

Serial Monitoring of the Inflammatory Response in Resectable Lung Cancer

**A thesis presented for the degree of
Doctor of Medicine (M.D.)**

at

Trinity College Dublin, the University of Dublin

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Year Awarded: 2026

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Acknowledgments

I would like to express my deepest gratitude to all who have offered their time, support, and expertise throughout this journey.

I am very grateful to the Royal City of Dublin Hospital Trust and AstraZeneca for their generous financial support, which made this research possible.

My sincere thanks to Professor Derek Doherty, who generously took time out of his busy schedule to advise on antibody panel design, provide training in cell culture, antibody staining, and flow cytometry, and to review my thesis. Navigating the complexities of flow cytometry would not have been possible without your invaluable help, as well as Julie David, Gráinne Jameson, and Aoife Killgallon — thank you all for your patience and guidance.

I am deeply grateful to the Thoracic Oncology Research Group for their tremendous support, kindness, friendship, and advice. It has been a privilege to work alongside such talented and dedicated individuals. Special thanks to Volga M. Saini, Sinéad Hurley, Éabha O'Sullivan, Christina Cahill, Brian Henderson, Martin Barr, Ezgi Öner Bozkurt, and Rebecca O'Brien. I will miss you all dearly and wish you every success in your future careers. I have no doubt you will continue to advance this incredible work in thoracic research.

My appreciation also goes to Rhieya Rahul for assistance with data collection and the reformatting of countless Excel spreadsheets, your help made a real difference. I am also so grateful to Fiona Boland, for her assistance with biostatistical design and interpretation.

I would like to extend heartfelt thanks to the staff of Keith Shaw Ward and theatre 1 & 2 and to the patients who kindly consented to take part in this research. Your generosity and willingness to contribute are sincerely appreciated.

To my supervisor, Dr. Kathy Gately, thank you for being an incredible mentor, advocate, and constant source of support. Your belief in me has been invaluable, and I am deeply appreciative of all your help, advice, patience and reassurance. Special thanks to Mr. Gary Fitzmaurice for your guidance and mentorship since my internship in 2020 and now as PI for my MD. Your encouragement has meant so much, and I am truly grateful.

To my parents, Marian and Jim Staunton, thank you for being my unwavering supporters. Your advice and guidance in the past, present, and future means the world to me. To my brothers Brian, Liam, and Sèamus, my nieces Ella, Niamh and Clíodhna and god daughter Katie; thank you for always being there for me and for all your support through the years. I would also like to thank my in-laws for their kindness, advice and enthusiasm. All my family and friends, you are a true and continual source of strength, motivation, and happiness.

Finally, to my wonderful husband, Chris Cannon, thank you for being my constant supporter through every academic endeavour, from returning to study medicine, completing my master's, enduring the endless exams, call shifts, conferences, and now my MD. Without your love and encouragement, none of this would have been possible. My success is truly ours.

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ABBREVIATIONS

1FU: First Follow Up
2FU: Second Follow Up
ADC: Antibody-Drug Conjugate
AREG: Amphiregulin
ANOVA: Analysis of Variance
BTLA: B and T Lymphocyte Associated Attenuator
CD4+ T cells: Helper T cells with CD4+ marker
CD8+ T cells: Cytotoxic T cells with CD8+ marker
CI: Confidence Interval
CIC: Circulating Immune Complex
CCL: Chemokine Ligand cc motif
CLuB: All Ireland Cancer Liquid Biopsies Consortium
CM: Central memory (T cells)
COPD: Chronic Obstructive Pulmonary Disease
CT: Computed Topography
CTC: Circulating Tumour Cell
ctDNA: circulating tumour DNA
CTLA-4: Cytotoxic T cell Antigen 4
CXCL: Chemokine Ligand cxc motif
CXCR3: Chemokine Receptor 3
DAMP: Disease associated molecular patterns
EM: Effector Memory (Tcells)
ERAS: Enhanced Recovery After Surgery
FBC: Full Blood Count
FDA: Food and Drug Administration
FGF23: Fibroblast Growth Factor 23
Gal-1: Galectin 1
Gal-3: Galectin 3
Gal-9: Galectin 9
HALP index: Haemoglobin Albumin Lymphocyte and platelet index
HEA: Higher Education Authority
HVEM: Herpes Virus Entry Mediator

ICI: Immune Checkpoint Inhibitors

ICOS: Inducible Co-Stimulatory Receptor

ICP: Immune Checkpoint Protein

ICU: Intensive Care Unit

IFN: Interferon

IL: Interleukin

IL-2RA: IL-2 receptor subunit α

iNKT: invariant Natural Killer T cells

LAG-3: Lymphocyte Activation Gene-3

LMR: Lymphocyte-to-Monocyte Ratio

MAIT: Mucosal-associated invariant T cells

MDT: Multi-Disciplinary Team

MHC1: Major Histocompatibility Class I

NET: Neutrophil Extracellular Trap

NCHD: Non Consultant Hospital Doctor

NK: Natural Killer (cells)

NKT: Natural Killer T (cells)

NKG2D: Natural Killer Group 2, member D

NLST: National Lung Screening Trial

NLR: Neutrophil-to-Lymphocyte Ratio

NSCLC: Non-Small Cell Lung Cancer

MAIT: Mucosal-associated invariant T cells

MUC16: Mucin-16

PBMC: Peripheral Blood Mononuclear Cell

PD-1: Programmed Death 1

PET: Positron Emission Topography

PFS: Progression Free Survival

PLR: Platelet-to-Lymphocyte Ratio

PNI: Prognostic Nutritional Index

POP: Post-Operative Pneumonia

PTX3: Pentraxin-related protein

RCT: Randomised Controlled Trials

sBTLA: Soluble B and T Lymphocyte Associated Attenuator

SBRT: Stereotactic Body Radiation Therapy

SCLC: Small Cell Lung Cancer

SII: Systemic Immune-Inflammation Index

SIRI: Systemic Inflammatory Response Index

SIRS: Systemic Inflammatory Response Syndrome

sPD-L1: Soluble Programmed Death Ligand 1

STROBE: Strengthening the reporting of observational studies in epidemiology.

