of ovarian and endometrial cancer. The underlying genetic basis involves mutations in the DNA MisMatch Repair (MMR) genes and in such cases the risk of ovarian cancer is as high as 10% (Aarnio, Mecklin et al. 1995).

Reproductive

The lower risk of developing ovarian cancer with increasing parity has been well documented and is estimated to equate to a 16-22% reduction per birth (Risch, Marrett et al. 1994; Hankinson, Colditz et al. 1995; Purdie, Green et al. 1995). There has been no convincing risk reduction shown for women who choose to breast feed (Rosenblatt and Thomas 1993). When correcting for nulliparity, women who do not get pregnant following pharmacological ovulation induction have a higher risk of ovarian cancer though a causal link has not been demonstrated (Bristow and Karlan 1996). Tubal ligation and hysterectomy have been shown to reduce the risk of developing ovarian cancer for at least the first two decades following the procedure (Purdie, Green et al. 1995; Miracle-McMahill, Calle et al. 1997; Hankey, Drachenberg et al. 2002). Late menopause is associated with a higher risk of ovarian cancer but early menarche is not (Franceschi, La Vecchia et al. 1991).

Hormonal

Using the combined oral contraceptive pill (COCP) reduces the risk of ovarian cancer in both nulliparous and multiparous women. A recent re-analysis of 45 case-control studies concluded that lowest risk of ovarian cancer was in those that used the COCP for longer and that this benefit persisted for 30 years. After 10 years' use the number of cases/100 users developing ovarian cancer by the age of 75 fell from 1.2 to 0.8 with one death being prevented per 5000 women years (Beral, Doll et al. 2008). PolyCystic Ovarian Syndrome (PCOS) is linked to the development of endometrial cancer but a link with ovarian cancer is less certain, with some workers showing a marked increase in rates, especially for those cases where the COCP has not been used (Schildkraut, Schwingl et al. 1996), and others showing no association (Balen 2001). The Million Women study monitored 948 576 women between 1996 and 2001 and current HRT users were found to be at increased risk of ovarian cancer (RR 1.2) and death from ovarian cancer (RR 1.23) which persisted independently of known confounding factors such as parity and COCP use. Among a group of 3300 women taking HRT over a 5-year period one user will die from ovarian cancer solely as a result of its use (Beral, Bull et al. 2007).

Environmental

Eating eggs, lactose and cholesterol may be modestly associated with an increased risk of ovarian cancer while eating green vegetables may reduce risk (Kuchel, Tannenbaum et al. 2001), though a link between ovarian cancer and lactose consumption has not been found by all workers when parity and COCP use are corrected for (Risch, Jain et al. 1994). Obesity (when defined as a Body Mass Index >30) is not only associated with breast and endometrial cancer but increased the risk of ovarian cancer by 30% in a recent meta-analysis (Olsen, Green et al. 2007). Death from ovarian cancer was found to be related to cigarette smoking in the 6194 female doctors followed up from 1951 by Richard Doll and co-workers (Weiss, Lyon et al. 1982). On correcting for smoking, the consumption of coffee is linked to a modest increase in

the risk of developing ovarian cancer though a causal link has not been established (La Vecchia, Franceschi et al. 1984). Proving the precise mechanism remains obscure but the genital use of talcum powder has consistently been epidemiologically associated with the development of ovarian cancer (Weiss, Lyon et al. 1982; Purdie, Green et al. 1995). An increase in the number of ovarian malignancies expected was found in Swedish women who underwent radiotherapy for cervical carcinoma and survived to >10 years post-treatment (Weiss, Lyon et al. 1982).

1.1.2.3. Theories of causation

Four theories exist regarding the development of ovarian cancer which try to make sense of these risk factors.

Incessant ovulation

This theory suggests that physical trauma to the ovarian surface epithelium at ovulation and its subsequent exposure to viscous oestrogen are carcinogenic events (Fathalla 1971). It follows that the fewer ovulatory events that occur, the lower the chance of developing cancer. This explains why pregnancy and COCP use are protective and why hens are the only other animals to suffer from ovarian cancer in nature (Fredrickson 1987). A study supporting this estimated that each year's worth of ovulation within the 20-29 year age group equated to an estimated 20% increase in the risk of ovarian cancer (Purdie, Bain et al. 2003). Others regard this theory as an unlikely sole explanation of the epidemiology as it does not explain the protective effect of the progesterone only contraceptive pill (POP) which does not inhibit ovulation (Risch, Jain et al. 1994).

Gonadotrophin hypothesis

This theory postulates that exposure of the ovaries to high levels of gonadotrophins is carcinogenic (Cramer and Welch 1983). This explains why ovarian cancer is more common post-menopausally, the protective effect of pregnancy and the COCP and the effects of PCOS. However, others observe that it does not account for the high levels of oestrogen and HcG in seen in pregnancy and that gonadotrophins have

not been shown to be higher in post-menopausal women with ovarian cancer than their age matched controls (Helzlsouer, Alberg et al. 1995).

Hormonal hypothesis

This refinement of the last hypothesis attempts to bring all observations together by putting more emphasis on the roles of androgens and progesterone. The Graffian follicle contains a higher concentration of androgens than oestrogen, which could potentially be exposed to the surface epithelium during ovulation (Risch 1998). Androgens are present at higher levels in PCOS and are suppressed by the COCP and POP (Rosenburg 1994) (Gaspard 1983). Progesterone is anti-mitotic, present at large levels during pregnancy, is contained within the COCP and has been shown to increase wild type p53 tumour suppressor gene expression and induce apoptosis in human ovarian cancer cell lines (Bu 1997) (Riasch 1998). Finally, the rate of ovarian cancer in hens may be decreased by administering progesterone (Barnes, Berry et al. 2002).

Inflammation hypothesis

During ovulation the walls of the follicle are broken down under the influence of inflammatory mediators and connective tissue dissolving enzymes (Bonello, McKie et al. 1996). This process is likely to involve leukocyte inclusion, nitric oxide release, cytokine release, vasodilatation, DNA repair and tissue repair which are all potentially carcinogenic (Zackrisson, Mikuni et al. 1996).

As no single theory explains all epidemiological findings it is likely that each is in part correct, especially as ovarian cancer is a heterogeneous disease with a far from certain natural history (Fleming, Beaugie et al. 2006).

1.1.3. Pathology

1.1.3.1. Histological type

Primary neoplasia within the ovary can develop from a large variety of cell types and can behave in a benign, malignant or “low malignant potential” (borderline) manner. The degree of heterogeneity of disease

can be appreciated by examining the World Health Organisation (WHO) International Histological Classification of tumours which classifies neoplasia into at least 129 individual types with over 30 forms of frank cancer (Appendix 1) (Serov, Scully et al. 1973).

Epithelial ovarian cancer

Epithelial ovarian cancer accounts for over 90% of ovarian cancer and is thought to originate from invaginations of the ovarian surface epithelium.

Borderline cancers

Serous, mucinous, endometrioid or clear cell cancers of low malignant potential represent up to 15% of epithelial ovarian cancers but are histologically and biologically distinct from their frankly malignant counterparts (FIGO 1987). They exhibit the same proliferative features and cellular disorganisation but they do not generate a stromal reaction nor invade ovarian tissue, though they can spread, metastasise and invade distant organs (Scully 1977). They are staged in the same way as frank cancer but often present early (75% stage I) though, even correcting for this, they have a better overall 5-year survival of 89% compared to 51% for frank malignancy (Heintz, Odicino et al. 2001).

Malignant serous adenocarcinoma

Frankly malignant serous tumours are the most common form of ovarian cancer, accounting for 40-50% of cases (Heintz, Odicino et al. 2001; Quirk and Natarajan 2005; Beral, Doll et al. 2008). They are often felt to be associated with a poor prognosis but they present late and, when stage correction is performed, their outcome is not significantly different to endometrioid types (Makar, Baekelandt et al. 1995).

Malignant mucinous adenocarcinoma

Mucinous cancers account for 9-15% of epithelial ovarian cancers, often presenting at an early stage (55%) when they have a good prognosis (91% at 5 years) (Heintz, Odicino et al. 2001; Quirk and Natarajan 2005; Beral, Doll et al. 2008). However, when they present at a late stage they have a poor prognosis when compared to serous or

endometrioid cancers, due to their relative insensitivity to platinum chemotherapy (Harrison, Jameson et al. 2008).

Malignant endometrioid adenocarcinoma

Endometrioid cancers are the second most common type after serous, accounting for approximately 12-18% of all ovarian cancers and in 5-10% an origin within endometriosis may be demonstrated (Scully 1977; Heintz, Odicino et al. 2001; Quirk and Natarajan 2005; Beral, Doll et al. 2008). Endometrioid cancers occur synchronously with endometrial cancer in up to 15% of cases and in this situation the most common presenting symptom is post menopausal bleeding, often leading to an early diagnosis with disease confined to the pelvis (Soliman, Slomovitz et al. 2004).

Malignant clear cell adenocarcinoma

Clear cell cancers are relatively uncommon and account for 4-6% of ovarian cancers (Scully 1977; Heintz, Odicino et al. 2001; Quirk and Natarajan 2005; Beral, Doll et al. 2008). They are unexplainably two to three times more common within Japanese populations but a relative scarcity means that less data are available concerning their biology, though they have consistently been found to be associated with pelvic endometriosis (Kennedy, Biscotti et al. 1989; Komiyama, Aoki et al. 1999). Such circumstantial evidence may point to shared molecular aetiologies between the two conditions and, indeed, identical loss of heterozygositiy at 10q23 patterns and mutations in the phosphatase and tensin homolog (PTEN) tumour suppressor gene have been shown to exist (Sato, Tsunoda et al. 2000). Compared to serous ovarian cancer, clear cell cancers are more likely to present at an earlier stage but are more likely to recur and display platinum insensitivity, having a poorer prognosis on a sub-stage level as a result (Heintz, Odicino et al. 2001; Takano, Kikuchi et al. 2006; Itamochi, Kigawa et al. 2008).

Undifferentiated carcinomas

Undifferentiated ovarian carcinomas occur with a variable frequency of 5-25%, present at a late stage, advance rapidly and have the worst prognosis of all epithelial ovarian cancers with a 11-23% 5-year survival

(Scully 1977; Silva, Tornos et al. 1991; Heintz, Odicino et al. 2001; Quirk and Natarajan 2005).

Ovarian cancer is a heterogeneous disease and its morphological subtypes exhibit different prognoses, probably because of underlying molecular variations (Bell 2005). Epithelial cancers may be ranked borderline, endometrioid, mucinous, serous, clear cell, mixed and undifferentiated in order of worsening prognosis. While histological type is an important prognostic factor it is not classified by the International Union Against Cancer (UICC – Union Internationale Contre le Cancer) as having prognostic power independent of other factors which have prognostic power independent of each other (stage, patient performance status/age, degree of cytoreducibility and chemotherapy use). This is probably because the varying biology of the different histological subtypes concurrently influences stage at diagnosis, thus interlinking its prognostic power with that of a genuinely independent prognostic factor.

1.1.3.2. Histological grade

The histological grade of a neoplasm refers to the microscopic appearances of its constituent cells and is an attempt to objectively assess their rate of proliferation and degree of abnormality. The importance of poorly differentiated disease in determining outcome in ovarian cancer is less clear than at other sites such as breast (Elston and Ellis 1991). Using the FIGO grading system, tumours are categorised as either well (G1), moderately (G2) or poorly (G3) differentiated with 24% G1, 30% G2 and 47% G3 (Benedet 2000). Undifferentiated, Brenner and clear cell subtypes are often all assumed to be G3 (Silva and Gershenson 1998). Though grading does not have independent prognostic power, it predicts prognosis on a sub-stage level independently and can be a useful adjunct in individual cases – though it loses its predictive power in the most advanced cases (Gospodarowicz 2001; Heintz, Odicino et al. 2001).

1.1.3.3. Immunohistochemical profile

7% of ovarian tumours are metastatic, from a variety of cancer sites such as endometrium, pancreas, stomach and bowel. (Ulbright, Roth et al. 1984). As ovarian cancers are often poorly differentiated and may contain confusing cell types, it can be impossible to tell whether a cancer is a primary or secondary on the basis of light microscopy alone. While collateral information from the clinical work up may make the diagnosis clear, a number of immunohistochemical markers have also been studied in the attempt to precisely define the differences between morphologically identical pelvic cancers from different sources on the basis of their molecular variations.

Immunohistochemical expression of Cytokeratin 7 (CK7)

Keratins are a multi-gene family of at least 20 epithelium specific cytosolic polypeptides which form intermediate filaments within the cytoskeleton. Different epithelia express different patterns of cytokeratins, which are preserved following malignant transformation (Moll, Franke et al. 1982). CK7 is expressed by many normal tissues including the epithelia of mammary gland ducts, trachea, bladder, lung, cervix, bile duct, outer ovarian surface and fallopian tube. In contrast, hepatocytes and Gastro-Intestinal (GI) tract epithelia do not express CK7 (Moll, Franke et al. 1982; Ramaekers, van Niekerk et al. 1990). The immunohistochemical expression of CK7 has most often been examined using the OV-TL 12/30 monoclonal antibody and (though there has been considerable methodological variation between studies) it has consistently been found to be expressed in between 94% and 100% of ovarian cancers, with almost all serous and upwards of 80% of mucinous cancers expressing it (Ueda, Sawada et al. 1993; Berezowski, Stastny et al. 1996; Lagendijk, Mullink et al. 1998; Torenbeek, Lagendijk et al. 1998; Multhaupt, Arenas-Elliott et al. 1999; Chu, Wu et al. 2000; Cathro and Stoler 2002; Nishizuka, Chen et al. 2003; Dennis, Hvidsten et al. 2005; Heatley 2008). CK7 expression is commonly expressed in lung, breast, pancreatic and, to a lesser degree, gastric carcinoma. However, CK7 is rarely expressed in colonic or renal cancers. In addition, there is usually good correlation between

the expression of CK7 in the primary and metastatic sites (Tot 2002). There is no evidence that CK7 has any prognostic power in ovarian cancer.

Immunohistochemical expression of CytoKeratin 20 (CK20)

CK20 was discovered by Moll and co-workers in 1990 (Moll, Schiller et al. 1990). Initial data indicated that CK20 was widely expressed in the epithelia of normal small bowel and colon but not expressed in lung, breast, female genital tract, pancreatic or breast epithelia. CK20 expression pattern in tumours followed that seen in normal tissue and no non-mucinous ovarian cancers were found to express it, whereas most colonic cancers did. Accounting for methodological differences, subsequent surveys show the expression of CK20 in the general population of primary ovarian cancers to be approximately 0-36%, with serous cancers being almost exclusively negative and most CK20 positive cancers being of a mucinous subtype (Ueda, Sawada et al. 1993; Berezowski, Stastny et al. 1996; Lagendijk, Mullink et al. 1998; Torenbeek, Lagendijk et al. 1998; Lagendijk, Mullink et al. 1999; Multhaupt, Arenas-Elliott et al. 1999; Chu, Wu et al. 2000; Cathro and Stoler 2002; Nishizuka, Chen et al. 2003; Dennis, Hvidsten et al. 2005; Heatley 2008).

Immunohistochemical expression of Carcino Embryonic Antigen (CEA)

CEA is a highly glycosolated protein first described by Gold and co-workers in the human digestive tract (Gold and Freedman 1965). CEA is expressed in the normal squamous epithelial cells of the cervix, tongue and oesophagus and in mucous secreting upper and lower GI cells. Its expression is retained in colorectal, lung, pancreatic and endometrial tumours (Hammarstrom 1999). CEA is more variably expressed in ovarian cancers with 21-41% of all cases being reported as positive for CEA, though occasional studies on small numbers of selected cases have put this figure as high as 100% with the mucinous subtype most likely to express it (Macdonald, Bird et al. 1988; Breitenecker, Neunteufel et al. 1989; Neunteufel and Breitenecker 1989; Berezowski, Stastny et al. 1996; Torenbeek, Lagendijk et al. 1998; Keen, Szakacs et al. 1999; Lagendijk, Mullink et al. 1999). Serum

CEA may act as a tumour marker in colorectal cancer but there is no evidence that its immunohistochemical expression in ovarian cancer tissue has prognostic value.

Immunohistochemical expression of Cancer Antigen 125 (CA125)

CA125 was indirectly discovered in 1981 by Bast and co-workers. They developed a murine IgG1 monoclonal antibody to ovarian cancer cell lines that was found to stain ovarian cancer tissues and which they named OC125. The initial study indicated that OC125 was 100% sensitive and specific for ovarian cancer though subsequent work has found it to be present in less than 80% of ovarian cancers and more widely distributed in the normal and cancerous derivatives of the embryological coelomic epithelium (pleural, pericardial and peritoneal tissues) (Kabawat, Bast et al. 1983). Though the CA125 antigen proved difficult to investigate, its gene was finally cloned in 2001 by Yin and co-workers. They surmised that it was a new mucin species, the product of the MUC 16 gene (Yin and Lloyd 2001). Between 54% and 78% of the general population of ovarian cancers express CA125, with the proportion being higher for serous cancers (68-93%) compared to mucinous ones (25-43%) (Kabawat, Bast et al. 1983; Macdonald, Bird et al. 1988; Breitenecker, Neunteufel et al. 1989; Neunteufel and Breitenecker 1989; Loy, Quesenberry et al. 1992; Torenbeek, Lagendijk et al. 1998; Keen, Szakacs et al. 1999; Multhaupt, Arenas-Elliott et al. 1999; Dennis, Hvidsten et al. 2005). In specific circumstances, CA125 negative tumours may be associated with poorer outcome in late stage disease (Hogdall, Christensen et al. 2007) but there is little supportive evidence for CA125 tissue antigen expression being independently prognostic.

Immunohistochemical marker panels

While the expression of these four markers has been well characterised, used singly they are of limited use in determining the origin of an undifferentiated ovarian tumour. Using them together, as a panel, a tumour which is CK7(+)CK20(-) CEA(-)CA125(+) will most likely be of ovarian origin and not colonic origin (Tot 2002; Nishizuka, Chen et al. 2003; McCluggage and Young 2005) (Heatley 2008).

However, this panel is imperfect and the utility of a host of other supplementary markers has been assessed including CDX-2 (caudal related homeobox transcription factor 2), β-catenin, ER (oestrogen receptor), PR (progesterone receptor), WT1 (Wilms Tumour 1), P504s (alpha-Methylacyl-COA-racemase), p16 (cyclin dependent kinase inhibitor 2A) and DPC4 (Deleted in Pancreatic Cancer 4). Despite this, even extensive, complex panels do not approach 100% accuracy, with various combinations of markers from three (CEA, CK7, Vimentin) (Lagendijk, Mullink et al. 1998) to 10 (CA125, CDX2, CK7, CK20, ER, GCDFP-15 [Gross Cystic Disease Fluid Protein 15], lysozyme, mesothelin, PSA [Prostate Specific Antigen], TTF1 [Thyroid Transcription Factor 1]) (Dennis, Hvidsten et al. 2005) yielding accuracies of between 66% (Brown, Campagna et al. 1997) and 88% (Dennis, Hvidsten et al. 2005) in predicting the primary source of a tumour. The most recent review places emphasis on a panel including CK7, CK20, WT1, ER, PR, GCDFP-15, CDX2, DPC4, p16 and β-catenin in assessing the origin of cancers clearly metastatic to the ovary with CA125 and CK20 having a role in excluding possible colonic cancer metastases from probable primary ovarian cancer and CEA having no clear role (Mittal, Soslow et al. 2008).

1.1.3.4. Staging

The stage of the disease describes the extent to which it has advanced at the time of diagnosis and is assessed clinically, surgically, pathologically and, increasingly, radiologically. Ovarian cancer spreads by its direct extension, by the exfoliation and migration of cancer cells throughout the peritoneal cavities and through the movement of tumour emboli throughout the lymphatic system (Bartlett 2000). In 1973 FIGO published data to validate an ovarian cancer staging system which identified four distinct stages: confined locally (stage I), extending to neighbouring structures (stage II), more distant regional spread (stage III) and distant metastasis (stage IV) (Appendix 2). This was adopted by the American Joint Committee on Cancer (AJCC) in preference to an alternative system developed by UICC which describes the stage of all

cancers in the same terms of tumour characteristics (T), lymph node involvement (N) and metastasis (M). The FIGO system remains the internationally recognised standard for staging ovarian cancer but, as all three bodies collaborate closely, the two systems have converged to the point where they are now almost identical (Benedet 2000; Odicino, Pecorelli et al. 2008). Having a reproducible, validated and practical staging system is useful prognostically and facilitates meaningful scientific discussion with disease also potentially being more precisely described on a sub-stage level (Appendix 2). Reviewing cohorts of ovarian cancers that included cases presenting between 1982 and 1997 which were subsequently reported in the literature, the majority of series had a distribution of cases amongst the stages of 21-33% stage I, 4-11% stage II, 24-52% stage III and 12-37% stage IV (Table 1.1) (Hand, Fremgen et al. 1993; Yancik 1993; Brun, Feyler et al. 2000; Holschneider and Berek 2000; Heintz, Odicino et al. 2001; Quirk and Natarajan 2005).

1.1.4. Diagnosis

Screening

Diagnosing a disease in the population at risk through the widespread application of a test to asymptomatic individuals is the process of screening. The aim is to detect the disease at an earlier stage than it would otherwise be detected clinically, at a point in its natural history where intervention may be more successful and an improvement in mortality seen. This has been achieved by the national UK cervical screening programme but no national equivalent for ovarian cancer yet exists (Quinn, Babb et al. 1999). For the intended benefits of a screening programme to be achieved, most of its targeted population needs to participate and so the test employed must be simple and acceptable. As accurate diagnostic tests are often unpleasant, acceptable screening tests often have to compromise on accuracy, producing false negative results (leading to individuals being falsely reassured) and false positive results (leading to individuals undergoing unnecessary diagnostic testing or treatment). A good screening test

Table 1.1 FIGO stage and related 5-year survival
Data derived from cohorts recruited within the time period covered by this study (1982 – 1997) which were reported in the literature.

	Series							
	Wolfe (1997)	**Granai (1997)**	**Berek (2000)**	**Yancik (1992)**	**Quirk (2001)**	**Hand (1993)**	**Brun (2000)**	**Heintz (2001)**
Number of cases	118	137	12316	21000	23484	2977	323	3758
Location	U.K	U.S	U.S	U.S	U.S	U.S	E.U	Global
Time period	1991	1987 1992	1983 1988	1973 1987	1992 1999	1983 1988	1975 1995	1993 1995
% cases by stage								
I	13	47	32	26	26	29	21	33
II	66	14	11	4	11	11	7	9
III	53	27	32	70	59	24	52	46
IV		12	25			37	20	12
5-year survival by stage								
I		89	93			76-80	76	80-89
II		66	70			61	42	64 -70
III		39	37			21-28	21	29-59
IV		6	25			15-1	6	17

seeks to balance the benefits of acceptability with the risks of inaccuracy. Two tests for ovarian cancer have been most investigated, serum CA125 and Trans Vaginal ultrasound Scanning (TVS). Serum CA125 levels above the upper limit of normal are found in 80-90% of patients with epithelial ovarian cancer (Hogdall, Christensen et al. 2007). Though lacking in formal consensus, a level of 35 U/ml has become the widely accepted value for delimiting normality with a level of >50 U/ml being associated with malignancy in 94% of instances (Jacobs, Oram et al. 1990; Einhorn, Sjovall et al. 1992; Hogdall, Christensen et al. 2007). However, raised levels (of up to 5000 U/ml) may also be seen in benign situations such as menstruation, pregnancy, endometriosis, pelvic inflammatory disease, peritonitis and in association with any cause of ascites or pleural effusion (Daoud and Bodor 1991). In addition, many mucinous epithelial ovarian cancers and up to 50% of stage I epithelial ovarian cancers have a normal serum CA125 (Jacobs and Bast 1989; Einhorn, Sjovall et al. 1992). For this reason a single abnormal measurement of CA125 on its own has an unacceptable sensitivity (81%) and specificity (75%) for screening. With a relatively low prevalence of ovarian cancer in the general population at risk, using such a test would be associated with a large number of false positive results and consequently unacceptable expense and morbidity (Jacobs, Oram et al. 1990). The use of serial measurements and a risk score improves accuracy slightly to a sensitivity of 86% and specificity of 98% (Skates, Menon et al. 2003). A series of over 25 000 women received annual TVS screening by a group at the University of Kentucky with a sensitivity of 85%, specificity of 98.7%, Positive Predictive Value (PPV) of 0.14 and a Negative Predictive Value (NPV) of 0.99 being reported. There was evidence of a downward shift of stage at diagnosis with 82% being diagnosed at stage I/II. Additionally, 5-year Overall Survival (OS) was higher in this group (77%) than in the local registry population (49%). However, the low sensitivity and PPV of TVS means that it may not be accurate enough to act as a lone tool in national screening (van Nagell, DePriest et al. 2007). The large scale feasibility of first line TVS alone or following abnormal CA125

measurements is therefore being assessed in a randomised multi-centred trial that benefits from an unscreened control group, a cohort of 200 000 initially asymptomatic women having been recruited. It will report in 2012 (United Kingdom Collaborative Trial of Ovarian Cancer Screening – UKCTOCS) (Menon, Skates et al. 2005).

Symptoms

The clichéd description of ovarian cancer as "the silent killer" is inaccurate as it implies that it is asymptomatic. In fact, patients retrospectively recall a variety of symptoms including increasing abdominal size (63%), abdominal bloating (68%), abdominal pain (50-58%), pelvic pain (26-41%), fatigue (50%) urinary symptoms (34%), along with upper gastrointestinal symptoms such as indigestion, feeling full quickly and reduced appetite which precede the diagnosis by many months (Bankhead, Kehoe et al. 2005). A more accurate summation might be that ovarian cancer is associated with a variety of commonly seen non-specific symptoms, the significance of which is usually only appreciated retrospectively by the majority of patients (Smith and Anderson 1985). One group that reported data collected prospectively from cases and controls concluded that the onset, duration and frequency of symptoms discriminated between ovarian cancer and non-specific conditions. New onset symptoms of <12 months' duration (abdominal pain, increasing abdominal size, urinary symptoms and feeling full) occurring >12 times per month had a sensitivity of 87% and specificity of 87% in the >50 year age group for detecting ovarian cancer (Goff, Mandel et al. 2007).

Investigation

Surgery for ovarian cancer is usually undertaken before a definitive tissue diagnosis has been made and, for that reason, supplementary tests are performed in order to reduce the likelihood of false positives.

<u>Incidentally discovered masses</u>

Such adnexal massed are investigated by TVS and the measurement of the serum CA125. These data are used to compute the risk of malignancy index (RMI), a validated scoring system originally

developed by Jacobs and co-workers which assesses the malignant potential of the mass (Jacobs, Oram et al. 1990; Scottish Intercollegiate Guidelines Network. 2003) (Figure 1.1). Some radiologists advise that a single high resolution 3 Teslar Magnetic Resonance (MR) scan can now adequately define the nature of masses accurately enough to remove the need for this approach (Spencer 2008).

Clinical Ovarian cancer

Where ovarian cancer has been diagnosed clinically, baseline tumour markers are sent which include CA125 and markers which help exclude alternate primaries including CA153 (breast) (Duffy 2006), CA19-9 (pancreatic) (Goonetilleke and Siriwardena 2007) and CEA (colorectal) (Moreno Carretero, Cerdan Miguel et al. 1998). To assess the extent of the disease, radiological imaging of the chest, upper abdomen and pelvis is required. Ultrasound (USS), CT and MR have similar accuracies in determining whether disease has spread beyond the pelvis (Kurtz, Tsimikas et al. 1999) but CT scanning has been shown to be superior to USS and equivalent to 1.5 Teslar MR scanning in approximating the stage of advanced disease. Cytological samples may be taken from ascites or from effusions prior to surgery to delineate spread and these imaging modalities can be used to guide the taking of these samples and the removal of biopsy tissue where necessary.

1.1.5. Treatment of epithelial ovarian cancer

The successful treatment or longer term palliation of ovarian cancer combines surgical and medical approaches.

1.1.5.1. Surgical treatment

Surgery combines the expert assessment of the extent of the disease (staging) with its removal (debulking).

Surgical staging

An adequate staging operation demands a vertical mid-line abdominal incision, the collection of samples for cytology, the complete inspection and palpation of all of the abdominal contents, the taking of biopsies from suspicious sites and random ones from all other peritoneal surfaces, the inspection of the ovaries for signs of capsular rupture, the

Figure 1.1 The Risk of Malignancy Index (RMI)
Developed by Jacobs et al for assessing the likelihood of ovarian pathology detected at ultrasound being cancerous (Jacobs, Oram et al. 1990.

Parameter	Score
Ultrasound appearances	no abnormality = 0
Multilocular cyst	1 abnormality = 1
Solid areas	
Bilateral lesions	2 or more abnormalities = 4
Ascites	
Intra-abdominal metastases	
Menopausal status	
Post-menopausal	4
Pre-menopausal	1
CA125 level	U/ml

RMI = ultrasound score x menopausal score x CA125

Using a cut-off of 200; Positive predictive value = 80%; Sensitivity 74-80%; Specificity 89-92% (Scottish Intercollegiate Guidelines Network. 2003)

removal of all visible tumour, the removal of the omentum and the removal of the regional lymph nodes to the level of the renal hilar. By the end of the operation a total hysterectomy and bilateral salpingo-oophrectomy should have been completed (Benedet 2000; Te Linde, Rock et al. 2003). Despite these recommendations, concerns about surgical morbidity mean that most patients have historically been incompletely staged, with the lymphadenectomy and random biopsies being the most frequently omitted steps (Trimbos, Schueler et al. 1990; Murakami, Nagashima et al. 2002). As lymph node infiltration (Stage III) occurs in up to 38% of apparently otherwise stage I cases, such incomplete staging can lead to under treatment and unexpectedly poor outcomes in this group (Knapp and Friedman 1974; Morice, Joulie et al. 2003). The risks of operative complications have therefore to be set in context and most authorities recommend that a full staging operation which includes loco-regional lymphadenectomy is undertaken in apparent stage I cases (di Re, Baiocchi et al. 1996; Benedet 2000; Angioli, Plotti et al. 2008).

Surgical debulking

Solid tumours are conventionally treated surgically if it can be reasonably certain that all the disease will be removed, the potential for cure offsetting the risks of complex surgery. In ovarian cancer this principle is distorted due to the massive tumour volumes involved. Attempting to remove all of the tumour may be impossible in advanced disease but aiming to remove as much of the tumour as possible (debulking) will improve a patient's performance status, quality of life and the subsequent effectiveness of chemotherapy (Blythe and Wahl 1982; Hoskins and Rubin 1991). Minimal residual disease (tumour remaining at the end of debulking surgery) is an end-point which has been variously defined as the presence of cancer deposits with a diameter of <2cm, <1cm, <0.5cm or the absence of any visible disease (maximal macroscopic cytoreduction). The lower the amount of residual disease the better the OS and the absence of any visible disease is an important independent prognostic factor, with the persistence of residual deposits ≥2cm following surgery conferring no improvement in

OS above that of a simple diagnostic surgical procedure (Gospodarowicz 2001) (Makar, Baekelandt et al. 1995). If a strategy of debulking surgery is to be aggressively pursued, it must thus aim to attain a level of cytoreduction below this and preferably to the level of maximal cytoreduction. If this is achieved, the morbidity of even complex procedures (extensive bowel resection, splenectomy, cystectomy, nephrectomy, hepatic lobectomy) can be offset in even the most advanced cases by improvements in survival and quality of life (Curtin, Malik et al. 1997; Liu, Benjamin et al. 1997; Bristow, Montz et al. 1999; Benedet 2000). Though maximal cytoreduction is associated with the best outcomes, debulking the disease to a maximal point possible below 2cm is the accepted surgical standard (Omura, Brady et al. 1991; Hoskins, McGuire et al. 1994). It has not been discerned whether achieving maximal cytoreduction is made possible by biologically indolent cancer, or if the surgery itself alters the biology of the disease. However, the prognostic power in multivariate models of residual disease has diminished following the adoption of platinum based chemotherapy, demonstrating some interdependence between the two (Hoskins and Rubin 1991; Hunter, Alexander et al. 1992; Venesmaa 1994; Kehoe 1996; Brun, Feyler et al. 2000). Alterations in chemo-sensitivity may explain an interdependency as poor blood supply and the presence of quiescent or resistant clones are features of both high volume disease and chemo-resistance that should theoretically respond to its surgical reduction (Bartlett 2000).

Surgical sub-specialisation

Several studies have shown that cases managed by general surgeons have a poorer OS than those managed by gynaecologists, though improvements when a sub-specialist gynae-oncologist performs the surgery have been less clearly demonstrated (Nguyen, Averette et al. 1993; Kehoe, Powell et al. 1994; Junor, Hole et al. 1999). This variation in survival may be explained by surgical differences (gynaecologists performing more accurate staging, more hysterectomies and more lymphadenectomies) or through the improved coordination of care seen

when gynaecological cancers are treated within a gynaecological department (Earle, Schrag et al. 2006).

1.1.5.2. Medical treatment

The specific medical therapy employed depends on the origin (non-epithelial/epithelial) and stage (early/advanced) of the disease and has evolved over time with the retirement of certain modalities (radiotherapy) and the ever more imaginative delivery of successive novel chemotherapeutic agents. Monitoring response to treatment is the key to detecting resistance and newer strategies and agents continue to be developed to deal with cases which fail to respond to the best first line treatments.

Early stage

Assuming that the disease has been correctly staged, well differentiated stage Ia and Ib tumours have a >90% chance of cure through surgery alone with the remainder of early stage cancers having a high risk of recurrence (40-50%). Trials which have investigated the use of single agent platinum chemotherapy in early stage ovarian cancer concluded that it confers significant benefit in all Ic cases and those Ia and Ib cases showing certain high risk features on histological assessment. However, most patients in the high risk group would survive without chemotherapy and as 18% will have recurrent disease despite receiving it the current system is imperfect (Trimbos, Parmar et al. 2003; Ozols 2005).

Advanced stage

First line medical treatment of advanced epithelial ovarian cancer is usually commenced following surgical debulking and the most accepted practice is to administer 6 cycles of carboplatin combined with paclitaxel at three-weekly intervals. Ovarian cancer is sensitive to chemotherapy and the majority (75%) of cases will initially respond, though the disease is characterised by high levels of recurrence (75%) and eventual death following the development of resistance to platinum based chemotherapies (Lister-Sharp, McDonagh et al. 2000; Ozols, Bundy et al. 2003).

Evolution of chemotherapeutic regimens

The practice of using single alkylating agents was abandoned from the late 1970s onwards as evidence that the use of platinum was superior accumulated (Lambert and Berry 1985). Platinum based drugs interact with DNA forming inter-strand cross-links and DNA-Platinum-Protein Complexes (DPCLs) which trigger cell death through as yet imperfectly defined mechanisms, though the final common pathway is likely to be apoptosis (Bartlett 2000; Chvalova, Brabec et al. 2007). A regimen combining cisplatin and cyclophosphamide found favour following the accumulation of evidence, eventually distilled in a Cochrane meta-analysis (2000), that this regimen improved survival above the single agent regimen. A meta-analysis in 1995 looking at the effect of the anthrocyclin doxyrubicin supported using this drug in combination with platinum, the standard treatment having become Cyclophosphamide-Anthracyclin-Platinum (CAP) (A'Hern and Gore 1995). Carboplatin was then found to be equivalent to CAP and to cause less toxicity (1998). Paclitaxel is a member of a family of drugs derived from the bark of the Pacific Yew tree (taxanes) that achieve cell killing by promoting the assembly and persistence of microtubules within the cell, leading on to mitotic arrest and apoptosis (Blagosklonny and Fojo 1999). Paclitaxel was shown to have activity in non-responding cases (McGuire, Rowinsky et al. 1989) and to improve tolerability and response rates when used in combination with cisplatin (Muggia, Braly et al. 2000). The use of combined paclitaxel and platinum regimens was extensively assessed, with many workers discovering improved outcomes (McGuire, Hoskins et al. 1996; Piccart, Bertelsen et al. 2000) (du Bois, Luck et al. 2003; Ozols, Bundy et al. 2003). However, this was not a universal finding and the large International Collaborative Ovarian Neoplasia (ICON) 3 trial found no clear benefit from adding paclitaxel to carboplatin and some evidence of worse toxicity (2002). Despite this, the consensus statement on the management of ovarian cancer issued by the Gynaecologic Cancer Inter Group (GCIG) concluded that best practice regimen to which new therapies should be compared should be carboplatin (AUC 5-7.5) and paclitaxel 175mg/m^2/3h given every

three weeks for 6 courses for FIGO stages IIb-IV (du Bois, Quinn et al. 2005). Guidance in the UK currently falls short of recommending this unless the clinician and patient feel that the benefits outweigh the risks (NICE 2003).

Novel chemotherapy delivery and scheduling

Investigating ways of delivering established agents more effectively can be an economical way of improving efficacy.

Intra-peritoneal administration

There is evidence that administering chemotherapy directly into the peritoneal cavity (IP) improves OS in optimally debulked patients and since 2006 the National Cancer Institute (NCI) has recommended that it be considered in such circumstances. Disadvantages include increased toxicity, unfamiliarity with the technology, catheter related problems and a perception of IP administration of chemotherapy as old fashioned (Trimble and Christian 2008). However, a meta-analysis of 8 phase III trials estimated an increase in survival of up to 17 months through administering the platinum component of standard therapy IP (Jaaback and Johnson 2006) and it is likely to become more widely used.

Delaying primary surgery

In cases where optimal debulking of the disease is considered unlikely (due to radiological or clinical findings or patient factors) the option exists to initiate primary chemotherapy and to reassess the case for surgery thereafter, the argument being that surgical debulking might become possible and be more complete in such cases if neo-adjuvant chemotherapy is used (Jacob, Gershenson et al. 1991). The data suggest that neo-adjuvant chemotherapy followed by surgery is no worse than a conventional approach, although survival is worse if surgery is still not attempted following it (Schwartz, Rutherford et al. 1999; Morrison, Swanton et al. 2007). An ongoing randomised, controlled Medical Research Council (MRC) trial – CHemotherapy OR Upfront Surgery in ovarian cancer patients (CHORUS) should determine which philosophy is superior.

Maintenance schedules

Extended courses of single agent cisplatin of up to 12 cycles have not been shown to improve OS or Progression Free Survival (PFS) but do increase toxic side effects (Jaaback and Johnson 2006). However, one randomised phase III trial has looked at continuing paclitaxel over a 12-month period following standard chemotherapy in patients with a complete response to primary therapy and has shown an improvement in PFS. Paclitaxel may work well as a long term drug circumventing platinum resistance though a definite role for using it in this manner is yet to be defined (Markman 2008).

Response to treatment, detection of recurrence and long-term follow-up

Detecting refractory or recurrent disease allows salvage chemotherapy to be initiated at the earliest opportunity. Response to treatment and the detection of recurrence is measured clinically, radiologically and biochemically with guidelines for objectively measuring these parameters having been developed. The first guidelines were developed by the WHO (Miller, Hoogstraten et al. 1981) but updated as technology improved into the Response Evaluation Criteria In Solid Tumours (RECIST) criteria which place emphasis on measuring the tumour volume radiologically in reproducible sites over time (no less than 4 weeks, usually 6-8 weeks). This allows treatment effects to be objectified as either complete response (CR) to treatment (no tumour detected at >4 week intervals), partial response (PR) (>50% decrease in tumour burden), stable disease (SD) (>25% but ≤50% decrease in tumour burden) or progressive disease (PD) (new lesions or >25% increase in existing lesions) (Therasse, Arbuck et al. 2000).

As the miliary nature of ovarian cancer may not be appreciated radiologically, the response to treatment may be assessed by a “second-look” operation. The findings at such procedures correlate with survival though undertaking them does not confer a survival benefit (Nicoletto, Tumolo et al. 1997; Benedet 2000).

A 50% fall in the serum CA125 level from its pre-treatment value in the initial treatment phase which is maintained for at least 28 days indicates a PR to treatment (providing that the initial level was at least twice the

upper limit of normal) while rising post-treatment levels approach 100% specificity for PD. In the context of longer term follow-up, rising serial CA125 levels have been validated, predicting relapse before clinical signs become apparent and having a specificity approaching 98% when there is also a clinical suspicion of recurrence (Rustin, Nelstrop et al. 1992; Rustin, Nelstrop et al. 1996). Some clinicians commence the treatment of recurrent/progressive disease solely on the basis of CA125 levels, arguing that few unnecessary treatments occur (Markman, Rothman et al. 1991; Guppy and Rustin 2002) and an MRC trial (OV05) is under way to assess this management strategy.

The follow-up schedule after the successful treatment of advanced ovarian cancer needs to balance the presumed need to detect recurrence early with the creation of unnecessary patient anxiety. FIGO recommends that follow-up should occur at 4-6 month intervals, after an initial year of three-monthly assessments, followed by annual reviews after 5 years of survival (Benedet 2000). The routine use of radiological tests is eschewed, with their role remaining confined to the investigation of clinically or biochemically suspected recurrence. There remains, however, variation and individualisation of follow-up arrangements as a robust evidence base for a prescriptive schedule is lacking (Vaidya and Curtin 2003).

Treatment of recurrent and unresponsive disease

Though recurrent or refractory disease is incurable, salvage therapies have been developed which prolong survival.

Platinum sensitive recurrent disease

Where an initial response to platinum has been demonstrated and a platinum free interval of >6 months has been observed, recurrent disease may still respond to platinum (Sabbatini and Spriggs 1998). Further courses of platinum based chemotherapy are therefore offered to patients with suspected platinum sensitive disease until it develops resistance or until quality of life issues predominate (Markman 1998). In this scenario standard first line combination chemotherapy has been shown to improve median OS by 5 months and median PFS by three

months when compared to single agent platinum chemotherapy (Thigpen, Blessing et al. 1994; Parmar, Ledermann et al. 2003).

Conventional second line treatment

Disease which progresses during initial therapy or on supplementary courses of first line chemotherapy is termed platinum refractory and platinum resistant respectively. Such cases have the salvage option of using a variety of established second line agents such as paclitaxel, topotecan, etoposide and Pegylated Liposomal Doxorubicin (PLD). However, as no second line regimen has been shown to be superior, the goals of second line therapy seek to balance modest survival gains with quality of life and the need to assess the effect of new treatments in clinical trials (Vermorken 2008).

Novel treatments directed against platinum resistance mechanisms

When intrinsic cellular resistance to platinum occurs, this may only be overcome by developing novel drugs which either circumvent or antagonise the mechanisms involved.

Valspodar abrogates the effect of P-glycoprotein (PgP), which mediates multi-drug resistance (MDR) by encouraging trans-membrane efflux of drug but, so far, only a modest clinical impact has been observed in phase II trials (Fracasso, Brady et al. 2001). Gemcitabine is a false nucleotide which inhibits Excision Repair Cross-Complementation group 1 (ERCC1) mediated DNA repair and acts synergistically with platinum therapies; it has been shown to have some effect in phase II studies and is being evaluated in the phase III ICON5 trial (Lorusso, Di Stefano et al. 2006). Decitabine reverses the epigenetic hyper-methylation of DNA about the Mut-L -Homolog 1 gene (MLH1) that can lead to its silencing, the incorrect functioning of the DNA MisMatch Repair (MMR) family of genes and then platinum resistance. Similarly, DNA polymerase alpha inhibitors disrupt the function of the NER (Nucloetide Excision Repair) pathway that confers platinum resistance through the repair of the platinum DNA adducts that trigger apoptosis. However, both of these drug types have currently only been completely

assessed in phase I trials (Sessa, Zucchetti et al. 1991; Sargent, Elgie et al. 1996; Appleton, Mackay et al. 2007). Over-expression of the ErbB2 and ErbB1 tyrosine kinase members of the Epidermal Growth Factor Receptor (EGFR) signalling family are implicated in the progression of cancer and have been shown by some groups to be associated with poorer outcome in ovarian cancer (Lassus, Leminen et al. 2004; Psyrri, Kassar et al. 2005). Small molecules targeting this pathway such as Erlotininb have shown only marginal effects in ovarian cancer so far (Gordon, Finkler et al. 2005). Many chemoresistant tumours exhibit resistance to a range of agents with the final common pathway of apoptosis with one of its most important regulators, p53, being associated with chemoresistance in ovarian cancer when the non-functional mutant form is expressed (Righetti, Della Torre et al. 1996). Therapies specific to cells lacking functional p53 (Onyx-015 oncolytic adenovirus) or directed at reviving functional p53 expression in mutant p53 expressing cells (Adenoviral vector p53 – Ad-p53) have been shown to be safe and to exert a modest effect (Buller, Shahin et al. 2002; Vasey, Shulman et al. 2002).

The development of drug resistance depends upon the production of a population of resistant cells. This may occur due to the selection and then proliferation of intrinsically resistant clones by therapy but, as cytotoxic chemotherapy is targeted against rapidly dividing cells, potentially sensitive cells outside the cell cycle will also show a degree of chemo-resistance. This may help explain the often poor translation of laboratory findings into the clinic. Developing therapies designed to target quiescent cells is an important field of research, as is the development of predictive markers that might inform first line therapies for individual patients (Agarwal and Kaye 2003; Agarwal, Linch et al. 2006).

1.1.6. Survival

Reporting survival data

Various forms of survival data exist against which the prognostic effect of age, histological cell type, histological grading, stage of disease, the

use of adjuvant treatment and the expression of established and novel biomarkers can be assessed.

The simplest data are whether the patient is alive or dead. This is crucial and easily confirmed but on its own does not discriminate between those who die as a result of cancer and those who die of other causes such as peri-operative events, accidents, co-morbidities and “old age". While accurate peri-operative mortality data are objectively generated (percentage of patients who die within 28 or 30 days of surgery), objective data regarding specific cause of death are more difficult to compile, given that the disease specific causes of death are many and subjective (malignant bowel obstruction, inadequate nutrition and electrolyte imbalances, obstructed urinary system, thromboembolic disorders, respiratory complications, etc) and the accuracy of data collected from registries and death certificates is inconsistent (Gagliardi, Fung et al. 2005). Despite such issues, cause specific survival data are key in calculating standard comparable measures of disease specific deaths such as fatality and mortality rates. The fatality rate is the percentage of cases dying from the disease and the mortality rate is the annual number of deaths from a disease per year/100 000 population at risk. European Standard Rate (ESR) mortality is the same measure age-adjusted to reflect the average European population, allowing meaningful direct comparison between regions. A measure of survival which accounts for deaths from other causes without a need to measure cause specific deaths is the relative age-adjusted survival rate. This compares the percentage of deaths within a cohort of patients with the percentage of deaths in the aged matched unaffected population at a fixed time point following treatment. This returns a ratio of two percentages, itself expressed as a percentage – the relative age-adjusted 1-, 3- or 5-year survival.

The length of time between treatment and death from any cause is termed the Overall Survival (OS), with the median or mean value being quoted for cohorts of patients. Using a fixed time point, 1-, 3- or 5-year OS can be expressed as a percentage of the total cohort in a crude non-adjusted manner. Large epidemiological studies most commonly

use relative, age-adjusted, standardised data, whereas studies comparing molecular marker expression with prognosis within a series most commonly use crude OS data (Quinn 2001). Crude OS data have the advantage of being easily acquired and objective and are clinically useful for patients, as they are interested in their chances of being alive at a future point rather than their chances of dying from a specific cause.

Trends in survival

Ovarian cancer represents 4% of cancers in women but accounts for 6% of all cancer deaths and this disproportion is due to its high fatality. Overall ESR mortality in 1997 was 11.6/100 000 in the UK and this has altered little over the last 50 years. Though mortality increases with age (as for all cancers), when this is corrected for ovarian cancer mortality also varies by decade of birth, with cohorts up to the 1930s having a higher mortality than the after coming ones (Quinn 2001). Peri-operative mortality has not been systematically reported for individual ovarian cancer cases but retrospective figures in the literature suggest a range of 0-5% depending on the size and co-morbidity of the population (Jalêuvka 1980; Elit, Bondy et al. 2002; Ben-Ami, Vaknin et al. 2006).

The observed country-weighted 1- and 5-year survival for ovarian cancer in Europe for patients diagnosed from 1990-1994 (Eurocare3) were 65% and 34% respectively with relative survival equivalents of 66% and 38%. The low proportions surviving to 5 years and the small size difference between observed and relative data illustrate the high fatality of the disease. For the UK the relative age-adjusted 1- and 5-year survival data were 59% and 32% compared to Austria (best outcomes) – 81.9% and 49.3% – and Estonia (worst outcomes) – 53.3% and 25.6%. These marked differences in survival data across Europe (which persist in Eurocare4) are thought to be due to variations in the stage at diagnosis, the inclusion or exclusion of borderline cases, the quality of registry data and the quality of cancer care (Sant, Aareleid et al. 2003; Berrino, De Angelis et al. 2007). There is some evidence

from Eurocare4 that relative 5-year survival data for the UK may be improving slightly though its survival figures still compare unfavourably to the rest of Europe when the former Soviet and Eastern Bloc countries are discounted (Berrino, De Angelis et al. 2007).

These survival data are adjusted for age and background mortality but are not corrected for the influence of independent prognostic factors such as FIGO stage (Gospodarowicz 2001). This is important, as survival between stages and the distribution of stage of disease geographically varies considerably. Reviewing international series of ovarian cancers that included patients presenting within the period 1982-1997 reveals relative 5-year survival figures of 76-93% for stage I, 42-70% for stage II, 21-59% for stage III and 6-25% for stage IV (Table 1.1) (Hand, Fremgen et al. 1993; Yancik 1993; Ilekis, Gariti et al. 1997; Wolfe, Tilling et al. 1997; Brun, Feyler et al. 2000; Holschneider and Berek 2000; Heintz, Odicino et al. 2001; Quirk and Natarajan 2005). However, on an individual level, even when age, stage, the use of chemotherapy and the degree of residual disease are corrected for, seemingly identical cases continue to have divergent outcomes and this points to the existence of other as yet undiscovered independent prognostic (probably molecular) factors which may also help explain the regional variations seen in outcome.

Overall, a review of the evidence compiled by FIGO indicates that 5-year survival improved internationally from 27% (1958-1962) to 35% (1982-1986) and then dramatically to 48% (1993-1995) following the introduction of platinum based regimens, this improvement occurring for all stages (Heintz, Odicino et al. 2001).

1.2. Immunological biomarkers

The purpose of this section is to outline why biomarkers are researched, the logic behind selecting particular biomarkers for investigation, why tissue microarrays may be employed, the importance of the immune system in human cancers and the rationale behind investigating biomarkers of an immunological nature in ovarian cancer.

1.2.1. Biomarkers in general

1.2.1.1. The importance of prognosis and prognostic factors

Making a prognosis necessitates reasonably forecasting the course, pattern, progression, duration and termination of a disease for an individual patient (Gospodarowicz 2001).

Cancer is a heterogeneous disease and prognostic factors are the variables which account for differences in the expected course of the disease that this produces. Commonly used prognostic factors in oncology can be split into tumour, host and environmental factors. In ovarian cancer they include stage and degree of cytoreduction (tumour), performance status/age (host) and the delivery of optimal treatment (environmental/geographic). Though factors associated with variation in response to treatment are termed predictive rather than prognostic, the predicted response to treatment remains the de facto prognostic factor upon which patients rely in making a decision whether to undergo or decline treatment. (Henderson and Patek 1998). The foremost role for prognostic factors is in counselling patients. However, a knowledge of them also aids learning from experience, informs the allocation of public health resources and allows outcome end points (PFS/OS) to be meaningfully compared between centres whilst additionally identifying patients who may benefit from novel therapies most. Furthermore, applying equal stratification for known prognostic factors within all experimental arms in clinical trials allows for the effect of the intervention to be more independently assessed (Gospodarowicz 2001). New independent prognostic factors are those which predict prognosis in a way which does not depend upon an association with a currently clinically employed independent 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 power may therefore reveal the importance of a particular intracellular process and identify a target to which novel therapy can be developed and directed. However, for a molecular marker to make the transition to the clinic it must be externally

validated, employ reproducible assays, have substantial predictive or prognostic power and have therapeutic implications. Therefore, the systematic study of molecular markers demands a logical approach rooted in the biology of cancer (Gospodarowicz 2001).

1.2.1.2. The hallmarks of cancer

The American Cancer Society (ACS) defines cancer as a group of diseases characterised by uncontrolled growth and the spread of abnormal cells which, if not controlled, can result in death. The essential properties or hallmarks of cancer which lead to this type of behaviour comprise self sufficiency regarding growth signalling, insensitivity to inhibitory growth signals, evasion of apoptosis, limitless replication potential, sustained angiogenesis, tissue invasion and metastasis (Hanahan and Weinberg 2000). The drivers of this behaviour are molecular.

1.2.1.3. Examples of molecular processes involved in carcinogenesis

A cancer cell may take control of its own growth by upregulating its secretion of growth signals, instructing nearby cells to secrete growth factors in a paracrine fashion and by upregulating its expression of growth factor receptors such as Erythroblastic leukemia viral oncogene homolog 2 (ErbB2) (Skobe and Fusenig 1998; Yarden and Sliwkowski 2001). A major effector mechanism of anti-proliferative signals is the Retinoblastoma protein (pRb) which blocks cell cycle progression whilst unphosphorylated. As phosphorylation of this protein is controlled by Transforming Growth Factor β (TGFβ), disruption of its receptor, loss of pRb itself or loss of intermediate signalling molecules can all lead to loss of responsiveness to anti-proliferative signals (Fynan and Reiss 1993). Apoptosis balances cell proliferation by causing cells to dismantle themselves in response to effector stimuli such as the binding of fasLigand (fasL) to its receptor. Apoptosis is under the control of numerous intracellular mediators, of which two of the most important are the p53 tumour suppressor gene and Bcl-2. The disruption of these can lead to an acquired resistance to this process (Wyllie, Kerr et al.

1980). The majority of cell populations have a finite ability to divide mediated partly by a shorting of telomeres which occurs at each division (Hayflick 1997). Most cancers (91% of ovarian) exhibit telomerase activity, an enzyme which maintains telomere length through telomeric DNA synthesis. It is thought that this contributes to the development of immortality (Shay and Bacchetti 1997). Tumour growth is disordered and with cells often lying >100μm from capillary vessels, hypoxia is common. Tumours attempt to correct this using angiogenic factors (Hanahan and Weinberg 2000). Vascular Endothelial Growth Factor (VEGF) is a cytokine which is a stimulus for angiogenesis and which is commonly secreted by neoplastic cells. It stimulates neovascularisation through binding to VEGF receptors (VEGFR) on endothelial cells, relieving hypoxia (Neufeld, Tessler et al. 1994; Bamberger and Perrett 2002). Successful proliferation, immortality and angiogenesis leads on naturally to invasion and metastasis, the cause of the majority of cancer deaths (Sporn 1996). Disruption of cell adhesion molecules such as E-Cadherin and the upregulation of protease producing genes are key processes by which the cancer cell may manipulate its immediate environment to achieve its invasive and metastatic potential (Werb 1997; Semb and Christofori 1998).

These molecular processes are examples of the many routes through which cancer cells can develop these 6 key abilities, a situation favoured by their genetic instability. This predisposes their genome to mutational events which otherwise occur only rarely and is engendered by the disruption of DNA repair mechanisms (Lengauer, Kinzler et al. 1998).

1.2.1.4. Biomarker research strategy

The NIH defines a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”. With respect to cancer, studying the molecular changes associated with the development of its hallmarks is the logical way of

searching for molecular biomarkers which may have independent prognostic power that may thereafter direct clinical and research efforts. Molecular markers may be found in bodily fluids (urine, saliva, serum, etc), cancer cell lines or cancer tissue (fresh, frozen, Formaldehyde Fixed Paraffin Embedded [FFPE]). When researching post-surgical prognostication, the search for biomarkers expressed in tissue is a common strategy. Molecular alterations associated with cancer may be studied along any point of the axis leading from gene (genomics/ epigenomics), through its transcription (transcriptomics) to the final protein responsible for cell function (proteomics) (He and Chiu 2003). The traditional starting point is the screening of the genome for alterations which can be followed through to the protein level. The disadvantage of this strategy is that many aberrations in the genome are not represented at the protein level, with single genetic aberrations in sporadic cancers usually requiring a second genomic “hit” before a cellular impact becomes evident (Knudson 1971). Variation in post-translational modification of peptides also means that single genomic changes map to a wide number of potential proteomic alterations (Cho 2007). Working backwards from the discovery of abnormal protein expression is the pragmatic alternative and several experimental platforms exist with which to investigate protein expression in cancer samples, including 2D-Gel Electrophoresis (Western blot), Enzyme Linked Immunosorbant Assay (ELISA), Mass Spectrometry (MS), protein microarrays and IHC, the uses of which are often complementary (Petricoin and Liotta 2002; Park, Kerbel et al. 2004). Regarding IHC, the high-throughput platform developed over recent years to survey large populations for prognostic biomarkers is the Tissue MicroArray (TMA) (Chung, Braunschweig et al. 2007).

1.2.1.5. The TMA platform

The development of TMAs (Figure 1.2)

Traditional experimental immunohistochemistry involves the staining of whole sections of tumour tissue. As only 20-40 slides can be stained by an average worker per day, in the past it was only possible to

Figure 1.2 The evolution of multi tumour tissue blocks (MTTB)

Please refer to Figure 2; Eguiluz, C., E. Viguera, et al. (2006). "Multitissue array review: a chronological description of tissue array techniques, applications and procedures." Pathol Res Pract 202(8): 561-8.

accumulate data for large cohorts over a number of experimental runs and so, historically, many studies were often either small and underpowered or subject to considerable intra- and inter-observer variability due to variation in experimental conditions. As the use of whole sections quickly renders a tissue block either unusable or non-diagnostic (many sections are discarded before one high quality section is acquired), work has to be performed in a very coordinated manner between groups in order to preserve tissue. A further disadvantage of the whole section approach is that each slide requires a full set of expensive reagents.

To address these points the principle of transferring small samples from many archived tumour blocks into a single Multi Tumour Tissue Block (MTTB) block was explored and first described in 1986 (Battifora 1986). This method involved deparaffinising archived tissue, cutting 1mm wide slivers from multiple specimens and then tying them together in a bundle before re-embedding them in wax. The result was termed a “sausage block”, due to its cross sectional appearance. This proved effective at reducing the amount of monoclonal antibody required but was only useful for quality control as individual tumours could not be identified. This technique was refined to the “chequerboard” block where the location of tumours was known (Battifora and Mehta 1990) but was ultimately superseded by a different method of producing a MTTB, developed by Wan and co-workers (Wan, Fortuna et al. 1987). This involved boring tissue cores from archived blocks using a 16-gauge needle, without deparaffinisation. More tumours could be re-embedded but tumour identification was still not possible. The following year Kraaz and colleagues described a technique of removing cores using a skin punch biopsy tool and then pushing them into known locations in a warm paraffin block though, due to tissue size, only 20 samples could be transferred (Kraaz, Risberg et al. 1988). The major advance came when Kononen and co-workers described the use of a custom built instrument (Tissue Microarrayer – Beecher instruments, Sun Prairie, WI, US) which allowed 0.6mm wide cylindrical tissue cores to be punched out from archived blocks and transferred immediately to

holes precisely punched out at known coordinates in a recipient paraffin block (Kononen, Bubendorf et al. 1998).

TMAs are now usually constructed using this method with up to 1000 cores being transferred into known locations without the need for deparaffinisation. As 3-4mm long cores are transferred and 4-8µm thick sections are cut, 200 slides can theoretically be obtained from each TMA block with each stained section providing data about many hundreds of cases for a given marker (Kononen, Bubendorf et al. 1998).

Validation of TMAs

Studies validating the use of TMAs aimed to show that the biochemical characteristics of a population of tumours as predicted by staining a TMA of small tumour biopsies would be the same as that predicted by whole sections.

Konnen and colleagues originally validated TMA use in breast cancer. Three hundred and seventy-two cancer tissue samples were assembled into a TMA and amplifications in three breast cancer oncogenes, ErbB2, MYC and Cyclin D1 (CCND1), were detected which concurred with published data. They also observed corresponding upregulations of mRNA and protein expression upstream. On examining ER, an 84% concordance was seen between the immunohistochemically and biochemically determined ER content – as would have been expected if whole sections had been used. The authors concluded that, despite the theoretical problem of using small samples of tissue to examine tumours which might express a marker heterogeneously, this technique could be used to produce scientifically valid results in place of whole sections as long as representative samples were selected. TMAs have subsequently been validated for use in prostate cancer (Mucci, Akdas et al. 2000), lymphoma (Hedvat, Hegde et al. 2002), gastric cancer (Gulmann, Butler et al. 2003), bladder cancer (Nocito, Bubendorf et al. 2001) and colorectal cancer (Fernebro, Dictor et al. 2002). The use of TMAs in ovarian cancer was validated by Rosen and co-workers. They stained 6 copies of a TMA

containing 45 ovarian cancers for Ki-67, mutant p53 and ER. A concordance of 98% was seen between the results from the whole sections and the TMA on analysing three cores, which did not increase on examining more copies. Overall agreement of expression data with that in published studies was also observed (Rosen, Huang et al. 2004).

1.2.1.6. Trastuzumab in breast cancer (Herceptin)

The development of Herceptin highlights the importance of biomarker discovery and how this can be translated into measurable clinical improvements (Shepard, Jin et al. 2008). ErbB2 (Her2/Neu) is a gene which encodes for a tyrosine kinase receptor implicated in cell proliferation, cell survival, angiogenesis and cell adhesion (Shepard, Jin et al. 2008). Its upregulation was found to be present in 20-30% of breast cancers and to be an independent prognostic and predictive factor (Slamon, Clark et al. 1987). Over-expression was subsequently found to be associated with the transformation of cells into a Tumour Necrosis Factor (TNF) α/macrophage resistant phenotype. However, this could be reversed using a murine monoclonal antibody (mAb) directed at the extracellular domain of ErbB2 (Hudziak, Lewis et al. 1989). Subsequently, Herceptin, a humanised version of this murine anti-$p185^{HER2}$ mAb, was developed. Herceptin mediates an anti-proliferative effect through its target receptor and the initiation of Antibody Dependent Cellular Cytotoxicity (ADCC) (Carter, Presta et al. 1992). Clinical trials subsequently established the clinical role of Herceptin as a first line agent in node positive, immunohistochemically strongly ErbB2 expressing breast cancers (Vogel, Cobleigh et al. 2002; Romond, Perez et al. 2005).

