A STUDY TO INVESTIGATE THE IMMUNE RESPONSE IN OVARIAN CANCER PATIENTS TREATED WITH CHEMOTHERAPY

A thesis submitted for the degree of Imperial College MDres

for

Shreelata Datta

Division of Cancer, Department of Surgery and Cancer

Reproductive Biology and Clinical Obstetrics and Gynaecology

Imperial College London

IRDB Building, Hammersmith Campus, Du Cane Road, London W12 0NN

DECLARATION OF ORIGINALITY

The work presented here was undertaken by the author, Shreelata Datta, unless stated otherwise in the text. The research was performed at the Department of Surgery and Cancer, Hammersmith Campus, Imperial College London. All external sources and contributions from other individuals have been duly acknowledged.

COPYRIGHT DECLARATION

ACKNOWLEDGEMENTS

The support and advice from my supervisors has been unrivalled. My deepest gratitude goes to my supervisors, Dr Sadaf Ghaem-Maghami and Dr Thilianagan. I was extremely grateful to receive their expert advice and constructive comments. I am thankful to Dr Sadaf Ghaem-Maghami for her scientific expertise, critical thinking and constant encouragement which have carried me through the study.

I wish to thank Jay Chatterjee, Lynsey Whilding, John Wahba and Pei Yin Teo for their help throughout this project, as well as Marina Natoli and Kirsty Flower for their review of this thesis. Thank you for sharing the FACs anxiety, computers and all the unforgettable moments in the office and the lab.

This study was supported by the British Medical Association and The Vandervell Foundation and I am very grateful for their belief in the research that I am conducting as well as their faith in me. I wish to acknowledge each of the individuals who participated in this research, whose support and continued optimism spurred me on when times were tough. My warm thanks to the staff at the Hammersmith Oncology clinic for their kind and cheerful assistance in practical matters such as database access.

My heartfelt thanks go to my family and friends who have supported me every step of the way from formulating experiments to writing up this thesis. More than anything, I must thank Harri Jaaskelainen, who within eighteen months has propelled my research further than I could ever imagined.

ABSTRACT

Women with ovarian cancer usually present with advanced stage disease and, although sensitive to chemotherapy initially, the majority will relapse following first line treatment. Resistance to chemotherapy contributes to poor prognosis in ovarian cancer. A potential strategy to prolong the response to treatment and reduce chemotherapy resistance is immune based therapies. These therapies are likely to be most effective when disease is minimal or subclinical, such as at completion of first line chemotherapy, but depend on the ability to mobilize a sufficient immune response.

Currently, little is known about the immune competence of ovarian cancer patients undergoing chemotherapy or upon its completion. The aim of this study was twofold. By measuring the cellular and humoral immune response to Hepatitis B vaccination we evaluated the immune competence of women once they had completed first-line chemotherapy for ovarian cancer. This provided important information about the optimal time of delivery and likely efficacy of future immune therapeutic strategies, suggesting a significant difference in B cell response between patients treated with chemotherapy and healthy age-matched control patients. A vaccine was used because it essentially replaces an initial infection but still leads to the generation of memory B cells.

Secondly, by quantifying peripheral blood mononuclear cell levels of the immune checkpoint inhibitory receptor PD-1 (in lymphocytes) and its ligand, PD-L1 (in monocytes) in ovarian cancer patients undergoing and completing chemotherapy, this study investigated the possibility of using PD-1 and PD-L1 as a biomarker in ovarian cancer treatment. Our results found a significant difference in lymphocytic PD-1 T cell count between patients who had recently completed chemotherapy and those who had completed chemotherapy over a year ago. This suggests further work to characterize lymphocytic PD1 changes after chemotherapy treatment is warranted, to optimize the timing of the delivery of future immunotherapy.

CONTENTS

Declaration 2
Copyright declaration 2
Acknowledgements 3
Abstract 4
Contents 5
List of tables 8
List of figures 9
Abbreviations 14
1. Introduction 17
1.1 Ovarian cancer 18
1.1.1 Epidemiology and natural history
1.1.2 Treatment
1.2 The immune environment in ovarian cancer 24
1.3 Immunotherapy in ovarian cancer 26
1.4 Immune response in advanced ovarian cancer following chemotherapy 31
1.5 Hepatitis B and the hepatitis B vaccine 32
1.6 Use of biomarkers in ovarian cancer 37
1.7 PD-1 and its ligands, PD-L1 and PD-L2 39
1.7.1 PD-1 and its ligands in ovarian cancer
1.8 Summary 41
1.9 Aims and objectives 42
2. Materials and methods 44
2.1 HBV vaccination study 44
2.1.1 Recruitment to vaccination study
2.1.1.1 Inclusion criteria
2.1.1.2 Exclusion criteria
2.1.2 Study protocol
2.1.3 Study endpoints

2.2 Recruitment of patients undergoing chemotherapy 49
2.2.1 Study endpoint
2.3 Data collection schedule 49
2.4 Laboratory Methodology 50
2.4.1 Buffers and solutions
2.4.2 Isolation of Human Peripheral Blood Mononuclear Cells
2.4.3 HLA-A2 tissue typing
2.4.4 Flow cytometry staining
2.4.5 Intracellular staining
2.4.5.1 Buffers and solutions
2.4.5.2 Method
2.4.6 T cell culture
2.4.7 ELISA sandwich detection of soluble free PD-L1
2.4.7.1 Buffers and materials
2.4.7.2 Assay materials
2.4.7.3 Method
2.4.7.3.1 Initial standardization
2.4.7.3.2 Assay procedure
2.5 Statistics and data analysis 57
2.5.1 Power calculation for vaccination study
2.5.2 Statistical analysis
3. Results 58
3.1 Hepatitis B serum antibody levels through the vaccination schedule 58
3.1.1 Aims
3.1.2 Patient characteristics
3.1.3 Blood markers for Group A and B participants
3.1.4 Serum antibody response for Group A and B participants
3.1.5 T cell response to vaccination
3.1.5.1 HLA-A2 typing
3.1.5.2 CD3+CD8+pentamer T cell response

3.1.5.3 CD19-pentamer T cell response
3.1.5.4 CD3+CD8+ pentamer T cell response following stimulation
3.1.6 CD45 RO/RA isoform expression
3.1.7 CD4/CD25/FOXP3 expression
3.2 PD-L1 and PD-1 as prognostic markers during chemotherapy 91
3.2.1 PD-L1 levels in the PBMC monocyte population
3.2.2 Quantification of PD-L1 levels in plasma
4. Discussion 103
4.1 Hepatitis B vaccination 103
4.2 PD-L1 as a prognostic marker 111
4.3 Summary and conclusion 115
5. Future work 116
Appendix 1: Flow cytometry gating 117
Appendix 2: Standard curve for ELISA 119
6. References 120

LIST OF TABLES

Table 1.1 Findings from phase I-II clinical trials of vaccine-based immunotherapy for ovarian cancer

Table 2.1 Buffers and solutions used in this project

Table 2.2 Antibodies used to stain cells for flow cytometry

Table 2.3 Isotype control antibodies used for flow cytometry

Table 2.4 Antibodies used as capture antibody for PD-L1 ELISA

Table 2.5 Antibodies used as detection antibody for PD-L1 ELISA

Table 2.6 PD-L1 recombinant proteins used to generate standard curve for PD-L1 ELISA

Table 3.1 Summary of pathology and management in Group A and Group B patients

Table 3.2 Summary of pathology and management in Group A and Group B patients who relapsed; in all cases, cancer type was ovarian.

Table 3.3 Hepatitis B serum antibody response to vaccination at month 6 in patients successfully vaccinated at the 6 month timepoint in tabular format. Results obtained in Group A and Group B participants was compared with historical control patients outlined in De Rave et al, 1994

Table 3.4 Stage of ovarian cancer in patients undergoing chemotherapy

Table 3.5 Summary of ovarian cancer subtype in participants undergoing chemotherapy

LIST OF FIGURES

Figure 1.1 Structure of the Hepatitis B virus

Figure 1.2 Serum antigen and antibody levels in relation to HBV infection

Figure 1.3 Anti-Hepatitis B antibody seroprotection rates and geometric mean titres among subjects following vaccination by interval from first dose using standard schedule monovalent hepatitis B vaccination

Figure 1.4 Potential cancer immunotherapy targets in the PD-L1 and PD-L2 pathway

Figure 2.1 Flowchart to show schedule of vaccination and sample collection:

Figure 2.2 Visit schedule undertaken by recruited participants and their group allocation.

Figure 3.1 Study recruitment, population and clinical outcomes of the study population

Figure 3.2 Stage of ovarian cancer in Group A and Group B patients

Figure 3.3 Serum CA125 level for Group A and Group B participants at the point of recruitment and end of study

Figure 3.4 Serum markers (white cell count, neutrophil, lymphocyte and monocytes count) for Group A and Group B participants at the point of recruitment and end of study

Figure 3.5 HBsAb levels obtained at months 2, 6 and 7 for a successfully vaccinated Group B participant in comparison to the serum antibody titre prior to receiving HBV vaccination (D0)

Figure 3.6 Serum antibody titres at the time of final vaccination in recently completed chemotherapy patients (Group B) and in women who had completed chemotherapy over a year ago (Group A)

Figure 3.7 Serum antibody titres at the time of final vaccination for women already successfully immune in recently completed chemotherapy patients (Group B) and in women who had completed chemotherapy over a year ago (Group A)

Figure 3.8 Geometric mean titres in responders obtained in Group A and Group B participants at the time of final vaccination in relation to those obtained in historical controls (De Rave, 1994)

Figure 3.9 Final HB serum antibody levels one month after completing the vaccination course in women in Group A and Group B. Bars indicate the arithmetic mean and standard deviation for each group; HBsAb levels above 10IU/L correlate with HBV immunity

Figure 3.9a Final HBs serum antibody levels one month after completing the vaccination course in full responders in Group A and Group B (i.e. HBsAb levels above 10IU/L only)

Figure 3.10 Hepatitis B serum antibody response to vaccination at month 7 in patients successfully vaccinated at the 7 month timepoint

Figure 3.11 Geometric mean titres in responders obtained in Group A and Group B participants at month 7 in relation to those obtained in historical controls (De Rave, 1994)

Figure 3.12 Hepatitis B serum antibody response to vaccination at month 7 when compared to CA125 serum levels, taken at the end of the study

Figure 3.13 Histogram from flow cytometry staining

Figure 3.14a FACS cytometry staining of CD3+CD8+ T cell population

Figure 3.14b FACs cytometry staining of CD3+CD8+ pentamer+ T cell population

Figure 3.15a FACS cytometry staining of the CD3+CD8 T cell population at month 6 for both Group A and Group B participants

Figure 3.15b Isolation of CD3+CD8 T cell population using FACS cytometry at month 7 for both Group A and Group B participants

Figure 3.16 FACS cytometry staining

Figure 3.17 Summary of percentage CD3+CD8+pentamer+ T cells seen on the lymphocyte gate in a Group A patient from recruitment to month 7

Figure 3.18 Percentage of CD3+CD8+pentamer+ T lymphocytes obtained in Group A and Group B patients at the point of final vaccination, on the lymphocyte gate

Figure 3.19 Percentage of CD3+CD8+pentamer+ T lymphocytes obtained in Group A and Group B patients once the vaccination schedule was completed, on the lymphocyte gate

Figure 3.20 Percentage of CD45RO+ T lymphocytes obtained in Group A and Group B patients once the vaccination schedule was completed, on the lymphocyte gate using FACS cytometry

Figure 3.21 Percentage of CD45RA+ T lymphocytes obtained in Group A and Group B patients once the vaccination schedule was completed, on the lymphocyte gate using flow cytometry

Figure 3.22 Percentage of CD4+ T lymphocytes obtained in Group A and Group B patients once the vaccination schedule was completed, on the lymphocyte gate

Figure 3.23 Percentage of CD4+CD25+ T lymphocytes obtained in Group A and Group B patients once the vaccination schedule was completed, on the lymphocyte gate

Figure 3.24 Percentage of CD4+CD25+FOXP3+ T lymphocytes obtained in Group A and Group B patients once the vaccination schedule was completed, on the lymphocyte gate

Figure 3.25 FACS cytometry staining in a patient prior to undergoing chemotherapy with stage 3C endometrioid ovarian cancer

Figure 3.26 Lymphocyte population prior to each chemotherapy cycle in patients with ovarian cancer, analysed using flow cytometry

Figure 3.27 Change in monocytes population identified by flow cytometry in participants undergoing chemotherapy unpaired two tailed t test between chemotherapy cycles 1, 3/4 and 6 as well as post-chemotherapy

Figure 3.28 CD14 monocyte count for patients undergoing chemotherapy (cycles 1, 3/4 and 6) and post-chemotherapy

Figure 3.29 Percentage of CD14+PD-L1+ cells seen, on the monocytes gate in relation to chemotherapy – prior to cycle 1, at cycles 3 or 4 and at cycle 6 – as well as following chemotherapy

Figure 3.30 CD3+ lymphocyte population prior to chemotherapy cycle 1, 3/4 and 6 and post-chemotherapy

Figure 3.31 CD3+/PD1+ lymphocyte population prior to chemotherapy cycles 1, 3 or 4 and 6 as well as post-chemotherapy

Figure 3.32 CD14+/PDL1+ monocyte populations identified at the start and end of vaccination for Group A and B participants

Figure 3.32a CD14+/PDL1+ monocyte populations identified at the start and end of vaccination for Group A and B participants who did not relapse

Figure 3.33 CD3+/PD1 T cell populations identified at the start and end of vaccination for Group A and B participants

Figure 3.33a CD3+/PD1 T cell populations identified at the start and end of vaccination for Group A and B non-relapsing participants

Figure 3.34 ELISA quantification of soluble PD-L1 in plasma in patients prior to chemotherapy cycle 1, during (cycle 3/4 and 6) and after chemotherapy (“post-chemo”) in a subgroup of patients

ABBREVIATIONS

APC	Allophycocyanin
APCs	Antigen presenting cells
BCR	B cell receptor
BSA	Bovine Serum Albumin
CA125	Cancer antigen 125
CTLA-4	CTL-associated antigen-4
CT	Computed tomography
CTLs	Cytotoxic T lymphocytes
DCs	Dendritic cells
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
EOC	Epithelial ovarian cancer
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Foetal Calf Serum
FIGO	International Federation of Gynaecology and Obstetrics
FITC	Fluorescein isothiocyanate
FOXP3	Fork head helix transcription factor
GM-CSF	Granulocyte-macrophage colony stimulating factor 18
HBsAb	Hepatitis B serum antibody
HBsAg	Hepatitis B serum antigen
HBV	Hepatitis B virus
HLA-DR	Human leukocyte antigen-DR molecule
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL-1	Interleukin-1
IL-2	Interleukin-2

IL-2R	Interleukin-2 receptor
IL-7	Interleukin-7
ISPF	2C-Methyl-D-erythritol 2, 4-cyclodiphosphate synthase
ITIM	Immunoreceptor tyrosine–based inhibitory motif
iTregs	Induced T regulatory cells 17
ITSM	Immunoreceptor tyrosine–based switch motif
mABs	Monoclonal antibody
MDSCs	Myeloid derived suppressor cells
MHC	Major Histocompatibility complex
mRNA	Messenger Ribonucleic acid
NK	Natural killer
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PD-1	Programme death-1
PD-L1	Programme death-ligand 1
PD-L2	Programme death-ligand 2
PE	Phycoerythrin
RCC	Renal cell carcinoma
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
TALS	Tumour associated lymphocytes
TAMs	Tumour associated macrophages
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th	T helper
Th1	T helper 1
Th2	T helper 2
TILs	Tumour infiltrating lymphocytes
TLR-4	Toll like receptor-4
TNF	Tumour necrosis factor

Tregs	T regulatory cells
UK	United Kingdom
VEGF	Vascular endothelial growth factors
WHO	World Health Organization

1. INTRODUCTION

Ovarian cancer is the fifth most common cancer in women in the UK and the second most common gynaecological cancer. There are approximately 6,600 new cases diagnosed annually in the United Kingdom but despite advances in treatment, the prognosis remains poor with an overall five year survival rate of approximately 40%. Ovarian cancer is surgically staged using the FIGO classification (Prat and Oncology 2014), which is based on the volume and extent of tumour spread. Over 60% of patients present with advanced stage disease, which has a 22% five year survival rate for stage III disease and less than 6% for those with stage IV disease. Stage III tumours are characterized by spread outside the pelvis into the abdominal cavity or retroperitoneal lymphatic system, whilst stage IV tumours are characterized by liver parenchymal metastasis or other distant metastases.

Treatment for ovarian cancer usually involves a combination of surgery and chemotherapy. Surgical outcome, tumour stage, the presence of residual disease after surgical debulking and the response to chemotherapy contribute to the prognosis, but there is wide variability in progression-free and overall survival among patients with similar clinical and pathological characteristics (Bristow R et al, 2009). In addition, certain sub-types of ovarian cancer, such as clear cell cancers do not respond as well as serous and endometrioid cancers to platinum- and taxane based chemotherapy regimens (Hennessey B et al, 2009). However, whilst the disease is heterogeneous, both at clinical and molecular levels, the treatment approach to all patients is generally uniform (NICE 2011) and little has changed in prognosis over the last twenty years.

Protective immune responses have been associated with a better prognosis in ovarian cancer (Zhang L et al, 2003; Preston et al, 2011). Whilst an anti-tumour reaction is naturally elicited against ovarian cancer, these responses may be suppressed by regulatory processes – for example, regulatory T cells in individuals with ovarian cancer

suppress T cell immunity and contribute to tumour growth in vivo (Curiel T et al, 2004). Alternatively, the tumour itself may release factors which blunt an immune response.

Although immune responses influence prognosis in many tumours including ovarian cancer (Coneja-Carcia et al, 2004; Tomsova M, 2008), there is currently no effective immunological treatment. Cellular immunotherapy may offer the prospect of treatment to prevent or delay recurrent metastatic disease, through therapeutic vaccination or novel immune checkpoint inhibitors. Recent advances in immunology make immunotherapy a promising area for future research in the quest to find an effective treatment for ovarian cancer.

1.1 OVARIAN CANCER

1.1.1 Epidemiology and natural history

The peak incidence of ovarian cancer is in post-menopausal women aged 60-64 years of age. The overall 5 year survival from the disease is currently around 46% (Office for National Statistics), due to late presentation, poor surgical outcomes and the development of chemotherapy resistance. By comparison, the five year survival rate of endometrial cancer is 87.9% and for cervical cancer is 71.5% (Ries et al 2007). The symptoms of ovarian cancer are often vague and are often mistakenly associated with common benign conditions before a diagnosis of ovarian cancer is considered. NICE recommendations for investigations include a CA125 blood test and a pelvic ultrasound scan (NICE 2011). Ovarian cancer is frequently diagnosed at an advanced stage, and although initially responsive to treatment, has a high rate of recurrence and mortality. The common histological categories of ovarian cancer include epithelial, germ-cell and sex-cord stromal tumours. 90% of ovarian cancers are epithelial in origin, which can be further categorized into high grade serous, endometrioid, clear cell, mucinous and low grade serous. Epithelial ovarian cancer presents later in life than germ-cell and sex-cord stromal tumours, which account for 3% and 5% of ovarian cancer respectively and are known to present at an earlier stage with a better prognosis (Arora et al 2012, Koonings et al 1989). High-grade serous tumours are the most common subtype (Clarke and Gilks 2011, Gurung et al 2013). Within each of these tumour subtypes, molecular

heterogeneity also contributes to treatment resistance; for example, clear cell tumours respond poorly to chemotherapy despite presenting at an earlier stage (Sugiyama et al 2000). However, currently, epithelial ovarian cancer is treated as a single disease entity, with a unified approach requiring little stratification of the different histological or molecular subtypes.

Risk factors for developing ovarian cancer include increasing age, genetic mutations such as BRCA1 and BRCA2 mutations, the presence of hereditary non-polyposis colorectal cancer, early menarche, late menopause, nulliparity and a positive family history (Cancer Research UK). Protective factors include the use of the oral contraceptive pill, pregnancy and breast-feeding (Sueblinvong and Carney, 2009).

Ovarian cancer is staged surgically according to the International Federation of Gynaecologists and Obstetricians criteria, whereby stage I describes tumours confined to the ovaries, stage II refers to tumours extending to the pelvis, stage III describes tumours which have spread beyond the pelvis, with or without the lymph nodes and stage IV, where distant metastasis has been found. Ovarian cancer is often asymptomatic in the early stages and over 75% of cases are therefore diagnosed with advanced stage disease – i.e. Stage III or stage IV (NICE 2011). Hoskins found that in patients with stage III or IV disease, 20-25% five year survival is seen despite appropriate treatment (Hoskins, Eisenhauer et al 2000).

1.1.2 Treatment

Current treatment of advanced ovarian cancer includes operative tumour debulking and chemotherapy, mainly using paclitaxel and platinum agents (Bristow R et al 2002, Mcguire W, 1996). Radiotherapy is of limited effectiveness and has adverse effects on other abdominal organs.

Surgery aims to confirm the diagnosis, provide symptom relief, remove or “debulk” the tumour, stage the disease and improve survival. Surgery is usually the first intervention used to treat the disease and is tailored to the individual patient’s age, tumour

resectability, stage and performance status. One of the most important prognostic factors is the presence of residual disease after initial debulking surgery (Chang S et al 2013).However, in most women complete removal of the tumour is not possible. This may be due to several influencing factors such as patient age and clinical morbidity, stage of disease, tumour bulk, subtype and extent of spread. In these cases, women may undergo a laparotomy for no benefit, delaying chemotherapy treatment until recovery from the operation. Thus, preoperative assessment to plan and predict surgical treatment is essential; early FIGO stage 1 and 2 disease are most likely to be completely excised (Wimberger et al 2007). This is performed by assessment of tumour load through tumour marker levels, ultrasound scans, CT and MRI. Whilst predictive index scores of operative success have been explored, these have not been validated (Axtell et al 2007, Gemer et al 2005). Operative tumour debulking is defined as a maximal diameter of residual tissue upto 10mm and is one of the most important factors in survival, irrespective of tumour histological subtype (Colombo et al 2009, Eisenhauer et al 2008). It can be performed before or after chemotherapy and usually involves total abdominal hysterectomy, bilateral salpingoophorectomy, omentectomy and in some cases lymphadenectomy. However, as at least 66% of patients present with advanced disease more aggressive surgery is often required to achieve maximal debulking – for example, peritoneal and diaphragmatic stripping, splenectomy and bowel resection. The ability to perform optimal surgical debulking depends on several influencing factors including FIGO stage of disease, patient performance status, age and additional co-morbidities can also influence surgical outcome (Wimberger et al 2007). Furthermore, tumour biology may well influence the ability to perform optimal debulking surgery but the multifactorial nature of surgical outcomes make identification of a single source difficult. The extent of operative treatment is known to have an independent impact on overall survival (Shih and Chi 2010), regardless of the tumour histological subtype (Takano et al, 2006), with a reported increase in overall survival of 14-28 months in patients with total debulking as compared to optimally debulked patients. Whilst there is no current predictability index score currently in use, the ability to predict surgical debulking success would ensure those who were predicted to have a lower chance of

optimal surgical debulking could be offered neoadjuvant chemotherapy with interval debulking. However, the multiple factors involved in the final surgical outcome make identification and interpretation of results difficult.

Chemotherapy is recommended in addition to operative management for women with stage 1C disease or higher and is most commonly administered after operative treatment. Patients receive post-operative chemotherapy usually within six to eight weeks of primary debulking surgery. Although chemotherapy is an important component of treatment in reducing tumour burden approximately 20-30% of patients who have advanced (stage IC-IV) ovarian cancer will ultimately survive tumour free (Franceschi S et al, 1993). The survival rate for women with epithelial ovarian cancer has changed little since the introduction of platinum-based chemotherapy over 30 years ago and further treatment options are needed (Coleman M et al 2011).

Recently, some studies have suggested that tumour resectability improved significantly after initial chemotherapy, with more patients having zero residual disease after delayed surgery than those who underwent primary surgery. Developing novel biomarkers which can predict surgical outcome in these patients may assist patient “triage” to the optimal primary treatment modality. Some studies have suggested that patients with advanced ovarian cancer who are initially treated with chemotherapy then surgery do equally well as those who have primary surgical treatment (Vergote, du Bois et al 2013). The most effective type of chemotherapy for ovarian cancer has been found to be a platinum based agent with or without paclitaxel (Lambert and Berry, 1985, NICE 2011). Cisplatin is a platinum containing anti-cancer drug, which binds to and stimulates DNA crosslinking, preventing replication and triggering apoptosis. It does not differ significantly in its efficacy or impact on survival when compared to carboplatin, but the two drugs do have markedly different side effects (Ozols et al, 2003) and over the last twenty years, carboplatin has consequently been used preferentially (Adams et al 1989). Paclitaxel is a cytotoxic taxane. It acts as a mitotic inhibitor, which therefore blocks cell division and results in cell death. In most cases, patients undergoing chemotherapy for ovarian cancer are given platinum based therapy, often in

combination with paclitaxel with better survival demonstrated using dual therapy (Sandercock et al 2002). Whilst platinum based agents induce cell death via molecular pathways that are distinct from those of the taxanes, the two groups of drugs are likely to share some of the molecular pathways associated with the apoptotic process (Pestell et al 2000). However, combination chemotherapy significantly increases the side effects experienced, including neutropenia, peripheral neurotoxicity and alopecia. As a result, NICE guidance recommends selecting the treatment of choice after a full discussion of the risks and benefits of each treatment option with the patient.

Initial response rates for standard chemotherapy regimes vary from 60-75% (Neijt et al 2000), with a proportion of patients relapsing within six months of completion of treatment. These women are likely to have platinum resistant disease (Gore et al 1990) and studies show that they have a response rate of 30% at best with second line agents (Harries and Kaye, 2001). In these women, disease may progress with an increase in tumour burden throughout chemotherapy cycles. 55 to 75% of women whose tumours respond to chemotherapy relapse within two years of completing treatment. The response to initial chemotherapy treatment is important as the time to relapse is predictive of response to second and subsequent courses of treatment with platinum based treatment. Currently, patients who have platinum-sensitive disease are retreated with a platinum compound. However, for women with platinum-refractory or resistant disease, non-platinum based regimens are usually used. Treatment failure may be related to somatic mutations, the survival and accumulation of quiescent cells, which cannot be targeted by cytotoxic agents and intrinsically resistant cells which proliferate (Agarwal and Kaye 2003). The chemotherapeutic drug itself may not be able to penetrate tumour cells or cause apoptosis. It may be cleared by the renal and hepatic systems in vascular tumours and suboptimally delivered to the target area. Choi et al found that in acute lymphocytic leukaemia, point mutations which result in resistance to chemotherapy drugs may have low prevalence prior to treatment, but become more prevalent during a relapse (Choi S e al 2007).

Women with a treatment free interval exceeding 12 months have a more favourable prognosis although the majority relapse within five years. Thus, developing more targeted tumour specific treatment as well as understanding the mechanism and reversal for this resistance is important. Eventually, ovarian tumours develop multi-drug resistance. The timeframe for this occurs varies from individual to individual and some women may experience long remission periods following each line of chemotherapy and survive for upto ten years with repeated treatments.

It is worth noting that in cases where primary debulking surgery is not possible (for example, due to the patient having multiple medical morbidities) neoadjuvant chemotherapy may be offered. Here, three cycles of chemotherapy is administered prior to surgery and a further three cycles after interval surgery. A systematic review by Bristow et al and a meta-analysis by Kang et Nam suggest there is little difference in survival, although a significant improvement in residual disease clearance was seen in this group (Bristow et al 2007, Kang et Nam 2009). Vergote et al found that there was no significant difference in overall survival in either group, although again, a larger proportion of patients were optimally debulked (Vergote et al 2010).

As well as the debates on the dosing regimens of chemotherapy, the merits of intraperitoneal chemotherapy are also being explored (Armstrong et al 2006) as well as the impact of different dosing regimens. Specific targeted therapies such as vascular endothelial growth factor (VEGF) inhibitors (Gaiskell et al 2011) are also being explored.

The current clinical challenges associated with ovarian cancer therefore include the need to develop an effective screening test to increase detection of early stage disease and better predictive tests for the success of surgical debulking – perhaps through a predictive index score – although methods used to achieve optimal surgical debulking through extensive upper abdominal surgery have been developed (Chi et al 2009a). Furthermore, better effective medical agents as well as reducing the rate of chemotherapy resistance remains important.

1.2 THE IMMUNE ENVIRONMENT IN OVARIAN CANCER

In cancer sites such as ovarian cancer, malignant tumours are known to be immunogenic and able to evade immune recognition, in part by “hijacking” the tumour microenvironment to become tumour-promoting. In addition, evidence suggests that immune suppression increases tumour incidence, as demonstrated by a higher incidence of cancer in renal transplant patients (Penn I, 2000). Inflammatory immune responses in chronic infections such as hepatitis B and pancreatitis are also known to promote tumour incidence. Tumour cells are known to express antigens which can be recognized by the immune system, which may provide targets for effective immune mediated rejection of the tumour. The immune system is composed of CD8+ lymphocytes, CD4+ lymphocytes, monocytes, dendritic cells and neutrophils. The balance between these immune regulatory cells and tumour cells dictates the immunosuppressive and pro-tumoral properties of the tumour microenvironment. Thus, understanding how the immune response is activated in ovarian cancer is essential to develop effective immunological strategies against it. The cancerous transformation of cells includes genetic alterations and epigenetic dysregulation, creating tumour associated antigens (TAAs) (Ferris R 2012). Tumour immune evasion is a complex mechanism of impaired processing and presentation of TAAs. These suppress immune effector cells by releasing suppressive factors from the tumour into the serum. The immune microenvironment also contains cytokines such as TGF-Beta and IL-10, which, with other immunosuppressive factors locally, reduce the effective anti-tumour cellular immune response and impair the functional capacity of tumour infiltrating cells (Taylor D, 2001). An example of how this is achieved is by up-regulating inhibitory molecules on APCs and tumour associated macrophages, thereby inhibiting the activation, proliferation and survival of cytotoxic T lymphocytes (Freedman and Deavers 2004). Where cytotoxic T lymphocytes are able to overcome this suppressive effect, they may fail to migrate to the tumour or indeed fail to initiate their effector response, due to inhibitory factors secreted directly by the tumour. These mechanisms of tumour induced immune suppression need to be overcome successfully for tumour regression. Uniquely, ovarian cancer has a predominantly locoregional pattern of disease spread – mainly to within the peritoneal cavity. The immune microenvironment of the peritoneum

in women with ovarian cancer has been investigated, showing for example, considerably higher numbers of dendritic cells than peripheral blood (Wertel F, 2008). Curiel et al found that dendritic cells accumulate in ovarian tumour ascites, inhibiting antitumour immunity. In vivo, these dendritic cells induced neovascularization by producing angiogenic cytokines (Curiel 2004). The human immune system naturally recognizes and attacks cancer cells as evidenced by the presence of tumour specific killer T cells within dissected tumours. This was established by Zhang et al in 2003, who found by immunohistochemistry performed on 186 specimens taken from patients with advanced ovarian cancer that the presence of tumour infiltrating CD8+ lymphocytes (TILS) correlated with delayed recurrence and better patient survival (Zhang L 2003, Conejo-Garcia Benencia et al 2004). Zhang et al found that patients whose tumours contained TILs had a five year overall survival rate of 38%, whilst those who lacked TILs had a five year survival rate of 4.5%, suggesting that the presence of immune response is associated with better prognosis. Furthermore, Curiel et al found that the presence of CD4+CD25+FOXP3+ T cells correlated to a poor clinical outcome in women with epithelial ovarian cancers, being found more readily in stage II to IV disease than stage I cancer. However, it is worth noting that Curiel's study involved a heterogeneous population of tumour histology, which may have affected results. By comparison, Zhang et al noted that immune suppression increases tumour incidence, most notable in malignancies with a viral aetiology, and that an inflammatory immune response can also promote tumour incidence (eg. Hepatitis B and liver cancer). Thus, tumour cells can express antigens that can be recognized by the immune system. These may provide targets for effective immune mediated rejection of the tumour. Interactions between the tumour and immune system can result in the immune mediated selection of the most resistant tumour cells and tumour mediated immune suppression. Therefore, to optimize the immune response to ovarian cancer antigens, tumour induced immune suppression should be overcome in conjunction with stimulation of CD8 cytotoxic T cells and CD4 helper T cell responses (Knutson, Curiel et al 2003). Recent work by Fialova et al found that the composition of TILs changed according to the stage of ovarian cancer (Fialova et al 2013). Patients with stage III and IV disease, high levels of activated T regulatory

cells were seen together with dendritic cells and monocytes. This was a shift from Th17 effector cells, noted in earlier stages of ovarian cancer.
To augment the immune response to ovarian cancer antigens, stimulation of CD8 cytotoxic T cells and CD4 helper T cell immunity is required as well as overcoming tumour induced suppression (Knutson K, 2003). The ultimate amplitude and quality of this T cell mediated response is controlled by co-stimulatory and inhibitory signals, and may be exploited by tumours.
These studies suggest that the immune response to ovarian cancer is an important independent factor which influences patient survival. Furthermore, it raises the possibility of harnessing the immune response to treat ovarian cancer and improve patient outcomes.

1.3 IMMUNOTHERAPY IN OVARIAN CANCER

Immune therapies – harnessing the body's own immune system to recognize and destroy tumour cells - seek to increase the immune system's attack on cancer by intensifying this response. Potential mechanisms employed by immune therapies include stimulating or blocking specific components of the immune system, depleting immune subsets, targeting chemotherapy to tumour antigens, genetically engineering immune cells to recognize tumour cells and injecting tumour specific immune cells. Procedures to activate immune reactivity and counteract immune suppression may also be considered.

Given the main cause of mortality in ovarian cancer is clinical relapse, immunotherapy may enhance the anti-tumour immune response following surgery and chemotherapy, thereby eradicating residual micrometastatic disease and preventing or delaying relapse. Immunotherapy may be an important treatment modality given the findings of Zhang et al regarding the impact of intratumoural T cells on survival and evidence of tumour associated antigens expressed by ovarian cancer can be used as a target for eliciting an immune response. Additionally, gauging the immune system response may be a crucial tool in diagnosis and in measuring the patient's treatment response.