TAM: Tumour Associated Macrophages

TAN: Tumour Associated Neutrophils

TCR: T Cell Receptor

TDEM: Terminally Differentiated Effector Memory (Tcells)

TGF: Transforming Growth Factor

Th: T Helper

TIM-3: T Cell Immunoglobulin Mucin-domain containing 3

TIL: Tumour Infiltrating Lymphocyte

TIME: Tumour Immune Microenvironment

TNF: Tumour Necrosis Factor

TME: Tumour Microenvironment

T Regs: T Regulatory Cells

NK cells: Natural Killer Cells

NKT cells: Natural Killer T cells

US: United States

VEGF: Vascular Endothelial Growth Factor

VTE: venous thromboembolism

YI: Youden's Index

SUMMARY

Background:

The perioperative period in non-small cell lung cancer (NSCLC) represents a critical window during which surgical stress, tumour biology, and host immunity interact, influencing post-operative complications and long-term outcomes. Understanding how immune cell populations, immune checkpoint proteins, and systemic inflammatory indexes change perioperatively may reveal predictive biomarkers for complications and disease recurrence.

Methods:

This longitudinal study of NSCLC patients undergoing lung resection, integrates analyses of adaptive and innate immune cell populations, inhibitory checkpoint protein expression and systemic inflammatory indexes. Samples were collected at baseline and multiple post-operative timepoints (2–4 days and 6–9 weeks). Associations between immune parameters, post-operative complications, and disease-free survival (DFS) were evaluated using regression and Cox proportional hazards models, adjusting for clinical covariates.

Results:

Early post-operative inflammatory indexes (Neutrophil-to-Lymphocyte ratio >4.9 , Systemic Immune-Inflammatory Index >1050 , Systemic Inflammatory Response Index >4) were predictive of post-operative complications. Immune profiles associated with post-operative complications included higher central memory CD4⁺ T cells, reduced CD8⁺ naïve T cells, and mucosal associated invariant (MAIT) cells. Perioperative expansion were observed in CD8⁺ T cells, CD4⁺ regulatory T cells, Natural Killer (NK), NKT-like, and MAIT cells. Surgery induced upregulation of inhibitory checkpoints across multiple cell types. Dynamic longitudinal changes in CD4⁺ central memory T cells, CD8⁺ naïve and effector memory T cells, B cells, NK cells, and checkpoint co-expression, baseline Haemoglobin Albumin Lymphocyte index (>54) and Prognostic Nutritional Index (>56) were independently associated with disease free survival. Advanced disease stage, tumour histology, age, and post-operative complications modulated these immune dynamics.

Conclusions:

This study demonstrates that surgical resection in NSCLC induces dynamic immune changes, with longitudinal monitoring having the potential to inform risk stratification and prognosis. Integrating immune profiling into postoperative care offers a minimally invasive approach to guide surveillance, tailor interventions, and mitigate recurrence risk. These findings highlight the potential of perioperative immunological assessment to optimize personalized management and improve long-term outcomes in lung cancer.

LAY SUMMARY

Lung cancer surgery is a critical time when the patient's immune system can be influenced by both the stress of surgery and the cancer itself. In this study, different immune cells were examined, alongside immune-regulating proteins, and markers of inflammation in the blood that change before and after surgery in patients with non-small cell lung cancer (NSCLC). We found that nearly half of patients experienced post-operative complications, such as lung or heart problems, blood clots, or wound infections. Early measurements of blood markers like NLR, SII, and SIRI were able to predict which patients were more likely to develop these complications. Certain immune cells, including helper and killer T cells, NK cells, and MAIT cells, showed significant changes after surgery, while others, such as B cells and some T cell types, remained largely stable. These changes were influenced by patient age, cancer stage, tumour type, and surgical approach.

The study also showed that surgery triggered an increase in proteins that temporarily suppress immune activity, which may reduce the body's ability to fight residual cancer cells. Patients with higher immune disturbances at baseline or who experienced post-operative complications were more likely to have cancer recurrence. Monitoring how immune cells and checkpoint proteins change over time provided valuable insight into which patients were at higher risk. Overall, the findings suggest that tracking immune and inflammatory changes around the time of surgery can help doctors predict complications, personalize patient care, and potentially improve long-term outcomes for lung cancer patients.

HYPOTHESIS, AIMS AND OBJECTIVES

Hypothesis

While the role of the immune response in the tumour microenvironment has been investigated a thorough understanding of the impact of the perioperative immune response is less well-defined and further studies are necessary. The interactions of immune cells, cytokines, and other peripheral immune markers may shift the immune response towards a conducive or inhibitory role in tumour defence. This study hypothesizes that perioperative immune alterations in NSCLC—encompassing changes in immune cell subsets, checkpoint protein expression, and systemic inflammatory indexes—predict post-operative complications and disease recurrence. The association between the peripheral immune response and clinical outcomes is essential for risk stratification and for developing effective immunotherapeutic strategies to improve long term patient outcomes. Understanding these dynamic immune profiles may identify biomarkers to guide personalized perioperative management.