1.2.1.7. Tissue expressed proteomic biomarkers in ovarian cancer

Candidate markers

A large number of tissue proteomic biomarker studies continue to be performed and promising candidates, including Mucin4 (MUC4), MatrixMetalloProteinase4 (MMP4), EPithelial Cell adhesion molecule (EpCam), CD24 and ErbB3, have been recently identified (Gagnon and

Ye 2008). Four of the most commonly studied molecular markers historically have been Epidermal Growth Factor Receptor (EGFR), ErbB2, Glutathione S-Transferase-pi (GSTpi) and mutant p53, which were reviewed by Crijns and co-workers in 2003 (Crijns, Boezen et al. 2003).

Of 11 studies reviewed concerning Epidermal Growth Factor Receptor (EGFR), in univariate analysis one showed a correlation with improved prognosis, four a correlation with worse survival and one a correlation with reduced response to chemotherapy; in multivariate analysis three studies showed EGFR to be an independently negative prognostic factor. Of 16 studies reviewed concerning ErbB2, in univariate analysis 8 showed a correlation with worse survival and three with a poorer response to chemotherapy; in multivariate analysis three showed ErbB2 to be an independently negative prognostic factor. Of 14 studies reviewed concerning GSTpi, in univariate analysis 5 showed a correlation with worse survival and worse response to chemotherapy. No study found it to be an independent prognostic factor. Of the 42 studies reviewed concerning mutant p53, in univariate analysis 24 showed a correlation with worse survival, 7 a correlation with shorter PFS, one an association with improved response to chemotherapy and 7 with worse response to chemotherapy; in multivariate analysis 12 studies showed mutant p53 expression to be a negative independent prognostic factor, with two showing it to have independent predictive power – one for a good response to chemotherapy and one for a poor response.

Translational difficulties

An ideal biomarker is linked to a biological phenomenon associated with the development of cancer, is easily detectable, is reproducible, has independent prognostic power, predicts outcome and response to treatment, has clinical relevance and is validated by several groups independently of each other (Gasparini, Pozza et al. 1993). However, as shown, the literature for biomarker studies is often inconsistent, with conflicting associations being the norm and the difficulty meeting these

criteria with the available evidence means that the only biomarker used clinically in ovarian cancer remains post-treatment serum CA125 (Agarwal and Kaye 2006). Efforts to standardise the reporting of biomarker studies may improve this, as the lack of a common systematic approach to biomarker studies has been cited as the most likely cause of the many unrepeatable, small, positive studies which confound the literature (McShane, Altman et al. 2006). However, as standard treatment is given to virtually all cases of ovarian cancer, and as few rational first line alternatives are currently available, part of the reason that the aggressive translation of markers into the clinic has not been pursued may be a perception that they will not influence the clinical decision making (Agarwal and Kaye 2005).

Despite this, the search for independent prognostic markers of prognosis remains important, though the logical selection of research markers is essential. Candidates should be biologically and clinically relevant and expected to have prognostic and therapeutic implications. Immunological molecular markers fulfil this brief.

1.2.2. The immune system in general

1.2.2.1. The innate immune system

Innate immunity non-specifically targets harmful agents which have not been encountered before. It results in a rapid, intense, generic response which is mediated by both cellular and humoural elements.

1.2.2.1.1. Cellular elements

Phagocytes

Macrophages and neutrophils are the main cells tasked with engulfing and destroying foreign material. They recognise the Pathogen Associated Molecular Patterns (PAMPs) expressed by foreign material through their expression of Pattern Recognition Receptors (PRR). PAMPs are molecular signatures characteristic of particular invaders such as the flagellin in bacteria and the double stranded DNA in virally infected cells. PRRs recognise particular PAMPs and comprise three main groups: Toll-Like Receptors (TLRs), C-type lectins and the

LipoPolySaccharide (LPS) binding CD14 scavenger molecule. The interaction between PRR and PAMP initiates phagocytosis, the release of cytokines and the acute inflammatory response, which attracts cells of the adaptive immune system to the site. The phagocyte employs a variety of mechanisms to effect actual cell killing, including the superoxide anion, nitric oxide, defensins, lysozyme and proteolytic enzymes (Roitt and Delves 2006).

Natural Killer (NK) cells

NK cells are lymphocytes defined by the expression of CD56 and the absence of expression of CD3 and are crucial effectors of innate immunity. NK cells kill and release cytokines without specific sensitisation and to prevent normal tissue from initiating this process they are tightly regulated through the expression of surface NK Receptors (NKRs). Several types of receptor exist, including Killer Immunoglobulin (Ig)-like Receptors (KIRs), the C-type lecthin family (including NKG2 and CD94), Ig-Like Transcripts (ILTs) and Natural Cytotoxicity Receptors (NCRs). NKRs may be inhibitory or activatory on binding, with the balance between the two determining the overall response. Generally, KIRs favour a state of inhibition unless the Human Leukocyte Antigen (HLA) class I allele that they bind to is absent or lost on a given cell (Vales-Gomez, Reyburn et al. 2000).

An activated NK cell kills by triggering apoptosis through the initiation of the capsase cascade. This is in part mediated by the release of perforin, which creates pores in the target cell's membrane through which pro-apoptotic factors such as granzyme B may pass (Hudig, Ewoldt et al. 1993). Apoptotic stimuli may also be transduced through the binding of fasL and TNF (Tumour Necrosis Factor) Related Apoptosis Inducing Ligand (TRAIL) to the corresponding death receptors on the target cell (Screpanti, Wallin et al. 2005). NK cells are also a rich source of tumouricidal cytokines such as interferon (IFN) ɣ, TNFβ, IL (Interleukin)-10, IL-13 and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF). NK cells may be either predominantly cytotoxic or cytokine producing dependent upon on their relative

expression of CD56 and CD16 (Cooper, Fehniger et al. 2001; Cooper, Fehniger et al. 2001; Roitt and Delves 2006).

Gammadelta (γδ) T cells

γδ T cells are distinct from αβ T cells as they respond to antigen without a need for its presentation with HLA molecules. The TCR in this subclass exhibits increased diversity and these cells each recognise a range of epitopes, lessening the need for clonal expansion when abnormal tissue is encountered. These cells are thought to play a role in the removal of stressed or damaged cells. It has been noted that mice deficient in this cell type are predisposed to the development of tumours (Girardi, Oppenheim et al. 2001; Hayday and Tigelaar 2003; Raspollini, Castiglione et al. 2005; Roitt and Delves 2006).

1.2.2.1.2. Humoural elements

Cytokines, chemokines and acute phase proteins

Cytokines are low molecular weight proteins which are secreted by T helper (Th) cells, NK cells, mast cells, stromal cells and monocytes. They consist of several families, including interleukins (IL1-33), Colony Stimulating Factors (CSFs), TNFs and IFNs, and they modulate the immune response on a local level. Typical actions include the control of cellular differentiation, the upregulation of receptors, the initiation of an inflammatory response, anti-tumour and anti-viral activity, direct cytotoxicity and the priming of the adaptive immune response. Chemotactic cytokines attract cells to areas of need, while IL-1 and IL-6 cause the release of acute phase proteins. Acute phase proteins such as C- Reactive Protein (CRP) and Mannose Binding Lectin (MBL) coat non-self material, opsonising them and fixing complement.

Complement (C)

Complement refers to a triggered enzyme system of more than 30 plasma proteins which mediate opsonisation, chemotaxis, activation of leukocytes, lysis of foreign cells, augmentation of antibody responses, enhancement of immunological memory, the disposal of immune complexes and the clearance of apoptosed cells.

Complement cascade

The key step in the activation of complement is the production of C3 convertase which formally cleaves the abundant component C3 into two pieces, C3a (small fragment) and C3b (large fragment). C3b then complexes with other complement components to form C5 convertase. This cleaves C5 into C5a and C5b with C5b then sequentially binding C6, C7 and C8 to form C5b-8 on the target cell membrane. This in turn facilitates the deposition and polymerisation of C9 to form a transmembrane channel termed the Membrane Attack Complex (MAC), which lyses the cell (Figure 1.3) (Muller-Eberhard 1986; Walport 2001).

The formation of MAC is the final common pathway of the complement cascade but the production of the convertases is driven by three distinct pathways, each activated in a different way.

The classical pathway

The classical pathway is initiated by antibody-antigen complexes (IgG and IgM), the presence of apoptotic cells, gram negative bacteria and the presence of CRP binding. C1 is activated by these stimuli, cleaving C2 and C4 into C2a, C2b, C4a and C4b. C4b and C2a then complex to form C3 convertase which complexes to the product of its action, C3b, to form C5 convertase (Figure 1.3).

The alternative pathway

C3b is continually produced by spontaneous hydrolysis in plasma but is normally converted into its inactive iC3b form by the presence of factors I and H. In the alternative pathway, factor b expressed on the surface of bacteria and fungi binds this C3b and forms C3 convertase under the influence of factor D. C3b production then enters a positive feedback loop amplifying the production of C3 convertase and C3b. These combine to form C5 convertase, leading to the formation of MAC (Figure 1.3).

The MBL pathway

The MBL pathway mediates the cleavage of C2 and C4 in a similar manner to the classical pathway and is initiated by the binding of the acute phase protein MBL to the terminal mannose groups present on the surface of bacteria. This forms complexes with MBL Associated

Figure 1.3 The complement cascade

A schematic illustrating how the classical, MBL and alternative pathways initiate and amplify the complement cascade and the final common pathway of MAC assembly which ultimately leads to cell lysis. The classical pathway is initiated by the presence of antigen-antibody complexes and the alternative pathway by the presence of factor B on the surface of pathogenic material.

Please refer to Figure 1; Walport, M. J. (2001). "Complement. First of two parts." N Engl J Med 344(14): 1058-66.

Serine Protease (MASP) 1 and 2; MASP2 then cleaves C2 and C4 with MASP1 cleaving C3 directly. The convertases are then produced in an analogous manner to the classical pathway (Figure 1.3) (Makrides 1998).

Anaphalaxtoxins

The small fragments C3a, C4a and C5a are called anaphalatoxins and mediate the acute inflammatory response to complement activation by causing mast cells to release histamine, chemokines, interleukins, leukotrienes, prostaglandins and thromboxanes. These mediators result in smooth muscle relaxation, increased vascular permeability and the recruitment of appropriate cells to the site of complement activation. C5a is the most potent of the three (Roitt and Delves 2006) (Gerard and Gerard 1994; Liszewski, Farries et al. 1996; Hartmann, Henz et al. 1997).

Complement regulation

Complement is a powerful humoural element of the innate immune system and, as its action is self perpetuating once triggered, a number of mechanisms exist to prevent damage to healthy normal tissue.

There are numerous soluble factors involved in regulating the activation and deposition of complement and MAC, such as C1 inhibitor, factor I, factor H, C4 binding protein, properdin, S protein, and clusterin (Liszewski, Farries et al. 1996). Cells may also express four membrane-bound complement regulatory proteins (mCRPs), CR1, CD46, CD55 and CD59. These are encoded for by genes lying within the Regulators of Complement Activation (RCA) cluster at 1q3.2 (Liszewski, Farries et al. 1996).

CD35

CR1 is a large 160-260 KDa glycoprotein which binds to C3b and C4b acting as a co-factor for their factor I mediated breakdown. The distribution of CR1 is restricted to erythrocytes and leukocytes with some expression on peripheral nerve cells and glomerular podocytes. Having a higher affinity for C3b than for C4b, it has a greater effect on the alternative pathway (Fearon 1979; Morgan and Meri 1994).

CD46

Also known as Membrane Co-factor Protein (MCP), this is a 48–68 KDa glycoprotein comprising four Short Consensus Repeats (SCRs), a Serine Threonine Proline (STP) rich region, a transmembrane domain and a cytoplasmic tail (Figure 1.4). It is expressed on the majority of cells, with the exception of erythrocytes, and it controls the availability of C3b through its action as a co-factor for its factor I mediated inactivation to iC3b. By controlling this precursor, it controls the levels of the C3 and C5 convertases produced by the alternative pathway (Figure 1.5) (Cole, Housley et al. 1985; Liszewski and Atkinson 1992; Morgan and Meri 1994). Additional roles of CD46 in intracellular signalling, the differentiation of naïve T cells into regulatory cells and in reproduction have also been suggested and the deficiency state is linked with the inherited predisposition to the development of Haemolytic Uraemic Syndrome (HUS) (Riley-Vargas, Gill et al. 2004).

CD55

Also known as Decay Accelerating Factor (DAF), CD55 is a 70-80 KDa glycoprotein featuring four SCRs, an STP rich region and a Glycosyl Phosphatidyl Inositol (GPI) anchor with which it attaches to the cell membrane (Figure 1.4). CD55 is widely distributed and present on all haemopoetic cells, vascular endothelium and epithelia of the gastrointestinal and genitourinary tracts. It accelerates the decomposition of C3 convertase in both the classical and alternative pathways by causing C2a and Bb to dissociate respectively (Figure 1.5) (Hoffman 1969; Medof, Walter et al. 1987; Morgan and Meri 1994; Nicholson-Weller and Wang 1994; Liszewski, Farries et al. 1996). There is evidence that CD55 may also inhibit NK cell lysis and apoptosis while its action as a ligand for CD97 may promote invasiveness, metastasis and angiogenesis in the tumour environment (Mikesch, Buerger et al. 2006). A lack of membrane bound CD55 is associated with the disease Paroxysmal Nocturnal Haemoglobinuria (PNH) (Jarva and Meri 1999).

Figure 1.4 The structure of the membrane bound complement regulatory proteins

Short Consensus Repeats (SRCs) are represented by square blocks. The Glycosyl Phosphatidyl Inositol (GPI) anchors of CD59 and CD55 are illustrated along with the transmembrane spanning domain of CD46 inserting into the cytosol (CY) and the Serine Threonine Proline rich areas of CD46 and CD55 (ST).

Please refer to Figure 2; Makrides, S. C. (1998). "Therapeutic inhibition of the complement system." Pharmacol Rev 50(1): 59-87.

Figure 1.5 The site of action of the mCRPs

CD55 (DAF) accelerates the decay of the C3 convertases, CD46 (MCP) acts as a co-factor for the factor I mediated conversion of C3b to iC3b and CD59 binds to the C5b-9 complex, preventing the polymerisation of C9 and the assembly of the MAC.

Please refer to Figure 2; Gasque, P. (2004). "Complement: a unique innate immune sensor for danger signals." Mol Immunol 41(11): 1089-98.

CD59

Otherwise known as protectin, CD59 is an 18-20 KDa single chain glycoprotein which links to the cell surface via a GPI anchor (Figure 1.4). CD59 is widely expressed on the majority of cells, including circulating cells, epithelia and endothelium, and it prevents the assembly of the MAC by binding the C5b-8+9 complex and preventing the polymerisation of C9 (Figure 1.5-6). The global deficiency of GPI in PNH means that membrane bound CD59 deficiency is also seen in this disease (Sugita, Nakano et al. 1988; Meri, Morgan et al. 1990; Meri, Morgan et al. 1990; Morgan and Meri 1994; Liszewski, Farries et al. 1996). Possible complement independent roles may include tyrosine kinase and LPS signalling and the regulation of NK-, T- and B-cells (Kimberley, Sivasankar et al. 2007). Nucleated cells may also resist the MAC mediated cell lysis through CD59 independent ways including membrane repair systems, mechanisms which physically remove MAC and intracellular metabolic processes (Morgan 1989).

1.2.2.2. The adaptive system

The adaptive immune system deals with pathogens which evade innate responses and produce a more specific response to foreign invasion. As with the innate immune system, both humoural and cell-mediated elements contribute.

1.2.2.2.1. Humoural elements

Antibodies

Antibodies, or immunoglobulins, bind the unique antigenic determinants of particular foreign organisms and initiate immune responses specifically directed against them. The antibody consists of two identical protein light chains and two identical protein heavy chains, often represented pictorially as a “Y” shaped molecule, the stem of which is called the Fragment crystalline (Fc) unit and each arm of which is termed a Fragment antigen binding (Fab) unit. The Fc unit binds effector molecules while the Fab units have regions which vary highly between antibodies and which each bind antigenic determinants unique to each antibody (Figure 1.7). Immunoglobulins come in 5 classes, IgG,

Figure 1.6 **The function of CD59**
A more detailed schematic illustrating how CD59 obstructs the unfolding and polymerisation of C9, thus preventing MAC assembly and cell lysis.

Please refer to Figure 2; Gorter, A. and S. Meri (1999). "Immune evasion of tumor cells using membrane-bound complement regulatory proteins." Immunol Today 20(12): 576-82.

Figure 1.7 The antibody structure
The immunoglobulin is made up of two identical heavy (H) and light (L) peptide chains which have both constant (C) and variable regions (V). The fragment crystalline (Fc) bears carbohydrate regions and is responsible for mediating the effector response whereas the Fragment antigen binding (Fab) recognises and binds antigen.

Please refer to Figure 2; Boenisch, T., A. J. Farmilo, et al., Eds. (2001). Handbook: Immunohistochemical Staining Methods. Gostrup, Denmark, Dako.

IgM, IgE, IgA and IgD. Each differs regarding its Fc unit with each triggering different effector mechanisms. The principal serum form of antibody is IgG, a monomer which comes in four subclasses, IgG1, 2, 3 and 4. Each fixes complement when complexed to antigen, with IgG3 performing this more potently than IgG1 and both performing this more potently than the other subclasses. Effector cells express Fc receptors for particular classes of immunoglobulin and antibodies therefore trigger specific immune responses through both cell mediated and humoural means. Each specific antibody originates from a single B-cell genetically predetermined to recognise a specific antigen (Roitt and Delves 2006).

1.2.2.2.2. Cellular elements

B-cells

B-cells develop in bone marrow and undergo a series of steps during their maturation which are associated with rearrangements within the genes encoding the variable regions of their surface immunoglobulin – the B-cell receptor (BCR). On encountering its corresponding antigen, BCR binding signals the B-cell to internalise the antigen, process it and express it complexed to HLA class II. B-cells may subsequently develop further into plasma cells, which can clonally expand to become many cells producing identical immunoglobulin in large quantities (Defrance, Casamayor-Palleja et al. 2002; Gold 2002; Roitt and Delves 2006).

Human Leukocyte Antigen

HLA is the human form of Major Histocompatability Complex (MHC) and its gene group on chromosome 7 encodes for three classes of molecules.

<u>HLA Class I region</u>

HLA class I itself is a heavy chain 44KDa polypeptide molecule consisting of three globular helical domains (α_1, α_2, and α_3) which are expressed on the cell surface and which inserts into the cytosol through the cell membrane. Its heavy chain is always associated with a lighter β2microglobulin (β2m) chain and three different class I antigens (-A, -B

and -C) are expressed, each of which displays marked allelic variation between individuals (Figure 1.8a).

HLA class I is expressed on virtually all cells and is responsible for the presentation of intracellular peptides associated with abnormal cellular conditions. Peptide undergoes processing by the proteosome and is transported by Transporters associated with Antigen Processing (TAP) 1 and 2 through the endoplasmic reticulum and Golgi apparatus to the cell surface. During this process it is complexed to β2m, arriving at the cell surface as an HLA class I antigen. This peptide-HLA complex then becomes a target for cytotoxic T-cells. Other non-classical HLA and HLA related molecules are encoded for in the HLA class I region including HLA- E, G, F and MHC Class I polypeptide-related sequence (MIC) A and B (Roitt and Delves 2006).

HLA class II region

HLA class II molecules themselves consist of two helical domains, (α_1+$\alpha_{2)}$ and two beta sheets (β_1+β_2) (Figure 1.8b). Its distribution is restricted to B-cells, Dendritic Cells (DCs), macrophages and thymic epithelium under normal circumstances with DP, DQ and DR subclasses in existence. Its role in Antigen Presenting Cells (APCs) is to present processed foreign antigen to Th cells (Roitt and Delves 2006).

HLA class III region

Non-classical molecules encoded within the class III region of the HLA gene group include C4, C2, factor B, Heat Shock Proteins (HSPs) (Roitt and Delves 2006).

Dendritic Cells

Cells which can present antigen in the context of HLA class II to Th cells are termed professional APCs and include the macrophages, B-cells and DCs. DCs originate in bone marrow and may be divided into two major subsets, myeloid (mDC) and plasmacytoid (pDC), on the basis of their differential expression of surface markers such as TLRs, CD8a, CD11b and CD11c. pDCs are found in blood and secondary lymphoid tissue and secrete large amounts of IFNɣ in response to virus.

Figure 1.8a **Structure of the HLA class I molecule**
The association of three globular helical domains with β_2m is shown along with the groove for peptide presentation. HLA class I is expressed on virtually all cells.

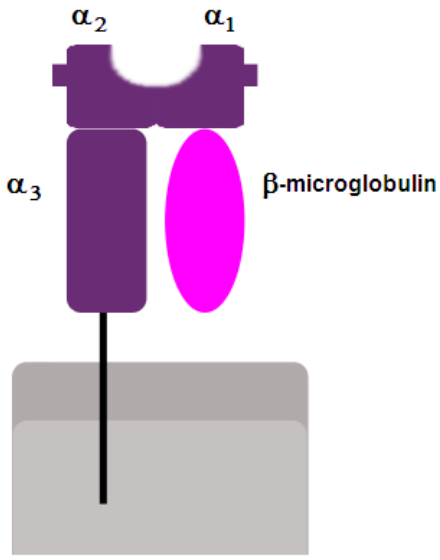
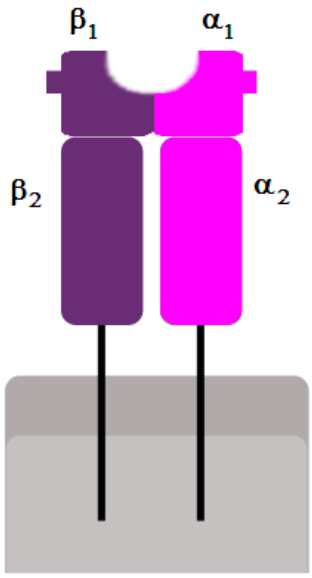


Figure 1.8b **Structure of the HLA class II molecule**
The two pleated sheets and two helical domains are shown. HLA class II expression is mainly restricted to APCs.



mDCs reside in skin and areas where they are likely to come in contact with foreign antigen. Here, stimuli such as PAMPs, apoptosis, necrosis, inflammation and the presence of other immune system cells can activate mDCs, causing them to begin a process of maturation and migration to the lymph nodes. When mDCs arrive at lymph nodes, those presenting self antigens are deleted, suppressed or rendered anergic (due to a lack of co-stimulation), which allows the peripheral tolerance of self. However, when foreign antigen is being presented, the co-stimulatory molecules expressed on mature DCs cause naïve T cells to become activated, facilitating a primary immune response which subsequently also directs the maturation of these T cells through the release of cytokines (Blanco, Palucka et al. 2008).

T cells

The two main types of T cell are the CD8+ (Tc, Cytotoxic T-Lymphocytes, CTLs) and the CD4+ (Th). Both originally derive from bone marrow but undergo most of their development in thymus where they differentiate from CD8+ CD4+ “double positive” cells into singly CD8+ or CD4+ cells. This process is driven by an individual cell’s strength of reaction when presented to HLA class I or class II complexed to self antigen in the thymus. Those cells which do not react with HLA of either class are deleted (positive selection) and those which overreact to HLA are either deleted or become regulatory T cells (Tregs), in order to prevent autoimmunity (negative selection). The surviving naïve CD8+ and CD4+ cells are therefore those which recognise HLA class I or HLA class II respectively and which do not express a TCR for self antigen (Roitt and Delves 2006).

<u>CD8+ cells</u>

These recognise antigen presented in conjunction with HLA class I and so recognise and deal with the abnormal intracellular processes responsible for such peptide presentation within all cells. Its cytotoxic mechanism is the same as that of NK cells (Roitt and Delves 2006).

CD4+ cells

Naïve T cells are thought to require two signals for their activation, with a lack of the second signal leading to a state of anergy. The first normally comes in the form of TCR binding to an HLA-peptide complex on the surface of a DC in the periphery. In the case of CD4+ cells, the second comes in the form of its CD28 molecule binding to the co-stimulatory molecules B7.1 and B7.2 on the surface of APCs. Depending on the nature of the pathogen, the resulting clonal expansion of CD4+ cells produced from the naïve cell may produce a pool of either Th1 or Th2 cells. Th1 cells produce cytokines which aid cellular immunity such as IFNɣ and IL-2 while the Th2 subset produces cytokines which aid humoural immunity such as IL-4, 5, 6,10 and IL-13. The resultant response to pathogen depends upon the relative sizes of each pool as the cytokine responses antagonise each other (Roitt and Delves 2006).

Regulatory T cells

CD4+ CD8+ naïve T cell progenitors which are neither apoptosed nor positively selected in the thymus are thought to undergo differentiation into Tregs. These are mainly CD4+ cells and are characterised phenotypically by the expression of the transcription factor Forkhead box p3 (Foxp3). Their role involves the suppression of excessive or inappropriate immune responses (hypersensitivity/autoimmunity) and the curtailing of the immune response once the original stimulus has been dealt with (Maloy and Powrie 2001; Roitt and Delves 2006).

1.2.3. The immune system and cancer

1.2.3.1. Immunosurveillance

The concept of the immune system influencing the growth of cancer crystallised into the theory of immunosurveillance first forwarded by Burnet and colleagues. This concept described a situation where cancer cells were constantly being produced and concurrently being continually removed by the immune system at the subclinical level (Burnet 1970).

Immunosurveillance in mice

This theory was tested by comparing the frequency of spontaneous cancers in over 10 000 inbred mice presumed to be immunocompetent with that found in their wild type counterpart. As no difference in frequency was found, this theory initially fell out of favour (Rygaard and Povlsen 1974). However, following the characterisation of the innate immune system it became clear that conclusions had been drawn using an imperfect model of immunocomprimise. The use of more genetically tailored murine models revealed that mice lacking NKT, T and B-cells developed spontaneous and induced cancers more readily (Strehlow 2000). Subsequently, it was found that an individual lack of these cells, perforin or IFNɣ was independently linked to increased tumour activity indicating important roles for them in a revived theory of immunosurveillance (Dunn, Bruce et al. 2002).

Immunosurveillance in humans

In humans the study of immunosurveillance in states of immunocomprimise is opportunistic. Evidence for the presence of immunosurveillance in humans has been gathered from observations in transplant patients, the comparison of clinical outcome data with the presence of immune cells within the tumours and the study of tumour reactive immunoglobulins in serum and their Tumour Associated Antigens (TAAs) (Dunn, Bruce et al. 2002).

Transplant data

A review of Scandinavian patients receiving renal transplants showed a 3- to 4-fold increase in non-viral cancers at sites such as colon and lung (Birkeland, Storm et al. 1995). A two-fold increase in the risk of malignant melanoma was also found in transplant patients in a US study (Penn 1996).

Tumour Infiltrating Lymphocytes

Cells of the adaptive immune system specific for antigens found to be restricted to particular cancers have been discovered, illustrating the inherent immunogenicity of tumours. The Cancer Testis (CT) antigen NY-ESO-1, which is restricted to a limited variety of normal tissues, has

been found in cancers at sites where it would not be expressed and has been investigated from its inappropriate genetic upregulation to the production of specific CD4+ and CD8+ cells. This is supporting evidence for the process of immunosurveillance in humans (Gnjatic, Nishikawa et al. 2006). MICA/B are ligands for NK, CD8+ and γδ T cells and are important in initiating target cell killing by these effectors in innate immunity (Pende, Cantoni et al. 2001). Their expression is mainly restricted to gastrointestinal epithelia but they can be upregulated by intracellular stress under the influence of HSPs and are more widely distributed in cancer (Groh, Bahram et al. 1996; Groh, Rhinehart et al. 1999). MICA/B expression on tumours has been shown to be associated with cell killing by γδ T cells, supporting the theory of immunosurveillance. As the expression of NKG2D on Tumour Infiltrating Lymphocytes (TILs) has been shown to be lower in MICA/B expressing tumours, this supports the existence not only of immunosurveillance but also the possibility of the development of mechanisms whereby tumours may avoid the attentions of the immune system (Dunn, Old et al. 2004). The infiltration of tumours by TILs has been seen in a variety of cancers and has been shown to be associated independently with improved prognosis in ovarian cancer (Zhang, Conejo-Garcia et al. 2003), while the presence of CD8+ cells has been independently associated with improved prognosis in colorectal and oesophageal cancer (Naito, Saito et al. 1998; Schumacher, Haensch et al. 2001). Similar findings have been shown for other cell types such as NK cells (Balch, Riley et al. 1990). The presence of TILs in tumours which is associated with outcome is further supporting evidence for the process of immunosurveillance in humans.

1.2.3.2. Immunoediting

The concept of immunosurveillance has, however, increasingly been superseded by a broader model of “immunoediting”, a refinement of its predecessor aimed at explaining observations such as the failure of immunosurveillance in clinically apparent tumours (Dunn, Bruce et al. 2002). Immunoediting maintains that the immune system interacts with

the tumour mass in complex ways in order to sculpt or edit it rather than simply to destroy it, the immune system providing a Darwinian selection pressure favouring the propagation of cells with less immunogenic phenotypes. In this way tumours may be produced which ultimately grow successfully in spite of a functioning immune system. Evidence for this in the murine model comes from the observation that tumours in mice with a deficiency in perforin rapidly grow but remain immunogenic enough to be rejected when transferred to wild type mice; conversely, when perforin functions normally the more immunogenic types are destroyed and these tumours are then not rejected following transfer to mice with an competent immune system (Street, Trapani et al. 2002).
The theoretical model of immunoediting envisages three states in which tumours may reside; elimination, equilibrium and escape (Dunn, Old et al. 2004).

Elimination

The elimination state equates to immunosurveillance inasmuch as the end result is the death of the tumour cell. The theoretical process begins with the development of a tumour cell which interacts abnormally with its environment due to its underlying deviant physiology (the 6 hallmarks of cancer). This would attract the attention of elements of the innate and adaptive immune system resulting in IFNɣ release. NK cells and macrophages would then synergistically release IL-12 and further IFNɣ, leading to a slowdown in proliferation, apoptosis and angiogenesis. Following tumour cell destruction by these cells, antigen would become available for presentation to the adaptive immune system via DCs which would migrate to the lymph nodes and stimulate the production of tumour specific CD4+ and CD8+ cells, leading to tumour cell killing and the release of more anti-tumour IFNɣ. This process would occur every time a microscopic tumour nest arose and become less efficient with senescence (Dunn, Old et al. 2004).

Equilibrium

The equilibrium phase equates to a stage of tumour development where the tumour population has been incompletely eliminated. During

this stage, the inherent genetic instability of cancer would mean that certain cells within the population would be immunogenic and be removed, whereas other less immunogenic cells would be more successful and propagate. It is envisaged that this phase would be the longest, the clinical exemplar perhaps being the long lead time observed between the development of genetically unstable Cervical Intraepithelial Neoplasia (CIN) and the occurrence of invasive cervical cancer. If this undetectable process were actively under way in transplant donors, it might explain the relatively rapid development of non-viral cancers in their immunosuppressed recipients (Dunn, Old et al. 2004).

Escape

The final state in immunoediting would be an escape phase, where the tumour mass would evolve to the point where it could continue to grow despite the presence of a functional immune system. A number of mechanisms known to occur in human cancers might underlie immunological escape such as loss of HLA class I expression, downregulation of tumour antigen, defects in death receptor pathways (fasL and TRAIL), a lack of co-stimulation, the presence of immunosuppressive cytokines, apoptosis of T cells, the effects of Tregs, the shedding of NKG2D ligand and IFNɣ insensitivity (Khong and Restifo 2002). It is likely that tumours develop strategies to escape both the innate and adaptive arms of the immune system.

1.2.3.3. Evidence for immunoediting in ovarian cancer

The concept of immunoediting remains largely theoretical in humans. However, altered expression of mCRPs and HLA class I, which both represent possible mechanisms of immunological escape, have been found to correlate independently with survival in breast cancer, which supports the model (Madjd, Pinder et al. 2003; Madjd, Durrant et al. 2004; Madjd, Spendlove et al. 2005). Evidence is also accumulating which suggests that immunoediting also occurs in human ovarian cancers.

Adaptive humoural reaction

Immunoglobulins to TAAs are found in serum such as mesothelin and cathepsin D (Taylor and Gercel-Taylor 1998; Ho, Hassan et al. 2005).

Innate cellular reaction

NK cells infiltrate ovarian tumours relatively sparsely and have been found to have a lower level of activity than normal in ovarian cancer patients. This activity becomes progressively less when observing the peripheral blood, ascitic and then TIL pools (Kabawat, Bast et al. 1983; Lotzova, Savary et al. 1984; Garzetti, Cignitti et al. 1993; Lutgendorf, Sood et al. 2005). A reduction in NK Cell Cytotoxicity (NKCC) has been shown to be associated with disease progression by one group (Garzetti, Cignitti et al. 1993), with an observed resistance of NK cells to IL-2 and their reduced secretion of IFNɣ possible underlying mechanisms (Lai, Rabinowich et al. 1996). Intriguingly, increased levels of psychological distress amongst ovarian cancer patients and poor social support appear to correlate with reduced NKCC in TIL. Though a link is biologically plausible, through neuroendocrinological mechanisms, causation has not, however, been established (Lutgendorf, Sood et al. 2005). This group also investigated the influence of psychological factors on the presence of NKT cells (which have been show to display potent cytotoxicity to ovarian cancer cell lines in vitro), finding that tumours accumulated NKT cells compared to their surroundings and that this was positively influenced by better psychological indicators (Gritzapis, Dimitroulopoulos et al. 2002; Lamkin, Lutgendorf et al. 2008). Tumour Associated Macrophages (TAMs) are generally abundant within the cancer microenvironment and interaction between the two is thought to foster a cytokine environment which actually promotes tumour growth in many cancers, including ovarian (Hagemann, Wilson et al. 2006; Allavena, Sica et al. 2008). In ovarian cancer up to 70% of cases are infiltrated by TAMs though this not been shown to predict outcome (Shah, Allison et al. 2008).

Innate humoural reaction

Complement deposition and associated cell lysis has been shown to occur by several groups on various tumours and has been shown to be inhibited by mCRPs (Niculescu, Rus et al. 1992; Yamakawa, Yamada et al. 1994; Donin, Jurianz et al. 2003). However, this has not invariably been the case with Niehans and colleagues finding that C5-9 deposition occurred in small cell lung cancers which were completely devoid of mCRPs. This group hypothesised that other MAC resistance strategies could be at work in tumour cells which had lost the expression of mCRPs. They also found that a change in expression pattern of mCRPs was common in cancer systems which varied by cancer site and could be an up- or downregulation of one or more mCRP (Niehans, Cherwitz et al. 1996). Examining 12 ovarian cancers in addition to ovarian cancer cell-lines, Børge and co-workers found that CD59 and CD46 were ubiquitously expressed whilst CD55 expression was more variably seen. By using an effector monoclonal antibody whilst variably blocking mCRP activity they were able to show that these molecules impede the Complement Dependent Cytotoxicity (CDC) of the innate immune system in vitro (Figure 1.9) (Bjorge, Hakulinen et al. 1997).

Adapative cell mediated reaction

Zhang et al examined 186 cases of advanced ovarian cancer and discovered that 55% were associated with a significant presence of CD3+ TILs, the presence of which was associated with a higher level of IFNɣ and IL-2. This suggested a functionally active infiltration and its presence was independently associated with improved 5-year OS and PFS (Zhang, Conejo-Garcia et al. 2003). This finding has been corroborated by a recent study showing the same prognostic association (Tomsova, Melichar et al. 2008) and replicated in a series of 95 advanced serous ovarian cancers where it was additionally discovered that an environment particularly enriched with ɣδ TCRs correlated with worse OS in a univariate analysis (Raspollini, Castiglione et al. 2005). Woo and co-workers found that CD8+ infiltrated ovarian cancers exhibited low levels of IL-2, IFNɣ and TNFβ

Figure 1.9 Effector mechanisms of the innate immune system provoked by mAb binding to antigen

Depending on class of IgG, a mAb may harness the innate immune system to effect cell killing by mediating Antibody Dependent Cellular Cytotoxicity (ADCC) or Complement Dependent Cytotoxicity (CDC) directly and by the indirect initiation of Complement Dependent Cellular Cytotoxicity (CDCC) via the rising C5a levels which follow the complement fixing effect of antibody-antigen binding. IgG3 and IgG1 fix complement most effectively and with much greater potency than IgG2 and IgG4.

Please refer to Figure 1; Gorter, A. and S. Meri (1999). "Immune evasion of tumor cells using membrane-bound complement regulatory proteins." Immunol Today 20(12): 576-82.

when TGFβ secreting Tregs were present, illustrating a potential immunological escape mechanism (Woo, Chu et al. 2001). This finding was supported by the work of Curiel et al which demonstrated that the presence of Foxp3 positive Tregs in the TIL was correlated with reduced OS. In addition, it was discovered that TAA directed T cell specific immunity was inhibited by the presence of Tregs (Curiel, Coukos et al. 2004). In contrast to Zhang and co-workers, Sato et al, on studying a series of 117 ovarian cancers, found that the presence of CD3+ cells within the TIL mass had no prognostic effect. However, this group did find that a high ratio of CD8+/Tregs was correlated with improved OS, supporting the notion that the adaptive response to tumour is regulated and that tumours become tolerated in the periphery (Sato, Olson et al. 2005). High expression of IFNɣ within TILs has been found to correlate with improved prognosis independently of other factors (Marth, Fiegl et al. 2004) and HLA class I expression has been shown to be downregulated in approximately 40% of ovarian cancers and non-independently associated with reduced OS in a series of 51 ovarian cancers (Vitale, Pelusi et al. 2005). A slightly smaller study of 29 patients where more allelic versions of HLA class I were investigated did not support this, however, with no cancers being found to have HLA class I downregulation and few showing any evidence of class II downregulation (Nijman, van Diest et al. 2001). Despite this, within TIL, reduced HLA class II expression and increased CD4+ presence has been associated with ovarian cancer progression (Tamiolakis, Kotini et al. 2003) and increased HLA Class II expression and CD8+ infiltration is associated with improved outcome (Matsushita, Ghazizadeh et al. 2003). Scant evidence has been accumulated concerning the expression of MICA/B in ovarian cancer, though a recent study found it to be expressed in one of 6 tumours (Carlsten, Bjorkstrom et al. 2007).

Summary of immunoediting in ovarian cancer

Ovarian cancer is immunogenic and elicits both humoural and cell-mediated reactions whilst mobilising both innate and adaptive arms. Immunoglobulins are produced against it and immunological cells of all

kinds infiltrate it. Cell killing may occur through complement dependent or purely cell mediated means and there is evidence that ovarian cancers interact with the immune system differently to normal tissue, lending support to the theory that immunoediting is active in this disease. The attenuation of NKCC, IFNɣ secretion and HLA class I expression are a few of the apparently active mechanisms of immunological escape, along with the infiltration of tumour by Tregs, TAMs and the ubiquitous expression of mCRPs. Direct prognostic evidence that some of these mechanisms have a clinical impact is slowly accruing though the prognostic effect of HLA class I expression and of the mCRPs in ovarian cancer has yet to be fully examined in the same way that it has at other sites such as breast cancer (Madjd, Pinder et al. 2003; Madjd, Durrant et al. 2004; Madjd, Spendlove et al. 2005) (Nelson 2008).

1.2.4. Immunotherapy in ovarian cancer

The aim of immunotherapy is to employ the host's immune system in order to achieve tumour cell killing, with two broad strategies existing. Active immunotherapy aims to produce long term antigen-antibody specific reactions to TAAs through vaccination, whereas passive immunotherapy seeks to produce a response through the transference of ready functional effector elements such as cytokines, CD8+ cells or immunoglobulins. Up to 20% of optimally debulked ovarian cancer cases are associated with micrometastatic disease in bone marrow and, as these cells are often in the G0 phase, they may represent a pool of chemoinsensitive cells and a source of recurrent disease (Barnhill, Piepkorn et al. 1998; Braun, Schindlbeck et al. 2001). As immunotherapy can also target non-dividing cells, this makes it an attractive adjuvant to standard therapy in ovarian cancer (Rubin 1993; Bjorge, Stoiber et al. 2006) though immunoediting processes which may have reduced tumour cell susceptibility must be taken into account (Schuster, Nechansky et al. 2006).

1.2.4.1. Vaccination

The principle behind active vaccination strategies is to challenge the immune system with TAA processed by APCs. This is then presented to CD8+/CD4+ TCRs in conjunction with HLA class I/II and co-stimulation to mediate CD8+ lysis and to recruit further innate and humoural responses. An example of a vaccination strategy under investigation in ovarian cancer is Oregovomab. This is a murine anti-CA125 antibody which when administered to humans in small doses stimulates the immune system to produce Human Anti-Mouse Antibodies (HAMA), anti-idiotypical antibodies and anti-CA125 antibodies – an "antibody as antigen" strategy. This approach stimulates both innate and specific arms of the immune system and is well tolerated. However, the results of phase II studies in recurrent disease are yet to show a clinical impact (Ehlen, Hoskins et al. 2005). As there are many ways in which vaccine strategies may fail in tumours that are immunoedited (including HLA Class I antigen loss and the infiltration of the tumour by Tregs), it is likely that co-administration of Oregovomab with cytokines and co-stimulatory molecules may prove more successful (Berek, Dorigo et al. 2003) (Sabbatini and Odunsi 2007).

1.2.4.2. Cytokines

IFNɣ has anti-tumour and anti-proliferative effects in vitro and has been shown to be safe to administer in combination with cisplatin and cyclophosphamide in ovarian cancer patients, with an improvement in PFS observed (Barton, Davies et al. 2005) (Windbichler, Hausmaninger et al. 2000). However, when this trial was repeated to facilitate comparison with current best practice chemotherapeutic regimens, though the safety profile remained the same OS was reduced in the IFNɣ group and the trial was abandoned early (Marth, Windbichler et al. 2006; Alberts, Marth et al. 2008). Potential explanations include a reduction in the dosage of standard chemotherapeutic agents in the IFNɣ arm due to tolerance issues or potentially the role of longer term IFNɣ administration in the proliferation of Tregs (Wood, Feng et al.

2007). However, the presence of IFNɣ remains clinically associated with improved outcome and it may have a role in improving the efficacy of HLA class I dependent immunotherapies, as it has been shown to upregulate the expression of HLA class I in ovarian cancer cell lines (Mobus, Asphal et al. 1993; Wood, Feng et al. 2007).

1.2.4.3. Adoptive T cell transfer

A passive method of initiating a cell mediated response involves the transfer of modified T cells. Kershaw and colleagues genetically re-engineered ex vivo autologous CD4+ and CD8+ cells to express anti-alpha folate receptor immunoglobulin, a TAA in ovarian cancer. However, there was little persistence of the T cells within the circulation as detected by Polymerase Chain Reaction (PCR) or localisation of the T cells within tumour as assessed by the activity of ^{111}In labelling. The failure of this approach was ascribed to the development of an inhibitory factor (Kershaw, Westwood et al. 2006).

1.2.4.4. Monoclonal antibodies

Passively initiating a primary immune response via the humoural arm of the immune system may be achieved through the use of monoclonal antibodies (mAbs). mAbs are intended to specifically target antigens associated with cancer or its progression. They can act through blocking pathways integral to the development of the hallmarks of cancer such as VEGF (Bevacizumab) and EGFR (Certuximab) or through binding TAAs to provoke an immune response via the complement cascade and NK cells. In reality, these mechanisms probably work synergistically (Figure 1.9) (Gorter and Meri 1999; Macor and Tedesco 2007). The use of mAbs in haematological malignancies is routine but their efficacy in ovarian cancers is disappointing. Oei et al recently reviewed 44 papers which included: 42 phase I/II trials and two phase III trials; 5 different TAAs (and VEGF); 15 different antibodies; 23 trials of unconjugated antibodies; 5 “antibodies as antigens” vaccination studies; and 16 studies concerning radioimmunotherapy. The TAAs studied were CA125, Human Milk Fat Globule (HMFG), ErbB2, MUC1 and Tumour Associated Glycoprotein72 (TAG72). The antibodies used

would be expected to effect responses through a combination of complement activation, innate and adaptive responses and possibly HAMA development (Oei, Sweep et al. 2008). The two most promising mAb clinically appear to be Bevacizumab and Oregovomab, each of which has a definite mode of action independent of fixation of complement. Any clinical effect of response to Herceptin (Trastuzumab) was abrogated by the infrequency of its over-expression in ovarian cancer (10% of cases) with the remaining unconjugated antibodies having no significant effect on OS (Bookman, Darcy et al. 2003) (Oei, Sweep et al. 2008). The lack of clinical responsiveness to simple unconjugated mAbs to TAAs, which is often at dissonance to animal work, may in part be due to an abrogation of complement fixation and deposition by the expression of mCRPs on human target tumour cells which do not exist in a murine model (Shin, Hansch et al. 1986). This theory is supported by in vitro work by several groups showing increased cell lysis in response to mAbs when mCRP function is experimentally disabled (Brasoveanu, Altomonte et al. 1996; Jurianz, Maslak et al. 1999; Bjorge, Stoiber et al. 2006).

1.2.4.5. Circumventing immunoediting escape mechanisms

Complement fixing mAbs theoretically have the advantage of being able to harness a powerful and ubiquitous soluble effector mechanism which can penetrate the disease well. However, for this strategy to be fully exploited, it is important for mechanisms of combining mAb therapy with the disabling of mCRP function in the clinical setting to be developed. As mCRPs are ubiquitously expressed, in order to prevent indiscriminate damage to normal tissues any such strategy needs to be more targeted than that used experimentally (Gorter and Meri 1999). One mechanism of achieving this may be through the use of antibodies bispecific for both TAAs and mCRPs, where the TAA Fab moiety is of sufficiently high affinity to restrict its deposition outside of tumour sites. There is preliminary in vitro evidence that this may prove effective (Gelderman, Blok et al. 2002; Gelderman, Kuppen et al. 2002). An alternative strategy might be through the use of anti-idiotypic antibodies

as antigens which not only bind TAA but also resemble mCRP, leading to the generation of antibodies which locate tumour and block mCRP simultaneously (Spendlove, Li et al. 2000) (Fishelson, Donin et al. 2003). mCRPs represent an obvious obstacle to the efficacy of unconjugated mAb to non-signalling TAA which rely on complement fixation for their effect. This perhaps explains why most ongoing mAb trials in ovarian cancer focus on mAb with a more discrete action. Of the 234 trials currently active as recorded by National Cancer Institute (accessed 23 June 2008 search: Epithelial Ovarian Cancer; All types; All stages; Treatment) there were several phase III trials of Bevacizumab (anti-VEGF), a phase I/II trial of Volociximab (anti-angiogenesis factor), a phase II trial of Catumaxomab (anti-EpCAM), a phase I trial of Almetuzamab (Anti-Vascular Leucocytes) and a phase I trial assessing the T Cell response to Trastuzumab (2008).

1.2.5. Immunological markers in ovarian cancer

Ovarian cancer is an immunogenic cancer but there is evidence that it presents clinically in an immunoedited form. An increased expression of mCRP and a decrease in the expression of HLA class I are two potential mechanisms by which escape from immune control might occur and the expression of these molecules in clinical cancers is therefore important. Immunotherapy is an emerging treatment strategy which would appear particularly suited to ovarian cancer, given its propensity to relapse late and the common presence of micrometastatic deposits which are likely to reside outside of the cell cycle. The high expression of mCRPs and the low expression of HLA class I also represent two potential barriers to the effective use of this emerging modality.

1.3. Study outline

The aims of this project were dual. The first aim was to produce a TMA representative of the ovarian cancer cases which had presented at Derby City General Hospital (DCGH) between 1982 and 1997 and which had been followed until the present day. The objectives were to identify and review each case pathologically, physically construct the

TMA, determine the optimal number of cores required to return valid expression data, confirm the consistency of the data in an associated clinical database and, having then adapted it appropriately, assess the validity of using the TMA and database in an integrated manner to assess not only marker expression but also its prognostic and clinicopathological associations. This would allow previously studied markers to be assessed using a new technique, allow the expression of novel markers to be rapidly surveyed in a large series and would facilitate future work by other researchers in the department. The second aim was to use the TMA to study immunological markers. The first objective was to stain the TMA for HLA class I and β_2m to determine the prognostic effect of their co-expression. The second objective was to immunohistochemically stain the TMA for CD59 using a commercial antibody and then for CD55 and CD46 using specially developed in-house antibodies, allowing the co-expression of these markers to be surveyed in a large series for the first time.

Chapter 2: Materials and Methods

This study aimed to construct a TMA to complement a clinical database. This would then be tested for its ability to produce data likely to be generally applicable to ovarian cancer before finally being used to investigate immunological markers for their prognostic value.

The following chapter describes the administrative tasks, generic techniques and the majority of the specific experimental and analytical methodologies used in achieving these aims. It details the importance of research governance in this project, examines the method behind the immunohistochemical examination of molecular marker expression and covers the statistical tests employed for analysis of data.

Further chapters will explain in more detail how the TMA and database were constructed and tested as well as reporting the findings of the experimental work.

2.1. Research governance

In order to perform this study, stored patient tissue in the form of paraffin embedded blocks had to be first identified and then removed from the pathology department archives at Derby City General Hospital (DCGH), from where they would be transferred to Nottingham's Queen's Medical Centre (QMC) and Nottingham City Hospital (NCH) for subsequent review and use. This necessitated that the prospective confidential database of patients' clinicopathological information be used in a linked manner in order that the correct samples be accurately identified, removed, processed and returned to storage.

The study would involve several key individuals and institutions: Ian Scott (I.Sc) – Consultant Obstetrician and Gynaecologist and local researcher, Derby City General Hospital; Philip Rolland (P.R) – DM student, University of Nottingham (UoN) and clinical research fellow NCH; Ian Spendlove (I.S) – Chief Investigator, Academic and Clinical

Department of Oncology (UoN) on the NCH campus and Suha Deen (S.D) – Gynaecological Pathologist, QMC, all of whom would require access to patient data and material.

The use of this type of material by multiple individuals at multiple separate institutions meant that managing the governance side of the project was labour intensive but important in complying with the Department of Health Research Governance Framework (RGF) (Davies 2005).

This meant embracing the overlapping requirements of two main areas: research ethics and research and development (R&D).

2.1.1. Ethics

This refers to the legal and moral responsibility of a researcher to protect the patients involved in the study.

2.1.1.1. Ethics and medical research

Following Nazi human experimentation during the Second World War, the need for a set of ethical standards by which to judge the accused led to the creation of the Nuremberg code of 1947. A need for more sophisticated ethical standards to guide medical research led to the development of this pragmatic tool and the Declaration of Helsinki at the assembly of the World Medical Association in 1964 (Markman and Markman 2007). Although this holds no legal authority itself, its principles, last updated in 2000 (2000), are embedded into national laws. Subsequently, the Belmont report helped to define key ethical principles underlying the involvement of patients in research and developed the idea of autonomy (respect for patients), beneficence (doing the most good and the least harm for patients) and justice (the even distribution of the burdens and benefits of research)(1979). These key documents led to specific practical consequences.

Research using human material should be performed with the informed consent of the individuals involved (which may be withdrawn at any time without prejudicing their care), provide information unobtainable by

other methods, be scientifically appropriately designed to achieve its aims and have objectives the benefits of which exceed the risks – its benefits being subsequently appropriately dissipated. There is also a requirement for independent ethical review bodies to provide approval for and oversight of research projects.

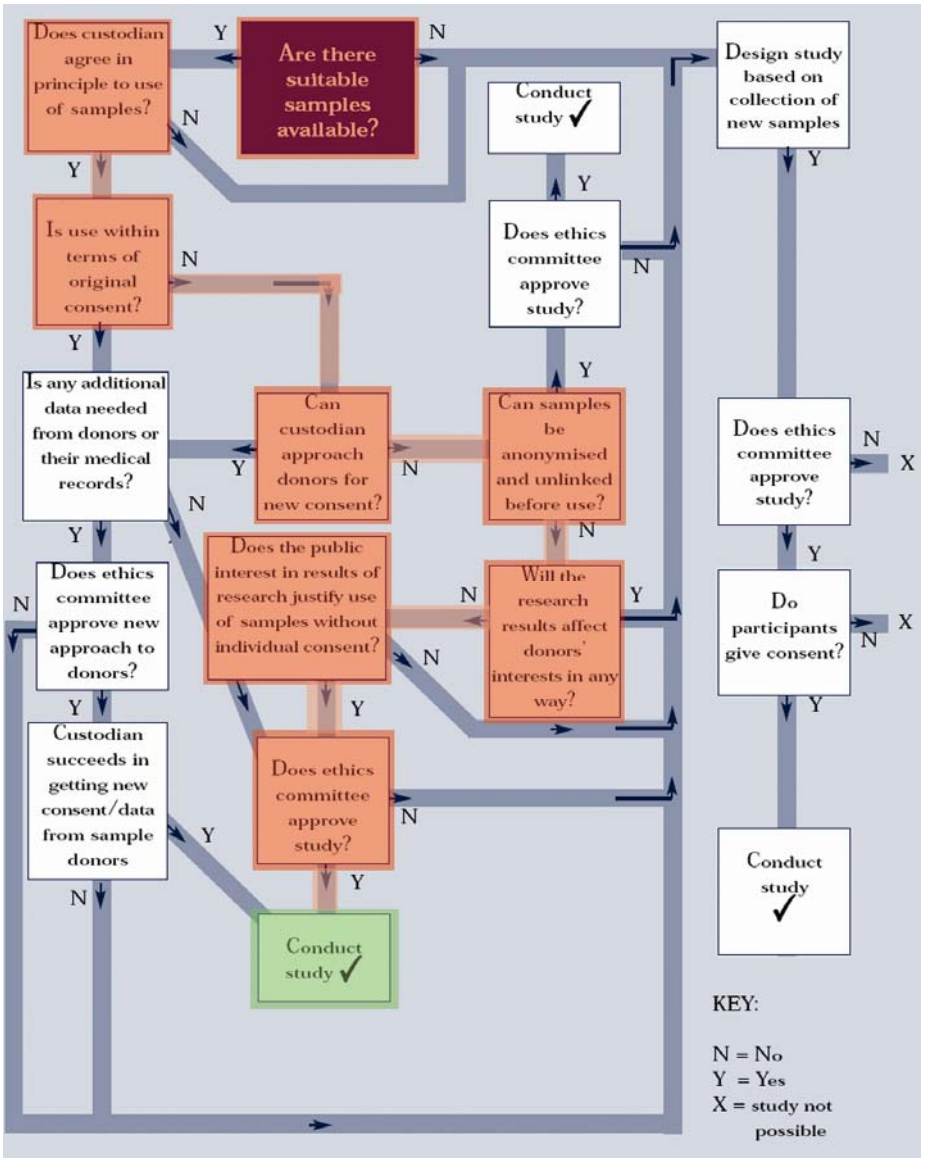
2.1.1.2. Review bodies

The first ethics committees were set up in the UK in 1966 and formal guidance for the establishment and operation of local research ethics committees (LRECs) by the district health authority followed in 1991 (1991). As research became more complex and collaborative, new multi-centred research ethics committees (MRECs) were established to deal with projects involving more than 5 sites. In addition, new authorities overseeing particular branches of research joined those wishing to approve research proposals (e.g. Gene Therapy Advisory Committee). Until recently, moderately sized pan-regional studies involving fewer than 5 sites have in practice needed to submit similar hard copy information in slightly different formats to multiple review bodies – the bureaucracy of which proved prohibitive. The submission of proposals has therefore been streamlined and computerised by the Central Office for Research Ethics Committees (COREC) followed by the National Research Ethics Service (NRES). Soon researchers will input information into the Integrated Research Application System (IRAS), which is designed to allow relevant information to be sent on to all necessary review bodies in the request through the filling of a single form.

2.1.1.3. Ethical committee approval

The application for ethical approval for this study pre-dated COREC and therefore a proposal entitled “The Significance of Complement Regulators in the Prognosis of Ovarian Cancer” was submitted to the Southern Derbyshire LREC on 5 June 2002, whereupon it was given the reference SDLREC:0205/495 and the R&D reference SDAH/2002/047.

Figure 2.1 The MRC established collections algorithm
Showing how the decision to approve the study was arrived at and why specific conditions concerning anonymisation were required.



The committee reviewed protocol version 1. In brief the unanonymised database of cases of ovarian cancer presenting to DCGH between 1982 and 1997 would be used to identify patients, the tissue would be removed from the archive and transferred to NCH for processing and the database at DCGH would be used on that site to analyse results.

Subsequently, the ethics committee gave the initial approval for the study to proceed in September 2003 (protocol version 2). Plans were made to review the project's progress on a yearly basis and the importance of amending the protocol prospectively if it needed to change was emphasised.

2.1.1.4. Ethical considerations and amendments

There were a number of ethical considerations which required protocol amendments before final approval could be given.

Consent

Before using a patient's biological data and/or tissue in research, that patient has to be given sufficient information in an appropriate form concerning the implications for themselves and/or others of participating in the study and be given the opportunity to ask questions. This respects a patient's autonomy and allows them to consent to involvement in an informed way. In this study, the majority of the patients had been prospectively consented for their cases to be used in research. However, as many cases dated back almost 20 years, it was felt that what was documented in the patients' notes did not represent adequate or valid consent in the context of modern standards and technologies. The options were to proceed without informed consent or to gain informed consent. The practicalities of gaining informed consent meant that it could not be considered as many of the patients were dead, there were over 400 cases and the risk of upsetting patients or their bereaved relatives by asking for retrospective consent many years after their treatment was real. Taking this into account, and using the MRC decision tree (Figure 2.1) (Johnstone 2001), approval was given to continue without consent. The main risk of causing harm by

proceeding without consent concerned the scenario of patients being made aware of potentially important information concerning their disease which could lead to difficult decisions either for themselves or their families, when they had never expected such information to come to light. The risks of their not being informed of new information about their case could also be damaging.

Anonymisation

To minimise the risk of harm to individual patients, while not losing the good that could come from using this prospectively gathered database, the LREC required that a new database be constructed that was anonymised and unlinked from the original by the removal of any field that could be used to reliably re-link them using reasonable methods (name, date of birth, address, local patient numbers, etc).

The unanonymised database was transferred to the trust server (DCGH NHS Trust) and access restricted to a small number of individuals based on site. A single worker was to identify a case from the database, acquire a pathology report for that case, update any missing fields in the database, acquire the appropriate tissue block and assign a unique study number to the tissue block and to the case on a copy of the unanonymised database. When the TMA had been constructed, the pathology reports would then be anonymised (photocopying the report with the unique fields covered, adding the TMA coordinates of the case to the copy and destroying the original). The copy of the unanonymised database would then be anonymised and unlinked by removing all unique fields including the study number. Linkage to the now anonymised pathology reports via the unique TMA coordinates would be maintained.

There would be no way that expression data generated off site could be re-linked to a specific patient. This had the advantage of eschewing a situation of generating data that should be made available to particular individuals. However, the disadvantages included the intensity of labour involved, the inability to update clinical data at a later date and the

potential for errors to occur when numbers and not names were being used.

No carte blanche

Ethical approval was given to study a small number of named molecules which needed to be amended if other ones were to be studied. Ethics committees are discouraged from giving researchers approval to study any marker they wish. They prefer that the scientific case for studying a new tranche of markers is made at intervals.

Human Tissue Act 2004

The Human Tissue Act (HT) was enacted in 2004 and came into force fully on 1 September 2006. The act replaced the Human Tissue Act 1962, the Anatomy Act 1984 and the Human Organs Transplants Act 1989. Its implementation is overseen by the Human Tissue Authority (HTA). When it was revealed that the storage and use of human tissue without consent was commonplace (Kennedy 2001; Redfern, Keeling et al. 2001) the existing law was deemed inadequate and, hence, the need for a new act apparent.

The HT covers all aspects of research on archived human tissue but does not apply to the tissue used in this study because it was already being used for a “scheduled” purpose before the HT was implemented. However, as the HT simply makes sound ethical standards a legal requirement, this study would easily meet the current legal requirements if an application for approval was made today (2004).

2.1.1.5. Subsequent amendments

Substantial

During the initial phase of the study a number of issues arose which necessitated the amendment of the terms of our ethical approval. Following the submission of an updated protocol (protocol version 3) a substantial amendment was granted by the LREC in June 2005 which covered three areas.

Access to the original unanonymised database was required continually by off-site researcher P.R in order to speed up the accurate extraction of relevant tissue and then to transform the database into its statistically useable form. In order to achieve this, permission was granted for P.R to have access to the original database on the DGCH trust server and a copy to use at NCH. The data were kept at the Department of Academic and Clinical Oncology and were password protected.

The database included patients up until 2002 but permission had only been requested to use cases up until 1997 (cases with 5 year survival data). As the study proceeded, 5 year survival data were becoming available for later cases and permission was granted to use the full database worth of cases to increase the power of the study further.

The study was renamed “The Significance of Biomarkers in Ovarian Cancer” and permission was given to continue research into other non-immune markers as long as a list of what was studied was periodically sent to the committee for review.

Non-substantial

The following procedural revisions did not require substantial amendments to the protocol but required the ethics committee to be informed by letter, which it was.

Identifying representative tissue blocks from archived cases could only be reliably done by making new slides and having them reviewed contemporaneously by S.D at QMC. We were given permission to use the tissue in this way. September 2004.

Coordinating the extraction of the correct tissue blocks was more complex than anticipated and required more man hours. It was agreed that P.R could extract the blocks from the archive. September 2004.

The database had missing fields which could be populated appropriately from the patient notes. Permission was granted for P.R to do this. September 2004.

Whole sections of ovarian tissue were needed to act as controls and for titrational experiments. Permission was granted to use the tissue blocks in this different manner. June 2005.

We had erroneously made 5 and not 4 copies of the array. The ethics committee granted us permission to use all 5. June 2005.

Having studied a breast cancer array for multiple markers and then analysed the results using an Artificial Neural Network (ANN) to good effect, we were granted permission to apply this technique to our results. June 2005.

2.1.2. Research and development

The Department of Health requires organisations providing care in England to be aware of any ongoing research within their body involving patients, tissue or data for which they are responsible. They must oversee the implementation of the RGF for each project and this has led to the establishment of R&D departments within each individual NHS organisation. Before a study can be conducted, permission from the appropriate R&D department is mandatory. As all the cases originated from DCGH, an application for approval was filed with the Southern Derbyshire Acute Hospitals (SDAH) NHS trust R&D department in June 2002. Final approval to commence the research was given on 16 September 2004. The time taken to get final approval reflects the time taken to assemble written evidence of the study's compliance with all the elements of the RGF for which the R&D department was providing oversight.