Immune modulators including monoclonal antibody therapy or cellular therapies can stimulate therapeutic immunity and tumour rejection. There are three potential immunotherapy approaches for ovarian cancer (Gavalas N, 2010):

1. Local, regional and systemic cytokine or targeted antibody therapies, including immune checkpoint inhibitors
2. Vaccine therapy (including DNA, peptide and dendritic cell vaccination)
3. Adoptive immunotherapy strategies

Current approaches applying immunotherapy include monoclonal antibodies, which may be conjugated (eg. Radiolabelled), cytokine therapy, prophylactic or therapeutic vaccination and adoptive transfer of immunological cells (eg. NK cells, TILs). Currently only two cytokines are FDA approved – IFN alpha, used as adjuvant therapy of stage III melanoma, and IL-2, which is used against metastatic melanoma and renal cell carcinoma and is thought to activate natural killer cells and T cells. IFN-alpha induces expression of MHC Class 1, mediates maturation of dendritic cells and can activate cytotoxic lymphocytes. However, most approaches with cytokine therapy are experimental and non-specific, with toxic side effects. Furthermore, most cytokines have dual function – for example, IL-2 activates CD4/CD8 cells but also mediates immune tolerance.

Monoclonal antibody therapy relies on the use of an antibody specific to one antigen, resulting in milder side effects compared to traditional chemotherapy. Known mechanisms of actions include blocking receptor mediated signaling for tumour cell survival or growth, blocking immunosuppressive pathways (eg. Checkpoint inhibitors such as anti-PD1 and anti-PD-L1), marking tumour cells for immune-mediated destruction and delivering targeted drugs via conjugation to the tumour mass. An example of monoclonal antibody therapy in cancer is Herceptin, which is used to treat HER2 positive tumours in breast and gastric cancers, by triggering HER2 internalisation and degradation, marking cells for NK cell attack and inhibiting the MAPK and PI3K pathways. However, mechanisms of resistance to Herceptin can develop by an altered

target expression (eg. Change in HER2 status) or an altered target (eg. Mutation in receptor which prevents antibody binding).

Vaccine-induced, antibody mediated prevention of infection has a well-established history of success. Some cancers are virally mediated – for example, cervical cancer. The implementation of vaccination programs against Human Papilloma Virus (HPV) show extremely favourable initial results against cervical carcinoma (Taira AV et al, 2004). However, therapeutic vaccines with a two-fold aim of preventing cancer developing and creating immunological memory to trigger an immune response to antigenic exposure during early relapse have been much less successful. The main challenge to developing an effective cancer vaccine comes in targeting an antigen specific to the tumour, which is distinct from auto-antigens. This is more challenging because a tumour may be composed of several different cell types, each with differing cell-surface antigens, some of which may not express foreign antigens. Thus, it may be difficult for the immune system to distinguish cancer cells from normal self-cells. Nonetheless, modulating the immune system has become an increasingly explored treatment option for solid tumours.

Ideally, an effective therapeutic vaccine will stimulate long-term memory and therefore prevent tumour recurrence. This requires overcoming immunosuppression associated with malignancy, stimulation of cellular immunity or antibody response and enhanced immunogenicity or presentation of relevant tumour antibody antigens. Cancer remission provides an ideal opportunity to treat with immune based vaccination strategies as the tumour burden is lower, leading to a small influence on the tumour microenvironment. Currently, cancer vaccines are being developed to expose patients to antigens to provoke an anti-tumour immune response usually in the presence of adjuvant and occasionally in combination with immunomodulation (Pollack S, 2011). Antigen-specific cancer immunotherapy (such as a vaccine) induces a tumour antigen specific anti-tumour immune response (Leffers N et al 2010). An example is Abagovomab, a monoclonal antibody targeted at CA125, which induced anti-anti-idiotypic antibody in patients, who showed significantly longer survival time (Reinartz S et al 2004).

However, whilst antibody-based and peptide vaccines are stable and easy to produce and have shown the most promise, they have poor immunogenicity and in the case of peptide vaccines, are HLA restricted. Table 1 summarizes the reported findings from key clinical trials of vaccine-based immunotherapy for ovarian cancer.

Strategy	Immune response	Clinical response	Reference
Antibody-based therapy			
Anti-CA125 (Oregovomab)	Higher antigen specific T cells	Improved survival	Berek et al 2008
Abagovomab	Mimics functional response to tumour cells with CA125	No change to recurrence free or overall survival	Sabbatini et al 2013
IgG1 antibody for VEGF (Bevacizumab)	Inhibits angiogenesis by targeting VEG-F	Improved progression free survival; no change in overall survival	Garcia et al 2013
Anti-MUC1 antibody	Humoral immune response potentiated	Improved survival	Nicholson et al 2004
Cytokine therapy			
IL-2	Mechanism unreported	Prolonged survival	Edwards RP et al 1997
IFN-alpha	Mechanism unreported	Response seen in 28%	Berek 1999
Dendritic cell based therapy	Ex-vivo derived DCs used with HER-2/neu or MUC-1 or tumour lysates	Limited response	Brossart et al, Wirthes et al 2001, Hernando, Part 2001,2002, Nguyen Pham TN et al 2012
Tumour cell vaccine: whole tumour cells	CD8 T cell response	No clinical response seen	Berd D et al 1998

Table 1.1: Findings from phase I-II clinical trials of vaccine-based immunotherapy for ovarian cancer

In contrast, non-specific immunomodulation induces anti-tumour immunity without exposing the patient to a target molecule or the need for an active/intact immune response. Immunotherapy using monoclonal antibodies (Mabs) and cytokines are in the more advanced stages of drug development (Gavalas N, 2010). Leffers et al found that the largest body of evidence currently available is for CA-125 targeted antibody therapy (15 studies: 1505 patients) (Leffers et al, 2010). Oei et al (Oei A, 2008) reviewed 44 published clinical trials investigating the effects of Mab vaccination and immunotherapy strategies directed towards the antigens CA 125, gp38, HER2, MUC1, TAG 72 or VEGF in patients with ovarian cancer. Of these Mabs, Oregovomab, a monoclonal antibody directed against CA 125, was tested in advanced phase trials but did not show any clinical benefit (Berek et al 2004). Abagovomab is a monoclonal antibody which does not bind directly to CA125 but directs the immune system to target tumour cells displaying the CA125 protein (Sabbatini et al 2013). Bevacizumab, an IgG1 Mab which inhibits VEGF, on the other hand is now used clinically for the treatment of ovarian cancer. Burger et al showed a 27-54% increase in progression-free survival in ovarian cancer patients treated with Bevacizumab in conjunction with chemotherapy (Burger et al 2010). In 2011, ipilimumab became the first Mab targeting the immune checkpoint cytotoxic T-lymphocyte antigen 4(CTLA4) to gain US Food and Drug Administration approval, following Phase III trials in patients with advanced melanoma (Hodi FS, 2010). CTLA4 binds to CD80 and CD86 ligands to stop T cell activation. Intra-peritoneal delivery of cytokines such as IFN-alpha or IL-2 has shown limited success in ovarian cancer (Freedman and Platsoucas 1996, Freedman Lenzi 1998).

Anti-suppressive approaches may benefit all cancer patients – specifically more advanced cases where higher levels of immune suppression and suppressive cells may be seen. New approaches to antagonise tumour-associated regulatory T-cell infiltration and redirection of self-antigen-driven regulatory T-cell activation may direct future strategies for cellular immunotherapy against ovarian cancer. Further progress in T-cell

specific immune responses requires the identification of specific ovarian cancer antigens that are processed and presented on the surface of tumour cells in the context of specific HLA molecules (Hwu et al, 2002). A combined immunological approach may be needed due to the number of immunological responses in ovarian cancers, including the simultaneous implementation of counter-suppression approaches. A further question remains as to the optimal time to introduce immune-based strategies, given that treatment in the form of surgery and chemotherapy are immunosuppressive (Hensler T et al 1997, Brune I et al 1999).

1.4 IMMUNE RESPONSE IN ADVANCED OVARIAN CANCER FOLLOWING CHEMOTHERAPY

As with many cancers, ovarian cancer is associated with suppression and inactivation of effector immune responses (Curiel T, 2003). For immunotherapy to be used clinically, it will most likely be used after standard therapy (surgery/chemotherapy). This may be the most efficacious time and setting for it to be administered, as the tumour burden will be at its minimum. Chemotherapeutic agents have varying effects on the immune response (Machiels JP et al 2007; Yamaeu H et al, 1991) and it has been long recognised that some chemotherapeutic agents can modulate the immune response (Polak L, 1974, Tsuda N et al, 2007) either by inducing lymphopenia (Solomayer E et al, 2003) or stimulating it (Coleman S et al, 2005). By comparison, Yamaeu found that tumour cell lysis by lymphokine activated killer cells was improved by cisplatin in vitro. Parente-Pereira et al also found that repeated administration of carboplatin followed by T4 targeted T cell therapy with a chimeric antigen receptor enhanced epithelial ovarian tumour regression, even in mice with advanced tumour burden (Parente-Pereira et al, 2013).

Tsuda et al found that taxol-induced tumour apoptosis, which releases tumour antigens, potentiated the immune response. However, the mechanism by which Taxol achieves this is unknown (Tsuda N et al, 2007). Recent studies show that ovarian cancer cells contain ISMMC (Immune stimulatory multi-molecular complexes) which activate proliferation and partial differentiation of HER-2- specific CD8+ cells. Taxol modified

ISMMC activated more T cells than native ISMMC (Tsuda, 2007), which may potentiate the immune response by increasing Taxol mediated tumour apoptosis and releasing immunogenic tumour antigen.

In another study looking at combination chemotherapy and immune capacity in advanced ovarian cancer, both a quantitative and functional depression in the primary humoral immune response was seen in these patients. These effects were rapidly reversible (Ten Berge R, 1984). Older studies also suggest that chemotherapy for ovarian cancer may not be significantly immunosuppressive against established levels of cell-mediated immunity and may have effects potentially beneficial to the host as evaluated by lymphocyte-mediated cytotoxicity and blocking factor studies (Mitchell, M.S., 1974). However, this has not been fully explored or confirmed and it must be noted that these studies did not involve the agents that are currently in common use.

The ability of women with advanced ovarian cancer to mount an effective immune response once they have completed chemotherapy has not been fully investigated. Wu et al found that OVCAR-3 cells underwent apoptosis within 24 hours of exposure to paclitaxel and carboplatin, triggering dendritic cells to activate T cells (Wu X et al 2010). They subsequently found a significant increase in subsets of effector T cells at S2 phase as well as INF-y secretion from CD8+ T cells during S2, suggesting that the anti-tumour immune response peaked at 12-14 days after chemotherapy. It is not known if the overall effect of chemotherapy on the global immune system is to up-regulate or down-regulate key immunological factors. So far the immune response to vaccination following successful completion of chemotherapy treatment has not been studied. It is important to understand when in the post-treatment phase the immune system recovers to provide a successful opportunity for employing other novel immune therapies.

1.5 HEPATITIS B AND THE HEPATITIS B VACCINE

Hepatitis B virus (HBV) is a DNA virus of the hepadnaviridae family of viruses. It replicates within infected hepatocytes via an RNA intermediate and utilizes reverse transcription in its replication strategy (Summers J 1982). The HBV genome is complex, with a partially double stranded DNA structure with overlapping open reading frames

encoding surface, core, polymerase and X gene (Figure 1.1). The infectious “Dane” particle consists of an inner core plus an outer surface coat composed of several proteins known collectively as HBs or surface proteins. This surrounds an inner protein shell – the core or capsid - which contains the viral DNA and DNA polymerase. Clinically, it can cause an acute illness which can lead to acute liver failure or lead to a chronic form of infection. The incubation of the Hepatitis B Virus (HBV) is between 6 to 25 weeks. Acutely infected individuals who successfully clear HBV develop vigorous CD4+ and CD8+ T cell responses to the virus. After infection and 1 to 6 weeks before symptoms occur HBsAg appears. A positive test for the presence of hepatitis B surface protein (HBsAg) is the standard currently taken to indicate current infection with hepatitis B. If HBsAg is present for more than 6 months this is generally taken to indicate chronic infection. Acute HBV infection is eliminated by a strong polyclonal T cell immune response (Bayard F et al 2010). Figure 1.2 illustrates the antigen and antibody levels seen over time after infection.

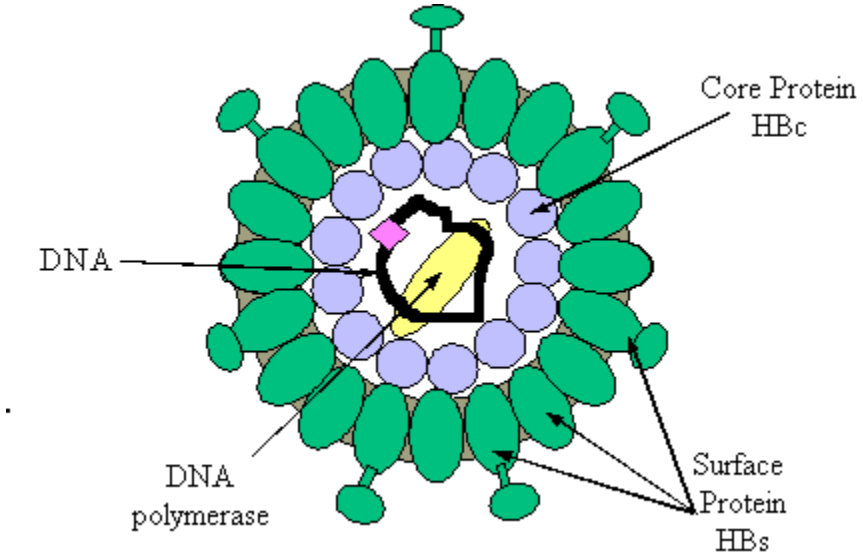


Figure 1.1 Structure of the Hepatitis B virus (Source: Health on the Net Foundation)

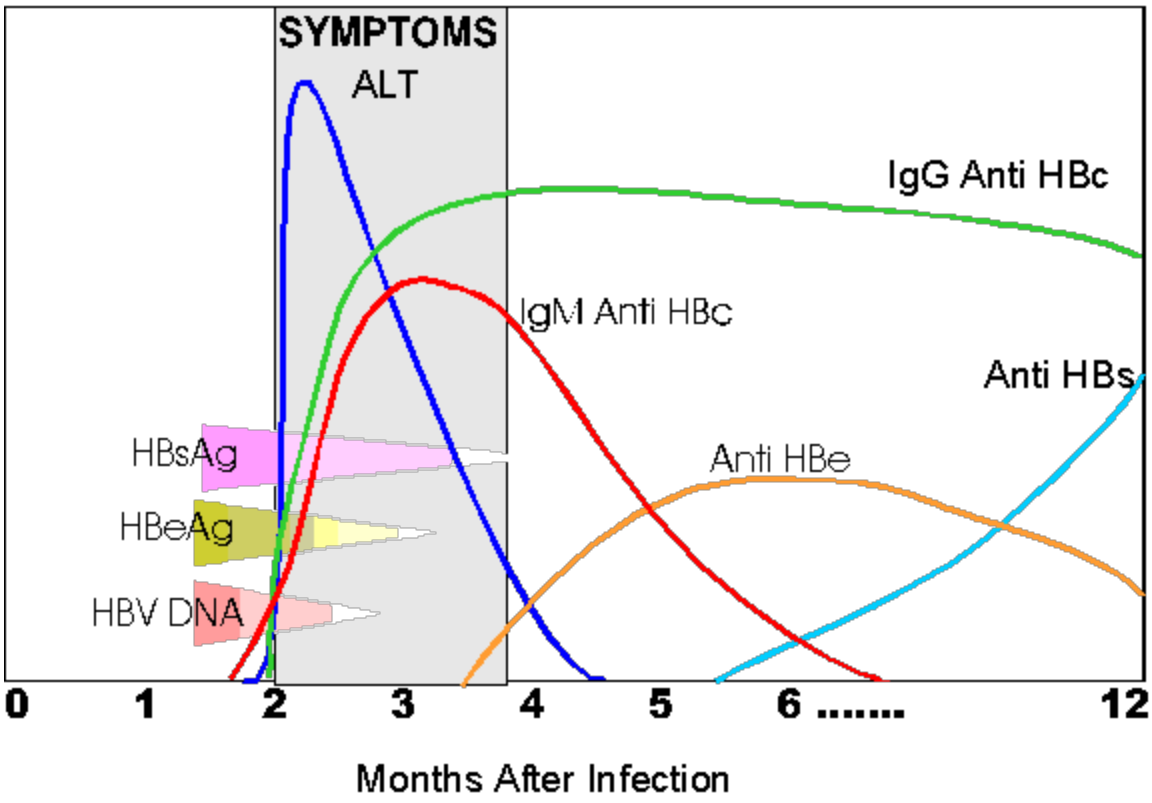


Figure 1.2 Serum antigen and antibody levels in relation to HBV infection (Source: Health on the Net Foundation)

During infection, a primary antibody response is launched to eliminate the pathogen. In addition to this, long-lived antigen-specific memory B cells are generated. These can be restimulated upon repeat exposure to the same pathogen, resulting in a faster and more intense secondary response.

Successful vaccine development requires a modification of a pathogen so that it can still produce a primary antibody response, but is non-infectious. Vaccines essentially replace the initial infection but still lead to the generation of memory B and T helper cells. In contrast, for memory killer T cells to be made, the attacker must infect an antigen-presenting cell. Types of vaccine include live attenuated, toxoid, subunit or protein vaccines. However, it is important to note that vaccines do not guarantee complete protection from a disease. Factors which may influence the efficacy of a vaccine include an inadequate response from the host immune system (eg due to immunosuppressive states such as diabetes, steroid use, HIV infection or age), or

factors such as adherence to the vaccination schedule, ethnicity and genetic predisposition (De Rave S, 1994; Looney R et al, 2000). One important feature of a vaccination is that its efficacy does not depend on the recipient altering his lifestyle. In some cases, the immune system may mount a sub-optimal response, resulting in a less severe infection, which is treated faster.

Safe and effective vaccines against Hepatitis B were developed in the 1980s. Hepatitis B vaccine (Engerix B- 20ug/ml) is a yeast derived recombinant vaccine used for immunisation against Hepatitis B infection. It is a DNA vaccine which stimulates both the cellular and the humoral component of the immune system, including CD8+ MHC class-I restricted cytotoxic lymphocytes (Roy M et al, 2001). Antigen specific CD8 T-cells create a pool of memory T-cells which is maintained in the absence of the antigen. Serum anti-HBsAg can be measured to indicate humeral response in Hepatitis B vaccination (Leroux-Roels G, 1994). This method of stimulating the immune system by vaccination enables the potential magnitude of immune response in ovarian cancer patients on completion of chemotherapy to be measured. Early field trials of the hepatitis B vaccine suggested successful vaccination in over 85% (Krugman 1981), although the WHO now quotes 95% success. Weber et al found that predictors of poor immunogenic response to hepatitis B vaccine included BMI and older age (Weber 1985). A potential drawback of the non-infectious HBV vaccine is that although it generates memory helper T cells and B cells (which can produce protective antibodies), memory killer T cells are not generated as antigen presenting cells are not infected.

One of the most widely available vaccines is the Engerix B HBV vaccine. This is a Hepatitis B recombinant vaccine which provides active immunization against HBV infection caused by all known subtypes in non-immune subjects. 1mL of Engerix B contains 20mcg of Hepatitis B surface antigen (HBsAg) adsorbed on aluminium hydroxide, hydrated. Engerix B provides immunity against HBV infection by inducing specific anti-HBs antibodies. In healthy individuals over the age of 16, seroprotection rates (defined as anti-HBs concentration of at least 10IU/L) were 96% at seven months

after the first dose, using the 0, 1 and 6 month schedule for Engerix B administration. 96% was also achieved using the 0, 1, 2 and 12 month immunization schedule.

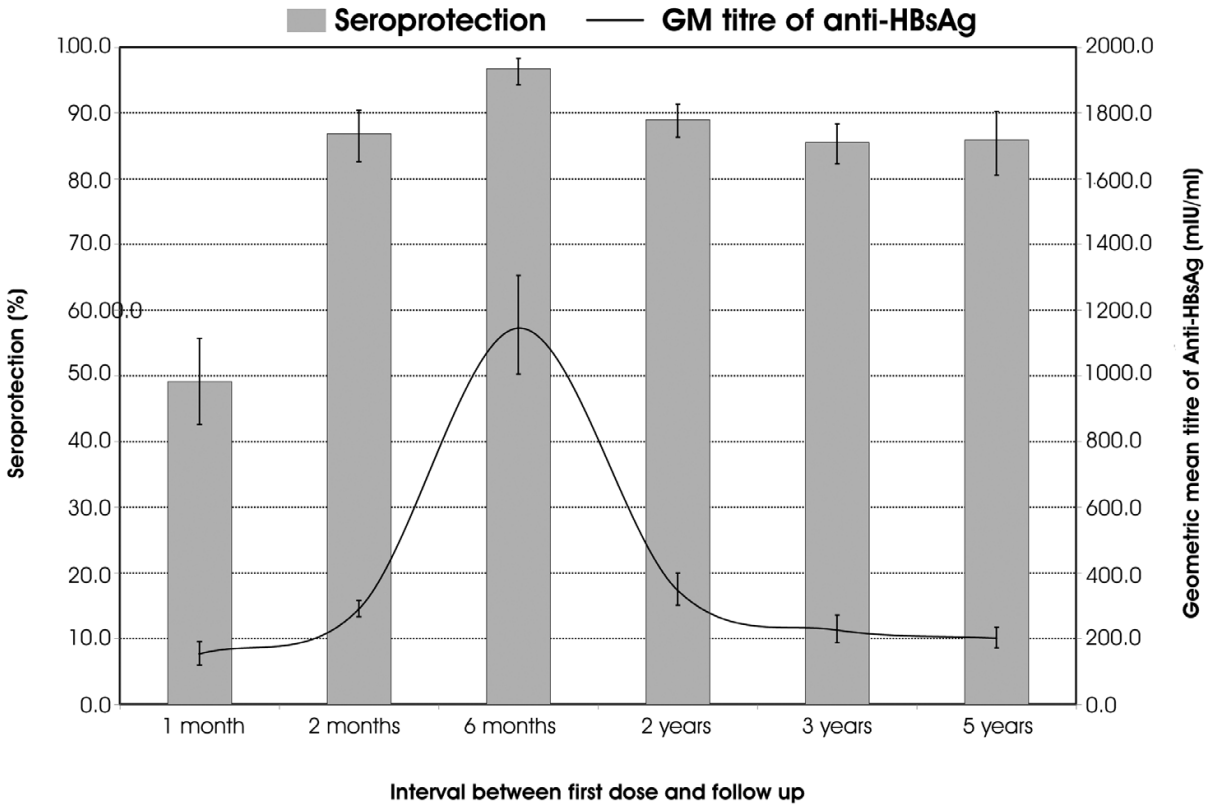


Figure 1.3 Anti-Hepatitis B antibody seroprotection rates and geometric mean titres among subjects following vaccination by interval from first dose using standard schedule monovalent hepatitis B vaccination (Sugunan AP et al, 2012)

The long incubation period of hepatitis B means that unrecognized infection may be present at the time of immunization. The vaccine may not prevent HBV infection in these cases. Separate to this, a protective immune response may not be elicited in all subjects. To optimize its efficacy, administration should be postponed in patients with acute severe febrile illness. Contraindications for Engerix B include known hypersensitivity to the active ingredients of the vaccine, or a previous reaction following Engerix B administration. Common side effects of Engerix B include local skin reactions (such as pain, induration, swelling or erythema), fatigue, fever, malaise, reduced appetite, drowsiness and headache.

Whilst studies on cancer patients receiving vaccination during chemotherapy have been performed – for example, Nordoy et al, who administered the influenza vaccine to cancer patients, the impact of the hepatitis B vaccine on women who have completed chemotherapy for ovarian cancer has not (Nordoy T et al 2002). The immune response to hepatitis B vaccine could thus be used to assess the ability of the immune system to mount a response following chemotherapy treatment.

1.6 THE USE OF BIOMARKERS IN OVARIAN CANCER

The National Cancer Institute defines a biomarker as "*A biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease. A biomarker may be used to see how well the body responds to a treatment for a disease or condition*."[1] It may be a quantifiable measurement of a biological process that can define what is normal or abnormal (Dalton and Friend 2006). It should be detected using well established technology, cost efficient without health risks and be acceptable to the patient.

In cancer research, biomarkers may be used in three ways:

1. To help diagnose conditions, as in the case of identifying early stage cancers
2. To forecast how aggressive a condition is, to determine a patient's prognosis without treatment
3. To predict a patient's response to treatment

In ovarian cancer, current approaches rely on the detection of proteins in the blood which are secreted from the tumour using ELISA. Whilst useful in clinical practice, these have poor sensitivity and specificity. The most widely used serum marker for ovarian cancer has been serum CA125, but this is elevated in 50% of early stage and 92% of late stage ovarian cancers. With a cutoff of 35IU/L, CA125 has a sensitivity of 82.5% and specificity of 67.3% (Hogdall, Christensen et al 2007). The positive predictive value of CA125 in the early detection of ovarian cancer is 57% (Kobayashi, Ueda et al 2012).

[1] NCI Dictionary of Cancer terms

Furthermore, it may be normal in non-serous ovarian cancers, such as clear cell and mucinous subtypes – or indeed elevated in benign conditions such as endometriosis and pelvic inflammatory disease. Therefore, there is a need to find a reliable biomarker to diagnose ovarian cancer which has a high sensitivity for early stage disease as well as high specificity (over 75% and 95% respectively), so that false positive surgical procedures are not performed unnecessarily. An effective screening test for ovarian cancer could increase detection of early stage disease, meaning that surgical treatment could be curative. The REMARK guidelines have been developed by the scientific community for reporting tumour marker prognostic studies (McShane et al 2005).

Equally, prognostic indices which consider tumour spread are required to better categorize patients with recurrent ovarian cancer into subsets according to predicted prognosis rather than treating all patients as one homogeneous group.

Currently there are no specific or sensitive biomarkers to diagnose ovarian cancer. NICE guidance recommends the measurement of Cancer Antigen-125 (CA125) in women suspected with ovarian cancer, but this is raised in 50% of women with early stage disease (Jacobs and Bast 1989). Furthermore, CA125 does not differentiate between the type of ovarian cancer (high levels may be seen in both serous epithelial and endometrioid ovarian cancer) and may even be normal in some subtypes (eg. Mucinous or borderline tumours). The positive predictive value of CA-125 in early detection of ovarian cancer is 57% (Kobayashi et al 2012). It is non-specific and may also be raised in other cancers such as endometrial, breast, pancreatic, gastrointestinal and lung cancers – as well as benign conditions such as endometriosis and pelvic inflammatory disease (Hogdall et al 2007). Therefore, the use of CA-125 as an accurate or specific marker which can be successfully used in the early screening of ovarian cancer is limited.

Ultrasound of the pelvis alone has a sensitivity of 69% in determining the extent of disease spread in ovarian cancer (Davies et al 1993)

Given the low prevalence of ovarian cancer (16.2/100000 in UK, 2008), an acceptable screening method must be highly sensitive for detection of early stage disease (i.e.

Over 75%) but also have high specificity (99.6%), ensuring that no more than 10 false positive surgical procedures are performed to diagnose a single case of ovarian cancer (Menon, Gentry-Maharaj 2009). The immune response seen in ovarian cancer may be used to develop diagnostic tests to identify the stage and volume of disease as well as its ability to disseminate and respond to treatment. One such important pathway may be the PD-1/PD-L1 pathway.

1.7 PD-1 AND ITS LIGANDS, PD-L1 AND PD-L2

Programmed death-1 (PD-1) is a 288 amino acid transmembrane protein (Keir ME 2008) which forms a member of the CD28 family (Dulos 1997). Its release is triggered by T cell activation (Ishida 1992) and it is expressed on immune cells including T, B and natural killer cells, monocytes and dendritic cells (Dong H 2002). It acts as an immune checkpoint inhibitory receptor and reduces T cell activity in the inflammatory response to infection (Ishida 1992). PD-1 has two known ligands: PD1 ligand (PD-L1 or CD274) and PD1 ligand 2 (PD-L2) (Latchman Y 2001). PD-L1 is the principle ligand of PD-1, with a similar pattern of expression as PD-1. However, it is widely expressed on macrophages and bone marrow derived mast cells (Weber J 2010). By comparison, PD-L2 is inducibly expressed in a more restricted fashion.

PD-L1 is a trans-membrane protein consisting of 290 amino acids (Keir ME 2008). Its expression on monocytes, activated T cells and dendritic cells is triggered by histological inflammatory signals (Pardoll DM 2012) and it enables down-regulation of T cell activity upon binding to PD1. It is upregulated in the tumour environment and numerous studies have found an association between increased PD-L1 expression on tumour infiltrating lymphocytes in malignant disease over benign disease (Zou W 2008). Work done in our laboratory has shown that increased PD-L1 expression on monocytes correlates with malignancy, advanced stage and surgical debulking status in ovarian cancer (Maine et al 2013). Furthermore, we have found high expression of PD-L1 at mRNA and protein level in all serous ovarian cancer cell lines. Wong et al showed that blockade of PD-1 expression on tumour infiltrating T cells in patients with melanoma increased T cell proliferation (Wong et al 2007). However, other external factors may influence prognosis and up-regulation of PD-L1. The PD ligand pathway inhibits T cell

function by engaging the PD-1 receptor on T cells, suppressing T cells during an immune response and modulating effector T cell responses during migration to the site of inflammation or in the target tissue itself.

1.7.1 PD-1 and its ligands in ovarian cancer

PD1 is expressed on activated T cells, B cells and macrophages. High levels of PD-1 on tumour infiltrating lymphocytes are seen in many different tumours (Sfanos KS 2009). PD-L1 regulation in tumours is thought to occur through innate and adaptive immune resistance (Pardoll 2012). Innate immune resistance involves the constitutive oncogenic ability to signal PD-L1 upregulation on tumour cells, independent of inflammatory signals in the tumour microenvironment. Adaptive immune resistance involves inducing PD-L1 expression in response to IFNs. Given that increased PD1 expression is seen on tumour infiltrating lymphocytes, with increased PD-L1 expression by tumour cells, antibody blockade of the PD1 pathway may potentiate the immune response to tumours. A recent multi-centre phase 1 trial found that antibody-mediated blockade of PD-L1 induced tumour regression and stabilization of disease for upto one year in patients with advanced stage cancers such as renal cell cancer (Brahmer J, 2012). Furthermore, Topalian et al found a complete or partial response to anti-PD1 antibody in patients with melanoma (28%) and non-small cell lung cancer (18%), with a response lasting over one year in 20/31 cases (Topalian et al 2012). It should be noted that not all tumours were found to respond – colon and pancreatic cancer patients showed no clinically significant response, suggesting that the tumour microenvironment influences the response to anti-PD1 antibody, warranting more understanding of the PD-1 pathway. Hamid et al found that Lambrolizumab, a monoclonal IgG4 PD-1 antibody administered to 85 patients with melanoma had a 51% response rate and a phase II trial at two dose levels is currently underway (Hamid O, Robert C et al 2013).
Hamanishi et al found that PD-L1 and PD-L2 expression in ovarian tumour cells correlated with overall survival as an independent prognostic marker for survival, as well as showing an inverse correlation for PD-L1 and CD8 count (which is known to be an independent prognostic marker of ovarian cancer) (Hamanishi J et al 2007).

Furthermore, Abiko et al also found that PD-L1 overexpression on tumour cells in ovarian cancer led to peritoneal spread of disease by suppression of cytotoxic T lymphocytes (Abiko K et al 2013).

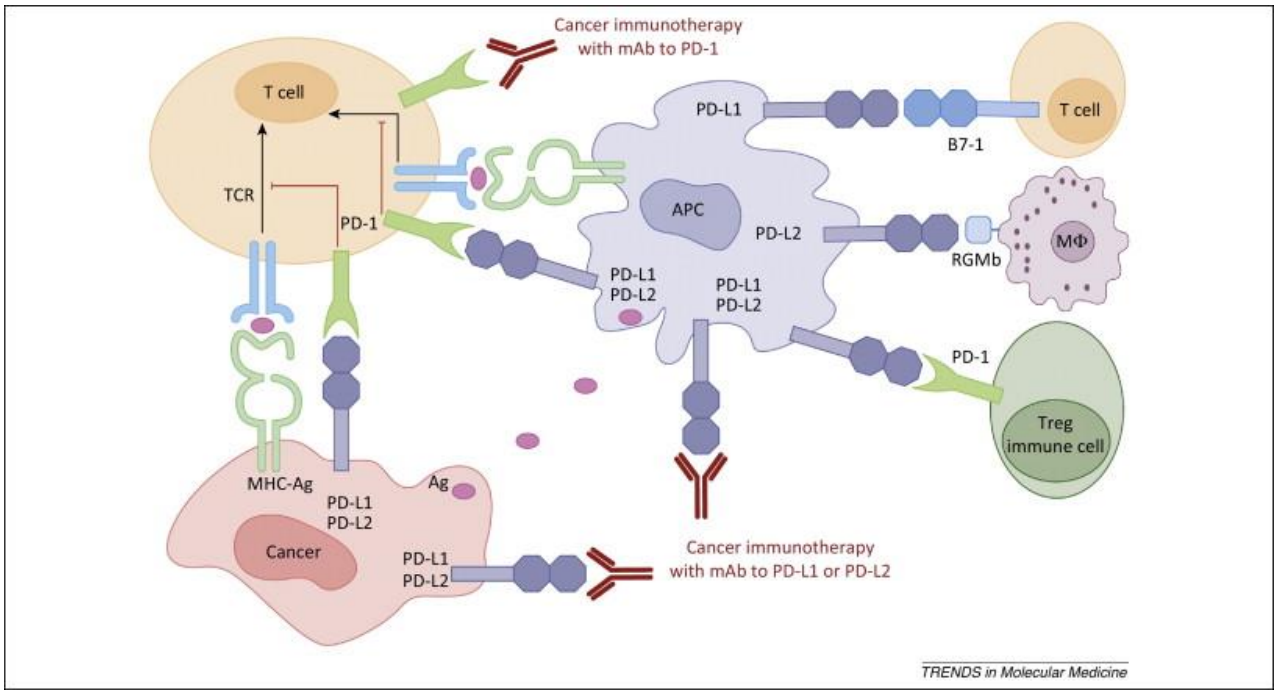


Figure 1.4 Potential cancer immunotherapy targets in the PD-L1 and PD-L2 pathway (Source: TRENDS in Molecular Medicine)

1.8 SUMMARY

A review of the literature confirms that further research is needed to fully understand the biology of ovarian cancer in order to apply a successful and individualized approach to treatment. Two of the most important prognostic factors for patients with ovarian cancer include surgical debulking success and response to primary chemotherapy. However, despite many advances, prognosis for ovarian cancer remains the same. Novel approaches for therapy are required and one which is currently under review is the use of immunotherapy after chemotherapy. This thesis focusses on the impact of primary chemotherapy on the immune system following successful surgical debulking.