Aims

The aims of this study is to

1. Monitor the peri-operative immune profile of patients with resectable NSCLC
2. Correlate the immune signature with patient clinicopathological parameters.
3. Characterise the relationship between immune signatures and postoperative complications
4. Examine the relationship between immune signatures and survival statistics

Objectives

1. Establish a biobank of longitudinal blood samples from patients with resectable NSCLC
2. Measure full blood counts to establish inflammatory indexes
3. Isolate Peripheral blood mononuclear cells and profile a panel of cell populations and immune markers by flow cytometry.
4. Correlate immune profiles to clinicopathological data, post-operative complications and survival data

VALUE OF RESEARCH

This research provides novel insights into how the immune system responds to lung cancer surgery, a critical period that influences both recovery and long-term outcomes. By tracking immune cell populations, immune checkpoint proteins, and systemic inflammatory markers before and after surgery, the study identifies key biomarkers that can predict post-operative complications and disease recurrence. Scientifically, it advances understanding of the complex interplay between surgical stress, tumour biology, and host immunity, highlighting cell- and checkpoint-specific dynamics that were previously underexplored in non-small cell lung cancer (NSCLC). The study also demonstrates the importance of longitudinal immune monitoring, showing that changes over time, rather than single measurements, better capture the patient's immune status and potential risk.

From a clinical perspective, these findings have direct relevance for patients and healthcare providers. Early identification of patients at higher risk of post-operative complications or recurrence allows clinicians to tailor perioperative care, implement preventive strategies, and potentially optimize timing for immunotherapies. For policymakers, the research underscores the value of integrating immune monitoring into surgical care pathways, which could improve patient outcomes and reduce healthcare costs associated with post-operative complications or recurrence. Beyond the clinic, this study contributes to society by improving understanding of how the body responds to cancer surgery, highlighting opportunities for more personalised, precise, and effective treatment strategies that may ultimately enhance survival and quality of life for lung cancer patients.

RESEARCH OUTPUTS

Poster Presentations

Irish Thoracic Society Conference 2024

- Staunton LM, Ryan R, Fitzmaurice GJ, Gately K. Lung Cancer Resection: Is there a role for perioperative inflammatory indexes?, 2024
- Staunton LM, Henderson B, Ryan R, Fitzmaurice GJ, Gately K. Perioperative lung cancer: To D-dimer or not to D-dimer that is the question?

07th European Conference of Immunology 2024

- Staunton LM, Doherty D, Ryan R, Fitzmaurice GJ, Gately K. Serial Monitoring of the Inflammatory Response in Resectable Lung Cancer, 2024

World Conference on Lung Cancer 2025

- Staunton LM, O'Doherty Derek, Ryan, R, Fitzmaurice GJ, Gately K. Peri-operative Immune Profile in Lung Cancer Resection: A Longitudinal Study

Oral Presentations

European Thoracic Oncology Platform Resident Workshop and Annual Meeting 2024

- Staunton LM, Doherty D, Ryan R, Fitzmaurice GJ, Gately K. Lung Cancer Surgery: Mapping the Peri-operative Immune Response, 2024

Early-Stage Lung Cancer International Expert Retreat 2025

- Staunton LM, Doherty D, Ryan R, Fitzmaurice GJ, Gately K. Lung Cancer Surgery: Exploring the Effect of Surgery on T cell Function in Patients with Early-Stage Lung Cancer, 2025

Prizes / Grants

Royal City of Dublin Hospital Trust

- Grant No.217, for research: Profiling the Perioperative Inflammatory Response in Resectable Lung Cancer, 2023, Awarded €96,000

Astrazenaca Ireland

- Grant for research: Profiling the Perioperative Inflammatory Response in Resectable Lung Cancer, 2025, Awarded €15,000
- Travel Grant for WCLC 2025

ETOP Travel Grant

- To attend and present at ETOP Residential Workshop and Annual Meeting, Barcelona, 2024, Awarded €400

ELIXR Travel Grant

- To attend and present at ELIXR25 annual meeting, Montreal, 2025, Awarded \$1500

CHAPTER 1

INTRODUCTION

1.1 NON-SMALL CELL LUNG CANCER

1.1.1 Epidemiology of lung cancer

Lung cancer is the main cause of cancer related diagnosis and mortality globally, causing 1.8 million deaths annually [1-4]. Lung cancer-related deaths are projected to rise to approximately 3.55 million annually by 2050, representing an estimated 95% increase [5]. Lung cancer diagnosis often occurs at advanced stages due to its relatively indolent presentation, which results in poor survival outcomes [1,3,4].

1.1.2 Histological classification

NSCLC with histopathological subtypes squamous cell, adenocarcinoma and large-cell carcinoma results in up to 80-85% of all lung cancer diagnosis [2,3]. Adenocarcinoma is the most common histological subtype of lung cancer and is can be associated with non-smokers [2,5]. Small cell lung cancer (SCLC) accounts for the remaining 10-15% of lung cancer cases [2]. Among NSCLC subtypes, adenocarcinoma represents the most common histology (40%), followed by squamous cell carcinoma (30%) and large cell carcinoma (10%) [2].

1.1.3 Risk factors for NSCLC

Smoking is the leading cause of lung cancer, with 30% of smokers developing the disease [1,2,4,5]. An estimated 85–90% of lung cancer incidences are attributable to tobacco smoking [5]. Lung cancer often develops in individuals with pre-existing pulmonary conditions, particularly chronic obstructive pulmonary disease (COPD), which is also associated with increased inflammatory response [2]. Other risk factors include air pollution, occupational exposure, age, previous history of lung cancer, family history and exposure to infectious organisms for example tuberculosis [1,4,6].

1.1.4 Staging of NSLC

The table below, adapted from the IASLC 9th edition of the Tumour, Node, Metastasis (TNM) staging system for lung cancer—first published in January 2025—illustrates tumour stage which then guides clinical management [6,7].