Ethical approval

The R&D department has a key responsibility to ensure that ethical approval has been granted by a body with the authority to grant it. Delays in obtaining ethical approval due to concerns over the use of human tissue without consent lead to delays with R&D approval.

Honorary contracts

Non-NHS employed researchers who perform research which potentially impacts on individuals cared for by NHS organisations normally have to hold an honorary NHS contract. The R&D department was more exacting and wanted all non-locally based researchers to hold a local NHS honorary contract. These were subsequently obtained for P.R and I.S, creating a minor delay.

Legal requirements

At the time of application, the development and enactment of the HT was in progress. In retrospect, this legislation does not affect the study but there was confusion about and unfamiliarity with the likely impact that this new law would have at the time. As the R&D department had a responsibility to ensure that the law would not be broken, a cautious approach to granting approval was taken.

Sponsorship

The R&D department required written proof that the study had a named sponsor. The sponsor is the individual or organisation responsible for ensuring that researchers have the necessary capabilities and resources to deliver the research, that the research is managed and monitored effectively, that external peer review has taken place and that a system is in place for the protection and division of intellectual property rights and the appropriate dissemination of findings. The sponsor is normally the employer of the chief investigator and therefore the University of Nottingham became the sponsor for this project.

External peer review

The ethics committee has a responsibility to ensure that the study is scientifically worthwhile and well designed but this is underwritten by the sponsor who, in turn, requires that the study is externally peer reviewed in addition to its own internal peer review system. The interrelations of these requirements lead to delays. The project was favourably externally peer reviewed by Dr M Illyas (senior lecturer in

pathology, Academic Unit of Pathology, Leeds General Infirmary) by June 2005.

Financial considerations

The involvement of a gynaepathologist at a separate NHS institution to review the project's slides during her special programmed activity sessions had cost implications. This £18 037 had to be signed off by the QMC NHS trust R&D. This required a separate application for R&D approval at this site, which caused a delay. The small costs incurred in the experimental arm of the project were met internally through a combination of the University of Nottingham, the three NHS trusts employing the co-researchers and a small donation of £4000 from the Derby City General Hospital Charitable Fund.

2.2. Immunohistochemistry

The main investigative method used in this work was ImmunoHistoChemistry (IHC), a technique which exploits the specific interaction between antigens and specially designed antibodies to estimate protein expression in tissue. It can be performed on freshly frozen tissue or Formalin Fixed Paraffin Embedded (FFPE) tissue which may have been archived for many years. It is quick, easily learned and reliable but subjective, and experience is required to know when staining is non-specific. The method used in this work comprised the following elements.

2.2.1. Tissue preparation

Following surgical removal, the tissue was fixed for 24 hours (exceptionally, up to a maximum of 72 hours for large specimens) in 10% w/v neutral buffered formalin (40% w/v formaldehyde) (NBF). The samples were then archived in an ambient environment for between 6 and 23 years.

Formaldehyde fixation preserves tissue morphology well but alters RNA, DNA and protein structure through cross linking functional groups such as amines (eg, lysine) and thiols (eg, cystein) (Fox, Johnson et al. 1985). These methylene bridges can only be broken down by drastic

hydrolysis (Puchtler and Meloan 1985). Therefore, when an antigen becomes changed by this process it becomes very stable, with little chance it will degrade even after long term storage. However, these conformational alterations often change immunoreactive epitopes to inert ones, complicating IHC techniques (Werner, Von Wasielewski et al. 1996).

There is evidence that the antigenicity of sectioned tissue decreases over time. This is worse in certain cancers, such as colorectal where it becomes obvious after 6 months. Degradation is influenced by the method used to fix the tissue but 10% NBF performs comparatively well (Atkins, Reiffen et al. 2004). Under-fixation (but not over-fixation) of tissues also leads to loss of antigenicity, (von Wasielewski, Mengel et al. 2002).Therefore, tissue should be fixed for at least 24 hours (at room temperature) and the case for using cut sections with minimal delay has been reinforced by work on breast cancer (Fergenbaum, Garcia-Closas et al. 2004). Therefore, 4 µm thick sections of TMA, whole sections of ovarian cancer and positive control tissue were cut as close to the date of staining as practical with all sections being utilised within 14 days. The sections were mounted onto positively charged slides to improve tissue adherence (Superfrost “Plus” – Surgipath Europe, Peterborough, UK). The control tissues were as recommended by the manufacturers of the primary antibodies or occasionally tumour known to express the marker well. Two copies of the array (8 sections) were prepared for each experiment in order to benefit from the advantages of the technique (minimising tissue usage, lowering reagent usage and staining all the tissue samples in a single run) while still aiming to achieve a high concordance with what would be expected in whole tissue sections. While some studies recommend staining 5 copies of the array (Fergenbaum, Garcia-Closas et al. 2004; Goethals, Perneel et al. 2006), most have used a single core, which is known to give a 91% concordance with whole sections. This increases to 96% with two copies and 98% with three copies, after which little improvement in

concordance occurs (Camp, Charette et al. 2000; Rosen, Huang et al. 2004).

To remove the paraffin-wax from the slides, they were heated in a dry oven at the melting point of wax (60ºC) for 10 minutes and then placed in xylene (BDH, Poole, UK) to dissolve the paraffin for a total of 20 minutes. The tissue was rehydrated by sequential bathing for 10 seconds in graded alcohol baths of 100%, 95% and 75% ethanol (Sigma-Aldrich, Gillingham, UK). The reagent baths were in a fume cupboard.

2.2.2. Antigen retrieval

Though chemically fixing tissue and heating it to 60º for embedding leads to the denaturement of the antigenic epitopes, treating tissue using antigen retrieval solutions and heat before the application of the primary antibody has been shown to help antigens regain immunogenicity (Shi, Key et al. 1991; Leong 1996), though precisely why this works still remains unclear (Leong and Leong 2007). In this work, the retrieval solutions used were either Ethylene Diamine Tetracetic Acid (EDTA) at pH 8.0-9.0 or Citrate buffer at pH 6.0 (Table 2.1). An 800w microwave was used as a source of heat radiation to bring the buffer to the boil.

2.2.3. Preventing non-specific antibody binding

A primary antibody is designed to bind a specific epitope in order to precisely locate a molecular marker. If there is no marker to bind, then there should be no staining. However, the antibody can undergo non-specific hydrophobic interactions with tissue in the absence of a specific epitope, leading to non-specific staining. To prevent this, normal swine serum (NSS) (X0901, Dako, Ely, UK) was applied to the sections and also used in diluting the primary antibody. NSS contains proteins which interact with and mask non-specific hydrophobic sites while not interacting with the experimental antibodies itself.

2.2.4. Application of the primary antibody

Selection

Primary antibodies for IHC on FFPE tissue should be designed to detect antigenic epitopes which can be effectively retrieved after the fixing and embedding process. In addition, they should have proven specificity, be available in sufficient quantities and ideally have been used in previous published work. Commercially available antibodies are preferred and usually meet these requirements but when none is available for a particular marker for use on FFPE tissue then the development and testing of an in-house antibody is possible.

Titration

The optimal working dilution for an antibody is defined as the dilution of antibody that gives the most intense specific staining with the least amount of background staining. It is affected by many factors, including how the tissues were originally fixed; the concentration, age and method of storing the antibody, the time between cutting the sections and the staining, the type of antigen retrieval method that is used and whether an avidin-biotin blocking step is performed (Boenisch, Farmilo et al. 2001). For this reason it varies between laboratories and was determined by performing titrational experiments: multiple sections of tissue found to stain with an antibody were stained at various dilutions. This titration preceded the full experiment, which could then be performed at optimal dilution for all slides. The primary antibody was titrated to a fixed application time of 60 minutes.

Negative control

The negative control had all the reagents applied to it apart from the primary antibody, confirming that all staining of experimental tissue was due to the primary antibody interacting with its specific epitope. In place of the primary antibody, non-immune immunoglobulin of the same subclass and from the same species was substituted. For commercial antibodies this was as recommended by the manufacturer.

2.2.5. Washing

The slides were mounted onto Shandon cover plates and placed into Shandon Sequenza™ slide racks. (Thermo Fischer Scientific Inc, Massachusetts, US). This system exploits a capillary gap method: holding slide and cover plate upright and apart by a fixed distance with reagents applied from a funnel arrangement above. This way, all sections are incubated in the same volume of reagent (Figure 2.2).

Thorough washing of the sections between steps with Tris Buffered Saline (TBS) was essential to prevent them drying out. This also limited artifactual staining by ensuring that each reagent's excess was washed off before the application of the next. See Table 2.1 for details of the preparation of the "in-house" reagents.

2.2.6. "ABC" method – amplification of the signal

The two basic principles behind IHC involve the specific recognition of a human antigen by an antibody raised in another species and the reaction between an oxidising enzyme such as Horse Radish Peroxidase (HRP) and a chromogen such as 3, 3'-DiAminoBenzidine (DAB).

If the primary antibody is labelled with HRP, it can be directly linked to the chromogen but this leads to only a small amount of staining at each site of interaction (Figure 2.3a). An increased amplitude of staining can be achieved by indirect linking of chromogen to the primary antibody via an intermediate, as in the "ABC" method this work employs (Hsu, Raine et al. 1981).

This method exploits a phenomenon whereby one molecule of avidin can bind four molecules of biotin (vitamin B7). After the incubation of the primary antibody, a secondary antibody "C" is applied which binds it and, indeed, all antibodies of that species (Figure 2.3b). This antibody is biotinylated; it is engineered to express multiple molecules of biotin, each able to bind avidin once. The next two reagents are mixed and given at least 30 minutes to associate; they are "A" streptavidin (a form of avidin better for IHC) and "B" – biotin complexed with HRP. When

Figure 2.2 The Shandon cover plate Sequenza™ system

Table 2.1 The 'in-house' preparation of solutions most commonly used in immunohistochemistry

Solution	Materials	Company	Quantity	Method
Citrate buffer concentrate	Citric acid. H_2O	Sigma-Aldrich, Gillingham, UK	21g	Working dilution: Take 50mls of the citric acid concentrate and make up to 500mls with dH_2O. Adjust the pH to 6.0 using 5-10 mmol HCl.
	NaOH pellets	Sigma-Aldrich, Gillingham, UK	10g	
	dH_2O	In-house	1000mls	
EDTA concentrate	TRIS	Sigma-Aldrich, Gillingham, UK	12.1g	Working dilution: Take 50ml of EDTA concentrate made up to 500mls with dH_2O. Adjust the pH to 8 – 9 using 5 -10 mmol NaOH / 5 – 10 mmol HCl.
	EDTA	Sigma-Aldrich, Gillingham, UK	374.24g	
	dH_2O	In-house	1000mls	
TBS	Tris Base	Sigma-Aldrich, Gillingham, UK	6.06g	To make 1l of 0.05m Tris 0.15m NaCl TBS: Dissolve the Tris base in 90ml of dH_2O. Adjust the pH with 10 mmol HCl to 7.6 and bring the whole up to 100mls with dH2O. Dissolve the NaCl in 100ml of dH2O. Add both solutions to the remaining 800mls of dH_2O. Add 2ml of Brij 35 to decrease the surface tension.
	NaCl	Sigma-Aldrich, Gillingham, UK	8.76g	
	dH_2O	In-house	1000mls	
	Brij 35	Sigma-Aldrich, Gillingham, UK	2mls	
0.5 % Copper Sulphate solution	$CuSO_4$	Sigma-Aldrich, Gillingham, UK	2.5g	To make 500mls of 0.5% $CuSO_4$ solution, 0.8% NaCl: Add 2.5g of $CuSO_4$ to 500mls dH_2O and add the 4.0g NaCl.
	NaCl	Sigma-Aldrich, Gillingham, UK	4.0g	
	dH_2O	In-house	500mls	

dH_2O: deionised water
TBS: TRIS Buffered Saline
Tris: TRIShydroxymethylaminomethane
EDTA: Ethylene Diamine Tetracetic Acid
NaOH: Sodium hydroxide
NaCl: Sodium Chloride
$CuSO_4$: Copper Sulphate

Figure 2.3 The molecular arrangement of reagents in immunohistochemistry

Figure 2.3a

The molecular arrangement of reagents in direct IHC and the presence of an HRP labelled primary antibody

Please refer to Figure 1; Boenisch, T., A. J. Farmilo, et al., Eds. (2001). Handbook: Immunohistochemical Staining Methods. Gostrup, Denmark, Dako.

Figure 2.3b

The molecular arrangement of reagents indirect IHC and the presence of a secondary antibody

Please refer to Figure 7; Boenisch, T., A. J. Farmilo, et al., Eds. (2001). Handbook: Immunohistochemical Staining Methods. Gostrup, Denmark, Dako.

Figure 2.3c

The molecular arrangement of reagents in the 'ABC' method
The streptavidin-biotin-HRP complexes are seen attached to a biotinylated secondary antibody.

Please refer to Figure 10; Boenisch, T., A. J. Farmilo, et al., Eds. (2001). Handbook: Immunohistochemical Staining Methods. Gostrup, Denmark, Dako.

associated into “AB”, each streptavidin molecule binds several biotin-HRP complexes. When this is added to the tissue each multi-HRP expressing complex can bind to each biotin site on the secondary antibody, multiplying the sites available for chromogen binding many times when compared to a direct method (Figure 2.3c). A commercially available kit was used for this work:

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

“A” = streptavidin in 0.01 mol/L phosphate buffered saline, 15mmol/L NaN_3; pH 7.2 diluted to 1:100

“B” = biotinylated horseradish peroxidase in 0.01 phosphate buffered saline, 15mmol NaN_3; pH 7.2 diluted to 1:100

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

Many normal tissues exhibit Endogenous Avidin Binding Activity (EABA) which can lead to non-specific binding of “AB”. Therefore, to prevent non-specific staining, before the primary antibody was applied the tissue was incubated with avidin which saturates its binding capacity, producing a layer that will bind biotin. By then incubating the tissue in excess biotin, all biotin binding sites are filled, preventing non-specific binding of “AB” as biotin can only bind avidin once (Wood and Warnke 1981) (Figure 2.4). 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

In order to visualise the location of the *primary antibody-secondary antibody-streptavidin-biotin-HRP* complex and thereby determine the antigen expression, the chromogen 3, 3’-diaminobenzidine (DAB) (K3465, Dako, Ely, UK) was used. On oxidisation by HRP, DAB produces a brown substrate which is clearly seen on microscopy.

Figure 2.4 The suppression of Endogenous Avidin Binding Activity (EABA)

Please refer to Figure 1e-f; Wood, G. S. and R. Warnke (1981). "Suppression of endogenous avidin-binding activity in tissues and its relevance to biotin-avidin detection systems." J Histochem Cytochem 29(10): 1196-204.

Endogenous peroxidase in tissues not labelled by primary antibody can non-specifically oxidise DAB. In order to prevent non-specific staining, the endogenous peroxidase activity was blocked before the antigen retrieval step using 0.3% hydrogen peroxide in methanol; 4mls of 30% hydrogen peroxide (Sigma-Aldrich, Gillingham, UK) was added to 400mls of methanol (Sigma-Aldrich, Gillingham, UK). A stronger 3% solution of hydrogen peroxide was not used in an attempt to preserve the integrity of cell surface antigens.

After the application of the DAB, the staining was darkened by the application of 0.5% copper sulphate in 0.8% sodium chloride (Table 2.1) and haematoxylin (Vector Laboratories Inc, California, US) used to counter stain the background blue.

To view the sections under the microscope, they were dehydrated in the graded alcohols (by reversing the order they proceeded through them), cleared of ethanol in xylene and Distyrene, Plasticiser and Xylene (DPX) (BDH, Poole, UK) was applied, followed by cover slips. This was performed in a fume cupboard and the slides were left to dry overnight.

2.2.8. IHC protocol

The principles described above are condensed into the following protocol.

1. Place the sections into a slide basket and then into a dry oven at 60º for 10 minutes.
2. Place the slide basket into a xylene bath (kept in a fume cupboard) for 10 minutes. Repeat this step using a different xylene bath.
3. Transfer the slide basket sequentially into three baths containing graded alcohols (kept in a fume cupboard) spending 10 seconds in each.
4. Place the slide basket into a bath of 0.3% hydrogen peroxide solution in methanol for 15 minutes (still within the fume cupboard).
5. Wash the slides in a container of tap water through which water is running.

6. Place the slide basket into a closed microwavable plastic container holding 500mls of the appropriate antigen retrieval fluid made up to the required dilution and pH. Microwave the container for 10 minutes on high power and then 10 minutes on low power.
7. Transfer the slide basket to a container of tap water through which water is running. Mount the slides onto cover plates and place them in a Sequenza™, taking care not to allow the sections to dry out.
8. Wash the sections with TBS.
9. Apply 100 µl of NSS diluted 1:5 in TBS to each slide for 15 minutes.
10. Apply 100 µl of avidin D solution to each section for 15 minutes and then rinse with TBS. Apply 100 µl of biotin solution for a further 15 minutes.
11. Rinse the sections three times, for 5 minutes each time, with TBS.
12. Apply 100 µl of optimally diluted antibody to each section, except the negative control, for 60 minutes.
13. Apply 100 µl of appropriately selected and diluted negative control immunoglobulin to the negative control sections for 60 minutes.
14. Wash the sections three times, for 5 minutes each time, with TBS.
15. Apply 100 µl of reagent “C”, diluted to 1:100 in TBS, to each slide for 30 minutes.
16. Wash the sections three times, for 5 minutes each time, with TBS.
17. Combine and dilute reagent “A” and reagent “B” to 1:100, by adding equal quantities to the appropriate volume of TBS. This must be at done at least 30 minutes before the “AB” can be used.
18. Apply 100 µl of the “AB” complex to each section for 60 minutes.
19. Wash the sections three times, for 5 minutes, with TBS.
20. Apply 100 µl of DAB, diluted 1:50 in its substrate buffer, to each section for 5 minutes. Repeat this step once.
21. Wash the sections three times, for 5 minutes each time, with TBS.
22. Apply 100 µl of 0.5% copper sulphate solution to each slide for 5 minutes. Repeat this step once.
23. Wash the sections three times, for 5 minutes each time, with TBS.
24. Apply 100 µl of haematoxylin to each slide for 5 minutes. Repeat this step once.

25. Remove the sections from the Sequenza™ and cover plates and wash in them in a container of tap water through which water is running.
26. Return the sections to a slide basket and dehydrate them by rotating them through the three sequential alcohol baths in reverse order, spending 10 seconds in each.
27. Place the sections into a xylene bath for 10 seconds and repeat this step in a different xylene bath.
28. Mount slide covers onto the slides using DPX and leave the slides in the fume cupboard to dry overnight.

2.2.9. Evaluation of the staining

The scoring of the tissue was performed concurrently by two experienced investigators, blinded to the clinical information (P.R and S.D), using a double-headed microscope. Whole sections and controls were initially reviewed to check that the location of the staining was correct. The TMA cores were then reviewed and scored using semi-quantitative systems.

Semi-quantitative systems

Within each core, only tumour cells were considered and if this represented <10% of the core, then the core was not scored.

The percentage number of cells staining was scored to the nearest 5%. This approach is supported by data showing that human observers can score the overall percentage of pink areas, that have been randomly superimposed onto a blue square in known quantities by a computer, with this level of accuracy (Cross 2001).

The intensity of the staining was scored if it appeared to vary between cases. The scoring system was 0 = 0; mild = 1; moderate = 2; strong = 3. Reference images of typical intensities were made to aid scoring.

Where the intensity and percentage of cells staining varied highly between cases, composite Intensity Scores (IS) were generated. IS = percentage cells stained x intensity (range = 0-300).

Concordance

The TMA matrices were scored by systematically following the array grids (Figure 3.6 a-d). Random cases were selected, scored and discussed, acting as a learning set. The arrays were then scored fully and independently, with consensus being reached if difficult cases were encountered. At the end of scoring each array grid, concordance was checked and was invariably >90%.

Post hoc data processing

Following scoring, all expression data (apart from intensity) needed appropriate grouping to facilitate statistical analysis. This was most commonly grouped into binary categories using justifiable cut-offs (high-low/positive-negative/either side of the median) or quartiles. Though initial allocation into discrete categories (positive/negative) is more reproducible when performing a semi-quantitative analysis than using more complex systems (eg, to the nearest 5%, H-score, etc) (Cross 1998), the opportunity was most often taken to collect more detailed data as there were no concerns regarding lack of concordance in this work. Cut-offs were informed by the median values, previous work and initial statistical analyses.

Quantitative scoring

Lack of standardisation in IHC is one reason why findings can be difficult to reproduce between centres. Semi-quantitative scoring is subjective and moves to objectify this and thereby improve standardisation have been made through introducing automated image analysis.

These systems produce a detailed digital image of the slide and are then be calibrated to analyse tumour areas within the cores, returning detailed quantitative data regarding intensity and the percentage of area stained. These systems have been used successfully by different groups studying ovarian cancer, both on whole sections and TMAs (Geisler, Geisler et al. 1997; Rosen, Huang et al. 2004). However, there are disadvantages. The set up of the system introduces variation

between operators which has been shown to reduce reproducibility. In addition, they are expensive, relatively slow and not widely available. As it can also be shown that semi quantitative scoring by a human observer is as accurate, quantitative scoring was not pursued in this work (Cross 2001; Goethals, Perneel et al. 2006). Some workers have argued that until standardisation of antigen retrieval and fixation is achieved, quantitative scoring will add relatively little to improving inter-laboratory variation (von Wasielewski, Mengel et al. 2002).

2.2.10. Pilot staining study at DCGH pathology laboratory

A pilot staining study was performed in order to acquire preliminary data about the staining characteristics of the array. Single copies of the array (copy 1) were stained for the markers which are routinely used to differentiate ovarian cancers from secondary ovarian deposits (colorectal, breast, etc). These comprised CK7, CK20, CEA and CA 125 (Lagendijk, Mullink et al. 1998).

The method used differed slightly from that detailed previously because the slides were included in a routine run of work performed at DCGH by Miss Andrea Gooding using an Optimax automated staining system (Biogenex, California, US). In brief, sections were mounted on 3-aminoPropyltriEthoxySilane (APES) treated slides and dewaxed in the normal way. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol. The antigen retrieval was as detailed previously with citrate buffer being used. The details of the primary antibodies are shown in Table 2.2. The negative control was by the omission of the primary antibody and its substitution with NSS. The amplification step was performed using Super Sensitive™ Polymer-HRP IHC Detection (Biogenex, California, US) and visualisation was by DAB. The cores were scored in the manner in which pathologists would normally score them – positive or negative, in binary fashion. Positive cases were effectively identified when >5% of cells stained within a core.

Following the results of the pilot study, four copies of the array were stained manually for CK7 and CEA to allow more detailed evaluation.

2.2.11. Manual staining details for individual markers

All manual staining was performed at NCH using the IHC protocol (2.2.8) with the following modifications for the individual markers (Table 2.3)

2.2.12. Staining the TMA for CD46

CD46 represented a special case, as no suitable antibody that had previously been used in published work to study CD46 expression in FFPE tissue could be found in sufficient quantities. Fortunately, prior work within the department performed by Dr Z Madjd had resulted in the successful production and validation of a suitable anti-CD46 antibody. This is detailed in her PhD thesis (Madjd 2004). As this antibody was used in this study, a brief summary of the steps involved in its creation and validation is provided below.

Generation of anti-CD46 monoclonal antibody

Monoclonal antibodies to CD46 were generated as previously described (Madjd, Durrant et al. 2004). Briefly, the peptide (CLKVSTSSTTKSPA), located on serine-threonine-proline rich (STP) regions of CD46 and was synthesised (Alta Bioscience, Birmingham) and conjugated to the carriers Keyhole Limpet Haemocyanin (KLH) and Bovine Serum Albumin (BSA) using sulpho-Maleimidobenzoyl-N-hydroxysulfosuccinimide (MBS) (Perbio Science, Cheshire, UK). The purified peptide-KLH conjugates were used to immunise Balb/c mice. Immune serum was tested by Enzyme Linked Immunosorbant Assay (ELISA) against peptide-BSA conjugates. Peak titre anti-sera was used in ELISA against peptide-BSA conjugate and compared against pre-immune sera (Figure 2.5). Splenocytes from the mouse showing greatest response were fused with NS0 (myeloma) 5 days after the final injection, seeded onto microtitre culture plates and suspended in Hypoxanthine-Aminopterine-in-Thymidine (HAT) (Sigma-Aldrich, Gillingham, UK) medium for 10 days before screening the supernatant

Table 2.2 The antibodies used in the pilot IHC TMA study
An automated staining system was used to stain the TMA slides (Optimax, Biogenix, California, U.S.).

Antibody	Type	Manufacturer	Clone Code	Optimal Dilution	References
CK 7	Mouse Monoclonal IgG1	Bond, Peloris & Novocastra Reagents, Newcastle, U.K	OV-TL-12/30 NCL-L-CK7-OVTL	1:20	(van Niekerk, Jap et al. 1991)
CK 20	Mouse Monoclonal IgG2a	Bond, Peloris & Novocastra Reagents, Newcastle, U.K	K_S20.8 NCL-L-CK20	1:50	(Moll, Lowe et al. 1992)
CEA	Mouse Monoclonal IgG1	Bond, Peloris & Novocastra Reagents, Newcastle, U.K	II-7	1:60	(Nap, Hammarstrom et al. 1992)
CA 125	Mouse Monoclonal IgG1	Bond, Peloris & Novocastra Reagents, Newcastle, U.K	OV185:1 NCL-L-CA125	1:20	(Keen, Szakacs et al. 1999)

Table 2.3 Immunohistochemical details of the individual marker studies (1 of 2)

Antigen	Antibody Type	Company	Clone Code	Dilution	Antigen Retrieval Solution	Positive Control Tissue	Negative control antibody	TMA copies stained	Scoring Data Collected Positive cut-off	Reference
CK7	IgG1 Monoclonal Mouse	Dako, Ely, UK	OV-TL 12/30 M7018	0.505mg/ml 1:200	Citrate pH 6.0	OVCA ANO 10	Non-immune murine IgG1 100mg/L 1:40 MCA928,Serotec, Oxford, UK	1,2,3,4	% Cells >5%	(Poels, Jap et al. 1989)
CD66ce (CEA)	IgG1 Monoclonal Mouse	Serotec, Oxford, UK	198 MCA1839	1mg/ml 1:400	Citrate pH 6.0	OVCA ANO 18	Non-immune murine IgG1 100mg/L 1:40 MCA928,Serotec, Oxford, UK	1,2,3,4	% Cells >5%	(Nap, Hammarstrom et al. 1992)
p53	IgG2a Monoclonal Mouse	Dako, Ely, UK	DO-7 M7001	390ml/L 1:30	Citrate pH 6.0	OVCA ANO 139	Non-immune murine IgG2b 100mg/L 1:8 X0944,Dako, Ely, UK	1,2	% Cells >10 %	(Lassus, Leminen et al. 2003)
Bcl-2	IgG1 Monoclonal Mouse	Dako, Ely, UK	124 M0887	280ml/L 1:50	EDTA pH 8.0	Tonsil	Non-immune murine IgG1 100mg/L 1:18 MCA928,Serotec, Oxford, UK	1,2	% Cells >10%	(Delia, Aiello et al. 1992)

Table 2.3 Immunohistochemical details of the individual marker studies (2 of 2)

Antigen	Antibody Type	Company	Clone Code	Dilution	Antigen Retrieval Solution	Positive Control Tissue	Negative control antibody	TMA copies stained	Scoring Data Collected Positive cut-off	Reference
HLA Class I	IgG2a Monoclonal Mouse	Gift: Prof. Ploegh Harvard Medical School, US	HC-10	1:10	Citrate pH 6.0	Tonsil	Omission of primary antibody Normal swine serum	1,4	% Cell membrane intensity >10% cells ≥ +1 intensity	(Stam, Spits et al. 1986)
β2-m	Polyclonal Rabbit	Dako, Ely, UK	A0072	1:3000	None	Tonsil	Omission of primary antibody Normal swine serum	1,3	>10% cells ≥ +1 intensity	(Madjd, Spendlove et al. 2005)
CD59	IgG2a Monoclonal Mouse	Serotec, Oxford, UK	MEM-43 MCA1054	1 mg/ml 1:7	Citrate pH 6.0	Tonsil	Non-immune murine IgG2a 100mg/L 1:1 MCA929 Serotec, Oxford, UK	1,2	% Cells ≥ 50%	(Madjd, Pinder et al. 2003)
CD55	IgG2b Monoclonal Mouse	'In-house'	RM1	1mg/ml 1:16	Citrate pH 6.0	Tonsil	Non-immune murine IgG2b 100mg/L 1:8 X0944,Dako, Ely, UK	3,4	Intensity Score ≥ 225	(Madjd, Durrant et al. 2004)
CD 46	IgG1 Monoclonal Mouse	'In-house'	MZ1	1mg/ml Undiluted	Citrate pH 6.0	Tonsil	Non-immune murine IgG1 100mg/L 1:1 MCA928,Serotec, Oxford, UK	3,4	Intensity Score ≥ 65	(Madjd 2005)

Figure 2.5 ELISA showing titration of MZ1 antiserum against purified CD46

ELISA plates were coated with 5 µ l/ml of purified CD46 overnight at 4°. Protein was detected with mouse antiserum and then rabbit anti-mouse HRP conjugate and OD measured at 405 nM.

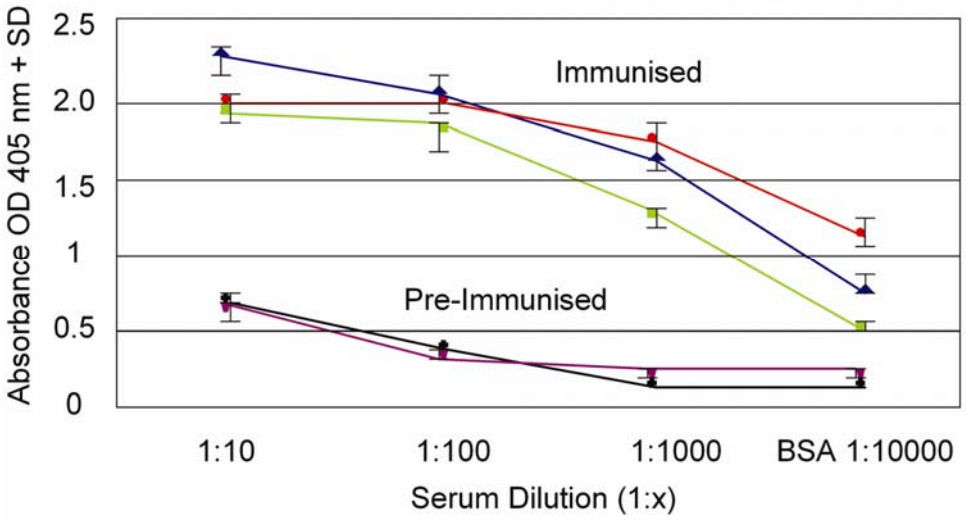
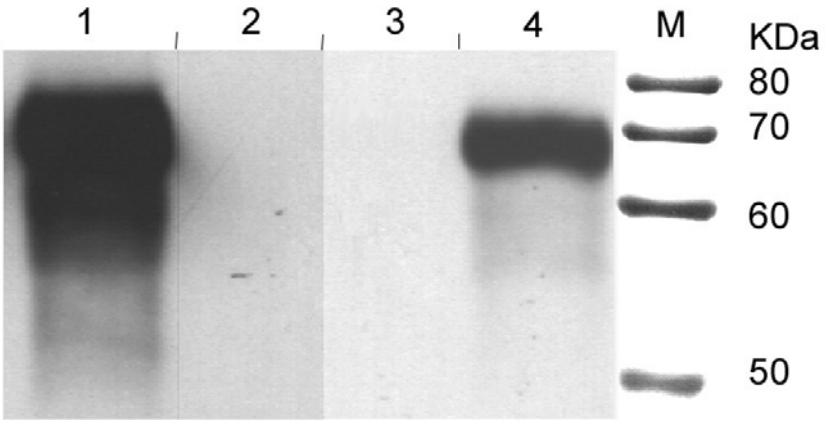


Figure 2.6 Western blot of protein samples from 791T cells

The cells were separated by SDS- (10%) and Western blotted. Lanes 1 and 2 represent non-reduced samples probed with rabbit polyclonal anti CD46 (lane 1) and MZ1 (lane 2). Lanes 3 and 4 represent reduced samples probed with MZ1 in the presence or absence of blocking peptide respectively. M represents 10KDa molecular weight markers.



against peptide-BSA conjugate. Positive wells were cloned at 3, 1 and 0.3 cells/well and re-screened for antibody producing clones. Second and third clonal expansions followed. Supernatant showing reactivity to the immunised peptide on ELISA was tested using a mouse isotyping kit (Serotec, Oxford, UK). IgG1 positive supernatant was discovered and purified to produce the MZ1 monoclonal antibody, the concentration of which was 1mg/ml.

Western blotting

The MZ1 monoclonal antibody was tested against the CD46 expressing cell line (791T) by reducing and non-reducing Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) and Western blotting. Samples were separated by standard 10% SDS-PAGE and Western blotted before probing with the anti-CD46 antibodies. The antibodies were either anti-CD46 monoclonal antibody (MZ1, 1/5) or the rabbit anti-human CD46 polyclonal antibody (1/200) (Gift: N Rushmere, University of Wales College of Medicine) (Singhrao, Neal et al. 1999; Madjd, Durrant et al. 2005). The Western blot was developed with 1/4000 rabbit anti Mouse or 1/2000 goat anti rabbit-HRP conjugated secondary antibodies (Dako, Denmark) and developed with ECL+ reagent (Amersham, UK). Polyclonal rabbit anti-CD46 was shown to detect a strong band by non-reducing SDS-PAGE while MZ1 was unable to identify any protein bands under these conditions. Under reducing conditions a band of approximately 65KDa was identified (lane 4) which could be blocked by the pre-incubation of MZ1 with peptide-BSA conjugate (lane 3), demonstrating specificity of the antibody for the 65KDa protein band (Fig. 2.6).

Peptide blocking of anti-CD46 monoclonal antibody on tissue sections

The specificity of MZ1 was demonstrated on a series of formalin fixed, paraffin embedded breast cancer sections using standard IHC protocols, the MZ1 antibody having been pre-incubated with varying concentrations of the Z154-BSA peptide conjugate to which it had been raised. A strong staining pattern to the MZ1 antibody was seen in

breast tumours (Fig 2.7a) which was completely inhibited when the monoclonal antibody was pre-incubated in the presence of the peptide conjugate (Fig. 2.7b).

Immunohistochemical staining of the ovarian TMA with MZ1

Having created the monoclonal MZ1 antibody, Dr Madjd also proved that it was specific for CD46 and that it could be used to study CD46 expression in FFPE tissues. The details of its use in this study are contained in Table 2.3.

2.3. Statistical tests

Following scoring, the expression data were statistically analysed. The majority of the statistical analyses were performed using the computer package SPSS version 11.0 & 13.0 (SPSS Inc, Chicago, Illinois, US).

2.3.1. p values

The null hypothesis is that no difference exists between groups. The alternate hypothesis is that a difference does exist between groups. Rejecting the null hypothesis means accepting the alternate hypothesis. When the null hypothesis is rejected when a true difference does not exist, this is a false positive and is type I error. The p value is the statistical chance of a false positive occurring. If a p value = 0.05, this means that the chance of a difference being a false positive is 1 in 20. If the p value is close to 1 there is a high probability that a result represents a false positive and if it is very small there is a low probability that accepting the result will represent a type I error. The level of chance of a false positive below which one will reject the null hypothesis is the alpha value and by convention is 0.05; therefore, a p value of <0.05 is considered to represent statistical significance. This is not the same as the difference between groups being large; it simply means that the probability of the difference having occurred by chance alone is relatively low. If 20 independent hypotheses are tested on the same data, with an alpha value of 0.05, one false positive association will occur. For this reason, where (n) number of hypotheses are being

Figure 2.7 **Peptide blocking of MZ1**
Immunohistochemical staining of serial breast sections stained with anti-CD46 monoclonal antibody.

Figure 2.7a Without pre-incubation with specific CD46 peptide.

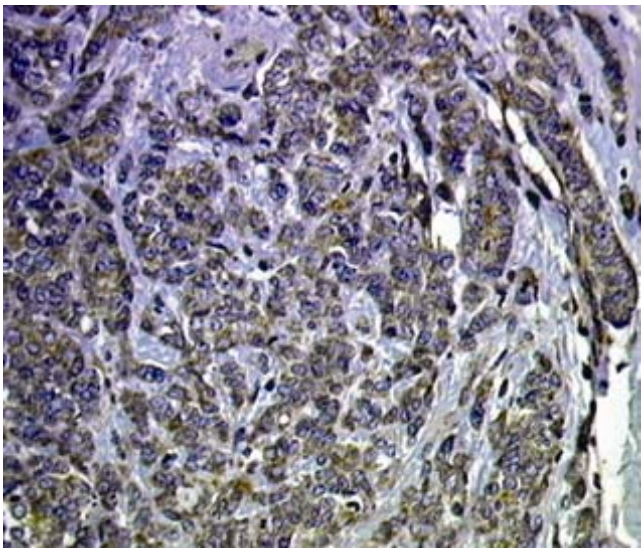
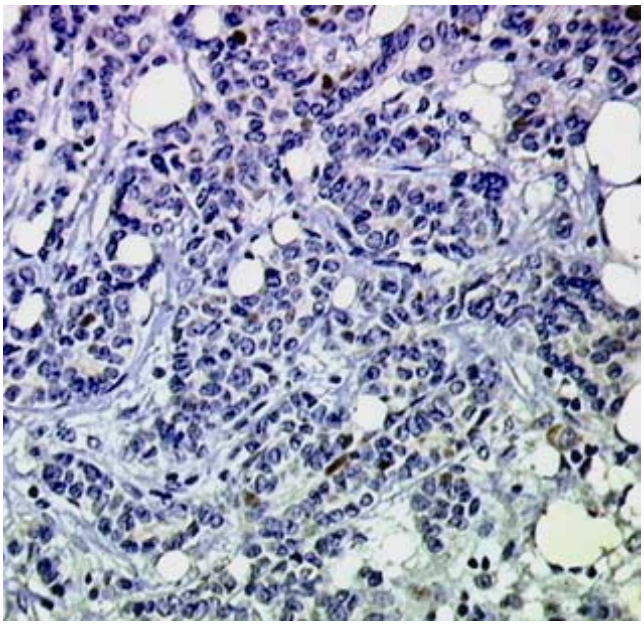


Figure 2.7b Following pre-incubation with specific CD46 peptide



tested, the alpha value should be adjusted to (0.05/n) – this is the Bonferroni correction (Swinscow, Campbell et al. 2002).

2.3.2. Univariate analysis of associations with clinicopathological criteria – Persons Chi squared (χ^2) test

The data for molecular marker expression and that representing the clinicopathological characteristics of the individual cases with which they would be compared were divided into categories.

The χ^2 test measures the probability that categorical variables have an effect on one another; for instance, that the positive expression of a marker would be associated with grade of disease. The categories are cross tabulated against each other forming tables the size of which range from 2 x 2 upwards, depending on the subdivision of the categories; positive and negative expression of a marker being compared with grade 1, 2 or 3 of disease would form a 2 x 3 table containing 6 individual cells. If there were no association between expression of a marker and grade, the proportions of cases with grade 1, 2 and 3 disease would be equal in both positive and negative marker groups. The χ^2 test measures the numerical difference between this expected scenario (E) and what is actually observed experimentally (O) in each cell. The sum of $([O-E]^2/E)$ is inputted into an χ^2 distribution which returns a p value.

The clinicopathological categories comprised age, FIGO stage, degree of cytoreduction, grade of disease, the use of chemotherapy and the histological subtype of the disease – 6 categories. The alpha value was therefore corrected to (0.05/6 = 0.012).

Some tables were considerably bigger than 2 x 2 and this was reflected in the degrees of freedom assigned to the calculations. The degrees of freedom = (number of columns - 1) x (number of rows - 1) – and in the example of grade this = (2-1) x (3-1) = 3. The number of degrees of freedom has a large effect on the p value returned. When large tables were generated the cases were spread more sparsely between the cells. The χ^2 test represents an approximation to the more accurate

though complex Fishers Exact test but for it to remain a good approximation each cell within the table needed to contain a minimum number of cases. No more than 1/5 of the cells were allowed <5 cases and none were allowed less than 1. In cases where the cells did not contain the appropriate number of cases the analysis defaulted to Fishers Exact test. In addition, when the numbers in a fourfold table were fairly small (<10 cases per cell or where the total number of cases was <100) Yates correction was applied. These methods are automatically appropriately applied by the statistical package SPSS vs 11 & 13 (Chicago, Illinois, US).

The limitation of the χ^2 was that it could only be used for categorical variables and these needed to be arranged in a valid way into the fewest number of categories possible. However, the main assumption, that each case contributes data to only one cell, appeared valid (Swinscow, Campbell et al. 2002).

2.3.3. Univariate analysis of associations with survival

Kaplan-Meier plot

Patients followed up over time from the point of treatment are at any given time point either alive, dead or lost to follow up. Ideally, no patients would be lost to follow up and the statistical analysis would only occur when all of the patients had died – of the disease or of other causes. However, this is impractical and the Kaplan-Meier method allows meaningful survival analysis with incomplete data.

The cases' survival times are ordered from smallest to largest in life tables from which the proportion of patients at risk of dying is then calculated at regular time points from treatment. For this method to be accurate, patients who are lost to follow up between intervals need to be removed – or censored – from subsequent analyses. The resulting cumulative survival is plotted against time as a Kaplan-Meier curve with censored cases being denoted by "+". If the effect of a binary marker on survival is being studied, two life tables can be constructed – one for

each category. The resulting curves may then be compared on the same axes where survival differences may be visually apparent.

This method assumes that the 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. It also assumes that patients entered into the study early have the same long term survival prospects as those entered later. Lastly, it assumes that the survival data are continuous and not grouped to the nearest follow up point (Bland and Altman 1998).

Log rank statistic

This test is used to determine whether there is a statistical difference in survival between two or more groups when the data are in continuous form and the Kaplan-Meier plot has been performed.

In the same way as when a Kaplan-Meier curve is plotted, the data are ranked in order of shortest to longest survival and sequential calculations of expected and observed numbers of deaths within each group are made each time a death occurs. When data are censored, they are excluded from subsequent analyses. The end result is the generation of a total number of expected and observed deaths within each group. This is then used to generate the log rank statistic, the p value for which is derived from referring to an χ^2 distribution table.

This method allowed differences in the survival experience over the whole of the study period to be investigated, rather than at arbitrary time points (such as 5 year survival). However, it makes the same assumptions regarding censored data as the Kaplan-Meier method. It also assumes that the relationship between the relative proportions of those dying within the different groups remains constant over time. If one group's rate of death at a point in time is twice that of another group, it should not increase to three times the rate of that group at a different time point – the ratio should be maintained through time. Practically speaking, if this assumption of proportional hazards is valid, this means that Kaplan-Meier curves would not cross. If the curves

cross then the log rank test cannot be performed and would in any event be unlikely to give a significant result (Bland and Altman 2004).

2.3.4. Multivariate analysis of associations with survival – Cox's proportional hazards model

The log rank test is a test of significance and does not compute size of effect or the independence of the effects of multiple factors on survival.

In order to do this, the Cox's regression was performed. This multivariate analysis measures the interrelationships between multiple variables simultaneously.

The model generates a baseline survival curve and calculates the hazard ratio (HR) for a variable by measuring the effect of removing it and adding it to the model. It determines the magnitude and independence of each variable's individual influence on the overall hazard rate. It returns a HR for each variable reflecting the increased likelihood of death at any time point due to the presence of that factor compared to the baseline.

Ninety-five percent confidence intervals (CI) are also generated. These delineate the range of values within which the true HR has a 0.95 chance of lying. An HR of <1 means that the factor will predict and improved prognosis. An HR of >1 means that the factor will predict a worse prognosis. An HR of 1 indicates that the factor has no influence on survival. Ninety-five percent CI must therefore confine their range to either wholly above or below 1 for a variable to be considered to have an effect on survival independently of other factors.

If a factor is associated with survival on univariate analysis but its HR 95% CI straddles 1 on Cox regression then this would suggest that this factor's effect on survival was due to an association with a more important, truly independent prognostic factor, such as FIGO stage.

The Cox model assumes that the effects of a factor's ability to predict outcome remains constant over time and that hazard rates remain proportionate between groups independently of time. Therefore,

variables which do not predict survival in a univariate analysis or factors with Kaplan-Meier curves that cross should not be included in this model, because in those situations this assumption is not valid (Cox 1972).

2.3.5. Power calculations

The power calculations for this study were performed in conjunction with an experienced biostatistician, Dr Sarah Lewis (UoN) using the EGRET package (1999) (Cytel Inc, Massachusetts, US).

The power calculations were made part way through the construction of the array when a full database was accessible. At this point it was possible to project the maximum number of cases which could be included in the study and to access information key to performing the calculations. Power calculations were undertaken in order to determine the importance of adding extra cases into the study and to project the size of effects that the future array would be able to detect.

The database revealed a total study period of 23 years with patients being accrued over 15 years. Of the 296 patients within a consecutive series of 358 who had died by November 2005, 40% had died within the first 12 months of their diagnosis, 88% had died by 60 months and 100% by 270 months. 62 were currently alive. Estimates of deaths over time were used as a guide to the baseline hazard function. From previous TMA work using other tissue types, it was estimated that a sample size of approximately 300 would be necessary to look for the relatively small effects that molecular markers exert on prognosis: HRs of between 1.5 and 2 being usual for molecular markers demonstrating independent prognostic power (Madjd, Spendlove et al. 2005; Watson, Spendlove et al. 2006; Rolland, Spendlove et al. 2007; Ullenhag, Mukherjee et al. 2007). Detailed information regarding the statistical variance for molecular marker expression in ovarian cancer as measured by TMA was not available from previous published work or pilot studies and so It was assumed that in most cases the molecular markers being studied would allocate the cases into one of two groups

(eg, high-low expression) and that the allocation of cases would often be unequal and range from 10:90 to 50:50. This range of potential allocation scenarios was investigated for between 300 and 360 cases. The alpha value was 0.05 and the power to detect an effect was set at 0.85 (Table 2.4).

Three hundred and thirty-nine cases would have an 85% chance of detecting a hazard ratio <0.67 and >1.5 with a false positive rate of 0.05 for an allocation of 10:90. Therefore, all available cases were added to the array and this information was then used in interpreting the results of the multivariate analyses.

The main assumptions of these calculations concern the estimations of HR and baseline hazard function. The limitations of using this method included the inelegance of the estimation of the molecular marker expression data (which limited the helpfulness of the calculations in predicting how the model would perform in situations where the data were not binary) and that the information could only be used to partially determine the optimal sample size (because the sample size was relatively fixed). The advantages of using power calculations were that it gave an idea of minimum sizes of effect that could be found in differing scenarios and informed a request to extend the series to eventually include cases up to 2002. The calculations also provided reassurance that the study was not overpowered, which could lead to the identification of very small differences unlikely to be of any clinical significance (Florey 1993).

Table 2.4 Power calculations

How detectable size of effect alters with increasing volume of cases and the proportional allocation of cases when the expression of a marker is binary.

Allocation ratio		**≈ 300+ Cases**			**≈ 300 Cases**	
		Nº Cases	Detectable HR		Nº Cases	Detectable HR
50:50		354	0.72> HR >1.38		300	0.67>HR >1.5
40:60		364	0.73>HR>1.38		288	0.7>HR>1.43
30:70		348	0.71>HR >1.40		300	0.71>HR>1.41
20:80		339	0.67>HR >1.49		300	0.67>HR>1.50
10:90		358	0.61>HR >1.65		300	0.59>HR>1.70

Assuming a fixed power of 0.85 and alpha value of 0.05

HR = Hazard Ratio

Chapter 3: Construction and Evaluation of the Tissue Microarray

This chapter deals with two distinct but inter-related processes vital to the production of a TMA which is to be used in the study of novel markers; firstly the physical construction of the tissue microarray and secondly the assessment of preliminary staining data derived from it.

The physical construction of the TMA involved identifying suitable cases, biopsying representative tissue and arraying and recording the position of this tissue in a systematic manner in order that the subsequently accrued expression data could be linked to clinicopathological data in order to provide meaningful analyses. The assessment of preliminary staining data from the array involved staining one copy of the array for four antigens (CK7, CK20, CEA and CA125) and comparing the expression data acquired with that widely available in the literature. Following this pilot evaluation of expression data, two antigens (CK7 and CEA) were studied in more detail in a full evaluation of the TMA. From the four copies of the array which were stained for these two antigens data were also generated from which the optimum number of copies to be used in the subsequent experimental work could be determined. Analysing this data also helped address potential concerns regarding the theoretical issue of tissue heterogeneity and sub-optimal tissue sampling in TMA work.

The aim of this chapter is therefore to document how the array was assembled and to give specifics about inclusions, exclusions, locations of particular samples and the pathological representativeness of the final cohort. Its aim is also to demonstrate that the TMA could produce staining data equivalent to that found within the literature through the use of two copies of the array which optimally balanced tissue core loss with accuracy of expression data and conservation of tissue and reagents

3.1. Construction of the TMA

3.1.1. Method

An overview of the steps involved in producing a TMA is outlined in Figure 3.1. Firstly potential cases for inclusion were determined through their identification on a prospectively maintained clinical database (Figure 3.2). The original pathology reports from these potential cases were then acquired from the patient record and subsequently anonymised (Figure 3.3). This allowed tissue to be located in the pathological archives which was then assessed contemporaneously by a gynaecological pathologist in order ultimately to identify representative tissue biopsy sites and to exclude inappropriate cases (Figure 3.4a-c). Tissue cores were then physically extracted from the appropriate block sites and inserted into the tissue array blocks in grid patterns (Figure 3.5a, b). A meticulous record of the positioning of the case tissue was produced (Figure 3.6a-d) and the final TMA blocks were then sectioned and H&E stained (Figure 3.7a, b)

3.1.2. Results

The cases included were recruited from those diagnosed between January 1st 1982 and December 31st 1997. The cohort of cases considered for inclusion numbered 424 after initial exclusions had been made (Figure 3.2), for which it was possible to find pathology reports for 403. Following contemporaneous review it was found that 66 of cases were unsuitable for inclusion and that 19 borderline cases had been included on the first array block. It was found that two cases had been physically included twice on the TMA under different study numbers (Figure 3.2). Ultimately tissue cores from a total of 339 surgically treated primary ovarian cancers were represented in four TMA blocks with five representative cores from each case being used to construct five copies of the TMA. 76 cases were represented on each of the TMA blocks 1-3 with 159 on the fourth block (Figure 3.6a-d).

The 339 cases included in the study were mostly epithelial ovarian cancers (98.5%). Serous adenocarcinomas (52.5% of the total) were the most numerous and clear cell adenocarcinomas (7.4% of the total)

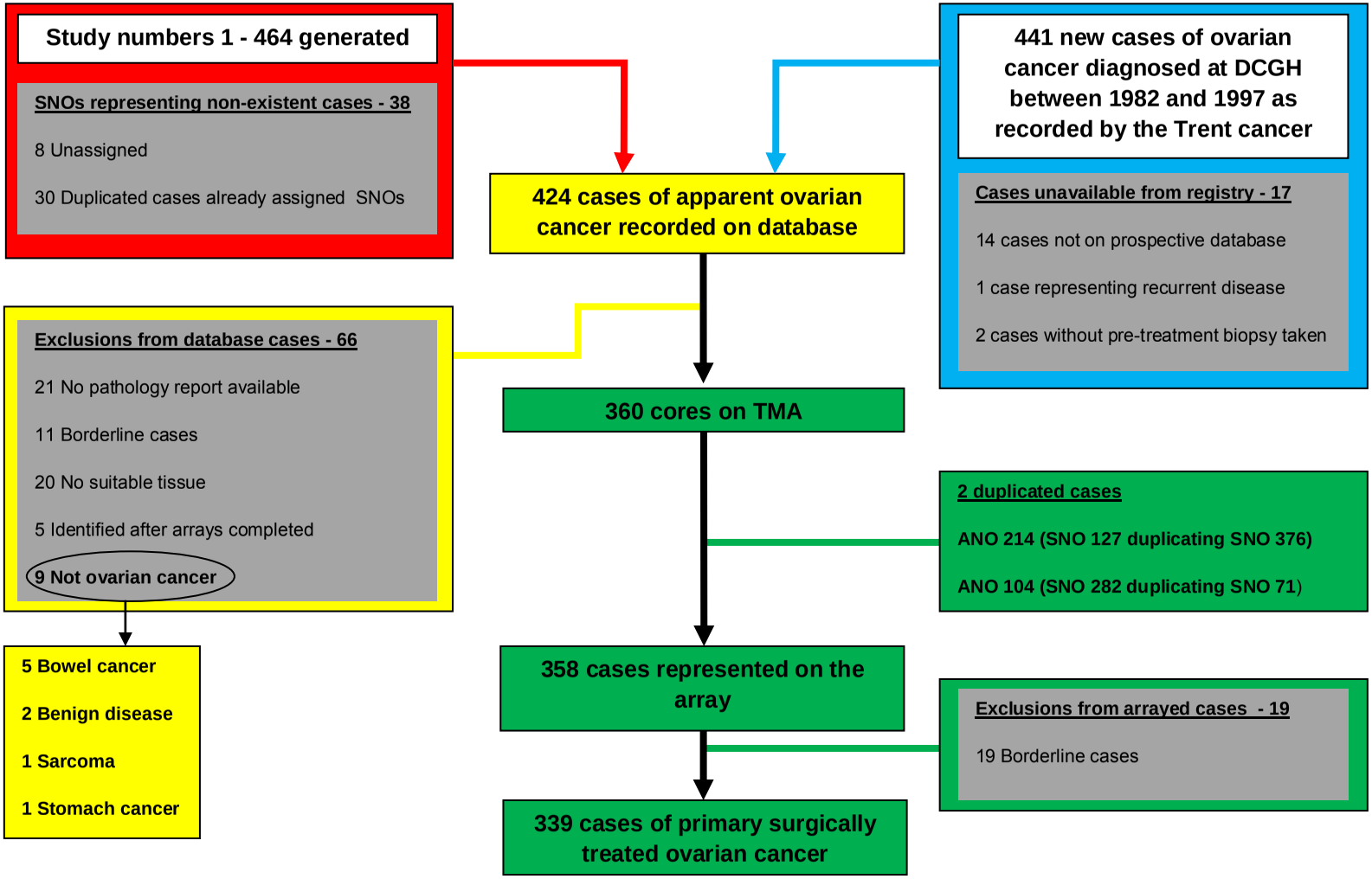
Figure 3.1 **The process of constructing and utilising a TMA**
The process of constructing a TMA begins with the identification of a suitable cohort of patients which may include many hundreds of cases. Cases are identified, retrieved and histopathologically reviewed after which representative tissue biopsies are physically taken from the archived blocks and transferred into a TMA format. The finished TMA is sectioned, stained for the marker being investigated and the staining evaluated. The expression data derived is then compared statistically with clinicopathological parameters including survival in a univariate and multivariate analysis.

Please refer to Figure 1; Rimm, D. L., R. L. Camp, et al. (2001). "Tissue microarray: a new technology for amplification of tissue resources." Cancer J 7(1): 24-31.

Figure 3.2

Logistics of identifying cases for the TMA

Restrictions placed on access to the complete dataset, a need for anonymisation and the staggered manner in which the tissue became available introduced errors which were identified and tracked. The study numbers were allocated before the database had been audited for duplicate cases and too many were issued. 441 cases of ovarian cancer were treated at DCGH in the recruitment period but only 424 cases could be included. 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 but this did not happen more frequently as cases usually had their suitable blocks temporarily removed from the archive during the arraying process. The most representative blocks were removed from the archive permanently and having punch holes to identify that the case had already been arrayed.



Despite the complex logistics, 339 individual cases of primary ovarian cancer treated surgically were represented on the 4 final array blocks from the investigated cohort of 441 presenting between 1982 and 1997 at DCGH.

Figure 3.3 **Anonymised pathology report**

When a case was identified for inclusion, a copy of the original pathology report was acquired which contained data essential for locating the archived tissue and then for narrowing down the number of blocks which needed to be reviewed by the pathologist. This was later anonymised and kept in the records. The unique pathology number could only be linked back to the patient through the original report in the patient's notes and therefore was allowed to remain by the ethics committee.

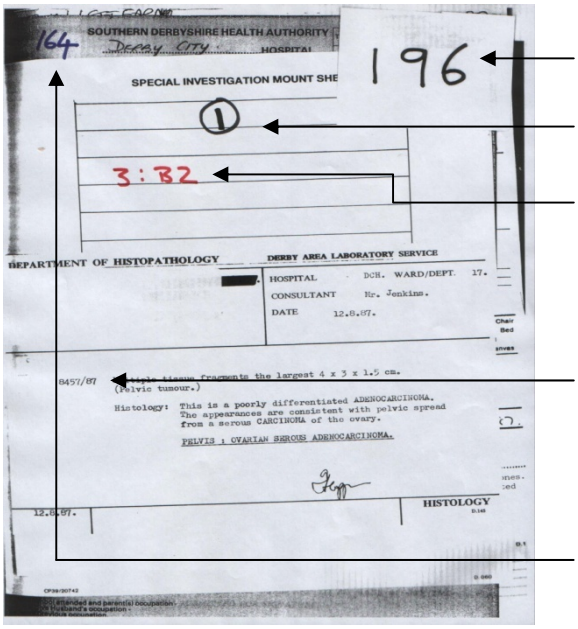


Figure 3.4 Identification of representative areas to include on the array

Figure 3.4a Archived tissue blocks

Each case to be included had up to 25 blocks pertaining to it within the archive. Blocks macroscopically appearing to contain cancer tissue were removed from the archive for review.

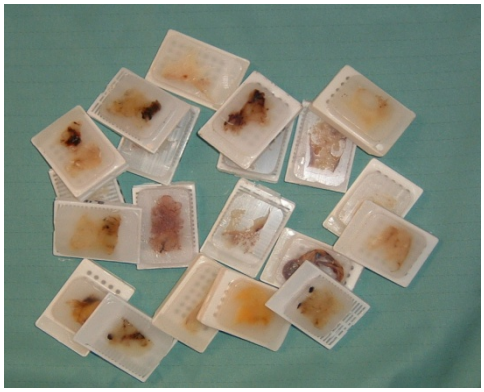


Figure 3.4b H&E sections

Section of these blocks were reviewed, typed and graded by a gynaecological pathologist. The most representative areas on the H&E slides were marked.

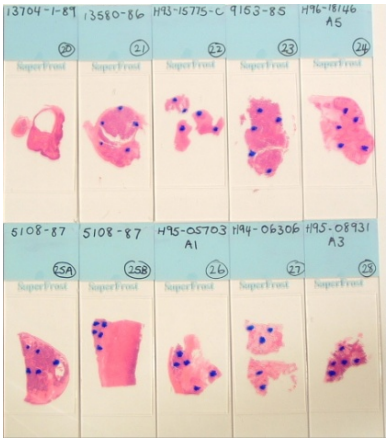


Figure 3.4c Marking the blocks

Using the H&E slides, the corresponding representative blocks identified were permanently separated from the main archive. The marker dots were transferred onto the blocks in preparation for the next stage; where the tissue was removed using the microarrayer.

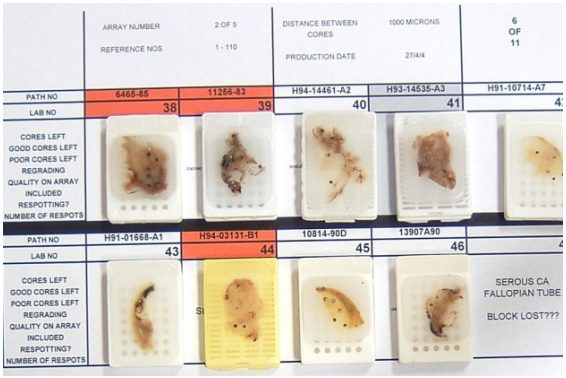


Figure 3.5 **The Beecher Instruments manual tissue microarrayer (Sun Prairie, WI, U.S.)**

Figure 3.5a **Frontal view**
Showing the positioning of a recipient block beneath the tissue microarrayer turret. The tissue is first precisely sampled and then precisely transferred using two separate (donor and recipient) needle punches mounted on the same swinging turret. The turret moves upwards and downwards, allowing the needles manoeuvrability in the Z axis. Ensuring that a correct depth of sample is transferred is dependent on the operator.

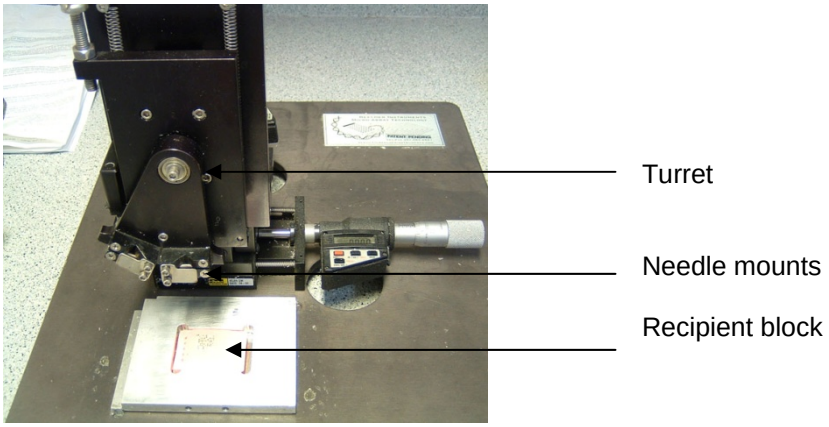


Figure 3.5b **Seen from above**
The perpendicular orientation of the micrometers is shown. These alter the turret's (and thereby the needle punches') alignment in precise increments. This allows each core to be precisely spaced from its neighbours (in this case 1mm) in the X and Y axes in order to produce a navigable grid pattern.