1.9 AIMS AND OBJECTIVES

AIM

The aim of this work was to assess the ability of the immune system in advanced ovarian cancer patients treated with chemotherapy to mount an effective response, whilst exploring the relevance of two potential biomarkers, PD-1 and PD-L1. The clinical relevance of this study was to explore the impact of primary chemotherapy on the immune system following surgery.

OBJECTIVES AND HYPOTHESIS

1. The primary objective was to assess the immune competence of patients who have completed chemotherapy for ovarian, fallopian tube or primary peritoneal cancer. This was quantified by measuring the cellular and humoral immune response to Hepatitis B vaccination, as a means of assessing the response to immune stimulation. A higher cellular and humoral immune response was hypothesized in women who had undergone chemotherapy over a year ago than those who had undergone chemotherapy more recently.

The number of patients recruited to each group was based on a power calculation using standard statistical tests that suggested a sample size of 44 per group was needed to show a 15% difference with a 5% significance level and 80% power. The study was powered so that a 15% logarithmic difference in outcome would be statistically significant.

2. A secondary objective was to explore the expression of PD-1 and its ligand PD-L1 in the lymphocytes and monocytes respectively of patients who have completed chemotherapy for ovarian, fallopian tube or primary peritoneal cancer and to correlate this to the response to Hepatitis B vaccination.

3. A third, exploratory objective was to assess the expression of PD-1 and its ligand PD-L1 in lymphocytes and monocytes respectively in women with advanced ovarian cancer undergoing chemotherapy.

STUDY ENDPOINTS

PRIMARY END POINTS:

1. To compare the serum hepatitis B antibody titres in Hepatitis B vaccinated ovarian cancer patients who have either had chemotherapy within the last three to six months or who have had chemotherapy over a year ago.
2. To quantify and compare the T cell response by analyzing Hepatitis-B specific CD8+ T cells in peripheral blood lymphocytes in a subgroup of HLA-A2 positive patients.

SECONDARY ENDPOINT: To explore the lymphocytic PD-1 and monocytic PD-L1 expression in those undergoing chemotherapy for ovarian cancer and Hepatitis B vaccinated ovarian cancer patients who have either had chemotherapy within the last three to six months or who have had chemotherapy over a year ago.

MATERIALS AND METHODS

2.1 HBV vaccination study

This was a prospective study of 34 patients at one tertiary referral centre with a confirmed diagnosis of ovarian cancer who had been staged and graded according to their histological type. All patients had no radiographic or serological evidence of disease on completion of chemotherapy. The number of patients recruited to each group was based on power calculations using statistical methods. Two groups of patients were recruited:

- Group A – minimum of 12 months after the completion of chemotherapy treatment.
- Group B – 3-6 months after the completion of chemotherapy treatment

2.1.1 Recruitment to HBV Vaccination study

Patients were recruited at the time of attending Medical Oncology follow-up clinics at Imperial College NHS Trust, Hammersmith Hospital as per the inclusion and exclusion criteria used in the study protocol. Information leaflets were given to potential participants and they will be given time and opportunity to ask questions about the research project.

There was no clinical, radiographic or serological evidence of disease at the time of recruitment: a recent CT and CA125 levels measured at the time of study entry were normal. All patients underwent an initial blood test to ensure that they had not been previously immunized against Hepatitis B infection or suffered from it in the past and they did not have any pre-disposing active immunosuppressive medical disorder (eg. HIV). Initial blood investigations were normal (Platelet count ≥ 100, 000/mL blood; creatinine ≤ 200 micro mol/l; bilirubin and ALT < 1.5 times upper limit of normal; Haemoglobin ≥ 10.0 g/dl; Haematocrit ≥30%; Lymphocyte count ≥ $1x10^9$/dl) prior to recruitment. Ethics for blood collection was approved by The Imperial College NHS

Trust Research and Ethics committee MREC/LREC 10/H0707/35, reference number JROHH0066.
All patients were recruited over a two year period using the same inclusion and exclusion criteria as those in the historical control patient group, based on De Rave's findings, with follow-up on their clinical status for a further year (De Rave et al, 1994).

2.1.1.1 Inclusion criteria

The inclusion criteria included patients with a history of ovarian cancer, fallopian tube or primary peritoneal cancer treated with surgery followed by chemotherapy or chemotherapy alone either 3 to 6 or 12 months plus from chemotherapy end at the time of recruitment. They also had a BMI below 45 (Ilavska S et al, 2012) and were at least 50 years of age, given De Rave's finding that a decline in immune response occurs with advancing age (De Rave S, 1994; Looney R et al, 2000).

Other inclusion criteria were:

- Disease free post chemotherapy with normal levels of CA125
- No pre-disposing immunosuppressive medical disorder
- No clinical or radiological evidence of disease at the time of recruitment
- Platelet count above 100 000/mL blood
- Adequate renal function (creatinine ≤ 200 micro mol/l)
- Adequate hepatic function (bilirubin and ALT < 1.5 times upper limit of normal)
- Haemoglobin ≥ 10.0 g/dl
- Haematocrit ≥30%
- Lymphocyte count ≥ $1x10^9$/dl

2.1.1.2 Exclusion criteria

Specific study exclusion criteria were:

- Pre-existing immunological disorder excluding vitiligo

- Age less than 50
- Previous immunity to Hepatitis B
- Previous Hepatitis B Infection
- Evidence of disease recurrence or persistence
- Inability to give informed consent
- Active intercurrent medical disease or infection
- Previous treatment with therapeutic cancer vaccines
- HIV positive or other immunosuppressive disorders.

2.1.2 Study protocol

Clinical data for each patient was collected retrospectively from the West London Gynaecological Cancer Centre database. Data collected included age at the point of recruitment, histological subtype, stage and grade, details of surgery and chemotherapy together with residual disease status.

Valid informed consent was taken in writing by the researchers, once patients gave their approval to participate in the study. All recruited patients were seen at 12 weeks and at least 52 weeks after completion of their chemotherapy when they attended the oncology clinic for follow-up. As well as blood tests to assess renal and hepatic function at the point of recruitment, routine laboratory blood tests for CA125, white cell count, monocyte, lymphocyte and neutrophil levels were performed for each participant at the point of recruitment and at the study endpoint. Both groups were vaccinated according to the adult hepatitis B vaccination schedule (see study schedule) using 1mL of Engerix B at each point, a Hepatitis B recombinant vaccine (20mcg of HBsAg, adsorbed on aluminium hydroxide, hydrated).

20mls of venous blood was collected at the first visit to check patient's existing immunity against hepatitis B infection using standardized routine clinical assays at Imperial College laboratories. In particular, Hepatitis B surface antigen (HBsAg) and anti-HB surface antibody (Anti-HBs) were tested for. If participants were not found to be

immune, they were invited to receive their first dose of vaccine. Subsequent doses of the vaccine were given at 1 and 6 months interval. 20mls of venous blood was taken at each visit prior to vaccination to measure anti-HBs antibody levels. Participants attended a month after having had the last dose of vaccine when again 20mls of blood was taken, to evaluate the response to vaccination. The Hepatitis B vaccine was administered via intramuscular injection into the upper arm (deltoid region). Anti-HBs antibody levels were measured on completing vaccination and patients were deemed to have sero-converted if their titre reached a concentration of ≥10iu/l. These values were measured by the standard diagnostic laboratory based at Hammersmith Hospital. The rate of sero-conversion as well as actual and mean geometric titres was then compared between the two groups to look for a difference in response.

Figure 2.1 Flowchart to show schedule of vaccination and sample collection

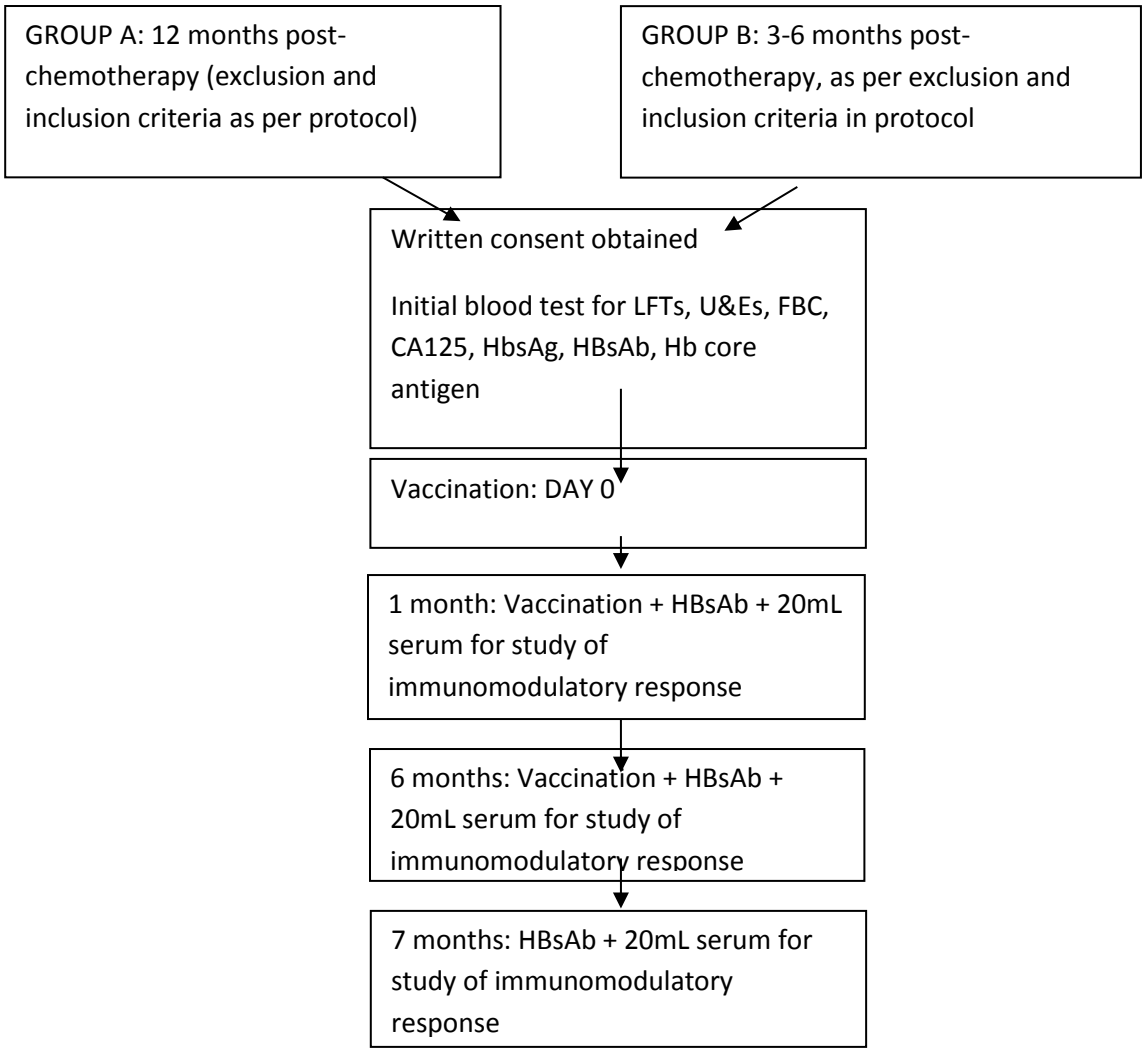


Fig 2.2 illustrates the vaccination schedule undertaken by recruited participants.

VISITS	GROUPA 12 MONTHS AFTER CHEMOTHERAPY	GROUPB 3 MONTHS AFTER CHEMOTHERAPY
FIRST	BLOOD TEST (to check preexisting immunity)	BLOOD TEST(to check preexisting immunity)
SECOND day 0	VACCINATION	VACCINATION
THIRD(1 MONTH after 1st vaccine)	VACCINATION AND BLOOD TEST (Anti-HBsAg antibody and pentamer staining)	VACCINATION AND BLOOD TEST(Anti-HBsAg antibody and pentamer staining)
FOURTH (6 MONTHS after first vaccine)	VACCINATION AND BLOOD TEST (Anti-HBsAg antibody and pentamer staining)	VACCINATION AND BLOOD TEST(Anti-HBsAg antibody and pentamer staining)
FIFTH (7 months after first vaccine)	BLOOD TEST (Anti-HBsAg antibody and pentamer staining)	BLOODTEST(Anti-HBsAg antibody and pentamer staining)

Figure 2.2 Visit schedule undertaken by recruited participants and their group allocation

In a subgroup of participants, selected according to their same HLA type (HLA02 and HLA23), Hepatitis B specific CD8+ T-cells were enumerated in peripheral blood lymphocytes (PBL) using peptide-HLA-pentamer staining and flow cytometry in our laboratory from the previously collected blood. Pentamer-positive cells were viewed by gating then analyzing on a two-colour plot showing CD3+CD8+ T lymphocytes on the y-axis and Pentamer on the x-axis. This was done with serum samples obtained at each visiting point in the study, once the initial vaccination had been undertaken (i.e. visits 3, 4 and 5 for both groups A and B - see figure 2.2, visit schedule table).

Patients with cancer recurrence requiring further chemotherapy during the course of vaccination were withdrawn from the study but the data collected was used in the final analysis.

2.1.3 Study endpoints

PRIMARY END POINT: to measure the serum hepatitis B antibody titres in Hepatitis B vaccinated ovarian cancer patients who have either had chemotherapy within the last three to six months or who have had chemotherapy over a year ago. This was also explored in relation to lymphocytic PD-1 and monocytic PD-L1 expression.
SECONDARY ENDPOINT: to quantify the immunomodulatory T cell response by analyzing Hepatitis-B specific CD8+ T cells in peripheral blood lymphocytes in a subgroup of HLA-A2 positive patients.

2.2 RECRUITMENT OF PATIENTS UNDERGOING CHEMOTHERAPY

Patients undergoing chemotherapy for ovarian cancer were invited to have blood taken prior to each chemotherapy cycle and upon completion of chemotherapy, in conjunction with the Metabonomics study at Imperial College NHS Trust. Blood was collected over a nine-month period. Clinical data on each patient was also collected retrospectively from the West London Gynaecological Cancer centre database on patients with a diagnosis of stage 3 and 4 ovarian, fallopian and primary peritoneal cancer who underwent surgery. Data collected included age at chemotherapy, histological subtype, stage and grade, details of surgery and residual disease status.

2.2.1 Study endpoint

PRIMARY END POINT: To monitor changes in the immune system by quantifying the expression of monocytic PD-L1 and lymphocytic PD-1 levels during chemotherapy.

2.3 DATA COLLECTION SCHEDULE

The recruitment of patients and laboratory experiments for the vaccination study was performed over 18 months; recruitment for patients undergoing chemotherapy was completed over 9 months. All personal information was anonymized so that researchers were blinded to this information and the NHS Code of Confidentiality was followed at all times. All experimental techniques were undertaken in our laboratory and are frequently used by the present research group. Ethical approval for blood collection was

approved by The Imperial College NHS Trust Research and Ethics committee (formally Hammersmith and Queen Charlotte's NHS Trust), reference

2.4 LABORATORY METHODOLOGY

2.4.1 Buffers and solutions

Table 2.1 summarizes the buffers and solutions used in this project.

Solution	Recipe
PBS	8g NaCl, 0.2g KCl, 1.44g Na2HPO4, 0.24g KH2PO4
PBS Tween	PBS, 0.1% v/v Tween 20
Fox P3 Fix/Perm buffer	Used for intracellular FoxP3 staining. Biolegend (Cat number 421401). 4X freshly diluted to 1x working solution with PBS prior to staining
Fox P3 Perm buffer	Used for intracellular FoxP3 staining. Biolegend (Cat number 421402). 10X freshly diluted to 1x working solution with PBS prior to staining
Ficoll-Histopaque	Polysucrose and sodium diatrizoate solution, adjusted to a density of 1.077g/mL (Sigma-Aldrich Biotechnology LP ad Sigma-Aldrich Co-10771)
Running buffer	12.5mM Tris Base, 142mM Glycine, 0.1% w/v SDS
Transfer buffer	12.5mM Tris Base, 142mM Glycine, 20% methanol

Table 2.1 Buffers and solutions used in this project

2.4.2 Isolation of Human Peripheral Blood Mononuclear Cells

Blood obtained from ovarian cancer patients who have undergone chemotherapy was first centrifuged at 1500rpm for 5 minutes to separate the plasma supernatant. The separated plasma supernatant was collected and stored in cryovials at -20^{o}C to -80^{o}C for future functional studies (see Section 2.4.6, Sandwich ELISA for detection of soluble free PD-L1). The plasma free blood was then layered over Histopaque /Ficoll (Axis Shield, Cambridge, UK) in a 1:2 ratio, after first diluting with PBS in a 1:1 ratio, and centrifuged at 2500 rpm for 25 minutes at room temperature with low brake. The mononuclear cells formed a white layer or the buffy coat which was removed using a pipette and transferred into a clean tube. Cells were washed in PBS and counted using a haemocytometer and Trypan blue 0.4% (Gibco) at 1:1 ratio. When not in use, cells

were stored in cryogenic vials using Fetal Calf Serum at -80^{0}C and then transferred to a liquid nitrogen storage bank.

2.4.3 HLA-A2 typing: flow cytometry staining

Cells were washed, counted and 1x10^5 cells were re-suspended in 100 µl PBS per well of a V-bottomed 96 well plate. All cells were first Fc receptor blocked using Fc receptor blocking reagent. HLA-A2 antibody and isotype were added at the appropriate dilution, and the cells were incubated on ice for 15 minutes. Following washing with PBS, cells were re-suspended in 100 µl of PBS. When staining for PD-L/PD-L1, a total of 10000 events were acquired; when staining for pentamer, 100000 events were acquired. The antibody stained cell suspension was then transferred to Facs tubes containing 300ul of PBS for FACS analysis using the FACScalibur (Becton Dickinson). Cell debris and dead cells were excluded from analysis and results were analyzed using the Flowjo software (Tree Star, Inc.).

2.4.4 Flow cytometry staining

Once the HLA-A2 status had been confirmed, isolated PBMCs obtained from blood taken at each vaccination timepoint were stained with anti-CD3, anti-CD8 and Pro5 pentamer in order to enumerate the CD3+CD8+ pentamer population. Whilst CD4 and CD8 can be used to identify the two major T cell populations, CD3, a T cell specific marker was used to differentiate T cells from other populations because CD4 and CD8 can be expressed by other cell types (CD8 can be expressed on NK cells, while CD4 can be expressed on populations of monocytes and dendritic cells). Pentameric peptide-HLA class I complexes (pentamers; ProImmune, Oxford UK) were used according to the manufacturer's instructions to stain antigen specific T cells. Cell surface staining was performed using anti-CD3-FITC and anti-CD8-APC and at least 100 000 lymphocyte gated events were acquired on a FACS Calibur flow cytometer and analyzed with FlowJo software. Flow cytometry was used to study the functional and phenotypic properties of these cell subsets because of the diversity of populations seen in PBMCs.

Cells were washed in PBS and counted. $1x10^5$ cells were re-suspended in 100µl PBS per well of a round-bottomed 96 well plate. All cells were Fc receptor blocked using Fc receptor blocking agent. Antibodies were added at the appropriate dilution (see table 1) and the cells were incubated on ice for 15 minutes. Optimal antibody concentration was based on titration experiments. Following washing with PBS, cells were incubated with secondary antibody. Where the primary antibody was directly conjugated, cells were resuspended in 100µl PBS. Cells which required a tertiary staining were washed after 15 minutes incubation on ice and resuspended in PBS with the tertiary antibody for a further 15 minutes on ice. Cells were again washed in PBS and resuspended in 100µL of PBS. The antibody stained cell suspension was then transferred to FACs tubes with PBS to a total of 300µL. FACS analysis was performed using FACScalibur (Becton Dickenson) and analyzed using Flowjo software (Tree Star Inc). The antibodies used are summarized in table 2.2 and isotype controls summarized in table 2.3.

Antibody	**Fluorochrome**	**Source**
FcR blocking agent	Pure	Bioscience
Mouse anti-human CD14	FITC	Serotec
Mouse anti-human CD8	APC	Bioscience
PD-1	R-PE	Bioscience
PD-L1	PE	eBioscience
Mouse anti-human CD3	FITC	Bioscience
CD19	APC	AbCam
Pro5-Pentamer	R-PE	Proimmune
CD4	FITC	Miltenyl Biotec
CD25		Miltenyl Biotec

Table 2.2 Antibodies used to stain cells for flow cytometry. Abbreviations: FITC: Fluorescein Isothiocyanate, R-PE: recombinant Phycoerythrin, APC: Allophycocyanin

Isotype	Conjugate	Company
Mouse IgG1	FITC and R-PE	Dako
Mouse IgG2b	APC	Biolegend
Mouse IgG1	FITC	BD Science

Table 2.3 Isotype control antibodies used for flow cytometry. Abbreviations: FITC: Fluorescein Isothiocyanate, R-PE: recombinant Phycoerythrin, APC: Allophycocyanin

2.4.4 Intracellular staining

2.4.4.1 Buffers and materials

The fixative solution was 4% paraformaldehyde (10ml PFA: 30mls PBS)

The wash buffer used was PBS with 0.1% Triton-X100, freshly prepared or used within 3 days of preparation, stored at 2-8^{o}C.

2.4.4.2 Method

After isolating PBMCs, adding FcR blocking agent and staining for CD4/CD25, cells were fixed with 100µl fixative in FACs tubes for 10 mins at room temperature and pressure. The cell solution was then permeabilized using 1ml ice-cold methanol at -20^{o}C for an incubation period of 10 minutes. Following washing in permeabilisation buffer (0.1% Triton-X100 in PBS) cells were re-suspended in 95µl of permeabilisation buffer and 5µl of FOXP3 antibody was applied directly. After an incubation period of 30 minutes in the dark at 2-8^{o}C, the cells were resuspended in 200µl permeabilisation buffer for FACS flow cytometry. When staining for FOXP3, a total of 10000 events were acquired. Cell debris and dead cells were excluded from analysis and results were analyzed using the Flowjo software (Tree Star, Inc.).

2.4.5 T cell culture

All cell culture was performed under aseptic technique in sterile plastic wares in a class II microbiological safety cabinet with vertical airflow. PBMCs were placed in culture and stimulated in a peptide-specific manner. Mononuclear cells were resuspended in T-cell medium [RPMI 1640 supplemented with 10% FCS, 1% penicillin/streptomycin, 2 mmol/L L-glutamine, and 50 mmol/L 2-mercaptoethanol (Sigma, Dorset, United Kingdom)] at a density of 3x10^{6} cells per 1 mL medium in a 24-well plate. The cytokines

interleukin-2 and interleukin-7 were added to the medium on day 0 at concentrations of 20 units/mL (Roche, Lewes, United Kingdom) and 2.5ng/mL (Roche), respectively. Peptide-specific stimulation was done by adding a 10 mmol/L concentration of either the pWT126 or the pMelan-A peptide directly to the medium. After a culture period of 8 days at 37^{o}C in a tissue culture incubator, the cells were re-stained for FACs analysis of CD3+CD8+pentamer+ T cell levels. Cell count was determined using a haemocytometer and Trypan blue 0.4% at 1:1 ratio.

2.4.6 Sandwich ELISA for detection of soluble free PD-L1

Antibody	**Capture/coating**	**Capture/coating**	**Capture/coating**
Catalogue number	10084-MM02	10084-RP01	LS-C60133/20065
Clone	4A7B1	Poly	Poly
Buffer	PBS 5% trehalose	PBS 55 trehalose	PBS
Company	Sino Biological	Sino Biological	Antibodies online

Table 2.4 Antibodies used as capture antibody for PD-L1 ELISA

Antibody	**Detection**	**Detection**	**Detection**
Catalogue number	329704	13-5983	BAF156
Clone	29E.2A3	MIH1	Poly
Buffer	PBS 5%	PBS	0.1% BSA
Company	Biolegend	eBioscience	R&D Systems

Table 2.5 Antibodies used as detection antibody for PD-L1 ELISA

	Standard	Standard
Recombinant protein	**1**	**2**
Catalogue number	**156-B7-100**	**10084-H08H**
Construct	**hB7-H1-H-Fc**	**hB7-H1-extra polyhistidine Tag**
Company	**R&D Systems**	**Sino Biological**
Buffer	**PBS and NACL**	**PBS 8% trehalose**
Reconstruction	**PBS**	**Assay Diluent**

Table 2.6 PD-L1 recombinant proteins used to generate standard curve for PD-L1 ELISA

2.4.6.1 Buffers and materials

The assay diluent used was PBS with 10% FCS (heat inactivated), at pH7.0. The coating buffer was made up of 0.1M Sodium carbonate, at pH 9.5, 8.40g$NaHCO_3$, 3.56g Na_2CO_3, q.s to 1.0L; at pH9.5

The wash buffer used was PBS with 0.05% Tween-20. Freshly prepared or used within 3 days of preparation, stored at 2-8^oC.

The stop solution used contained 1M H_3PO_4 or NH_2 SO_4 and substrate solution was TMB Reagent A+B (1+1).

2.4.6.2 Assay Materials:

(a) Immuno Maxisorb Plate (Nunc)- Surface with a high and uniform immunoglobulin binding capacity

(b) Capture antibody-

1. B7-H1 Protein (CD274) Rabbit anti-human polyclonal antibody, ABIN298935, Antibodies-online GmbH.

Concentration- 1ug/ml working concentration in coating buffer

2. B7-H1 (CD274) Rabbit anti-human polyclonal antibody, GTX104763, Source, BioScience

Concentration – 2ug/ml working concentration in coating buffer

(c) Plasma- Plasma samples obtained from patients, stored at -20^{O}C and thawed at RT

(d) Detection Antibody- Biotin- B7-H1 clone 29E.2A3, 329704, Biolegend, 1ug/ml working concentration in Assay Diluent

(e) Streptavidin-HRP complex- clone Streptavidin, 405210, Biolegend, 1:1000-1:3000 for Elisa working dilution

Working Detector: Detection antibody was added to Assay diluent 15 minutes prior to use add the required volume of Enzyme Reagent and vortexed.

(f) TMB (Tetramethylbenzidine) Substrate Reagent Set- 555214, Becton Dickinson

(g) Standard- Recombinant Human B7-H1/PDL-1 Fc Chimera, 156-b7-100, R&D systems.

2.4.6.3 Method

2.4.6.3.1 Initial standardization (example seen in Appendix 1)

A 3000ng/ml of standard was prepared from the stock standard and serial dilutions were done by adding in wells 2 to 7 in duplicate. Well number 8 was kept as blank or zero standard concentration, containing 100ul of Assay diluent only.

2.4.6.3.2 Assay Procedure

Maxisorb 96 microtiter plates (Nunc, GmbH, Germany) were coated with 100ul of Capture Antibody in each well (b), sealed and incubated overnight at 4^{o}C. Following this, the plate was washed 3 times with wash buffer and after the last wash, inverted and blotted on absorbent paper to remove any residual buffer. 200ul of assay diluent was added as a blocking agent to each well and incubated for 1 hour at RT. The plate was washed 3 times and 100ul of standard or sample (c and g) was added in duplicates

to each plate and incubated overnight at 4 ^{o}C. The plate was then washed 5 times and Streptavidin-HRP complex was added to each well and incubated for two hours. After washing the plate 5 times, 100ul of working detector (e) was added to each well and incubated for two hours. The plate was washed 7 times and 100ul of TMB Substrate was added to each well and incubated for half an hour in the dark. Following this 50ul of Stop solution was added to each well to stop the enzymatic reaction and absorbance at 450 nm was measured with reduction at 570 nm using ELISA plate reader within 30 minutes.

2.5 STATISTICS AND DATA ANALYSIS

2.5.1 Power calculation for vaccination study

The number of patients recruited to each group was based on a power calculation using accepted statistical methods. It was calculated using standard statistical tests that a sample size of 44 per group was needed to show a 15% difference with a 5% significance level and 80% power. The study was powered so that a 15% logarithmic difference in outcome would be statistically significant. The calculation was based on historical data obtained regarding titre range and geometric mean titre (GMT[2]) of healthy volunteers above the age of 50, who were vaccinated using the same vaccine and schedule (De Rave 1994).

2.5.2 Statistical analysis

Statistical analysis was performed using Graphpad 6 software (Prism, La Jolla, USA). Specific analysis included unpaired and two-tailed Student's T test, with results expressed as mean ±SEM or SD. P values were considered significant when $p<0.05$. Where statistical analysis of vaccination study data involved comparing results obtained for historical data, Group A patients and Group B participants, the Chi-squared test or the unpaired two-tailed Student's T test was used as indicated.

[2] Geometric mean titre is the simple arithmetic mean of the logarithm of the last positive dilution of each serum

3. RESULTS

3.1 Hepatitis B serum antibody levels through the vaccination schedule

3.1.1 Aims

The aim of this work was to assess the immunological response to vaccination in the post-operative post-chemotherapy patient with advanced ovarian cancer using Hepatitis B vaccination as a model to study the integrity of the cellular and humoral immune response.

3.1.2 Patient characteristics

The impact of administrating the Hepatitis B vaccination (HBV) on 34 patients with ovarian or fallopian tube cancer who had received this vaccination was evaluated. The median age of the patients was 66.5 years and the range was 52 to 82 years. None of the patients had received prior HBV vaccination.

Over an eighteen month period, 905 casenotes from a specialist Gynaecology Oncology clinic were reviewed and 290 patients were screened for eligibility into this study. 233 women declined immediately, withdrew consent prior to commencing vaccination, were found to be HBV immune, were involved in other studies, were found to have residual disease, other malignancies or relapsed. In total, 57 women were successfully recruited. Of these, 41 women participated in the study. Seven participants were excluded due to ovarian cancer relapse prior to or during vaccination, nine withdrew consent, two moved abroad, three could not attend the appointments schedule and two could not be contacted upon recruitment. Of the 17 women recruited in Group B, two women cancelled, one relapsed after undergoing the first vaccination and one was excluded after becoming fully immune to Hepatitis B following the initial vaccination. Figure 3.1 depicts recruitment, the study population and the outcome of the study population.

- Number of women who have completed chemotherapy at least one year ago (Group A): 21
- Number of women who had completed chemotherapy 3-6 months ago (Group B): 13

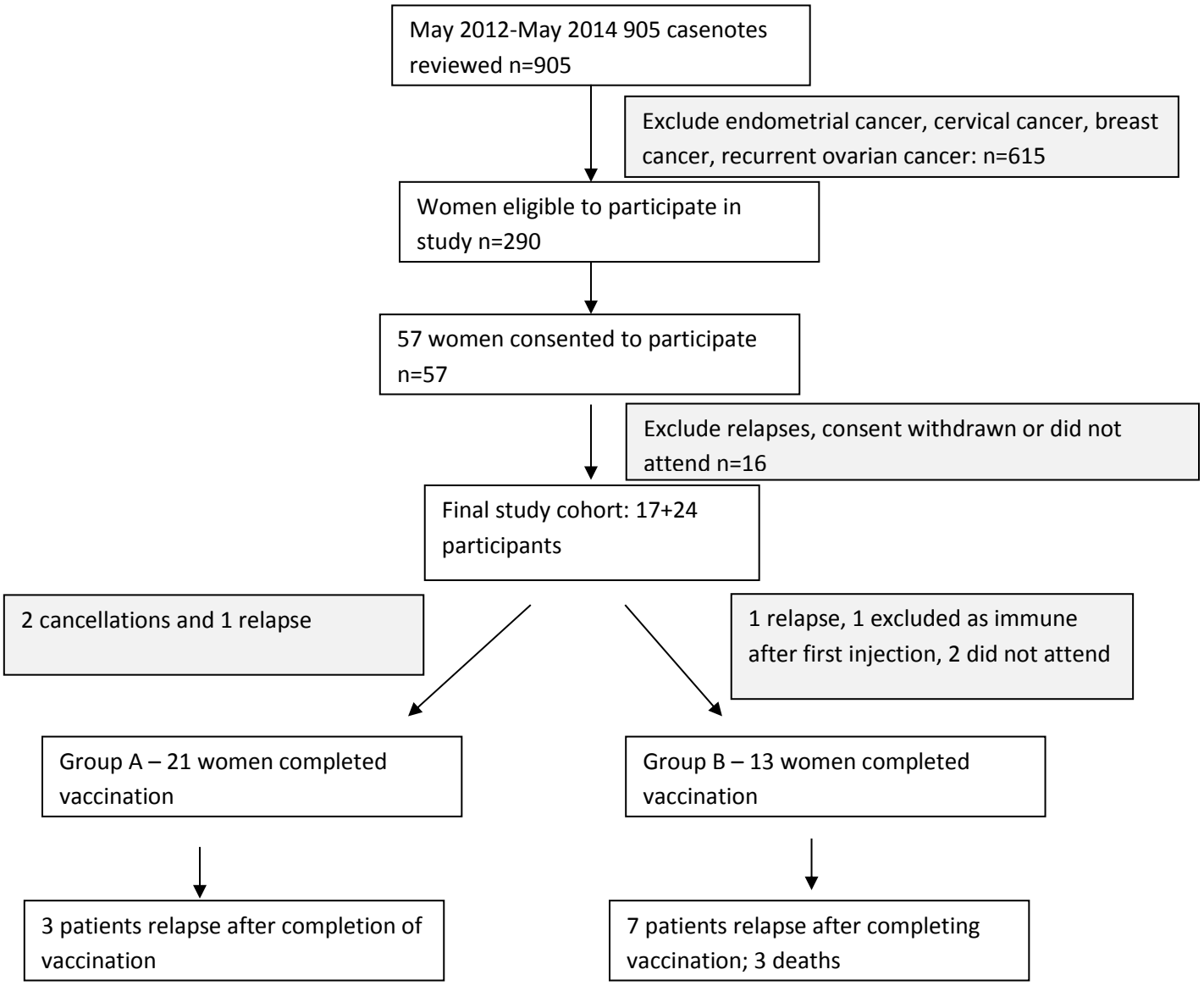


Figure 3.1 Study recruitment, population and clinical outcomes of the study population. Relapse indicates recurrence of malignancy within three years of recruitment.