Table 1.1: 9th edition of TNM staging for lung cancer

Stage	Size	Invasion	Tumour	Node	Metastasis
1A1	≤1cm	N/A	T1a	N0	M0
1A2	>1 to ≤2cm	N/A	T1b	N0	M0
1A3	>2 to ≤3cm	N/A	T1c	N0	M0
IB	>3 to ≤4cm	+/-visceral / central	T2a	N0	M0
IIA	≤1cm to ≤3cm	N/A	T1a to T1c	N1	M0
	>4 to ≤5cm	N/A	T2b	N0	M0
IIB	≤1cm to ≤3cm	N/A	T1a to T1c	N2a	M0
	>3 to ≤5cm	+/-visceral / central	T2a, T2b	N1	M0
	>5 to ≤7cm	+/-Nodules same lobe +/-visceral / central	T3	N0	M0
IIIA	≤1cm to ≤3cm	N/A	T1a to T1c	N2b	M0
	>3 to ≤5cm	+/-visceral / central	T2a, T2b	N2a	M0
	>5 to ≤7cm	+/-Nodules same lobe +/-visceral / central	T3	N1, N2a	M0
	>7cm	+/-visceral / central	T4	N0,N1	M0
IIIB	≤1cm to ≤3cm	N/A	T1a to T1c	N3	M0
	>3 to ≤5cm	+/-visceral / central	T2a, T2b	N2b,N3	M0
	>5 to ≤7cm	+/-Nodules same lobe +/-visceral / central	T3	N2b	M0
	>7cm	+/-visceral / central	T4	N2a, N2b	M0
IIIC	>5 to ≤7cm	+/-Nodules same lobe +/-visceral / central	T3	N3	M0
	>7cm	+/-visceral / central	T4	N3	M0
IVA	Any	Pleural, pericardial, nodules, Contralateral	T1a-T4	N0-N3	M1a
	Any	Single extrathoracic mets	T1a-T4	N0-N3	M1b
IVB	Any	Multiple mets in single organ	T1a-T4	N0-N3	M1c1
	Any	Multiple mets in multiple organ	T1a-T4	N0-N3	M1c2

Table 1: Mets (metastasis). Nodal disease, N1: Hilar station involvement, N2a: Single ipsilateral mediastinal station involvement. N2b multiple ipsilateral mediastinal station involvement, N3: distant nodal metastasis.

1.1.5 Screening for lung cancer

Screening of high-risk individuals as a strategy for secondary prevention and early-stage disease diagnosis is well-established in many developed countries [1,5]. Evidence from the national lung cancer screening trial (NLST) and Nelson trial demonstrates that screening programmes significantly improve patient outcomes with [1,4,5]. They showed that

screening using low-dose CT and based on age and smoking history, resulted in a 20–25% reduction in mortality [4,5].

There is currently no national lung cancer screening programme in Ireland. However, a pilot initiative titled the ‘Lung Health Check’ was launched in 2025 [8]. This programme aims to invite 30,000 high-risk individuals—primarily based on age and smoking history—for low-dose CT (LDCT) scans [8]. In addition to imaging, the study incorporates biomarker analysis using liquid biopsy, marking an important step towards integrating early detection and precision medicine into lung cancer care in Ireland [8].

1.1.6 Clinical Management

1.1.6.1 Introduction

Lung cancer management depends on the disease stage [1]. Treatment strategies include surgery, radiotherapy, chemotherapy, immunotherapy and targeted therapies [1,9]. Surgery is the standard of care for early stage disease [1,4,9]. Chemoimmunotherapy is increasingly being incorporated into perioperative regimens globally, although it is not yet universally available [1,9]. For advanced-stage cancer, it is widely used as a first-line treatment [1,9]. Radiation therapy, using various modalities, is also employed depending on the stage of the disease [1,9,10,11]. The use of targeted treatment is limited to individuals with actionable molecular alterations [1,4,5,9]. A broad spectrum of clinical trials are currently in progress, accompanied by the continuous emergence of new therapeutic agents and regular updates to clinical guidelines, thereby expanding available treatment options [4,5].

1.1.6.2 Symptoms

Symptoms include cough, dyspnoea, pain, haemoptysis, recurrent chest infections, fatigue and unexplained weight loss [1]. Patients frequently remain asymptomatic, with diagnoses often occurring incidentally or through screening programmes, which are not globally implemented [1].

1.1.6.3 Diagnosis

Investigations includes imaging including chest Xray, computed tomography (CT) Thorax, positron emission tomography (PET)-CT [1]. Histological confirmation remains essential, typically involving biopsy of the suspicious lesion when feasible [1,4,6]. In recent years, molecular testing for tumour biomarkers and genetic driver mutations has gained increasing

significance in guiding diagnosis and therapeutic decision-making. [1,6]. Staging biopsies may also be conducted, utilising techniques such as endoscopy or mediastinoscopy when indicated [6]. The scope of investigations is determined by the extent and location of the tumour, along with the suspected presence of mediastinal or distant metastases [6]. Currently validated diagnostic techniques for NSCLC are all invasive, and a notable gap remains in the development of liquid biopsy-based diagnostic approaches. This is due to limited research and the absence of approved methodologies [1,4,6].

1.1.6.4 Surgical management for early stage NSCLC

Surgical options include segmentectomy, wedge resection, lobectomy and pneumonectomy [2,6]. A systematic mediastinal lymph node dissection is also imperative for cancer staging [4]. Minimally invasive techniques, such as robotic-assisted thoracoscopic surgery (RATS) and video-assisted thoracoscopic surgery (VATS), are preferred when negative surgical margins can be achieved [6]. Entry into thoracic cavity via thoracotomy is performed if minimally invasive procedure not possible [6].

1.1.6.5 Post-operative complications

Postoperative complications occur in up to 45% of patients undergoing lung cancer surgery, with pulmonary complications the most frequent, affecting approximately 24 % of patients [12]. Postoperative pneumonia (POP) is the most commonly reported surgical complication and is associated with prolonged hospital stay, increased risk of intensive care unit (ICU) admission, and higher mortality rates [12]. Alongside co-morbidities including smoking history, age and pre-operative lung function, the invasiveness of the procedure and subsequent inflammatory response can result in pulmonary complications [12]. Minimally invasive procedures are shown to have fewer complications and a shorter length of hospital stay compared to open surgery [6].