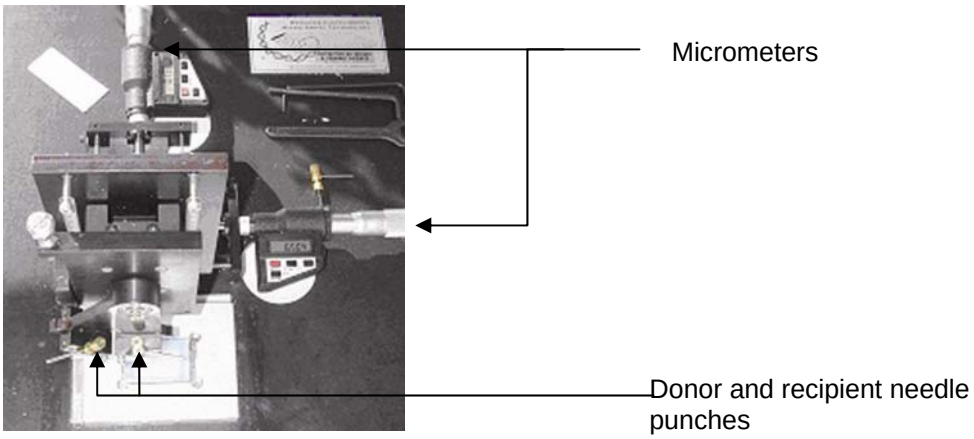


Figure 3.6 **The array grids mapping the location of tissue cores corresponding to each case from the 360 arrayed**

The 0.6mm tissue cores were extracted from donor blocks (which had had representative sites marked on them at histopathological review) using a Beecher Instruments™ (Sun Prairie, California, US) manual arrayer. The 339 cases were finally represented on four individual TMA blocks as illustrated bellow. For easier use, when the cases had all been arrayed their study number (SNO) was replaced by an array number (ANO), representing the case's sequential position on the array. This process was performed 5 times, producing 5 copies of the array. The individual block is identified as X:Y, where X = the TMA block within a copy of the array (1-4) and Y = the copy of the array being studied (1-5).

Figure 3.6a **Array block 1:1**

Showing the locations of the first 76 cases. The blanked out areas are the locations of borderline cases, arrayed but not used in this study. The first grid shows a case's SNO (the letters representing the block within that case's archive which arrayed) and the second grid shows its equivalent, final ANO. K represents a core of kidney, positioned to help orientation at microscopic evaluation.

J	105	106	107	108	109	110				
I	93	94	95	97	98	99	100	101	103B	104
H	81	82	83	84	85	87	89	90	91	92
G	69	70	71	72	73	74	76	78	78	79
F	55	56A	56B	57B	59	60	65	66	67	68A
E	44	45	46	48	49	50	51	52	53	54
D	32	33	35	36	37	38	39	40	42	43
C	24	25A	25B	26	27	28	29	30A	30B	31
B	13	14	15	16	17	18	19	21	22	23
A	1	2	3	4	5	6	8	10	11B	12
	1	2	3	4	5	6	7	8	9	10
	K									

J	72	73	74	75	76					
I	64		65	66	67		68	69	70	71
H	54	55	56	57	58	59	60	61	62	63
G	45		46	47	48	49	50	51	52	53
F	38	39			40	41	42	43	44	
E	31	32		33	34			35	36	37
D	23	24		25	26	27	28		29	30
C	17	18		19	20	21	22			
B	9	10		11	12	13		14	15	16
A	1	2	3	4	5	6		7	8	
	1	2	3	4	5	6	7	8	9	10
	K									

Figure 3.6b **Array block 2:1**
Showing the locations of the second 76 cases. The blanked out areas are the locations of cases arrayed wrongly. The first grid shows a case's SNO (the letters representing the block within that case's archive which arrayed) and the second grid shows its equivalent, final ANO. The boxed case, SNO 127, was found to be a duplicate of SNO 376 and was excluded from analysis. K represents a core of kidney, positioned to help orientation at microscopic evaluation.

J	K									
I										
H	174C	175A	176A	177	179B	180A				
G	164A	165	166A	167B	168A	169A	170A	171A	172A	173B
F	153A	154A	155A	156A	157A	158A	159A	161A	162C	163C
E	142B	143A	144C	145A	146A	147A	148A	149	150B	151C
D	131B	132A	133A	134A	135A	136B	138B	139F	140A	141B
C	120A	121A	122	123A	124B	125A	126	127A	128A	130A
B	80B	88D	111A	112A	113B	114A	115A	116A	117B	118A
A	8B	15C	20B	46B	47	50C	58B	62C	66C	77A
	1	2	3	4	5	6	7	8	9	10
	K									

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 3.6c **Array block 3:1**

Showing the locations of the third 76 cases. The first grid shows a case's SNO (the letters representing the block within that case's archive which arrayed) and the second grid shows its equivalent, final ANO. The boxed case, SNO 282C, was found to be a duplicate of SNO 71 and was excluded from analysis. K represents a core of kidney, positioned to help orientation at microscopic evaluation.

I										
H	286	287D	288A	288B	300B	301D				
G	287A	281B	282C	288B	287D	288B	292A	293A	294B	295A
F	255A	256C	257D	258D	259C	261D	262A	263A	264B	265A
E	240B	241A	242A	243C	244C	246B	248B	249A	250B	253A
D	226B	229C	230B	231A	232A	233A	235A	236A	236B*	237B*
C	208B	210B	211A	212C	213D	215D	216A	219A	221B	224B
B	194A	196B	197B	199D	200B	201B	202A	203D	205C	207D
A	116X	186Y	181B	184D	185B	186A	187B	188B	191B	193C
	1	2	3	4	5	6	7	8	9	10
	K									

I										
H	222	223	224	225	226	227				
G	212	213	214	215	216	217	218	219	220	221
F	203	204	205	206*	206*	207	208	209	210	211
E	193	194	195	196	197	198	199	200	201	202
D	183	184	185	186	187	188	189	190	191	192
C	173	174	175	176	177	178	179	180	181	182
B	163	164	165	166	167	168	169	170	171	172
A	153	154	155	156	157	158	159	160	161	162
	1	2	3	4	5	6	7	8	9	10
	K									

Figure 3.6d **Array block 4:1**
Showing the locations of the fourth 159 cases. The first grid shows a case's SNO (the letters representing the block within that case's archive arrayed) and the second grid shows its equivalent, final ANO. K represents a core of kidney, positioned to help orientation at microscopic evaluation.

N	448A	449A	450B							
M	219B	436B	439B	182A	440B	441A	442A	443A	445B	447B
L	439C	431D	176B	433B	434C	198C	435B	214D	438C	437A
K	422A	423B	424D	291A	425D	426D	427A	428B	110	192
J	419A	420A	365B	35	388B	367B	374C	262A	421C	94
I	406B	408B	409A	410C	412B	413D	189B	414B	417C	418B
H	397	398	70	299A	399A	400A	401D	402C	403A	405C
G	387	388A	389C	390A	288B	57B	391A	392A	394A	395A
F	40	376A	377B	379A	389C	382D	51	383A	384C	386
E	361B	363B	31	228B	364C	366D	369A	381C	382C	376A
D	335	337B	338A	340C	39A	342A	347A	223B	349B	350D
C	323B	324A	325C	326C	19	247B	327B	330A	333B	334B
B	313D	314B	316B	317B	319D	320C	321B	322B	222A	195A
A	303B	304A	305C	306D	307B	308C	309B	310D	312B	196B
	1	2	3	4	5	6	7	8	9	10
	K									

N	358	359	360							
M	348	349	350	351	352	353	354	355	356	357
L	338	339	340	341	342	343	344	345	346	347
K	328	329	330	331	332	333	334	335	336	337
J	318	319	320	321	322	323	324	325	326	327
I	308	309	310	311	312	313	314	315	316	317
H	298	299	300	301	302	303	304	305	306	307
G	288	289	290	291	292	293	294	295	296	297
F	278	279	280	281	282	283	284	285	286	287
E	268	269	270	271	272	273	274	275	276	277
D	258	259	260	261	262	263	264	265	266	267
C	248	249	250	251	252	253	254	255	256	257
B	238	239	240	241	242	243	244	245	246	247
A	228	229	230	231	232	233	234	235	236	237
	1	2	3	4	5	6	7	8	9	10
	K									

Figure 3.7 The TMA blocks

Figure 3.7a A final recipient TMA block
Once the donor cores had been precisely arrayed, the block was heated for 10-15 minutes at 37°C and the surface levelled using a clean glass slide. The block was then be sectioned using a standard microtome.

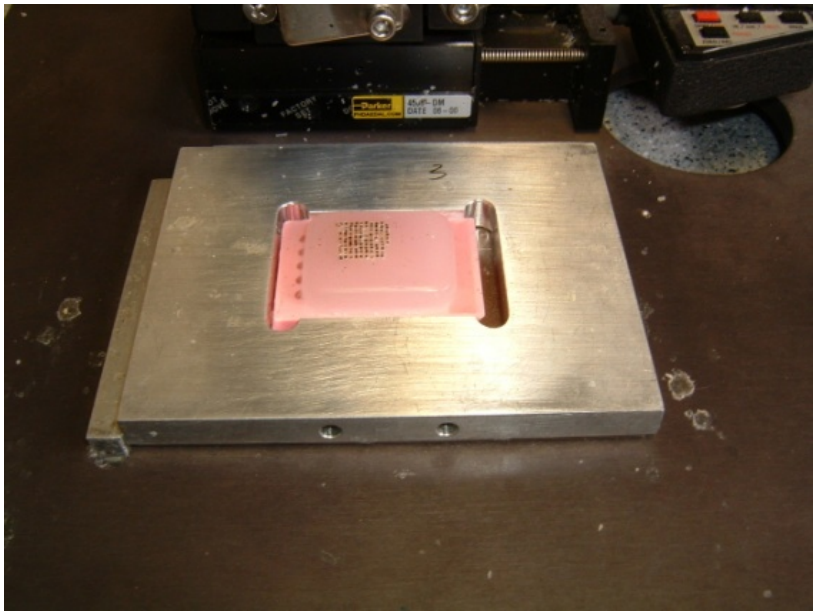
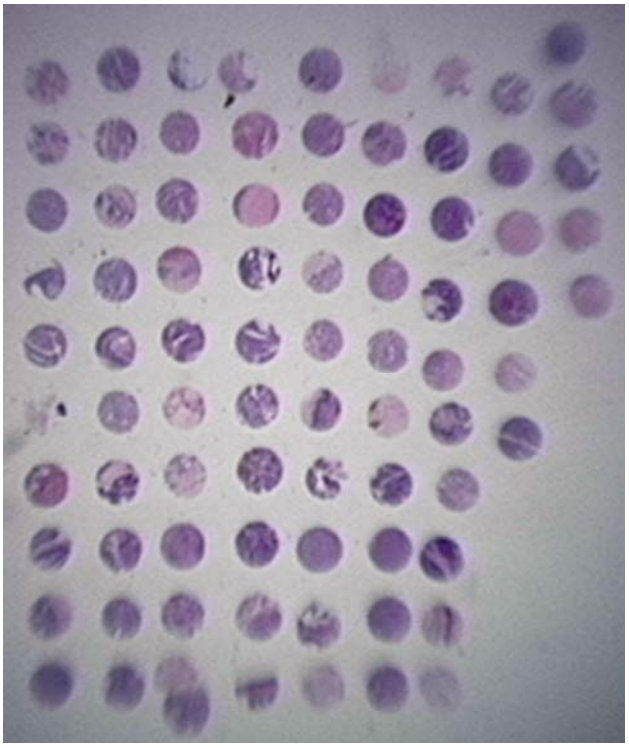


Figure 3.7b Section from an array block
Once the array block had been 'faced' sections were taken and mounted onto slides. One slide from each TMA block was H&E stained for evaluation, as shown below.



were the least common. The 30 borderline tumours represented 8% of the cases but were not included in subsequent analyses. The cancers were mostly high grade (66%) (Table 3.1).

3.1.3. Discussion

441 new diagnoses of cancer (ICD-10 C56-C57 (World Health Organization. 1992) + cases of uncertain malignant potential - borderline cases) from DCGH were registered with the Trent Cancer Registry (TCR) between 1982 and 1997, the time period from which cases were selected. In 1991 the county of Derbyshire had a population of approximately 459 000 at risk of ovarian cancer and during these 15 years that cases presented the incidence of ovarian cancer increased from 15 – 19 / 100 000 (Direct Age-Standardised Rate) with approximately 1087-1308 new cases of ovarian cancer being expected to present within the county during that time frame (Great Britain. Office of Population Censuses and Surveys. 1991; Quinn and Great Britain. Office for National Statistics. 2001). Since 2001, DCGH has had the clear remit to treat all cases of ovarian cancer within an 830 000 strong catchment (as cancer centre for the Derby/Burton cancer network) but prior to this reorganisation, following the Calman-Hine report in 1995, the provision of cancer services was more fragmented (Calman and Hine 1995). It had been normal for most patients with ovarian cancer to be treated at their nearest hospital, sometimes by non-specialists. This may explain why a relatively small proportion of the county's workload presented to DCGH during the recruitment period - which would not be the case today. From 1982-1997 the department served an immediate population at risk of ovarian cancer of approximately 117 000 (Derby City) and it would be expected that approximately 277- 333 cases would have presented from this catchment to the lone gynaecologist with an interest in cancer who performed the majority of the operations for ovarian cancer at that time (Great Britain. Office of Population Censuses and Surveys. 1991; Kehoe, Powell et al. 1994; Botha 2007). When taking these factors into account, a caseload of 441 seems an appropriate one for a single

Table 3.1 The distribution among the histological subtypes and grades of the cases within the final series

Histological type	Number	% of series (n=339)
Epithelial	334	99
Serous (total)	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 Müllerian	2	
Borderline cancers*	30 / 369	8
Grade	**Number**	**% of series (n=339)**
Ungradable §	2	1
G1	39	11
G2	73	22
G3 (High grade)	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 ungradable

handed gynaecological cancer service serving a mainly local population but receiving some county wide referrals in a sporadic fashion (Quinn and Great Britain. Office for National Statistics. 2001; Calvert 2007).

With 424 of the 441 cases originating from DCGH on the TCR (notification via hospital coding department, pathology department or death certificate) represented on the prospectively accrued database, the ascertainment rate had been particularly good considering that it had not been formally resourced. There is the possibility that some cases had presented and had not been recorded by the database or TCR. However as TCR has a good record concerning the ascertainment of cases (as measured indirectly by mortality : incidence ratios) and produces data which consistently meets stringent quality checks, the systematic non-registration of significant numbers of cases presenting to DCGH is unlikely (Parkin, World Health Organization. et al. 1997; Botha 2007).

Eurocare 4 highlighted the fact that compared to many European countries, U.K cancer registries have less information supplied to them from pathology departments, with an 83% microscopic verification rate of cancer cases (compared to 98% in Sweden) and a high proportion of cases being registered via the death of the patient (Death Certificate Only (DCO) cases = 6.7% :UK Association of Cancer Registries target = 2%) (Berrino, De Angelis et al. 2007). A recent peer review of TCR found that the DCO rate of 4.3% in 2002 fell to 3.2% in 2007 following the introduction of “live feeds” of electronic pathology data from individual laboratories implying that this problem has historically been problematic locally (Botha 2007). It was found during this study that difficulties in tracing pathology reports were exacerbated by the lack of a system in the past linking a patient’s pathology number to their unique identifying data and this may explain some of the problems previously experienced by TCR. Though this situation made anonymising the cases simpler, 21 cases could not be included due having no pathology number with which to locate archived tissue.

As others have observed, it was found that constructing the TMA itself took relatively little time compared with the collection and review of the

material during which a significant number of cases became unsuitable for inclusion (Simon, Mirlacher et al. 2004). This study concerns the behaviour of frankly malignant forms of ovarian cancer and cases found to be borderline were excluded. Because the pathological review lagged behind the assembly of the first TMA block it was found that 19 borderline cases had already been arrayed and they were excluded from the scoring grid instead. The potential remains to study the expression of markers in these 6% of cases should a suitable hypothesis develop. Twenty of the cases on the prospective database had little or no tissue available for inclusion in the TMA. This was either because the patient had been treated privately or because surgery had been abandoned for technical reasons with only a small biopsy being obtained for diagnostic purposes. During the pathological review, full immunohistochemical panels were occasionally generated to aid accurate diagnosis in difficult cases. Of the 380 cases reviewed, 9 were found to have been misdiagnosed as having ovarian cancer. Though the numbers involved were small, each patient may have come to harm through the inappropriate treatment of their true condition. The number of pathological misdiagnoses in the department is likely to have fallen to even lower levels with the routine use of complex marker panels in difficult cases, the sub-specialisation of pathologists and the review of cases by the Multi Disciplinary Team (MDT) but the importance of anonymisation in this studies design is emphasised by this finding (Hernandez, Bhagavan et al. 1984; Veenhuizen, De Wit et al. 1997). From a logistical point of view it would have simplest to have performed each step in the process for all cases before moving on to the next step but time constraints meant that multiple tasks had to be carried out concurrently for different cases. This used time efficiently and identified systematic problems at an early stage but the logistics were complex and errors did occur, the most obvious being the assignment of multiple study numbers to individual cases and the duplicate arraying of two cases. This way of working also meant that the tumours were arrayed in batches, leading to relatively few being located in each block. If all 339 tumours had been available together at the same time, two blocks

would have been enough to contain them. By making sure that when errors occurred they did not go unnoticed or uncorrected, the TMA was ultimately found to be easy to navigate and score by individuals unfamiliar with the complexities of its construction.

Of the 339 cases of ovarian cancer included, 98% of were found to be epithelial which is above the 90% normally seen in unselected series (Benedet 2000). A lower representation of non-epithelial cases probably reflects onward referral of this disparate group of rare cancers to the supra-regional centre, with only the occasional case remaining under the long term care of the department at DCGH. Though the series may have been partially selected in this respect, the breakdown of histological type within the series was otherwise typical. In line with the published frequencies (in brackets); 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%) (Scully 1977; Heintz, Odicino et al. 2001; Quirk and Natarajan 2005). Borderlines made up 8% of all cases reviewed (up to 15%) (FIGO 1987). This series was more likely to have cases with higher grade disease (66% G3) than generally reported (47% G3), (Heintz, Odicino et al. 2001). This may be a true difference but is more likely to reflect the well documented difficulty of objectively reproducing the commonly used grading systems for all histological types between observers (Hernandez, Bhagavan et al. 1984). There is variation in how ovarian cancers are graded by individual pathologists with some workers altering their grading system depending on the histological type (Silva and Gershenson 1998), others grading all subtypes with standard grading systems such as the WHO (Serov, Scully et al. 1973), Gynaecology Oncology Group (GOG) (Benda and Zaino 1994) or FIGO (Benedet 2000) methods, and others still attempting to develop reproducible, universal systems of their own (Bichel and Jakobsen 1989; Shimizu, Kamoi et al. 1998). For that reason conclusions regarding the number of highest grade cancers within our series are difficult to make.

3.2. Evaluation of the TMA

3.2.1. Pilot evaluation – one copy four antigens

When the first copy of the TMA had been constructed, a pilot evaluation of expression data generation was performed. The aim was to see if raw expression data for a number of markers could be acquired and compared to that in the literature, in order to reveal any major errors in the TMAs construction. As there was no useable database yet available, markers were selected that had no major known associations with clinicopathological factors and for which expression data from a number of sources was readily available. Though CK7, CK20, CEA and CA125 may not represent an ideal diagnostic ovarian panel, they met these criteria.

3.2.1.1. Method

Unlike the remainder of the IHC work, the four slides of the first copy of the TMA used in the pilot were not stained by the author but added to a partly automated run of routine work performed in the pathology department of DCGH. The materials and methods therefore differed from the general IHC protocol described in 2.2 and these differences and the method for the pilot study are recorded in detail in 2.2.10 and Table 2.2.

3.2.1.2. Results

Positive CK7 staining was seen mainly within the cytoplasm of the cells (Figure 3.8a-c). Scoring in binary fashion, approximately 64% of tumours were deemed positive for CK7 (Table 3.2). Loss of tissue cores effectively excluded 8% of cases from analysis.

Positive CK20 staining was seen mainly within the cytoplasm of the cells (Figure 3.9a-c). Scoring in binary fashion, approximately 5% of tumours were deemed positive for CK20 (Table 3.3). 8% of cases had too little tissue for analysis.

Figure 3.8 Examples of the expression patterns seen when staining for CK7

Figure 3.8a Whole sections of normal breast tissue (positive control) staining strongly for CK7
Section below seen at x 100 magnification staining cytoplasmically with no background staining seen.

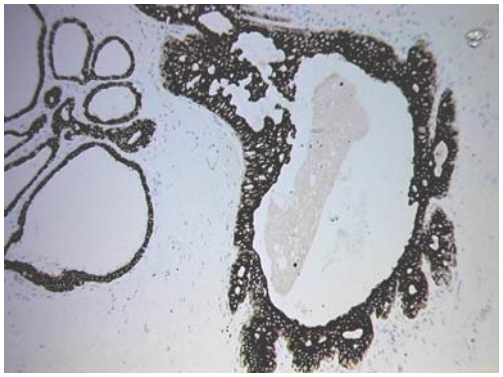


Figure 3.8b An example at x100 magnification of a TMA core staining very positively for CK7
As the staining was homogenous, the cores were scored by % cells to the nearest 5%, excluding normal tissue in the evaluation. A cut-off of 5% represented positivity.

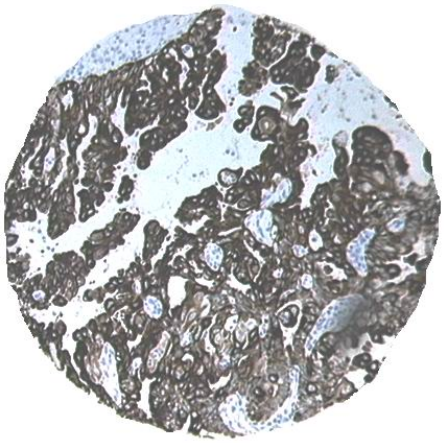


Figure 3.8c An example of a TMA core negative for CK 7 expression see at x100 magnification

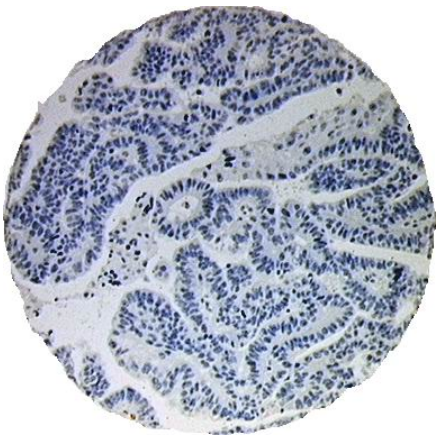


Table 3.2

The number of cases staining positive for CK7 through staining a single copy of the TMA at DCGH using an automated staining system.

The scoring system was binary. The % number of cases expected to stain positive, as discerned from the literature, is inset for comparison.

	CK7			
Histological type				
			n=313	
	Overall Number of cases	**Number of analysed cases**	**CK7 Positive % (n)**	**Cases expected CK7 Positive %**
Serous	178	168	71 (119)	100 **[1, 2]**
Mucinous	35	28	39 (11)	80-100 **[1, 2]**
Total	339	313	64 (199)	94-100 **[1, 3-7]**

1. Cathro, H.P. and M.H. Stoler, Expression of cytokeratins 7 and 20 in ovarian neoplasia. Am J Clin Pathol, 2002. 117(6): p. 944-51.
2. Wauters, C.C., et al., Keratins 7 and 20 as diagnostic markers of carcinomas metastatic to the ovary. Hum Pathol, 1995. 26(8): p. 852-5.
3. Nishizuka, S., et al., Diagnostic markers that distinguish colon and ovarian adenocarcinomas: identification by genomic, proteomic, and tissue array profiling. Cancer Res, 2003. 63(17): p. 5243-50.
4. Lagendijk, J.H., et al., Tracing the origin of adenocarcinomas with unknown primary using immunohistochemistry: differential diagnosis between colonic and ovarian carcinomas as primary sites. Hum Pathol, 1998. 29(5): p. 491-7.
5. Heatley, M.K., Immunohistochemical biomarkers of value in distinguishing primary ovarian carcinoma from gastric carcinoma: a systematic review with statistical meta-analysis. Histopathology, 2008. 52(3): p. 267-76.
6. Chu, P., E. Wu, and L.M. Weiss, Cytokeratin 7 and cytokeratin 20 expression in epithelial neoplasms: a survey of 435 cases. Mod Pathol, 2000. 13(9): p. 962-72.
7. Berezowski, K., J.F. Stastny, and M.J. Kornstein, Cytokeratins 7 and 20 and carcinoembryonic antigen in ovarian and colonic carcinoma. Mod Pathol, 1996. 9(4): p. 426-9.

Figure 3.9 Examples of the expression patterns seen when staining for CK20

Figure 3.9a Whole sections of bowel tissue (positive control) staining strongly for CK20
Cytoplasmic staining with no background staining is observed at x100 magnification.

Figure 3.9b An example at x100 magnification of a TMA core staining very positively for CK20
The scoring system was binary: positive if any tumour cells stained positive and negative if no cells stained positive.

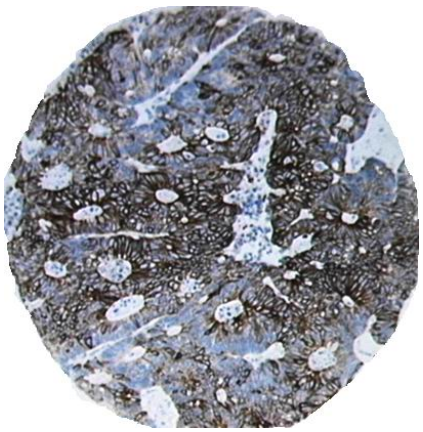


Figure 3.9c An example of a TMA core negative for CK20 expression seen at x100 magnification

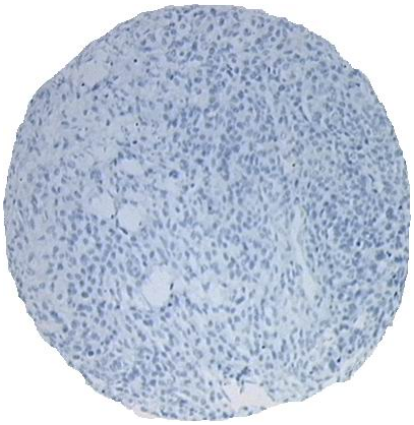


Table 3.3

The number of cases staining positive for CK20 through staining a single copy of the TMA at DCGH using an automated staining system

The scoring system was binary.The % number of cases expected to stain positive, as discerned from the literature, is inset for comparison.

	CK20			
Histological type				
			n=313	
	Overall Number of cases	**Number of analysed cases**	**CK20 Positive % (n)**	**Cases expected CK20 Positive %**
Serous	178	169	(1)	0 **[1-3]**
Mucinous	35	27	33 (9)	0-27 **[1-4]**
Total	339	313	5 (17)	2-36 **[1, 2, 4-8]**

1. Cathro, H.P. and M.H. Stoler, Expression of cytokeratins 7 and 20 in ovarian neoplasia. Am J Clin Pathol, 2002. 117(6): p. 944-51.
2. Berezowski, K., J.F. Stastny, and M.J. Kornstein, Cytokeratins 7 and 20 and carcinoembryonic antigen in ovarian and colonic carcinoma. Mod Pathol, 1996. 9(4): p. 426-9.
3. Wauters, C.C., et al., Keratins 7 and 20 as diagnostic markers of carcinomas metastatic to the ovary. Hum Pathol, 1995. 26(8): p. 852-5.
4. Moll, R., et al., Cytokeratin 20 in human carcinomas. A new histodiagnostic marker detected by monoclonal antibodies. Am J Pathol, 1992. 140(2): p. 427-47.
5. Chu, P., E. Wu, and L.M. Weiss, Cytokeratin 7 and cytokeratin 20 expression in epithelial neoplasms: a survey of 435 cases. Mod Pathol, 2000. 13(9): p. 962-72.
6. Nishizuka, S., et al., Diagnostic markers that distinguish colon and ovarian adenocarcinomas: identification by genomic, proteomic, and tissue array profiling. Cancer Res, 2003. 63(17): p. 5243-50.
7. Lagendijk, J.H., et al., Tracing the origin of adenocarcinomas with unknown primary using immunohistochemistry: differential diagnosis between colonic and ovarian carcinomas as primary sites. Hum Pathol, 1998. 29(5): p. 491-7.
8. Heatley, M.K., Immunohistochemical biomarkers of value in distinguishing primary ovarian carcinoma from gastric carcinoma: a systematic review with statistical meta-analysis. Histopathology, 2008. 52(3): p. 267-76.

Positive CEA staining was seen in the membrane and cytoplasm of those tumour cells which stained (Figure 3.10a-c). 36% of tumours stained positively for CEA when a binary scoring system was used (Table 3.4). Loss of tissue cores occurred in 8% of cases.

Positive CA125 staining was seen in the cell membrane and cytoplasm of strongly staining tumour cells (Figure 3.11a-c). 57% of tumours stained positively for CA125 when employing a binary scoring system (Table 3.5) 9% of cases had too little tissue to analyse.

3.2.1.3. Discussion

Overall the expression of CEA, CA125 and CK20 reflected that which would have been predicted by a body of published literature largely based on whole section staining. The results of the pilot staining for CK7 were however dissonant, with a low rate of expression (64%) for a marker which is usually more universally expressed (80-100%) (Wauters, Smedts et al. 1995; Cathro and Stoler 2002; Nishizuka, Chen et al. 2003; Heatley 2008). There were several possible reasons for this; a problem with the methodology of staining (wrong antibody, poor antigen retrieval etc), a genuine result and a new finding or a problem with tissue heterogeneity within the TMA.

The most concerning situation would be if tissue heterogeneity in the TMA had resulted in a very high rate of false negatives for this ubiquitously expressed marker, though on reviewing the literature this would seem unlikely. When TMAs were introduced, initially workers were concerned that analysing an extremely small percentage of the tumours (approximately 0.3%) might mean that false negatives were introduced if the expression of a marker was unevenly distributed throughout the tumour – tissue heterogeneity (Torhorst, Bucher et al. 2001). Work by Goethals and co-workers seemed to support this concern. This group studied 5 colorectal cancers and took 50 random TMA cores from each, staining them with a heterogeneously staining marker -carbonic anhydrase IX. On analysing the standard errors of the expression data they concluded that significant heterogeneity

Figure 3.10 **Examples of the expression patterns seen when staining for CEA**

Figure 3.10a **Whole sections of bowel tumours (positive control)**
Seen to stain cell membrane and cytoplasm strongly with no background staining seen at x 100 magnification.

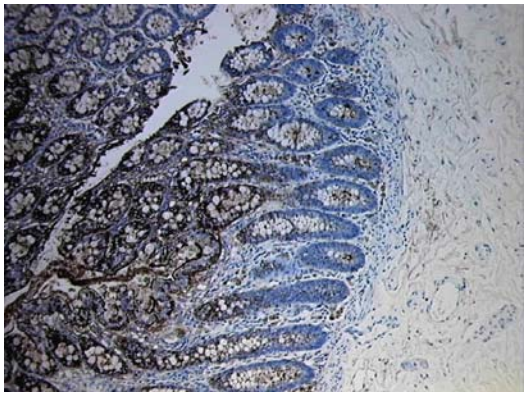


Figure 3.10b **An example at x200 magnification of a TMA core staining positively for CEA**
As the staining was homogenous, the cores were scored by % cells to the nearest 5%, excluding normal tissue in the evaluation. A cut-off of 5% represented positivity.

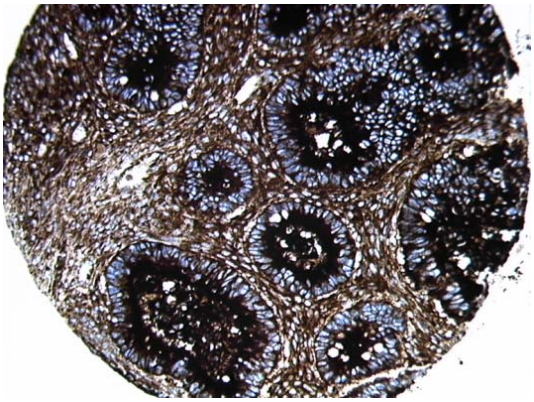


Figure 3.10c **An example of a TMA core negative for CEA expression seen at x100 magnification**

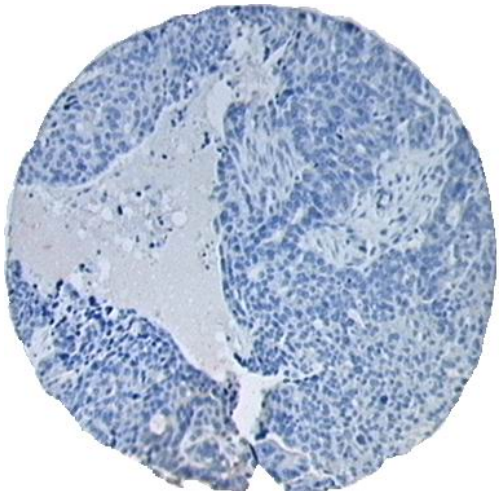


Table 3.4

The number of cases staining positive for CEA through staining a single copy of the TMA at DCGH using an automated staining system

The scoring system was binary. The % number of cases expected to stain positive, as discerned from the literature, is inset for comparison.

CEA				
Histological type				
			n=312	
	Overall Number of cases	**Number of analysed cases**	**CEA positive % (n)**	**Cases expected CEA positive %**
Serous	178	166	26 (43)	31-100 **[1-3]**
Mucinous	35	30	43 (13)	13-67 **[1, 3-5]**
Total	339	312	36 (113)	21-42 **[1, 3, 4, 6-10]**

1. Multhaupt, H.A., C.P. Arenas-Elliott, and M.J. Warhol, *Comparison of glycoprotein expression between ovarian and colon adenocarcinomas.* Arch Pathol Lab Med, 1999. 123(10): p. 909-16.
2. Kabawat, S.E., et al., *Immunopathologic characterization of a monoclonal antibody that recognizes common surface antigens of human ovarian tumors of serous, endometrioid, and clear cell types.* Am J Clin Pathol, 1983. 79(1): p. 98-104.
3. Macdonald, F., et al., *Expression of CEA, CA125, CA19-9 and human milk fat globule membrane antigen in ovarian tumours.* J Clin Pathol, 1988. 41(3): p. 260-4.
4. Lagendijk, J.H., et al., *Tracing the origin of adenocarcinomas with unknown primary using immunohistochemistry: differential diagnosis between colonic and ovarian carcinomas as primary sites.* Hum Pathol, 1998. 29(5): p. 491-7.
5. Keen, C.E., et al., *CA125 and thyroglobulin staining in papillary carcinomas of thyroid and ovarian origin is not completely specific for site of origin.* Histopathology, 1999. 34(2): p. 113-7.
6. Heatley, M.K., *Immunohistochemical biomarkers of value in distinguishing primary ovarian carcinoma from gastric carcinoma: a systematic review with statistical meta-analysis.* Histopathology, 2008. 52(3): p. 267-76.
7. Berezowski, K., J.F. Stastny, and M.J. Kornstein, *Cytokeratins 7 and 20 and carcinoembryonic antigen in ovarian and colonic carcinoma.* Mod Pathol, 1996. 9(4): p. 426-9.
8. Breitenecker, G., et al., *Comparison between tissue and serum content of CA 125, CA 19-9, and carcinoembryonic antigen in ovarian tumors.* Int J Gynecol Pathol, 1989. 8(2): p. 97-102.
9. Torenbeek, R., et al., *Value of a panel of antibodies to identify the primary origin of adenocarcinomas presenting as bladder carcinoma.* Histopathology, 1998. 32(1): p. 20-7.
10. Lagendijk, J.H., et al., *Immunohistochemical differentiation between primary adenocarcinomas of the ovary and ovarian metastases of colonic and breast origin. Comparison between a statistical and an intuitive approach.* J Clin Pathol, 1999. 52(4): p. 283-90.

Figure 3.11 **Examples of the expression patterns seen when staining for CA125**

Figure 3.11a **Whole sections of ovarian cancer tissue (positive control) staining appropriately**
Cell membrane staining was seen with no background staining observed at x100 magnification.

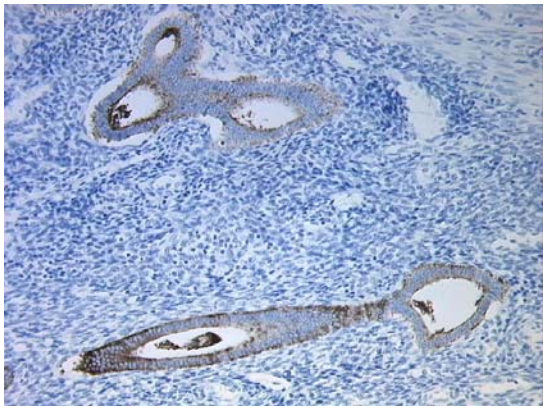


Figure 3.11b **An example at x200 magnification of a TMA core staining positively for CA125**
As the staining was homogenous, the cores were scored by % cells to the nearest 5%, excluding normal tissue in the evaluation. A cut-off of 5% represented positivity.

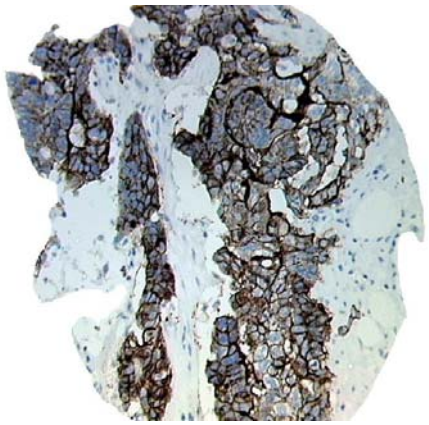


Figure 3.11c **An example of a TMA core negative for CA125 expression seen at x100 magnification**

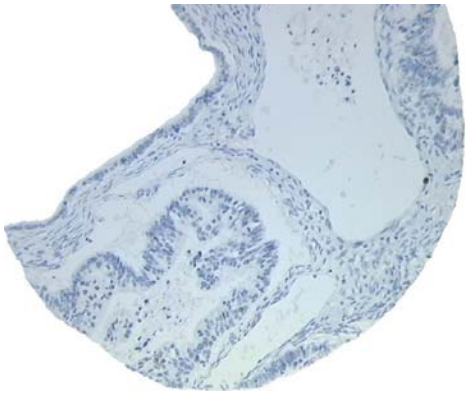


Table 3.5

The number of cases staining positive for CA125 through staining a single copy of the TMA at DCGH using an automated staining system

The scoring system was binary. The % number of cases expected to stain positive, as discerned from the literature, is inset for comparison.

CA125				
Histological type				
			n=307	
	Overall Number of cases	**Number of analysed cases**	**CA125 Positive % (n)**	**Cases expected CA125 Positive %**
Serous	178	162	76 (123)	68-93 **[1-6]**
Mucinous	35	28	14 (4)	25-43 **[2, 3, 6]**
Total	339	307	57 (174)	54-78 **[1-3, 6-10]**

1. Loy, T.S., J.T. Quesenberry, and S.C. Sharp, *Distribution of CA 125 in adenocarcinomas. An immunohistochemical study of 481 cases.* Am J Clin Pathol, 1992. 98(2): p. 175-9.
2. Dennis, J.L., et al., *Markers of adenocarcinoma characteristic of the site of origin: development of a diagnostic algorithm.* Clin Cancer Res, 2005. 11(10): p. 3766-72.
3. Multhaupt, H.A., C.P. Arenas-Elliott, and M.J. Warhol, *Comparison of glycoprotein expression between ovarian and colon adenocarcinomas.* Arch Pathol Lab Med, 1999. 123(10): p. 909-16.
4. Neunteufel, W. and G. Breitenecker, *Tissue expression of CA 125 in benign and malignant lesions of ovary and fallopian tube: a comparison with CA 19-9 and CEA.* Gynecol Oncol, 1989. 32(3): p. 297-302.
5. Keen, C.E., et al., *CA125 and thyroglobulin staining in papillary carcinomas of thyroid and ovarian origin is not completely specific for site of origin.* Histopathology, 1999. 34(2): p. 113-7.
6. Macdonald, F., et al., *Expression of CEA, CA125, CA19-9 and human milk fat globule membrane antigen in ovarian tumours.* J Clin Pathol, 1988. 41(3): p. 260-4.
7. Bast, R.C., Jr., et al., *Reactivity of a monoclonal antibody with human ovarian carcinoma.* J Clin Invest, 1981. 68(5): p. 1331-7.
8. Breitenecker, G., et al., *Comparison between tissue and serum content of CA 125, CA 19-9, and carcinoembryonic antigen in ovarian tumors.* Int J Gynecol Pathol, 1989. 8(2): p. 97-102.
9. Torenbeek, R., et al., *Value of a panel of antibodies to identify the primary origin of adenocarcinomas presenting as bladder carcinoma.* Histopathology, 1998. 32(1): p. 20-7.
10. Kabawat, S.E., et al., *Immunopathologic characterization of a monoclonal antibody that recognizes common surface antigens of human ovarian tumors of serous, endometrioid, and clear cell types.* Am J Clin Pathol, 1983. 79(1): p. 98-104.

existed, which could only be reduced to acceptable limits if five separate cores were used in assessing cases (Goethals, Perneel et al. 2006). Conversely, most other workers have found high correlations between whole section data (considered the gold standard) and that produced by TMAs when tissue cores are purposefully selected from non-random, representative tumour sites: most usually there has been a 91% concordance with single core evaluation, 95-96% on evaluating two cores, 98% on evaluating three cores and diminishing returns thereafter (Camp, Charette et al. 2000; Hoos and Cordon-Cardo 2001; Rosen, Huang et al. 2004). It has always been realised that TMAs could have a theoretically higher false negative rate than full sections which might have great clinical impact if an attempt was made to use them diagnostically but false negatives aside, it was also felt that the data obtained from staining of hundreds of tumours would give a more accurate assessment of the true expression of a marker within the general population of cancers than the very accurate assessment of just a few cases by whole section. TMAs have been shown to generate accurate expression data across populations due to this power, while they may not have the resolution to provide accurate information about individual cases (Kononen, Bubendorf et al. 1998; Mucci, Akdas et al. 2000; Rubin, Dunn et al. 2002). Concerns about tissue heterogeneity in TMA work have therefore receded with increasing experience and the assessment of five cores per case as suggested by Goethals et al is uncommon. Some workers do however continue to examine three cores (Fernebro, Dictor et al. 2002; Rubin, Dunn et al. 2002; Rosen, Huang et al. 2004) though many argue for less cores to be used per case, as long as there are enough tumour cells present within each sample and enough cases within the series (Nocito, Bubendorf et al. 2001; Hedvat, Hegde et al. 2002). It is interesting to note that the group who validated TMAs in ovarian cancer using triplicate cores moved to using two cores in their subsequent work (Rosen, Yang et al. 2006) and of the five most recent TMA publications (PubMed search “Tissue Micro Array” 24 April 2008), three evaluated a single core, one examined two cores and a single study investigated triplicate cores (Prentice, Klausen

et al. 2007; Cohen, Prus et al. 2008; Harris, La Cerda et al. 2008; Preusser, Janzer et al. 2008; Yang, Hung et al. 2008).In addition, the use of larger tissue cores has been mathematically discredited (Nocito, Kononen et al. 2001).

3.2.2. Full evaluation – four copies two antigens

The results of the CK7 pilot staining were therefore unlikely to be due to tissue heterogeneity and more likely to be due to experimenter error (incorrect antibody, mixing up of slides etc) and/or methodological variances such as the use of a different manufacturer of primary antibody or the use of an automated system at DCGH to perform the amplification step. In order to investigate this further, four copies (not five as the fifth copy had been constructed in error and ethical approval for its use at that point had not been obtained) of the TMA were manually re-stained for CK7, along with CEA for comparison. Importantly this additionally allowed the effect of staining multiple copies of the array on overall case loss and accuracy of data to be assessed.

3.2.2.1. Method

As described in detail in 2.2 and table 2.2

3.2.2.2. Results

CK7 (Table 3.6)

When evaluating a single copy of the TMA, 85-88% of cases were positive for CK7 when using a cut off of 5% to allocate cases to the positive group. Depending on the TMA studied, between 16 and 33% of cases could not be assessed due to tissue core loss. The pattern of distribution among the quartiles followed a general pattern - approximately 1/5 of the cases in the first quartile, 1/8 in the second quartile, 1/4 in the third quartile and 2/5 in the highest centile. The distribution of expression data when arranged in to quartiles varied

Table 3.6 A complete breakdown of the expression data for CK7 on staining four copies of the TMA

Quartiles, % positive cases and the number of cores available to form average expression data are shown. The data were generated for each possible combination of the arrays (1-4) to observe the effects of staining more than a single copy on power, accuracy and reproducibility.

	Quartile % cells CK Positive							Number of cores contributing to average			
	0-25	**26-50**	**51-75**	**76-100**	**> 5% = Positive**	**N º Cases with data**	**0**	**1**	**2**	**3**	**4**
Single core											
1	23	13	14	51	**86**	245	94	245	X	X	X
2	24	11	10	56	**85**	228	111	228	X	X	X
3	21	14	16	48	**87**	286	53	286	X	X	X
4	21	20	13	45	**88**	271	68	271	X	X	X
Two cores											
1+2	21	13	19	47	**86**	290	49	89	201	X	X
1+3	21	14	21	44	**87**	312	27	93	219	X	X
1+4	20	15	24	41	**87**	310	29	104	206	X	X
2+3	22	16	20	43	**86**	307	32	100	207	X	X
2+4	22	15	21	43	**87**	297	42	95	202	X	X
3+4	20	16	24	40	**90**	317	22	50	267	X	X
Three cores											
1+2+3	20	16	20	44	**86**	321	18	54	96	171	X
1+2+4	19	17	20	44	**88**	319	20	60	93	166	X
1+3+4	17	15	27	40	**88**	328	11	44	94	190	X
2+3+4	21	15	24	40	**88**	320	19	38	99	183	X
Four cores											
1+2+3+4	19	16	27	39	**88**	328	11	27	56	90	155

most between TMA copies within the middle two centiles but largely followed this same pattern.

When evaluating two copies of the TMA and averaging the data, five of the possible six combinations resulted in 86-87% of the cases being categorised positive. A sixth combination (copy 3+4) classified a higher proportion, 90%, of cases positive. The majority of cases had an average derived from two tissue cores (60-79%). Data from a single core was available for many of the remaining cases (14-30%) while cases with no data available represented between 6-14% of the whole. The distribution of the expression data amongst the quartiles followed the same pattern irrespective of the combinations analysed.

When evaluating three copies of the TMA and averaging the expression data, three of the four combinations resulted in 88% of the cases being categorised positive. The fourth combination classified 86% of tumours positive. Depending on combination 3-6% of cases could not be evaluated due to core loss. However 49-56% had all three cores available for averaging, 27-29% had two and 1-18% of cases were evaluated on the basis of a single core. The distribution of expression data among the quartiles, while still remaining within the general pattern was less uniform than when two cores had been evaluated.

When evaluating all four copies of the TMA and averaging the expression data, 88% of the cases were classified positive. 3% of cases had no expression data available. 46% had all four cores available for averaging, 27% had three, 17% two and 8% of cases were evaluated on the basis of a single core. The distribution of expression data among the quartiles remained within the general pattern.

CEA (Table 3.7)

When evaluating a single copy of the TMA, three of the four copies classified 19% of the cases as positive for CEA on the basis of a 5% cut-off. The fourth copy classified 17% of cases positive. Depending on which TMA was evaluated 10-19% of cases had insufficient material to be assessed following processing of the TMA. The distribution of expression data amongst the quartiles followed a general pattern of 8-9

Table 3.7 A complete breakdown of the expression data for CEA on staining four copies of the TMA

Quartiles, % positive cases and the number of cores available to form average expression data are shown. The data were generated for each possible combination of the arrays (1-4) to compare the effects of staining more than a single copy on power, accuracy and reproducibility.

	Quartile % cells CEA Positive						**Number of cores contributing to average**				
	0-25	**26-50**	**51-75**	**76-100**	**> 5% = Positive**	**N º Cases with data**	**0**	**1**	**2**	**3**	**4**
Single Array											
1	87	5	2	7	**19**	274	65	274	X	X	X
2	89	2	2	8	**17**	255	84	255	X	X	X
3	88	3	2	8	**19**	304	35	304	X	X	X
4	88	2	2	9	**19**	289	50	289	X	X	X
Two Arrays											
1+2	88	4	2	7	**21**	311	28	93	218	X	X
1+3	87	5	2	6	**21**	321	18	64	257	X	X
1+4	87	4	2	7	**20**	322	17	81	241	X	X
2+3	87	4	2	7	**20**	321	18	83	238	X	X
2+4	88	3	2	7	**20**	306	33	68	238	X	X
3+4	87	4	3	6	**20**	324	15	55	269	X	X
Three Arrays											
1+2+3	87	4	3	6	**21**	327	12	28	92	207	X
1+2+4	87	3	3	7	**21**	326	13	39	82	205	X
1+3+4	86	5	3	6	**22**	329	10	20	80	229	X
2+3+4	87	4	3	6	**22**	326	13	27	76	223	X
Four Arrays											
1+2+3+4	87	4	4	5	**22**	331	8	16	33	88	194

/ 10 of cases within the first quartile, up to 1/10 within the last quartile - the few remaining of cases being distributed amongst to the middle quartiles with proportionately more in the second quartile.

When evaluating two copies of the TMA the percentage of tumours staining positive was distributed from 20-21% and altering the precise combination of data analysed had little effect on this. Insufficient material was available for analysis in 4-9% of cases. Similarly the distribution of the expression data amongst the quartiles followed the general pattern previously described. The majority of cases (64-79%) had data from two cores available for averaging with evaluation on the basis of a single core necessary in 16-27% of cases, depending on the combination of TMAs assessed.

When evaluating three copies of the TMA, 21-22% of tumours were determined positive for CEA on the basis of their averaged expression data. 3-4% of cases could not be assessed due to a lack of a single core to evaluate. The majority of cases (61-68%) had all three cores available for averaging, 22-27% had two and 5-11% of tumours were evaluated on the basis of a single core. The distribution of expression data between the quartiles followed the general pattern described previously.

When four copies of the TMA were evaluated, 22% of the tumours were found to be positive for CEA on the basis of their averaged expression data with only 2.3% of cases having no tissue available for evaluation. The majority of cases (57%) had all four cores available when calculating averaged data, 26% had three, 10% had two and 5% were assessed on the basis of a single core.

3.2.2.3. Discussion

The cores were scored by percentage to allow averaging of data. Irrespective of how many or which copies of the TMA were analysed, the expression data for CK7 was the same and agreed with the published literature, normally being positive in around 88% of cases (range 85-90%). A similar result was seen with CEA, with the

expression data varying little between combinations of copies of the TMA and agreeing although with the published literature. It is therefore a possibility that the low expression of CK7 determined in the pilot study was due to methodological errors, supporting the use of manual staining by a single investigator in the subsequent work.

In line with other studies, it was found that tissue was frequently lost during the processing of the TMA slides and that occasionally, unusable or insufficient tissue had been transferred. In the literature this occurs in 2-25% of cases and in the pilot study it occurred in 8% (Mucci, Akdas et al. 2000; Hoos and Cordon-Cardo 2001; Rosen, Yang et al. 2006). By staining more than one copy of the TMA it was discovered that over 300 cases consistently had tissue available for analysis, the optimal number determined by previous power calculations. Therefore it was concluded that staining two copies of the TMA in further experiments would strike a good balance between limiting tissue wastage, maximising data quality and maintaining the power of the study. It was also discovered that tissue loss was exacerbated if EDTA was used as the antigen retrieval solution and so its use was avoided where possible in subsequent work (Figure 3.12).

During this chapter it has been shown that a typical series of 339 surgically treated primary ovarian cancers was assembled into a TMA. It has been shown that the staining of two copies could accurately reproduce appropriate raw expression data for a series of well studied markers whilst also ensuring that sufficient cases were included in the study. The next chapter will describe the results of the evaluation and transformation of the prospective database into a form which could be used in conjunction with the TMA to study marker associations with clinicopathological characteristics.

Figure 3.12 **Loss of tissue cores**

Loss of tissue cores occurred with greater frequency at the beginning and, as shown below, near the end of the array block. The type of antigen retrieval solution also affected tissue loss. The slides bellow show consecutive TMA sections which underwent citrate and EDTA antigen retrieval respectively.

Tissue loss worse towards the base of the TMA block

Tissue loss compounded by EDTA based antigen retrieval

Chapter 4: Development and Evaluation of the Database

This chapter covers the management and testing of the electronic data available for the cases in the study cohort concerning events during a 23 year period that extended from 1st January 1982 up to the study database being frozen on 31st November 2005. The aim of this section is to substantiate the steps involved in transforming the raw clinical database into its leaner form - the analysis database - which could be used to analyse expression data in a valid way. The administration of the database, evidence for the data's consistency and the clinicopathological characteristics of the series are described.

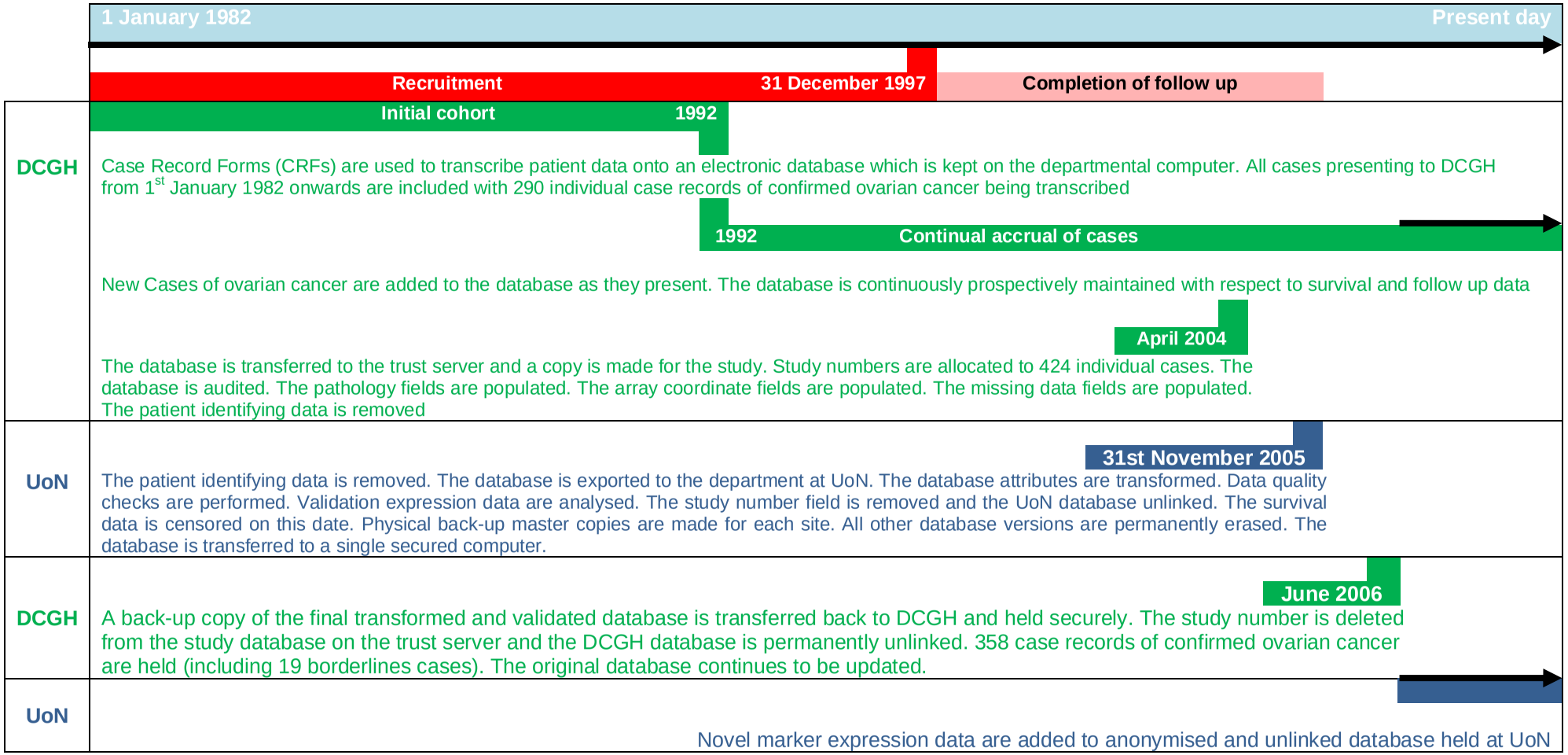
4.1. Database management

The database was originally populated using Case Record Forms (CRFs) in 1992 with data being retrospectively extracted from the clinical record (Figure 4.1). The stimulus to collect the data in this way was a need to perform a departmental audit and the rapid expansion in the availability of sophisticated data management programs to the average user that was occurring (Crockett, Scott et al. 1992). Prospective data were added from 1992 onwards (new cases and follow up data) with the currently preferred electronic format being Microsoft Access 2003. A copy of this database became the original study database which was kept at DCGH. This underwent multiple transformations through multiple formats before single validated SPPS vs.11.0 file containing anonymous clinicopathological data was produced that could be used to analyse expression data and then compare it to published work; the analysis database. Multiple copies and back-ups were generated during this process and when the analysis database had been validated they were collected to together onto two identical CD-ROMs (one held securely at each site) with the original files being overwritten and deleted from their primary sources. The timelines involved in managing the data are illustrated in figure 4.2.

Figure 4.1 The Case Record Form (CRF)
The form below was used to extract data from the clinical record and to transcribe it onto the electronic database in 1992. These data are prospectively maintained and new cases added.

Data	Values				
First name					
Surname					
Hospital number					
Year of diagnosis	(1982-1997)				
Date of surgery	(1st January 1982 - 31st December 1997)				
Age at diagnosis	> 0				
Consultant	Gynae-oncologist	Gynaecologist	Other Surgeon		Missing
Pre-op CA125	Units / ml				Missing
FIGO Stage	Ia-Ib-Ic	IIa-IIb-IIc	IIIa-IIIb-IIIc	IV	Missing
Residual disease	None	<2cm	> 2cm		Missing
Grade	Undifferentiated	Poor	Moderate	Well	Missing
Histological type	Serous	Mucinous	Endometrioid	Other	Missing
Treatment	Nothing	Platinum	Carboplatin	Other	Missing
Date of last follow up	(1st January 1982 – 31st November 2005)				Missing
Date of death	(1st January 1982 – 31st November 2005)				Missing

Figure 4.2 **Timeline illustrating the processes involved in producing an anonymised and unlinked database of clinicopathological parameters**



The consistency of the data was checked in a number of ways. Within the data columns (attributes) containing continuous values; the range, mean, median and outlying values were checked. Data were frequently categorised or re-categorised en route to the production of a final analysis database and the distribution of data within final categorical datasets was compared to that documented in published series. The completeness of data was assessed by observing the number of cells (fields) with missing values. The validity of the clinical data was checked by assessing its ability to reproduce known clinicopathological and survival associations. The correctness of data was assessed during formal audits where clean data obtained contemporaneously from the medical record were compared to that in the database. A continuous process of testing the logic of each case entry into the database was manually undertaken.

4.2. Database attributes

Each individual attribute was assessed for its consistency and this occurred simultaneously for multiple variables, as demonstrating the inter-relationships between the clinicopathological data was essential in validating it. For this reason the results reported bellow occasionally references associations with data the consistency of which is documented later in the chapter. A complete record of the data attributes held on the analysis database can be found in appendix 3.

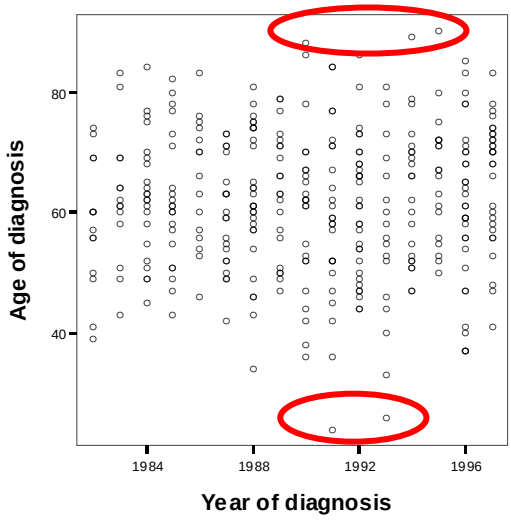
4.2.1. Patient characteristics

4.2.1.1. Results

Age

The age at diagnosis data were recorded on the original clinical database, included unchanged on the analysis database and were in the range 24 – 90 years with one missing field present. The data were plotted on a scatterplot (Figure 4.3). Outlying results were checked and found to be correct with the logic of these entries assessed and found to be in order. The median age of presentation was 62.0 years (mean = 61.9). The distribution of data amongst the age categories used by

Figure 4.3 A scatterplot illustrating the distribution of age data within the analysis database.
The data is separated out by year of presentation in order to distinguish outliers more clearly. The data highlighted were assessed for correctness and the logic of their case entries and were found to be consistent.



FIGO (Figure 4.4a) (Table 4.1a) (Heintz, Odicino et al. 2001), the ONS (Figure 4.4b) (Table 4.1b) (Quinn and Great Britain. Office for National Statistics. 2001) and the Surveillance, Epidemiology and End Results programme (SEER) (Table 4.1c) (Quirk and Natarajan 2005) was similar when comparing the analysis database to these large epidemiological studies. On testing the data in SEER categories, increasing age was seen to be associated with reduced survival though in this form age was not an independent prognostic factor (Figure 4.5) (Table 4.2). However assessing the data in its continuous form, age did exert independent prognostic power (Table 4.3).

4.2.1.2. Discussion

The age related data on the database were similar to each of the series it was compared with and most similar to the U.K. data (Quinn and Great Britain. Office for National Statistics. 2001) (Holschneider and Berek 2000; Heintz, Odicino et al. 2001). This series was slightly older than that seen in the more international studies and this may reflect the effect of race, differing birth cohort characteristics and a later stage at presentation in the U.K.(Berrino, De Angelis et al. 2007). The data were transformed into SEER categories in order to investigate if age was associated with survival in a univariate analysis as this categorisation has been used to describe the age distribution in the largest number of ovarian cancer patients and separates the data into a small number of clinically sensible categories. In this form increasing age was seen to be a non-independent prognostic factor which is consistent with other groups findings (DiSilvestro, Peipert et al. 1997; Heintz, Odicino et al. 2001) though on using the data in its continuous form age did have independent prognostic power (Figure 4.3). This in turn agrees with other workers findings and further supports the validity of the dataset (Thigpen, Brady et al. 1993; Crijns, Boezen et al. 2003). As the HR was small and as a comparable univariate analysis of the continuous data was not technically possible, the result of this multivariate analysis should be treated with caution but the result was intuitively correct. Therefore the continuous data were included in the multivariate model

Figure 4.4a **Bar graph showing the distribution of cases within FIGO age categories**
Data re-categorised and compared to the evidence provided to FIGO: a similar distribution is observed (Heintz, Odicino et al. 2001).