Clinical data collected included age at diagnosis, histological subtype, stage and grade and details of treatment. The vast majority of patients either had platinum based chemotherapy alone or platinum and taxane based combination treatment. The mean age of patients in Group A was 70.4 years (range 50-82), whilst the mean age of

patients in Group B was 65.1 years (range 50-82). Table 3.1 summarizes the patient characteristics for both Group A (n= 21) and Group B (n=13).

Study participants were followed up longitudinally and three women in Group A were found to have cancer relapse after completing the vaccination course (14%); in Group B, seven women had cancer relapse (54%), with three subsequent deaths. Relapse was defined as either clinical or radiological recurrence. All outcomes data were retrospectively reviewed and analyzed until the study was closed.

Table 3.2 summarizes the patient characteristics of patients who developed cancer recurrence in both groups, whilst Figure 3.2 highlights the stage of ovarian cancer, within both groups.

Patient characteristics	Group A (n=21)	Group B (n=13)
Primary disease		
Ovarian	19	12
Fallopian (serous)	2	1
Histological subtype (ovarian)		
Serous	7	7
Clear	3	1
Endometrioid	6	2
MMMT	2	0
Papillary	1	1
Carcinosarcoma	0	1
Stage		
1	11	1
2	3	1
3A	2	2
3B	0	2
3C	3	5
4	2	2
Grade		
1	2	1
2	7	0
3	12	12
Surgery		
Delayed primary debulking	2	1
Primary TAH/BSO/Omentectomy	17	12
Other primary debulking	2	0
Chemotherapy: time		
Neoadjuvant	2 (11)	1 (8)
Secondary	19 (89)	12 (92)
Chemotherapy regimen		
Carboplatin	5	3
Carboplatin/paclitaxel	14	10
Other combination	2	0

Table 3.1 Summary of pathology and management in Group A and Group B patients

Patient characteristics	Group A (n=3)	Group B (n=5)
Histological subtype		
Serous	1	1
Clear	0	1
Endometrioid	0	2
MMMT	1	0
Papillary	1	1
Stage		
1	1	0
2	0	1
3A	0	1
3B	0	1
3C	1	1
4	1	1
Grade		
1	0	1
2	0	0
3	3	4
Surgery		
Delayed primary debulking	1	0
Primary TAH/BSO/Omentectomy	2	5
Chemotherapy regimen		
Carboplatin/paclitaxel	1	5
Other combination	2*	0

Table 3.2 Summary of pathology and management in Group A and Group B patients who relapsed; in all cases, cancer type was ovarian. *Other chemotherapy combinations used were carboplatin, paclitaxel and gemcitibine and 3 cycles of carboplatin and paclitaxel, followed by 3 cycles of carboplatin and coelyx due to poor patient tolerance.

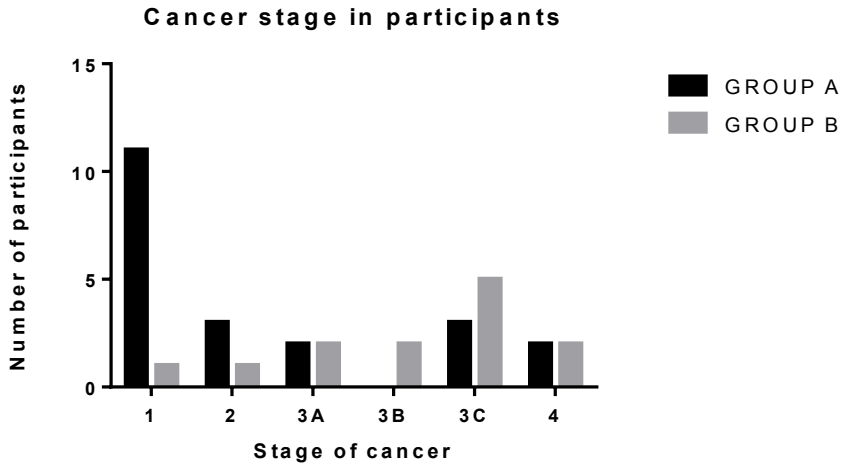


Figure 3.2 Stage of ovarian cancer in Group A and Group B patients. A non-statistically significant difference in staging for the two groups was found using the unpaired two tailed t-test (p=0.4449)

3.1.3 Blood markers for Group A and B participants

CA125 levels were found to be higher and significantly different at the start and end points of the study for Group B participants (see Fig 3.3, p=0.0335, unpaired t-test). In all cases, data based from all recruited patients is displayed unless stated otherwise. Furthermore, CA125 levels at the end of the study were significantly different between groups A and B (see Fig 3.3, p= 0.032). This may relate to the higher relapse rate and number of deaths seen in this cohort of patients. However, the CA125 levels at the point of recruitment for Group A and B were not statistically different (p=0.057, two-tailed p value).

In analyzing the data from Group A start and end points (paired t test), the two-tailed p value is 0.1527 and therefore there is no statistical difference between the CA125 levels at the start and end of the study for Group A control patients. However, a statistically significant difference was noted to be found in the CA125 levels at the start and end of the study for Group B patients (p=0.0335).

Routine laboratory blood tests for white cell count, monocyte, lymphocyte and neutrophil levels were performed for each participant at the point of recruitment and at the study endpoint. These suggested that white cell and white cell subset levels were similar across both groups, with no acute inflammatory response. Figure 3.4 highlights the non-

significant difference in white cell count, neutrophil count, lymphocyte and monocyte count for participants in both groups at the point of recruitment to the study (month 0) and upon completing vaccination (month 7). This provides evidence of no significant change in immune response over the course of the study and supports the fact that the immune response was equitable in both groups.

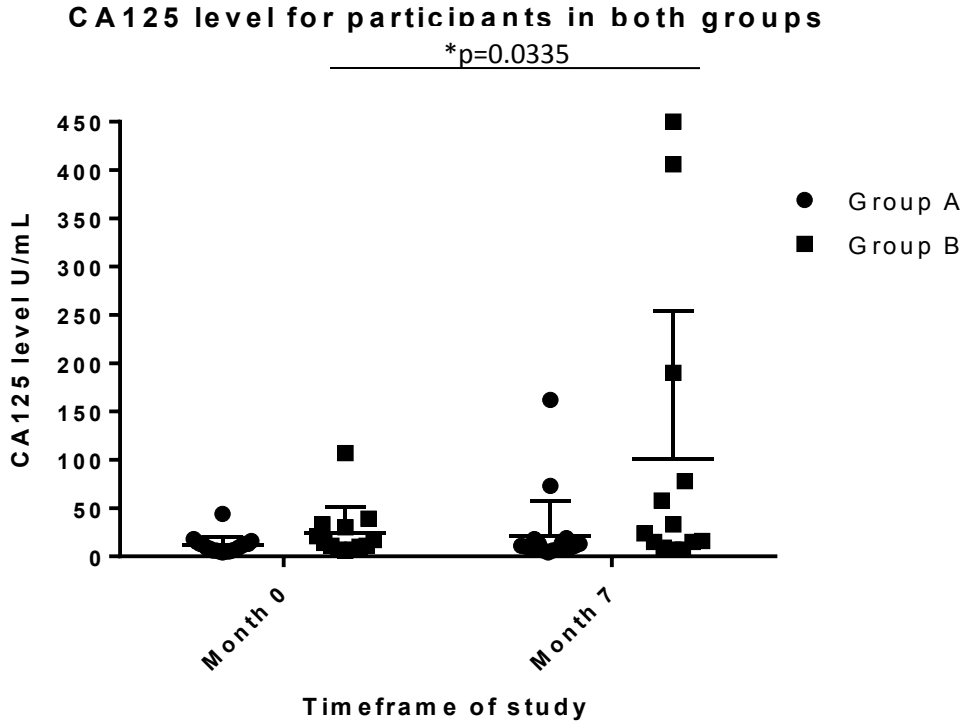
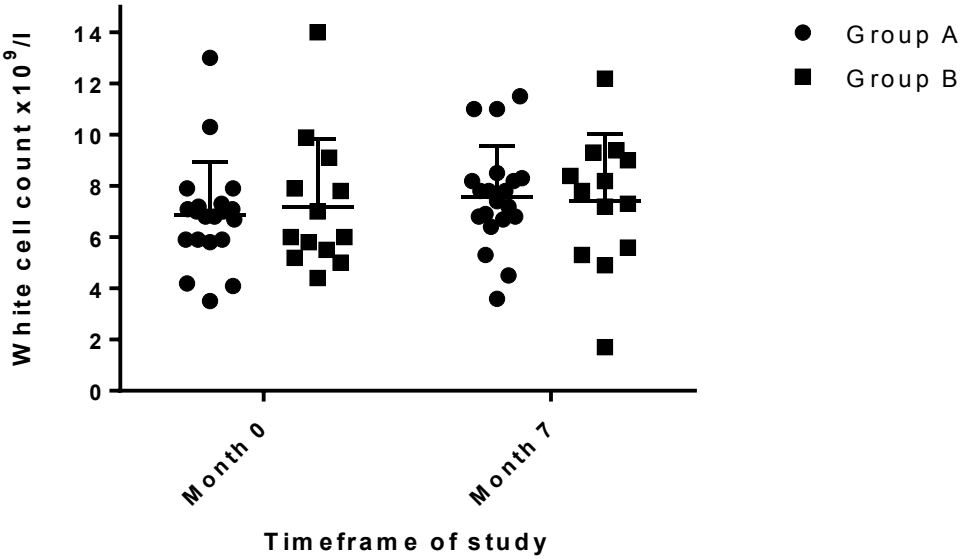
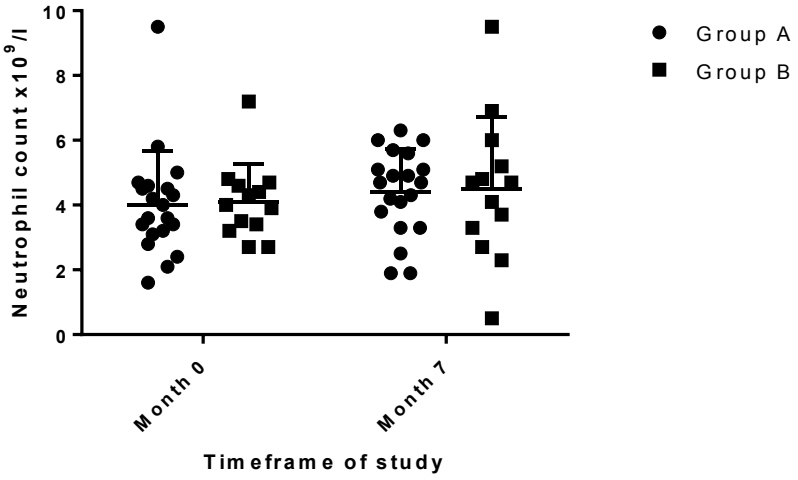


Figure 3.3 Serum CA125 level for Group A and Group B participants at the point of recruitment and end of study. Bars represent mean CA125 levels and standard deviation. CA125 levels were found to be statistically significantly higher at the start and end points of the study for Group B participants, using the two-tailed paired t test (p=0.0335).

White cell count for participants in both groups



Neutrophil count for participants in both groups



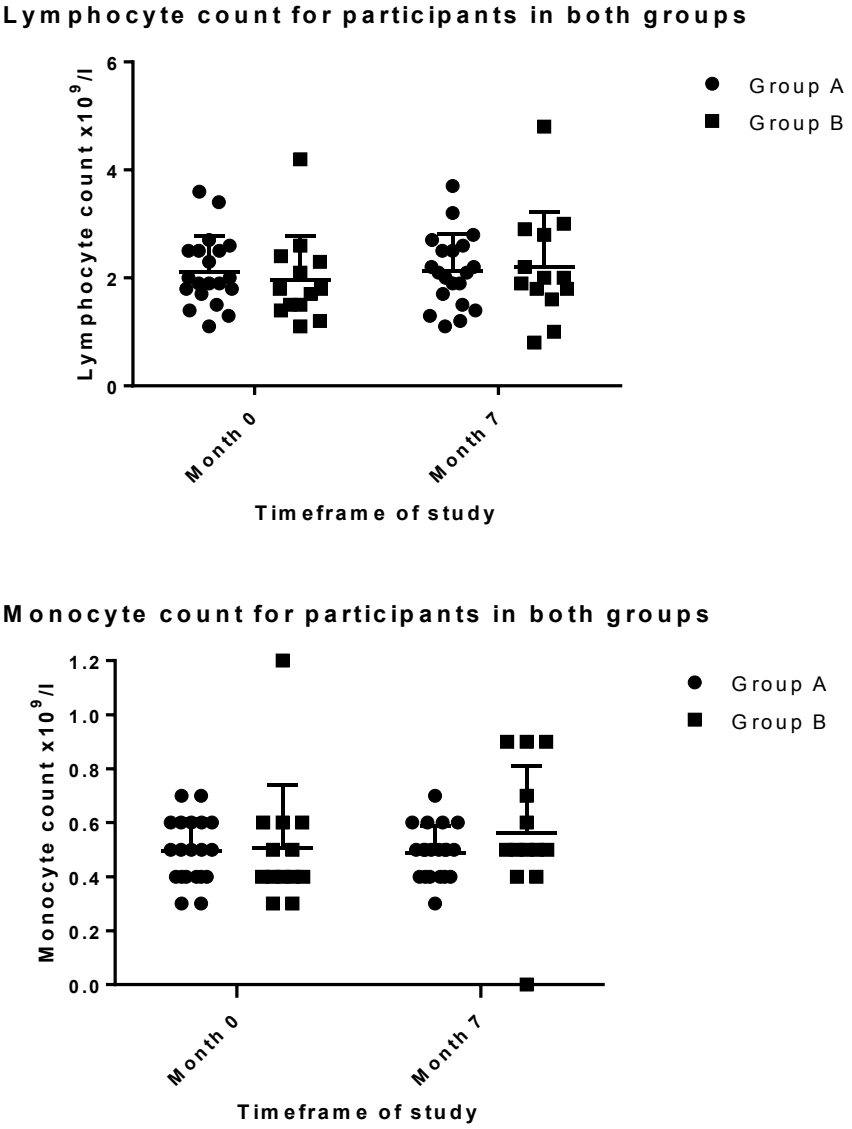


Figure 3.4 Serum markers (white cell count, neutrophil, lymphocyte and monocytes count) for Group A and Group B participants at the point of recruitment and end of study. Bars represent mean levels and standard deviation. No statistically significant difference in serum markers was seen between Group A and Group B participants at the two different timepoints.

3.1.4 Serum antibody response for Group A and Group B participants

Prior to vaccination and at each timepoint of HBV vaccination 5mL blood was taken from each participant and sent to Hammersmith Hospital pathology laboratory for HBsAb analysis. 34 women successfully completed the full vaccination schedule (21

women in Group A, 13 women in Group B). Figure 3.5 summarizes the HBsAb levels obtained at months 2, 6 and 7 for a single Group B participant in graphical format. This is displayed alongside serum antibody titres measured prior to vaccination (d0). In all cases, HBsAb titres were measured prior to vaccination to ensure all participants had not been previously vaccinated or exposed to Hepatitis B virus.

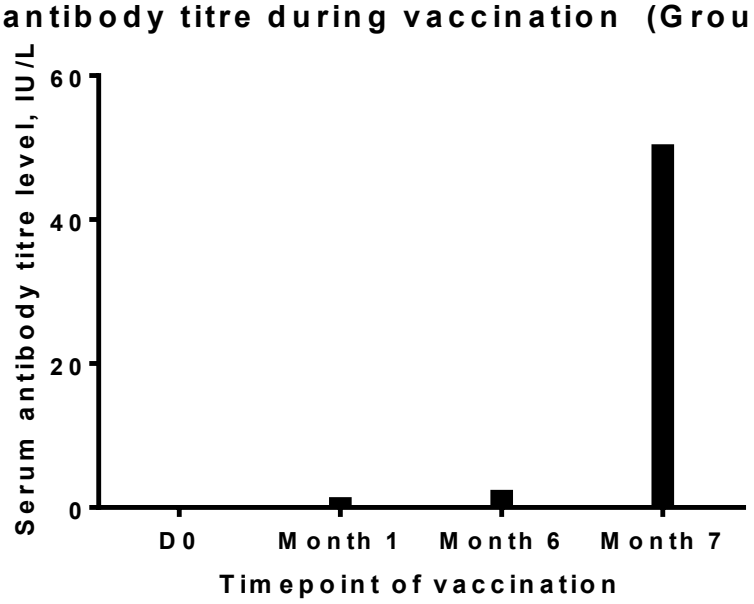


Figure 3.5 HBsAb levels obtained at months 2, 6 and 7 for a successfully vaccinated Group B participant in comparison to the serum antibody titre prior to receiving HBV vaccination (D0).

At the point of second vaccination (month 1), a serum antibody response was seen in 14% of Group A patients and 15% of Group B patients. Only three Group A participants and two Group B participants showed a change in serum antibody titre at this timepoint. In all cases, the HBsAb titre level observed was less than 2IU/L.

At month 6 (point of final vaccination), 29% of Group A patients were noted to be fully immune with serum antibody titres at or above 10IU/L, whilst 41% of Group B patients had serum antibody titres at or above 10IU/L. HBsAb titres at the time of final vaccination (i.e. month 6) for both Group A and Group B participants are illustrated in Figure 3.6. Of the six women who had been successfully immunized at month 6 in Group A (Figure 3.7), the most common stage of ovarian cancer was stage I (66%), with

the most common subtype being endometrioid ovarian cancer. Of the five responding women in Group B (Figure 3.7), four were noted to have stage 3 ovarian cancer, with the most common subtypes being serous and endometrioid ovarian cancers. Table 3.3 and figure 3.8 demonstrate the hepatitis B serum antibody response to vaccination and geometric mean serum titres at month 6 in patients successfully vaccinated at this timepoint, as compared to historical age-matched control patients outlined in De Rave et al. Figure 3.8 confirms a statistically significant difference in geometric serum antibody titre between the historical control group and each of the participant groups, using the single sample T test.

After completing the three-part vaccination, 57% of women in Group A were successfully immune at the 7 month-timepoint (i.e. HBS antibody titre≥10IU/L), noting that lower levels of HBsAb were found in Group A than in Group B – although this was not significant overall. Of the twelve responders in Group A at month 7, seven had stage I ovarian cancer, two had stage three and three women had stage two ovarian cancer. The most common subtype of ovarian cancer was serous ovarian cancer, followed by endometrioid cancer. Of the nine responders in Group B at month 7, seven had stage 3 ovarian cancer, one had stage 2 and one had stage 4 ovarian cancer. The most common subtype of ovarian cancer in Group B immunized patients was serous ovarian cancer followed by endometrioid ovarian cancer. Furthermore, a full HBsAb response was seen earlier in the vaccination schedule in Group B participants (38%, 5/13 women) with HBS antibody titre ≥10IU/L, compared to 29% (6/21 women) in Group A, although this difference was not statistically significant (p=0.4424, two-tailed p value).

Of the three patients who relapsed in Group A, only one patient was found to be successfully immunized. Of the seven patients who relapsed in Group B, five had been successfully immunized, with four patients having a mean serum antibody titre above 1000IU/L.

Figure 3.9 summarizes the individual and arithmetic mean serum antibody titres obtained for each group of patients at month 7, following completion of the vaccination

schedule. Figure 3.9a looks at the serum antibody levels at month 7 of successfully vaccinated patients.

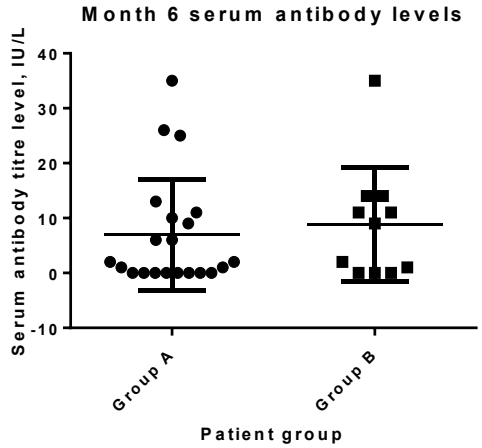


Figure 3.6 Serum antibody titres at the time of final vaccination in recently completed chemotherapy patients (Group B) and in women who had completed chemotherapy over a year ago (Group A). Bars indicate mean antibody titres and standard deviation. A non-significant difference was seen between the two groups using a two-tailed unpaired t-test (p= 0.6365). For this analysis, in Group A, n=21 and in Group B n=11.

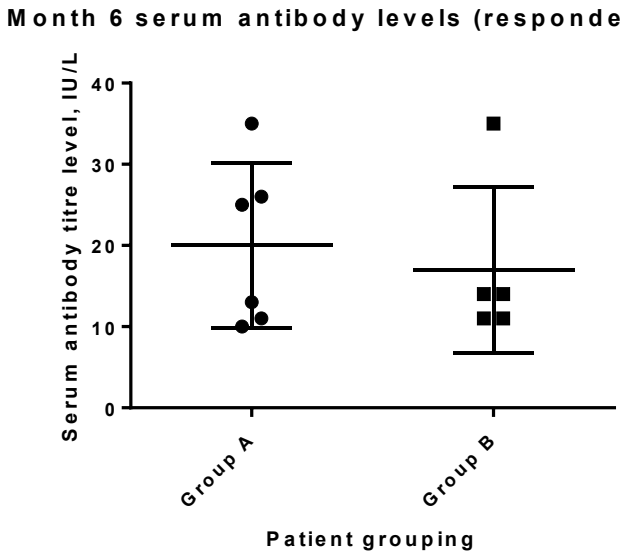


Figure 3.7 Serum antibody titres at the time of final vaccination for women already successfully immune in recently completed chemotherapy patients (Group B) and in women who had completed chemotherapy over a year ago (Group A). Bars indicate mean antibody titres and

standard deviation. A non-significant difference was seen between the two groups using a two-tailed unpaired t-test (p= 0.6374).

	Responders (%)	Non-responders (%)
Historical control	**26**	**74**
Group A	**29**	**71**
Group B	**41**	**59**

Table 3.3 Hepatitis B serum antibody response to vaccination at month 6 in patients successfully vaccinated at this timepoint in tabular format. Results obtained in Group A and Group B participants was compared with historical control patients outlined in De Rave et al, 1994. Fisher's exact test showed a statistically significant difference between the historical control patient group and group B (p=0.0356); the difference between historical control and group A was not statistically significant.

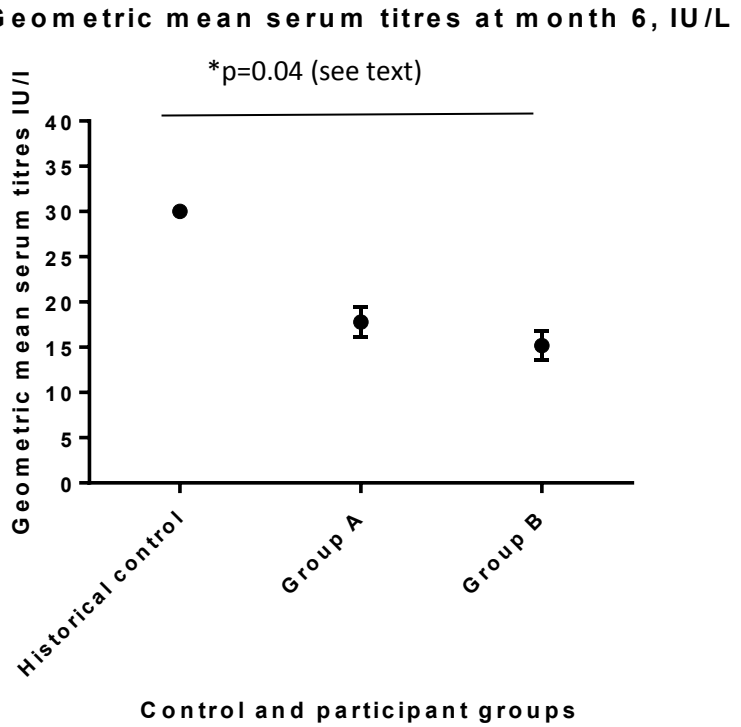


Figure 3.8 Geometric mean titres in responders obtained in Group A and Group B participants at the time of final vaccination (ie. Month 6) in relation to those obtained in historical controls (De Rave, 1994). Error bars denoting the standard deviation is also noted for each participant group. Single sample two-tailed t test showed a statistically significant difference between the historical control patient group and group B (p=0.04); the difference between historical control and group A was not statistically significant when using the two-tailed T test. Values for standard deviation was not available in the historical control data, however, the range of serum antibody titres was 13-93IU/L (geometric mean serum titre 30IU/L). By comparison, the range of serum antibody titres for Group A patients was 0-35 IU/L (geometric mean serum titre 17.87IU/L) and for Group B patients was from 10-35IU/L (geometric mean serum titre 15.27IU/L)

As the historical data available from De Rave et al did not include the full data set, simply the geometric mean serum titre, a single sample t test was used after consultation with a statistician to compare the participant data obtained with the mean of that within the historical control group, as the best approximation of a test of significance.

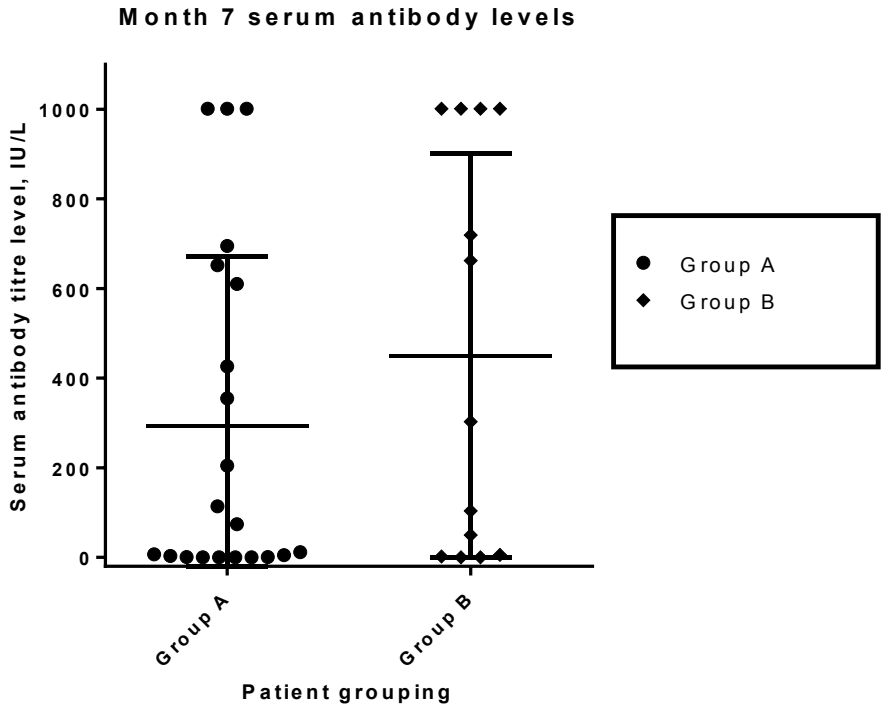


Figure 3.9 Final HB serum antibody levels one month after completing the vaccination course in women in Group A and Group B. Bars indicate the arithmetic mean and standard deviation for each group; HBsAb levels above 10IU/L correlate with HBV immunity. There was no statistically significant difference seen between Group A and Group B participants using the unpaired two-tailed t-test (p=0.2831).

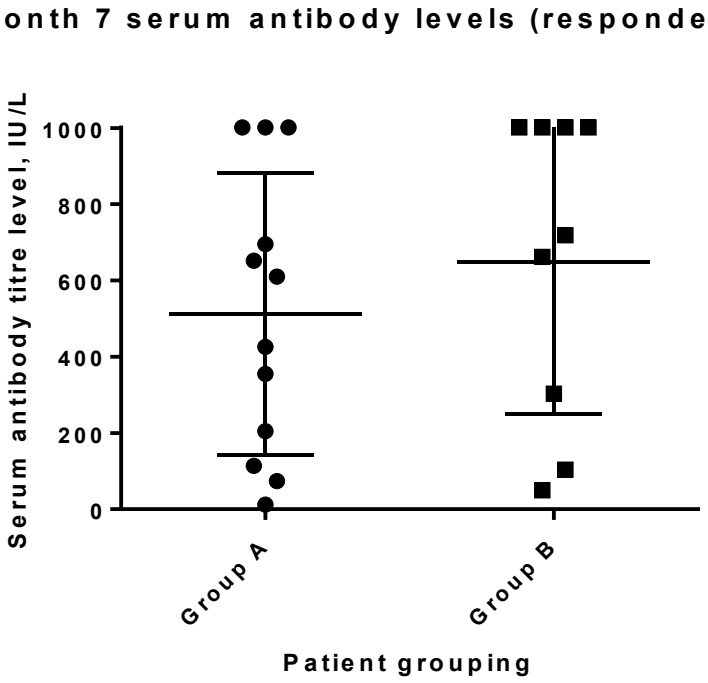


Figure 3.9a Final HBs serum antibody levels one month after completing the vaccination course in full responders in Group A and Group B (i.e. HBsAb levels above 10IU/L only). Bars indicate arithmetic mean values and standard deviation for each group. There was no statistically significant difference seen between Group A and Group B participants using the unpaired two-tailed t-test (p=0.427).

Serum antibody titres obtained at month 7, following vaccination were compared with historical control data outlined in De Rave et al, 1994. In all cases, the same inclusion and exclusion criteria were applied to all study participants, thereby ensuring the sole difference between historical control participants and our study patients was the diagnosis and treatment of ovarian cancer. Figure 3.10 shows the difference between the serum antibody titres seen in Group A and Group B patients, together with the level of serum antibody titres observed in historical control patients. This was noted to be non-significant using ANOVA analysis (p=0.6744). The frequency of HBsAb titres seen over 100IU/L in Group B participants was also noted to be higher than those observed in Group A patients. It should be noted that Hammersmith laboratories reported serum antibody titres upto and including 1000IU/L only.

Figure 3.11 illustrates the geometric mean titres obtained in Group A and Group B participants in relation to those observed in historical controls. Here, a non-significant difference is seen in the mean titres between Group A and Group B participants at the end of the study (p=0.2684), but a statistically significant difference was seen between the mean titres in the historical control group and each of the participant groups. Again, serum titres measured in the laboratory at Hammersmith was limited to 1001IU/L. No significant difference was found when comparing HBV serum antibody response to vaccination within stratified CA125 serum levels at the end of the study, as illustrated by Figure 3.12; however, with few data points in CA125 levels above 200, no firm conclusions can be drawn.

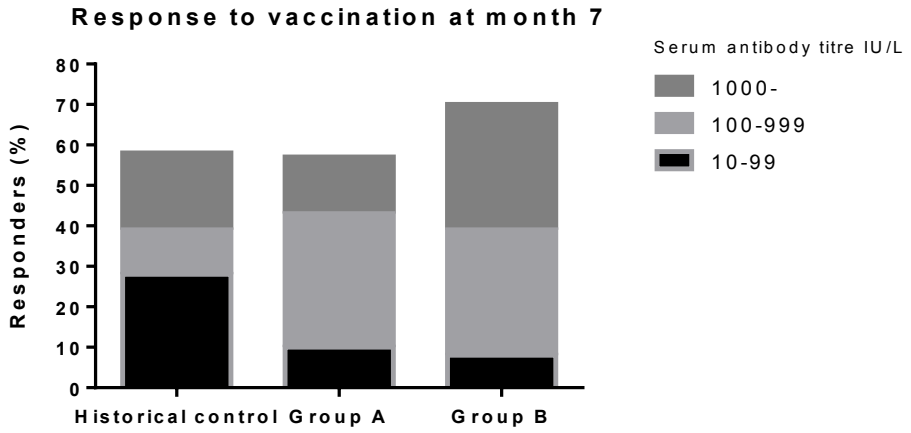


Figure 3.10 Hepatitis B serum antibody response to vaccination at month 7 in patients successfully vaccinated at the 7 month timepoint. Results obtained in Group A and Group B participants were compared with historical control patients outlined in De Rave et al, 1994. p<0.0001 for both comparison between responders in the >10-99IU serum antibody titre category in historical control and Group A and historical control and Group B (two-tailed Fisher's exact unpaired test); similarly, p<0.0001 for both comparison between responders in the >100-999IU serum antibody titre category in historical control and Group A and historical control and Group B (two-tailed Fisher's exact unpaired test). There was no significant difference between responders in the >1000IU serum antibody titre category in historical control and Group A and historical control and Group B (p=0.4107 and p=0.2062 respectively, two-tailed Fisher's exact unpaired test).