1.1.6.6 Medical oncological management of NSCLC

1.1.6.6.1 Introduction

Chemotherapy, immunotherapy, and/or targeted therapy may be administered depending on the tumour's molecular profile [1]. Recent advances have positioned immunotherapies as vital tools in cancer treatment, with a multi-modal approach including; immune checkpoint inhibitors (ICIs), cytokines, monoclonal antibodies, vaccines and chimeric

antigen receptor (CAR) T cell therapies, bispecific antibodies and antibody-drug conjugates (ADCs) which are either approved or in clinical trials [2,14,15].

ICIs have become the most widely used form of immunotherapy for NSCLC, since the Phase II CheckMate 063 trial which was published in 2015 [2,3,14,16]. ICIs target the immune inhibitory checkpoint interaction on surface of immune cell and tumour cell, thereby causing re-activation of the immune cell to target tumour cell [14-19]. See Figure 1.1 for details. This form of therapy aims not at killing cancer cells directly, but at removing the inhibitory signals that suppress T cell activity, thereby enabling a more effective immune response against the tumour [2,7,18,19]. ICIs exhibit variable and patient dependent efficacy, with some studies reporting efficacy rates below 40% [20]. Inflammation-related adverse effects are also a key consideration in the use of ICIs [6].

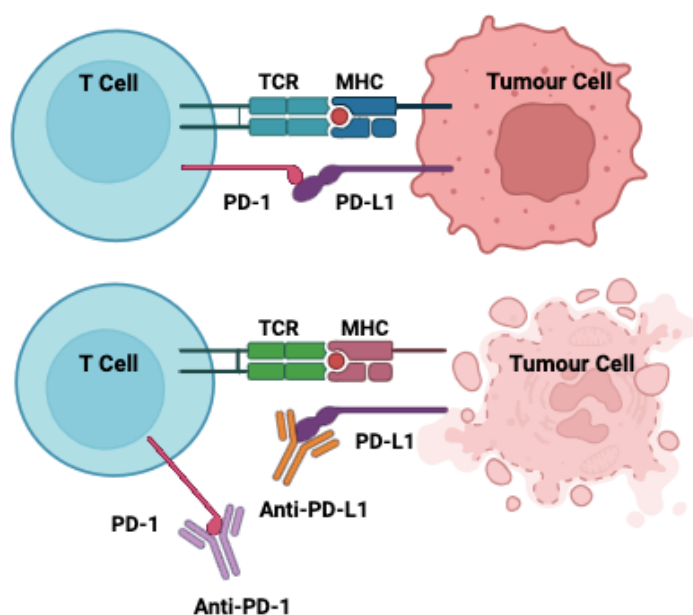


Figure 1.1. Schematic diagram showing mechanism of action of immune checkpoint inhibitors. Anti-PD-1 (for example pembrolizumab) and anti-PD-L1 (for example durvalumab) are monoclonal antibodies that target PD-1 or PD-L1 and enhance T cell response against tumour cells. Figure created with Biorender.com

1.1.6.6.2 Early Stage NSCLC

Medical oncological management options for early-stage lung cancer include neoadjuvant, perioperative, and adjuvant treatment regimens [1]. Neoadjuvant therapy, comprising of immunotherapy and/or chemotherapy administered prior to surgical resection, is typically

indicated for larger tumours (>4 cm) and cases involving nodal disease as demonstrated in the CheckMate 816 and Neocoast 2 trials [1,4]. The addition of ADCs have also shown promise [4]. Adjuvant chemoimmunotherapy can be administered following surgical resection, depending on disease stage [1,6]. Clinical trials (e.g. Impower010) with ICIs alone, have failed to yield a significant increase, in overall survival [6,4]. Cisplatin based chemotherapy regimens are the current gold standard treatment post-surgical resection of stage II-III disease and high risk stage I [4,6]. In patients with high risk stage IB and stages II-III with molecular markers for example EGFR, targeted therapy is recommended [4,6]. A perioperative regimen comprises neoadjuvant therapy, followed by surgical intervention, and subsequently adjuvant treatment, trial examples include KEYNOTE-671, CheckMate 77T and AEGEAN [1,6], which include dual therapies with ICIs and chemotherapy. Additional research is necessary to determine which patients benefit from neoadjuvant therapy alone versus those who require adjuvant therapy [4]. In Ireland currently, Nivolumab is utilised as per the CheckMate 816 trial, which is the only neoadjuvant regimen to include ICI and reimbursed by the HSE [22].

1.1.6.6.3 Late stage unresectable NSCLC

Blockade against immune checkpoint proteins (ICPs) PD1 / PDL1 are approved for use by the Food and Drug Administration (FDA) in the United States (US) [9]. ICI's including pembrolizumab, nivolumab and durvalumab are first line in advanced cases in combination with chemotherapy usually platinum based in Ireland [9,22]. For late stage disease, the current established approach involves a combination of chemoradiotherapy followed by immune checkpoint inhibition, as determined by the PACIFIC trial [4]. This approach differs in the presence of targetable mutations [4].

Anti-CTLA4 therapies, either as monotherapy or in combination with anti-PD-1/PD-L1 agents, have demonstrated treatment efficacy, with combination therapies showing superior clinical outcomes in lung cancer [9,14,15]. Ipilimumab is one such anti-CTLA therapy [9,22] which works through lymphocyte activation [9,22]. In pre-clinical trials monoclonal antibodies targeting LAG3, TIM3 and BTLA are also underway [14]. Similar to PD1/PD-L1 inhibitors, anti-LAG3 help to restore T cells function, whilst also reducing the immunosuppressive activity of Tregs [23,24]. Dual combination with anti-PD1/PDL1 inhibitors leads to a synergistic effect [22-24]. Similar effects were noticed with anti-BTLA therapies [25].