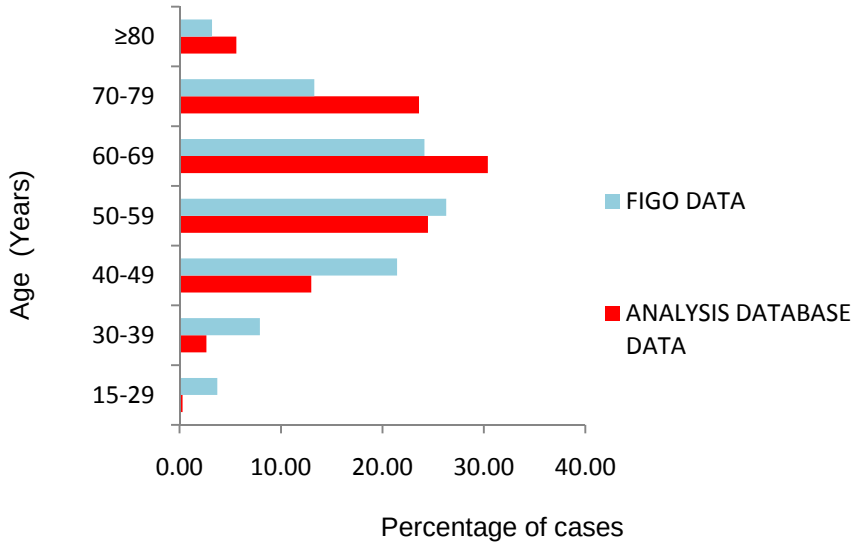


Figure 4.4b **Bar graph showing the distribution of cases within ONS categories**
Data re-categorised and compared to the ONS data for 1997: similar trends are seen (Quinn and Great Britain. Office for National Statistics. 2001).

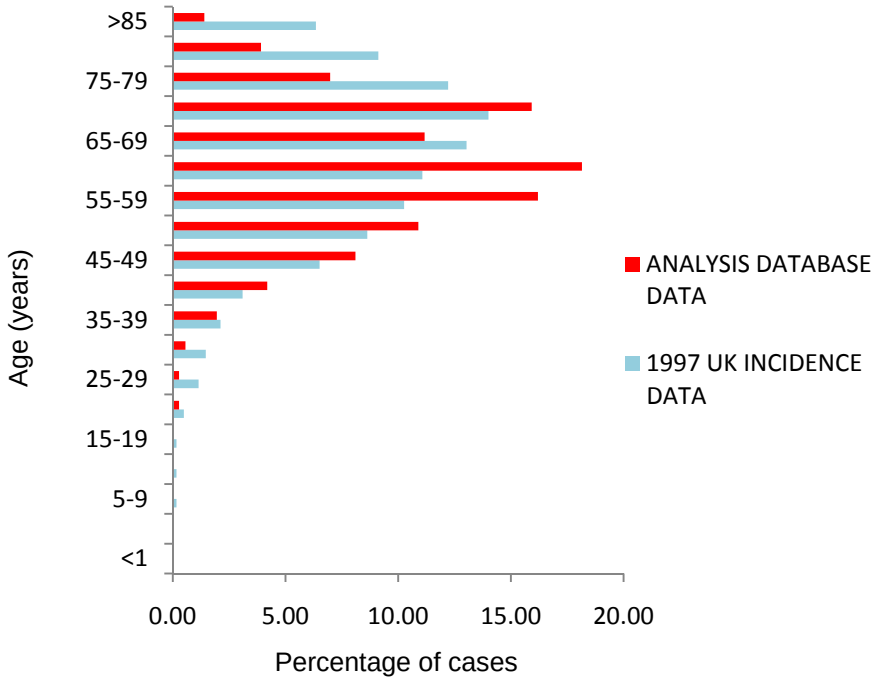


Table 4.1a **The distribution of age data on the analysis database when re-categorised to allow comparison with the evidence supplied to FIGO by Heinz and co-workers (Heintz, Odicino et al. 2001)**

Age categories	Number of cases in the evidence provided to FIGO	Distribution by % in each category	Number of cases on the analysis database	Distribution by % in each category
15-29	127	**4**	1	
30-39	270	**8**	8	**2**
40-49	731	**21**	44	**13**
50-59	896	**26**	83	**25**
60-69	823	**24**	103	**31**
70-79	453	**13**	80	**24**
≥80	109	**3**	19	**6**
Totals	**3409**		**338**	

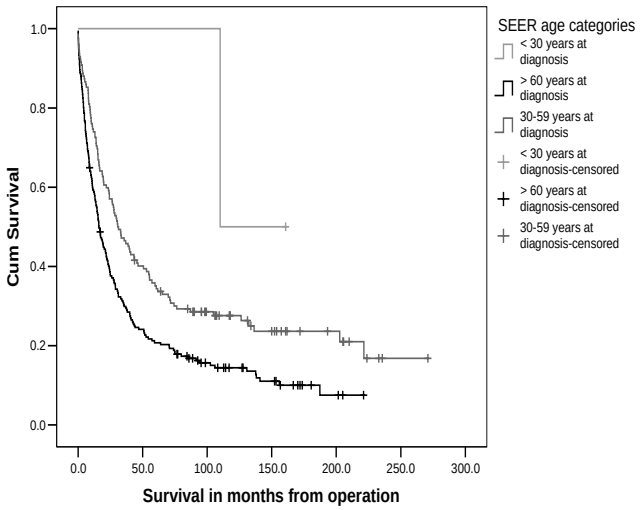
Table 4.1b The distribution of the age data amongst the cases on the analysis database when re-categorised to allow comparison with the ONS data in Cancer Trends 1997 (Quinn and Great Britain. Office for National Statistics. 2001)

Age categories	Number of cases in the UK - 1997	Distribution by % in each category	Number of cases on the analysis database	Distribution by % in each category
<1	0	**0**	0	**0**
1-4	0	**0**	0	**0**
5-9	10		0	**0**
10-14	10		0	**0**
15-19	10		0	**0**
20-24	30	**1**	1	
25-29	70	**1**	0	**0**
30-34	90	**2**	1	
35-39	130	**2**	7	**2**
40-44	190	**3**	14	**4**
45-49	400	**7**	28	**8**
50-54	530	**9**	34	**10**
55-59	630	**10**	57	**17**
60-64	680	**11**	63	**19**
65-69	800	**13**	38	**11**
70-74	860	**14**	53	**16**
75-79	750	**12**	25	**7**
80-84	560	**9**	12	**4**
≥85	390	**6**	5	**2**
Total	**6140**		**338**	

Table 4.1c **The distribution of age data on the analysis database when re-categorised to allow comparison to the data recorded by the Surveillance, Epidemiology and End Results (SEER) programme of the National Cancer Institute in the U.S (epithelial ovarian cancer)**

Age categories	Number of cases in SEER series	Distribution by % in each category	Number of cases on the analysis database	Distribution by % in each category
< 30	783	**4**	1	
30-59	9690	**43**	135	**40**
≥ 60	11905	**53**	202	**60**
Totals	**22 378**		**338**	

Figure 4.5 **A Kaplan-Meier plot of age within the analysis database when reclassified into the categories used in reporting the SEER data**
A statistically significant worsening of survival with advancing age is shown.



Log rank = 15.84; p<0.001

Table 4.2 **Cox's multivariate analysis of independent prognostic factors and age data in SEER categories**
Within the analysis database age was not an independent prognostic factor when re-categorised this way

	Hazard Ratios (95% Confidence intervals)	**Significance (p)**
FIGO Stage		
1		0.001
2	2.424 (1.426 – 4.121)	<0.001
3	4.621 (2.864 – 7.456)	<0.001
4	5.901 (3.394 – 10.261)	<0.001
Residual macroscopic disease	2.238 (1.600 – 3.131)	<0.001
Patient received chemotherapy	0.441 (0.315-0.617)	<0.001
Age at diagnosis (years) SEER categories		
< 30		0.001
30 -59	2.070 (0.286 – 14.992)	0.471
≥ 60	3.242 (0.449 – 23.417)	0.244

Table 4.3 Cox's multivariate analysis of independently prognostic factors showing age data within the analysis database in its continuous form to be an independent prognostic factor

	Hazard Ratios (95% Confidence intervals)	Significance (p)
FIGO Stage		
1		0.001
2	2.567 (1.503 - 4.383)	<0.001
3	4.719 (2.914 – 7.643)	<0.001
4	6.562 (3.737 – 11.523)	<0.001
Residual macroscopic disease	2.131 (1.519 – 2.991)	<0.001
Patient received chemotherapy	0.437(0.312 – 0.613)	<0.001
Age at diagnosis (years)		
Continuous data	1.024 (1.014-1.035)	**<0.001**

as it was important not to disregard a possible independent prognostic factor when assessing new markers. Overall, the data for age within the analysis database were complete, within an expected distribution, behaved in a valid manner and were appeared consistent.

4.2.2. Pathology

4.2.2.1. Results

Histological type

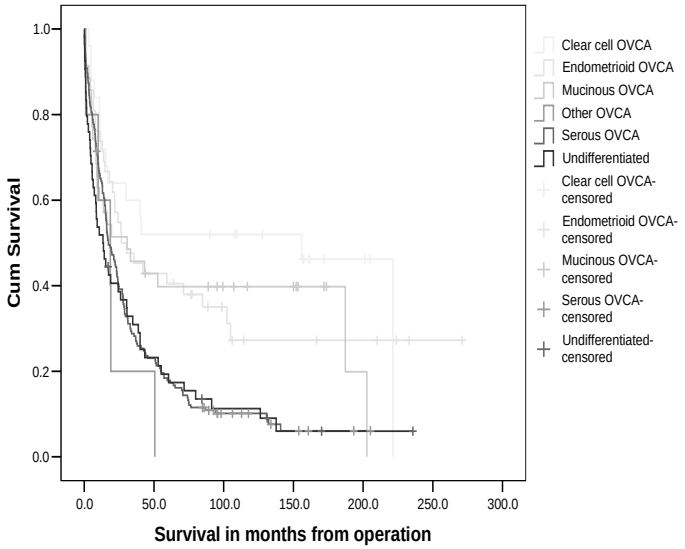
The data were generated by the contemporaneous histological review of the cases and there were no missing fields. The distribution of cases amongst the histological types is as detailed in 3.1.1 and Table 3.1. The logic of individual entries was checked by ensuring that non-epithelial types were presenting in appropriate age groups and that types usually associated poorly differentiated disease (clear cell and undifferentiated) were appropriately graded. Inconsistencies in logic were checked against the clinical record and with further pathological review on a number of occasions with no inaccuracies being detected. A univariate analysis showed that histological type predicted survival and ranked the subtypes; the order of increasing lethalness was clear cell, mucinous, endometrioid, serous and undifferentiated (Figure 4.6). In a multivariate analysis including known independent prognostic factors, the prognostic effect of histological type did not persist (Table 4.4).

Histological grade

The data were generated by the contemporaneous histological review of the cases and there were no missing fields though there were two instances where tumours were deemed ungradeable. The distribution of grades amongst the cases is as described in 3.1.1 and Table 3.1. When analysed in its three original categories (G1, G2, G3) and in a high/ low grouping (G3 = high grade; G1+G2 = low grade); more poorly differentiated disease was associated with poorer outcome in a statistically significant manner (Figure. 4.7 a, b). In a multivariate model including recognised independent prognostic factors, grade did not retain this prognostic power (Table 4.5 a, b).

Figure 4.6 Kaplan-Meier plot showing the differing survival characteristics between the histological types

Clear cell, mucinous and endometrioid types have a relatively good prognosis whereas serous, undifferentiated and non-epithelial types have a poorer prognosis. Histological type predicts prognosis with statistical significance in a univariate analysis.

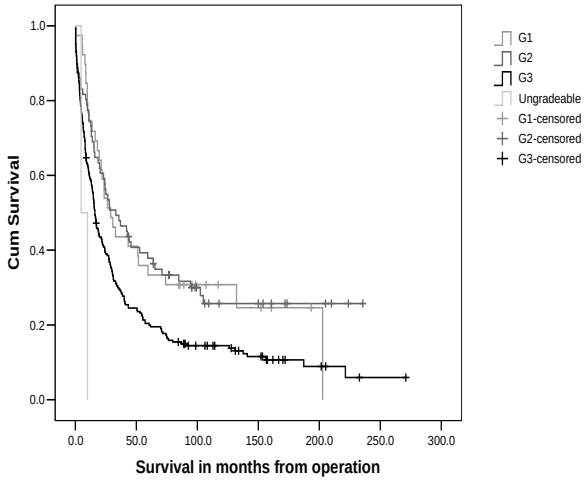


Log rank = 29.36; p<0.001

Table 4.4 **Cox's multivariate analysis of independent prognostic factors including histological type in order of worsening prognosis**
There is a loss of predictive power on multivariate analysis and it is not an independent prognostic factor.

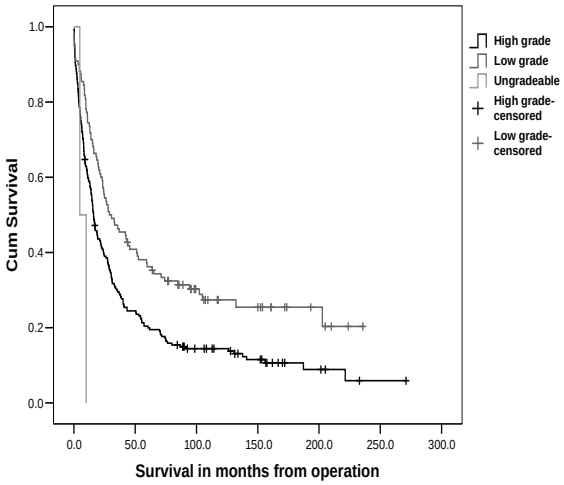
	Hazard Ratios (95% Confidence intervals)	**Significance (p)**
Age (Continuous)	1.026 (1.015-1.037)	<0.001
FIGO Stage		
1		<0.001
2	2.345 (1.316 -4.176)	0.004
3	4.494 (2.657 – 7.599)	<0.001
4	6.472 (3.567 – 11.743)	<0.001
Residual macroscopic disease	1.957 (1.380 – 2.776)	<0.001
Patient received chemotherapy	0.445 (0.315 – 0.630)	<0.001
Histological type		
Clear cell		0.479
Mucinous	0.957 (0.604 – 1.518)	0.853
Endometrioid	1.022 (0.617 – 1.693)	0.934
Serous	1.081 (0.773 – 1.512)	0.648
Undifferentiated	0.600 (0.327 – 1.103)	0.100
Other	1.696 (0.624 – 4.611)	0.301

Figure 4.7a Kaplan-Meier plot showing the difference in survival between the three grades of disease
G1+G2 disease behave almost identically to each other whereas G3 predicts a worse survival in univariate analysis.



Log Rank = 17.04; p<0.001

Figure 4.7b A Kaplan-Meier plot showing the difference in survival between high and low grade disease



Log rank= 17.04; p=0.001

Table 4.5a Cox's multivariate analysis of independent prognostic factors including grade of disease in its three traditional categories
Grade does not have prognostic power independently of other factors

	Hazard Ratios (95% Confidence intervals)	**Significance (p)**
Age (Continuous)	1.027 (1.016-1.039)	<0.001
FIGO Stage		
1		<0.001
2	2.471 (1.428-4.276)	0.001
3	4.686 (2.866 – 7.661)	<0.001
4	6.220 (3.510 – 11.023)	<0.001
Residual macroscopic disease	2.024 (1.436 – 2.853)	<0.001
Patient received chemotherapy	0.441 (0.314 – 0.620)	<0.001
Histological grade		
G1		0.244
G2	1.309 (0.807 – 2.123)	0.275
G3	1.431 (0.937 – 2.186)	0.097

Table 4.5b Cox's multivariate analysis of independent prognostic factors including high/low grade disease
This factor loses the prognostic power it has on univariate analysis and is not an independent prognostic factor.

	Hazard Ratios (95% Confidence intervals)	**Significance (p)**
Age (Continuous)	1.027 (1.016-1.038)	<0.001
FIGO Stage		
1		<0.001
2	2.447 (1.416-4.230)	0.001
3	4.688 (2.866 – 7.667)	<0.001
4	6.235 (3.517 – 11.051)	<0.001
Residual macroscopic disease	2.005 (1.423 – 2.825)	<0.001
Patient received chemotherapy	0.436 (0.310 – 0.614)	<0.001
High / Low grade disease	1.205 (0.916 – 1.584)	0.128

Stage

The data was detailed to a sub-stage level in the study database and in the analysis database was additionally extrapolated to generate a category of the major FIGO groupings I-IV. The more detailed sub-stage data were incomplete in 76 cases but there only 9 cases with data missing to a FIGO Stage level. A quarter of cases were stage I, half were stage III with the remaining cases equally divided among the stage II and stage IV categories (Figure 4.8a,b)(Table 4.6). Assessing the association of stage with other clinicopathological variables using the χ^2 test revealed no association of stage or sub-stage with age (in SEER categories) though worsening stage was associated with worse histological grade (13.56; p=0.035) and worse stage or sub-stage was significantly statistically associated with poorer prognosis histological subtype (Stage – 87.5; p<0.01: Sub-stage – 141.8; p<0.01). Increasing stage and sub-stage were statistically significantly associated with worse overall survival in univariate analysis, though the relationship was not linear on a sub-stage level and the survival plots crossed frequently (Figure 4.9 a, b). A multivariate analysis showed FIGO stage was an independent prognostic factor (Table 4.7)

Specimen type

A note was kept of the tissue source from which the arrayed cancer specimens were removed; 7% originated from omental metastases, 5% from pelvic metastases and 88% from ovarian tissue.

4.2.2.2. Discussion

The histological subtype had been recorded on the study database following its prior extraction from the original pathology reports. As all of the cases were reviewed contemporaneously by a specialist gynaepathologist, rather than general pathologists of the type who had produced the original reports, this original data were not assessed and were removed from the database. Though interesting, comparing historic and modern findings was unnecessary, especially as conflicts would be resolved in favour of the specialist gynaepathologist. The rankings of the tumour types in order of lethalness may appear counter-

Figure 4.8a **Histogram showing the relative distribution of cases within the FIGO stage groupings**
Stage III was the most common and stage II the least common.

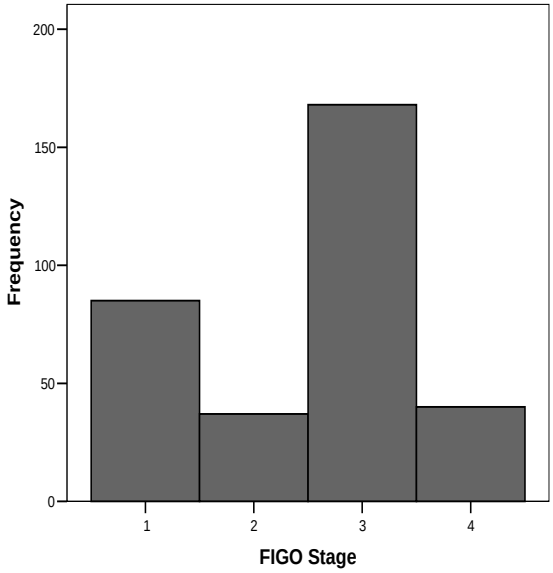


Figure 4.8b **Histogram showing the distribution of cases within the FIGO sub-stage groupings**
Stage IIIa was the most common and Ib the least common.

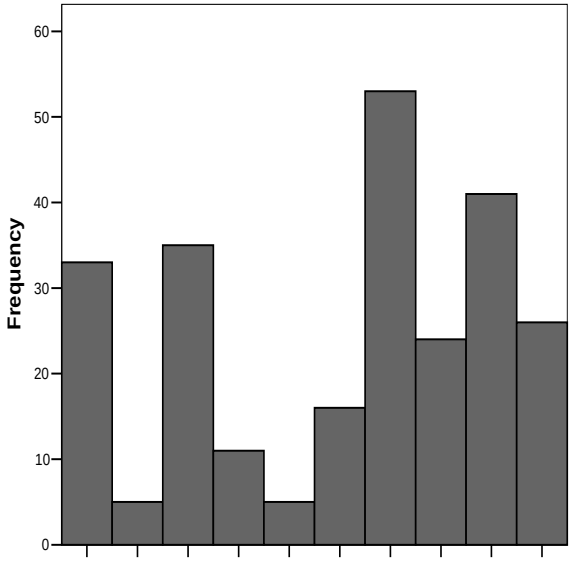
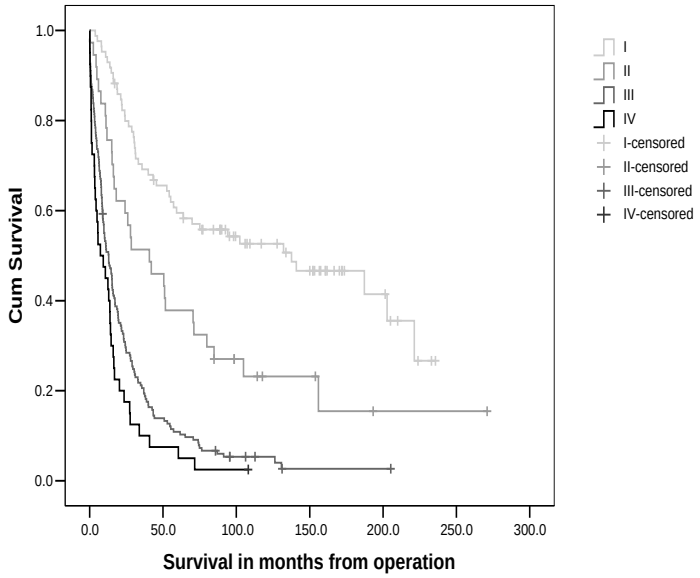


Table 4.6 The distribution of cases by stage
There were less data available concerning the more detailed sub-stage categorisation of the cases.

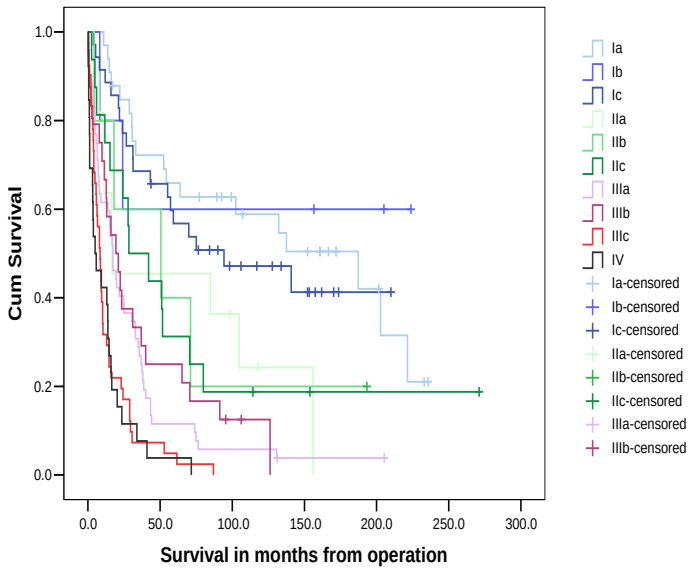
	n (%) cases	Mean survival (months)	Median survival (months)	Approximate 5-year overall survival
FIGO Stage				
Missing data	9 / 339 (3)			
n=330				
I	85 (26)	129.4	137.6	**60%**
II	37 (11)	79.3	40.7	**38%**
III	168 (51)	26.3	13	**10%**
IV	40 (12)	15.4	7.3	**5%**
FIGO Sub-stage				
Missing data	76 (22)			
n = 263				
Ia	33 (13)	136.8	187.2	**63%**
Ib	5 (2)	140.8		**60%**
Ic	35 (13)	114.8	94.2	**56%**
Stage but no sub-stage data	12			
IIa	11 (4)	63.9	16.7	**40%**
IIb	5 (2)	67.5	50.6	**30%**
IIc	16 (6)	76.8	28.4	**30%**
Stage but no sub-stage data	5			
IIIa	53 (20)	30.3	17.3	**10%**
IIIb	24 (9)	36.6	19.4	**20%**
IIIc	41 (16)	14.1	8.4	**2%**
Stage but no sub-stage data	50			
IV	40 (15.2)	12.2	5	**5%**

Figure 4.9a **Kaplan-Meier plot showing the statistically significant association between increasing FIGO stage and worsening survival**



Log rank = 114.55; p<0.001

Figure 4.9b **Kaplan-Meier plot showing the statistically significant association between FIGO sub-stage**
In general, survival becomes worse with increasing sub-stage but as there are multiple curve crossing points an assumption of proportional hazards may not be valid.



Log rank 117.33; p<0.001

Table 4.7 Cox's multivariate analysis of independent prognostic factors including FIGO stage

When other independent factors are included into a multivariate model, FIGO stage retains its prognostic power and is an independent prognostic factor in ovarian cancer.

	Hazard Ratios (95% Confidence intervals)	**Significance (p)**
Age (Continuous)	1.025 (1.014 - 1.037)	<0.001
Residual macroscopic disease	1.990 (1.414 – 2.801)	<0.001
Patient received chemotherapy	0.439 (0.312 – 0.618)	<0.001
FIGO Stage		
1		<0.001
2	2.506 (1.449 - 4.333)	0.001
3	4.902 (2.992 – 8.031)	<0.001
4	6.624 (3.743 – 11.723)	<0.001

intuitive, as clear cell and mucinous cancers are anecdotally associated with a poor prognosis. However they often present at an early stage and overall are associated with a relatively good prognosis, unlike undifferentiated and serous types which are often present later leading to a poor prognosis The final ranking of tumours and the observation that this prognostic affect disappeared on a multivariate analysis substantiates this and validates the dataset through its agreement with the wider literature (Silva, Tornos et al. 1991; Makar, Baekelandt et al. 1995; Gospodarowicz 2001; Heintz, Odicino et al. 2001; Harrison, Jameson et al. 2008; Itamochi, Kigawa et al. 2008). Overall the data for histological type were consistent and the data were subsequently used in univariate (but not multivariate) analyses when investigating molecular marker expression. Further discussion concerning histological type is found in 3.2.

The grading data in the original database was also removed with contemporaneous data being substituted. Histological grading has important prognostic power but this is inseparable from other aspects of disease biology such as stage and response to chemotherapy. Therefore it was appropriate that grade was a prognostic indicator in a univariate but not multivariate analysis in this dataset, supporting its validity (Gospodarowicz 2001). A larger than expected number of cases were high grade (G3) in this series but the fact that this category was of prognostic importance in univariate analysis when compared to G1 and G2 makes investigator inaccuracy an unlikely cause of this. Though a series with above average numbers of poorly differentiated cancers may have occurred by chance there is another explanation. It is possible that the higher number of G3 cases was at the expense of G2 cancers with truly moderately differentiated cancers being unconsciously allocated to the G3 category; explaining why G2 cancers behaved indistinguishably from G1 cancers regarding survival. This would be understandable as objectifying the grading of ovarian cancer has proved elusive and would reflect a sensibly cautious approach to reporting disease as low grade (Hernandez, Bhagavan et al. 1984).

Overall the data for histological grade were not inconsistent and were included in univariate but not multivariate analyses when assessing new markers. Further discussion regarding grade is found in 3.2.

The level of missing data within the major categories of FIGO stage was small but the more detailed description of the disease in the form of sub-stage classification was often incomplete, possibly due to difficulties in extracting this data the clinical record. Another explanation is that the disease may not have been assessed clinically in some cases on a sub-stage level by some surgeons with the required pathological elements of cytology and lymph nodes not being sent. Despite the absence of some details, the distribution of cases amongst the FIGO stages in their major groupings was similar to that of contemporaneous cohorts (Hand, Fremgen et al. 1993; Yancik 1993; Ilekis, Gariti et al. 1997; Wolfe, Tilling et al. 1997; Brun, Feyler et al. 2000; Holschneider and Berek 2000; Heintz, Odicino et al. 2001; Quirk and Natarajan 2005). The assumption of proportional hazards was not valid for the sub-stage categorisation as the survival curves crossed and so FIGO stage was assessed in the multivariate model in only its major groupings, where it predicted prognosis independently of other factors. As stage is a de facto independent prognostic factor, this finding validates both the stage and survival data (Gospodarowicz 2001; Heintz, Odicino et al. 2001). Overall the data were deemed consistent.

As the source of tissue available for contemporaneous review depended upon the extent of the original surgery, some minimally debulked cases had only metastatic material available within the archive. Differential expression of molecular markers between primary and metastatic samples within an individual case may theoretically occur; as previously noted CK20 expression varies between anatomical sites whereas there is much better correlation concerning CK7 expression (Tot 2002). This could perhaps most intuitively be the case with regards to molecules implicated biologically in metastasis such as cell adhesion molecules, growth factor receptors and those involved in

angiogenesis. There is evidence that E-cadherin is expressed more frequently in metastatic deposits than in paired primary samples (Elloul, Silins et al. 2006) and a discrepancy between vascular endothelial growth factor (VEGF) expression in 8 out of 30 cases where paired samples were compared has also been observed (Fujimoto, Sakaguchi et al. 2001), though other workers have found no significant differences in E-cadherin, β-catenin, EGF and ERB2 expression between different anatomical sites (Davidson, Gotlieb et al. 2000). A relative lack of studies examining the immunohistochemical expression of markers in paired samples means that it is hard to be certain of the impact of using metastatic samples in this experimental work but it is likely to vary according to the individual marker (Davidson 2007). Having a small proportion of metastatic samples in the series did however keep any theoretical impact to a minimum and it was possible to investigate any significant difficulties ascribable to sample related false negative or false positives in detail with full sections if this became necessary.

4.2.3. Surgery

4.2.3.1. Results

Surgical debulking

13 cases had data missing concerning the level of cytoreduction. The logic of the entries were checked by reviewing the early stage cases, where no instances of residual disease were expected and the stage IV cancers where a majority of cases were expected to have residual disease; these checks revealing no inconsistent results.130 (40%) of cases had no macroscopic disease remaining after surgery (optimally debulked), 89 cases (27%) had macroscopic disease with lesions of a maximal size of <2cm and 107 cases (33%) had bulky residual disease with lesions of ≥ 2cm. Regarding overall survival, optimally debulked disease had the best prognosis, cases with ≥2cm sized deposits of tumour had the worst survival and those cases having deposits <2cm residual had an intermediate prognosis (Figure 4.10a). Optimally debulked disease used singly also predicted a better prognosis (Figure

4.10b) and both ways of sub-categorising residual disease were prognostic for survival independently of other prognostic factors (Table 4.8a, b).

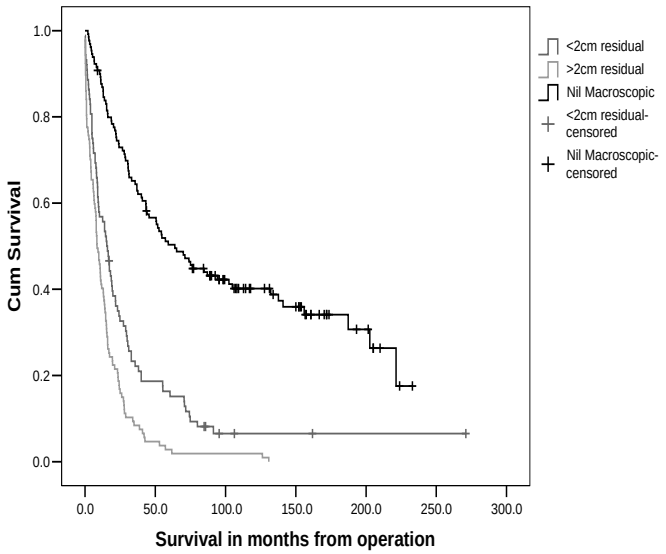
Pre-operative serum CA125

217 cases had pre-operative serum CA125 values recorded with 36% having this data absent and no cases having serial measurements available for analysis. The values ranged from 0 – 14200. 42% of cases had an elevated serum CA125 above 35 U /ml; 2 % had a value between 35 - 60 U / ml, 7% a value between 60-150 U /ml, 12% a value between 150-500 U /ml and 21% a value above 500 U /ml. Using these cut-off points to investigate its prognostic effect on overall survival in a univariate analysis revealed that a worse prognosis was predicted by a serum CA125 > 500 U /ml (Figure 4.11a, b). Using the χ^2 test to look for associations with other clinicopathological factors revealed statistically significant associations between this definition of high CA125 and increasing FIGO stage (17.34; p=0.01), sub-optimally debulked disease (9.15; p=0.002) and more lethal histological type (15.58; p=0.008). In a multivariate analysis including other known independently prognostic factors, pre-operative high pre-operative serum CA125 level did not retain independent prognostic power (Table 4.9).

Surgical sub-specialisation

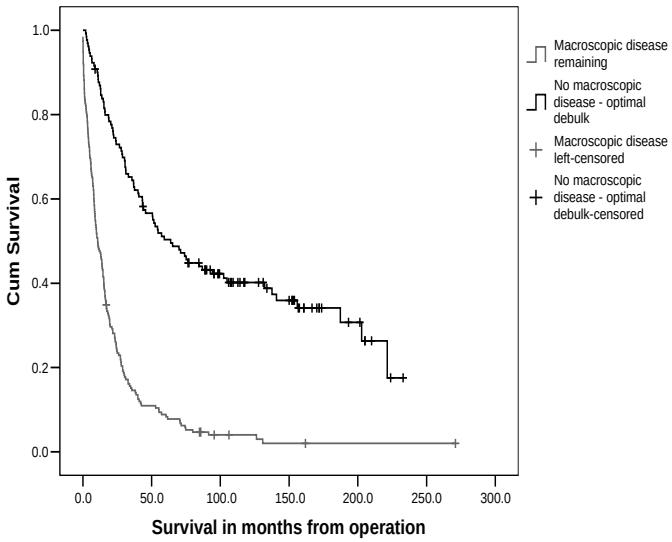
Of the 339 operations performed, data concerning the degree of specialisation of the operating surgeon were available for 321 cases (missing data = 5%). Of these cases, 42% were performed by a gynae-oncologist, 54% were performed by an unspecified gynaecologist and 4% were treated by a general surgeon. There were no statistically significant variations between the surgeons concerning FIGO stage of disease (14.19; p=0.12), the presence of visible residual disease at the end of surgery (7.26; p=0.06) or the subsequent use of adjuvant chemotherapy (5.71; p=0.13). The survival of patients treated by the general surgeons was worse than that seen when a general gynaecologists or gynae-oncologist operated (Figure 4.12a) though this was not an independent prognostic factor on multivariate analysis

Figure 4.10a Kaplan-Meier plot showing the statistically significant univariate association between the increasing amount of residual disease left at the end of surgery and worsening survival. The groups are categorised >2cm, <2cm (>0) and no macroscopic residual



Log rank = 130.40; p<0.001

Figure 4.10b Kaplan-Meier plot with residual disease re-categorised into two broad categories (any macroscopic residual vs. no macroscopic residual) showing the strong statistical association between the presence of residual disease and poorer outcome in a univariate analysis



Log rank = 107.89; P<0.001

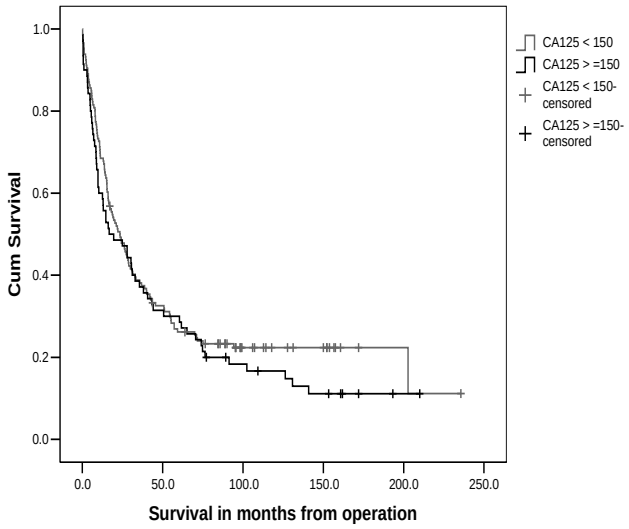
Table 4.8a Cox's multivariate analysis of independent prognostic factors amount of residual disease using a threshold of 2cm sized deposits

	Hazard Ratios (95% Confidence intervals)	Significance (p)
Age (Continuous)	1.024 (1.013 - 1.035)	<0.001
FIGO Stage		
1		<0.001
2	2.624 (1.512 – 4.554)	0.001
3	4.830 (2.928 – 7.968)	<0.001
4	6.284 (3.516 – 11.23)	<0.001
Patient received chemotherapy	0.452 (0.319 - 0.642)	<0.001
Residual disease		
No residual		<0.001
<2cm residual	1.673 (1.151 – 2.430)	0.007
≥2cm residual	2.542 (1.733 – 3.728)	<0.001

Table 4.8b Cox's multivariate analysis including the absence of any macroscopic residual disease

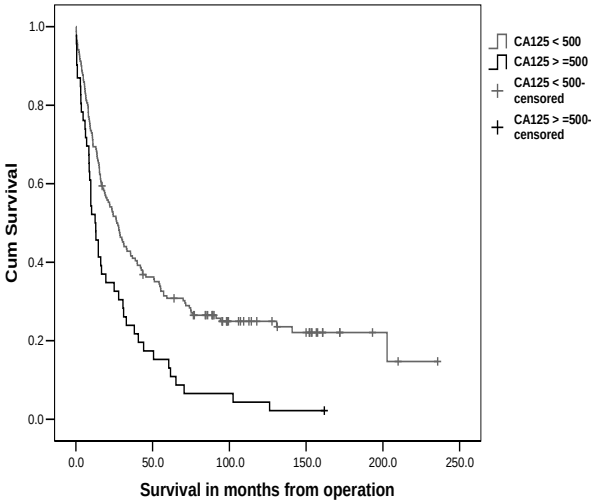
	Hazard Ratios (95% Confidence intervals)	Significance (p)
Age (Continuous)	1.025 (1.014 - 1.037)	<0.001
FIGO Stage		
1		<0.001
2	2.506 (1.449 – 4.333)	0.001
3	4.902 (2.992 – 8.031)	<0.001
4	6.624 (3.743 – 11.723)	<0.001
Patient received chemotherapy	0.439 (0.312 - 0.618)	<0.001
Residual disease		
		<0.001
Macroscopic disease remaining	1.990 (1.414 – 2.801)	<0.001

Figure 4.11a Kaplan-Meier graph showing the predicting of survival by pre-operative CA125
Cut offs of 35, 60 and 150 U/ml (bellow) were not associated with statistically significant differences in survival in a univariate analysis.



Log rank = 1.003; p=0.316

Figure 4.11b Kaplan-Meier graph showing the predicting of survival by pre-operative CA125 using a cut-off of 500 U/ml
The two groups had a statistically significant difference in survival – a high CA125 predicting a poorer overall survival in a univariate analysis.



Log rank 14.167; p<0.001

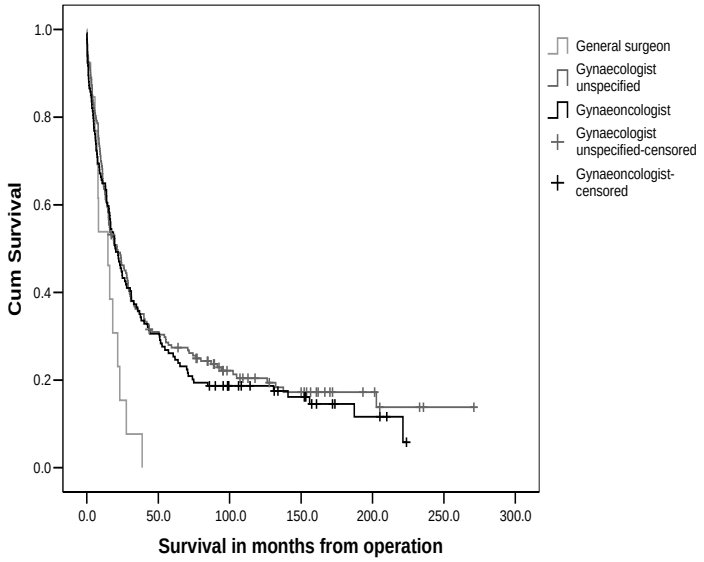
Table 4.9 **Cox's multivariate analysis including high pre-operative CA125**
CA125 >500 U/ ml did not retain the prognostic power it exhibited in univariate analysis.

	Hazard Ratios (95% Confidence intervals)	**Significance (p)**
Age (Continuous)	1.028 (1.013-1.043)	<0.001
FIGO Stage		
1		<0.001
2	2.807 (1.383 – 5.697)	0.004
3	4.613 (2.484 – 8.565)	<0.001
4	8.360 (4.059– 17.221)	<0.001
Residual macroscopic disease	2.119 (1.354 – 3.316)	<0.001
Patient received chemotherapy	0.457 (0.301 – 0.695)	<0.001
CA125		
CA125 > 500 U / ml	1.320 (0.925 – 1.884)	0.126

Table 4.10 **Cox's multivariate analysis including degree of specialisation of the surgeon operating**
This was not an independent prognostic factor in this series.

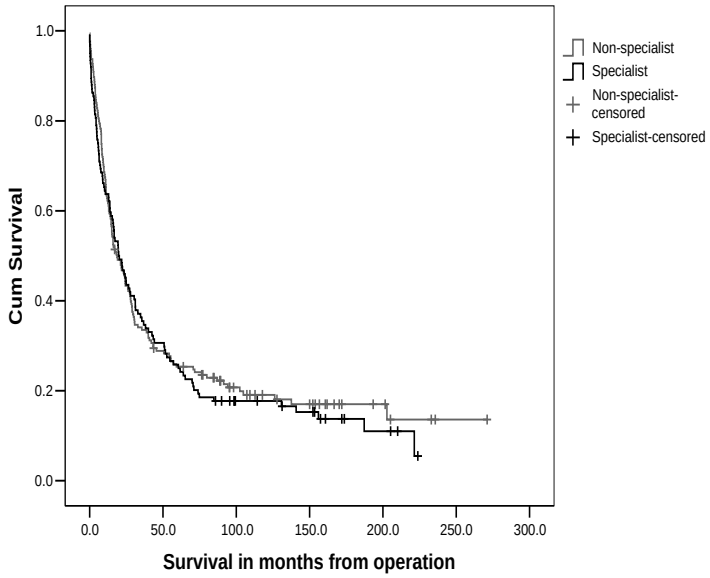
	Hazard Ratios (95% Confidence intervals)	**Significance (p)**
Age (Continuous)	1.026 (1.015-1.037)	<0.001
FIGO Stage		
1		<0.001
2	2.384 (1.379 – 4.120)	0.002
3	4.890 (2.963 – 8.072)	<0.001
4	6.953 (3.891 – 12.422)	<0.001
Residual macroscopic disease	1.902 (1.348 – 2.684)	<0.001
Patient received chemotherapy	0.452 (0.319 - 0.642)	<0.001
Specialty of surgeon		
Gynae-oncologist		0.241
General gynaecologist	1.186 (0.917 – 1.533)	0.194
General surgeon	1.505 (0.837 – 2.705)	0.172

Figure 4.12a Kaplan-Meier plot showing the effect of degree of surgical specialisation on survival
Cases managed by general surgeons had the worst outcome and the difference was statistically significant.



Log rank = 7.057; p=0.029

Figure 4.12b Kaplan-Meier plot showing the association of gynae-oncologist performing the surgery with survival
As only 13 cases were managed by general surgeons, these were combined with the cases managed by general gynaecologists to investigate the influence of the gynae-oncologist's practice on overall survival. There was no association found.



Log rank =2.787; p=0.248

(Table 4.10). There was no difference in overall survival seen between the cases attended by the general gynaecologist or specialist gynaecologist (Figure 4.12b).

Operation performed

On reviewing the pathology reports a note was made of the specimens examined by the pathologist. There were no missing data. 17% had only biopsy material removed, 14% had a single ovary removed, 20% had both ovaries removed, 13% had ovaries and uterus removed with 35% of cases having uterus, ovaries and omentum removed.

4.2.3.2. Discussion

The proportion of cases debulked to <2cm in the literature range from 36- to 87% (Venesmaa 1994; Makar, Baekelandt et al. 1995; Kehoe 1996; Heintz, Odicino et al. 2001) and to the point of no visible macroscopic disease from 15 – 85% (Venesmaa 1994; Eisenkop, Friedman et al. 1998; Bristow, Montz et al. 1999; Brun, Feyler et al. 2000; Heintz, Odicino et al. 2001). This series data were well within these ranges, supporting its consistency. Optimal debulking by either standard is an accepted independent prognostic factor , which was found to be the case in this series, validating the dataset (Gospodarowicz 2001). Overall the data were deemed consistent and as the simpler classification of optimal debulking (no macroscopic disease) is now the widely accepted standard, the data were categorised in this way for the subsequent multivariate analyses.

The data for pre-operative serum CA125 were to a large degree incomplete. The majority of cases missing data were from the early 1980s when the routine use of serum CA125 was not established. None of the values were out of the range expected for cases of ovarian cancer but only 42% of cases exhibited a raised CA125 despite it usually being elevated in 80-90% of epithelial ovarian cancers (Hogdall, Christensen et al. 2007). Potential explanations include bias introduced through the missing data, an unusual case series or the incorrect extraction of data from the clinical record. If all of the missing cases had

a raised serum CA125 then this would elevate the proportion to 77% for the whole series but this seems the least plausible explanation. A raised serum CA125 is found less frequently in mucinous epithelial ovarian cancers and in only 50% of stage I cancers but there was no evidence that such cases were disproportionately represented in the group with CA125 data available (Jacobs and Bast 1989; Einhorn, Sjovall et al. 1992). The most likely possibility is that post- and not pre-operative CA125 levels were inadvertently recorded. In order to compare the data with other clinicopathological factors and overall survival, previously published cut-off points were empirically analysed at 35, 60, 150 and 500 U / ml Jacobs, Oram et al. 1990; Einhorn, Sjovall et al. 1992; Hogdall, Christensen et al. 2007). Using a cut-off of 500 U/ ml cut-off for this series reproduced the findings of Cooper et al except for an association with histological grade and the independent effect of its prognostic power (Cooper, Sood et al. 2002) while also reproducing an association with optimally debulked disease found by other groups (Chi, Venkatraman et al. 2000; Gemer, Segal et al. 2001). The lack of association with grade in this series is in keeping with other publications though might also have been influenced by the high number of G3 cases in this series (Rossi, Di Vagno et al. 2004). The survival data were congruent to the findings of Makar et al who found pre-operative CA125 to be associated with poorer survival in univariate but not multivariate analysis (Makar, Baekelandt et al. 1995). In general the data reproduced known associations but it was incomplete and some fundamental inconsistencies were present. It may have been possible to more completely and accurately re-extract the data from the clinical record but the relative unimportance of pre-operative serum CA125 meant that resources were prioritised elsewhere and the existing data were not used further (Hunter, Daly et al. 1990; Gospodarowicz 2001) (Meier, Stieber et al. 1997).

42% of cases had their operations performed by a gynae-oncologist and though this is a higher figure than has been seen in other series (21% of cases within a U.S series of over 12 000 cases presenting

during a similar time period that were treated by a sub-specialist) it is at dissonance with the perception that the majority of cases had been treated by a single sub-specialist (Nguyen, Averette et al. 1993). There is some evidence suggesting that outcomes should be better when cases are treated by gynae-oncologists and not general gynaecologists and this might support the theory that a large body of cases in this series had been inaccurately allocated; leading to homogeneity of outcomes and the observed dissonance between the anecdotal and objective evidence (Junor, Hole et al. 1999). Inaccurate allocation may have occurred in cases where the gynae-oncologist was the assistant (but in effect senior operator) or where the denomination of the surgeon was unknown. However it is just as likely that inaccurate recall was the explanation as other groups have found no difference in outcomes between gynae-oncologist and general gynaecologist (Nguyen, Averette et al. 1993; Kehoe, Powell et al. 1994; Earle, Schrag et al. 2006). However all groups agree that cases treated by general surgeons do worst of all and despite the small numbers involved, this was also the case in this series. Though inconsistent with the anecdotal evidence concerning the lead surgeon the data are valid but were not used further. As the degree of sub-specialisation of the surgeon is not an accepted independent prognostic factor and it did not predict prognosis independently in this multivariate model (Gospodarowicz 2001).

Though it was intended that noting the organs inspected by the pathologist might give an insight into the completeness of the operation performed, the lack of information concerning previous surgeries for benign conditions made this an unreliable indirect measure. A better measure of radicalness of surgery is residual disease. This attribute might therefore be better titled “specimens examined by original pathologist “. It was decided that the usefulness of this data was limited and it was not used in any formal analysis.

4.2.4. Medical treatment

4.2.4.1. Results

Adjuvant treatment

73% of patients received adjuvant treatment, no cases received neo-adjuvant treatment and data were missing in seven instances. Outlying data points were found to be accurate on cross referencing with the medical record (Figure 4.13). 72% of patients underwent a course of chemotherapy, 50% of patients received platinum based chemotherapy and 2% of cases received combined platinum and paclitaxel chemotherapy. 28% of patients were treated by surgery alone. On univariate analysis, age (in SEER categories) was not seen to influence the initiation of adjuvant therapy. The use of adjuvant therapy was strongly influenced by stage of the disease (Table 4.11). The univariate Kaplan-Meier plots inferred that a decision to initiate chemotherapy would be associated with a long term survival disadvantage but the log rank test revealed no statistical association between the use of adjuvant therapy, or chemotherapy in general and overall survival in the 339 cancers of all stages, (Figure 4.14 a, b). On excluding stage I cancers from the univariate survival analysis it is shown that the use of specific adjuvant treatments and of chemotherapy in general was associated with statistically significant improvements in survival (Figure 4.14 c, d). Analysing the effect on survival of both of these sub-categorisations revealed that both were independent prognostic factors, though radiotherapy was of borderline statistical significance in the more specific multivariate analysis of adjuvant treatment (Table 4.12a, b).

ICON trials

59 patients participated in the first three ICON studies that recruited in the 1990s (Figure 4.15). A single case was diagnosed several years before ICON2 commenced and is seen as an outlier on the scatterplot.

Figure 4.13 **A scatterplot illustrating the distribution of adjuvant therapy received by cases within the series**
The consistency of the outlying data was checked. A move toward the use of carboplatin and then carboplatin and paclitaxel in combination in the late nineties is seen. Radiotherapy was used rarely and discontinued in the 1990s.

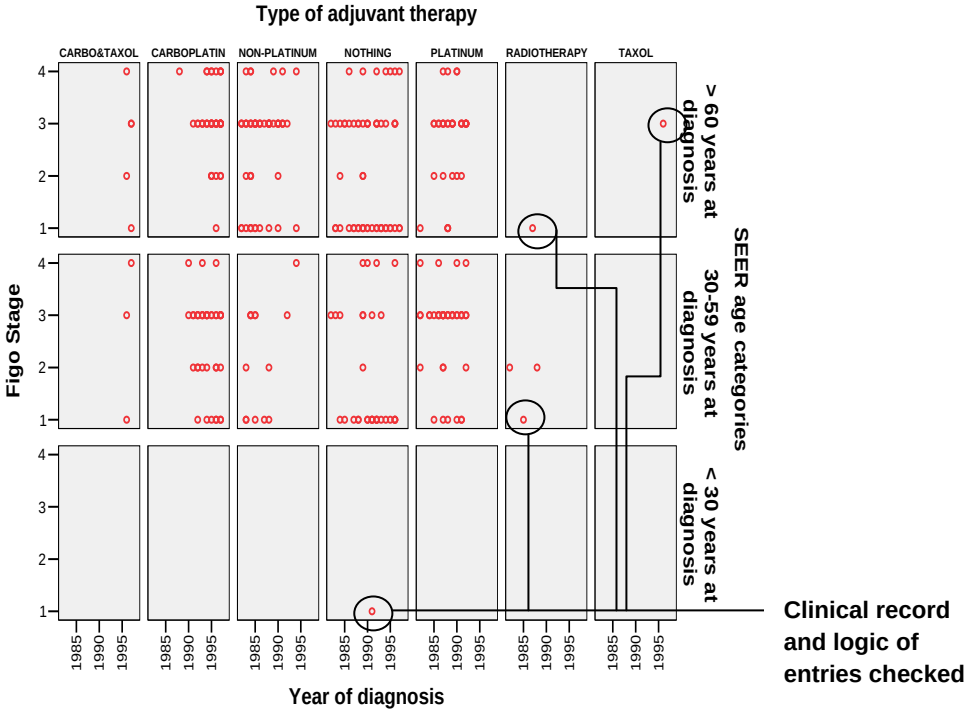
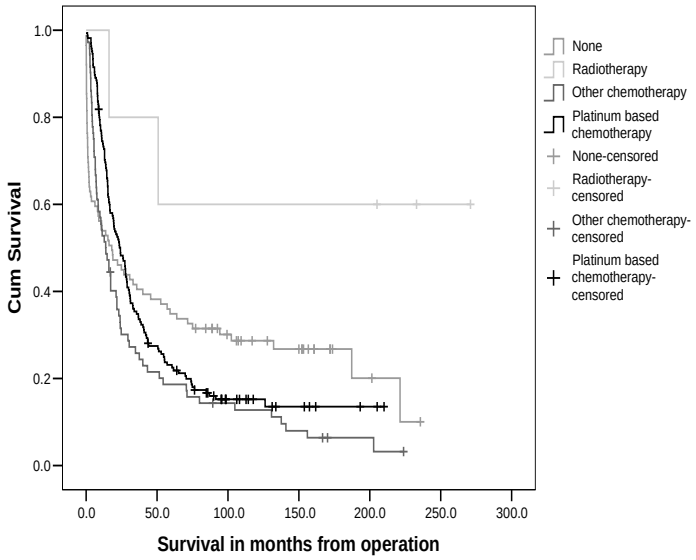


Table 4.11 The proportion of patients undergoing specific forms of post-operative medical treatment

For the purposes of subsequent analyses the raw data were regrouped into logical categories prior to their prognostic properties being assessed. While the age of the patient did not influence the decision to start medical therapy, the stage of the disease did.

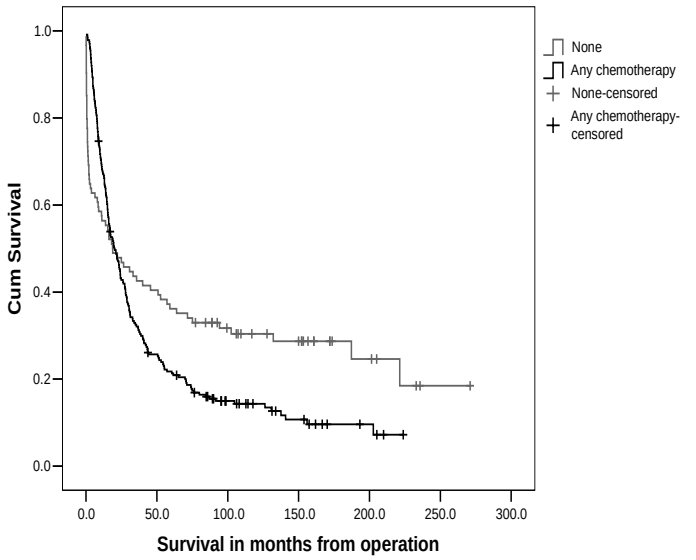
	Frequency	Univariate associations	
Type of treatment	**n= (%)**	**Age (SEER)**	**Stage (FIGO)**
Missing data	7 (2)	7 (2)	9 (3)
Original categories (n= 332)		**χ^2 (p=)**	**χ^2 (p=)**
Non-platinum treatment	72 (22)		
Platinum (unspecified)	74 (22)		
Carboplatin	84 (25)		
Carboplatin+ Paclitaxel	8 (2)		
Single agent Paclitaxel	1		
Radiotherapy	4 (1)		
Surgery only	89 (27)		
Re-categorised - Platinum (n=332)		**22.4 (0.001)**	**56.4 (<0.001)**
Platinum based chemotherapy	166 (50)		
Non-platinum based chemotherapy	73 (22)		
No chemotherapy given	93 (28)		
Re-categorised - Any chemotherapy (n=332)		3.13 (0.209)	**45.83 (<0.001)**
Any chemotherapy given	239 (72)		
No chemotherapy given	93 (28)		

Figure 4.14a **A Kaplan-Meier plot showing the association between adjuvant treatment and survival**
Within the series of 339 cases that included all stages of disease a statistically significant difference in survival is not present and the survival lines cross at multiple points.



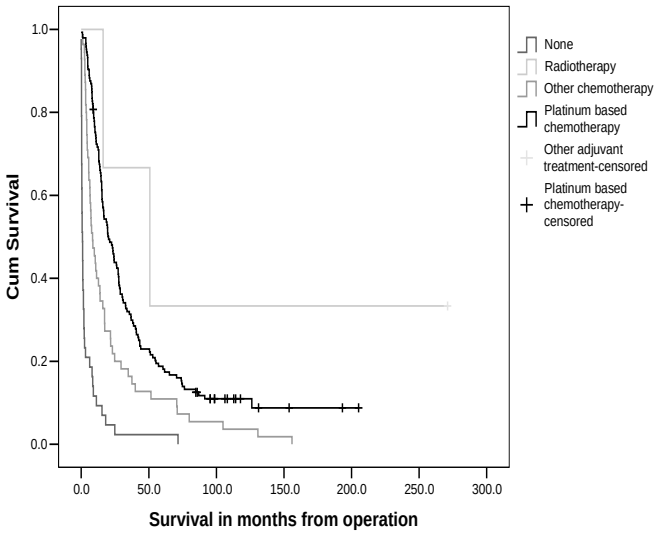
Log rank = 11.59; p= 0.09

Figure 4.14b **A Kaplan-Meier plot seems to show the association of use of chemotherapy with overall survival rates for all stage disease**
As this dataset was not designed to study differences between particular modalities of adjuvant treatment the decision to initiate chemotherapy in general was instead assessed. No statistically significant differences were seen when all stages were included.



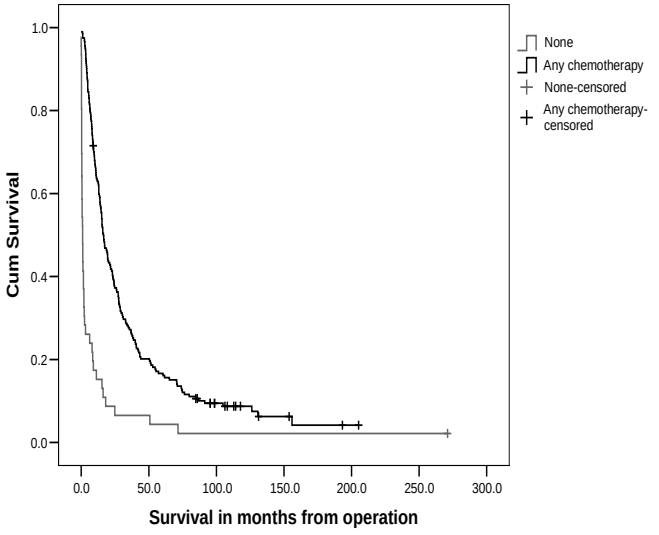
Log rank = 3.53; p=0.6

Figure 4.14c A Kaplan-Meier plot bellow showing the effect of adjuvant treatment on survival for stage II-IV cancers
In these cases, the decision not to give therapy would have been made only if the patient was deemed too unfit to receive it. Adjuvant treatment is statistically associated with a survival benefit for this group.



Log rank =123.86; p<0.01

Figure 4.14d A Kaplan-Meier plot showing the association of the decision to initiate chemotherapy with improved prognosis in stage II-IV cancers



Log rank = 57.2; p=<0.001

Table 4.12a Cox's proportional hazards model including type of adjuvant therapy
This was differentially associated with improved survival independently of other factors.

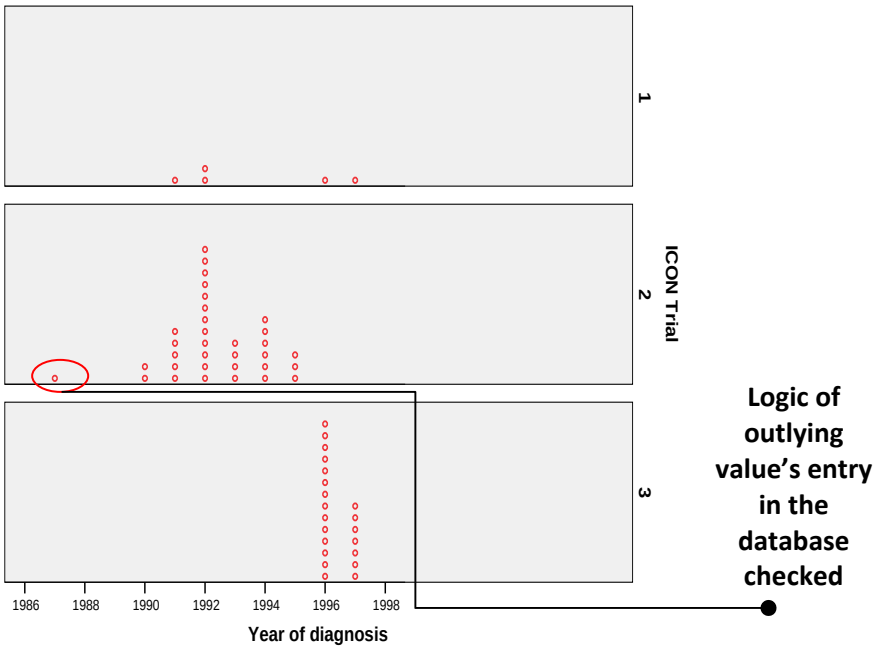
	Hazard Ratios (95% Confidence intervals)	Significance (p)
Age at diagnosis (years)	1.019 (1.007-1.030)	0.001
FIGO Stage		
1		<0.001
2	3.505 (1.994 – 6.162)	0.001
3	6.321 (4.566 -14.795)	<0.001
4	8.220 (4.566-14.795)	<0.001
Residual macroscopic disease	1.945 (1.360 – 2.782)	<0.001
Adjuvant treatment		
None		<0.001
Non-Platinum chemotherapy	0.292 (0.201-0.424)	<0.01
Platinum based chemotherapy	0.494 (333-0.733)	<0.01
Radiotherapy	0.115 (0.026 – 0.516)	0.05

Table 4.12b Cox's proportional hazard model including the decision to initiate chemotherapy
This was associated with improved survival independently of other independent prognostic factors.