The difference between the percentage responders and non-responders in historical control group and B was non-statistically different (p=0.104)

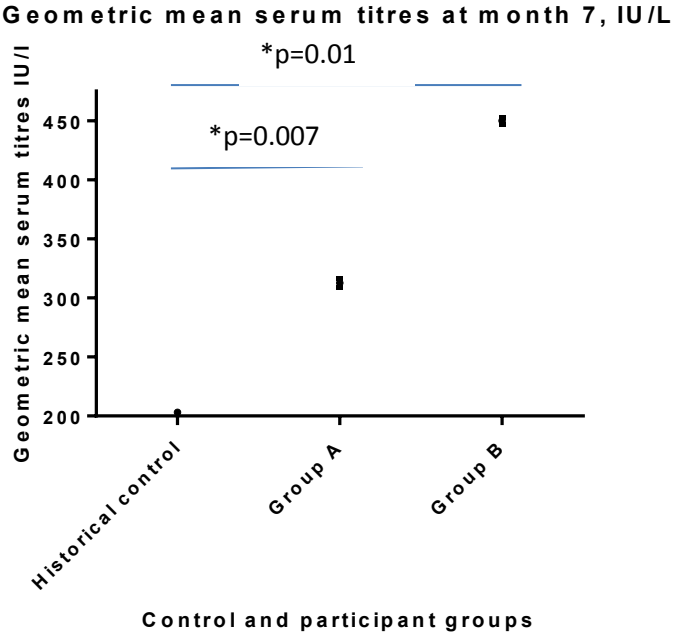


Figure 3.11 Geometric mean titres in responders obtained in Group A and Group B participants at month 7 in relation to those obtained in historical controls (De Rave, 1994). Error bars to highlight standard deviation for participant groups are also shown. Single sample two-tailed t tests showed a statistically significant difference between the historical control patient group and group B (p=0.01); the difference between historical control and group A was also statistically significantly different using the two-tailed t test (p= 0.007). A single sample t test was used as the full data set for the historical control data published by De Rave could not be obtained, after discussion with a statistician

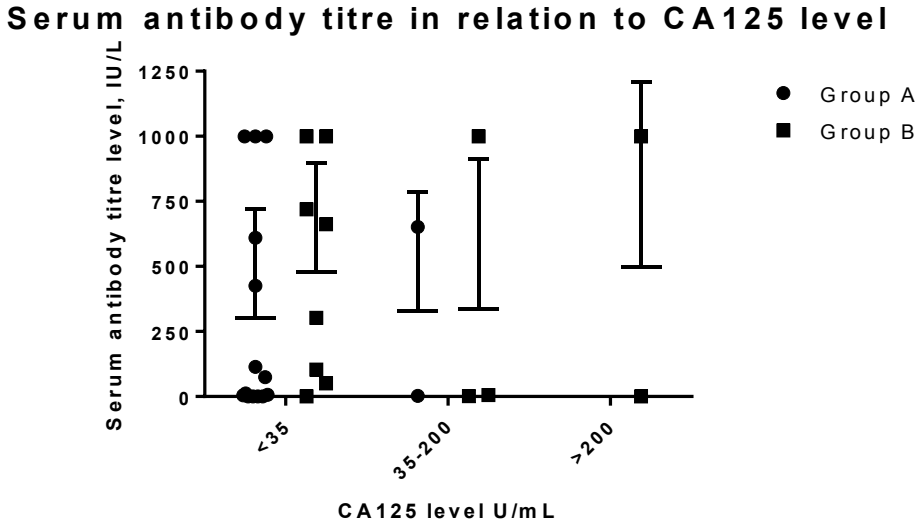


Figure 3.12 Hepatitis B serum antibody response to vaccination at month 7 when compared to CA125 serum levels, taken at the end of the study. Bars indicate mean titre levels and standard deviation. There was no statistically significant difference seen between Group A and Group B participants, using multiple t-tests (at CA125 levels <35, p=0.35 and 35-200 p=0.987)

In group A, 3 women were noted to relapse upon followup after completion of chemotherapy. Of these women, only 1 had been deemed to be successfully immunised using the HBV vaccine. In group B, 7 women relapsed and three deaths noted within 18 months of study completion. Of the seven women who relapsed, 6 were considered to be fully immune to the Hepatitis B vaccination. Of the three deceased women, two had responded to the Hepatitis B vaccine successfully.

3.1.5 T cell response to vaccination

One of the benefits of the DNA HBV vaccination is its ability to elicit a CD8+ T cell response. As well as measuring the serum antibody titre, we enumerated antigen-specific CD8+ T lymphocytes in HLA-A2 positive individuals. CD3+CD8+ T cells were stained with Pro5 Pentamer and analyzed by flow cytometric analysis in order to determine the frequency of antigen-specific T cells.

3.1.5.1 HLA-A2 typing

HLA-A2 typing was undertaken for all of the 34 patients recruited. Using flow cytometry, 9 patients were found to be HLA A2 negative. Figure 3.13 illustrates a HLA A2 positive result. Women with a HLA A2 positive result underwent further pentamer staining to quantify the CD8 T cell response to HBV vaccination.

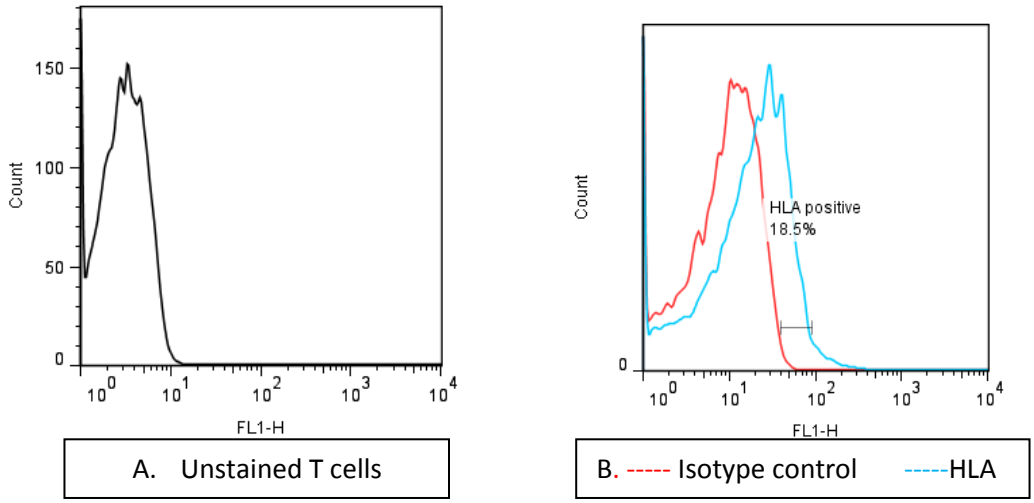


Figure 3.13 Histogram from flow cytometry staining; Panel A shows unstained T cells, Panel B shows 18.5% HLA positive result when compared to the isotype control

3.1.5.2 CD3+CD8+pentamer T cell response

Once the HLA-A2 status had been confirmed, isolated PBMCs obtained from blood taken at each vaccination timepoint were stained with anti-CD3, anti-CD8 and Pro5 pentamer in order to enumerate the CD3+CD8+ pentamer population. Cell surface staining was performed using anti-CD3-FITC and anti-CD8-APC and at least 100 000 lymphocyte gated events were acquired on a FACS Calibur flow cytometer and analyzed with FlowJo software. Flow cytometry was used to study the functional and phenotypic properties of these cell subsets because of the diversity of populations seen in PBMCs. Values were considered to be positive when at least 0.02% of CD3+CD8+ pentamer stained T cells were counted as positively stained. Figure 3.14a and Figure 3.14b illustrates the steps performed to isolate CD3+CD8+pentamer+ T cell population

in each case. There was no significant difference in the CD3+CD8+ populations isolated for Group A and Group B patients at months 6 and 7, as illustrated by Figures 3.15a and 3.15b. Furthermore, there was no significant difference in CD3+CD8+ populations at month 6 and 7 for either Group A or Group B participants.

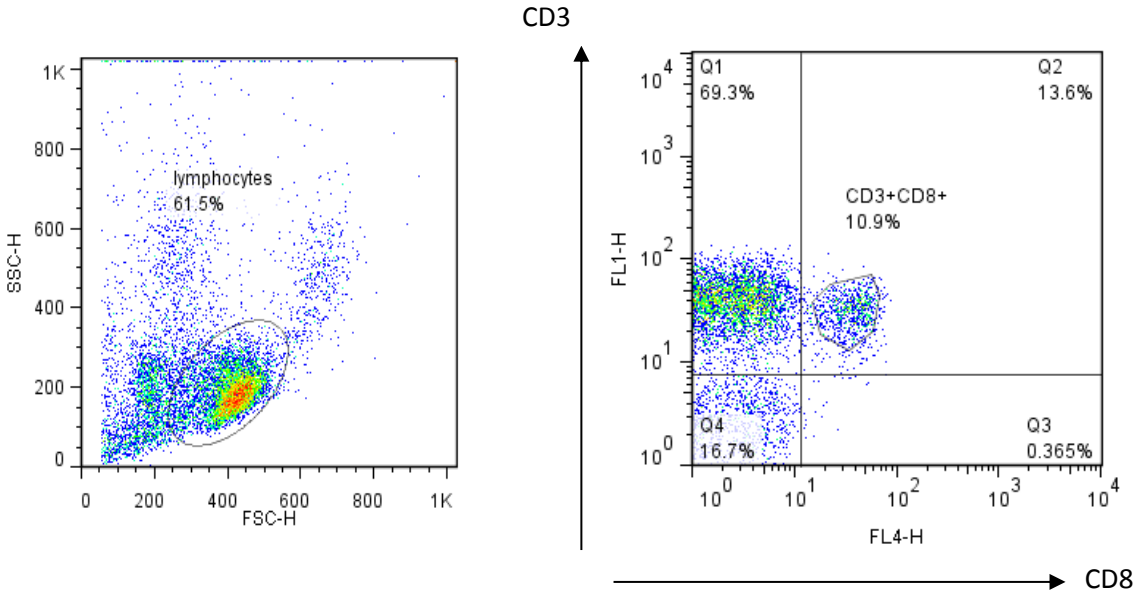


Figure 3.14a FACS cytometry staining of CD3+CD8+ T cell population. Panel A illustrates the lymphocyte population, whilst Panel B gates CD3+CD8+ T lymphocytes. Gates were set with appropriate isotype controls.

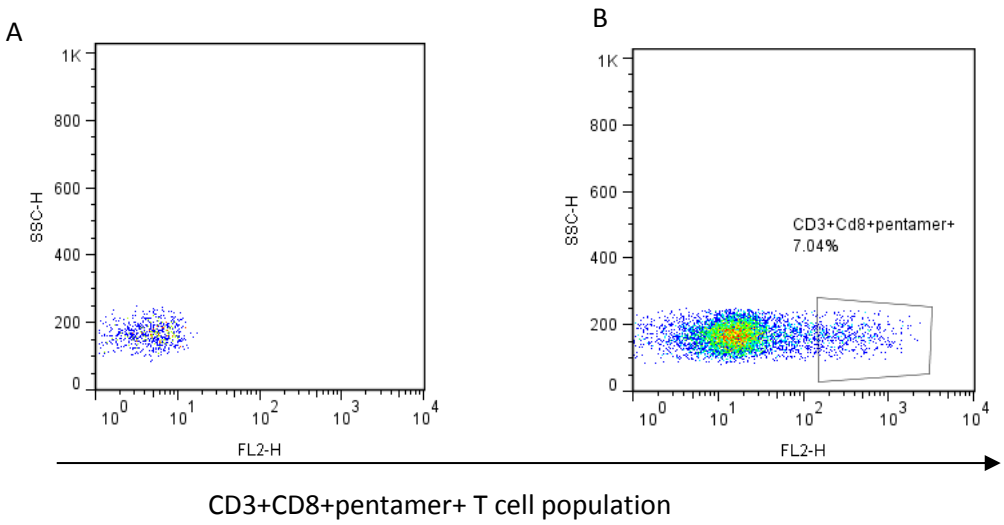


Figure 3.14b FACs cytometry staining of CD3+CD8+ pentamer+ T cell population. Panel A illustrates the CD3+CD8+ lymphocyte population which has not been stained with Pro5 pentamer (10 000 lymphocyte gated events), whilst Panel B isolates CD3+CD8+T lymphocytes which have stained positively for Pro5-pentamer (7.04%, 100 000 lymphocyte gated events). Where indicated, gates were set with appropriate isotype controls.

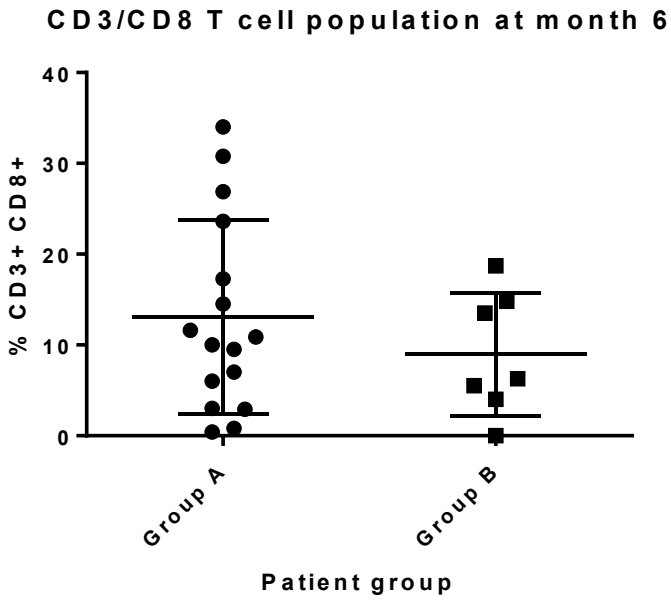


Figure 3.15a FACS cytometry staining of the CD3+CD8 T cell population at month 6 for both Group A and Group B participants. Bars indicate the mean expression and standard deviation for each group. There was no statistically significant difference seen between Group A and Group B participants using the unpaired two-tailed t-test (p=0.361).

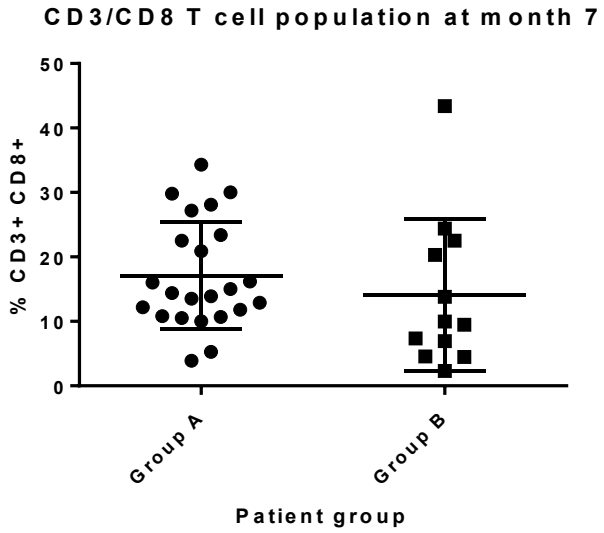


Figure 3.15b Isolation of CD3+CD8 T cell population using FACS cytometry at month 7 for both Group A and Group B participants. Bars indicate the mean expression and standard deviation for each group. There was no statistically significant difference seen between Group A and Group B participants using the unpaired two-tailed t-test (p=0.394).

3.1.5.3 CD19-pentamer T cell response

In order to validate the results obtained, in addition to enumerating CD3+CD8+pentamer+ T lymphocytes, pentamer positive T lymphocytes were also quantified by identifying and excluding CD19 B cells. Figure 3.16 illustrates the staining and exclusion of CD19+ cells to quantify pentamer positive T cells.

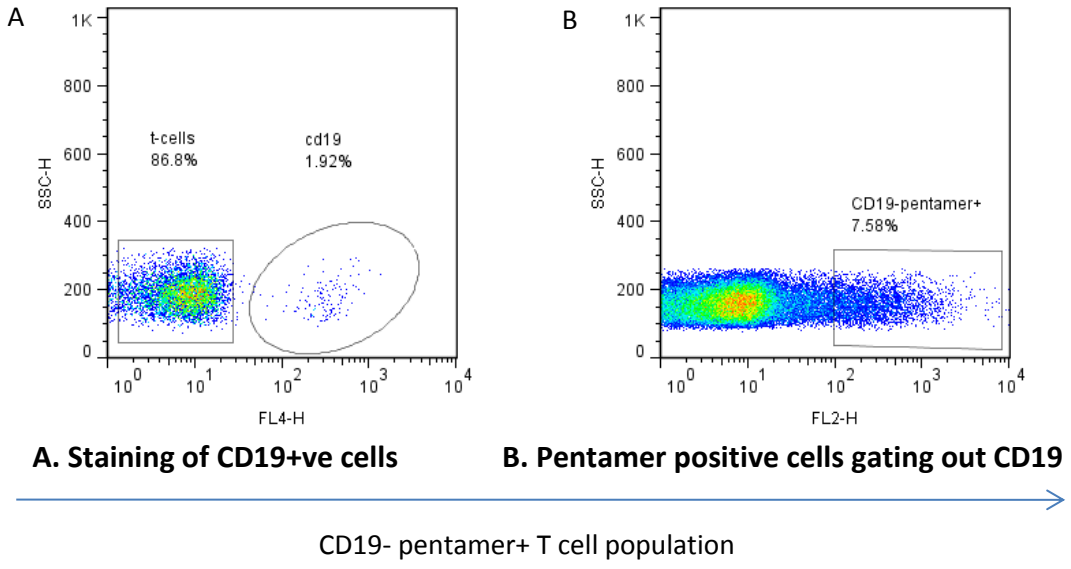


Figure 3.16 FACS cytometry staining. Panel A highlights the CD19+ population in SSC/APC channels (1.92%), gated on the lymphocyte population. Panel B gates on pentamer positive lymphocytes (7.58%), having excluded the CD19+ affiliated non-specific staining. Gates were set with appropriate isotype controls.

3.1.5.4 CD3+CD8+pentamer T cell response following stimulation

Given that the initial results obtained for CD3+CD8+pentamer+ lymphocytes was less than 10%, in a subgroup of patients, PMBCs were stimulated with IL-2 and IL-7 in culture as described in section 2.4.5. Figure 3.17 tracks the pentamer positive staining CD3+CD8+ T cell population in a participant treated for bilateral ovarian poorly differentiated serous papillary adenocarcinoma who has undergone the HBV vaccination series and is HLA A2 positive.

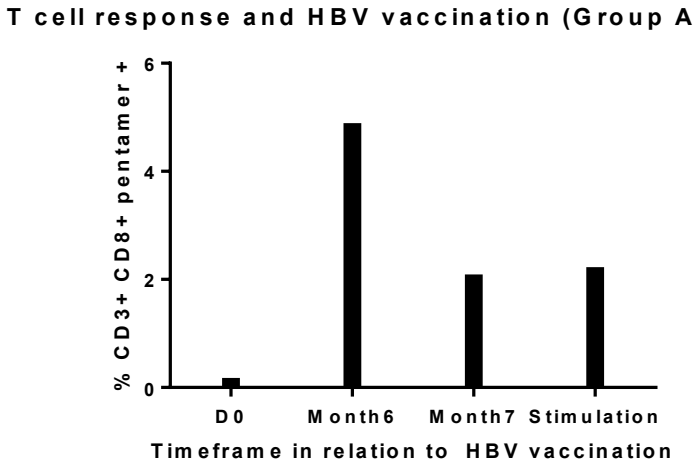


Figure 3.17 Summary of percentage CD3+CD8+pentamer+ T cells seen on the lymphocyte gate in a Group A patient from recruitment to month 7. The final column illustrates the percentage of CD3+CD8+pentamer positive lymphocyte population seen following 8 day stimulation with IL-2 and IL-7.

Figure 3.18 illustrates the percentage of CD3+CD8+pentamer+ T lymphocytes obtained at month 6, at the final point of the vaccination schedule. Here, the difference in mean percentages for Group A and Group B patients were noted to be non-significant. Figure 3.19 illustrates the percentage of CD3+CD8+pentamer+ T lymphocytes obtained once the vaccination schedule was completed. This was noted to be significantly higher in Group A patients than in Group B patients. A two-tailed paired t test found an insignificant difference between the values obtained at months 6 and 7 in Group A ($p=0.3340$) and similarly for group B patients ($p=0.1053$). A non-significant difference was also seen in the results obtained at months 6 and 7 when comparing Group A and Group B responders.

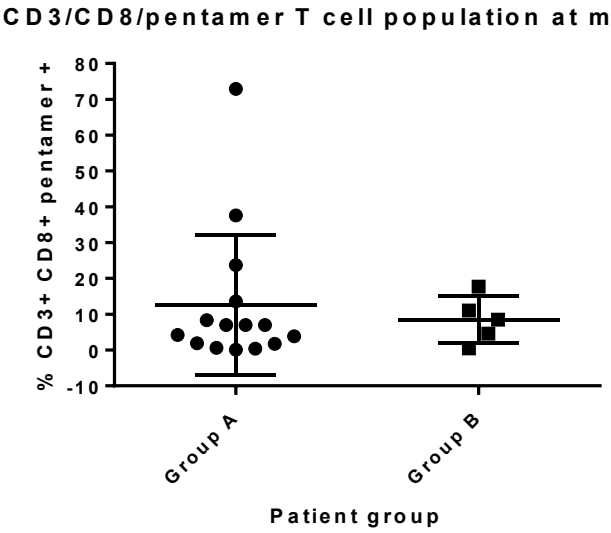


Figure 3.18 Percentage of CD3+CD8+pentamer+ T lymphocytes obtained in Group A and Group B patients at the point of final vaccination, on the lymphocyte gate. Bars indicate the mean expression and standard deviation for each group. There was no statistically significant difference seen between Group A and Group B participants using the unpaired two-tailed t-test (p=0.6466).

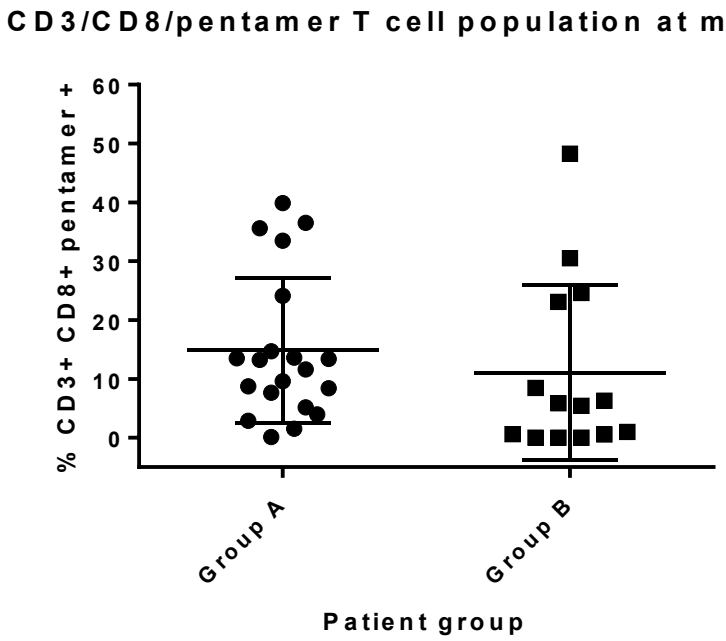


Figure 3.19 Percentage of CD3+CD8+pentamer+ T lymphocytes obtained in Group A and Group B patients once the vaccination schedule was completed, on the lymphocyte gate. Bars indicate the mean expression and standard deviation for each group. There was no statistically significant

difference seen between Group A and Group B participants using the unpaired two-tailed t-test (p=0.418).

3.1.6 CD45RO/RA isoform expression

CD4 and CD8 T cells are naïve or antigen experienced populations which include central memory, effector memory and effectors. Central memory and effector memory populations differ in their effector functions and ability to migrate to different anatomical sites. CD45 is a protein tyrosine phosphatase expressed on all hematopoietic cells which can be used to differentiate between naïve and memory cells. CD45RA and CD45RO are expressed on peripheral blood T lymphocytes within both the CD4 and CD8 subsets. Expression of the CD45RA and RO isoforms on T cells is thought to reflect the differentiation state of the cells, CD45RA denoting “naïve” T cells, as well as effector cells in both CD4 and CD8. After antigen experience, central and effector memory T cells gain expression of CD45RO and lose expression of CD45RA (Warren HS et al 1999). In a subset of our patients who had completed the vaccination schedule, we analyzed the proportion of CD45RA T cells and CD45RO T cells in order to ascertain the ratio of naïve to memory T cells. Figure 3.20 shows the RO+ T cell population obtained for Group A and Group B patients upon completing the vaccination schedule, with a non-significant difference was seen in the proportion of CD45RO+ T cells in Group A and Group B patients. Figure 3.21 illustrates a non-significant difference seen in the proportion of CD45RA+ T cells in Group A and Group B patients at the end of the vaccination schedule.

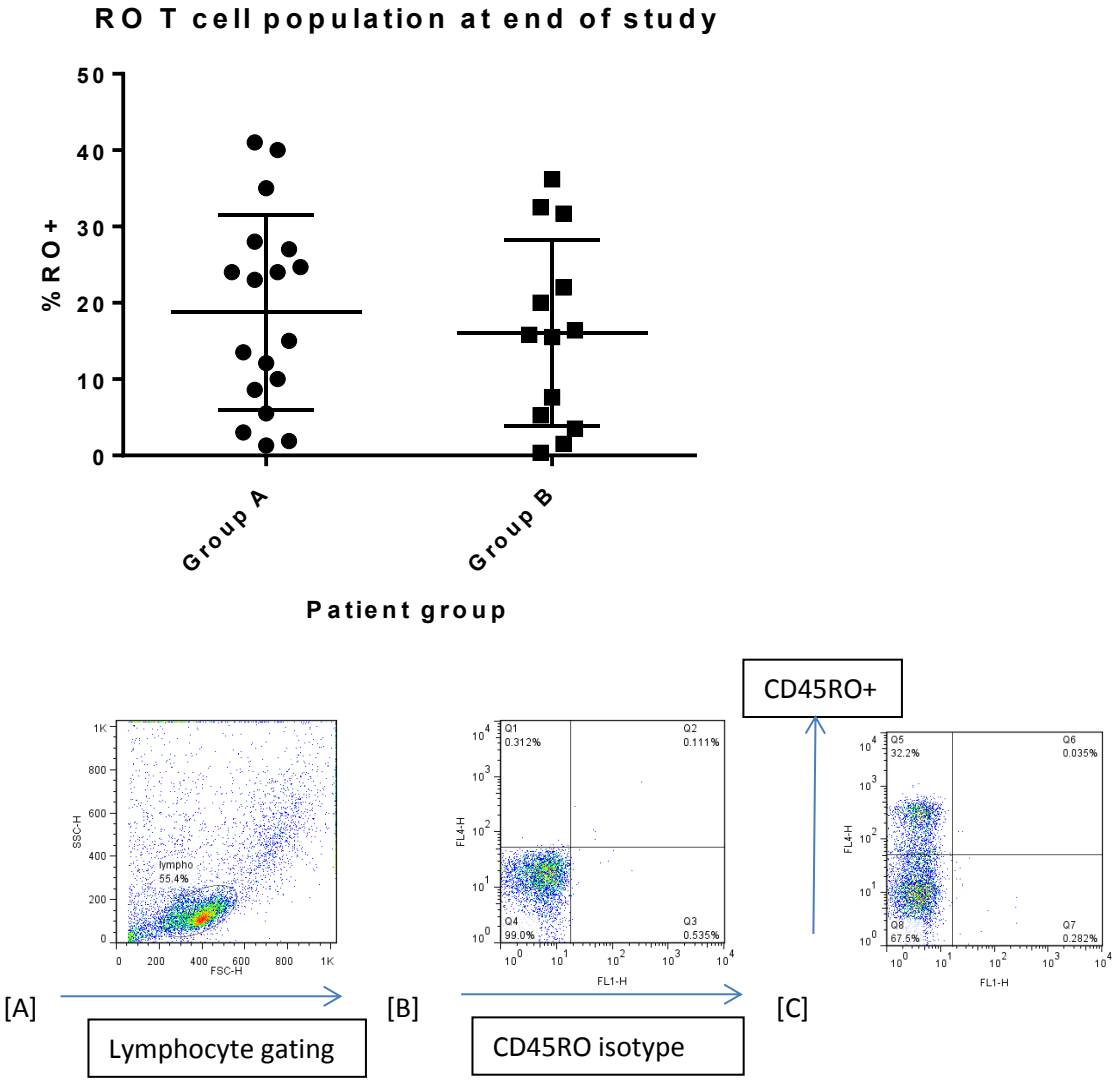


Figure 3.20 Percentage of CD45RO+ T lymphocytes obtained in Group A and Group B patients once the vaccination schedule was completed, on the lymphocyte gate using FACS cytometry. Bars indicate the mean expression and standard deviation for each group. A non-significant difference was seen between Group A and Group B patients using two tailed unpaired t test (p=0.2515). Panel A illustrates gating of the lymphocyte population which has not been stained with CD45RO (10 000 lymphocyte gated events), whilst Panel B demonstrates isotype staining. Panel C isolates the CD45RO+ T lymphocytic population which has stained positively (10 000 lymphocyte gated events).

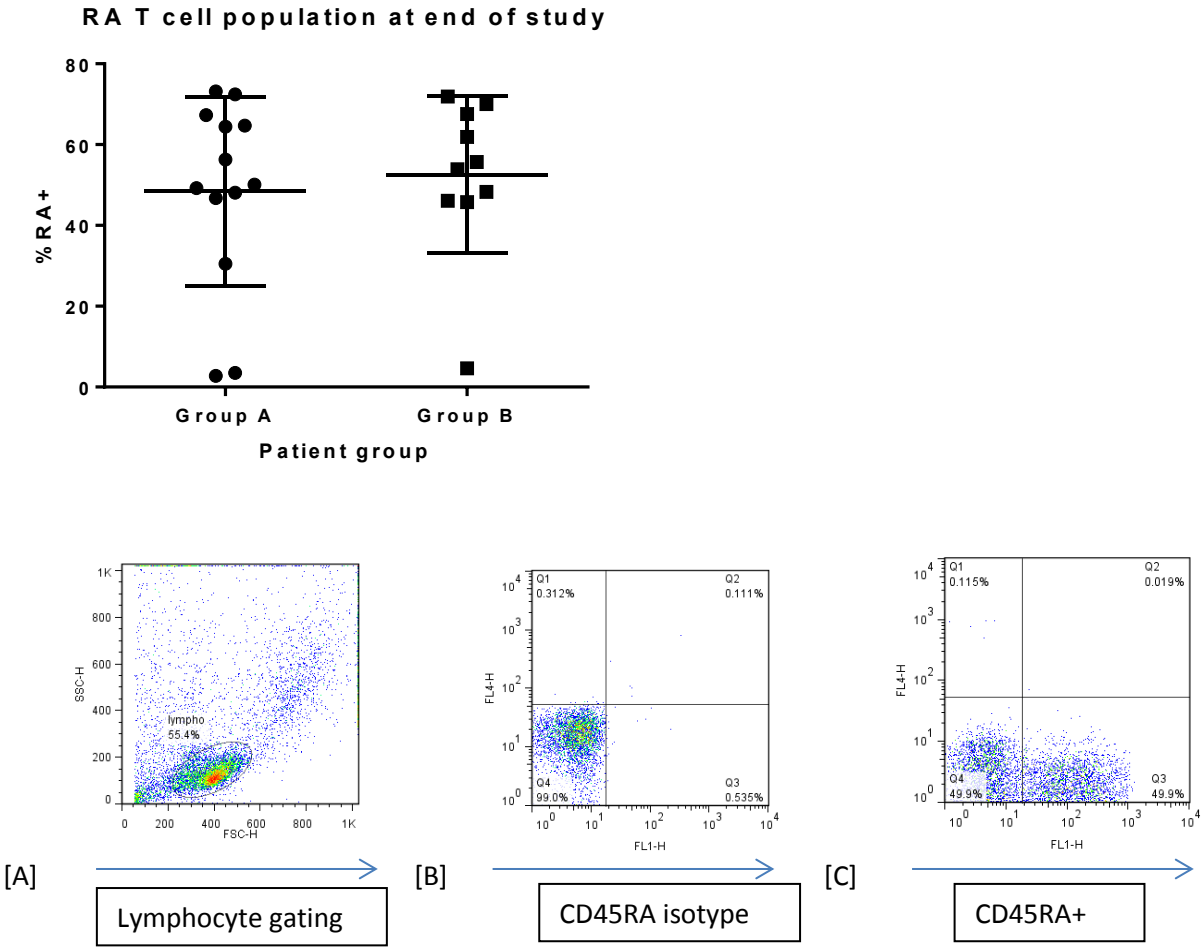


Figure 3.21 Percentage of CD45RA+ T lymphocytes obtained in Group A and Group B patients once the vaccination schedule was completed, on the lymphocyte gate using flow cytometry. Bars indicate the mean expression and standard deviation for each group. A non-significant difference was seen in Group A and Group B using two tailed unpaired t test (p=0.3425). Panel A illustrates gating of the lymphocyte population which has not been stained with CD45RA (10 000 lymphocyte gated events), whilst Panel B demonstrates isotype staining. Panel C isolates the CD45RA+ T lymphocytic population which has stained positively (10 000 lymphocyte gated events).

3.1.7 CD4/CD25/FOXP3 expression

The main focus of this part of our study was to examine the difference in the type of immune response mounted by the two patient groups. In a subgroup of vaccination

patients, CD45 RO/RA levels were enumerated in order to identify whether the T cell response seen was mounted by mature or immature T cells. In addition, we also conducted CD4+CD25+FOXP3 intracellular staining in the two patient group samples. Forkhead box P3 (Foxp3) is a member of the fork-head/winged-helix transcription factor family. It is the key regulatory gene and specific molecular marker for the development and function of CD4+CD25+ regulatory T cells (Tregs) and can be used as molecular marker for regulatory T cells (Hori S 2003; Ahmadzadeh et al 2006). In vivo and in vitro studies have shown that FOXP3 converts normal naïve T cells to T reg-like cells with a suppressive function (Sakaguchi S et al 2005).To evaluate any changes in the number of circulating T cells, the absolute lymphocyte count was measured at each point in the study. Whilst there are no reliable surface markers exclusively expressed on regulatory T cells, expression of FOXP3 was used as a molecular marker for regulatory T cells.
T regs are thought to mediate immune suppression in the tumour microenvironment. They are a heterogeneous CD4+ T cell population involved in suppressing the function of activated T cells. High levels of Foxp3 and Treg expression in tumour and blood of cancer patients has been associated with tumour progression, with studies showing that Tregs may enhance tumour immune evasion by inhibiting the local immune response (Woo E et al 2002). In addition, Foxp3 is expressed within tumour cells and may mimic the function of Tregs. Li et al studied the expression of Foxp3 in mouse Lewis lung cancer cells and found that Foxp3 reduces the sensitivity of lung cancer cells to chemotherapy agents, thus promoting tumour progression (Li C et al 2012). Foxp3 was found to upregulate the expression of multidrug resistance protein 1 mRNA and its protein product P-glycoprotein (P-gp), suggesting a new mechanism of resistance to chemotherapy in non-small cell lung cancer. Ahmadzadeh et al found that IL-2 administration on the CD4 T cell population of patients with metastatic melanoma and renal cell cancer increased the proportion of CD4+CD25 T cells expressing FOXP3, suggesting that the elimination of these cells may improve the response to IL-2 therapy (Ahmadzadeh et al 2006). Curiel et al found a strong association of CD4+CD25+ T cells with poor survival (Curiel TJ et al, 2004). Figure 3.22 highlights the similar CD4 T cell population in Group A and B patients at the end of the vaccination schedule, with a non-

significant difference also seen in CD4+/CD25+ and CD4+/CD25+/FOXP3 T cell population (Figures 3.23 and 3.24).