Cytokines have also been used to treat lung cancer, with several limitations. Cytokines can induce multiple, sometimes opposing effects, including both pro- and anti-inflammatory responses [2,26]. They are subject to compensatory mechanisms, which may render native cytokines less effective or even redundant [26]. Cytokines often have a short half-life and can be toxic at higher concentrations, which limits clinical use [15, 26,75]. Nevertheless trials are ongoing for cytokine therapy, either alone or in combination with ICIs [2,6,15,28]. IFN- α 2b therapy is used to treat melanoma and haematological tumours. IL-12 and IL-15 therapy are also in clinical trials [15]. Therapeutic use of IL-2, CCL21 and CCL25 in NSCLC has been shown to slow disease development [11,28]. Targeted immunotherapy to molecular markers is beyond the scope of this study [11].

1.1.6.7 Radiological Management

Stereotactic Body Radiation Therapy (SBRT) is offered to patients with early-stage lung cancer who are not candidates for surgical resection [1]. Radiation therapy is predominantly utilized as a palliative modality for managing metastatic disease in advanced-stage cancer, including brain metastasis [1,6]. Radiation therapy is not utilized as part of the surgical treatment approach and will therefore not be discussed further.

1.1.7 Survival statistics

Recurrence rates for NSCLC even after curative-intent surgery, the current standard of care, remains high at 14-19% for Stage I, 50% for Stage II and 66% at Stage III at 5 years [4,27]. Recurrence can be local or metastatic, likely due to spread of CTCs alongside post-operative immunosuppression [18]. Despite advancement in management, the 5-year overall survival rates remain low overall at 29% 65-70% for stage I, 45-55% for stage II at and 13–36% for stage III [2,3, 6,17].

1.1.8 Summary

NSCLC accounts for 80-85% of lung cancer cases and is the leading cause of cancer-related deaths worldwide, with poor survival rates due to late diagnosis. Major risk factors include smoking, COPD, and environmental exposures. Diagnosis involves imaging and biopsy, with staging guiding treatment, which ranges from surgery (with or without chemoimmunotherapy) in early stages to chemoimmunotherapy and targeted therapies in advanced disease. Despite advances, recurrence rates remain high and 5-year survival is generally low.

1.2 CANCER IMMUNITY

1.2.1 Introduction

Inflammation can act as a contributing factor to cancer development and a consequence of tumour growth and progression [2,17,30,31]. The immune system is essential in the fight against cancer [14,15]. Cell populations of the innate immune response including NK cells, monocytes and neutrophils work, alongside adaptive immune response including, CD8⁺ and CD4⁺ T cells and B cells, to try and eradicate the malignant cells [1,2,15]. The different stages of the immune response to cancer is known as ‘Immunoediting theory’, i.e: eradication, control and evasion [21]. The inflammation in the tumour microenvironment (TME) spreads to the systemic circulation [2,17,30,31]. Tumour cells are capable of subverting the immune system for their own advantage [15,25]. Therapeutic strategies—including surgery, radiotherapy, chemotherapy, and immunotherapy—aim to restore immune competence and facilitate tumour elimination [13,25].

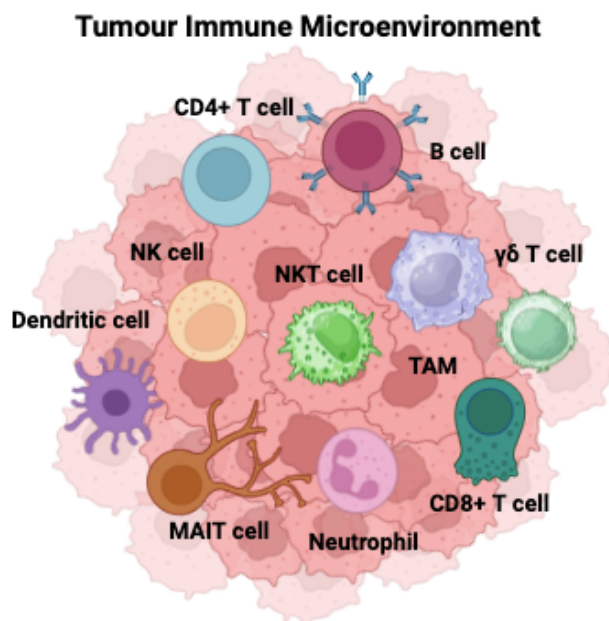


Figure 1.3. Illustration of Tumour Immune Microenvironment. Immune cells including CD4⁺ T cells, CD8⁺ T cells, Natural Killer T (NKT) cells, MAIT cells, $\gamma\delta$ T cells, Natural Killer (NK) cells, Neutrophils, Dendritic cells, tumour-associated macrophages (TAMs) and B cells constitute the immune defence in the tumour microenvironment. Figure created with Biorender.com

1.2.2 Innate Immune Response

1.2.2.1 Myeloid cells

Myeloid cells encompass a diverse group of immune cells, including monocytes, macrophages, neutrophils, eosinophils, basophils, and dendritic cells (DCs) [31,32].

Depending on their specific subtype and functional state, myeloid cells can exert either pro-tumour or anti-tumour effects [31,32].

Monocytes evolve into macrophages in tissue which are known as tumour-associated macrophages (TAM) in the TME [11,32]. Monocytes can also develop into dendritic cells under appropriate growth factor conditions [32,33]. Markers to identify monocytes include CD14 and CD16 [32,33]. Myeloid derived suppressor cells (MDSCs) play an immunosuppressive role within the TME and are activated from monocytes or neutrophils [32,33]. As peripheral blood is the focus of this project, tissue macrophages or MDSCs are not further discussed.