	Hazard Ratios (95% Confidence intervals)	Significance (p)
Age at diagnosis (years)	1.025 (1.014-1.037)	<0.001
FIGO Stage		
1		<0.001
2	2.506 (1.449 – 4.333)	0.001
3	4.901 (2.992 -8.031)	<0.001
4	6.624 (3.743-11.723)	<0.001
Residual macroscopic disease	1.990 (1.414 – 2.801)	<0.001
Any chemotherapy given	0.439 (0.312 – 0.618)	<0.01

Figure 4.15 Cases recruited to the ICON trials

Of the 239 patients in the series who received chemotherapy, 59 were randomised within ICON trials. Cases were recruited to ICON I (1991 – 2000; The use of adjuvant chemotherapy in early stage disease), ICON II (1991 – 1996; Carboplatin compared with CAP regimen) and ICON III (1995 - Combined carboplatin and paclitaxel versus other conventional regimens). The recruitment of cases is shown in the scatterplot below.



4.2.4.2. Discussion

As the study cohort was selected from the years where significant alterations in best practice had occurred, the patients experienced a full spectrum of chemotherapeutic regimens ranging from single agent cyclophosphamide through to the current standard of 5-6 cycles of carboplatin (cycles AUC 6 over 60 minutes 3 weeks apart) combined with Paclitaxel (175mg/m^2 over 3 hours) (Benedet 2000). The regimens used were however all acceptable practices for the day and were initiated whenever physically practicable to do so (Appendix 4). Despite this, approximately 30% of cases did not receive chemotherapy and comparing the sub-stage distribution of cases not receiving chemotherapy with the limited data available in the literature regarding this; 57% of stage I cases in our series (48% literature), 16% of stage II (10%), 17% stage III (10%) and 27% (14%) received no chemotherapy (Heintz, Odicino et al. 2001). This suggests a general tendency toward under-treatment rather than any over representation of stage I cancers in this series (low risk stage Ia and Ib cancers routinely being treated by surgery alone). As the database did not contain detailed information concerning the medical management of these cases it is not possible to know precisely why they did not receive treatment, though institutional issues and concerns surrounding performance status would be possible explanations. Conversely, as the comparison data is multi-centred, international and from a slightly later time period an apparent under-treatment could also be artifactual. It is regrettable that an objective prospective record of medical treatment had not been assembled as this information would have allowed more detailed marker associations to be studied. An attempt was made to retrieve this data for the patients that had been enrolled into the ICON studies but the MRC steering committee could not release this data. Consideration was given to the retrospective collection of this data but the resources to locate and extract the data accurately were unavailable.

Ovarian cancer treatment has evolved so as to preclude the study of the non-use of chemotherapy in advanced disease, though evidence

from observational work and studies on early epithelial ovarian cancer support the axiom that chemotherapy slows disease progression, prolongs survival and palliates symptoms and that it is not curative on its own and may not reduce overall mortality (Heintz, Odicino et al. 2001) (Trimbos, Parmar et al. 2003). A univariate association between the use of adjuvant treatment and improved overall survival would therefore be expected, as has been seen by other workers (Lassus, Leminen et al. 2003; Rosen, Yang et al. 2006), some of who have found the use of chemotherapy to have independent prognostic power (Heintz, Odicino et al. 2001; Psyrri, Kountourakis et al. 2007). Counter-intuitively, the use of chemotherapy initially seemed to predict a poor prognosis in this series. However, this is likely to be due to the inclusion of stage I cases in the first univariate analysis, as most of these cases did not receive adjuvant treatment because they already had an excellent prognosis - in variance to more advanced cases who have a better prognosis because they receive it. The subsequent analysis of stage II-IV cases supports this assertion as the use of adjuvant treatment improved overall survival in a statistically significant way, in this group which was expected to benefit most from it. A multivariate analysis was performed, including other independent factors, using broad groupings (received chemotherapy or did not receive chemotherapy) and a more specific adjuvant therapy category. Both groupings showed the use of adjuvant therapy to independently predict a better survival. Though intuitively this would be ascribed to the effect of the drugs themselves, a lack of large randomised controlled trials comparing surgery alone with combined medical and surgical treatment in advanced ovarian cancer raises the possibility that it was the decision to initiate chemotherapy that was independently prognostic rather than any biological actions. The lack of objective data concerning performance status in this series makes it impossible to know whether such selection bias into the group receiving adjuvant therapy on the basis of fitness was occurring. However whatever the mechanism, from a practical point of view it was important to include the use of adjuvant treatment as a factor in subsequent multivariate analyses and it was

used in the same grouping that has been shown to be independently prognostic in observational studies – “received chemotherapy / did not receive chemotherapy” (Heintz, Odicino et al. 2001; Psyrri, Kountourakis et al. 2007). Overall, the data within the database concerning adjuvant treatment appeared consistent, almost 100% complete but limited in terms of objectively documented response to treatment.

The fact that a case has been included in an ICON trial was an indicator of randomised treatment allocation and acceptable performance status but was otherwise of limited use as it was not possible to link the database to MRC held outcome data. The case which was randomised in to ICON I but diagnosed many years before the start of this trial was an early stage cancer incidentally discovered after following an oophrectomy where subsequent full pelvic clearance was not undertaken and where the initial decision to not give chemotherapy was subsequently reversed. Though this is unusual, interestingly it did not contravene the study protocol.

4.2.5. Survival

4.2.5.1. Results

Date of operation

Dates of operation ranged appropriately within the recruitment phase from *.2.82 - *.10.97. This data equated to the date of initiation of treatment and as the follow up period spanned two millennia, they were transformed manually into DD/MM/YYYY format in order to facilitate the accurate electronic calculation of OS. Concurrently, the entries were checked to ensure that the dates of surgery were logical; correlating with year of diagnosis and being earlier than the latest survival data. Data were 100% complete.

Year of diagnosis

Year of diagnosis data were contemporaneously extracted from the pathology reports in order to act as an external check for the operation

date. The Inaccuracies which occasionally occurred were largely due to keying errors but all discrepancies were explored and then satisfactorily resolved. The number of cases treated varied from year to year within an acceptable range with a trend toward treating more cases developing over time (Figure 4.16). Data were 100% complete.

Date of last follow up

Patients were followed up according to a local protocol, three monthly for two years, 6 monthly for 5 years and thereafter annually and indefinitely with the patient seeing the consultant or nurse specialist at alternating visits and with an USS being performed every six months for the first 5 years and a serum CA125 and CEA being sent at every visit. The date of latest follow up was recorded in the database and ranged from *.05.82 - *.08. 05; which included clinic visits and in-patient deaths. The data were recoded into DD/MM/YYYY format to facilitate calculation of latest known survival data. The data were manually checked to ensure that no dates in this attribute preceded the year and date of diagnosis, the few errors discovered being attributable to keying errors introduced during the manual transformation of the date format. Data were available for 336 cases (99% complete).

Date of death

Data concerning date of death were also available for 99% of cases and ranged from *.05.1982 - *.04.2004. The data were transformed into a DD/MM/YYYY format to facilitate subsequent calculations. Using this data, patient survival status was coded 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 dates of death were checked to ensue that they were later than the dates of diagnosis, with only keying errors discovered.

Figure 4.16 **Histogram showing the number of cases presenting in each year of the recruitment phase of the study**
The year to year variation in workload is shown.

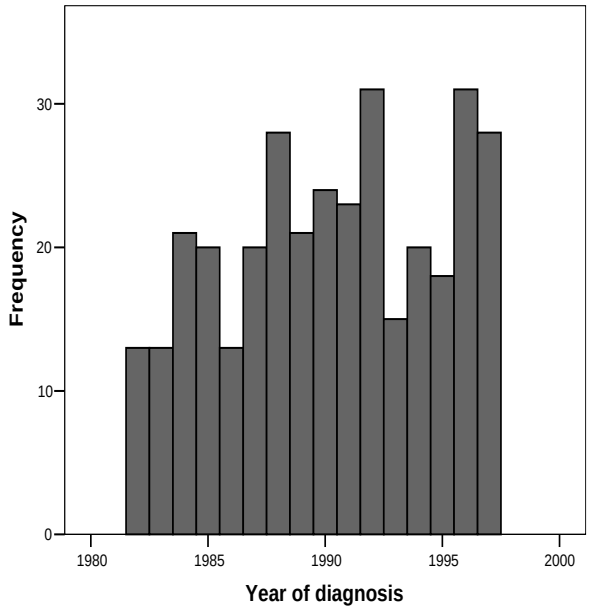
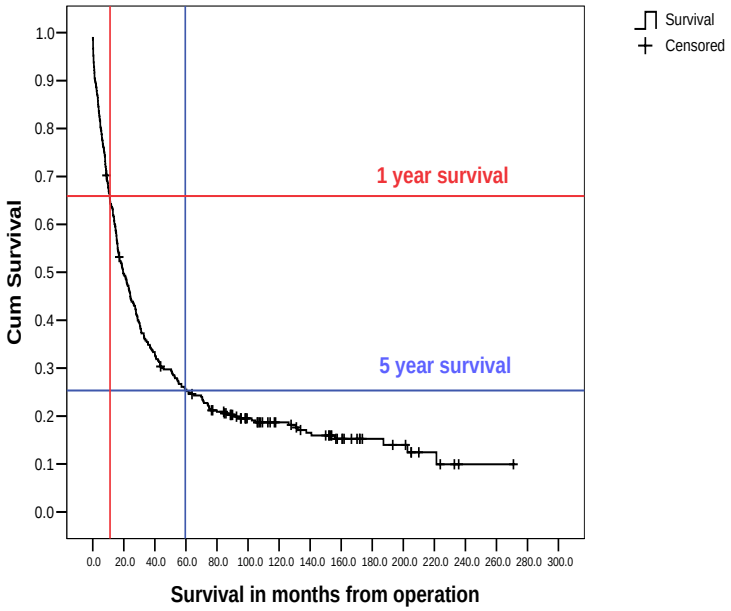


Figure 4.17 **A Kaplan-Meier curve showing raw survival data for the series**
Overall survival at 1-year (65%) and at 5-years (25%) is indicated.



Survival

The data concerning date of diagnosis, latest follow-up, date of death and survival status were used to generate crude OS data up to the time of locking the database along with 1-year survival and 5-year survival rates. There were three instances of missing data (99% complete). 24 patients (7%) died peri-operatively 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; S.E 5.1) and median OS of 19.5 months (95% CI 15.5 – 23.5; S.E 2.1). The crude one year overall survival was 63.7% and the corresponding five year measurement 25% (Figure 4.17). Though graphically later year of diagnosis appeared to be associated with improved peri-operative (Figure 4.18a), 1- year (Figure 4.18b) and 5-year OS (Figure 4.18c), no statistically significant differences were seen. 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 4.6).

4.2.5.2. Discussion

The intensity of follow up was similar to that recommended by FIGO and NIH and its indefinite nature on an annual basis was understandable in a time when changes to first line treatment were occurring regularly. 68% of all recurrences in cases of advanced ovarian cancer documented within the 24th annual report compiled by FIGO occurred within the first 2 years of treatment with the relative survival at the 4th and 5th year of follow up being almost the same for most stages (Heintz, Odicino et al. 2001).This data lends support to decreasing the intensity of review following the first 2 years and to the argument that risk of death from ovarian cancer returns to background after 5 years. However, few clinicians would discharge patients with treated advanced ovarian cancer this soon after treatment and as FIGO is not prescriptive on this point the schedule is often individualised. The routine use of USS in an asymptomatic follow up population without

Figure 4.18a **Scatterplot showing the variation of deaths within 30 days of surgery by year of surgery**
There was no statistical association between the two (17.7; p=0.28).

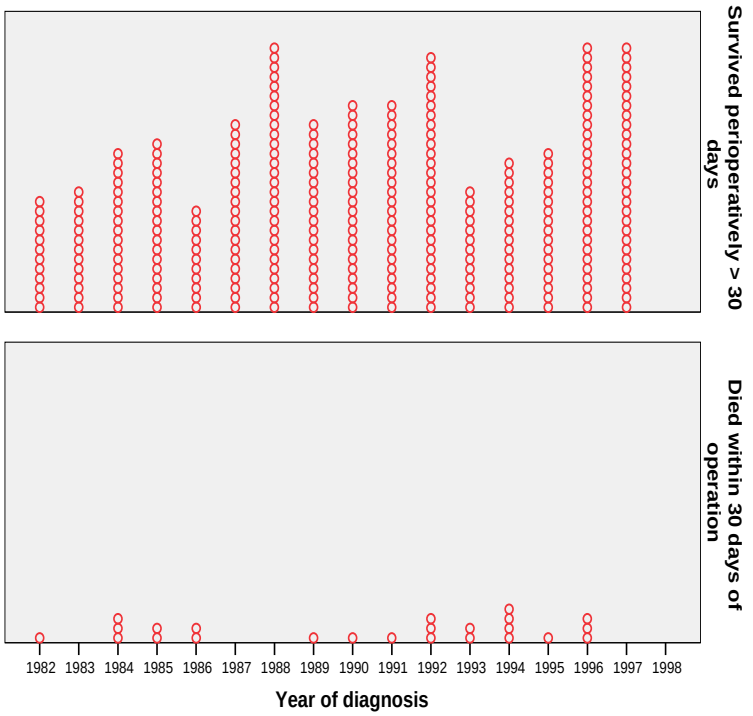


Figure 4.18b **Scatterplot showing the variation of 1 year survival by year of surgery**
There was no statistical association between the two (11.47;p=0.72).

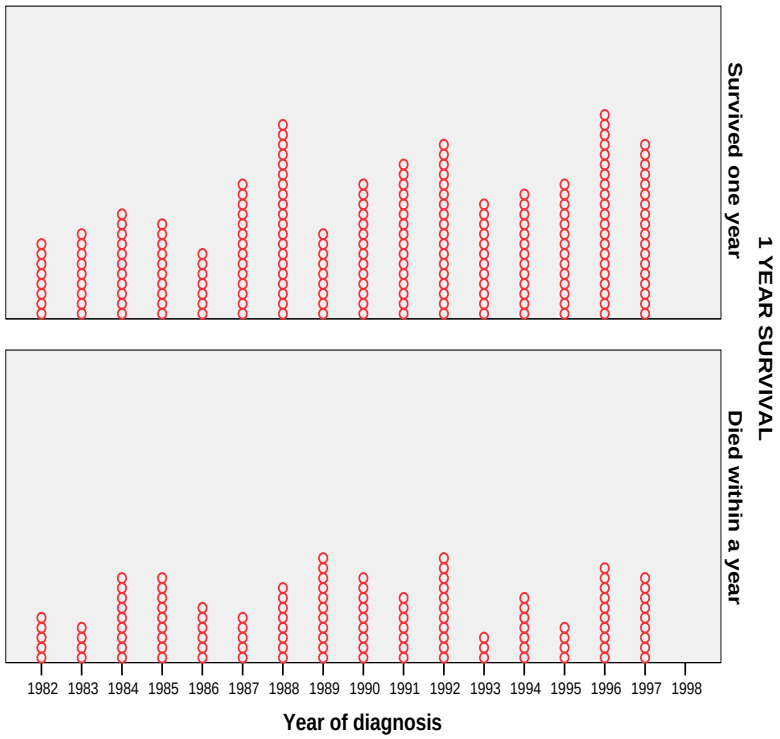
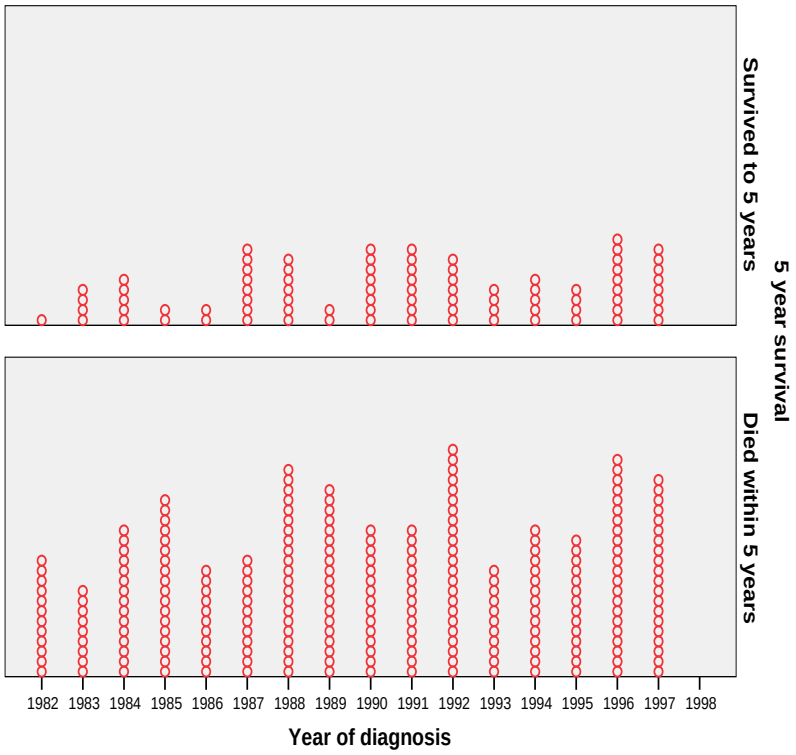


Figure 4.18c **Scatterplot showing the variation in five year survival by year of diagnosis**
There was no statistical association between the two (13.57; p=0.56)



other evidence of recurrence is controversial and would not recommended by most authorities, though it may have a specific role in the follow up of early and borderline cases where fertility sparing surgery has been performed (1995; Benedet 2000; Zanetta, Rota et al. 2001; Scottish Intercollegiate Guidelines Network. 2003). However, with an indefinite time span to the long term follow up, clinical examination by a consultant specialist would not have been possible for all patients in this series at all visits and the use of this highly sensitive (though likely less specific) modality may have been used as a catch all.

The data concerning date of death were complete but the occasional inaccuracies reinforce the advantages of double keying entries ; which has been shown to reduce the number of transcription errors from approximately 50 / 10 000 keystrokes to 15 / 10 000 (Reynolds-Haertle and McBride 1992; Blumenstein 1993; Horbar and Leahy 1995).

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, but the case fatality rate appears higher than expected given the oft quoted fatality rate of approximately 2/3 (Jolles 1985; Nagle, Bain et al. 2006). Large long term studies reporting fatality rates at time periods in excess of 5 years within this study's time frame are few but one American study estimates the fatality rate for the 38 012 patients diagnosed between 1973 and 1997 to be 56%; even lower than 2/3, and reflecting the historically lower mortality of the disease in the U.S (Nagle, Bain et al. 2006). One explanation for this series high fatality rate might be if it was untypical in terms of its clinicopathological make up, though there was no evidence that it differed significantly from other series in terms of the distribution of age, stage and residual disease (independently prognostic factors). However, the tendency toward under-treatment with chemotherapy could have resulted in a failure to prevent more deaths during this period; although such under treatment might have been expected to influence length of survival rather than mortality itself. A further explanation might be that a larger proportion of cases were

dying from non-cancer related causes than appreciated. Checking this could only be done by scrutinizing death certificates. This would have presented ethical, financial and (as TCR could not provide the data) logistical obstacles which would have been surmountable only if the accuracy of the data acquired offset them and if the data were crucial. It was permissible for the cohort to have a high fatality rate and still be valid and consistent for use in assessing novel expression, as long as the overall survival data behaved normally, reproducing known associations with clinicopathological factors with accepted independent prognostic factors being consistently identified. As has been shown earlier in this chapter, such associations were frequently reproduced – validating both the clinicopathological and survival data. Therefore it was decided to accept that this series had a relatively high fatality rate compared to other series and the complex attempt to correct for potential non-cause specific deaths, which were likely to be few in number, was not undertaken.

The OS data were complete with only positive figures being generated, confirming that no systematic errors had been incurred. In addition they consistently produced valid associations with other clinicopathological factors, identifying the anticipated independent prognostic factors correctly. However, the proportion of deaths within 30 days of operation is slightly higher, though of the same order of magnitude, as seen in the few other studies which have reported this (Jalêuvka 1980; Elit, Bondy et al. 2002; Ben-Ami, Vaknin et al. 2006). This variation could theoretically have been caused by inferior peri-operative care though if this was the cause then improvement peri-operative death rates would have been expected over time as medical care advanced, which was not seen within this series. This variation may therefore be more convincingly explained by heterogeneity between the studies' cohorts. As there were no instances encountered in the literature where peri-operative deaths had been corrected for in the marker expression survival analyses, the OS data were used unadjusted. As expected, OS decreased with increasing stage but the overall all stage 5- year OS of

25% was poor despite a typical distribution of cases among the stages. Reviewing contemporaneous data from Eurocare 3, the observed European average 5- year survival around this time period was 34%. The 1- year OS statistics, which are often taken as a measure of diagnosis-treatment delay, are similar at 63% for this series against the European average of 65% and this suggests that superior treatments in continental Europe account for the difference at 5-years. Reviewing survival on a stage level; 5- year survival for Stage I cancers is reported in the literature as 90% but in this series only 60% survived to this point along with 38% of stage II cases (42-70% in the literature), 10% for stage III (21 – 59% in the literature) and 5% for stage IV cancers (6-25% in the literature) (Hand, Fremgen et al. 1993; Yancik 1993; Ilekis, Gariti et al. 1997; Wolfe, Tilling et al. 1997; Brun, Feyler et al. 2000; Holschneider and Berek 2000; Heintz, Odicino et al. 2001; Quirk and Natarajan 2005). The width of these ranges is in part because they are a mixture of relative and crude five year OS, with crude OS data usually returning a lower value than its relative equivalent (as deaths from other causes are not included). However, this series' crude figures are even lower than the bottom of these ranges; as was seen with the fatality data. 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, delay in diagnosis). Intriguingly, these five year OS data are similar to those collated for the 19th FIGO Annual Report on the Results of Treatment in Gynecologic Cancer for the decade preceding the one from which these patients were recruited (1973-1975) (Heintz, Odicino et al. 2001). This again suggests that the cancer service needed to be improved; which was recognised nationally at the time.

4.3. Auditing of data

The accuracy of the data kept on the database was assessed through three audit processes, the details of which are outlined in (Figure 4.19).

4.3.1. Results

Study database - audit 1

Once the irregularities had been explained (Figure 4.19), clean data were collected from medical records of the non-excluded cases. The original audit tool (Figure 4.20) allowed the most crucial data to be collected as it appeared in the study database for comparison to this clean data. Of the 168 fields assessed for the 21 cases, 23 were not reliably verifiable through inspecting the medical record. Missing data within the study database for this sample was found in 7 individual instances (96% complete. Of the 138 fields that could be assessed for correctness (168 minus the data fields that were missing or for which clean data were unavailable), 2 were found to be incorrect. One concerned the degree of extent of cytoreduction, one concerned the adjuvant treatment and both were significant inaccuracies. Assuming the data in the database which could not be verified from the clinical record was also incorrect, 81% of data were definitely correct in 96% of the entries. The outcome data, survival and death, were 100% accurate (Table 4.13).

Analysis database – audit 2

A new audit tool was required to assess the data within the analysis database as it had been extensively modified and this was created by refining the original version (Figure 4.21). Six of the cases had medical records which were inaccessible. Of the 98 fields assessed within the remaining 14 cases, 6 were not reliably verifiable from clean data. No instances of missing data occurred within the analysis database for these cases (100% complete). 5 of the assessable 92 fields were incorrect and assuming data which was non-verifiable was also incorrect, the cases assessed were 89% correct overall in a dataset which was 100% complete. The outcome data was 100% correct in the 13 cases with verifiable data equating to a rate of correctness of 93% if unverifiable data is considered inaccurate (Table 4.13). There was no evidence that the database columns had become misaligned.

Figure 4.19 **Auditing of the database** Three assessments of the accuracy of the database were performed at various stages during its evolution. During the first assessment, 21 of the 54 cases selected from the original study database for audit had their important fields analysed using an audit tool appropriate for that database. 33 of the 54 took no part in this audit (many cases selected for this review were those likely to be unsuitable for inclusion into the study). Following the production of the analysis database containing restructured clinicopathological data, a second audit was performed using an appropriately refined version of the original audit tool. The clean data from the first audit was then re-categorised following the same principals used to generate the analysis database from the study database and the original 21 cases were re-audited using the refined audit tool. A third audit combined the data from both sample sets to increase the numbers analysed while simultaneously screening for systematic errors that may have occurred during the various database manipulations that had occurred.

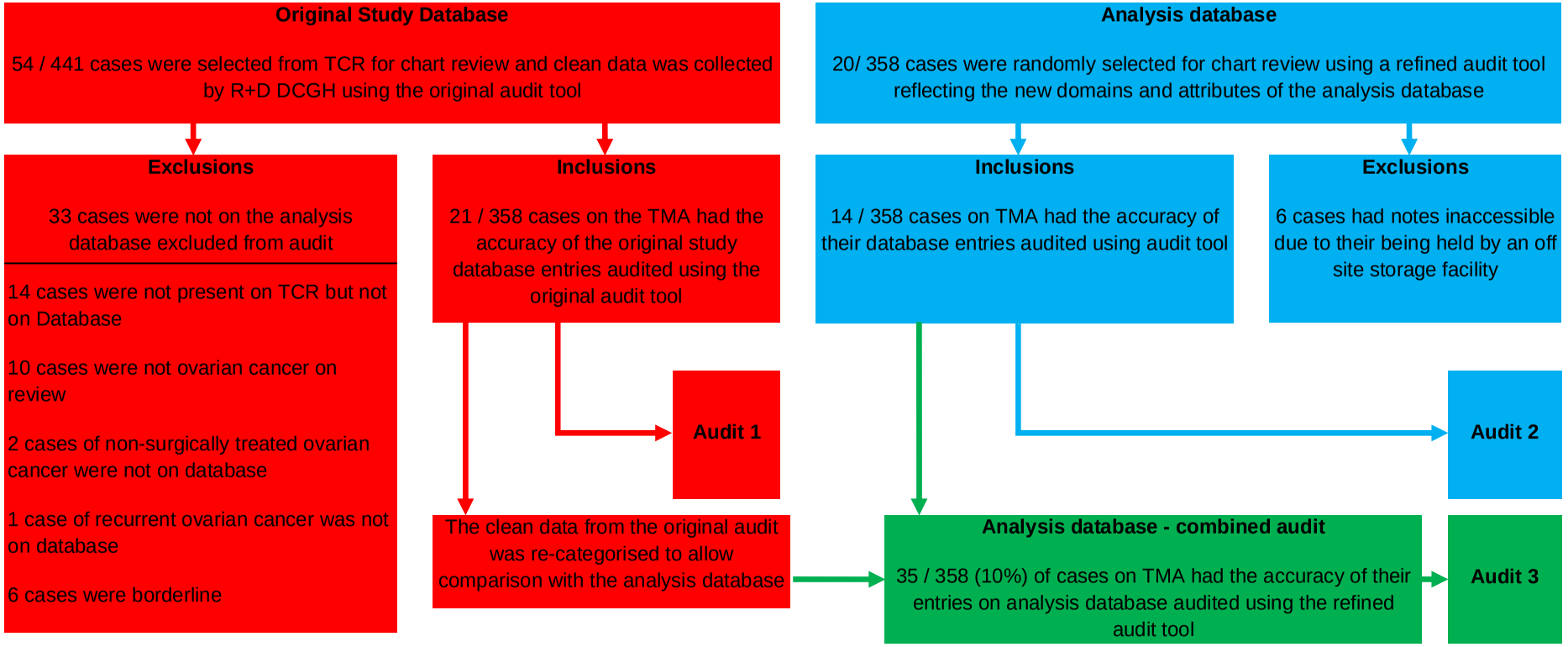


Figure 4.20 The original audit tool

This was used extract clean data from the clinical records (medical record and PAS) which was compared to the study database record. The database was compared to the Trent Cancer Registry (TCR) and the case notes of ovarian cancer cases not on the database reviewed. Cases on the database that did not appear to be ovarian cancer were also reviewed. A randomly selected sample of other cases was identified and in total 54 of a possible 441 sets of notes was inspected. This audit was performed by an R+D officer independently of the investigative team.

Study Number						Agreement with information on database
Date of surgery	Date	Missing				Y / N
Age at surgery	Years	Missing				Y / N
Type of tumour	Serous	Mucinous	Endometrioid	other	Missing	Y / N
Grade	Well	Moderate	Poorly	Undifferentiated	Missing	Y / N
Stage of tumour	Stage 1	Stage 2	Stage 3	Stage 4	Missing	Y / N
Sub-stage of tumour	a b c	a b c	a b c	Missing		Y / N
Residual	None	< 2cm	> 2cm	Missing		Y / N
Treatment	None	Platinum	Carboplatin	Non-platinum	Missing	Y / N
Follow up	Deceased	Date of Death	Missing			Y / N
	Alive	Date Last Seen	Missing			Y / N

Table 4.13 The audit results

Noted are the instances where clean data could not be clearly found within the clinical record, data was missing from the database or where data was frankly inaccurate. The final column represents a worst case scenario where data in the database for which clean data could not be extracted is assumed inaccurate. There were no changes in the data extracted from the database for the 21 cases examined pre-and post-restructuring from study to analysis database.

	Number of fields n=	Clean data unavailable n= (%)	Missing database data n= (%)	Inaccurate data n= (%)	Definitely complete and correct data n= (%)
			Audit 1		
Date of surgery	21	0	0	0	21 (100)
Age at surgery	21	0	0	0	21 (100)
Stage of tumour	21	7	2	0	12 (57)
Sub-stage of tumour	21	8	5	0	8 (38)
Residual	21	7	0	1	13 (62)
Adjuvant Treatment	21	1	0	1	19 (90)
Date last seen	21	0	0	0	21 (100)
Date of death	21	0	0	0	21 (100)
Total	**168**	**23 / 168 (6)**	**7 /168 (4)**	**2 / 138 (2)**	**136 / 168 (81)**
			Audit 2		
Date of diagnosis	14	0	0	0	14 (100)
Age at diagnosis	14	0	0	2	12 (86)
Stage of tumour	14	3	0	0	11 (79)
Residual	14	3	0	0	11 (79)
Adjuvant treatment	14	0	0	1	13 (93)
Date last seen	14	0	0	1	13 (93)
Date of death	14	0	0	1	13 (93)
Total	**98**	**6**	**0**	**5 / 92 (5)**	**92 / 98 (89)**
			Audit 3		
Date of diagnosis	35	0	0	0	35 (100)
Age at diagnosis	35	0	0	2	33 (94)
Stage of tumour	35	10	2	0	23 (66)
Residual	35	10	0	1	24 (69)
Adjuvant treatment	35	1	0	2	32 (91)
Date last seen	35	0	0	1	34 (97)
Date of death	35	0	0	1	34 (97)
Total	**245**	**21**	**2**	**7 / 222 (3)**	**218 / 245 (89)**

Figure 4.21 The refined audit tool

In response to the initial audit of the study database at DCGH, it was updated; missing fields were populated and data were rearranged into analytically useful categories - producing the analysis database. Subsequently a further audit of 20 randomly selected cases from the 358 cases on the TMA was performed by P.R using the audit tool bellow. The clean data collected were then used to assess the levels of correctness and completeness and thereby the accuracy of the analysis database.

Array Number						Agreement with information on database
Date of Diagnosis	Date	Missing				Y / N
Age at Diagnosis	Years	Missing				Y / N
Stage of tumour	Stage 1	Stage 2	Stage 3	Stage 4	Missing	Y / N
Residual	No macroscopic disease	Macroscopic disease present	Missing			Y / N
Treatment	No adjuvant chemotherapy	Adjuvant chemotherapy given	Missing			Y / N
Follow up	Deceased	Date of Death	Missing			Y / N
	Alive	Date Last Seen	Missing			Y / N

Analysis database – audit 3

Pooling the data, 35 cases and 245 individual items of data were assessed using this refined audit tool. 21 pieces of individual data could not be verified due to a lack of clean data. 2 items of data (<1%) were missing. 7 items of data were definitely incorrect, with an overall rate of incorrectness of 89% if non-verifiable data are assumed to be inaccurate in a 99% complete sample of the analysis database. Applying the same standard, the outcome data were 97% correct. There was no evidence that the database columns had become misaligned.

4.3.2. Discussion

Though little specific evidence exists in the literature concerning the assessment of a retrospective database, guidelines have been suggested by one group. They suggest that data be extracted from the clinical records using a CRF by independent, specifically trained paramedical staff. They suggest that 20% of the cases are checked for 100% of the most important data and that 100% of cases have 20% of their data checked. They arbitrarily suggest that with regards to clinicopathological data >80% should be correctly recorded and that 95% of clinical outcomes should be correct (Jansen, van Aalst-Cohen et al. 2005). In terms of accuracy of the records it is also important to set the correctness of entries in context by knowing the level of completeness of the database; an empty database being 100% correct (Hogan and Wagner 1997). Hogan and co-workers also suggest that data accuracy should be over 80% and that unbiased sample should be assessed by blinded auditors.

At the point that the initial audit of the study database was performed, the main investigators were not allowed access to the clinical records or the database. Therefore the audit was performed by the R+D team and was designed to meet the study's governance requirements. It had dual aims – to investigate apparent irregularities in the database (e.g. where the type of tumour entered was not ovarian cancer) and to assess the accuracy of a random sample of notes relating to genuine primary

ovarian cancer cases. The strength of this first audit was that it was performed by a sufficiently qualified paramedical person who was independent of the investigative team, who had received some training as to how to interpret the medical notes from an ovarian cancer point of view. However, the original audit cases selected cannot be said to be an unbiased sample of the whole as many were investigated because of a suspicion that they should not be on the database at all. As a result, many of these cases became irrelevant and were excluded; leaving a small sample of 21 cases for assessment. Ideally the audit would have been performed on a larger (20%) random sample of cases more likely to be included in the study. 23 items of data on the database could not be verified from the medical record and this may have been due to the relative unfamiliarity of the worker with ovarian cancer cases, illegibility of the notes, loss of parts of the paper record, accurate information having been obtained from an unknown source or inappropriate entries in database. Assuming that unverifiable entries were the same as frankly inaccurate entries, the data were over 80% correct in a highly complete sample. The data collected concerning histology and grade were not analysed as the wholesale review and updating of these attributes was being undertaken. The outcome data were above the 95% recommended, being 100% accurate. As these results reflect a worst case scenario (with some of the unverifiable entries likely to be correct), it provided initial reassurance about the accuracy of the database, albeit in a small sample. In addition, the inconsistencies between the database and TCR had been explained by the end of the process.

The second assessment also had dual aims – to assess the accuracy of a random set of cases from the newly generated analysis database and to screen for any systematic errors whereby columns of data had become misaligned during their rearrangement. This audit used a randomly selected sample of cases, revealing a problem with the logistics of auditing the database using the clinical notes as a gold standard. As the time span of the study was so long, many of the

patient's original notes had been sent to off-site storage if they were alive or disposed of if the patient had died. The limited accessibility of notes therefore introduced bias, limited sample size and increased the resources required to perform the audit. A sample size of 20 had been selected to complement the 21 cases already audited but 6 of these cases had to be subsequently excluded due to lack of the original clinical record. Of these 14 cases 6 fields could not be verified which was the same proportion seen in the first audit and suggested that this was not particular to the operator. There were no missing fields in this audit, which suggests that the general update of the missing data that had occurred during the production of the analysis data base had been a success. Overall the clinicopathological data met a >80% standard of accuracy though the outcome data were slightly bellow a 95% standard (93%) due to one entry's data being unverifiable. However, overall this audit provided reassurance concerning the integrity of the data following the considerable transformations that had occurred between versions.

The main aim of the third audit was to use the clean data from audit 1 to re-audit the analysis database and to pool this data with that from audit 2 to assess an increased sample size. A second aim was to screen once more for major errors introduced by erroneous sorting of the data. The final audit's sample size was smaller than the 20% hoped for and approximately 10% of cases from the whole series were assessed. The level of inaccuracy seen in the third audit within the clean data from the first audit (all inaccuracies discovered during these audits being corrected as a final step before locking the analysis database) fell, reflecting a reduction in the contribution of inaccuracies from retired data attributes such as sub-stage. This audit confirmed a level of correctness for clinicopathological and outcome data above the 80% and 95% standards. Reassuringly the multiple rearrangements of the data were achieved without introducing any errors within the alignment of the database columns. Though individually a patient's electronic record should be as close to 100% accurate as possible, for a study

designed to survey large populations of tumours this level of accuracy was a strong supporter of the consistency of the data.

4.4. General discussion

The final analysis database contained both prospective and retrospectively collected data. Though the prospective collection of data is widely felt to be superior to its retrospective collection in terms of quality the latter technique is increasing, as new techniques such as TMAs seek to make use of archived biological material. Reassuringly, results as accurate as prospectively executed work may be obtained if sufficient quality controls are in place (Concato, Shah et al. 2000). The database anonymisation protocol aimed to unlink the data so that subsequent attempts to re-identify patients using reasonable methods would be unsuccessful. Though obvious patient identifying fields such as surname were removed from the database, combinations of seemingly anonymous data could conceivably still be used by knowledgeable or malicious users to re-identify patients. If the data was to be anonymised to the standard required for general access, it would require encrypting, transforming into a query only database and protecting by mathematical techniques that prevent queries being run that can return patient identifiable combinations of data (Chiang, Hsu et al. 2003; Malin 2007). As this was not feasible, the decision was made to physically restrict access to the database and the final SPSS database master copy was transferred to Professor Durant's personal computer for day to day use in the laboratory. It is unlikely that anyone could re-identify patients without a combination of malicious intent, particular clinical knowledge and multi-site privileges. In addition, the chances of harm resulting from the unauthorised re-linking of these data are slight, as many of the patients are deceased and much of the newly generated data is not be useful on the individual patient level as it is derived from TMA studies.

The main aim of this chapter has been to report how the data were handled and to provide evidence for its consistency and substantiate the final analysis database and multivariate model that would be used

to investigate the prognostic effect of novel markers. The data crucial to these analyses are shown in Table 4.14. The data were over 98% complete and met the required standards of correctness at audit. The data were re-categorised in valid ways and used in that form reproduced known associations. Age, FIGO stage, residual disease and the use of chemotherapy were all independent prognostic factors; as predicted by the literature. Grade and histological subtype were important prognostic factors but without independence of effects, again as expected from the literature. Compared with other series, this cohort had a slightly higher median age, a higher number of G3 cases, was probably under treated with platinum based chemotherapy had a poor overall survival and was limited by having no objective response to chemotherapy data included. However the data was consistent, being correct, complete and valid, and suited to the investigation of the prognostic effect of molecular markers.

In the previous chapter it was shown that the TMA would produce valid expression data and in this chapter it has been shown that the database was valid. In the following chapter the integration of the two to produce meaningful associations will be evaluated.

Table 4.14 The essential data in the final analysis database
This data were used for analysing the prognostic power of novel markers in univariate (UV) and multivariate (MV) analysis. The factors used in the multivariate model were all independent prognostic factors. The statistically essential data are emboldened. From a total possible number of fields of 1356 within these four factors, 33 pieces of data were missing. The essential data in the analysis database was 98% complete.

Data	Column	Data type	Categories	Missing Data
Identifier	ANO	Continuous	1-360	0
Age	AGEDIAGNOSED **(MV)**	Continuous	24 - 90	1
	age - recode-SEER **(UV)**	Ordinal	1:< 30 years 2: 30-59 3: ≥ 60 years	1
Stage	FIGO Stage **(UV) (MV)**	Ordinal	1 :Stage I 2: Stage II 3: Stage III 4: Stage IV	9
Residual	residual-recode-MACRO **(UV) (MV)**	Ordinal	0: Residual disease 1: Residual disease	13
Adjuvant treatment	adjuvant-recode-ANYCHEMO **(UV) (MV)**	Ordinal	0: No chemotherapy 1: Chemotherapy	7
Outcome	SURVIVALMONTHS	Continuous	0-271	3
	DEAD	Categorical	0: Alive 1: Dead	3
Grade	TUMOURGRADE **(UV)**	Ordinal	0: Ungradeable 1: Grade 1 2: Grade 2 3: Grade 3	0
Histological Type	tumourtype-recode-BESTWORST **(UV)**	Ordinal	1: Clear cell 2: Mucinous 3: Endometrioid 4: Serous 5: Undifferentiated 6: other	0

Chapter 5: Evaluating the Integrated Tissue Microarray

In the two preceding chapters the ability of the TMA to generate reliable expression data and the consistency of the data in the analysis database were confirmed. In this chapter the integration of these two elements is assessed.

The successful integration of TMA and database and the validity of using this as a tool to investigate the prognostic power of novel markers would be confirmed if it were able to reproduce the prognostic associations between molecular markers and outcome that other high quality research has identified. The ideal single molecular marker to validate the TMA in this way would be an accepted independently prognostic marker that has been confirmed as such by separate groups and which is prognostic in a multivariate model including all accepted independently prognostic clinicopathological factors. Though a plethora of individual studies has reported the independent prognostic power of immunohistochemically detected molecular markers, including CD24, kallikrein 9 and Actinin 4, none has yet met the criteria for being accepted into clinical or research practice, though CycloOXygenase2 (COX2) and oestrogen/progesterone receptors have recently begun to emerge as promising candidates (Yousef, Kyriakopoulou et al. 2001; Kristiansen, Denkert et al. 2002; Hogdall, Christensen et al. 2007; Steffensen, Waldstrom et al. 2007; Yamamoto, Tsuda et al. 2007). This lack of translation into the clinic setting has generally been due to a difficulty in consistently replicating results and the use of multivariate models by researchers subsequently considered too incomplete to determine the independence of effects (Agarwal and Kaye 2005). As a single gold-standard molecular marker could not be identified against which to validate the TMA, the prognostic properties of two biomarkers, which have at least been widely studied and about which much is

known both within and without ovarian cancer, were investigated instead: p53 and Bcl-2.

5.1. Introduction

5.1.1. Immunohistochemical staining for p53

p53 is the 53KDa gene product of the gene of the same name located on the short arm of chromosome 17 (17p.13) which was first discovered and described over 30 years ago (Linzer and Levine 1979; Lane 1992). With the discovery that loss of heterozygosity within chromosome 17 was associated with the development of lung (Takahashi, Nau et al. 1989), colonic (Baker, Fearon et al. 1989), breast (Cropp, Lidereau et al. 1990) and ovarian cancer (Russell, Hickey et al. 1990; Sato, Saito et al. 1991), researchers began to investigate a tumour suppressor role for the p53 gene and its product. This was subsequently confirmed by a massive body of work which followed. It is now known that mutations within the p53 tumour suppressor oncogene are present in the majority of cancers and mostly lie within a highly conserved region between exons 5 and 8 and are mainly of a missense type (Greenblatt, Bennett et al. 1994).

The normal, “wild type” p53 (WTp53) exists in relatively small amounts within the cell and degrades rapidly under the control of the murine double minute 2 gene (MDM2) (Jones, Roe et al. 1995). Under circumstances of cellular stress, such as hypoxia or exposure to cytotoxic drugs like cisplatin, levels of p53 increase, leading to cell cycle arrest and subsequent DNA repair or apoptosis via a variety of downstream mechanisms designed to maintain the overall stability of the genome (Ferreira, Tolis et al. 1999). However, missense mutations in the p53 gene lead to the production of a mutant form of p53 which, as well as being functionally inactive, is stable enough to accumulate within the nucleus where it can be visualised using standard immunohistochemical techniques (Iggo, Gatter et al. 1990). The most commonly employed monoclonal anti-p53 antibody is the DO-7 clone, which stains both mutant and WTp53. Though tumours staining positive

with DO-7 may be expressing either form of p53, strong nuclear staining is widely regarded as indicating the presence of the inactive mutant form, as WTp53 is so rapidly degraded that its expression is unlikely to be reliably observed using immunohistochemistry (Pillai, Roberts et al. 2003). Tumour kinetics are dependent on the balance between cell proliferation and cell death – which within cancer systems may largely be due to apoptosis; a mechanism distinct from necrosis which is the final common pathway of action of many cytotoxic drugs (White 1996). As mutant p53 cannot trigger apoptosis, tumours expressing strong nuclear staining for p53 on immunohistochemistry should theoretically be more aggressive. This reasoning is supported by considering mutant p53's other functions which include the induction of vascular endothelial growth factor (Kieser, Weich et al. 1994) and macrophage colony stimulating factor (Asschert, Vellenga et al. 1997) – both of which are implicated in carcinogenesis.

The mutant pattern of immunohistochemical expression of p53 has subsequently been shown to be associated with poorer outcome in a variety of cancers, including ovarian cancer, where it has been shown to display both prognostic (regarding OS) (Hartmann, Podratz et al. 1994; Henriksen, Wilander et al. 1995; Klemi, Pylkkanen et al. 1995; Eltabbakh, Belinson et al. 1997; Geisler, Geisler et al. 1997; Anttila, Ji et al. 1999; Crijns, Duiker et al. 2006) and predictive (regarding response to chemotherapy) power (Anttila, Ji et al. 1999; Ferrandina, Fagotti et al. 1999; Levesque, Katsaros et al. 2000; Reles, Wen et al. 2001). Furthermore, occasional studies have shown this prognostic power to be retained following multivariate analysis (Klemi, Pylkkanen et al. 1995; Geisler, Geisler et al. 1997)**.** However, these associations have not invariably been established and some studies have failed to discover such prognostic (Marks, Davidoff et al. 1991; Sheridan, Silcocks et al. 1994; Allan, Campbell et al. 1996; Rhei, Bogomolniy et al. 1998; Levesque, Katsaros et al. 2000) and predictive powers (Silvestrini, Daidone et al. 1998; Fallows, Price et al. 2001; Kupryjanczyk, Szymanska et al. 2003).

Almost all immunohistochemical studies on mutant p53 in ovarian cancer have been performed on whole sections, though at least two have now been performed using tissue microarrays. The earlier study, by Lassus et al, included 500 sequential cases of serous ovarian cancer arranged on a tissue microarray and stained for p53 using the most commonly employed techniques and the DO-7 antibody. The authors felt that there were three distinct types of staining. There was weak diffuse staining similar to that seen in normal tissue, strong nuclear staining and tumours which were entirely negative for p53. They concluded that tumours staining completely negative for p53 and those staining strongly for p53 represented an abnormally functioning p53 gene and they grouped them together in a category termed “aberrant” p53. Tumours with >20% of cells or <50%of cells staining positive for p53 were considered to be expressing WTp53 and were considered a separate, normal entity. Using these criteria, aberrant p53 expression was found to be associated with reduced OS following multivariate analysis (Lassus, Leminen et al. 2003). The second study by the van der Zee group combined populations from two centres and stained 5 copies of an array containing 476 patients using the DO-7 antibody at a low concentration, 1:2000. This team set their cut-off at 50%, due to the presence of weak background staining in their control tissues (presumed to indicate WTp53). They found no associations with OS and were unable to replicate the Lassus study’s findings (de Graeff, Hall et al. 2006).

In the current study the TMA was stained with the most commonly employed technique. The DO-7 antibody was selected and nuclear staining in ≥ 10% of cells within a core defined positivity for mutant p53 with other staining assumed to indicate the presence of WTp53 (Kerns, Jordan et al. 1992; Baas, Mulder et al. 1994; Kmet, Cook et al. 2003). After the staining data had been acquired, the serous tumours were considered separately and the staining data were re-categorised using the cut-offs suggested by Lassus and co-workers to define aberrant p53 expression.

The hypothesis was that the expression data from this TMA would reproduce the clinicopathological associations published by other groups.

5.1.2. Immunohistochemical staining for Bcl-2

Bcl-2 is an oncoprotein encoded by the gene of the same name which is located on chromosome 18 and which was first described in the context of B-Cell Lymphoid tumours over 20 years ago (Cleary, Smith et al. 1986). The gene encodes not one but a family of proteins, within the so-called Bcl-2 family, which regulate apoptosis. Some are pro-apoptotic (Bax, Bak, BAD) (Oltvai, Milliman et al. 1993; Farrow, White et al. 1995) while others are anti-apoptotic (Bcl-2, Bcl-x) (Hockenbery, Nunez et al. 1990) (Vaux, Cory et al. 1988; Boise, Gonzalez-Garcia et al. 1993). Bcl-2 itself prevents apoptotic stimuli from orchestrating the release of cytochrome c from mitochondria and thereafter the downstream activation of capsases – the irreversible final common pathway of apoptosis which leads to the demolition of the cell (Reed 1997; Youle and Strasser 2008). In non-lymphoid tissues, the expression of Bcl-2 is generally restricted to tissues such as neurones (post-mitotic long lived cells), skin (long lived stem cells) or breast (glandular tissue under hormonal control regarding involution) with Bcl-2 not generally being seen within normal ovarian tissue (Hockenbery, Zutter et al. 1991).

Bcl-2 expression has, however, been widely studied in ovarian cancer with respect to its prognostic and predictive properties and may be visualised immunohistochemically in between 20% and 50% of ovarian cancers, depending on the antibody employed and the specific methods employed to stain and evaluate tissue sections (Baekelandt, Kristensen et al. 1999; Mano, Kikuchi et al. 1999; Kupryjanczyk, Szymanska et al. 2003; Eltabbakh, Mount et al. 2004; Lee, Park et al. 2005). Being a protein involved in antagonising apoptosis, Bcl-2 expression might be expected to be associated with ovarian cancers that proliferate aggressively, poorer survival and greater resistance to chemotherapy. While this has occasionally been shown to be the case

(Mano, Kikuchi et al. 1999), Bcl-2 expression has more often been shown to either have no effect on response to chemotherapy and survival (Marone, Scambia et al. 1998; Silvestrini, Daidone et al. 1998; Geisler, Geisler et al. 2000; Kupryjanczyk, Szymanska et al. 2003) or have a counterintuitively positive effect on them (Henriksen, Wilander et al. 1995; Herod, Eliopoulos et al. 1996; Marx, Binder et al. 1997; Baekelandt, Kristensen et al. 1999; Diebold 2003; Ferlini, Raspaglio et al. 2003)

In the current study the TMA was stained for Bcl-2 using standard techniques and reagents to investigate whether it would produce associations between clinicopathological and expression data which were concordant with those in the literature. Cases with an average of >10% of cells staining positive for Bcl-2 in the cytoplasm were considered positive for Bcl-2 expression, a cut-off used by other workers (Nezhat, Cohen et al. 2002; Lee, Park et al. 2005).

5.1.3. Analysis of the combined p53/Bcl-2 immunophenotype

In breast and colorectal cancer it has been shown that co-expression of markers may reveal independently prognostic information that is not apparent on examination of the individual markers themselves. In breast cancer, the p53 (+) Bcl-2 (-) immunophenotype represents a state independently prognostic of poor prognosis (Rolland, Spendlove et al. 2007). In colorectal cancer, the p53(-) Bcl-2(+) immunophenotype was found to be independently prognostic of good prognosis (Watson, Madjd et al. 2005). Therefore, in this study the data generated in 5.2 and 5.3 are combined and the prognostic power of the four possible p53 (+/-) Bcl-2 (+/-) immunophenotypes is investigated with respect to the hypothesis that a p53 (+) Bcl-2 (-) immunophenotype would be associated with an aggressive phenotype.

The expression data were combined to generate immunophenotypes and Kaplan-Meier plots were produced for each. The immunophenotypes were subsequently regrouped into two groups on the basis of their prognostic behaviour – relatively good or relatively

poor – and these groups were then used to investigate univariate associations with clinicopathological factors and survival and to test for independence of effects.

5.2. Results

5.2.1. Immunohistochemical staining of p53

The location of staining was as expected, within the nucleus (Figure 5.1a, b) with negative controls not staining (Figure 5.1c) (Kerns, Jordan et al. 1992). Concordance approached 100%. The median percentage of cells expressed was <5% (Figure 5.1d). Of the 339 cases of ovarian cancer included in the study, 306 had p53 expression data available for analysis, indicating a rate of core loss of 10% (Figure 5.2). Of the cases analysed, 44% were p53 positive as defined by >10% of cells staining positive per case (Table 5.1). There were no obvious major differences between the clinicopathological characteristics of the whole series of 395 cases and the analysed group of 306 (Table 5.2). On univariate analysis using the χ^2 method p53 positive status was found to be associated with advancing FIGO stage, sub-optimally debulked cases, higher grade cancers and those cases having a worse histological subtype (Table 5.2). The Kaplan-Meier plot (Figure 5.3) illustrates that p53 positive tumours had a poorer survival. This association with survival was not independent of other clinicopathological parameters on Cox's regression (Table 5.3); though as expected patient age, FIGO stage, degree of cytoreduction and the use of chemotherapy were independently prognostic of OS.

Concerning the sub-analysis of the serous ovarian cancers to facilitate comparison with the results of the Lassus group, 164 cases were available for analysis of which 138 (84%) expressed the aberrant form of p53 (Figure 5.4) (Table 5.4). The Kaplan-Meier plot (Figure 5.5) showed a relationship between aberrant p53 expression and improved survival. This prognostic power was not independent of other clinicopathological factors (Table 5.5) (Lassus, Leminen et al. 2003).

Figure 5.1 p53 expression

Figure 5.1a Example of nuclear p53 staining seen at x 200 magnification on the positive control tissue

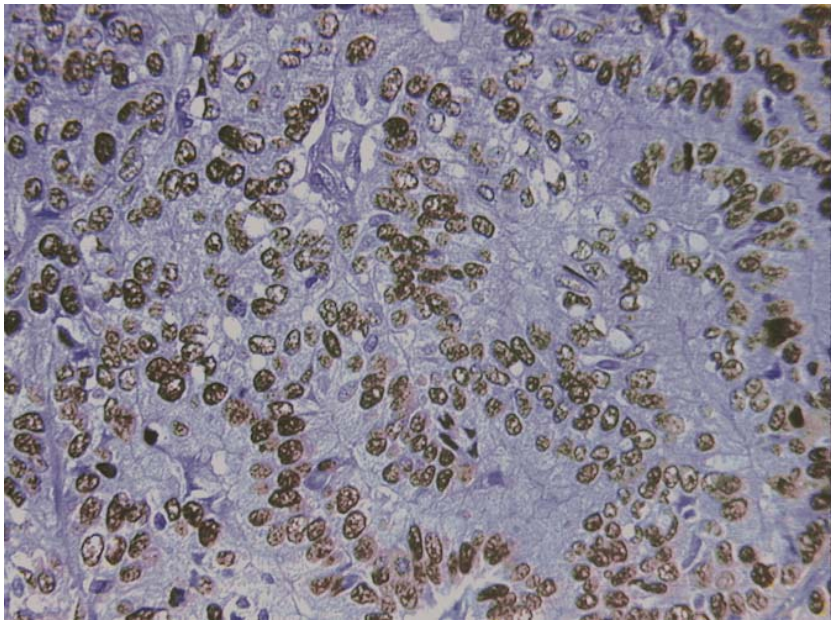


Figure 5.1b Example of nuclear p53 staining seen at x 400 magnification on the positive control tissue

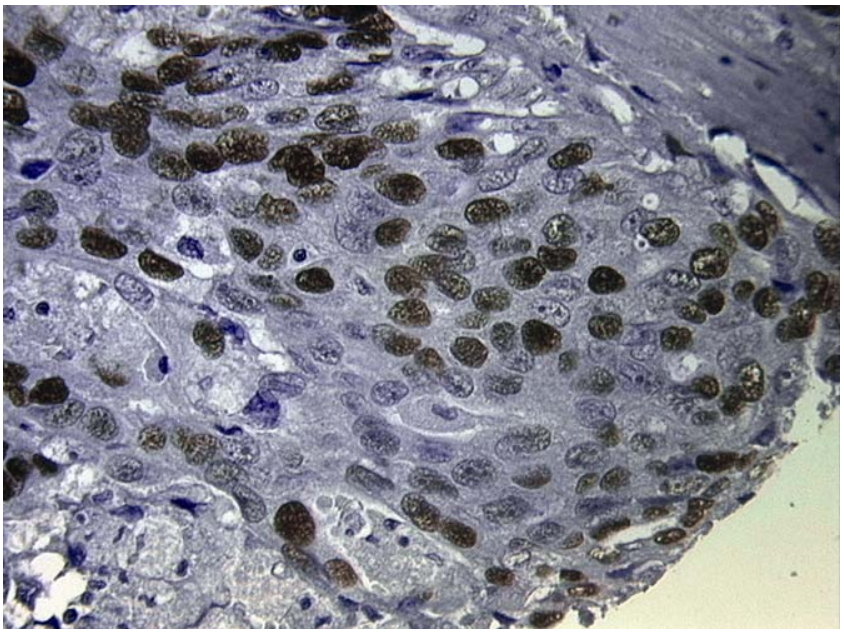


Figure 5.1c The negative control in the p53 staining arm showed no staining – seen at x 200 magnification

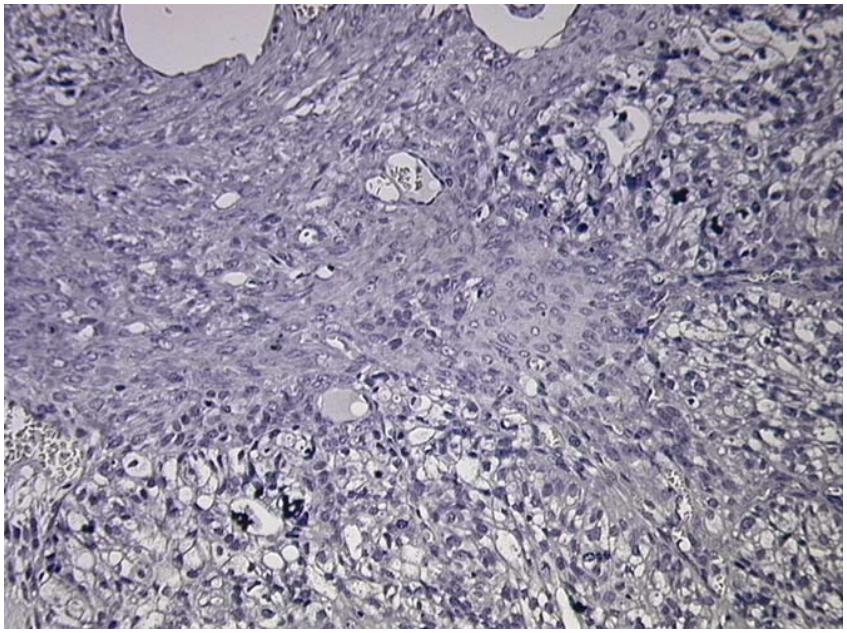


Figure 5.1d Histogram showing the degree of p53 expression within the series

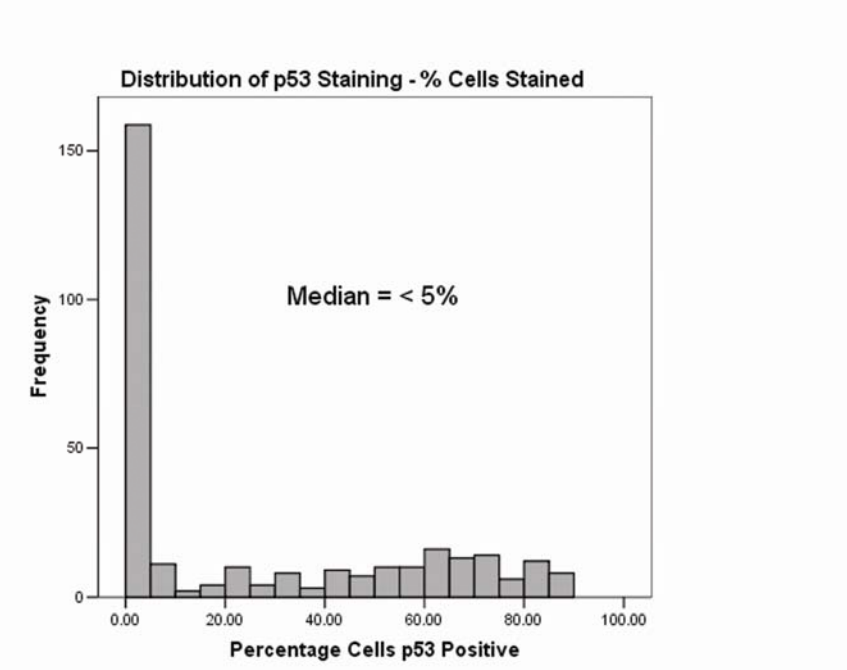


Figure 5.2 Flow diagram showing how a group of 298 tumours with data available concerning p53 and Bcl-2 expression was generated

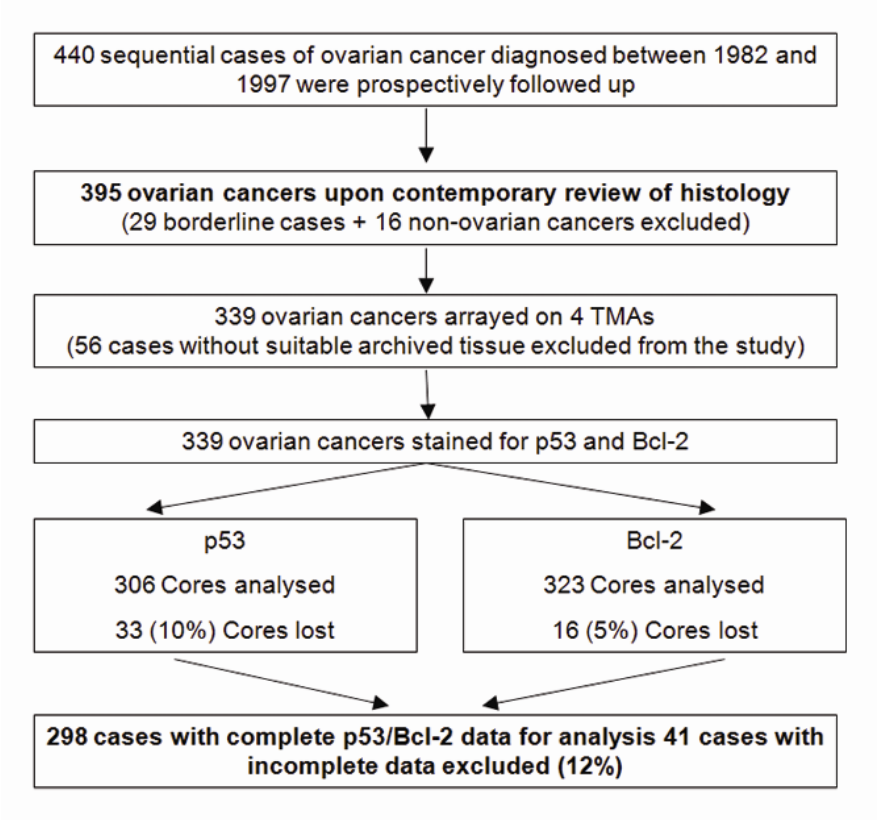


Figure 5.3 Kaplan – Meier curve showing the negative effect of positive p53 expression status on survival

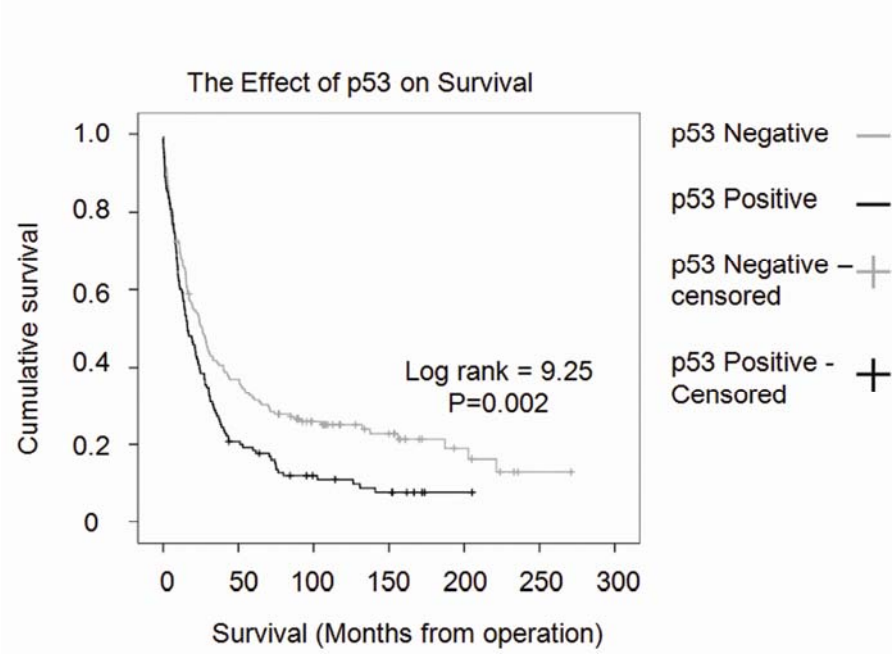


Table 5.1 The distribution of the immunohistochemical expression of p53, Bcl-2 and their combined phenotypes

	n° cases	**% of cases**
p53 % cells staining	n=306	
0-25	190	62
26-50	37	12
51-75	59	19
76-100	20	7
p53 Positive (>10% cells staining)	136	44
Bcl-2 % cells staining	n=323	
0-25	257	80
26-50	37	12
51-75	16	5
76-100	13	4
Bcl-2 Positive (>10% cells positive)	142	44
p53 Bcl-2 Phenotypes	n=298	
Improved prognosis phenotype		
p53(-) Bcl-2 (+)	77	26
Other phenotypes		
p53(-) Bcl-2 (-)	87	29
p53(+) Bcl-2 (+)	82	27
p53(+) Bcl-2 (-)	52	18
Combined other phenotypes	221	74

Table 5.2 The main clinicopathological characteristics of the entire series and those of the tumours expressing p53
A univariate analysis by χ^2 of p53 status vs. clinicopathological criteria is also shown.