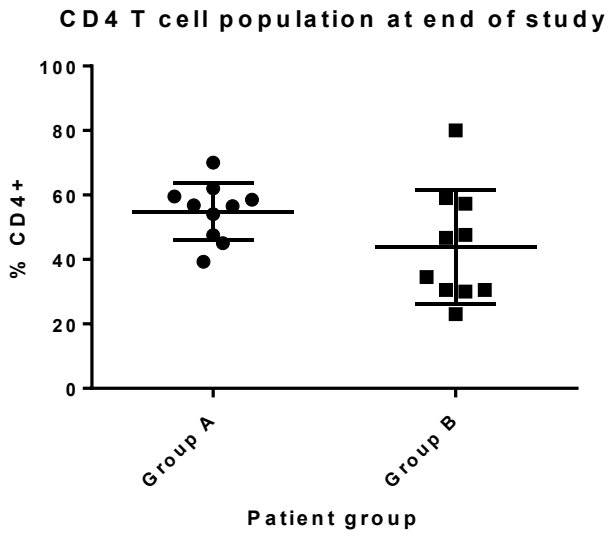


Figure 3.22 Percentage of CD4+ T lymphocytes obtained in Group A and Group B patients once the vaccination schedule was completed, on the lymphocyte gate. Bars indicate the mean expression and standard deviation for each group. There was no statistically significant difference seen using the unpaired t test two tailed (p=0.092). Note: CD4 T cell population was only reviewed at the end of the study in a subgroup of patients.

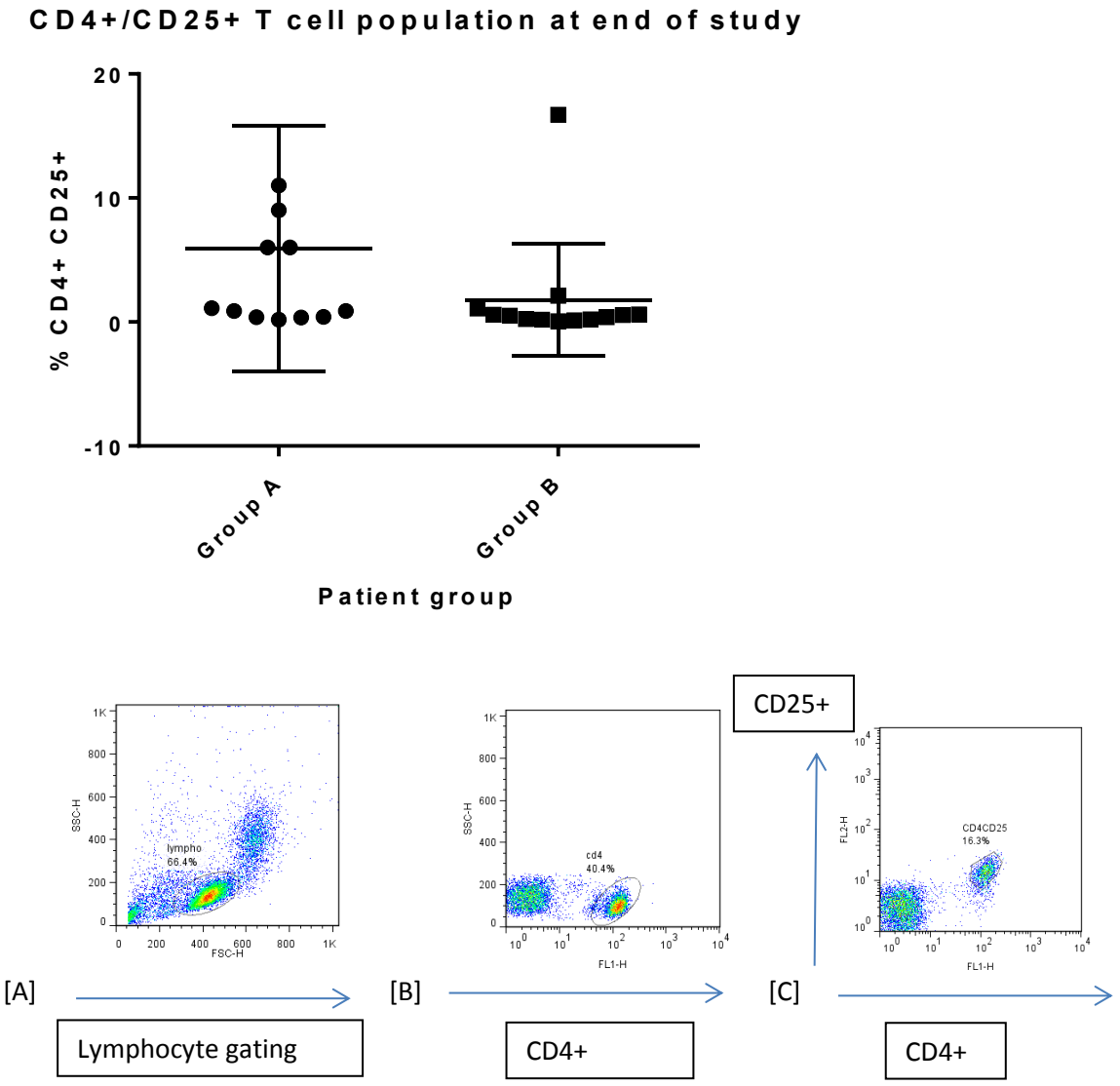


Figure 3.23 Percentage of CD4+CD25+ T lymphocytes obtained in Group A and Group B patients once the vaccination schedule was completed, on the lymphocyte gate. Bars indicate the mean expression and standard deviation for each group. A non-significant difference was seen in Group A and Group B using two tailed unpaired t test (p=0.1861). Panel A highlights the lymphocytic population, panel B highlights the CD4+ T cell population and panel C highlights the CD4+CD25+ population in FITC/PE channels, gated on the lymphocyte population. Gates were set with appropriate isotype controls.

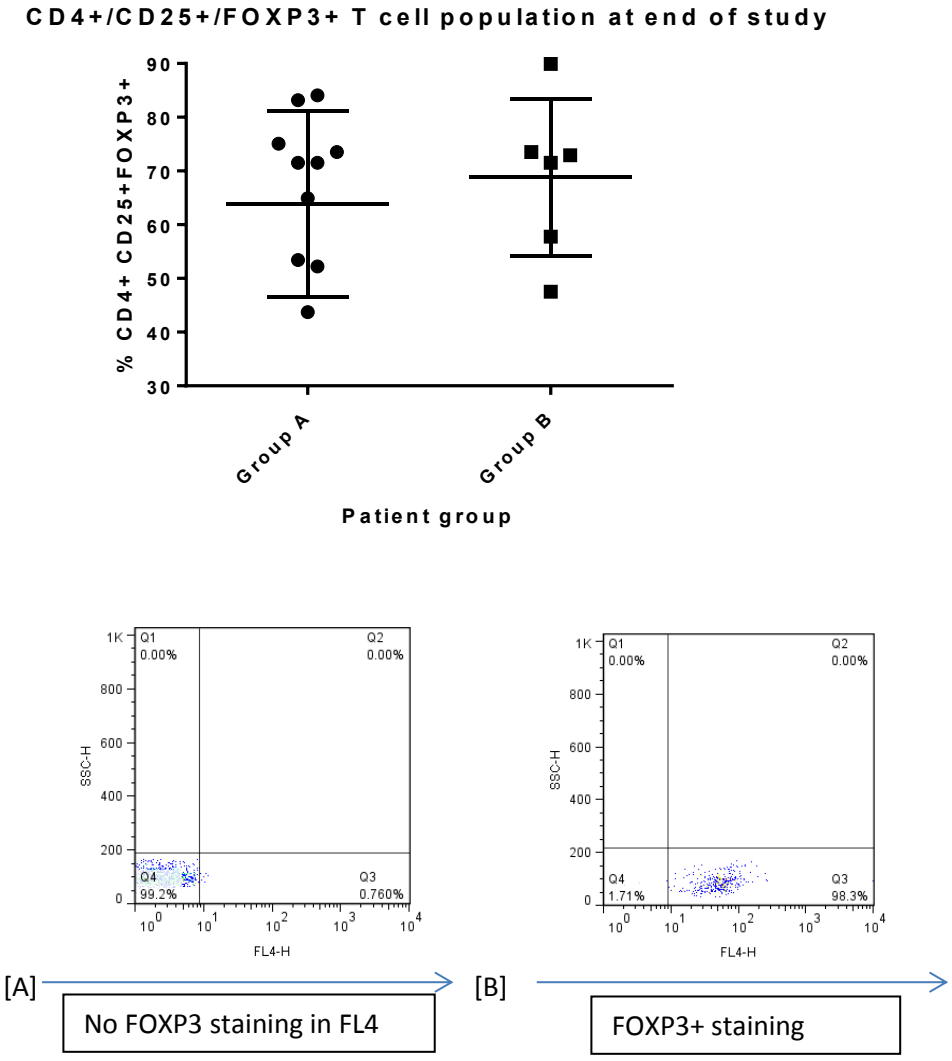


Figure 3.24 Percentage of CD4+CD25+FOXP3+ T lymphocytes obtained in Group A and Group B patients once the vaccination schedule was completed, on the lymphocyte gate. Bars indicate the mean expression and standard deviation for each group. There was no statistically significant difference between Groups A and B using the two-tailed unpaired t-test (p= 0.557). FACS cytometry staining in panel A highlights the CD4+CD25+ population in SSC/APC channels prior to intracellular FOXP3 staining, gated on the lymphocyte population. Panel B highlights the CD4+CD25+FOXP3+ population, gating on the lymphocytic CD4+CD25+ population (figure 3.23 panels A and B highlights gating of T lymphocytes and the CD4+ T cell population). Gates were set with the appropriate isotype control.

3.2 PD-L1 and PD-1 as a prognostic marker during chemotherapy

As outlined in the introduction of this thesis, the clinical need for highly sensitive and specific biomarkers for the diagnosis and prognosis of ovarian cancer is well established. In successfully treating and following up women with ovarian cancer, biomarkers which direct clinical decision making and prognosis would be of clinical benefit. Work in our laboratory has previously shown higher expression of PD-L1 on monocytes obtained from blood and ascites of patients with ovarian cancer when compared to patients with benign ovarian tumours. The purpose of this experiment was to explore the possible role of the PD-1/PD-L1 pathway as a prognostic marker in patients with ovarian cancer who were undergoing chemotherapy.

Blood samples were obtained over an 18 month period from 45 women with differing subtypes, stages and grades of ovarian cancer undergoing chemotherapy, with an average age of 64. Tables 3.4 and 3.5 summarize the patient demographics and cancer subtypes. Chemotherapy regimens included cisplatin and paclitaxol and blood was taken prior to each chemotherapy cycle. Wherever possible, individual patients were tracked through each chemotherapy cycle.

Stage of cancer	Number of patients
1a	2
1b	0
1c	3
2	2
3*	4
3a	3
3b	4
3c	17
4	10

Table 3.4 Stage of ovarian cancer in patients undergoing chemotherapy. (*stage 3 – undefined substaging)

Histological subtype	Number of patients
Serous	30
MMMT	4
Clear cell	3
Endometrioid	3
Mullerian	2
Large cell NE	1
Unknown	2

Table 3.5 Summary of ovarian cancer subtype in participants undergoing chemotherapy (NE – neuroendocrine; MMMT – malignant mullerian mixed tumours)

3.2.1 PD-L1 levels in the PBMC monocyte population

Immune cells present in blood were separated by Histopaque and checked for viability using trypan blue staining and microscopic examination. Immunological marker staining was then performed and analyzed by flow cytometry. CD14/PD-L1 monocytic levels have been investigated in these samples using flow cytometry and analyzed using Flowjo. A representative flow cytometry profile of a patient prior to their first cycle of chemotherapy diagnosed to have poorly differentiated primary peritoneal cancer is seen in Figure 3.25. Panel B shows a 78.2% CD14+PD-L1 positive monocyte population, which is higher than expected from a participant without tumour.

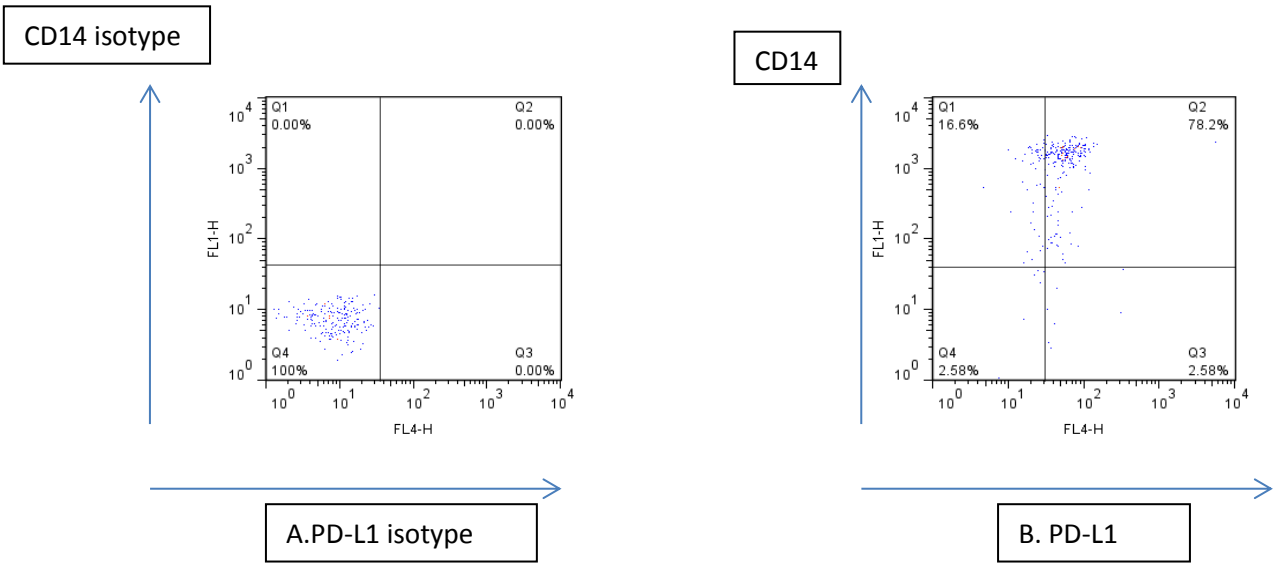


Figure 3.25 FACS cytometry staining in a patient prior to undergoing chemotherapy with stage 3C endometrioid ovarian cancer. This is representative of multiple experiments which are summarized in later figures. Gates were set with appropriate isotype controls. Panel A shows the PD-L1 isotype and CD14 isotype cell population. Panel B shows a 78.2% CD14+PD-L1+ population, gated on the monocyte population.

Figure 3.26 and 3.27 summarise the lymphocyte and monocyte populations seen prior to cycles 1, 3 or 4 and 6 of chemotherapy as well as post-chemotherapy (non-significant difference was obtained between cycles 1 and 6, p=0.3246 and p=0.1495).

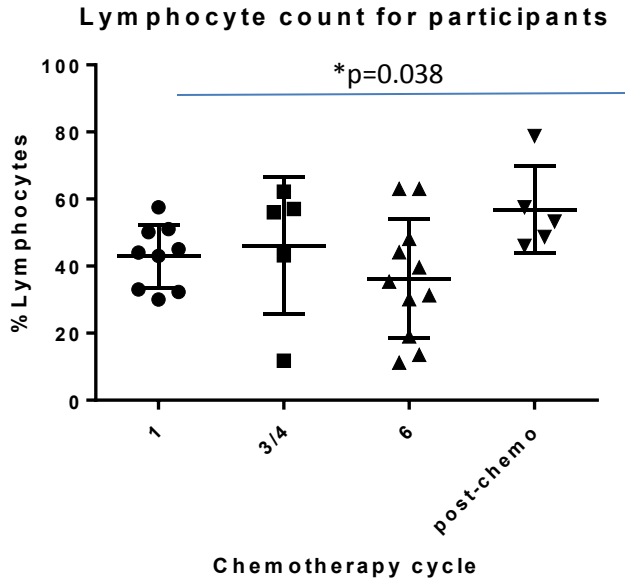


Figure 3.26 Lymphocyte population prior to each chemotherapy cycle in patients with ovarian cancer, analyzed using flow cytometry. Bars indicate mean and standard deviation values. A statistically non-significant difference was seen between cycles 1 and 6 using the unpaired two-tailed t-test (p=0.3246). A statistically significant difference was seen in lymphocyte count for participants prior to chemotherapy cycle 1 and post-chemotherapy using the unpaired two-tailed t-test (p=0.0382).

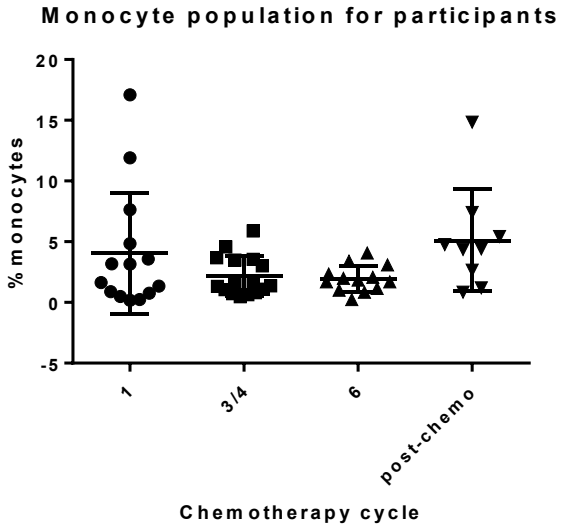


Figure 3.27 Change in monocytes population identified by flow cytometry in participants undergoing chemotherapy unpaired two tailed t test between chemotherapy cycles 1, 3/4 and 6 as

well as post-chemotherapy. Bars indicate mean expression and standard deviation for each group. A non –significant difference was seen in expression between chemotherapy cycle 1 and 6 using the unpaired two-tailed t-test (p=0.1495) as well as between chemotherapy cycle 1 and post-chemotherapy (p=0.610).

Figure 3.28 illustrates a non-significant difference in CD14 monocyte populations in participants prior to cycles 1, 3 or 4 and 6 of chemotherapy. Figure 3.29 summarises the CD14+PD-L1+ monocyte population seen prior to cycle 1 of chemotherapy and during chemotherapy. Again, a non-significant difference was seen.

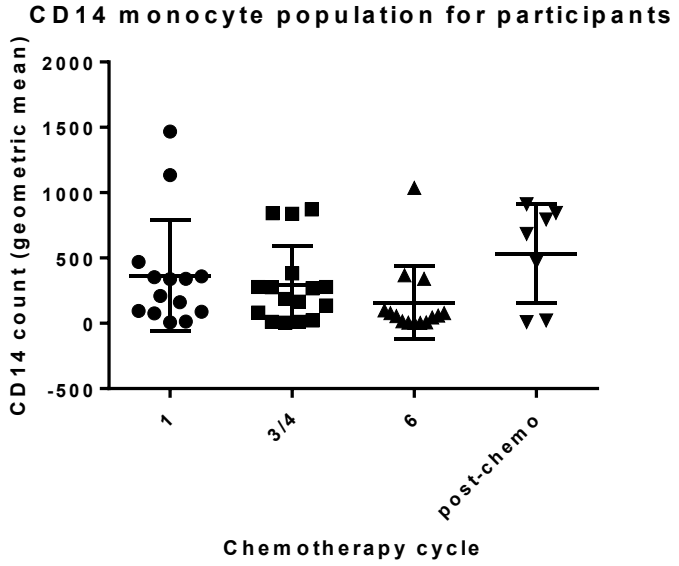


Figure 3.28 CD14 monocyte count for patients undergoing chemotherapy (cycles 1, 3/4 and 6) and post-chemotherapy. Bars indicate the mean expression and standard deviation for each group; minimum number in each group was five. A non –significant difference was seen in expression between chemotherapy cycle 1 and 6 using the unpaired two-tailed t-test (p=0.2766). A non-significant difference was also see when comparlng CD14 count between cycle 1 of chemotherapy and post-chemotherapy using the unpaired two-tailed t-test (p=0.8798).

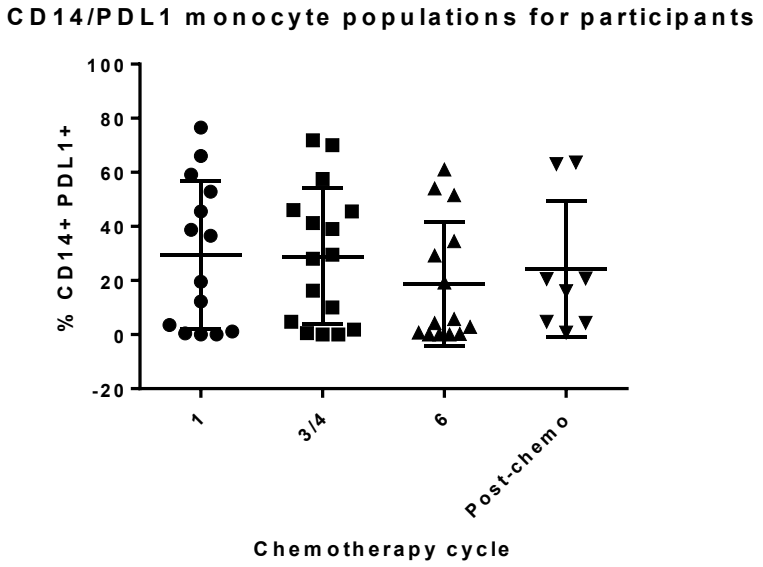


Figure 3.29 Percentage of CD14+PD-L1+ cells seen, on the monocytes gate in relation to chemotherapy – prior to cycle 1, at cycles 3 or 4 and at cycle 6 – as well as following chemotherapy. In each group, the minimum number of patients obtained was 5, maximum 10. Bars indicate mean and standard deviation values. Using the percentage of PD-L1 positive and CD14 positive cells for statistical analysis, an unpaired two tailed t test between cycles 1 and 6 showed a non –significant difference (p=0.2773). There was also a non-statistical difference in the CD14+PDL1+ population seen prior to cycle 1 chemotherapy and post-chemotherapy using an unpaired two tailed t-test (p=0.6632).

Having analyzed monocytic PD-L1 levels in women during chemotherapy using flow cytometry, PD-1 levels in serum samples taken from this group were also analyzed. PD-1 is expressed on the surface of activated T cells (Agata, Kawasaki et al 1996, Keir, Francisco et al 2007). PD-1 expression on lymphocytes was enumerated in patients undergoing chemotherapy for ovarian cancer, using cell separation, cell staining and flow cytometry. Figures 3.30 and 3.31 highlight the CD3 T cell populations and CD3/PD1 T cell populations prior to cycles 1, 3 or 4 and cycle 6 of chemotherapy. No significant difference was seen in CD3 or CD3/PD1 levels at the start of cycles 1 and 6.

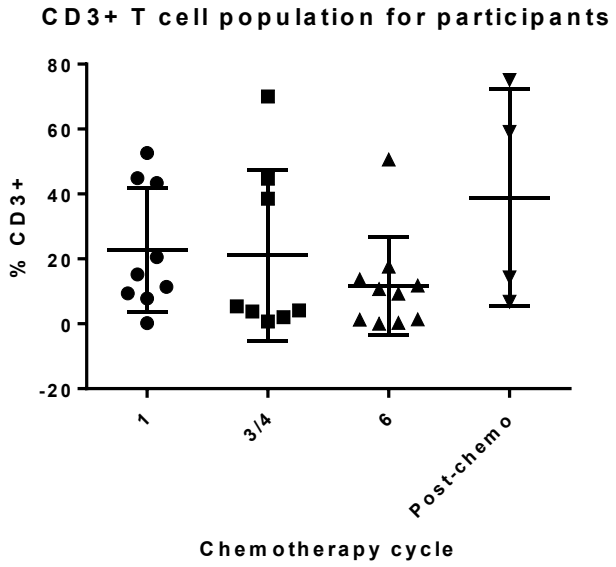


Figure 3.30 CD3+ lymphocyte population prior to chemotherapy cycle 1, 3/4 and 6 and post-chemotherapy. Bars indicate mean and standard deviation values. Using the percentage of CD3 positive T cells for statistical analysis, an unpaired two tailed t test between cycles 1 and 6 showed a non –significant difference (p=0.206). A non-statistically significant difference was also noted in the CD3+ population seen prior to cycle 1 chemotherapy and post-chemotherapy using the unpaired two-tailed t-test (p=0.2882).

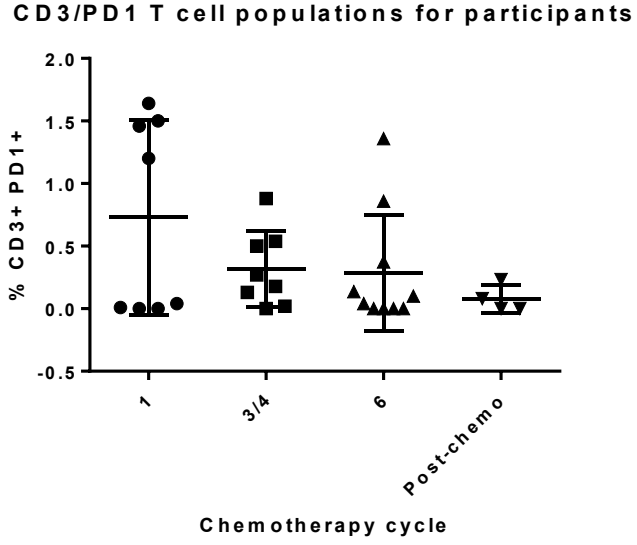


Figure 3.31 CD3+/PD1+ lymphocyte population prior to chemotherapy cycles 1,3 or 4 and 6 as well as post-chemotherapy. The minimum number of samples analyzed was four. Bars represent mean

and standard deviation values. Using the percentage of CD3 positive PD1 positive T cells for statistical analysis, an unpaired two tailed t test between cycles 1 and 6 revealed a non – significant difference p=0.151. There was also a non-statistical difference in the CD3+/PD1+ population seen prior to cycle 1 chemotherapy and post-chemotherapy (p=0.1338, unpaired two tailed t test).

As well as considering the lymphocytic and monocytic changes seen in patients undergoing chemotherapy, in a small cohort of patients, these experiments were repeated upon completion of chemotherapy. No significant difference was found in monocyte count, CD3, CD14+/PDL1+ or CD3+/PD1+ pre- and post-chemotherapy; a statistically significant difference was seen pre- and post-chemotherapy in lymphocytic count only (Figure 3.26).

In addition, CD14/PDL1 monocyte populations were reviewed for a subgroup of our patient cohort undergoing HBV vaccination – specifically, at the end of the schedule, using flow cytometry. This was done to investigate a correlation in the response to Hepatitis B vaccination and PD-L1 expression on monocytes. No significant difference was found in CD14/PD1 monocyte levels between group A and B patients at month 0 or 7, as demonstrated by Figure 3.32.

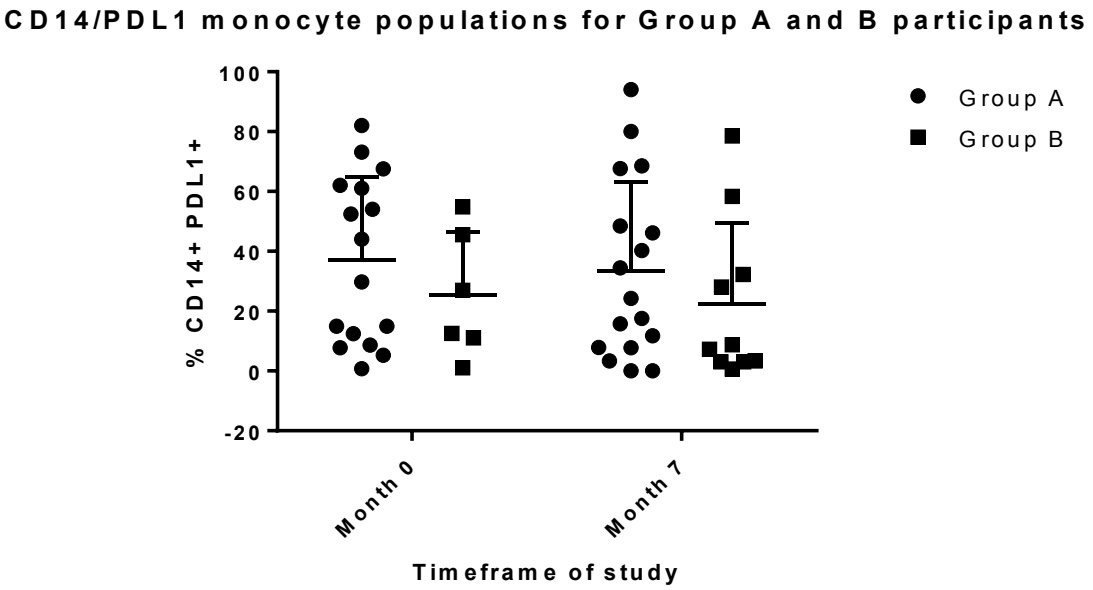


Figure 3.32 CD14+/PDL1+ monocyte populations identified at the start and end of vaccination for Group A and B participants. Bars indicate the mean expression and standard deviation values for each group. No significant difference was seen using ANOVA or Fisher's exact test on the mean values for each group at each time interval – p=1.0

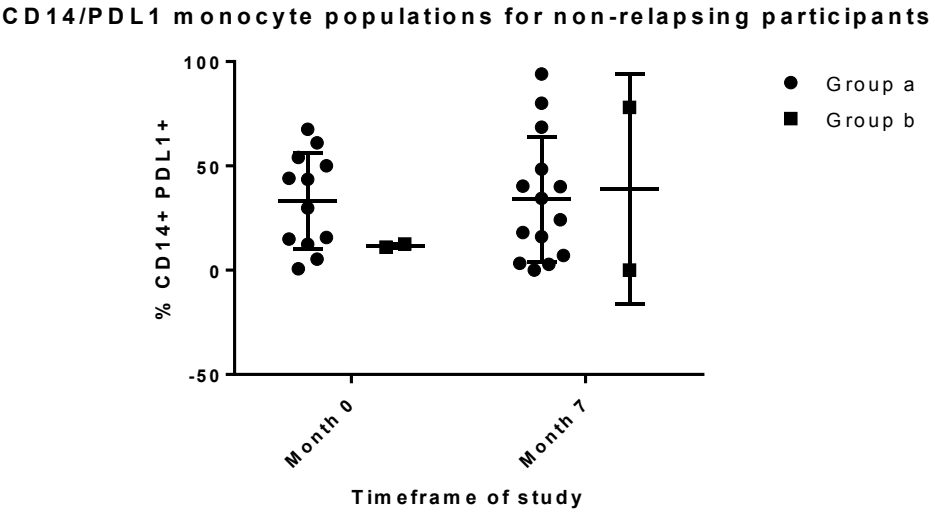


Figure 3.32a CD14+/PDL1+ monocyte populations identified at the start and end of vaccination for Group A and B participants who did not relapse. Bars indicate the mean expression and standard deviation for each group; the minimum sample size in each group was two. No significant

difference was seen using multiple unpaired t test – at month 0, p= 0.225214 between Group A and Group B participants and at month 7, p= 0.8437.

In addition, CD3/PD1 lymphocyte populations were reviewed for a subgroup of our patient cohort undergoing HBV vaccination – specifically, at the start and end of the schedule, using flow cytometry. This was done to investigate a correlation in the response to Hepatitis B vaccination and PD1 expression on lymphocytes. A significant difference was found in CD3/PD1 lymphocyte levels between group A and B patients at month 0, as demonstrated by Figure 3.33. This was also reiterated when focusing on non-relapsing participants (Figure 3.33a).

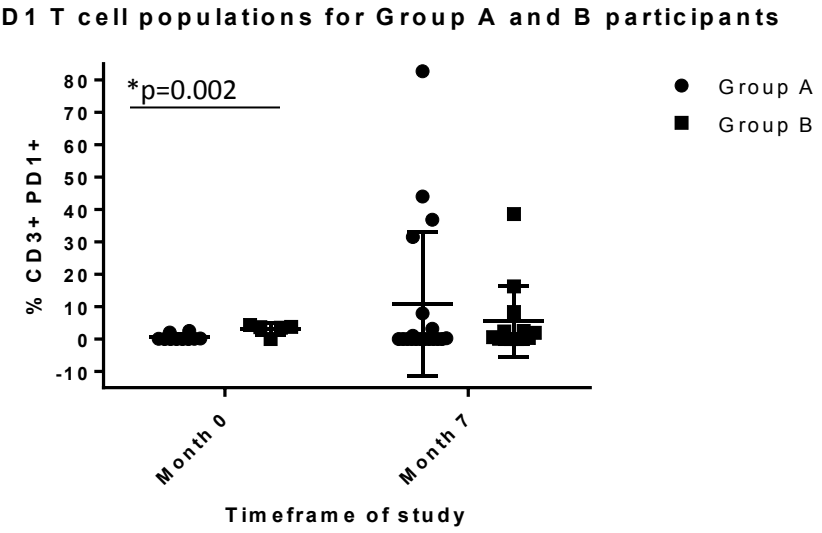


Figure 3.33 CD3+/PD1 T cell populations identified at the start and end of vaccination for Group A and B participants. Bars indicate the mean expression and standard deviation for each group. Whilst a statistically significant difference was found between Groups A and Group B participants at month 0 (p=0.002), no statistically significant difference was found between the two groups at month 7, using two-way ANOVA analysis. No significant difference was seen between months 0 and 7 for either Group A or Group B participants.