Eosinophils play a dual role in immunity, with their effects varying based on the cancer type and the TME [32]. Elevated eosinophil levels in both the TME and peripheral blood serve as prognostic indicators in various cancers and are utilized to predict responses to ICI therapy [32]. Eosinophilia has been shown to be a good prognostic indicator in several solid tumours [32]. DCs, as antigen-presenting cells, contribute to anti-tumour immunity by inhibiting metastatic dissemination and reestablishing compromised immune function [32]. Tumour-associated dendritic cells exhibit pro-tumorigenic properties through the secretion of immunomodulatory cytokines [32]. Eosinophils and DCs are not further addressed in this study, as the primary focus is on lymphocytes and monocytes along with their expression markers.

Neutrophils promote angiogenesis by secreting angiogenic factors and enhancing endothelial cell proliferation, while also indirectly stimulating the pro-angiogenic activity of other immune cells [32,34]. Several functionally and phenotypically diverse neutrophil populations have been identified in the peripheral blood of cancer patients [6,34,35]. Neutrophilia is associated with a poor prognosis in cancer [6,34,35]. Neutrophil extracellular traps (NETs) are web-like structures composed of DNA, histones, and granule proteins, released by neutrophils during systemic inflammation as a defence mechanism against pathogens [6,34,35]. NETs contribute to tumour progression by supporting local invasion, disrupting vascular integrity, and promoting immune escape [6,34,35]. Tumor associated neutrophils (TANs) can have anti-tumour (N1) or pro-tumour (N2) properties within the TME, with loss of CXCR3 receptor leading to progression [34,35]. Migration of

immunosuppressive neutrophil populations to the TME is mediated through chemokines CXCL1, CXCL2, CXCL5, CXCL6 and CXCL8 [34,35].

1.2.2.2 Natural Killer cells

NK cells are a subset of lymphocytes characterized by the CD3-CD56⁺ phenotype, accounting for approximately 5–15% of peripheral blood lymphocytes [11,36]. These large cytotoxic cells cause tumour cell apoptosis, without the need for prior antigen exposure and are a powerful defence against cancers [34,36]. CD16 can be used to highlight subsets of NK cells, CD16⁺ NK cells are characterized by a more potent cytotoxic profile [36]. Variability in NK cell activity exists within the general population, with individuals exhibiting differing levels of activity as a result of genetic and environmental influences [35,37]. NK cells produce a variety of proteins including Interferon (IFN)- γ and nitric oxide [35,36]. Cytokines including interleukin (IL)-2, IL-12, IL-15, and IL-18 stimulate these cells, further augmenting their anti-tumour efficacy [35]. NK cells also contribute to immune regulation by preventing excessive immune activation through the targeted killing of immune cells [35,36]. NK cells positively influence senescent T cells; with studies showing that NK cell infusions can reduce inhibitory receptors and number of senescent T cells [35]. NK cells in the TME however become dysfunctional and inactivate [35,36].

1.2.3 Adaptive Immune Response

1.2.3.1 T cells

All T cells are produced in the bone marrow and mature in the thymic gland and are positive for CD3 [11]. The main T cell populations include Helper T cells with CD4⁺ marker (CD4⁺ T cells) and Cytotoxic T cells with CD8⁺ marker (CD8⁺ T cells) [11,41,42]. In addition to the major T cell subsets, smaller populations such as regulatory T cells (Tregs), mucosal-associated invariant T cells (MAIT), Natural Killer T cells (NKT) and $\gamma\delta$ T cells also contribute to immune regulation and defence [11,37-42]. T cells are the most important immune cells to act in defence of cancer growth and progression and are the target of many studies [37-42].

T cells become activated following antigen exposure and differentiate from naïve to central memory (CM) and effector memory (EM) to terminally differentiated effector memory (TDEM) [11,40,42]. These four populations of cells are differentiated from each other using CD45RA and CCR7 alongside T cell markers [40]. T-cell dysfunction, through the

mechanisms of anergy, exhaustion & senescence, occurs when immune response is disrupted and has impaired ability to respond to activation [16,35,38,39,41,42]. These processes serve to protect normal tissues from damage [35,37-39,41,42]. As cancer progresses, T-cell impairment occurs, resulting in a reduced number of functional T cells, downregulation of effector activity and therefore ineffective immune response [11,18,35,37].

T cell exhaustion arises from prolonged antigen exposure following chronic activation [11,40,41,42]. T cell exhaustion is characterized by the progressive loss of effector functions and the upregulation of inhibitory receptors on the T cell surface [11,40,41,42]. A hallmark of T cell exhaustion is the upregulation of multiple surface immune checkpoint proteins (ICPs), [11,40,41,42]. which can be reversed by ICIs [38,42,43]. T cell anergy occurs when T cells lack responsiveness to antigens leading to low IL2 production, likely to prevent autoimmunity [11,38,39]. This results from transcriptional downregulation and insufficient CD28-mediated co-stimulation [11,40,41,42]. Anergic cells cannot be identified via cell surface markers [11,40,41,42]. Senescence is the result of shortening of telomeres, arrest of cell cycle, CD28/CD27/CCR7 loss and CD45RA upregulation, typically as a consequence of chronic activation and replication [39,41,42]. These T cells lack the ability to proliferate, but unlike exhausted T cells, they retain the capacity to produce pro-inflammatory cytokines [37,39,41,42].