	Whole series	**Analysed cases**	**p53 (+)**	**p53 (-)**	**χ^2 (p=)**
	nº cases (%)	nº cases (%)	nº cases (%)	nº cases (%)	
FIGO Stage	n=375	n=297	n=131	n=166	
1	99 (26)	75 (25)	18 (14)	57 (34)	
2	46 (12)	35 (12)	12 (9)	23 (14)	
3	188 (50)	152 (51)	79 (60)	73 (44)	**22.48**
4	42 (11)	35 (12)	22 (17)	13 (8)	**(<0.001)**
Optimally Debulked	n=376	n=294	n=130	n=164	
Yes	157 (42)	119 (41)	32 (25)	87 (53)	**24.33**
No	219 (58)	175 (60)	98 (75)	77 (47)	**(<0.001)**
Grade	n=376	n=304	n=135	n=169	
1	50(13)	34 (10)	10 (7)	24 (14)	
2	93 (25)	66 (22)	25 (19)	41 (24)	**5.99**
3	233 (62)	204 (67)	100 (74)	104 (62)	**(<0.001)**
Histological Type	n=395	n=306	n=136	n=170	
Serous	203 (51)	164 (54)	81 (60)	83 (49)	
Endometrioid	46 (12)	38 (12)	11 (8)	27 (16)	
Mucinous	50 (13)	30 (10)	11 (8)	19 (11)	
Undifferentiated	65 (17)	47 (15)	26 (19)	21 (12)	
Clear cell	26 (7)	23 (8)	4 (3)	19 (11)	**16.64**
Other	5 (1)	4 (1)	3 (2)	1	**(0.001)**

Table 5.3 Multivariate analysis of p53 expression by Cox's regression
p53 status does not predict prognosis independently of other clinicopathological parameters.

	Hazard Ratios (95% Confidence intervals)	**Significance (p)**
Age at diagnosis (years)	1.028 (1.017-1.040)	<0.001
FIGO Stage		
1		<0.001
2	2.754 (1.565 – 4.845)	0.001
3	5.250 (3.149 -8.751)	<0.001
4	6.623 (3.637-12.062)	<0.001
Residual macroscopic disease	2.167 (1.522 – 3.085)	<0.001
Patient received chemotherapy	0.494 (0.342 – 0.713)	<0.001
p53 Positive	0.949 (0.73-1.233)	0.695

Figure 5.4 **Flow diagram illustrating how a group of 164 serous ovarian cancers became available for analysis and the distribution of aberrant p53 staining within that group**

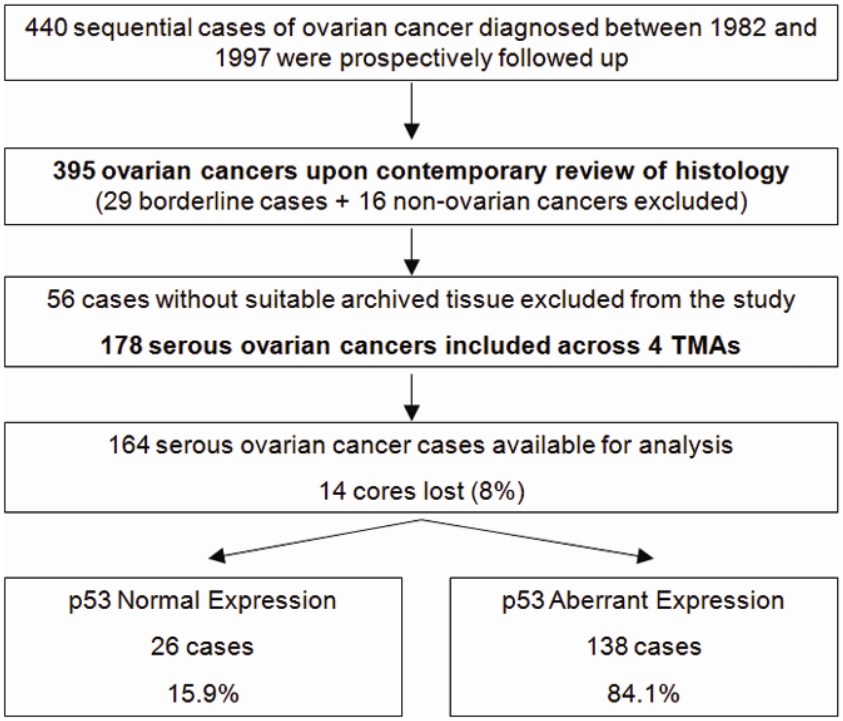


Figure 5.5 **Kaplan-Meier curve showing the positive effect of aberrant p53 expression status on survival**

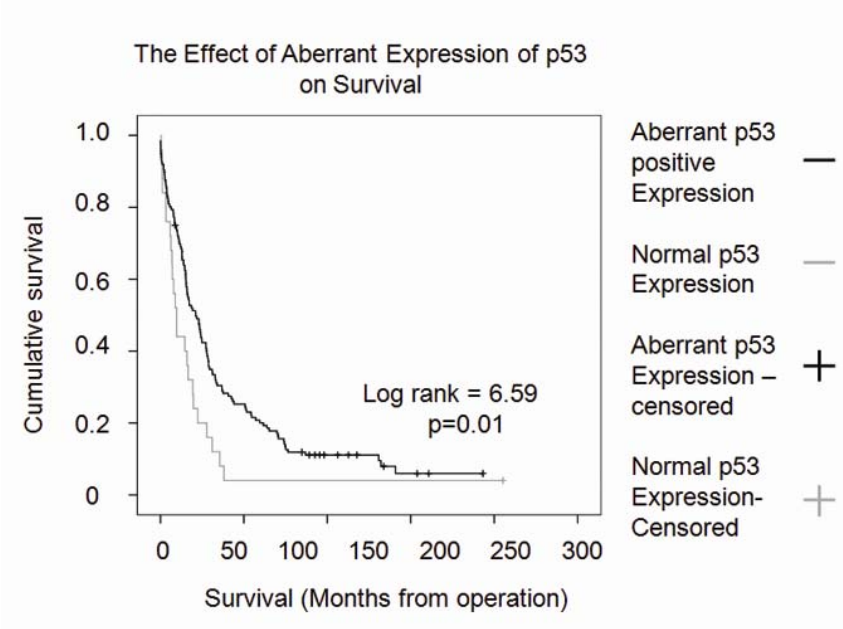


Table 5.4 The distribution of cases into aberrant and normal p53 expression within the serous ovarian cancer subset using the criteria of Lassus et al (Lassus, Leminen et al. 2003)

	nº cases	% of cases
p53 % cells staining	164	
≤ 20 (Negative)	89	54
> 20 ≤ 50 (Normal)	26	16
> 50	49	30
≤ 20 or > 50 (Aberrant p53 expression)	138	84

Table 5.5 Multivariate analysis by Cox's regression of aberrant p53 expression described by Lassus et al (Lassus, Leminen et al. 2003) This classification does not retain independent prognostic significance when analysed in conjunction with other clinicopathological factors in our model.

	Hazard Ratios (95% Confidence intervals)	Significance (p)
Age at diagnosis (years)	1.026 (1.009-1.044)	0.002
FIGO Stage		
1		0.004
2	2.008 (0.864 – 4.664)	0.105
3	2.973 (1.439 – 6.142)	0.003
4	5.102 (2.090 – 12.454)	<0.001
Residual macroscopic disease	2.313 (1.441 – 3.714)	0.002
Patient received chemotherapy	0.321 (0.195 – 0.531)	<0.001
p53 – Aberrant Expression	0.791 (0.500 – 1.252)	0.317

5.2.2. Immunohistochemical staining of Bcl-2

The staining was located as expected, within the endoplasmic reticulum and mitochondrial membrane (Figure 5.6a) (Hockenbery, Nunez et al. 1990). The negative controls did not stain (Figure 5.6b). Concordance was evaluated and found to be in excess of 90%. The median percentage cell staining within tumours was 5% (Figure 5.6c). 323 cases had Bcl-2 expression data available for analysis, indicating a core loss of 5% (Figure 5.2). Of the cases analysed 142 (44%) were Bcl-2 positive (Table 5.1). There were no obvious major differences between the clinicopathological characteristics of the whole series of 394 and the analysed group of 323 (Table 5.6). On univariate analysis, Bcl-2 positive status had no statistically significant associations with clinicopathological parameters (Table 5.6). Though the Kaplan-Meier plot (Figure 5.7) initially appeared to indicate that Bcl-2 status stratified the cases into separate prognostic groups, there was no statistically significant difference found and so a multivariate analysis was not performed.

5.2.3. Analysis of the combined p53/Bcl-2 immunophenotype

298 cases possessed the data concerning p53 and Bcl-2 expression required to be included in the co-analysis (Figure 5.2). There were no concerns that this was an unrepresentative sample of the entire series on comparing clinicopathological data (Table 5.7). There was no association between the expression of p53 and the expression of Bcl-2 (1.93; P=0.159). The cases were numerically evenly distributed between the four possible immunophenotypes (Table 5.1) and the Kaplan-Meier curves (Figure 5.8) demonstrated that these groupings were prognostic, with three of the immunophenotypes performing similarly with respect to prognosis while a fourth (the p53 (-) Bcl-2 (+) phenotype) behaved differently and predicted a better prognosis (Figure 5.9). On univariate analysis the possession of this better prognostic phenotype was not associated with patient age, FIGO stage, histological subtype or the use of chemotherapy. However, it was associated with a lower grade of disease and an increased likelihood of

Figure 5.6 **The expression of Bcl-2**

Figure 5.6a **Example of a tissue microarray core staining positive for Bcl-2 seen at x 200 magnification**

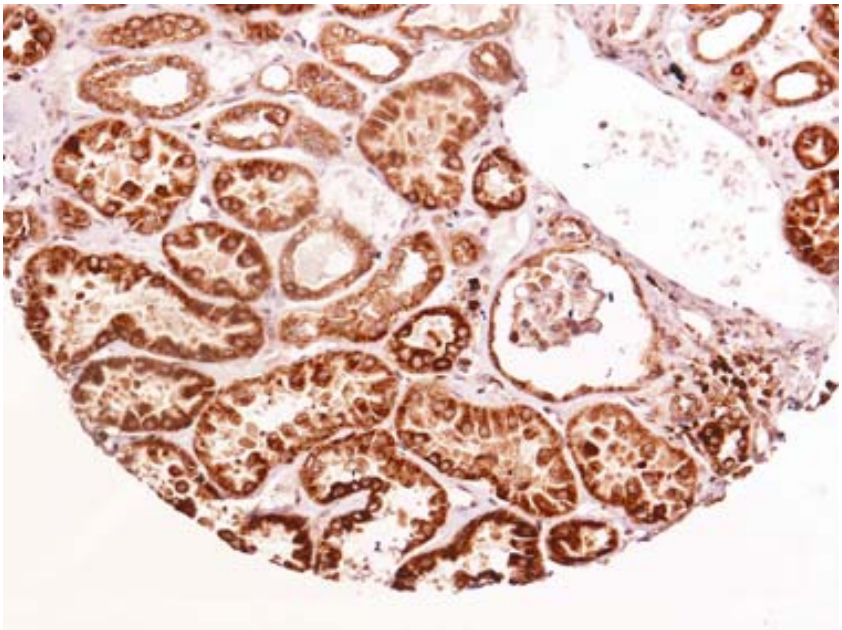


Figure 5.6b **The tonsillar tissue used as a negative control in the Bcl-2 arm did not stain – seen at x 200 magnification**

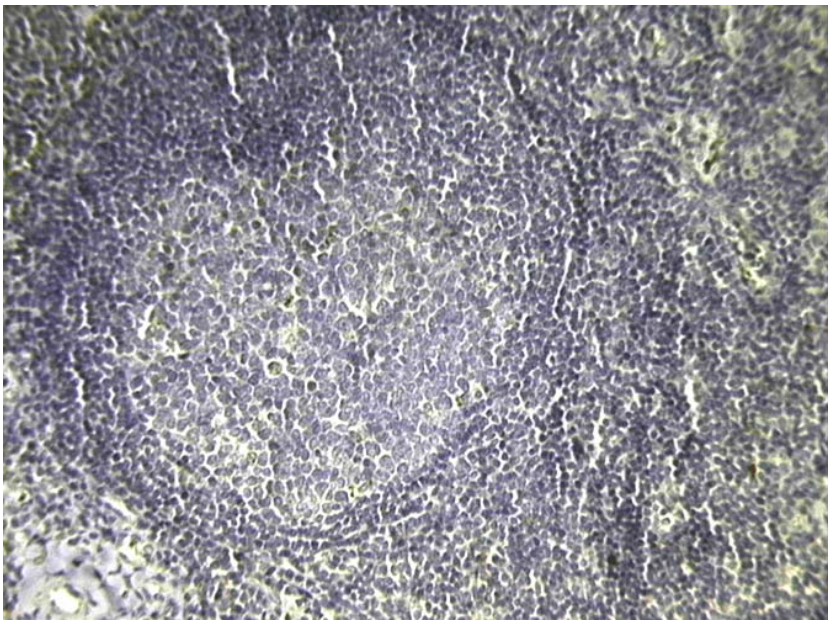


Figure 5.6c **Histogram showing the range of percentage of cells staining with the Bcl-2 antibody within the cases and the median value**

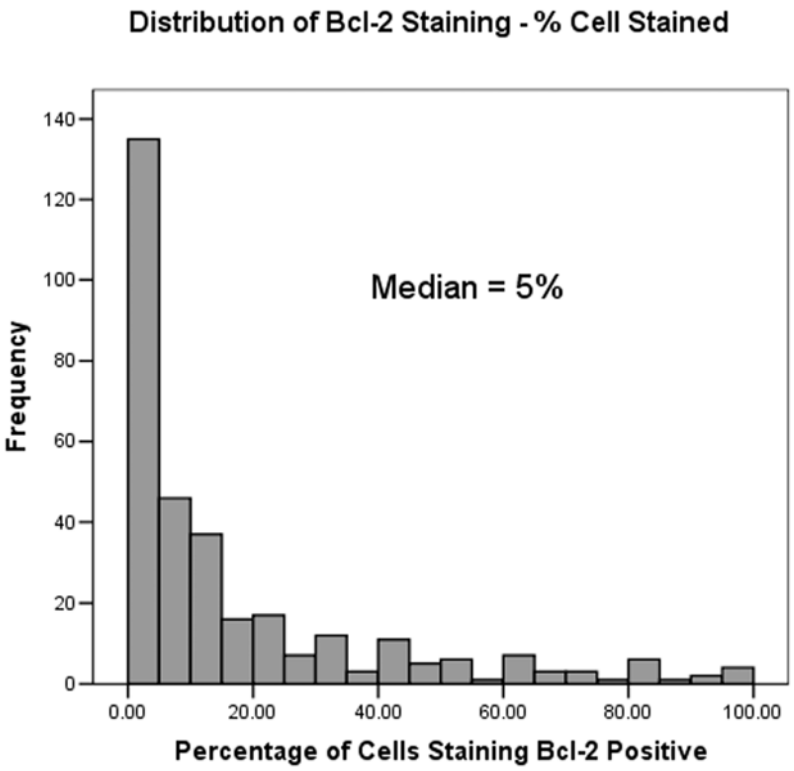


Table 5.6 **The main clinicopathological characteristics of the entire series and those of the tumours expressing Bcl-2**
Univariate analysis by χ^2 of Bcl-2 status vs. clinicopathological criteria is also shown.

	Whole series	**Analysed cases**	**Bcl-2 Positive**	**Bcl-2 Negative**	**χ^2 (p=)**
	n° cases (%)	n° cases (%)	n° cases (%)	n° cases (%)	
Age	n=394	n=322	n=142	n=180	
< 30	1	1	1	0	
30 – 59	167 (42)	130 (40)	54 (38)	76 (42)	1.78
>60	226 (57)	191(59)	87 (61)	104 (58)	(0.41)
FIGO Stage	n=375	n=314	n=140	n=174	
1	99 (26)	81 (26)	38 (27)	43 (25)	
2	46 (12)	34 (11)	17 (12)	17 (10)	
3	188 (50)	162 (52)	71 (51)	91 (52)	1.30
4	42 (11)	37 (12)	14 (10)	23 (13)	(0.73)
Optimally Debulked	n=376	n=311	n=136	n=175	
Yes	157 (42)	125 (40)	59 (43)	66 (38)	1.02
No	219 (58)	186 (60)	77 (57)	109 (62)	(0.31)
Chemotherapy	n=388	n=316	n=140	n=176	
Yes	283 (73)	228 (72)	99 (71)	129 (73)	
No	104 (27)	88 (28)	41 (29)	47 (27)	0.26
Platinum	195 (50)	160 (51)	70 (50)	90 (51)	(0.61)

Figure 5.7 Kaplan-Meier curve showing the lack of association between Bcl-2 expression and survival

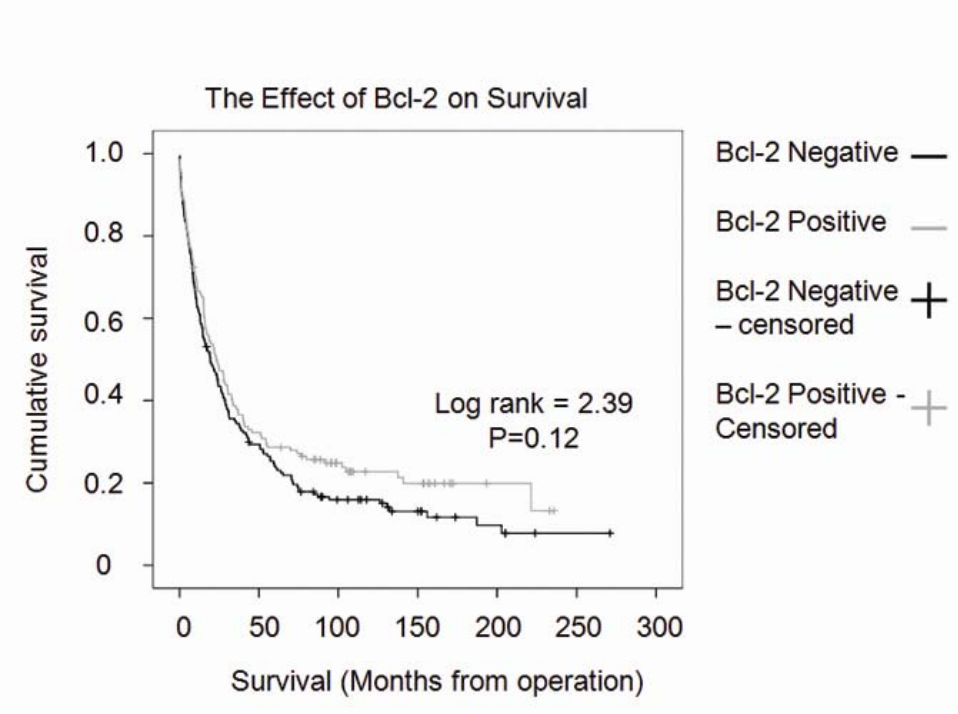


Table 5.7 The main clinicopathological characteristics of the entire series and those of the tumours expressing the p53(-) Bcl-2 (+) phenotype
A univariate analysis by χ^2 of this phenotype status vs. clinicopathological criteria is also shown.

	Whole series	**Analysed cases**	**Other Phenotypes**	**p53 (-) Bcl-2 (+)**	**χ^2 (p=)**
	nº cases (%)	nº cases (%)	nº cases (%)	nº cases (%)	
FIGO Stage	n=375	n=289	n=213	n=76	
1	99 (26)	74 (26)	47 (22)	27 (36)	
2	46 (12)	34 (12)	24 (11)	10 (13)	
3	188 (50)	148 (51)	114 (54)	34 (45)	7.09
4	42 (11)	33 (11)	28 (13)	5 (7)	(0.07)
Optimally Debulked	n=376	n=286	n=212	n=74	
Yes	157 (42)	117 (41)	77 (36)	40 (54)	**7.14**
No	219 (58)	169 (59)	135 (64)	34 (46)	**(0.008)**
Grade	n=376	n=296	n=219	n=77	
1	50(13)	34 (12)	19 (9)	15 (20)	
2	93 (25)	64 (22)	45 (21)	19 (25)	**8.138**
3	233 (62)	198 (67)	155 (71)	43 (56)	**(0.002)**
Chemotherapy	n=386	n=291	n=215	n=76	
Yes	276 (72)	214 (74)	163 (76)	51 (67)	
No	110 (29)	77 (26)	52 (24)	25 (33)	2.19
Platinum	195 (50)	149 (51)	114 (50)	35 (46)	(0.14)

Figure 5.8 Kaplan-Meier curve showing the differential effect on survival of the four different p53 (+/-) Bcl-2 (+/-) immunophenotypes
The p53 (-) Bcl-2 (+) phenotype was associated with an improved prognosis.

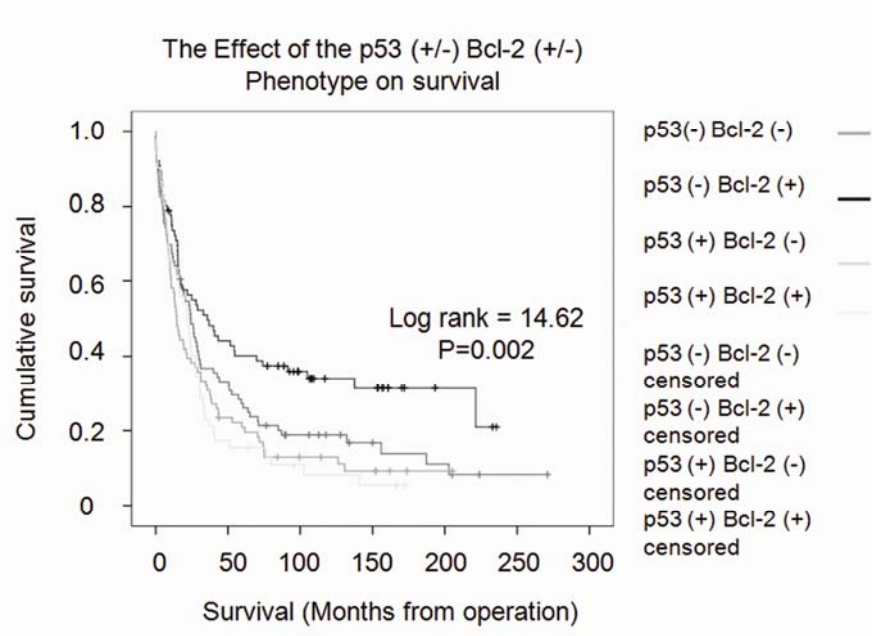
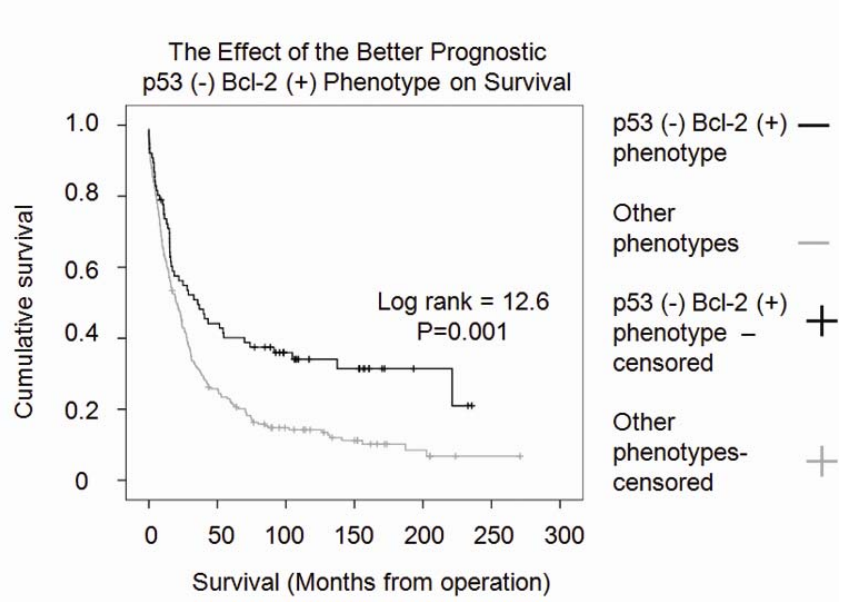


Figure 5.9 Kaplan-Meier curve showing the association of a p53 (-) Bcl-2 (+) immunophenotype with improved survival



optimal cytoreduction at operation (Table 5.7). The association with improved survival found on univariate analysis was not maintained when looking for the independence of effects using Cox's proportional hazards model (Table 5.8).

5.3. Discussion

p53

In this study we found that 44% of cases stained positive for p53, which compares well to what has been found in other studies (43-47%) (Kmet, Cook et al. 2003)[Reviewed]. p53 positive tumours were associated with the clinicopathological features of aggressive disease, which has been found by most studies investigating the prognostic significance of the immunohistochemical expression of p53 in ovarian cancer (Marks, Davidoff et al. 1991; Klemi, Pylkkanen et al. 1995; Allan, Campbell et al. 1996; Diebold, Baretton et al. 1996; Eltabbakh, Belinson et al. 1997; Anttila, Ji et al. 1999). p53 was associated with reduced survival in a univariate analysis which agrees with many studies (Hartmann, Podratz et al. 1994; Henriksen, Wilander et al. 1995; Klemi, Pylkkanen et al. 1995; Eltabbakh, Belinson et al. 1997; Geisler, Geisler et al. 1997; Anttila, Ji et al. 1999; Crijns, Duiker et al. 2006) but is at dissonance with others (Marks, Davidoff et al. 1991; Sheridan, Silcocks et al. 1994; Allan, Campbell et al. 1996; Rhei, Bogomolniy et al. 1998; Levesque, Katsaros et al. 2000). p53 did not have independent prognostic power in this series, though this has been found in other work (Klemi, Pylkkanen et al. 1995; Geisler, Geisler et al. 1997). In this multivariate model, chemotherapy was found to be an independent prognostic factor. Though no data were available concerning response to chemotherapy in this series, other workers have found p53 abnormalities to be associated with a poorer response to chemotherapy, as would be biologically plausible (Anttila, Ji et al. 1999; Ferrandina, Fagotti et al. 1999; Levesque, Katsaros et al. 2000; Reles, Wen et al. 2001). An intrinsic link between p53 expression, response to chemotherapy and survival might explain why p53 expression lost its prognostic power on multivariate analysis in this series, though it must

Table 5.8 Multivariate analysis by Cox's regression
The p53 (-) Bcl-2 (+) phenotype, which predicts good prognosis, does not retain independent prognostic power when analysed together with other clinicopathological parameters.

	Hazard Ratios (95% Confidence intervals)	**Significance (p)**
Age at diagnosis (years)	1.028 (1.016-1.041)	<0.001
FIGO Stage		
1		<0.001
2	2.042 (1.150 – 3.628)	0.015
3	3.773 (2.253 – 6.317)	<0.001
4	5.010 (2.700 – 9.297)	<0.001
Residual macroscopic disease	1.886 (1.321 – 2.693)	<0.001
Patient received chemotherapy	0.836 (0.543 – 1.288)	**0.836**
Good prognosis phenotype p53(-) Bcl-2 (+)	0.746 (0.538 – 1.033)	**0.695**

be noted that the ability of p53 to predict response to chemotherapy has not been universally found (Silvestrini, Daidone et al. 1998; Fallows, Price et al. 2001; Kupryjanczyk, Szymanska et al. 2003).

Within the subset of 164 serous ovarian cancers, employing the criteria of Lassus et al, 54% of tumours were defined negative for p53 and 84% expressed aberrant p53. There was a statistically significant association between this aberrant form and survival but it was the reverse of what was found by the Lassus group, as it predicted improved survival and did not persist following multivariate analysis. The effect of dividing our group by Lassus' criteria was to transfer many tumours that were considered p53 positive on the basis of standard criteria, ≥10% of nuclei staining strongly (which were associated with a poorer prognosis in this series), into a "normal" category. This may explain why the polarity of our findings in this analysis is the reverse of the Lassus group. Lassus et al describe a system that may account for the expression of WTp53 detected by DO-7 and, though this makes biological sense, it could be that their criteria for allocating cases were more subtle than a numerical cut-off could describe. Though the findings of the Lassus group could not be reproduced, this is in keeping with the van der Zee group (Crijns, Duiker et al. 2006). This second study's findings were replicated by this work regarding the univariate associations between p53 expression and higher FIGO stage, grade and chance of suboptimal surgery. While they did not demonstrate a similar association with survival, this could explained by their setting of the cut-off for abnormal p53 expression at 50%, which is a high level compared to most previously published studies.

The lack of consistency between the findings of published work might be explained by methodological differences such as the type of tissue used, the antibodies employed, the scoring systems employed, differences between the studied groups and the factors included in the multivariate model (Shahin, Hughes et al. 2000). Another explanation remains: the use of immunohistochemistry as a tool for investigating disruption of p53 function. Although immunohistochemistry has been

shown to have a high correlation with gene sequencing for detecting missense mutations in the p53 gene (Sheridan, Silcocks et al. 1994) the immunohistochemically determined rate of non-functional p53 is lower than the actual rate that would be detected on full sequencing of the gene from exon 2 through 11 looking for null mutations not associated with the production of a stable mutant p53 gene product (Skilling, Sood et al. 1996). While missense mutations make up the majority of p53 mutations amongst ovarian cancers, immunohistochemistry has been shown to have superior prognostic power when sequencing data concerning null mutations are combined with it (Shahin, Hughes et al. 2000). However, with immunohistochemical detection of p53 having quite a high negative predictive value for p53 mutations (81%) (Wang, Helland et al. 2004) researchers have been undeterred and there are currently over 150 citations including (p53; Ovarian cancer; Prognosis) (PubMed 2008), 40 of which were of sufficient quality to be included in a meta-analysis (Crijns, Boezen et al. 2003).

In summary, the TMA was able to reproduce the findings of a significant body of work into the immunohistochemical expression of p53 in ovarian cancer though, due to inconsistency in the literature, doing this inherently contradicted the findings of other work. The state of the current literature highlights the importance of attempting to adhere to as standardised a method as possible when investigating p53 immunohistochemically and following the ReMARK guidelines is to be recommended (McShane, Altman et al. 2006).

Bcl-2

In this study, 44% of tumours stained positively for Bcl-2 which lies within the expected range of 20-50% suggested by other groups' work (Baekelandt, Kristensen et al. 1999; Mano, Kikuchi et al. 1999; Kupryjanczyk, Szymanska et al. 2003; Eltabbakh, Mount et al. 2004; Lee, Park et al. 2005). In line with other studies it was found that Bcl-2 was not associated with any clinicopathological factors. Although this made it more likely that any prognostic power would be independent in

nature, as in these other studies a statistically significant difference in OS was not found (Mano, Kikuchi et al. 1999; Schuyer, van der Burg et al. 2001; Kupryjanczyk, Szymanska et al. 2003). It was, therefore, inappropriate to include Bcl-2 expression into the multivariate model as this may have led to a type I error.

Considering the biology of Bcl-2 in more depth can help explain why no associations were found by this work and that of other groups and can also help explain why when associations between Bcl-2 expression and survival have been found they have indicated a counterintuitive link with better outcome (Henriksen, Wilander et al. 1995; Herod, Eliopoulos et al. 1996; Marx, Binder et al. 1997; Baekelandt, Kristensen et al. 1999; Diebold 2003; Ferlini, Raspaglio et al. 2003). Firstly, Bcl-2 has been shown not only to block apoptosis but to have a function independent of this whereby it can slow the cell cycle at various points, thereby inhibiting proliferation which leads to tumour survival but in an indolent form (Mazel, Burtrum et al. 1996; Vairo, Innes et al. 1996; Lind, Wayne et al. 1999; Vairo, Soos et al. 2000; Belanger, Cote et al. 2005). Secondly, given their ability to form hetero- and homo-dimmers, it is now thought that a more complicated story explains the Bcl-2 family protein's control over apoptosis whereby the relative levels of their members determine which of their individual functions predominate (Youle and Strasser 2008). The ratio of Bcl-2 to Bax is important and it has been shown that Bax triggers cell death more effectively in tumours where Bcl-2 is expressed more highly and that outcome may be improved in these circumstances (Oltvai, Milliman et al. 1993; Marx, Binder et al. 1997; Schuyer, van der Burg et al. 2001). Therefore, considering the co-staining of Bax and Bcl-2 in our series may have yielded more prognostically useful information. Finally, it has been noted that cleavage of a regulatory loop domain of Bcl-2 by capsases can convert it into a Bax-like death effector initiating a self potentiating cascade toward apoptosis. This would occur more readily in environments of relative Bcl-2 abundance (Cheng, Kirsch et al. 1997).

While the findings of the TMA staining with Bcl-2 were unremarkable they were, however, consistent with a large body of immunohistochemical work performed in this area and further support the validity of the findings produced using the integrated TMA.

p53 / Bcl-2 immunophenotypes

The loss of tissue cores in tissue microarray work can be problematic when co-expression data for cases are being analysed. In this study, 10% of cores were lost in the p53 experiment and 5% were lost in the Bcl-2 arm, which were individually within acceptable limits for TMA work (Hoos and Cordon-Cardo 2001; Henshall 2003). However, though a maximum loss of cases of 15% through studying these two markers together did not occur, 12% of cases had incomplete co-expression data which was still at the high end of an acceptable range. Fortunately, despite this loss of data, the power of the study was unaffected (approximately 300 cases were still available) and this tissue loss appeared random in nature with no skewing of the clinicopathological data detected.

The results show that one immunophenotype has a different prognosis to the others. The p53 (-) Bcl-2 (+) expressing tumours have the best prognosis with a 5-year survival, almost double that of the other phenotypes combined (39.5% vs. 21.5%). In this work, no relationship between the expression of p53 and Bcl-2 was found, which is at variance to work in breast and colorectal cancer and one study involving small numbers of ovarian cancers where inverse relationships have been described (Rolland, Spendlove et al. 2006; Watson, Durrant et al. 2006) (Henriksen, Wilander et al. 1995). However, it is in keeping with the majority of work in ovarian cancer where this association has been investigated (Herod, Eliopoulos et al. 1996; Baekelandt, Kristensen et al. 1999; Geisler, Geisler et al. 2000; Schuyer, van der Burg et al. 2001; Kupryjanczyk, Szymanska et al. 2003; Lee, Park et al. 2005). This study's hypothesis, that the p53 (+) Bcl-2 (-) phenotype would lead to particularly aggressive behaviour as seen in breast cancer, remained unsubstantiated. Ovarian cancer appears to have

more in common with colorectal cancer, where the Bcl-2 positive tumours within the p53 negative group also had the best prognosis. This may be understandable, given the occasional clinical mimicry of the two diseases which can sometimes be impossible to resolve. Though the prognostic information provided by co-expression data does not reach independent significance, its univariate association is less likely to have occurred when compared to the prognostic capability of p53 alone ($p=0.01$ vs. $p=0.02$). An additional advantage of considering Bcl-2 expression in combination with p53 data is that this identifies cases with a prognosis better than normal, whereas p53 status alone stratifies cases into two groups of differently poor prognoses. Considering the multivariate analysis, it is interesting to note that the independent prognostic power of chemotherapy use becomes non-independent on the addition of this new prognostic group, implying that their influences on outcome are mechanistically interrelated. This connection is not predicted by a univariate association and so it may be that this immunophenotype has the power to predict response to chemotherapy rather than whether a patient would receive it. However, a lack of data concerning response to chemotherapy in this series makes it impossible to investigate this hypothesis further. These findings concerning the co-expression of p53 and Bcl-2 support previous work in stage III ovarian cancers (Baekelandt, Kristensen et al. 1999). This group also found that p53 expression (but not Bcl-2 expression) was predictive of OS and, in addition, they concluded that the co-expression of these factors was a better predictor of prognosis than the individual markers themselves. They found that 21% of their cases expressed a p53 (-) Bcl-2 (+) phenotype and that this predicted better prognosis independently of other clinicopathological factors. The slightly lower frequency of this immunophenotype compared to that found in the current study (26%) may be due to the addition of all stages (including better prognostic stage I and II cases), which would also explain why this immunophenotype was not independently prognostic in this work. Importantly, the Baekelandt group did not

discover any predictive capabilities for these markers when used alone or in combination with regard to response to chemotherapy.

It is biologically plausible that a combination of differential p53/Bcl-2 expression data should prove synergistic: p53 negative tumours may retain a functional apoptotic pathway and tumours expressing Bcl-2 have a protein present which slows their proliferation, which are both potentially potent anti-tumour processes. However, in addition, WT p53 may trigger apoptosis by upregulating the expression of Bax more effectively in the presence of higher levels of Bcl-2 (Oltvai, Milliman et al. 1993; Miyashita, Kitada et al. 1995; Miyashita and Reed 1995; Jones, Turner et al. 1998) and this functional interdependence due to cross-talk between p53, Bax and Bcl-2 has been established for some time (Eliopoulos, Kerr et al. 1995).

It is, therefore, logical that p53 (-) Bcl-2 (+) tumours should have the best prognosis and, while their response to chemotherapy is also likely to be better, this may be better established by suitably designed prospective work.

5.4. General discussion

The principal aim of this section was to evaluate whether this TMA and database were linked in such a way as to be able to effectively reproduce prognostic information generated in other studies. From this point of view, it is interesting to note that the co-staining data, generated principally to facilitate comparison with published work in breast and colon cancer, produced an unexpected finding which, on subsequent review of the literature, had incidentally almost completely successfully replicated a previous high quality study in ovarian cancer (Baekelandt, Kristensen et al. 1999).

Given the volume of published data concerning these two markers in ovarian cancer and the irresolvable methodological differences between studies, it is only possible to decide what should have been found in this large series of ovarian cancers by observing what has been found by a majority of workers. In this context it is not

controversial to state that p53 is usually associated with aggressive disease and worse survival and is not an independent marker for prognosis in ovarian cancer while Bcl-2 expression is probably not a prognostic marker on its own, though it might have some prognostic influence for improved outcome in p53 negative tumours. Our findings are, therefore, in line with the main body of work in this area. One of the major conclusions of this section is the importance of adopting a consistent approach to the investigation of molecular markers. For that reason, this study has sought to follow practice which would allow its data to be replicated or incorporated into a future meta-analysis (McShane, Altman et al. 2006).

As there was no gold-standard marker that could be employed in order to incontrovertibly prove the effectiveness of the integration of the TMA, this section aimed instead to replicate meaningful data for two commonly studied molecular markers in order to support the use of the TMA in studying novel markers about which little is known. While this has been successful it has also identified interesting avenues for further investigation, particularly regarding the predictive power of p53 (+/-) Bcl-2 (+/-) phenotypes within the clinical setting.

Having confirmed the validity of the TMA in producing expression data, the consistency of the database and the validity of using the two together, the following chapters will describe the expression and prognostic significance of novel immunological molecular markers.

Chapter 6: HLA Class I Antigen Expression is an Independent Prognostic Factor in Ovarian Cancer

6.1. Introduction

Ovarian cancer is the most common gynaecological cancer in the UK and represents the fourth most common cancer site in women. Its low 5-year survival of 29% has altered little over the last 40 years despite advances in treatment (Quinn 2001). Improving our understanding of the molecular events which underlie the divergent outcomes of apparently identical cases through the search for biomarkers which predict prognosis independently of traditional criteria is fundamental to the development and subsequent targeting of novel treatments.

Downregulation of Human Leucocyte Antigen (HLA) class I antigen expression has been documented in a variety of tumours, including ovarian cancer, and is often, though not exclusively, associated with features of aggressive disease and a poorer prognosis (Kabawat, Bast et al. 1983; Stein, Momburg et al. 1988; Concha, Esteban et al. 1991; Levin, Klein et al. 1991; Hicklin, Marincola et al. 1999; Vitale, Pelusi et al. 2005; Norell, Carlsten et al. 2006). HLA class I antigen downregulation as a biomarker has also been shown to have prognostic powers in breast and colon cancer independent of the traditional prognostic markers for these diseases (Madjd, Spendlove et al. 2005; Watson, Ramage et al. 2006). In ovarian cancer the presence of tumour specific cytotoxic T-cells (CTL) within the tumour infiltrating lymphocytes of advanced ovarian cancer suggest an important role for HLA class I antigens (Santin, Bellone et al. 2004). However, while HLA class I antigen downregulation has been shown to predict poor prognosis in aneuploid types (Moore, Fowler et al. 1990), the independence of this prognostic power is as yet unreported in unselected ovarian neoplasms.

The aim of this study was thus to investigate whether HLA class I antigen downregulation predicts prognosis in unselected ovarian cancer, the hypothesis being that this would be associated with poorer

survival. We used established tissue microarray (TMA) techniques and immuno-histochemistry to study 339 sequential cases of ovarian cancer (Kononen, Bubendorf et al. 1998; Hoos, Urist et al. 2001; Rosen, Huang et al. 2004).

The mouse anti-human monoclonal antibody to HLA class I heavy chain (HC-10–Gift: Prof. H Ploegh, Harvard Medical School, USA) has been raised to denatured HLA class I heavy chains freed from the β_2 Microglobulin light chain (β_2-m) element of denatured native HLA class I antigen and preferentially recognises HLA-B and HLA-C on formalin fixed paraffin embedded tissue (Stam, Spits et al. 1986). HC-10 is the single antibody with the widest sensitivity for HLA class I heavy chain types. HLA class I antigens require the presence of β_2-m in order to function and co-staining tumours for β_2-m using a commercial polyclonal rabbit anti-human antibody allows the generation of HC-10/β_2-m phenotypes.

6.2. Results

The HC-10 positive tumours were scored (0-3) regarding the intensity of their cell membrane staining and the β_2-m positive tumours were scored regarding the percentage of cell membranes staining (Figure 6.1 a, b, c, d).

Average values for intensity of membranes staining HC-10 positive were determined over zero fields (28/339; 8.3% cases), one field (82/339; 24.2% cases) and two fields (229/339; 67.6% cases). Average values for percentage of cells β_2-m positive were determined over zero fields (24/339; 7.1% cases), one field (102/339; 37.2% cases) and two fields (213/339; 62.8% cases). In defining positivity (to allow for co-expression analysis of both markers), any degree of positive staining was considered to indicate positive expression – thereby minimising false negatives while also adopting a reproducible system. This equated to a cut off of $\geq$10% for β_2-m staining (cores scored to within the nearest 10%) and $\geq$1 intensity for HC-10. $\geq$10% of cell membranes staining with $\geq$1 intensity effectively defined positivity for both markers.

Figure 6.1 **HC-10 and β2 Microglobulin expression patterns**

Figure 6.1a **Ovarian cancer tissue core staining weakly (+1) with HC-10 in the cell membrane**

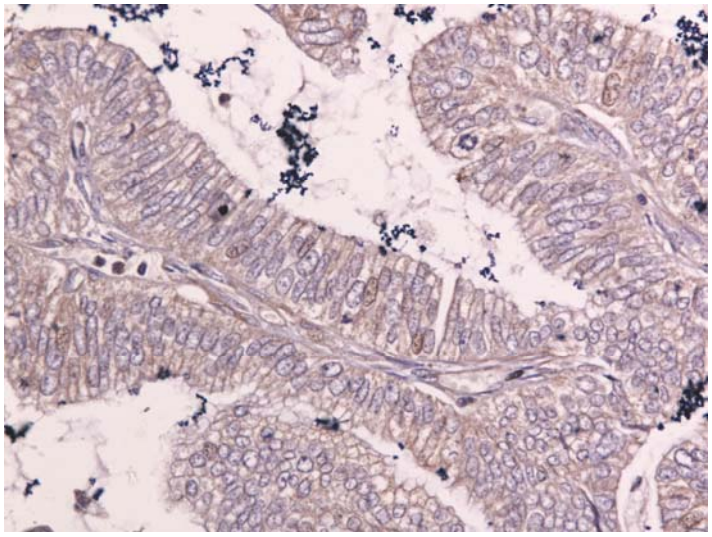


Figure 6.1b **Ovarian cancer tissue core staining moderately (+2) with HC-10 in the cell membrane**

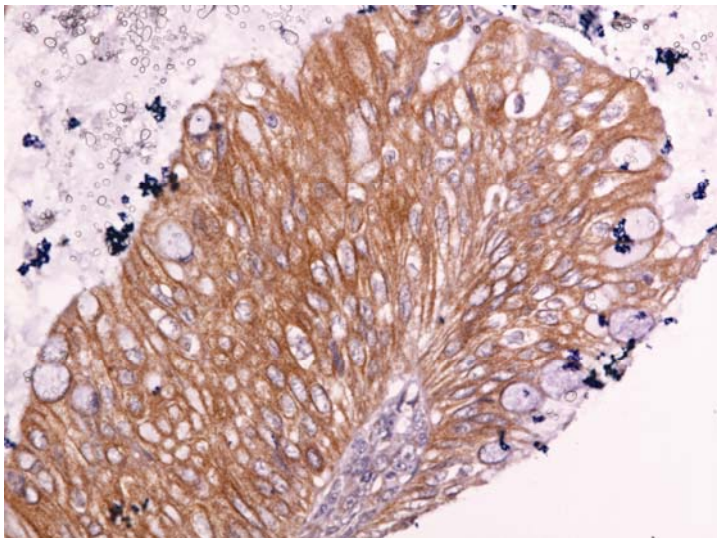


Figure 6.1c **Ovarian cancer tissue core staining strongly (+3) with HC-10 in the cell membrane**

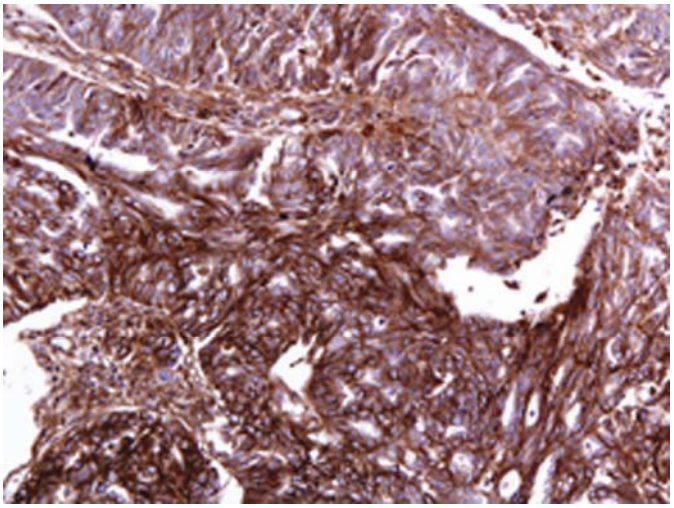
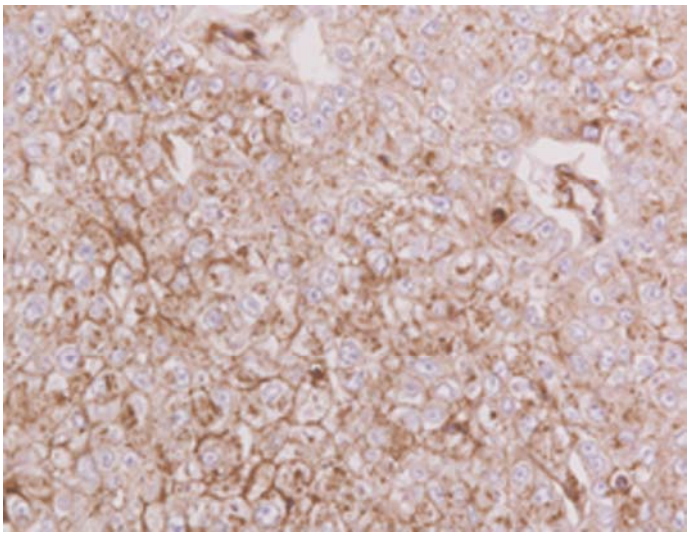


Figure 6.1d **Ovarian cancer tissue core staining positive for β2 Microglobulin in the cell membrane of 100% of the cells**



Neither the degree of HC-10 nor the degree of β_2-m expression showed any correlation with clinicopathological characteristics (data not shown) or prognosis [Log rank = 1.18; p=0.28 and Log rank = 1.89; p=0.17 respectively] and, as intended, subsequent analyses focused on their combined phenotypes. Of the series of 395 patients, 298 had expression data complete enough to allow for co-analysis (Figure.6.2). Any variation from an expected HC-10(+)β_2-m(+) phenotype was considered a downregulation of the HLA class I antigen. An HC-10(+)β_2-m(-) phenotype is likely to represent a state of genuinely free, non-functional HLA Class I heavy chain and an HC-10(-)β_2-m(-) phenotype complete loss of HLA class I antigen. An HC-10(-)β_2-m(+) phenotype may represent downregulation of HLA class I antigen and/or the presence of other HLA class I heavy chain locus products not identified by HC-10. 32.8% (102/298) of cases were negative for HC-10 and 55.6% (175/298) of cases were negative for β_2-m. With only 34.6% (103/298) of cases HC-10(+)β_2-m(+), 65.4% (195/298) of cases had phenotypes suggestive of HLA class I antigen downregulation, revealing this to be a common event in this series (Table 6.1).

There were no striking differences between the clinicopathological characteristics of the entire series and this apparently randomly selected 298-strong subgroup of cases (Table 6.2). The median age of diagnosis for these cases was 62 years, the median follow up for this group was 21.7 months (Range = 0-271 months) and the 5-year survival was 27.1%. This group's characteristics are similar to those of large scale reports (Yancik 1993; 1998; Brun, Feyler et al. 2000; Quinn 2001; Quirk and Natarajan 2005). On univariate analysis using the χ^2 method, the HC-10(+)β_2-m(+) phenotype was not found to be associated with age (using the Surveillance, Epidemiology, and End Results programme of the National Cancer Institute subdivisions (Quirk and Natarajan 2005)]), FIGO stage, degree of cytoreduction, grade, histological type or the use of chemotherapy (Table 6.3). However, as seen in the Kaplan-Meier plot (Figure 6.3), those patients without this phenotype did have a worse prognosis [Log Rank = 5.69 (p=0.017)]. These cases with phenotypes indicating downregulation of HLA class I

Figure 6.2 Flow diagram illustrating the derivation of the study cohort
298 cases from the original series of ovarian cancers were analysed immunohistochemically for functional HLA class I antigen expression.

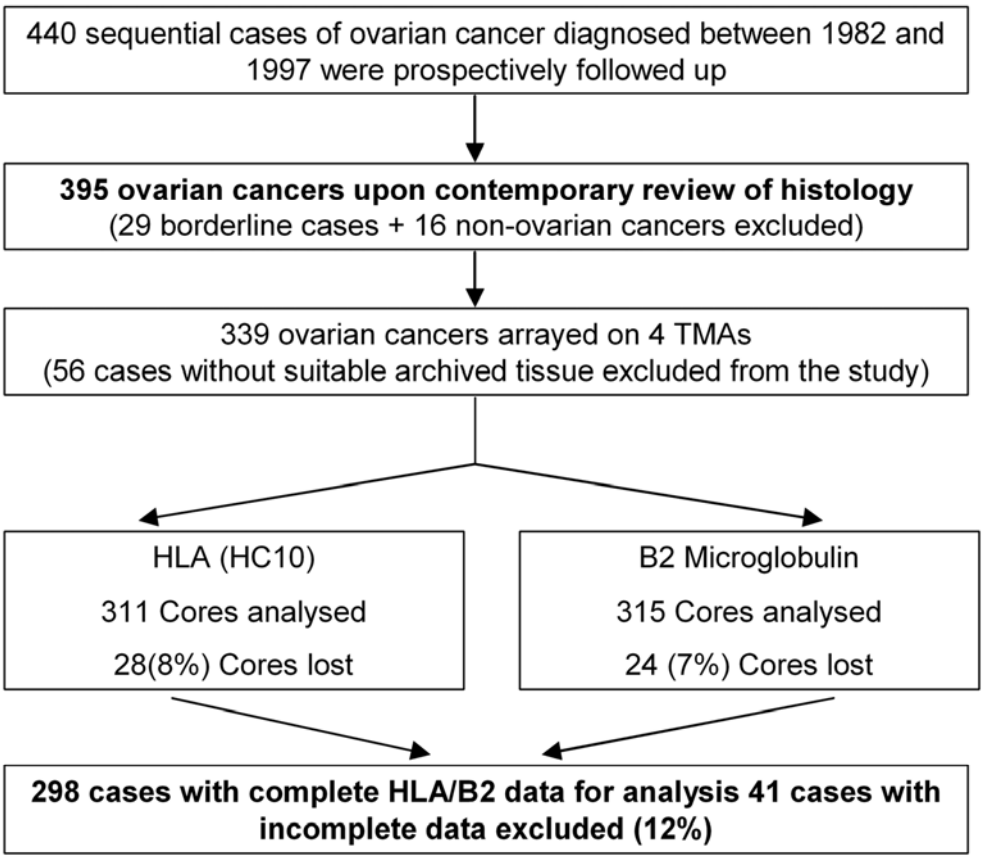


Table 6.1 The immunohistochemical expression of HC-10, β_2-m and their combined phenotypes

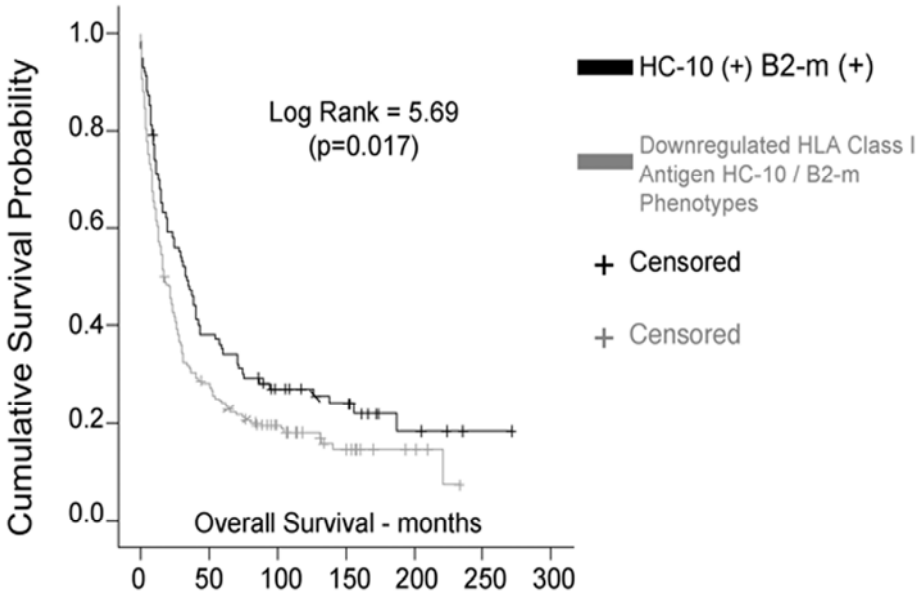
	Nº Cases	**% of Cases**
HLA class I heavy chain (HC-10) intensity	n=311	
<1	102	32.8
1-2	85	27.3
2-3	124	39.9
HLA class I heavy chain (HC-10) positive (≥1 intensity)	209	67.2
β_2 microglobulin light chain % cells positive	n=315	
0-25	242	76.8
26-50	42	13.3
51-75	19	6
76-100	12	3.8
β_2 microglobulin light chain positive (>10% cells)	140	44.4
HLA Class I antigen expression phenotypes	n=298	
HC-10 (+) β_2-m (+)	103	34.6
Downregulated	195	65.4
HC-10 (+) β_2-m (-)	98	32.9
HC-10 (-) β_2-m (+)	68	23.8
HC-10 (-) β_2-m (-)	29	9.7

Table 6.2 Association of the HC-10(+)β_2-m(+) phenotype with clinicopathological features in a univariate analysis

	Whole series n = 395	**Analysed cases n = 298**	**HC-10(+) β_2-m (+) n=103**	**Downregulated Phenotypes n=195**	
	N^o cases (%)	**N^o cases (%)**	**N^o cases (%)**	**N^o cases (%)**	**χ^2 (p=)**
Age	n=394	n=297	n=103	n=194	
< 30	1 (0.3)	1 (0.3)	0	1 (0.5)	
30 – 59	167 (42.4)	119 (40.1)	42 (40.8)	77 (39.7)	0.554
>60	226 (57.4)	177 (59.6)	61 (59.2)	116 (59.8)	(0.758)
FIGO Stage	n=375	n=290	n=99	n=191	
1	99 (26.4)	77 (26.6)	27 (27.3)	50 (26.2)	
2	46 (12.3)	33 (11.4)	9 (9.1)	24 (12.6)	
3	188 (50.1)	144 (49.7)	51 (51.5)	93 (48.7)	0.836
4	42 (11.2)	36 (12.4)	12 (12.1)	24 (12.6)	(0.841)
Optimally Debulked	n=376	n=287	n=99	n=188	0.057
Yes	157 (41.8)	119 (41.5)	42 (42.4)	77 (41.0)	(0.811)
Grade	n=376	n=296	n=99	n=195	
1	50(13.3)	32 (10.8)	11 (10.9)	21 (10.8)	
2	93 (24.7)	66 (22.3)	19 (18.8)	47 (24.1)	1.102
3	233 (62)	198 (66.9)	71 (70.3)	127 (65.1)	(0.576)
Histological Type	n=395	n=298	n=103	n=195	
Serous	203 (51.4)	162 (54.4)	52 (50.5)	110 (56.4)	
Endometrioid	46 (11.7)	37 (12.4)	11 (10.7)	26 (13.3)	
Mucinous	50 (12.7)	26 (8.7)	12 (11.7)	14 (7.2)	
Undifferentiated	65 (16.5)	45 (15.1)	17 (16.5)	28 (14.4)	
Clear cell	26 (6.6)	24 (8.1)	10 (9.7)	14 (7.2)	3.264
Other	5 (1.3)	4 (1.4)	1 (1)	3 (1.5)	(0.659)
Chemotherapy	n=388	n=292	n=100	n=192	0.228
Yes	283 (73.2)	214 (73.3)	75 (75)	140 (72.9)	(0.633)

Figure 6.3 HC-10 (+) β2-m (+) phenotype and overall survival

Kaplan-Meier plot showing the improved overall survival for patients with HC-10 (+) β2-m (+) ovarian cancers.



antigen had a 5-year survival of 23.2% compared with 34.7% when both HC-10 and β_2-m were expressed. Factors in this series found to predict prognosis independently of each other were age (as a continuous variable), FIGO stage, the absence of residual macroscopic disease following primary surgery and whether the patient received chemotherapy of any form. These factors were included in the Cox's model along with the HLA class I antigen expression phenotype. The presence of the HC-10(+)β_2-m(+) phenotype was found to retain its power to predict an improved prognosis independently of the other factors [Hazard Ratio 0.587 ($p<0.001$; 95% Confidence intervals 0.442-0.781)] (Table 6.3). When the other phenotypes were analysed in a similar fashion, no correlations were found in either univariate or multivariate analysis (data not shown), indicating that it is the lack of an expected HC-10(+)β_2-m(+) phenotype that is deleterious in ovarian cancer.

6.3. Discussion

As with all TMA work, the final group analysed (298) is smaller than the original series identified (395) due to incomplete data and missing tissue. Studying the co-expression of two markers exacerbated this. Significant selection bias within the final analysed group could potentially have been introduced by non-random loss of tissue but, as the final group varies little from the original series regarding its clinicopathological characteristics, this is unlikely to have occurred. With 298 cases available the power of the study was retained.