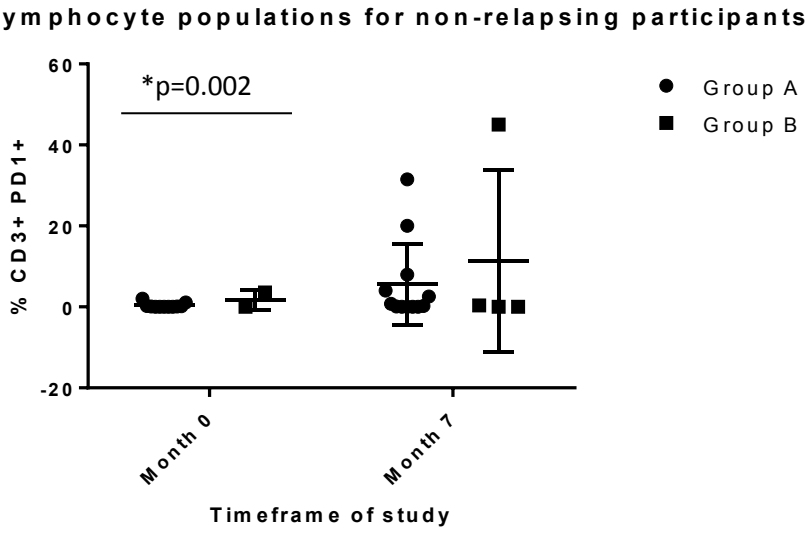


Figure 3.33a CD3+/PD1 T cell populations identified at the start and end of vaccination for Group A and B non-relapsing participants. Bars indicate the mean expression and standard deviation for each group; minimum number in each group was two. Whilst a statistically significant difference was found between Groups A and Group B participants at month 0 (p=0.002), no significant difference was found between the two groups at month 7, using two-way ANOVA analysis and multiple unpaired t tests. No significant difference was seen between months 0 and 7 for either Group A or Group B participants.

3.2.2 Quantification of PD-L1 levels in plasma

As well as enumerating CD14+PD-L1 levels in PMBC monocytes, in a subgroup of patients, we also quantified free soluble PD-L1 in plasma of women undergoing chemotherapy by using sandwich ELISA, developed and optimized within our laboratory as described in section 2.3.5. Samples of plasma were identified which had been stored at the time patients were recruited and analyzed for PD-L1 expression using ELISA. Figure 3.34 illustrates the mean and standard deviation of soluble PD-L1 serum levels obtained at each timepoint prior to starting, during and after chemotherapy.

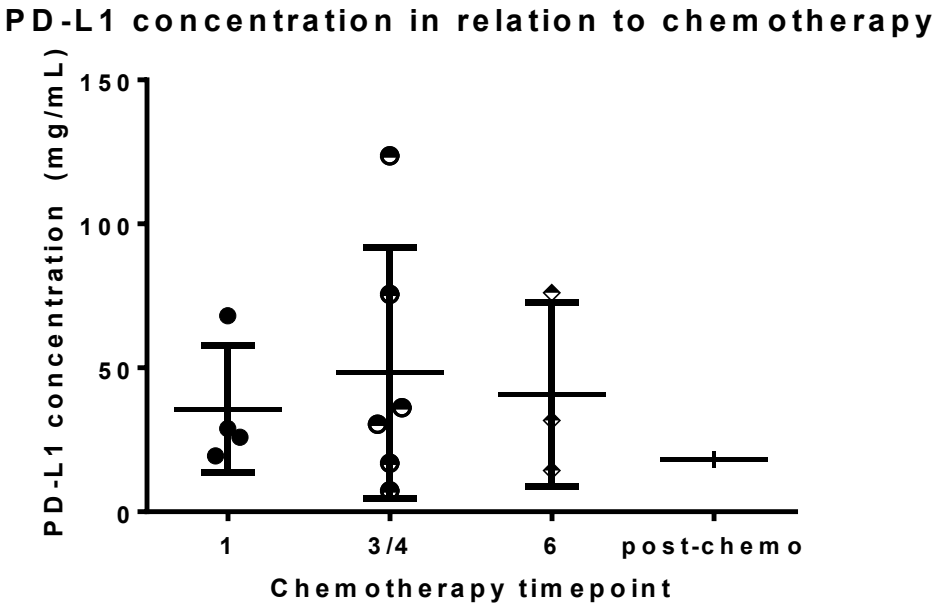


Figure 3.34 ELISA quantification of soluble PD-L1 in plasma in patients prior to chemotherapy cycle 1, during (cycle 3/4 and 6) and after chemotherapy ("post-chemo") in a subgroup of patients. Bars indicate the mean expression and standard deviation for each group; minimum number in each group was one. No significant difference in soluble PD-L1 concentration was seen between cycle 1 and cycle 6 using the two-tailed unpaired T test (p=0.8076); given the small sample size, no comparison could be made in the post-chemotherapy group.

4. DISCUSSION

4.1 Hepatitis B vaccination

There is a paucity of information on the effects of standard chemotherapy for cancer treatment on immune potential (particularly T cells) over time and hence when to optimally combine it with novel therapies such as immunotherapy. The degree of immunosuppression associated with chemotherapy treatment is influenced by several factors – including the subtype of ovarian malignancy, the treatment regimen and the patient's individual response to chemotherapy agents. Wu et al found that OVCAR-3 cells underwent apoptosis within 24 hours of exposure to paclitaxel and carboplatin, triggering dendritic cells to activate T cells (Wu X et al 2010). They subsequently found a significant increase in subsets of effector T cells at S2 phase, suggesting that the anti-tumour immune response peaked at 12-14 days after chemotherapy. Potentially therefore, immunotherapy –for example, a vaccine - could be given during S2 cell cycle, when the function of CD8+ T cells is already partially restored, potentiating the impact of effector CD8+ T cells to eradicate residual cancer cells most effectively. This work set out to investigate the immune response in women who have recently completed chemotherapy for ovarian cancer using HBV vaccination.

To investigate when the immune system recovers to mount an effective immune response following chemotherapy treatment for ovarian cancer, we compared patients who had recently completed chemotherapy (within three months – Group B) with those who had completed chemotherapy over a year ago (Group A), referring to the data published by De Rave et al for comparative healthy control patient data, without a diagnosis of ovarian cancer (De Rave et al 1994). Previous studies suggest a recovery in T cell function following chemotherapy for ovarian cancer (Tsuda et al, 2003). Our hypothesis therefore was that patients who had completed chemotherapy over a year ago would mount a stronger response to HBV vaccination. HBV vaccination was used as it is a synthetic yeast derived recombinant protein vaccination which elicits a measurable B cell and T cell response (Roy M et al 2000; Leroux-Roels G, 1994). One of the benefits of the DNA HBV vaccination is that it allows quantification of both the

serum antibody B cell response as well as T lymphocyte response. Extensive efficacy studies of HBV vaccines have demonstrated that a serum anti-HBsAg antibody concentration of 10 IU/L is associated with effective prophylaxis. We therefore firstly investigated the hepatitis antibody titres and secondly enumerated the T cell response during and upon completion of HBV vaccination. The Pro5 Pentamer+ T lymphocyte population was measured in two ways, by isolating CD3+CD8+pentamer+ T cells or by eliminating CD19 B cells prior to staining lymphocytes with Pro5 Pentamer and in all cases the values obtained were comparable. Negative controls were established by pentamer staining on samples obtained prior to first HBV vaccination.

Hepatitis-naïve participants were recruited on the basis of ovarian cancer diagnosis and treatment, medical history, haematology and chemistry results as well as negative serology for hepatitis B (Figure 3.1). The clinical characteristics of both patient groups were similar to each other and representative of ovarian cancer patients in the general population, with comparable ages ($p=0.3767$). Primary disease in both groups was predominantly either ovarian or serous fallopian tube cancer, but also included primary peritoneal cancer – all of which fall under the broad diagnosis of ovarian cancer and are treated as one entity (Table 3.1). Whilst there was no statistically significant difference in the two groups, the majority of the patients recruited in Group A were noted to have stage 1 and 2 disease, whilst those in Group B were noted to have stage 3 and 4 disease. The most common subtype of ovarian cancer in both groups was serous epithelial ovarian cancer – as was expected, given this is the most prevalent subtype (Bell DA, 2005). Stage IC was the most common stage in Group A patients, whilst stage 3C was more common in Group B patients (Figure 3.2). In both groups, grade 3 cancer was the most common grade, with the majority of patients in both groups undergoing primary surgical debulking followed by secondary chemotherapy. Patients who had received high-dose or non-standard chemotherapy were ineligible to participate. All patients had received a minimum of four to six months' of chemotherapy treatment, typically a platinum based agent with or without paclitaxel. Although HBV is not a live vaccine and therefore safe for immunocompromised patients, patients who were found to be immunocompromised were excluded from this study.

In a healthy adult population, over 90% achieve protective antibody titres against HBV following vaccination. De Rave et al found that age influenced the immune response to recombinant HBsAg, despite not using a control younger group (De Rave et al, 1994). Patients in both groups were clinically stable, with no statistically significant difference in common immune serum markers as highlighted in Figure 3.4, with the exception of CA125 levels (Figure 3.3). CA125 levels were significantly different at both timepoints between each group, and indeed between recruitment and month 7 in Group B patients (Figure 3.3). Whilst the limitations of CA125 as a prognostic marker have been outlined previously, this may be explained by the fact that the majority of patients recruited in this group have stage 3 or 4 malignancy (Table 3.2, Figure 3.2), or may relate to the higher relapse rate seen in Group B patients – perhaps due to a higher stage of disease at the point of recruitment. Again however, the difference in relapse rates between the two groups was non-significant (p=0.21). Nustad et al have shown that when stratified by disease stage, elevated CA125 levels were found in over 90% of patients with advanced stage ovarian cancer, but in only 50% of patients with stage I disease (Nustad K et al, 1996). In keeping with the literature on the response time to hepatitis B vaccination, our patients showed the highest antibody titre response to vaccination at month 7, as illustrated by Figures 3.5 and 3.9. This suggests a similar timeframe of B cell response to vaccination after chemotherapy treatment to healthy control patients.

Whilst this work included a wider age range of patients and focused on female patients only than that used by De Rave, a higher serum antibody response was demonstrated in Group B patients who had completed chemotherapy recently at both months 6 and 7, which was statistically significant when compared with De Rave's data (Table 3.3 and Figure 3.10). This was reiterated when comparing geometric mean values (Figures 3.8 and 3.11). Far more compelling was the fact that at month 7, a statistically significant difference was also seen between the serum antibody titres of 10-999 between Group A patients and De Rave's data, suggesting that antibody response is stimulated following chemotherapy – or potentially bone marrow function may be reset, stimulating a heightened response (Figure 3.10). This was reconfirmed when comparing geometric mean data for Group A patients and De Rave at month 7 (Figure 3.11). The yeast

derived recombinant Engerix B was given in both studies. In addition, mean HBsAb titres were seen to increase earlier in the vaccination schedule in patients who had completed chemotherapy recently (Group B), although the difference in response by Group A participants at month 6 was not statistically significant (Table 3.3, Figure 3.6). The 69% vaccination success rate in Group B patients suggests an effective B cell immune response can be mounted, 3 months following chemotherapy. Our results suggested that whilst the percentage of responders was similar in both patient groups, a statistically significant difference in response was seen when compared to healthy control patients. The mechanism for immune response may be hyperacute in women who have recently completed chemotherapy. Alternatively, the size of this response may be reflective of a system which has not fully matured. Coleman et al found that after a full course of chemotherapy, women in remission displayed potent CD8+ T cell responses, whilst those who progressed displayed low or decreasing T cell responses (Coleman S et al, 2005). However, Coleman related CD8+ T cell function to CA125 levels, which may limit the reliability of the findings. In our study, we did not find a significant correlation between normal or moderately raised (i.e. 35-200U/mL) CA125 serum levels at the end of the study and final serum antibody response to vaccination (Figure 3.12). However, interpretation is limited by the sample size in the CA125>200U/mL category.

In our study, the difference in magnitude of response for both patient groups in relation to historical controls in a similar age group is demonstrated in Table 3.3 and Figure 3.10. Table 3.3 shows a similar response rate between the historical control and group A patients, with a significant difference with group B patients, suggesting that the B cell response had occurred earlier in patients who had recently undergone chemotherapy. However, the magnitude of the actual serum levels were noted to be significantly different between the historical control group and both Group A and B patients. This may be due to the different laboratory methods used to assess serum antibody levels or indeed explained by the fact that patients in the historical control group have not been immunochallenged at any point.

Serum hepatitis B antibody titre levels provide a binary result for vaccination status, hence the use of the geometric mean serum antibody titres. This again confirmed a difference in serum antibody levels between historical control data and both Group A and B patients. More importantly, there was no significant difference in B and T cell response between Group A and Group B patients, suggesting that the immune response recovers within the first six months after chemotherapy treatment.

Although it could be argued that the sample size of the cohort investigated was small, the findings of both serum titres and geometric mean data are statistically significant and the study sample size is comparable to other studies recruiting cancer patients undergoing chemotherapy – for example, Nordoy et al recruited 35 patients in total (Nordoy T et al, 2002). The calculated number of patients was 44 per group to achieve a 15% difference with a 5% significance level and 80% power. Our results may be influenced by individual patient factors – for example, Group B Patient 2 had a raised BMI, although less than 45 – as well as tumour heterogeneity. In addition, a suboptimal immune response may have been mounted, as was seen in Group B Patient 3, who was noted to have a final serum antibody titre of 7mIU/L.

A further point to consider is the fact that approximately 5-10% of immunized individuals fail to elicit detectable specific antibodies and thus remain at risk of HBV infection (Coates et al 2001, Szmuness et al 1980). These individuals are unable to develop detectable or protective anti-HBs titres (Leroux-Roels et al 1997). Factors which have been proposed to explain the absence of response include the absence of ThI cytokine or Th2 production (Larsen et al 2000). Goncalves et al suggested that an overall inability of T cells to be inactivated could result in differences in MHC/antigen presentation. In our study, an altered T helper response may therefore have been seen in a minority of patients if vaccinated prior to cancer diagnosis and this response may have been exacerbated by diagnosis and treatment.

Intriguingly, the cell-mediated CD8+ T cell responses detected at months 6 and 7 displayed a non-significant difference between the two groups, suggesting that the T cell response had recovered in those who had recently completed chemotherapy

(Group B patients, Figures 3.18 and 3.19). The number of patients in these cohorts was smaller due to the need to be HLA-A2 positive to be compatible with pentamer detection as well as optimization of pentamer staining. This may have limited the reliability of the results obtained. Equally, given Ferrandina's findings, those with higher CD8+ T cells may be associated with better survival and may simply reflect the immunological features associated with Group A patients, who remain in remission one year after completing chemotherapy.

One potential mechanism to explain the differences in cell-mediated CD8+ T cell response may be that this response could be dependent on a mature immune system response, which may be depleted following immunosuppression due to chemotherapy. On the other hand, whilst the T cell response was not investigated in healthy age matched controls, there was no significant difference in antibody titre response between those who had recently completed chemotherapy and those who had completed chemotherapy over a year ago. Multiple factors – such as the impact of the regime and duration of chemotherapy, or tumour grade – may have influenced the immune response seen for patients who had recently completed chemotherapy.

In relation to the T lymphocyte response mounted to HBV vaccination, this was measured using Pro5 Pentamer, either by excluding CD19 B cells or specifically isolating CD3+CD8+ T lymphocytes. We found that the percentage of Pro5 pentamer positive cells were comparable but slightly higher when measuring Pro5 pentamer levels by excluding CD19 B cells (Figure 3.16). This may be due to the fact that a more specific population is isolated by focusing on CD3+CD8+ T lymphocytes, as opposed to merely excluding the B cell CD19 population (Figure 3.14). When comparing the percent of CD3+CD8+ and CD3+CD8+pentamer+ T cells at the end of the HBV vaccination schedule, a non-significant difference in response was seen between Group A and Group B patients (Figures 3.15a and 3.18, 3.15b and 3.19). Similar percentages of CD3+CD8+pentamer staining at month 7 and following stimulation suggest that whilst cell stimulation with IL-2 may have led to cell proliferation, the proportion of CD3+CD8+pentamer remained constant (Figure 3.17).

The nature of T cell response to vaccination was then further investigated in a subset of patients by enumerating the percentage of CD45 RO+ T cells to provide an indication of the maturity of the T cells involved in the immune response. Our work is unique in immunophenotypically characterizing lymphocyte recovery within the first year after chemotherapy treatment for ovarian cancer. Our results found a higher percentage of naïve CD45RA T cell population in Group B patients, although not statistically significant - potentially limited by the sample size (Figure 3.21). Similarly, a higher percentage of mature CD45RO T cells were found in Group A patients – although again not statistically significant (Figure 3.20). This suggests that whilst the overall T cell response seen is similar in patients who have recently completed chemotherapy, there is difference in the cellular make-up of that response.

Further to this, we also conducted CD4+CD25+FOXP3 intracellular staining. The tumour microenvironment involves the interaction between several cell types, as well as the cytokines secreted among them. Forkhead Box P3 (FoxP3) is thought to be a key transcription factor which controls regulatory T cell development and function and is expressed in several tumour cells. However, its precise role in ovarian cancer has yet to be established, with both tumour cells and T regs expressing FoxP3, thus complicating the relationship. Ma et al found that FoxP3 expression in gastric cancer cells suggested a better prognosis, due to interaction between tumour cells, immune cells and molecules such as TGF-β (Ma GF et al 2014). FoxP3 may therefore result in longer survival in these patients, opening up potential new strategies for immunotherapeutic treatment. However, more studies are required to further explore which immune cells are crucial to this interaction. In vivo and in vitro studies have shown that the FOXP3 gene converts normal naïve T cells to T reg-like cells with a suppressive function (Sakaguchi S et al 2005). Our results showed that there was no significant difference in the CD4+ T cell population, CD4+CD25+ T cell population or indeed the CD4+CD25+FOXP3 T cell population in group A and group B patients at the end of the study (Figures 3.22-24).

This quantitative study contributes important experiential insights to a limited body of knowledge around the immune response seen following chemotherapy treatment for women with a diagnosis of ovarian cancer. The results presented suggest a novel and safe way of assessing the function of the immune system for patients following chemotherapy. However, the response to HBV vaccination may vary according to tumour subtype and indeed the type of cancer. In addition, clinical vaccine efficacy usually relies on surrogate markers – namely, the level of specific serum antibodies. The relevance of these parameters may be questioned; indeed, this was reported by Kempe et al, who found that 24% of children with cancer developed influenza despite having antibody titres considered to be protective in healthy controls (Kempe A et al 1989).

Further validation is required for our results given the limited sample size; perhaps also a different timepoint could also be investigated – for example, the vaccination of patients who have completed chemotherapy six months ago. Recruiting chemotherapy participants is known to be difficult and given the focus of the current study, women were wary of the impact of further medication when considering participation. Furthermore, a significant limitation of the study was the participants were not tested for seropositivity for the HLA class I A2 subtype, which limited the number of participants for whom pentamer specific CD8+ T cells could be explored.

A particular strength of the study is the homogeneous composition of the patient sample. All the participants in this research had similar immune function when commencing the study, with the exception of a significant difference in CA125 levels, as noted earlier. However, our study may have been affected by selection bias as we only included women who had been referred to a tertiary centre. This may have led to an over-representation of more complex pathology of cancer. Equally, over-representation may have been present in both groups, leveling these effects.

A possible source of bias is the absence of a healthy age matched control group, which limits the opportunity to compare groups with a healthy population. Nonetheless, as we intended to explore differences between the responses of two groups of patients who

underwent chemotherapy, the addition of a control group is perhaps less relevant as it pertains to a healthy population. Furthermore, this was partly addressed by referring to historical control data, as published by De Rave et al.

Through the development of this work, it has become apparent that we have relied heavily on complete purification of the flow cytometry sorting scheme. Whilst each experiment was duplicated or replicated in triplicate wherever possible, a different laboratory technique such as ELISPOT analysis could also have been considered.

4.2 PD-L1 as a prognostic marker in women undergoing chemotherapy

PD-L1 is a cell surface immunoglobulin originating from the B7 superfamily. It is expressed on a wide range of cells of haematopoietic lineage, including activated T cells, B cells, monocytes, dendritic cells and macrophages, as well as the surface of many tumour cells. It is likely to suppress anti-tumour immune responses, thus promoting tumour growth and enabling tumour cells to evade immune surveillance.

The present diagnostic tools of ultrasound scanning and serum CA-125 levels fail to accurately distinguish between benign and malignant ovarian cancers – and furthermore, do not indicate the stage of cancer. Previous work in our laboratory has shown that CD14+PD-L1+ expression on monocytes in blood and ascites correlates significantly with the diagnosis of ovarian cancer. Indeed, PD-L1 expression on monocytes from ascites and blood from patients with ovarian cancer was significantly higher than in patients with borderline or benign ovarian tumours. However, there is no doubt that further work is needed to identify whether PD-L1 is differentially expressed according to stage and tumour histology.

Hatziveis et al examined the impact of paclitaxel and carboplatin combination chemotherapy on the CD4 and CD4/CD8 count in patients with ovarian cancer before, during and after chemotherapy. They found that there was a statistically significant difference of CD4 and CD4/CD8 after chemotherapy, suggesting that an increase in T-helper cells after chemotherapy had a positive impact on survival (Hatziveis K et al 2012).

It is important to reiterate the finding that blocking the immunosuppressive PD1 pathway, which is known to be dysregulated in epithelial ovarian cancer, may improve T cell longevity and function. Given the recent clinical success of Anti-PDL1 and anti-PD1 therapy as demonstrated by Brahmer and Topalian, the role of anti-PD1 and anti-PDL1 antibody in treating ovarian cancer needs to be explored further – namely, its toxicity as a single therapeutic agent as well as its efficacy in combination therapy with traditional chemotherapy agents.

Whilst work within our laboratory has shown increased expression of total serum monocytic CD14+PD-L1+ correlated with suboptimal surgical outcome, we considered the impact of PD-L1 as a prognostic marker during and after chemotherapy. This was measured using FACS cytometry to identify a CD14+PD-L1+ monocytic population during and after chemotherapy as well as quantifying soluble plasma PD-L1 levels in a subgroup of these patients. A pilot study by Park et al observed increased levels of recently activated T regs with tumour migrating ability, with Tregs consistently returned to pre-chemotherapy levels at the end of treatment (Park A et al, 2012). These results indicated that T cell subset distribution returned to pre-chemotherapeutic levels, not to that seen in healthy women. However, the sample size was small. In our study, the majority of the patients recruited had either stage 3 or 4 ovarian cancer, most commonly serous subtype (tables 3.4 and 3.5). Non-significant differences were seen in lymphocyte count, monocyte population and the CD14+ monocytic population between cycles 1 and 6 of chemotherapy. In keeping with this, no significant difference was seen between cycles 1 and 6 of chemotherapy or indeed pre- and post chemotherapy in the CD3+, CD14+ PD-L1 monocytic population or CD3+PD1 lymphocytic population. Given soluble PD-L1 and cell surface PD-L1 have different mechanisms of action, this was also considered using ELISA quantification of soluble PD-L1 in plasma in patients prior to chemotherapy and during chemotherapy. However, a small sample size at the post-chemotherapy timepoint limited statistical analysis (Figure 3.34).

By comparison, a significant difference was seen in the lymphocyte count prior to cycle 1 of chemotherapy and after chemotherapy (Figure 3.26). Whilst there is a paucity of

data on the implications of acute lymphocyte count recovery after chemotherapy in ovarian cancer, Behl et al found that a high absolute lymphocyte count after induction chemotherapy (but prior to consolidation chemotherapy) in patients with acute myeloid leukaemia had a superior overall survival (Behl et al, 2006).This may be due to thymic-dependent T-cell development or peripheral expansion of circulating T lymphocytes that survive chemotherapy and warrants further investigation. Coleman acknowledges that tumour progression can suppress CD8+ T cell responses, and that successful chemotherapy can reverse this. Ferrandina et al found that CD3+, CD4+ and CD8+ count one year after chemotherapy was an independent marker of longer time to progression, but did not quantify when this recovery occurs (Ferrandina G et al 2003).

In a subgroup of HBV vaccinated patients, CD14/PD-L1 monocyte and CD3/PD-1 lymphocyte levels were investigated to identify whether a significant difference exists between Group A and Group B patients pre- and post-vaccination following chemotherapy. When comparing the CD14+PD-L1 monocytic population in women undergoing HBV vaccination at the end of their vaccination schedule, no significant difference was seen (Figures 3.32 and 3.32a). By comparison, the CD3+PD-1 levels were noted to be significantly different for patients in Group A and B at the start of the vaccination study (responders and non-responders), although not significant at the end of the study (Figures 3.33 and 3.33a). This may be explained again by a recovering immune system, which may have fully recovered by month 7 – i.e. approximately 10-12 months after the completion of chemotherapy. Previous work in our laboratory has shown over-expression of PD-1 on T cells to be associated with ovarian cancer; however, this is the first time that PD-1 has been used as a prognostic marker following dual therapy by surgery and chemotherapy. These findings correlate with Zhang's recent retrospective study investigating the expression of PD-1 in CD3+ T cells in patients with diffuse large B cell lymphomas. Here, results indicated that PD-1 expression may be used as an indicator of chemotherapeutic efficacy, albeit in B cell lymphoma (Zhang L et al 2015). Our results support the need for further research on the role of Nivolumab, a PD-1 blocking antibody currently used in unresectable or metastatic melanoma as well as non-small cell lung cancer. Nivolumab blocks the

interaction between PD-1 and its ligands PD-L1 and PD-L2 (found on T cells), inhibiting T cell proliferation and cytokine production. This prevents PD-1 pathway mediated inhibition of the immune response, including the anti-tumour immune response, resulting in decreased tumour growth. Similar support for the use of a humanized antibody interacting with PD-1 in patients with haematological malignancies has previously been made by Berger et al (Berger R et al, 2008).

It is important to bear in mind the fact that the PD-1 and PD-L1 levels seen may be influenced by tumour type, stage and chemotherapy regimen undertaken. Another factor may be the timepoint at which blood was obtained at the end of the treatment regimen. Through the development of this work it has become apparent that perhaps a clinically more relevant question is whether there is clinical benefit in measuring serial PD-L1 monocytic levels and PD1 lymphocytic levels at regular intervals after chemotherapy in ovarian cancer patients, which remains unreported.

This work has not been validated in those who develop chemotherapy resistance after first line treatment. As tumour cells are continually evolving, the mechanisms of primary chemotherapy resistance and secondary resistance may well be different and further work is needed to explore this, in women before and after developing resistance.

Overall summary and conclusion

The field of immunotherapy as a whole has advanced hugely over the last decade. Recent clinical trials have demonstrated the potential for using immunotherapy as a therapeutic agent in ovarian cancer. This work has highlighted how the process of regeneration of the immune system and its function can alter the T cell and B cell response to vaccination, depending on the time from chemotherapy. Vaccination in this case has been successfully applied to quantify the immune response of cancer patients after treatment, but may also be used as a platform to discover new cancer therapy targets.

We have further examined our understanding of some of the pathways involved in the potential role of PD-1 as a prognostic marker in women who have undergone chemotherapy treatment for ovarian cancer, suggesting the potential need for further clinical exploration of the role of anti-PD1 antibody therapy.

Whilst there is no doubt that further work is needed, the findings from this study further increase our understanding of some of the cellular responses and molecular pathways involved in oncological treatment response. Despite the amount of work still required, there is no doubt that immunotherapy is now a serious contender as a novel therapy for ovarian cancer.

5. FUTURE WORK

Further recruitment to the HBV vaccination study to strengthen and confirm our serum antibody findings is warranted. In relation to the B cell serum antibody response, whilst a low level response may have been detected one month after vaccination, T cell priming may have occurred at this point and thus warrents further exploration. Whilst we have shown a significantly higher T cell response to HBV vaccination by patients who have recently completed chemotherapy, given the non-significant differences in CD45RO, CD45RA and CD4+CD25+FOXP3 populations, the underlying mechanism warrants further investigation. Furthermore, the higher rate of non-responders to vaccination also warrants exploration – for example, what is the Th1 and Th2 profile of these participants? Whilst historical patient data was available for healthy patients when comparing antibody response, no historical control data was available when characterizing the T cell response to HBV vaccination and therefore recruitment of healthy age matched controls could be considered.

This study confirms the complex role of the PD-1 and PD-L1 pathways in regulating ovarian cancer. Whilst PD-L1 has been shown to be a potential diagnostic biomarker in human ovarian cancer, its role as a prognostic marker for patients undergoing chemotherapy for ovarian cancer is less certain. PD-1 may have a more significant role and further investigation of its potential use as a prognostic marker is merited. Clinically, our findings suggest further exploration of anti-PD-1 monoclonal antibody therapy may be more successful in ovarian cancer patients. In addition, the early lymphocytic recovery seen in patients following chemotherapy warrants further characterization and relationship analysis with overall survival.

Further work would involve a comparison with age-matched patients diagnosed with benign ovarian tumours and indeed healthy controls.

APPENDIX 1: FLOW CYTOMETRY GATING

The diagram below outlines the gating principles using for flow cytometry analysis in peripheral blood.

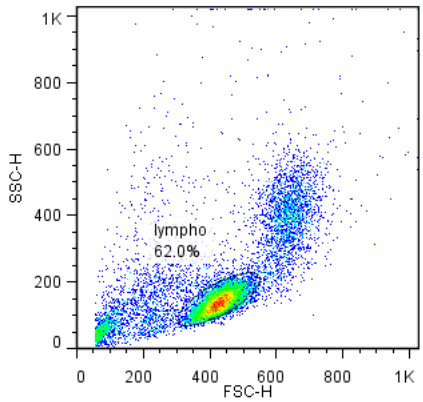


Figure 1: Gating strategy to identify lymphocytic population in peripheral blood for flow cytometry analysis.

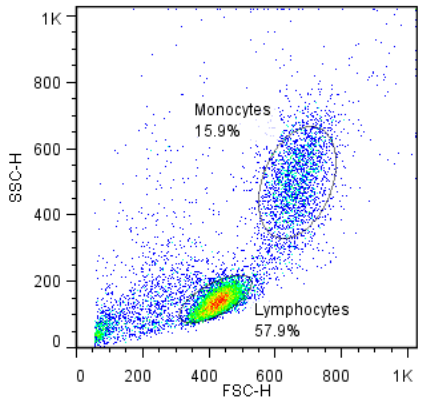


Figure 2: Gating strategy to identify monocytic and lymphocytic T cell population for flow cytometry analysis.

Forward and sideward scatter plots were used to set initial gates to identify lymphocyte and monocytic populations clearly. Dead cell population was identified and excluded using PI staining. Subsequent gates were set using the relevant isotype control for all corresponding fluorochromes, with 99% of the gated cell population isolated in the

bottom left quadrant. An example of isotype gating for lymphocyte population stained using FITC and APC isotypes is shown below (Figure 3).

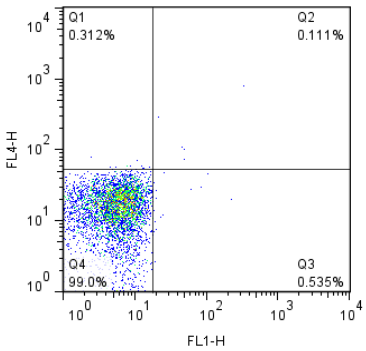
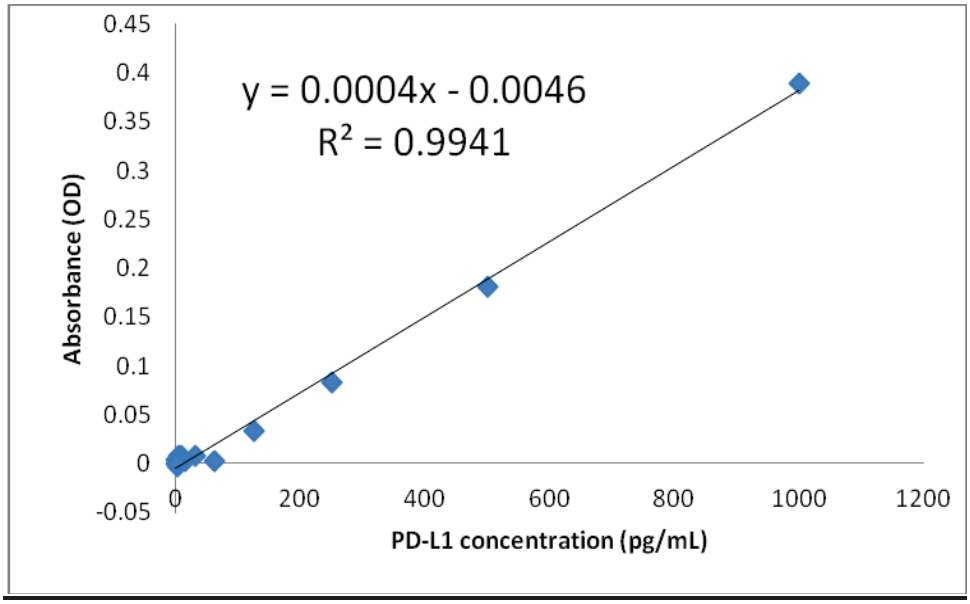


Figure 3: Flow cytometry analysis of isotype staining using FITC and APC.