In this study we show, along with other work, that downregulation of HLA class I antigen is a common event in ovarian cancer (Vitale, Pelusi et al. 2005; Norell, Carlsten et al. 2006). The 34.6% of tumours which retained a complete HC-10(+)β_2-m(+) phenotype had a better prognosis than other cases and, furthermore, the expression/loss of this phenotype is an independent prognostic factor in ovarian cancer of a similar magnitude to the presence/absence of residual macroscopic disease (Table 6.3). The absence of complete HLA class I antigen expression leading to a worse prognosis supports work by Vitale et al (Vitale, Pelusi et al. 2005) which concluded that downregulation of HLA

Table 6.3 Multivariate analysis by Cox's regression using the HC-10(+) β_2-m (+) phenotype as a factor
The analysis shows that a retained HC-10(+) β_2-m (+) phenotype is an independent prognostic factor for good prognosis

	Hazard Ratios (95% Confidence intervals)	**Significance (p)**
Age at diagnosis (years)	1.027 (1.015-1.038)	<0.001
FIGO Stage		
1		<0.001
2	2.934 (1.591 – 5.414)	0.001
3	5.796 (3.311-10.146)	<0.001
4	8.071(4.247-15.338)	<0.001
Residual macroscopic disease	2.026 (1.405-2.921)	<0.001
Patient received chemotherapy	0.430 (0.287 – 0.644)	<0.001
HC-10 (+) β_2-m (+) phenotype	**0.587 (0.442-0.781)**	**<0.001**

type I loss was associated with worse disease – in this case stage III cancers. Differences in our methodologies (regarding powering, biomarker co-analysis and the use of UICC independent prognostic factors (Gospodarowicz 2001) in statistical analyses) meant that, while we should not duplicate this specific finding, we were better positioned to demonstrate a novel and important independent association with prognosis.

Cancer cells may be eliminated by, be in equilibrium with or escape innate or adaptive immunological control mechanisms via immunediting (Ochsenbein, Klenerman et al. 1999; Dunn, Bruce et al. 2002) and an important mechanism by which the immune system eliminates cancer cells is via a CTL based response to abnormal peptide presented in conjunction with HLA class I antigen on tumour cells (Seliger, Maeurer et al. 2000). Given the genetic instability of cancer, such a selective pressure might result in the evolution and ultimate protection of tumours from this process via downregulation of HLA class I antigen expression, as either an early or multi-step process (Klein and Klein 1985). This is likely to be important in the clinical course of ovarian cancer (Algarra, Cabrera et al. 2000) and evidence for such tumour sculpting comes from the observation that highly tumour specific CTL exist within the tumour infiltrating lymphocyte population of advanced disease whose function can be inhibited by anti-HLA class I monoclonal antibodies but which fail to elicit a clinically adequate immune response (Santin, Hermonat et al. 2000; Santin, Bellone et al. 2004). That downregulation of HLA class I antigen should associate with worse survival, as we found, is therefore logical.

When HLA class I antigen expression is lost, killer-cell inhibitory receptors on the surface of natural killer (NK) cells (which produce an inhibitory signal when bound to HLA class I antigen) no longer function. The “missing self” hypothesis concludes that a resultant increase in NK cell killing activity will compensate for diminished T-cell killing in these circumstances (Ljunggren and Karre 1990). This may occur clinically in breast and colorectal cancer and explain the improvement in prognosis of those tumours with total loss of HLA class I antigen expression

(Madjd, Spendlove et al. 2005; Watson, Ramage et al. 2006). However, our data suggest that this mechanism must be less effective in ovarian cancers. This may be due to a concurrent acquired resistance to NK cell killing (Garrido, Ruiz-Cabello et al. 1997), a lack of NK cell presence within the tumour infiltrating lymphocytes (Kabawat, Bast et al. 1983; Ferguson, Moore et al. 1985) or the rapid exhaustion of this mechanism by the large volume nature of ovarian disease (Ochsenbein, Klenerman et al. 1999).

There are 7 reported mechanisms by which a state of downregulated HLA class I antigen might occur: total HLA class I antigen loss due to the disrupted synthesis of antigen-processing machinery components such as β_2-m and transporter associated with antigen processing; haplotypic loss of either one or two haplotypes due to chromosomal events; HLA A, B or C locus downregulation due to altered HLA class I antigen gene transcription; individual HLA allelic loss due to gene point mutations or deletions; a compound state of two or more of these events; an unresponsiveness to interferons due to downregulation of the interferon response sequence element and subsequent pathway disruption; and, finally, downregulation of HLA A-B-C molecules with a concurrent upregulation of HLA-E. The most frequent event observed in this series was loss of β_2-m. This contributed to over 55% of the loss of HLA class I antigen. The mechanisms for β_2-m loss in cancer are, like those for heavy chain, varied but commonly include loss of mRNA stability (Hicklin, Wang et al. 1998) and mutational silencing (Bicknell, Kaklamanis et al. 1996). However, genomic loss due to chromosomal defects, particularly in chromosome 15, are not frequently observed in ovarian cancer. In melanoma, β_2-m mutations are primarily related to extensive loss of chromosome 15 material (Paschen, Arens et al. 2006) but, though alterations in chromosome 15 in ovarian cancer have been well documented, there are no reports of frequent alterations in the β_2-m locus (15q21). These mechanisms may relate to ovarian cancer where we have demonstrated a significant loss and clear clinical correlation with poor outcome. However, other reports suggest that HLA class I antigen downregulation in ovarian cancer is a post translational

event that can be restored upon interferon-ɣ treatment (Boyer, Dawson et al. 1989; Mobus, Asphal et al. 1993; Santin, Rose et al. 1996; Freedman, Kudelka et al. 2000). The exact mechanism of HLA-class I antigen loss in ovarian cancer warrants further investigation and would be most successfully achieved on freshly isolated tumour samples.

Our work shows that ovarian cancers not expressing the HC-10(+)β_2-m(+) phenotype have a poorer prognosis irrespective of their gross appearance. Future work could also test whether foreknowledge of this phenotype might predict those cases which will respond best to CTL or interferon-ɣ based immunotherapies.

Chapter 7: The Prognostic Significance of Membrane Bound Complement Regulatory Protein Expression in Ovarian Cancer

7.1. Introduction

Ovarian cancer is the most common gynaecological cancer in the UK and represents the fourth most common cancer site for women. Its low 5-year survival of 29% has altered little over the last 40 years (Quinn 2001). Novel immunotherapies will demand an improved understanding of the tumour-host immunological interface if they are to reach their potential to improve prognosis in this disease.

The complement cascade is an important effector mechanism of the innate immune system which, via the classical, lectin or alternative pathways, can lead to cell lysis and Complement Dependent Cytotoxicity (CDC) via the deposition of membrane attack complex (MAC) (Muller-Eberhard 1986; Walport 2001; Walport 2001). Most normal tissues protect themselves from non-specific bystander effects through the expression of membrane bound complement regulatory proteins (mCRP) (Morgan 1989; Morgan and Meri 1994). These include CD46 (membrane co-factor protein) which inhibits the cascade by acting as a cofactor for factor I in the cleavage of C3b and C4b in the alternate pathway (Cole, Housley et al. 1985; Seya, Ballard et al. 1988; Devaux, Christiansen et al. 1999); CD55 (Decay Accelerating Factor) which accelerates the decay of the C3 and C5 convertases (Medof, Lublin et al. 1987; Nicholson-Weller and Wang 1994); and CD59 (Protectin) which interferes with the assembly of MAC by binding to C8 and C9 (Meri, Morgan et al. 1990; Rollins and Sims 1990).

The distribution of mCRP in cancers often differs to that in analogous normal tissues which may alter their susceptibility to CDC (McConnell, Klein et al. 1978; Magyarlaki, Mosolits et al. 1996; Bjorge, Hakulinen et al. 2005). CD46 is upregulated in breast cancer (Hofman, Hsi et al. 1994) and colorectal cancer but downregulated in renal cancers (Niehans, Cherwitz et al. 1996; Blok, Daha et al. 2000). CD55 is

upregulated in colorectal and renal cancers but expressed at lower levels in breast cancers (Niehans, Cherwitz et al. 1996; Li, Spendlove et al. 2001). CD59 expression is upregulated in poorly differentiated colorectal and renal cancers (Koretz, Bruderlein et al. 1993; Niehans, Cherwitz et al. 1996) but lost in gastric cancers (Kiso, Mizuno et al. 2002). Additionally, same-site heterogeneity of CD55 and CD59 expression predicts survival in breast cancer (Madjd, Pinder et al. 2003; Madjd, Durrant et al. 2004) and colorectal cancer cases (Durrant, Chapman et al. 2003; Watson, Durrant et al. 2006).

In ovarian cancer cell lines and a small series of tumours, CD46 and CD59 showed wide expression in vitro and in vivo in both benign and malignant neoplasms, while CD55 was variably expressed. Furthermore, the presence of high levels of mCRP represents a method of immune protection from complement activating antibodies to tumour associated antigens (Bjorge, Hakulinen et al. 1997; Bjorge, Hakulinen et al. 2005). We therefore investigated the hypothesis that ovarian tumours with differentially high expression of mCRP expression would be the most protected from complement mediated lysis and would display the worst prognosis and most aggressive behaviour.

7.2. Results

7.2.1. Immunohistochemistry

The expression of CD46 varied in intensity and in the percentage of expression within the cell membranes of different tumours but 95% of the 316 tumours examined expressed CD46 to some degree (Figure 7.1a). The distribution of expression of CD55 was similar to CD46 and all of the 318 tumours examined expressed this marker to some extent (Figure 7.1b). The intensity of staining for CD59 was of homogenous intensity but varied regarding the percentage number of cells staining with 86% of the 293 tumours examined expressing some CD59 (Figure 7.1c). With such ubiquitous positive expression evident, relative high versus low expression states were determined as described previously to stratify tumours further (Table 7.1). As tissue was lost during

Figure 7 **CD46, CD55 and CD59 expression patterns**

Figure 7.1a **A core from an ovarian tumour staining positive for CD46 at x 100 magnification with an insert at x 200 magnification**
There was diffuse cytoplasmic staining, granular cell membrane staining and no nuclear staining. Both intensity and % cells staining varied between tumours and so each was recorded

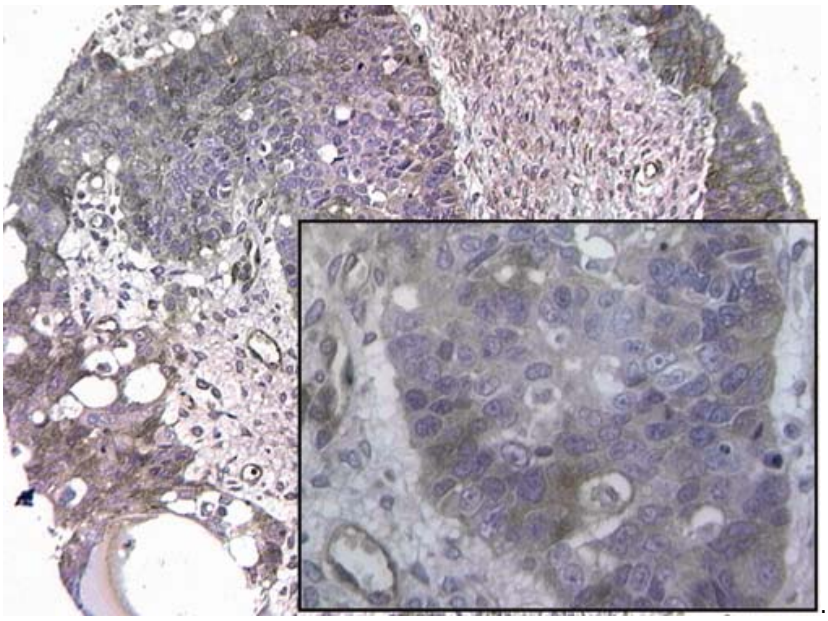


Figure 7.1b **A core from an ovarian tumour staining positive for CD55 at x 100 magnification with an insert at x 200 magnification**
The staining pattern seen was similar to that of CD46 and expression data were collected in the same way

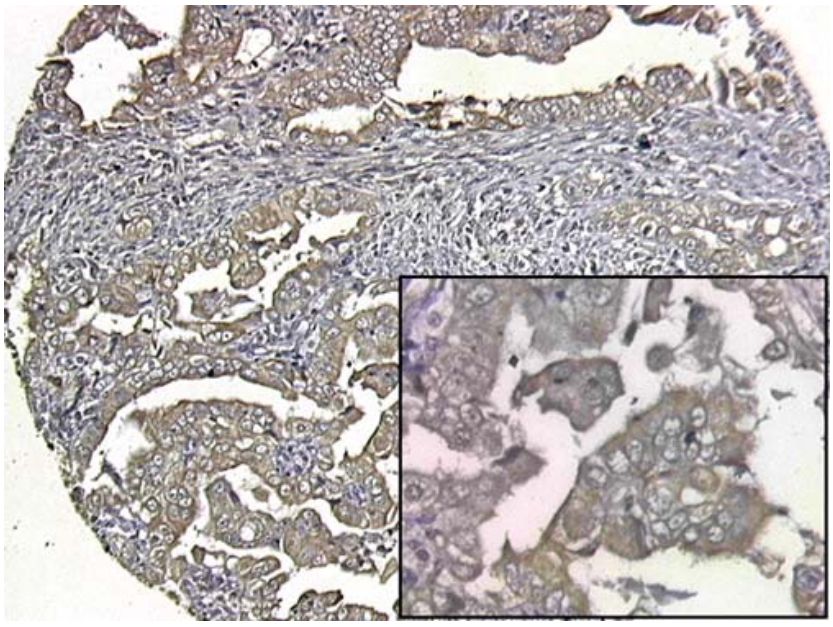


Figure 7.1c **A core from an ovarian tumour staining positive for CD59 at x 100 magnification with an insert at x 200 magnification**
The staining was granular, in the cell membrane and with some distribution cytoplasmically. There was no nuclear staining. There was no variation in the intensity of the staining between positive tumours and so only percentage of cells staining was recorded for this marker

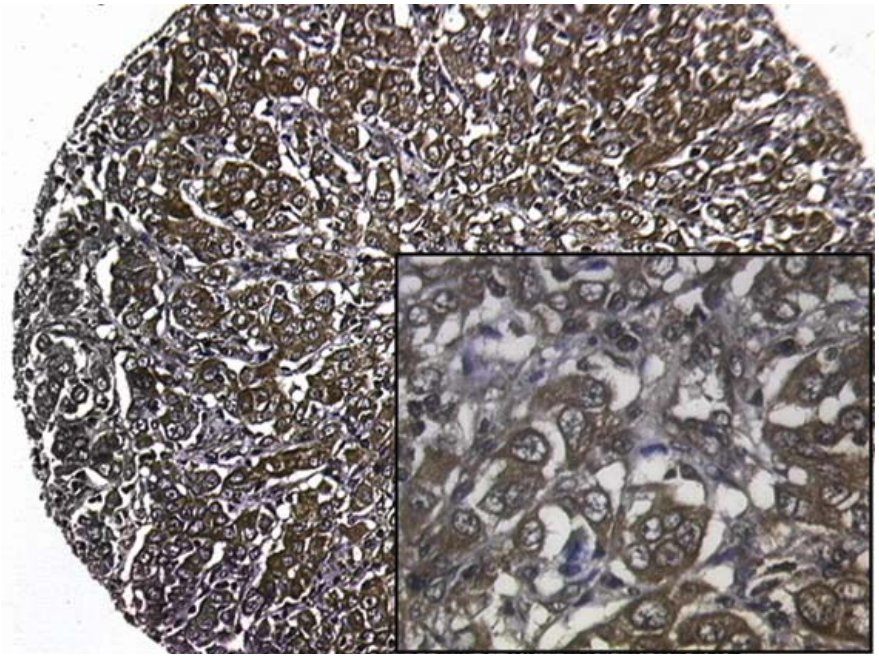


Table 7.1 Summary of the immunohistochemical expression data for CD46, CD55 and CD59

	CD46	**CD55**	**CD59**
	no cases (%)	no cases (%)	no cases (%)
	n=316	n=318	n=293
Average % Cells			
0	16 (5)	0	41 (14)
1-5	5 (2)	0	34 (12)
6-25	19 (6)	2 (1)	82 (28)
26-50	91 (29)	3 (1)	74 (25)
51-75	104 (33)	22 (7)	44 (15)
76-100	81 (26)	291 (92)	18 (6)
Average Intensity Score			
0-100	268 (85)	8 (3)	
101-200	44 (14)	147 (46)	
201 – 300	4 (1)	163 (51)	
Positive expression	300 (95)	318 (100)	252 (86)
High expression	156 (49)	143 (45)	82 (28)

processing, average values for CD46 expression were calculated using zero cores in 7%, one core in 23% and two cores in 70% of cases; for CD55 expression, zero cores in 6%, one core in 25% and two cores in 69% of cases; and for CD59, zero cores in 14%, one core in 30% and two cores in 56% of cases. 276 (81%) of cases had relative high-low expression data for all three markers available. 11% of these cases displayed universally high mCRP expression and 29% had universally low expression (Figure 7.2). There were no striking differences between the clinicopathological characteristics of the entire series and the sets of expression data generated (Table 7.2).

7.2.2. Comparison of differential mCRP expression with clinicopathological factors – univariate analysis using the χ^2 method

Relatively high CD59 expression was associated with a more advanced FIGO stage, the presence of post-surgical macroscopic disease, worse histological type and less chance that the patient would have received chemotherapy. Relatively high CD55 expression was associated with advanced FIGO stage, the presence of post-surgical macroscopic disease and worse histological type (Table 7.3). There were no associations discovered between differential CD46 expression and clinicopathological factors and this was also the case for tumours expressing relatively low levels of all three mCRP. However, low expression of CD55 and low expression of CD59 were associated with one another (16.16; $p<0.001$) and CD59 [-] CD55 [-] cases (low co-expression tumours, 45% of the whole) were associated with earlier FIGO stage, a higher chance of optimal debulking and better histological type (Table 7.4).

7.2.3. Association of differential mCRP expression with overall survival

Individually, relatively high expression states of CD59 or CD55 were associated with worse prognosis in a univariate analysis (Figure 7.3a, b). Low co-expression of CD59 and CD55 was associated with the best prognosis (Figure 7.3c). There was no association between

Figure 7.2 **The distribution of tumours between the eight immunophenotypes that combined high [+] and low [-] CD46- CD55-CD59 expression data generates**

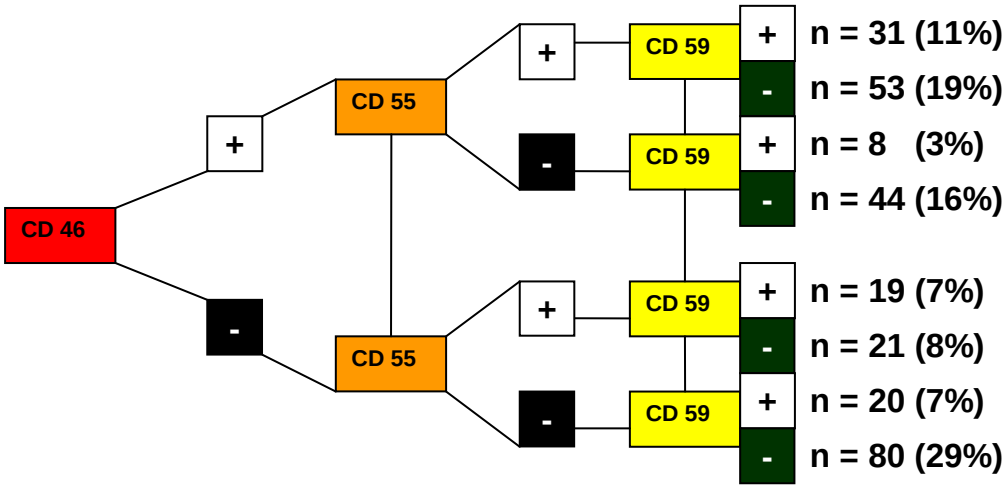


Table 7.2 The major clinicopathological features of each of the analysed groups

	Whole series	**CD46 cases analysed**	**CD55 cases analysed**	**CD59 cases analysed**	**Cases with complete mCRP data**
	nº cases (%)	nº cases (%)	nº cases (%)	nº cases (%)	nº cases (%)
	n = 395	n=316	n=318	n=293	n=276
Age	n=394	n=315	n=317	n=292	n=275
< 30	1	1	1	1	1
30 – 59	167 (42)	124 (39)	124 (39)	116 (40)	106 (39)
>60	226 (57)	190 (60)	192 (61)	175 (60)	168 (61)
FIGO Stage	n=375	n=308	n=311	n=284	n=269
1	99 (26)	81 (26)	79 (25)	74 (26)	69 (26)
2	46 (12)	33 (11)	34 (11)	34 (12)	31 (12)
3	188 (50)	159 (52)	162 (52)	144 (51)	141 (52)
4	42 (11)	35 (11)	36 (12)	32 (11)	28 (10)
Optimally Debulked	n=376	n=304	n=307	n=281	n=266
	157 (42)	123 (41)	122 (40)	116 (41)	110 (41)
Received Chemotherapy	n=388	n=310	n=313	n=286	n=271
	283 (73)	224 (72)	228 (73)	208 (73)	200 (74)

Table 7.3 A univariate analysis showing statistical associations between the major clinicopathological characteristics and differential CD59 and CD55 expression

	CD59 low	**CD59 high**	**χ2 (p=)**	**CD55 low**	**CD55 high**	**χ2 (p=)**
	nº cases (%)	nº cases (%)		nº cases (%)	nº cases (%)	
FIGO Stage	n=284			n=311		
1	60 (30)	14 (17)		50 (29)	29 (21)	
2	28 (14)	6 (7)		23 (14)	11 (8)	
3	92 (45)	52 (64)	**9.39**	75 (44)	87 (62)	**9.87**
4	23 (11)	9 (11)	**(0.03)**	22 (13)	14 (10)	**(0.02)**
Optimally Debulked	n=281			n=307		
Yes	91 (45)	25 (32)	**4.21**	76 (45)	46 (33)	**4.30**
No	111 (55)	54 (68)	**(0.04)**	93 (55)	92 (67)	**(0.04)**
Histological Type	n=293			n=318		
Serous	103 (49)	55 (67)		88 (50)	81 (57)	
Endometrioid	27 (13)	9 (11)		22 (13)	18 (13)	
Mucinous	26 (12)	4 (5)		17 (10)	12 (8)	
Undifferentiated	32 (15)	12 (15)		24 (14)	27 (19)	
Clear cell	19 (9)	2 (2)	**12.12**	22 (13)	3 (2)	**13.08**
Other	4 (2)	0	**(0.03)**	2 (1)	2 (1)	**(0.02)**
Chemotherapy	n=286			n=313		
Yes	142 (70)	66 (82)	**4.34**	129 (75)	99 (70)	
No	63 (31)	15 (19)	**(0.04)**	43 (25)	42 (30)	(0.34)

Table 7.4 Association of clinicopathological factors in a univariate analysis with the CD59 [+/-] CD55 [+/-] immunophenotypes

	CD59 [-] CD55 [-]	**Other Immunophenotypes**	**χ^2 (p=)**
	no cases (%)	no cases (%)	
FIGO Stage	n=273		
1	39 (32)	30 (20)	
2	19 (16)	13 (9)	
3	46 (38)	97 (64)	
4	17(6)	12 (8)	**18.06 (<0.001)**
Optimally Debulked	n=270		
Yes	58 (48)	52 (35)	**4.7 (0.03)**
No	63 (52)	97 (65)	
Histological Type	n=280		
Serous	57 (45)	95 (62)	
Endometrioid	19 (15)	16 (10)	
Mucinous	12 (10)	13 (8)	
Undifferentiated	19 (15)	25 (16)	
Clear cell	17 (14)	4 (3)	**16.36 (0.01)**
Other	2 (2)	1	

Figure 7.3c **A Kaplan-Meier plot showing that tumours expressing relatively low levels of both CD59 and CD55 have the best prognosis**

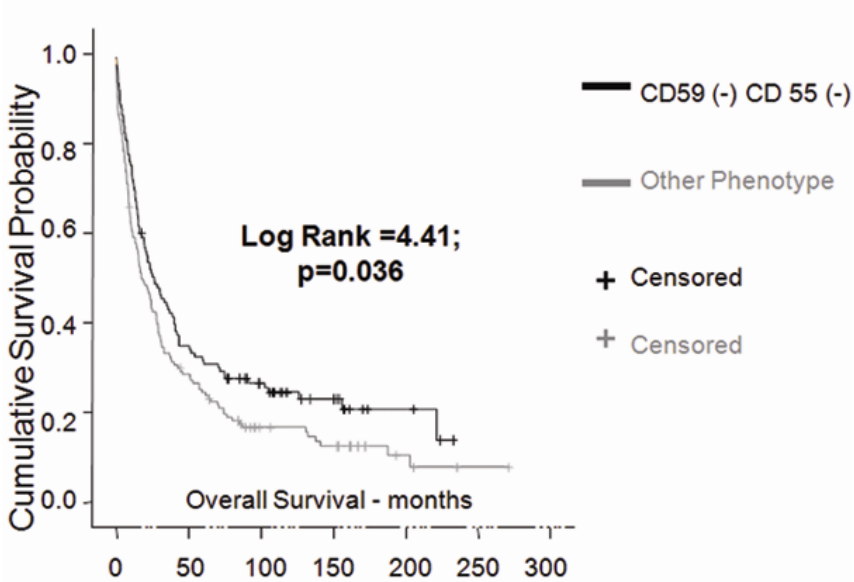


Figure 7.3 **Univariate associations between the expression of mCRP and overall survival**

Figure 7.3a **A Kaplan-Meier showing that high CD55 expression is associated with a reduction in overall survival**

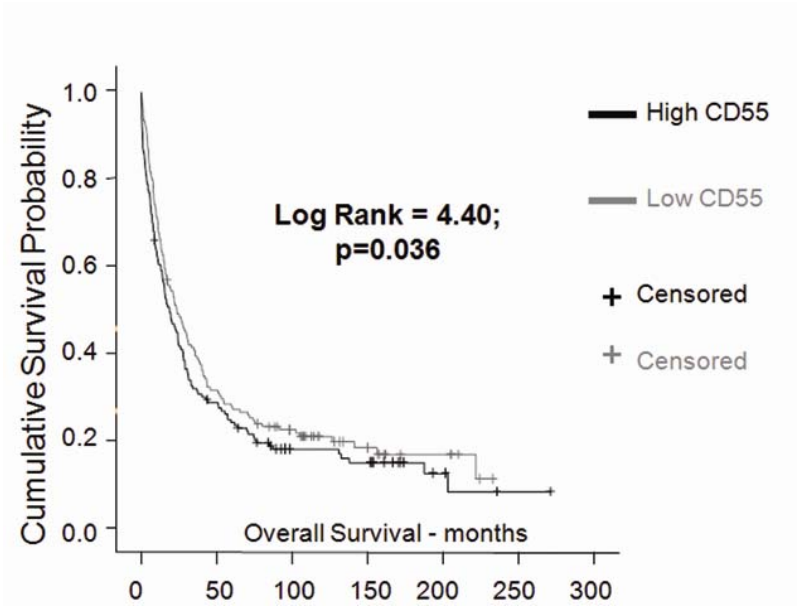
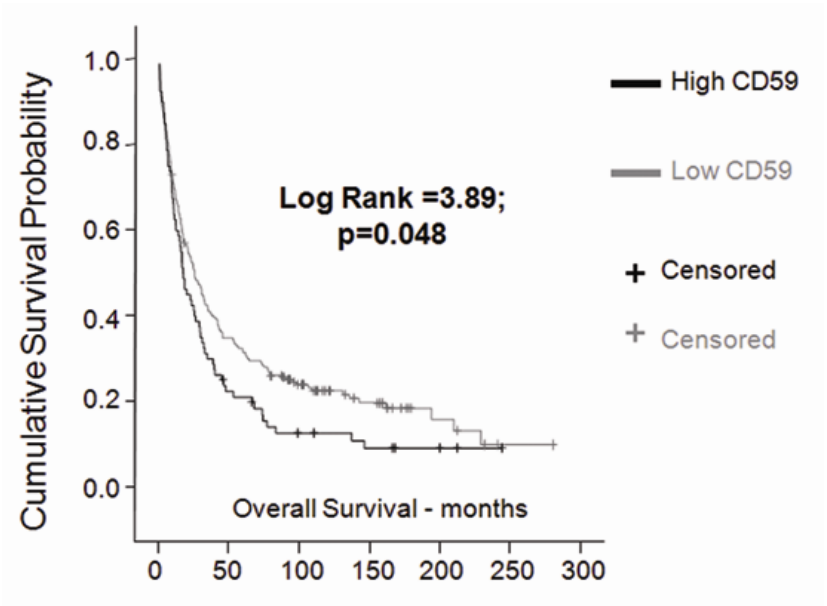


Figure 7.3b **A Kaplan-Meier plot showing that high CD59 expression is associated with a reduction in overall survival**



CD46 expression or low expression of all three mCRP and survival. Factors in this series found to predict prognosis independently of each other were age (as a continuous variable), FIGO stage, the absence of post-surgical residual macroscopic disease and whether the patient received chemotherapy. The survival associations found on univariate analysis did not persist following Cox's multivariate analysis when these independent prognostic factors were included in the model (CD59 p=0.262; CD55 p=0.364; CD59 [-] CD55 [-] p=0.386).

7.3. Discussion

In this work a novel anti-CD46 antibody was employed in order to study the co-expression of three mCRP in Formalin Fixed Paraffin Embedded (FFPE) tissue. For the first time, this allowed a large historical series to be fully surveyed and the biologically plausible hypothesis that differential expression of mCRP stratifies prognosis to be tested.

The immunohistochemical expression of these three mCRP in frozen normal ovarian tissue and adenomas has previously been described by two groups. Though the numbers studied have been small, CD59 and CD46 seem to be universally expressed, with CD55 being present more variably (Bjorge, Hakulinen et al. 1997). Within the Müllerian elements of the female genital tract, mCRP upregulation and overexpression has been shown to be associated with the development of cervical and endometrial cancers (Simpson, Jones et al. 1997) (Murray, Mathure et al. 2000). This agrees with observations at other cancer sites but, though the suspicion is that similar changes occur during ovarian carcinogenesis, direct evidence that upregulation of mCRP is part of its natural history is currently lacking (Cheung, Walter et al. 1988). The limited indirect evidence available suggests that CD59 is equally universally and highly expressed in both carcinomas and normal tissue, that CD55 is only expressed in 75% of ovarian cancers and that CD46 expression is unchanged following malignant transformation (Bjorge, Hakulinen et al. 1997; Macor, Mezzanzanica et al. 2006). In this study CD59 was widely and strongly expressed and CD46 was moderately

expressed, which is in keeping with other work. However, in this series tumours were universally CD55 positive and, while this finding is at variance to the work of Bjorge et al, it is supported by other data (Symonds, Perkins et al. 1985). Methodological variations make it impossible to say whether CD55 expression in this series represented overexpression and upregulation relative to other studies and normal tissue, though a methodological variation in itself could explain the discrepancy as the definition of positive expression in this series was very sensitive and included cases displaying even weak staining. Despite this, CD55 may be more widely expressed in the ovarian cancer population than previously appreciated.

Given that deposition of complement is associated with tumour cell killing (Perlmann, Perlmann et al. 1981; Bara and Lint 1987; Fishelson, Donin et al. 2003) and that resistance to this can be overcome when CD55 and CD59 are blocked (Gorter, Blok et al. 1996; Bjorge, Hakulinen et al. 1997; Donin, Jurianz et al. 2003), the almost ubiquitous expression of mCRP we observed may indicate an immunological evasion tactic, particularly if carcinogenesis and upregulation are intertwined. In such circumstances tumours expressing relatively low levels of mCRP might be expected to be associated with an improved prognosis (Gorter and Meri 1999).

However, in this study differential CD46 expression did not correlate with survival. While this may represent a limitation of factor I availability, a pre-requisite for its activity, this lack of clinical impact might also be explained by the modest contribution of the alternative pathway to complement activation in cancer. In this situation, CD55 and CD59 expression would effectively become the co-determinants of complement inhibition and immune evasion, which is concordant with the suspicions of other workers (Bjorge, Hakulinen et al. 1997; Devaux, Christiansen et al. 1999; Madjd, Durrant et al. 2005). This hypothesis is supported by this work's other findings – that relative high expression of CD59 and CD55 predicted an aggressive phenotype and reduced survival while tumours expressing neither in high levels had the best outcomes.

The modest, non-independent prognostic effect of mCRP expression in ovarian cancer might be explained if only very low absolute levels of mCRP are required to protect the developing tumour from the effects of complement, as data from CD55 transgenic pigs would suggest, or if other mechanisms of complement defence prevail (Morgan 1989; Carrington and dos Santos Cruz 2001; Donin, Jurianz et al. 2003). As mCRP are implicated in T cell regulation and the inhibition of T cell activation, it is also conceivable that the clinical behaviour of tumours would be less predictable than hypothesised, while it is additionally possible that these clinically apparent tumours may have passed through the phase where their behaviour could be predicted by their relationship with the immune system (Astier, Trescol-Biemont et al. 2000; Dunn, Bruce et al. 2002; Marcenaro, Augugliaro et al. 2003; Capasso, Durrant et al. 2006; Spendlove, Ramage et al. 2006). Considering the fall in the number of cases from over 300 to 273 that occurred prior to the multivariate analysis of the co-expression data being performed, a final explanation for the non-independent prognostic effects observed remains a type II error associated with reduction in statistical power.

Though the prognostic power of mCRP was of borderline clinical significance, the ubiquitous presence of mCRP in a large series of ovarian cancers represents important corroboratory clinical evidence. Ovarian cancer appears particularly suited to immunotherapy, with strategies employing CDC being particularly intuitive as the soluble components of the innate immune system have been shown to penetrate the disease well (Rubin 1993; Gorter and Meri 1999; Fishelson, Donin et al. 2003; Bjorge, Hakulinen et al. 2005). However, as CD55 and CD59 have important functional roles in antagonising CDC, such immunotherapy may need to routinely incorporate a parallel mCRP blocking strategy in order to achieve clinically effective cell killing (Bjorge, Hakulinen et al. 1997; Bjorge, Junnikkala et al. 1997; Macor, Mezzanzanica et al. 2006). Important future work might focus on more directly studying the changes in mCRP expression through carcinogenesis and progression in a large series and the power of

mCRP expression to predict response to immunotherapeutic and conventional medical treatments.

Chapter 8: General Discussion

Ovarian cancer is the fourth most common cancer in British women, and accounted for over 6000 new cancer registrations in 1997 and over 4000 deaths in 1999. Despite improvements in life-extending treatments, its high mortality has remained static over recent decades, in contrast to what has been seen in breast cancer (Quinn 2001). This is due to a persisting combination of late stage at diagnosis and tendency toward the development of resistance to conventional chemotherapy (Heintz, Odicino et al. 2001; Ozols, Bundy et al. 2003). Though making diagnoses earlier and achieving "stage shift" will theoretically do most to improve outcomes, this requires the establishment of a mass screening programme, the precise methodology and efficacy of which are yet to be defined (Jacobs, Mackay et al. 2006). Improvements in treatment are therefore also required, with the systematic investigation of molecular markers expressed in tissue having the potential to aid drug development and the targeting and tailoring of therapy through the discovery of independent markers of prognosis – as has been the case for Herceptin in breast cancer (Shepard, Jin et al. 2008). The impact of Herceptin was assessed in recurrent ovarian cancer and, though little clinical impact was seen for this mAb (Bookman, Darcy et al. 2003), immunotherapy remains particularly theoretically suited for use in ovarian cancer as it circumvents traditional mechanisms of chemoresistance and targets microscopic residual disease which may be outside the cell cycle (Bjorge, Stoiber et al. 2006). There is evidence, however, that the immune system edits ovarian cancer which may make it less susceptible to de novo and artificial immune attack, with escape mechanisms likely to be directed against both the cell mediated and humoural modalities (Schuster, Nechansky et al. 2006). Two potential mechanisms of escaping immune attack might include the downregulation of HLA class I and the expression of mCRPs (Dunn, Old et al. 2004), the expression of which have both been shown to have independent prognostic power in colorectal cancer (Watson, Durrant et

al. 2006; Watson, Ramage et al. 2006). It was therefore logical in this thesis to study the biomarker potential of HLA class I and mCRP expression in ovarian cancer tissue. Interestingly, the role of the immune system was overlooked by the landmark paper by Hanahan et al which reviewed the fundamental steps in carcinogenesis (Hanahan and Weinberg 2000) and this probably reflects the fact that the theory of immunosurveillance had historically been discredited while its resurgence, in the form of immunoediting, was yet to be widely appreciated (Dunn, Bruce et al. 2002). The importance of the immune system in both the development of cancer and its treatment has subsequently led to the suggestion that immunoediting be termed the seventh hallmark of cancer (Smyth, Dunn et al. 2006).

Despite the surfeit of studies examining tissue biomarkers in ovarian cancer, no immunohistochemical marker has yet been translated into clinical practice (Agarwal and Kaye 2006). Part of the reason for this is that many single, small studies are often unrepeatable or examine markers of limited clinical relevance (Gospodarowicz 2001). Difficulties in externally validating research often stem from inconsistencies in approach, a point which has been emphasised each time the literature surrounding a marker has been reviewed in this thesis. Attempts to standardise the reporting of research by developing consensus statements have been made for randomised trials (CONSORT - Consolidated Standards of Reporting Trials) (Moher, Schulz et al. 2001), diagnostic tests (STARD - Standards for Reporting of Diagnostic Accuracy) (Bossuyt, Reitsma et al. 2003) and, latterly, molecular markers of prognosis (REMARK - Reporting recommendations for tumour marker prognostic studies) (McShane, Altman et al. 2006). This thesis attempted to follow these guidelines closely so that the results would have greater reproducibility and so that the data would be useable in potential future meta-analyses. To this end, the cohort that was gathered for this study has been described in detail with explanations being given as to which cases were excluded and why, along with a description of how treatments were allocated. The samples

used, their processing, their storage and their histological review have been described and the assays used, which have been accurately recorded, sought to replicate the most common practices found within the literature. The assessment of the staining was consistent, blinded and concordant. The study design has been described in some depth as have the endpoints (OS – no data on PFS), the candidate variables in the multivariate model and the rationale for the sample size (related to power calculations and convenience). All statistical methods have been described along with any limitations, assumptions and missing data issues. A detailed description of how the data were re-categorised along with the logic behind the creation of cut-off points has been given. The data have been thoroughly assessed and a diagram of flow through the study produced showing the numbers of cases dropping out at each stage, along with the clinicopathological and demographic characteristics of each subgroup. The relation of the markers to standard prognostic variables has been recorded and an estimation of sizes of effect using HR has been reported along with Kaplan Meier plots checking the assumption of proportional hazards. All confidence intervals have been reported for all variables in the final multivariate model. Importantly, an attempt has been made at all points to emphasise the limitations of the work and to set it in its clinical context. Studying a large population and addressing best practice in this way supports the main findings of the thesis.

TMAs have become established as a valid high throughput platform for the study of biomarkers in tissue and may be used in researching genomics, transcriptomics and proteomics (Kononen, Bubendorf et al. 1998). The cohort available for this thesis numbered in excess of 400 cases and this technique represented an attractive method of reducing the amount of antibody which would be required whilst also allowing all tumours to be stained in single standardised runs (Kononen, Bubendorf et al. 1998). The physical construction of the TMA, as has been found by others, represented a small amount of work compared to that involved in assembling the appropriate data and tissue for this work

(Simon, Mirlacher et al. 2004). Logistical, ethical and bureaucratic considerations occasionally introduced errors and delays, though these were often anticipated and managed, with the study finally being completed to time and with no major systematic errors detected. By fostering a productive relationship with the R&D department at DCGH, this thesis was also able to adhere to best practice regarding research governance issues. Since the inception of TMAs, concerns regarding tissue heterogeneity and the ability of a tissue core to be representative of a whole tumour have been recorded (Kononen, Bubendorf et al. 1998; Goethals, Perneel et al. 2006). This concern was thoroughly addressed during an extensive validation process for TMAs and has mostly been shown to be unfounded where large populations have been surveyed (Kononen, Bubendorf et al. 1998; Mucci, Akdas et al. 2000; Fernebro, Dictor et al. 2002; Hedvat, Hegde et al. 2002). However, there is variation between researchers regarding the optimal number of cores to use per case, with between one and 5 being variously employed. In this thesis the use of two cores was shown to be optimal in balancing the loss of cases and statistical power due to loss of tissue, the need to conserve resources and the improved accuracy seen when using higher numbers of cores. An accurate and complete database of the main clinical prognostic factors and outcome measures was essential in providing reassurance that this series was typical whilst also fundamental to the investigation of associations between markers and outcome. The dataset was found to be consistent and the multivariate model developed included the factors which may have been anticipated a priori: stage, residual disease, the use of chemotherapy and age. Though the data were consistent, they were limited inasmuch as no objective response to chemotherapy had been recorded. Showing that an independent prognostic marker mediates its effect through response to treatment provides important biological insights and can prove useful in the design of clinical trials and potentially in the tailoring of treatments. Though this was not correctable in this thesis, the impact of its absence may perhaps at

least be attenuated by the fact that no replacement first-line treatments for ovarian cancer are yet being considered (Agarwal and Kaye 2005).

As no tissue biomarker for ovarian cancer has yet been introduced into clinical practice, this could not be used to validate the integration of the TMA and clinicopathological data (Agarwal and Kaye 2006). Notwithstanding this, several individual studies have been published which show particular markers as having independent prognostic factors in ovarian cancer (Kristiansen, Denkert et al. 2002; Yamamoto, Tsuda et al. 2007). The known difficulty in replicating the findings of single studies in biomarker research meant that attempting this for validation of the integrated TMA was an unattractive strategy with a high chance of failure. It therefore appeared more logical to study the expression of p53 and Bcl-2 for this purpose, as they have been exhaustively examined in cancer research at all sites. As the literature is hopelessly divided on the clinical relevance of the expression of these markers in ovarian cancer, this strategy had the disadvantage of being inherently unable to provide 100% assurance as to the validity of the TMA. However, the advantage of using these markers was that the large amount of data available made it possible to develop a broad perspective concerning which associations should be found most commonly. Duplicating these associations was likely to provide more accurate validation than duplicating single studies, where the false positive and negative correlation rates would be high. The integrated TMA ultimately produced data supportive of its validity by reproducing the most common findings within the body of literature reviewed concerning p53 and Bcl-2.

On examining the prognostic effect of immunological molecular markers, deviation from the co-expression of HC-10 and β_2m was found to occur in 65% of cases and correlated independently with reduced survival. The conclusion is that loss of a phenotype compatible with successful antigen presentation is an immune escape mechanism correlated with poor prognosis. This novel finding agrees with data collected at other cancer sites and preliminary work in ovarian cancer,

is intuitive and provides evidence that immunoediting occurs in ovarian cancer (Vitale, Pelusi et al. 2005; Watson, Ramage et al. 2006). It also has implications for the targeting and development of immunotherapies heavily reliant on CD8+ cells for their clinical effect, such as vaccines. However, such conclusions must be interpreted in the light of certain caveats. Firstly, as HC-10 does not identify all HLA class I haplotypes or allelic variations and, as the patients' individual tissue types were not known, it is conceivable that there was a pool of cases within the "Downregulated HLA class I" group which continued to express a phenotype compatible with successful antigen presentation. Unfortunately, a satisfactory panel of antibodies with the required specificities does not exist with which to exhaustively test this theory immunohistochemically on FFPE tissue (Cabrera, Lopez-Nevot et al. 2003) and it remains plausible that the identification of such cases may have resulted in either an augmentation or attenuation of the prognostic effects revealed by this work. Secondly, though the HC-10 (+) β_2m (+) phenotype represented the immunohistochemical expression of a phenotype compatible with intact Antigen Processing Machinery (APM), it did not confirm that antigen presentation was actually occurring, for which functional studies would be required. Despite these methodological constraints, the independent association of the HC-10 (+) β_2m (+) phenotype with improved prognosis supports other work on immunoediting in ovarian cancer while implying that this phenotype approximates the identification of functional/non-functional HLA class I well (Vitale, Pelusi et al. 2005). Han and co-workers recently reported the results of studying several APM elements using immunohistochemistry, including HC-10, β_2m, TAP1, TAP2 and tapsin, within a cohort of 150 epithelial ovarian cancers. Though the co-expression immunophenotypes were not studied, the individual expression data were concordant with that presented in this thesis. The loss of individual APM elements correlated with each other, with a state of loss of all APM elements being shown to be an independent prognostic factor. Though these findings validate the observations in this thesis, this group also discovered that a loss of APM elements

correlated with a reduction in TILs, with the presence of TILs being found to be a positive independent prognostic factor, as in other studies (Han, Fletcher et al. 2008). The use of the TMA constructed in this thesis to study the prognostic effect of TILs would therefore represent an interesting future application, as TILs have not yet been surveyed in detail in a large population of ovarian cancers using this technique. As proof of principle, this platform has been used to study TILs in breast and colorectal cancer, (Pages, Berger et al. 2005; Theurillat, Ingold et al. 2007). As the absence of intra-tumoral T cells is related to higher levels of serum VEGF, acquiring these data would also allow cross comparison with recent data generated by other workers using this TMA which shows that high VEGFR expression is a negative independent prognostic factor in ovarian cancer (Cooper, Ritchie et al. 2002; Duncan, Al-Attar et al. 2008).

IFNɣ has been shown to upregulate HLA class I and improve CD8+ recognition (Raffaghello, Prigione et al. 2005) and though it also has the effect of lowering NK cell cytotoxicity, it is interesting to note that recent work on the TMA constructed during this thesis by other workers has shown that loss of the IFNɣ receptor is associated with a poor prognosis (Duncan, Rolland et al. 2007). This may indicate a limited role for NK cells in ovarian cancer systems, reflect the multiple anti-tumour effects of intact IFNɣ signalling, or both. A minor role for NK cell killing in ovarian cancer might be explained if concurrent immune escape mechanisms develop in parallel with HLA class I loss which abrogates the NK stimulating effect of this state of “missing self”. One such mechanism might be through the inappropriate expression of non-classical class I HLA molecules such as HLA-G. The expression of this molecule is normally restricted to a small number of cell types which includes trophoblastic cells. However, it is abnormally expressed in up to 70% of ovarian cancers, where it has been shown to inhibit NK cell lysis though not predicting prognosis independently of other factors (Lin, Yan et al. 2007) (Sheu and Shih Ie 2007). Investigating the expression of this marker in tandem with TILs and the existent HLA

class I data could provide interesting insights into the nature of immunoediting in this large population of ovarian cancers. Other non-classical HLA markers which might contribute to improved understanding if studied might include the MICA and MICB. These markers have been little studied in ovarian cancer but may represent an additional way in which stressed cells can stimulate the immune system in the absence of HLA class I: through the binding of NK cells via the NKG2D receptor and the binding of γδ T cells via their TCR (Bauer, Groh et al. 1999; Qi, Zhang et al. 2003). Indeed, relative over-expression of MICA has been shown to be a positive independent prognostic factor in colorectal cancer, though similar observations have not been made in breast cancer (Madjd 2005; Watson, Spendlove et al. 2006). Intriguingly, bacterial adhesins released by diarrhoea inducing Escherichia coli strains have been shown to upregulate MICA in normal gastrointestinal epithelium by signalling via CD55, in one of its non-complement dependent roles, justifying its future study further in this series (Tieng, Le Bouguenec et al. 2002).

The study of the co-expression of mCRPs and their prognostic effect in a large population of ovarian cancers was a novel undertaking and generated important population data. However, the prognostic effects, though consistent with the theory that mCRP expression represents an immune escape mechanism, were of borderline significance. Carcinogenesis is generally felt to be associated with an increase in mCRP expression and function, with the upregulation of one mCRP potentially being able to cover for loss of another (Koretz, Bruderlein et al. 1993; Gorter and Meri 1999). The ubiquitous expression of the mCRP in this series supports this assertion and may also represent a further mechanism of resistance to chemotherapy. Conventional chemotherapy is thought to effect cell death through a sterile process of apoptosis. During this process, apoptotic bodies are removed by macrophages which secrete powerful mediators in order to prevent the overwhelming inflammation and potential auto-immunity that might otherwise ensue. However, evidence is accumulating that

chemotherapeutic cytotoxicity is, in fact, immunogenic with both innate and adaptive immune systems being implicated along with the triggering of a systemic inflammatory response (Zitvogel, Apetoh et al. 2008). mCRP expression may therefore attenuate the local effects of inflammation on cancer cells and may thereby be linked to responsiveness to conventional chemotherapy as well as immunotherapy. Unfortunately, methodological constraints prevented the testing of this theory in this series but it could be studied in series with access to PFS data.

The TMA constructed and validated in this thesis was used to study molecular markers in a novel way and provides new data. Though these studies are individual, the use of a large number of specimens and an adherence to best practice provides reassurance that the findings are correct, while setting a standard that if repeated by other groups may ensure that the literature does not become a mass of contradictory reports. The strength of this approach is that, once the integrated TMA was produced, expression data could be assessed for multiple markers in a homogenous, high-throughput manner which was systematic and which used resources efficiently. Indeed, subsequently it has been used by other workers in the study of a variety of markers. Once a larger number of molecular markers have been assembled it may become possible to employ sophisticated bioinformatics that include hierarchical clustering and Artificial Neural Networks (ANNs) in order to identify new prognostic groups using the co-expression data (Gospodarowicz 2001). Ellis and co-workers employed this technique in a large series of breast cancers. Using expression data for 25 markers, this group discovered 5 new prognostic groups defined by the expression of 9 key antigens (Abd El-Rehim, Ball et al. 2005). The molecular characterisation of ovarian cancer in this and other ways is likely to be crucial to the aim of mapping the proteomic circuit within the cell and the overall aim of oncological care: the development of treatment specific to an individual's disease (Calvo, Liotta et al. 2005).

Appendices

Appendix 1 The World Health Organisation International Histological Classification of tumours N°9
Histological Typing of Ovarian Tumours, World Health Organisation, 1973 (Serov, Scully et al. 1973) – abridged.

I. Common "Epithelial" Tumours

A. Serous Tumours

1. Benign
   a) Cystadenoma and papillary cystadenoma
   b) Surface papilloma
   c) Adenofibroma and cystadenofibroma
2. Carcinomas of low malignant potential (borderline)
   a) Cystadenoma and papillary cystadenoma
   b) Surface papilloma
   c) Adenofibroma and cystadenofibroma
3. Malignant
   a) Adenocarcinoma, papillary adenocarcinomas and papillary cystadenocarcinoma
   b) Surface papillary carcinoma
   c) Malignant adenofibroma and cystadenofibroma

B. Mucinous Tumours

1. Benign
   a) Cystadenoma
   b) Adenofibroma and cystadenofibroma
2. Carcinomas of low malignant potential (borderline)
   a) Cystadenoma
   b) Adenofibroma and cystadenofibroma
3. Malignant
   a) Adenocarcinoma and cystadenocarcinoma
   b) Malignant adenofibroma and cystadenofibroma

C. Endometrioid Tumours

1. Benign
    a) Adenoma and cystadenoma
    b) Adenofibroma and cystadenofibroma
2. Carcinomas of low malignant potential (borderline)
    a) Adenoma and cystadenoma
    b) Adenofibroma and cystadenofibroma
3. Malignant
    a) Carcinoma
        (1) Adenocarcinoma
        (2) Adenocanthoma
        (3) Malignant adenofibroma and cystadenofibroma
    b) Endometrioid stromal sarcomas
    c) Mesodermal Müllerian Mixed Tumours (Homologous / Heterologous)

D. Clear Cell Tumours

1. Benign: Adenofibroma
2. Carcinomas of low malignant potential (borderline)
3. Malignant: Carcinoma and adenocarcinomas

E. Brenner Tumours

1. Benign
2. Of borderline malignancy (proliferating)
3. Malignant

F. Mixed Epithelial Tumours

1. Benign
2. Of borderline malignancy
3. Malignant

G. Undifferentiated carcinoma

H. Unclassified epithelial tumours

II. Sex Cord Stromal Tumours

A. Granulosa - stromal tumours

1. Granulosa cell tumour
2. Tumours in the thecoma - fibroma group
   a) Thecoma
   b) Fibroma
   c) Unclassified

B. Androblastomas: Sertoli - Leydig cell tumours

1. Well differentiated
   a) Tubular androblastoma: Sertoli cell tumour
   b) Tubular androblastoma with lipid storage: Sertoli cell tumour with lipid storage
   c) Sertoli-Leydig cell tumour
   d) Leydig cell tumour: Hilus cell tumour
2. Of intermediate differentiation
3. Poorly differentiated (sarcomatoid)
4. With heterologous elements

C. Gynadoblastoma

D. Unclassified

III. Lipid (Lipoid) Cell Tumours

IV. Germ Cell Tumours

A. Dysgerminoma

B. Endodermal sinus tumour

C. Embryonal Carcinoma

D. Polyembryoma

E. Choriocarcinoma

F. Teratoma

1. Immature

2. Mature

a) Solid

b) Cystic

(1) Dermoid cyst (Mature cystic teratoma)

(2) Dermoid cyst with malignant transformation

c) Monodermal and highly specialised

(1) Struma Ovarii

(2) Carcinoid

(3) Struma Ovarii and carcinoid

(4) Others

G. Mixed forms

V. Gonadoblastoma

A. Pure

B. Mixed with dysgerminoma or other form of germ cell tumour

VI. Soft tissue tumours not specific to the ovary

VII. Unclassified tumours

VIII. Secondary (Metastatic) Tumours

IX. Tumour-Like conditions

A. Pregnancy luteoma

B. Hyperplasia of ovarian stroma and hyperthecosis

C. Massive oedema

D. Solitary follicle cyst and corpus luteum cyst

E. Multiple follicle cyst (polycystic ovaries)

F. Multiple luteinised follicle cysts and/or corpus lutea

G. Endometriosis

H. Surface-epithelial inclusion cysts (germinal inclusion cysts)

I. Simple cysts

J. Inflammatory lesions

K. Parovarian cysts

Appendix 2 Staging of ovarian cancer by the FIGO and TNM classifications (Benedet 2000)

FIGO			TNM	
		Primary tumour cannot be assessed		**TX**
0		No evidence of primary tumour		**T0**
I		**Tumour confined to the ovaries**		**T1**
	IA	Tumour limited to one ovary, capsule intact No tumour on ovarian surface No malignant cells in ascites or peritoneal washings	T1a	
	IIB	Tumour limited to both ovaries, capsules intact No tumour on ovarian surface No malignant cells in ascites or peritoneal washings	T1b	
	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	T1c	
II		**Tumour involves one or both ovaries with pelvic extension**		**T2**
	IIA	Extension and/ or implants in uterus and/or tubes No malignant cells in the ascites or peritoneal washings	T2a	
	IIB	Extension to other pelvic organs No malignant cells in the ascites or peritoneal washings	T2b	
	IIC	IIA/B with positive malignant cells in the ascites or positive peritoneal washings	T2c	
III		**Tumour involves one or both ovaries with microscopically confirmed peritoneal metastasis outside the pelvis and/or regional lymph node metastasis**		**T3 or N1**
	IIIA	Microscopic peritoneal metastasis beyond the pelvis	T3a	
	IIIB	Macroscopic peritoneal metastasis beyond the pelvis 2cm or less in dimension	T3b	
	IIIC	Peritoneal metastases beyond the pelvis more than 2cm in greatest dimension and/or regional lymph node metastasis	T3c or N1	
IV		**Distant metastasis beyond the peritoneal cavity** (including parenchymal liver metastasis and pleural effusion with positive cytology)		**M1**

	Regional Nodes
Regional lymph nodes cannot be assessed	**Nx**
No regional lymph node metastasis	**N0**
Regional lymph node metastasis	**N1**

	Distant Metastasis	**Mx**
	Distant metastasis cannot be assessed	**M0**
	No distant metastasis	**M1**
	Distant metastasis (excluding peritoneal metastasis)	

Appendix 3
1 of 5

A record of all the data attributes contained within the final analysis database. A total of 339 frank ovarian cancer cases and 19 borderline cases were represented. Emboldened data columns represent data extracted from the original study database. Attributes used in the statistical analyses are also highlighted.

Data	Column	Data type	Categories	Missing	Used in final analyses	Notes
Identifying numbers	ANO (Array number)	Continuous	1-360	0	**Yes**	
	PATHNUMBER	Ordinal	6*****82 – H97****7	0	No	From the pathology form
	ARRAY COORDINATES	Ordinal	1:A1 – 4:N3	0		Derived from and present on the scoring grids
Date treatment initiated	**DATEOFOP** (operation)	Ordinal	*/02/1982 – */10/1997	0		Used to in determining overall survival but could be removed
	YEAROFOP	Continuous	1992 - 1997	0		Derived from DATEOFOP
	YEARDIAGNOSED	Continuous	1992 - 1997	0		Derived from pathology form
Age diagnosis made	**AGEDIAGNOSED**	Continuous	24 - 90	1	**Yes**	
	ageORIGINAL	Ordinal	1: < 54 years at diagnosis 2: 54-70 years at diagnosis 3: ≥ 70 years at diagnosis	1	No	First empirical subdivision of age into groupings
	agerecodeSEER	Ordinal	1:< 30 years at diagnosis 2: 30-59 years at diagnosis 3: ≥ 60 years at diagnosis	1	**Yes**	Age regrouped into categories used by SEER
	agerecodeICON	Ordinal	1: < 55 years at diagnosis 2: 55-65 years at diagnosis 3: ≥ 65 years at diagnosis	1	No	Age re-categorised into categories used in the ICON trials
	agerecodeEUROPE	Ordinal	1: <40 years at diagnosis 2: 40-60 years at diagnosis 3: ≥ 60 years at diagnosis	1		Groupings used by a small European series (Brun, Feyler et al. 2000)

Data	Column	Data type	Categories	Missing	Used in final analyses	Notes
Operating consultant's specialty	**SPECIALISTSURGEON**	Nominal	General surgeon General gynaecologist Gynae-oncologist	39	No	The importance of operator is that they have an interest specifically in gynaecological cancer
	specialistsurgeonrecode	Categorical	0: Non-Gynae-oncologist 1:Gynaeoncologist	39		
Stage of disease	**STAGE**	Ordinal	1 :Stage I 2: Stage II 3: Stage III 4: Stage IV	9	**Yes**	Staged according to FIGO classification
	SUBSTAGE	Nominal	Stage Ia, Ib, Ic Stage IIa, IIb, IIc Stage IIIa, IIIb, IIIc Stage IV	116	No	The sub-stage category was incomplete and of uncertain accuracy.
Operation performed	OPERATION oprecode	Categorical	1: Biopsy 2: Single oophrectomy 3: Bilateral oophrectomy (BSO) 4: Total hysterectomy (TH) + BSO 5: TH + BSO + Omentectomy	0		Data derived from the original pathology report and reflective of this more than radicalness of surgery
Residual disease	**RESIDUAL DISEASE**	Nominal	> 2cm < 2cm Nothing	13		Residual disease was re-categorised on a more stringent criteria whereby any visible remaining disease is considered sub-optimally debulked
	residualrecodeMACRO	Ordinal	0: Macroscopic disease present 1: No macroscopic disease present	13	**Yes**	

Data	Column	Data type	Categories	Missing	Used in final analyses	Notes
Tissue reviewed by pathologist	tissuesourcerecodeRAW	Nominal	1: Omental metastasis 2: Pelvic Metastasis 3: Ovary	1		Collected at the time of contemporaneous review of the tumours to help explain any unusual results should they occur due to the potential differential expression of markers between primary and secondary sources of tissue
Tumour cell type	TUMOURTYPE	Nominal	Serous Serous papillary Endometrioid Mucinous Undifferentiated Clear cell Granulosa cell Mixed Müllerian cell Teratoma Yolk sac	0	No	The nominal data in the study database were replaced with that from the contemporaneous review and were logically re-categorised, following which a survival analysis performed. The categories were then ranked in order of increasingly poor prognosis with borderline cases excluded (which had the best prognosis of all) from the final analysis database
	tumourtype-recodeBESTWORST	Ordinal	1: Clear cell 2: Mucinous 3: Endometrioid 4: Serous 5: Undifferentiated 6: other	0	**Yes**	

Appendix 3 Continued
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Data	Column	Data type	Categories	Missing	Used in final analyses	Notes
Tumour grade	TUMOURGRADE	Ordinal	0:Ungradeable 1: Grade 1 2: Grade 2 3: Grade 3	0	**Yes**	The study database data were replaced by the data from the contemporaneous review
Post-operative medical therapy	**ADJUVANTTHERAPY**	Nominal	CARBO&TAXOL (Carboplatin and paclitaxel) CARBOPLATIN NON-PLATINUM (Chemotherapy) RADIOTHERAPY TAXOL (Paclitaxel)	7	No	As explained formally in chapter 2, the optimal re-categorisation of data concerning adjuvant treatment in this series was whether the case had received chemotherapy or not
	adjuvantrecodePLAT	Categorical	1: No chemotherapy 2: Platinum based chemotherapy 3: Other chemotherapy 4: Other adjuvant treatment	7		
	adjuvantrecodeANYCHEMO	Ordinal	0: No chemotherapy received 1: Chemotherapy received	7	**Yes**	
Participation in trials	ICONTRIAL	Categorical	1: ICON I 2: ICON II 3: ICON III	0	No	Information concerning the ICON trial participation was obtained from the MRC before the database was unlinked

Appendix 3 Continued
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Data	Column	Data type	Categories	Missing	Essential for final analysis	Notes
Outcome data	**LASTFOLLOWUP**	Ordinal	*/03/1992 – */08/2005	N/A	No	LASTFOLLOWUP and DATEOFDEATH data each concerned those cases either alive or dead and so missing data in the latestsurvival column better reflects the completeness of these attributes in the database.
	DATEOFDEATH	Ordinal	*/05/1982 – */04/2004	N/A		
	LATESTSURVIVAL	Ordinal	*/05/1982 - */08/2005	3		Using the data above, the latest confirmed survival for each patient could be determined
	SURVIVALMONTHS	Continuous	0-271	3	**Yes**	Using DATEOFOP and LATESTSURVIVAL allowed survival data to be generated
	DEAD	Categorical	0: Alive 1: Dead	3	**Yes**	Knowing the survival status of the patient allowed data to be censored in the survival analyses
	survivalrecode5YEAR	Categorical	0: Died within 5 years of diagnosis 1: Survived 5 years after diagnosis	3	No	Data generated to aid comparison with other series
	survivalrecodePERIOP	Categorical	0: Survived beyond 30 days post – op 1: Died within days 30 post- op	3	No	Some authorities use 28 days

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