APPENDIX 2: ELISA STANDARD CURVE

An example of the standard curve for in-house sandwich PD-L1 ELISA quantification:



6. REFERENCES

Office for National Statistics. Cancer Statistics registrations: Registrations of cancer diagnosed in 2006, England. Series MB1 no.37 2008

Abiko K, Mandai M, Hamanishi J, Yoshioka Y, Matsumura N, Baba T, Yamaguchi K, Murakiami R, Yamamoto A, Kharma B, Kosaka K, Konishi I. PD-L1 on tumour cells is induced in ascites and promotes peritoneal dissemination of ovarian cancer through CTL dysfunction. Clin Cancer Res 2013; 19 (6): 1363-74

Adams M, Kerby I, Rocker I, Evans A, Johansen K, Franks C. A comparison of the toxicity and efficacy of cisplatin and carboplatin in advanced ovarian cancer. The Swons Gynaecological Cancer Group 1989; 28 (1): 57-60

Agarwal R, Kaye S. Ovarian cancer: strategies for overcoming resistance to chemotherapy. Nat Rev Cancer 2003; 3 (7): 502-16

Ahmadzadeh M, Rosenberg S. IL-2 administration increases CD4+CD25FOXP3 regulatory T cells in cancer patients. Blood 2006; 107 (6): 2409-14

Agata Y, Kawasaki A, Nishimura H, Ishida Y, Tsubata T, Yagita H, Honjo T. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. Int Immunol 1996; 8(5): 765-772

Armstrong D, Bundy B, Wenzel L, Huang H, Baergen R, Lele S, Copeland L, Walker JL, Burger RA. Intraperitoneal Cisplatin and Paclitaxel in Ovarian Cancer. N Engl J Med 2006; 354: 34-43

Arora R, Alston R, Eden T, Geraci M, Birch J. Comparative incidence patterns and trends of gonadal and extragonadal germ cell tumours in England, 1979 to 2003. Cancer 2012; 118 (17): 4290-7

Axtell AE, Lee MH, Bristow RE, Dowdy SC, Cliby WA, Raman S, Weaver JP, Gabbay M, Ngo M, Lentz S, Cass I, Li AJ, Karlan BY, Holschneier CH. Multi-institutional reciprocal validation study of computed tomography predictors of suboptimal primary

cytoreduction in patients with advanced ovarian cancer. J Clin Oncol 2007; 25 (4): 384-9

Bayard F, Malmassari S, Deng Q, Lone YC, Michel M. Hepatitis B virus derived DRB1*0101-restricted CD4 T cell epitopes help in the development of HBV specific CD8+ T cells in vivo. Vaccine 2010; 28 (22): 3818-3826

Behl D, Porrata LF, Markovic SN, Letendre L, Pruthi RK, Hook CC, Tefferi A, Elliot MA, Kaufmann SH, Mesa RA, Litzow MR. Absolute lymphocyte count recovery after induction chemotherapy predicts superior survival in acute myelogenous leukaemia. Leukemia 2006; 20 (1): 29-34

Bell DA. Origins and molecular pathology of ovarian cancer. Mod Pathol. 2005; 18 Suppl 2: S19-32

Berd D, Kairys J, Dunton C, Mastrangelo MJ, Sato T, Maguire HC. Autologous, hapten-modified vaccine as a treatment for human cancers. Semin Oncol 1998; 25: 646-653.

Berek JS, Taylor PT, Nicodemus CF. CA125 velocity at relapse is a highly significant predictor of survival post relapse: results of a 5-year follow-up survey to a randomized placebo-controlled study of maintenance oregovomab immunotherapy in advanced ovarian cancer. J Immunother 2008; 31: 207-214.

Berek JS, Markman M, Stonebraker B, Lentz SS, Adelson MD, DeGeest K, Moore D: Intraperitoneal interferon-alpha in residual ovarian carcinoma: a phase II gynecologic oncology group study. Gynecol Oncol. 1999; 75: 10-14.

Berger R, Rotem-Yehudar R, Siama G, Landes S, Kneller A, Leiba M, Koren-Michowitz, Shimoni A, Nagler A. Phase I Safety and pharmacokinetic study of CT-011, a humanized antibody interacting with PD-1, in patients with advanced haematologic malignancies. Clin Cancer Res 2008; 14 (10) 3044-51

Borley J, Wilhelm-Benartzi C, Brown R, Ghaem-Maghami S. Does tumour biology determine surgical success in the treatment of epithelial ovarian cancer? A systematic literature review. Br J Cancer 2012; 107 (7): 1069-74

Brahmer J, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, Drake CG, Camacho LH, Kauh J, Odunsi K, Pitot HC, Hamid O, Bhatia S, Martins R, Eaton K, Chen S, Salay TM, Alaparthy S, Grosso JF, Korman AJ, Parker SM, Agrawal S, Goldberg SM, Pardoll DM, Gupta A, Wigginton JM. Safety and Activity of Anti-PD-L1 Antibody in Patients with Advanced Cancer. NEJM 2012; 366:2455-65

Bristow RE, Puri I, Chi DS. Cytoreductive surgery for recurrent ovarian cancer: a meta-analysis. Gynaecol Oncol 2009; 112 (1): 265-74

Bristow RE, Santillan A, Chi DS. Delaying the primary surgical effort for advanced ovarian cancer: a systematic review of neoadjuvant chemotherapy and interval cytoreduction. Gynaecologic Oncology 2007; 104: 480-90

Bristow RE, Tomacruz R, Armstrong DK, Trimble EL, Montz FJ. Survival effect of maximal cytoreductive surgery for advanced ovarian carcinoma during the platinum era: a meta-analysis. J Clin Oncol 2002; 20: 1248-1259

Brune I, Wilke W, Hensler T, Holzmann B, Jorg-Rudiger S. Downregulation of T helper type 1 immune response and altered pro-inflammatory and anti-inflammatory T cell cytokine balance following conventional but not laparoscopic surgery. Am J Surg 1999; 177 (1): 55-60

Burger RA, Brady MF, Bookman MA, Walker JL, Homesley HD, Fowler J, Monk BJ, Greer BE, Boente M, Liang SX. Phase III trial of Bevacizumab in the primary treatment of advanced epithelial ovarian cancer, primary peritoneal cancer or fallopian tube cancer: A Gynaecologic Oncology Group Study. J Clin Oncol 2010; 28:7 (June 20 Suppl): 946s (Abstract LBA1)

Cannon MJ, O'Brien TJ. Cellular immunotherapy for ovarian cancer. Expert Opin Biol Ther 2009 Jun; 9(6): 677-88

Chang S, Hodeib M, Chang J, Bristow R. Survival impact of complete cytoreduction to no gross residual disease for advanced-stage ovarian cancer: a meta-analysis. Gynaecol Oncol 2013 130 (3): 493-98

Chi DS, Eisenhauer EL, Zivanovic O, Sonada Y, Abu-Rustum NR, Levine DA, Guile MW, Bristow RE, Aghajanian C, Barakat RR. Improved progression-free and overall survival in advanced ovarian cancer as a result of a change in surgical paradigm. Gynaecol Oncol 2009a; 114 (1): 26-31

Choi S, Henderson MJ, Kwan E, Beesley AH, Sutton R, Bahar AY, Giles J, Venn NC, Pozza LD, Baker DL, Marshall GM, Kees UR, Haber M, Norris MD. Relapse in children with acute lymphoblastic leukaemia involving selection of a pre-existing drug-resistant subclone. Blood 2007; 110: 632-39

Clarke B, Gilks B. Ovarian carcinoma: Recent developments in classification of tumour histological subtypes. Canadian J Path 2011; 33-42

Coates T, Wilson R, Patrick G, Andre F, Watson V. Hepatitis B vaccines; assessment of the seroprotective efficacy of two recombinant DNA vaccines. Clin Ther 2001; 23: 392-403

Coleman MP, Forman D, Bryant H, Butler J, Rachet B, Maringe C, Nur U, Tracey E, Coory M, Hatcher J, McGahan CE, Turner D, Marrett L, Gjerstorff ML, Johannesen TB, Adolfsson J, Lambe M, Lawrence G, Meechan D, Morris EJ, Middleton R, Steward J, Richards MA. Cancer survival in Australia, Canada, Denmark, Norway, Sweden and the UK, 1995-2007: An analysis of population-based cancer registry data. Lancet 2011; 377: 127-138

Coleman S, Clayton A, Mason M, Jasani B, Adams M, Tabi Z. Recovery of CD8+ T cell function during systemic chemotherapy in advanced ovarian cancer. Cancer Res 2005; 65 (15): 7000-6

Colombo PE, Mourregot A, Fabbro M, Gutowski M, Saint-Aubert B, Quenet F, Gourgou S, Rouanet P. Aggressive surgical strategies in advanced ovarian cancer: a monocentric study of 203 stage IIIc and IV patients. Eur J Surg Oncol 2009; 35 (2): 135-43

Coneja-Garcia J, Benencia F, Courreges M, Gimotty P, Khang E, Buckanovich R, Frauwirth K, Zhang L, Katsaros D, Thompson C, Levine B, Coukos G. Ovarian carcinoma expresses the NKG2D ligand and promotes the survival and expansion of CD28 anti-tumour T cells. Cancer Res 2004; 64 (6): 2175-82

Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen K, Zou W. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med, 2004; 10 (9):942-9

Dalton WS, Friend SH. Cancer biomarkers – an invitation to the table. Science 2006; 312 (5777): 1165-8

Davies A, Jacobs I, Woolas R, Fish A, Oram D. The adnexal mass: benign or malignant? Evaluation of a risk of malignancy index. Br J Obstet Gynaecol 1993; 100 (10): 927-31

De Rave S, Heijtink RA, Bakker-Bendik M, Boot J, Schalm SW. Immunogenicity of standard and low dose vaccination using yeast-derived recombinant hepatitis B surface antigen in elderly volunteers. Vaccine 1994; 12(6): 532-534

Edwards RP, Gooding W, Lembersky BC, Colonello K, Hammond R, Paradise C, Kowal CD, Kunschner AJ, Baldisseri M, Kirkwood JM, Herberman RB. Comparison of toxicity and survival following intraperitoneal recombinant interleukin-2 for persistent ovarian cancer after platinum: twenty-four-hour versus 7-day infusion. J Clin Oncol 1997; 15: 3399-3407.

Eisenhauer EL, Abu-Rustum NR, Sonoda Y, Aghajanian C, Barakat RR, Chi DS. The effect of maximal surgical cytoreduction on sensitivity to platinum-taxane chemotherapy and subsequent survival in patients with advanced ovarian cancer. Gynaecol Oncol 2008; 108 (2): 276-81

Ferrandian G, Pierelli L, Perillo A, Rutella S, Ludovisi M, Leone , Mancuso S, Scambia G. Lymphocyte recovery in advanced ovarian cancer patients after high-dose chemotherapy and peripheral blood stem cell plus growth factor support. Clin Cancer Res 2003; 9(1): 195-200

Ferris R. PD-1 targetting in cancer immunotherapy. Cancer 2012; 119 (23): E1-3

Fialova A, Partlova S, Sojka L, Hromadkova H, Brtnicky T, Fucikova J, Kocian P, Rob L, Bartunkova J, Spisek R. Dynamics of T-cell infiltration during the course of ovarian cancer: the gradual shift from a Th17 effector cell response to a predominant infiltration by regulatory T cells. Int J Cancer 2013; 132 (5); 1070-79

Franceschi S, Levi F, La Vecchia C. Survival after ovarian cancer treatment. JAMA 1993, 270 (10): 1196-7

Freedman R, Deavers M, Liu J, Wang E. Peritoneal inflammation – a microenvironment for epithelial ovarian cancer. J Transl Med (2004) 2 (1): 23

Freedman RS, Platsoucas CD. Immunotherapy for peritoneal ovarian carcinoma metastasis using ex vivo expanded tumour infiltrating lymphocytes. Cancer Treat Res 1996; 82: 115-46

Freedman RS, Lenzi R, Kudelka AP, Lawrence DD, Rosenblum M, Platsoucas CD. Intraperitoneal immunotherapy of peritoneal carcinomatosis. Cytokines Cell Mol Ther 1998 Jun; 4 (2): 121-40

Gaistskell K, Martinek I, Bryant A, Kehoe S, Nicum S, Morrison J. Angiogenesis inhibitors for the treatment of ovarian cancer. Cochrane Database Syst Rev 2011; 9 Cd007930

Garcia A, Singh H. Bevacizumab and ovarian cancer. Ther Adv Med Oncol (2013) Mar; 5 (2): 133-41

Gemer O, Lurian M, Gdalevich M, Kapustian V, Piura E, Schneider D, Lavie O, Levy T, Fishman A, Dgani R, Levavi H, Beller U. A multicentre study of CA125 level as a

predictor of non-optimal primary cytoreduction of advanced epithelial ovarian cancer. Eur J Surg Oncol 2005; 31 (9) 1006-10

Goncalves L, Albarran B, Salmen S, Bores L, Fields H, Montes H, Soyano A, Diaz Y, Berrueta L. The nonresponse to hepatitis B vaccination is associated with impaired lymphocyte activation. Viroloy 2004; 326(1): 20-28

Gurung A, Hung T, Morin J, Gilks CB. Molecular abnormalities in ovarian carcinonoma: clinical, morphological and therapeutic correlates. Histopathology 2013; 62: 59-70

Hamanishi J, Mandai M, Iwasaki M, Okazaki T, Tanaka Y, Yamaguchi K, Higuchi T, Yagi H, Takakura K, Minato N, Honjo T, Fujii S. Programmed cell death 1 ligand and tumour infiltrating CD8+ T lymphocytes are prognostic factors of human ovarian cancer. Proc Natl Acad Sci 2007; 104 (9): 3360-65

Hamid O, Robert C, Daud A, Hodi S, Hwu W, Kefford R, Wolchok J, Hersey P, Joseph R, Weber J, Dronca R, Gangadhar T, Patnaik A, Zarour H, Joshua A, Gergich K, Elassaiss-Schaap J, Algazi A, Mateus C, Boasberg P, Tumeh P, Chmielowski B, Ebbinghaus S, Li X, Kang S, Ribass A. Safety and tumour responses with lambrolizumab (anti-PD-1) in melanoma. N Engl J Med 2013; 369 (2): 134-144

Harries M, Kaye SB. Recent advances in the treatment of epithelial ovarian cancer. Expert Opin Investig Drugs 2001; 10 (9): 1715-24

Hatziveis K, Tourlakis D, Hountis P, Kyriazanos I, Sougleri M, Ginopoulos P, Camoutis C. Effects on the immune system and toxicity of carboplatin/paclitaxel combination chemotherapy in patients with stage III-IV ovarian and non-small cell lung cancer and its role in survival and toxicity. J Buon 2012; 17 (1): 143-8

Hensler T, Hecker H, Heeg K, Heidecke CD, Bartels H, Barthlen W, Wagner H, Siewert JR, Holzman B. Distinct mechanisms of immunosuppression as a consequence of major surgery. Infect Immun 1997; 65 (6): 2283-91

Hennessy B, Coleman R, Markman M. Ovarian Cancer. Lancet 2009; 374 (9698):1371-82

Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbe C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ. Improved survival with ipilimumab in patients with metastatic melanoma. NEJM 2010; 363 (8): 711-23

Hogdall EV, Christensen L, Kjaer SK, Blaakaer J, Kjaerbye-Thygesen A, Gayther S, Jacobs IJ, Hogdall CK. CA125 expression pattern, prognosis and correlation with serum CA125 in ovarian tumour patients. From the Danish "MALOVA" Ovarian Cancer Study. Gynaecol Oncol 2007; 104 930: 508-15

Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003; 299: 1057-61

Hoskins P, Eisenhauer E, Vergote I, Dubuc-Lissoir J, Fisher B, Grimshaw R, Oza A, Plante M, Stuart G, Vermorken J. Phase II feasibility study of sequential couplets of cisplatin/topotecan followed by paclitaxel/cisplatin as primary treatment for advanced epithelial ovarian cancer: a National Cancer Institute of Canada Clinical Trials Group Study. J Clin Oncol 2000; 18 (24); 4038-44

Hwu P, Freedman RS. The immunotherapy of patients with ovarian cancer. J Immunother 2002 May-Jun; 25(3):189-201

Ilavska S, Horvathova M, Szabova M, Nemessanyi T, Jahnova E, Tulinska J, Liskova A, Wsolova L. Association between the human immune response and body mass index. Human Immunology 2012; 73 (5) 480-5

Jacobs I, Bast RC. The CA125 tumour-associated antigen: a review of the literature. Hum Reprod 1989; 4 (1): 1-12

Kang S, Nam BH. Does neoadjuvant chemotherapy increase optimal cytoreduction rate in advanced ovarian cancer? Meta-analysis of 21 studies. Ann Surg Oncol 2009; 16 (8): 2315-20

Keir M, Francisco L, Sharpe A. PD-1 and its ligands in T cell immunity. Curr Opin Immunol 2007; 19(3): 309-14

Kempe A, Hall CB, MacDonald NE, Foye HR. J Paediatr 1989; 115:33-9

Knutson K, Curiel TJ, Salazar L, Disis M. Immunologic principles and immunotherapeutic approaches in ovarian cancer. Haematol Oncol Clin N Am. 2003; 17: 1051-73

Kobayashi E, Ueda Y, Matsuzaki S, Yokoyama T, Kimura T, Yoshino K, Fujita M, Kimura T, Enomoto T. Biomarkers for screening, diagnosis and monitoring of ovarian cancer. Cancer Epid Biomarkers Prev 2012; 21 (11): 1902-12

Koonings PP, Campbell K, Mishell DR, Grimes DA. Relative frequency of primary ovarian neoplasms; a 10- year review. Obstet Gynaecol 1989; 74 (6): 921-6

Krugman S, Holley HP, Davidson M, Simberkoff MS, Matsaniotis N. Immunogenic effect of inactivated hepatitis B vaccine: Comparison of 20mcg and 40mcg doses. J Med Virol 1981; 8: 119-21

Lambert HE, Berry RJ. High dose cisplatin compared with high dose cyclophosphamide in the management of advanced epithelial ovarian cancer (FIGO stages III and IV): report from the North Thames Co-operative Group. Br Med J (Clin Res Ed) 1985; 290 (6472): 889-93

Larsen C, Xu J, Lee S, Dubey D, Uko G, Yunis E, Alper C. Complex cytokine responses to hepatitis B surface antigen and tetanus toxoid in responders, nonresponders and subjects naïve to hepatitis B surface antigen. Vaccine 2000; 18: 3021-30

Leffers N, Daemen T, Helfrich W, Boezen HM, Cohlen BJ, Melief K, Nijman HW. Antigen-specific active immunotherapy for ovarian cancer. Cochrane Database Syst Rev. 2010 Jan 20;(1):CD007287

Leroux-Roels G, Desombere I, Cobbaut L, Petit MA, Desmons P, Hauser P, Delem A, De Grave D, Safary A. Hepatitis B vaccine containing surface antigen and selected

preS1 and preS2 sequences. Immunogenicity in poor responders to hepatitis B vaccines. Vaccine 1997; 15; 1732-36

Leroux-Roels G, Van Hecke E, Michielsen W, Voet P, Hauser P, Petre J. Correlation between in vivo humoral and in vitro cellular immune responses following immunization with hepatitis B surface antigen (HBsAg) vaccines. Vaccine, Volume 12, Issue 9, 1994, Pages 812-818.

Li C, Yang W, Gai X, Zhang Y, Li Y, Fu H. Foxp3 overexpression decreases sensitivity to chemotherapy in mouse Lewis lung cancer cells. Molecular medicine reports 2012; 6: 977-82

Looney R, Hassan M, Coffin D, Campbell D, Falsey A, Kolassa J, Agosti J, Abraham G, Evans T. Hepatitis B Immunization of Healthy Elderly Adults: Relationship between naïve CD4+ T cells and primary immune response and evaluation of GM-CSF as an adjuvant. J Clinical Immunology 2001; 21 (1): 30-36

Ma GF, Miao Q, Liu YM, Gao H, Lian JJ, Wang YN, Zeng XQ, Luo TC, Ma LL, Shen ZB, Sun YH, Chen SY. High FoxP3 expression in tumour cells predict better survival in gastric cancer and its role in tumour microenvironment. Br J Cancer 2014; 110: 1552-60

Machiels JP, Reilly RT, Emens LA, Ercolini AM, Lei RY, Weintraub D, Okoye FI, Jaffee EM. Cyclophosphamide, doxorubicin and paclitaxel enhance the antitumour response in granulocyte/macrophage-colony stimulating factor-secreting who cell vaccines in HER-2/neu tolerized mice. Cancer Res 2001; 61 (9):3689-97.

Maine CJ, Aziz N, Chatterjee J, Hayford C, Brewig N, Whilding L, George JT, Ghaem-Maghami S. Programmed death ligand-1 overexpression correlates with malignancy and contributes to immune regulation in ovarian cancer. Cancer Immunology, Immunotherapy 2014; 63 (3): 215-224

McGuire W, Hoskins W, Brady MF, Kucera PR, Partridge EE, Look KY, Clarke-Pearson DL, Davidson M. Cyclophosphamide and cisplatin versus paclitaxel and cisplatin: a

phase III randomized trial in patients with suboptimal stage III/IV ovarian cancer. Semin Oncol 1996; 23: 40-47

McShane LM, Althman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. Diagnostics SSotN-EWCoC. Reporting recommendations for tumour MARKer prognostic studies (REMARK). Nat Clin Pract Urol 2005; 2 (8): 416-22

Menon U, Gentry- Maharaj A, Hallett R, Ryan A, Burnell M, Sharma A, Lewis S, Davies S, Philpott S, Lopes A, Godfrey K, Oram D, Herod J, Williamson K, Seif M, Scott I, Mould T, Woolas R, Murdoch J, Dobbs S, Amso N, Leeson S, Cruickshank D, McGuire A, Campbell S, Fallowfield L, Singh N, Dawnay A, Skates S, Parmar M, Jacobs I. Sensitivity and specificity of multimodal and ultrasound screening for ovarian cancer and stage distribution of detected cancers: results of the prevalence screen of the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS). Lancet Oncol 2009; 10 (4): 327-40

Mitchell MS, Kohorn EL. Cell-mediated immunity and blocking factor in ovarian carcinoma. Obstetrics and Gynaecology. 1976 Nov; 48(5): 590-7

Morrison J, Haldar K, Kehoe S, Lawrie TA. Chemotherapy versus surgery for initial treatment in advanced ovarian epithelial cancer. Cochrane Database Syst Rev 2012 (8): CD005343

Neijt JP, Engelholm SA, Tuxen MK, Sorensen PG, Hansen M, Sessa C, de Swart CA, Hirsch FR, Lund B, van Houwelingen HC. Exploratory phase III study of paclitaxel and cisplatin versus paclitaxel and carboplatin in advanced ovarian cancer. J Clin Oncol 2000; 18 (17): 3084-92

NICE (2011) Ovarian Cancer: the recognition and initial management of ovarian cancer (CG122). London: National Institute for Health and Care Excellence

Nicholson S, Bomphray CC, Thomas H, McIndoe A, Barton D, Gore M, George AJ: A phase I trial of idiotypic vaccination with HMFG1 in ovarian cancer. Cancer Immunol Immunother. 2004, 53: 809-816.

Nordoy T, Aaberge I, Husebekk A, Samdal H, Steinert S, Melby H, Kolstad A. Cancer patients undergoing Chemotherapy show adequate serological response to vaccinations against influenza virus and Streptococcus pneumonia. Med Onc 2002; 19 (2): 71-8

Nustad K, Bast RC Jr, Brien TJ, Nilsson O, Seguin P, Suresh MR, Saga T, Nozawa S, Bormer OP, de Bruijn HW, Nap M, Vitali A, Gadell M, Clark J, Shigemasa K, Karisson B, Kreutz FT, Jette D, Sakahara H, Endo K, Paus E, Warre D, Hammarstrom S, Kenemans P, Hilgers J. Specificity and affinity of 26 monoclonal antibodies against the CA125 antigen: first report from the ISOBM TD-1 workshop. International Society for Oncodevelopmental Biology and Medicine. Tumour Biol 1996; 17: 196-219

Oei AL, Sweep FC, Thomas CM, Boerman OC, Massuger LF. The use of monoclonal antibodies for the treatment of epithelial ovarian cancer (review). Int J Oncol 2008 Jun; 32(6):1145-57

Ozols RE, Bundy BN, Greer BE, Fowler JM, Clarke-Pearson D, Burger RA, Mannel RS, DeGeest K, Hartenbach EM, Baergen R, Gynecologic Oncology Group. Phase III trial of carboplatin and paclitaxel compared with cisplatin and paclitaxel in patients with optimally resected stage III ovarian cancer; a Gynecologic Oncology Group study. J Clin Oncol 2003; Sep 1; 21 (17):3194-200

Park A, Govindaraj C, Xiang S, Halo J, Quinn M, Scalzo-Inguanti K, Plebanski M. Substantially Modified Ratios of Effector to Regulatory T cells During Chemotherapy in ovarian Cancer patients Return to Pre-Treatment Levels at Completion: Implications for Immunotherapy Cancers 2012; 4: 581-600

Parente-Pereira AC, Whilding LM, Brewing N, van der Stegen SJ, Davies DM, Wilkie S, van Schalkwyk MC, Ghaem-Maghami S, Maher J. Synergistic chemoimmunotherapy of EOC using ErbB Retargeted T cells combined with Carboplatin. J Immunol 2013; 191: 2437-45

Penn I. Post-transplant malignancy: the role of immunosuppression. Drug Saf 2000; 23: 101-113

Perkins FT. A Ready Reckoner for the Calculation of Geometric Mean Antibody Titres. J Gen Microbiol 1958; 19: 540-41

Pestell KE, Hobbs SM, Titley JC, Kelland LR, Walton MI. Effects of p53 status on sensitivity to platinum complexes in a human ovarian cancer cell line. Mol Pharmacol 2000; 57: 503-11

Polak L, Turk JL. Reversal of immunological tolerance by cyclophosphamide through inhibition of suppressor cell activity. Nature 1974; 249:654–656

Pollack SM, Loggers ET, Rodler ET, Yee C, Jones RL. Immune-based therapies for sarcoma. Sarcoma 2011; ID 438940

Prat J, FCOG Committee on Gynaecologic Oncology. Staging classification for cancer of the ovary, fallopian tube and peritoneum. Int J Gynaecol Obs 2014; 124 (1): 1-5

Preston C, Goode EL, Hartmann LC, Kalli KR, Knutson KL. Immunity and immune suppression in human ovarian cancer. Immunotherapy 2011; 3 (4): 539-56

Reiartz S, Kohler S, Schlebusch H, Krista K, Giffels P, Renke K, Huober J, Mobus V, Kreieberg R, DuBois A, Sabbatini P, Wagner U. Vaccination of patients with advanced ovarian carcinoma with the anti-idiotype ACA125: immunological response and survival (phase Ib/II). Clin Cancer Res 2004; 10 (5): 1580-7

Ries L, Young J, Keel G, Eisner M, Lin Y, Horner M. SEER Survival Monograph: Cancer Survival Among Adults: US SEER Program, 1988-2001, patient and tumour characteristics. Nat Cancer Institute 2007.

Roy M, Wu M, Barr L et al. Induction of antigen-specific CD8+ T cells, T helper cells, and protective antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine. Vaccine 2001; 19: 764-778

Sabbatini P, Harter P, Scambia G, Sehouli J, Meier W, Wimberger P, Baumann KH, Kurzeder C, Schmalfeldt B, Cibula D, Bidzinski M, Casado A, Martoni A, Colombo N, Holloway RW, Selvaggi L, Li A, del Campo J, Cwiertka K, Pinter T, Vermorken JB,

Pujade-Lauraine E, Scartoni S, Bertolotti M, Simonelli C, Capriati A, Maggi CA, Berek JS, Pfisterer J. Abagovomab as maintenance therapy in patients with epithelial ovarian cancer: a phase III trial of the AGO OVAR, COGI, GINECO, and GEICO--the MIMOSA study. J Clin Oncol. 2013 Apr 20;31(12):1554-61.

Sakaguchi S, Ko K, Yamazaki S, Nakamura K, Nishioka T, Hirota K, Yamaguchi T, Shimizu J, Nomura T, Chiba T. Treatment of advanced tumors with agonistic anti-GITR mAb and its effects on tumor-infiltrating Foxp3+CD25+CD4+ regulatory T cells. J. Exp. Med. 2005; 202: 885-891.

Sandercock J, Parmar MK, Torri V, Qian W. First-line treatment for advanced ovarian cancer: Paclitaxel, platinum and the evidence. Br J Cancer 2002; 87 (8): 815-24

Shih KK, Chi DS. Maximal cytoreductive effort in epithelial ovarian cancer surgery. J Gynaecol Oncol 2010; 21 (2): 75-80

Solomayer E, Feuerer M, Bai L, Umansky V, Beckhove P, Meyberg GC, Bastert G, Schirmacher V, Diel IJ. Influence of Adjuvant Hormone Therapy and Chemotherapy on the Immune System Analysed in the Bone Marrow of Patients with Breast Cancer. Clin Cancer Res 2003; 9:174-80

Sueblinvong T, Carney ME. Current understanding of risk factors for ovarian cancer. Curr Treat Options Oncol 2009; 10 (1-2): 67-81

Sugiyama T, Kamura T, Kigawa J, Terakawa N, Kikuchi Y, Kita T, Suzuki M, Sato I, Taguchi K. Clinical characteristics of clear cell carcinoma of the ovary. Cancer 2000; 88: 2584-89

Sugunan AP, Bhattacharya H, Bhattacharya D, Mandal A, Ghosal SR, Rao RC, Mandal RK. Impact of hepatitis B immunization among the Nicobarese tribe – antibody titres and seroprotection five years after vaccination. Indian J Med Res 2014; 139: 2014, p427-30

Summers J, Mason W. Replication of the genome of a hepatitis B like virus by reverse transcription of an RNA intermediate. Cell 1982; 29(2): 403-15

Szumeness W, Stevens CE, Harley EJ, Zang EA, Oleszko WR, William DC, Sadovsky R, Morrison JM, Kellner A. Hepatitis B vaccine: demonstration of efficacy in a controlled clinical trial in a high-risk population in the United States. N Engl J Med 1980; 303: 833-41

Takano M, Kikuchi Y, Yaegashi N, Kuzuya K, Ueki M, Tsuda H, Suzuki M, Kigawa J, Takeuchi S, Moriya T, Sugiyama T. Clear cell carcinoma of the ovary: a retrospective multicentre experience of 254 patients with complete surgical staging. Br J Cancer 2006; 94 (10): 1369-74

Taira AV, Neukermans C, Sanders G. Evaluating Human Papillomavirus Vaccination Programs. Emerg Infect Dis 2004 Nov; 10 (11): 1915-23

Taylor DD, Bender DP, Gercel-Taylor C, Stanson J, Whiteside TL. Modulation of TcR/CD3-zeta chain expression by a circulating factor derived from ovarian cancer patients. Br J Cancer 2001: 84 (12); 1824-9

Ten Berge RJ, Schellekens PT, Hamerlynck JV, Bruning PF. Combination Chemotherapy and immune capacity in advanced ovarian carcinoma. Eur J Cancer Clin Oncol 1984: Jan; 20 (1): 91-8

Tomsova M, Melichar B, Sedlakova I, Steiner I. Prognostic significance of CD3+ tumour infiltrating lymphocytes in ovarian carcinoma. Oncology 2008: 108 (2); 415-420

Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DN, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, Leming PD, Spigel DR, Antonia SJ, Horn L, Drake CG, Pardoll DM, Chen L, Sharfman WH, Anders RA, Taube JM, McMiller TL, Xu H, Korman AJ, Jure-Kunkel M, Agrawal S, McDonald D, Kollia GD, Gupta A, Wigginton JM, Sznol M. Safety, activity and immune correlates of Anti-PD1 antibody in cancer. NEJM 2012; 366: 2443-54

Tsuda N, Change DZ, Mine T, Efferson C, Garcia-Sastre A, Wang X, Ferrone S, Ioannides CG. Taxol increases the Amount and T-cell Activating Ability of Self-Immune

Stimulatory Multimolecular Complexes Found in Ovarian Cancer Cells. Cancer Res, 2007; 67(17): p8378-87

Vergote I, du Bois A, Amant F, Heitz F, Leunen K, Hartner P. Neoadjuvant chemotherapy in advanced ovarian cancer: On what do we agree and disagree? Gynaecol Oncol 2013; 128 (1): 6-11

Vergote I, Trope CG, Amant F, Kristensen GB, Ehlen T, Johnson N, Verheijen RH, van der Burg ME, Lacave AJ, Panici PB, Kenter GG, Casado A, Mendiola C, Coens C, Verleye L, Stuart GC, Pecorelli S, Reed NS, Group EOfRaToC-GC, Group NCT. Neoadjuvant chemotherapy or primary surgery in stage IIIC or IV ovarian cancer. N Engl J Med 2010; 363 (10): 943-53

Yamaue H, Tanimura H, Noquchi K, Iwahashi M, Tsunoda T, Tani M, Tamai M, Hotta T, Mizobata S, Arii K. Cisplatin treatment renders tumour cells more susceptible to attack by lymphokine-activated killer cells. J Clin Lab Immunol 1991; 35:165-70.

Warren HS, Skipsey LJ. Loss of activation-induced CD45RO with maintenance of CD45RA expression during prolonged culture of T cells and NK cells. Immunology 1991; 74: 78-85

Weber D, Rutala WA, Shamsa G, Santimaw JE, Lemon SM. Obesity as a Predictor of Poor Antibody Response to Hepatitis B Plasma Vaccine JAMA 1985; 254: 22: 3187-3189

Wertel I, Polak G, Bednarek W, Barcynski B, Rolinski J, Kotarski J. Dendritic Cells subsets in the peritoneal fluid and peripheral blood of women suffering from ovarian cancer. Cytometry Part B 2008; 74B: 251-8

Wimberger P, Lehmann N, Kimmig R, Burges A, Meier W, Du Bois A, Group AGOOCS. Prognostic factors for complete debulking in advanced ovarian cancer and its impact on survival. An exploratory analysis of a prospectively randomized phase III study of the Arbeitsgemeinschaft Gynaecokologische Onkologie Ovarian Cancer Study Group (AGO-OVAR). Gynaecol Oncol 2007; 106 (1): 69-74

Wong R, Scotland R, Lau R, Wang C, Korman A, Kast W, Weber J. Programmed death – 1 blockade enhances expansion and functional capacity of human melanoma antigen-specific CTLs. Int Immunol 2007; 19 (10): 1223-4

Woo EY, Yeh H, Chu CS, Schlienger K, Carroll RG, Riley JL, Kaiser LR, June CH. Cutting edge: regulatory T cells from lung patients directly inhibit autologous T cell proliferation. J Immunol 2002; 168: 4272-76.

Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, Makrigiannakis A, Gray H, Schlienger K, Liebman MN, Rubin SC, Coukos G. Intra-tumoural T cells, recurrence and survival in epithelial ovarian cancer. N Eng J Med 2003; 348:203-13

Zhang L, Du H, Xiao TW, Liu JZ, Liu Z, Wang JX, Li GY, Wang LX. Prognostic value of PD-1 and TIM-3 on CD3+ T cells from diffuse large B cell lymphoma. Biomed Pharmacother 2015; Oct (75): 83-7

Zou W, Chen L. Inhibitory B7-family molecules in the tumour microenvironment. Nature Reviews. Immunology 2008; 8 (6): 467-77