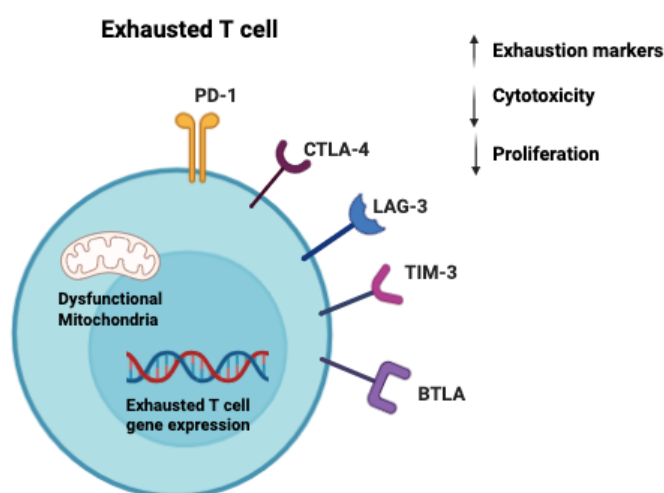


Figure 1.3. Exhausted T cell. Immune checkpoint proteins including PD-1, CTLA-4, TIM-3, LAG-3 and BTLA are surface markers that are expressed following chronic antigen stimulation. Dysfunction occurs with reduced cytotoxicity, proliferation and effector function. Figure created with Biorender.com

1.2.3.1.1 T Helper Cells (CD4+)

CD4⁺ T cells, play a pivotal role in modulating the immune response by activating and orchestrating the function of various immune cell subsets, thereby facilitating effective host defence against pathogens [11,37,44]. Subsets include Th1, Th2, Th17, Treg cells [11,44], which are defined by the cytokines that they release. Th1 cells release IFN- γ and TNF- α and mediate immunity against intracellular pathogens and tumours [11,44]. Th2 cells release produce IL-4, IL-5, IL-10 and IL-31 and mediate immunity against worms and parasites by recruiting and activating mast cells and eosinophils [11,44]. Th17 cells release IL-17A, IL-17F and IL-22, which promote defence against extracellular bacteria and fungi [11,44]. Treg cells release IL-10 and TGF- β , which regulate Th1, Th2 and Th17 cell differentiation [11,37,42].

1.2.3.1.2 Cytotoxic T cells (CD8+)

CD8⁺ T cells play a critical role in tumour cell elimination through their cytotoxic activity. [11,35,44]. CD8⁺ T cells mediate tumour cell killing through antigen recognition by their T-cell receptors (TCRs) presented on major histocompatibility complex class I (MHC I) molecules, followed by the release of cytotoxic granules that induce target cell death [11,44]. Antigen loss on cancer cells allows for recurrence [11,44]. CD8⁺ T cells undergo senescence earlier, likely due to their lower mitochondrial biogenesis [37,38,40,41,42]. Elevated levels of CD8⁺ T cells are a good prognostic indicator in the TME [35].

1.2.3.1.3 T Regulatory Cells

Tregs are immunosuppressive cells that constitute 5-10% of blood [11,37,42]. They can be identified by expression of different cell surface markers, in this study CD4⁺/CD8⁺CD25⁺CD127⁻ are utilised [11,37]. Elevated levels of Tregs, which can be induced by the tumour, are a poor prognostic indicator, as they facilitate disease progression [11,37,42]. They can express cell surface receptors including ICPs and produce cytokines for example IL6 and TGF- β [3,37,43]. They also negatively impact the function of immune cells including T and NK cells [11,37,42].

1.2.3.1.4 $\gamma\delta$ T cells

$\gamma\delta$ T cells constitute 1–5% of total T cells in peripheral blood and their genome has similarities to T cell and NK cell populations [40,45,46,47]. The two principal $\gamma\delta$ T cell

subpopulations are V δ 1, which predominantly reside in epithelial tissues, and V δ 2, which constitute the majority of $\gamma\delta$ T cells in peripheral blood [46,47]. V δ 2 T cells have been shown to be more vulnerable to apoptosis and functional exhaustion compared to V δ 1 T cells [46,47]. $\gamma\delta$ T cells play a key role in immune surveillance by responding to antigens through the production of cytokines and the mediation of cytotoxic activity [11,46,47]. Preclinical trials are underway to activate $\gamma\delta$ T V δ 2 cells using IL-2 and bisphosphonates, leveraging their potent cytotoxic capabilities against tumour cells [11,46,47].

1.2.3.1.5 Natural Killer T cells

Natural Killer T (NKT) cells exhibit features of both CD4⁺ and CD8⁺ T cells, as well as NK cells and originate from T cell precursors in the thymus. [11,49,50]. NKT cells typically represent less than 1% of peripheral blood lymphocytes but can comprise up to 5% in some healthy individuals, reflecting inter-individual variability [49,50]. NKT cells play a key role in tumour immunosurveillance, primarily through direct cytotoxic activity and by promoting the activation of other immune cells, particularly T cells [49,50]. Similar to T cells, chronic antigen exposure can lead to prolonged activation of NKT cells, resulting in anergy and subsequent cell death [49,50]. Reduced numbers of NKT cells have been observed in different cancers [49,50,55]. NKT cells comprise heterogeneous subsets. Type I NKT cells, also referred to as invariant NKT (iNKT) cells, express an invariant T-cell receptor (TCR) α -chain, V α 24J α 18, whereas type II NKT cells lack this invariant chain [11,49,50]. NKT-like cells represent a subset of type II NKT cells and are characterized by the co-expression of the surface markers CD3 and CD56 (CD3⁺CD56⁺) [11,49,50]. Additionally, CD161 expression may be observed on NKT cells. In the present study, NKT-like cells exhibiting the CD3⁺CD56⁺ phenotype will be analysed.

1.2.3.1.6 Mucosal-associated invariant T cells

Mucosal-associated invariant T (MAIT) cells express an invariant V α 7.2-J α 33 TCR α -chain and CD161⁺ [11,51,52]. These cells recognise small metabolites, presented by the antigen presenting molecule MR1 [11,51,52]. MAIT cells are reduced in the peripheral blood of cancer patients compared to healthy individuals [51,52]. They are predominantly located at mucosal surfaces, including the lungs, where they play an important part of the immune surveillance and response [51,52]. Through various signalling pathways, they secrete cytokines that facilitate immune cell recruitment or respond to cytokines themselves, leading to their activation [51,52]. Owing to the diverse range of cytokines

they secrete, MAIT cells exhibit dual roles, contributing to both pro-tumorigenic and anti-tumorigenic effects [51,52]. Through IFN- γ induction, MAIT cells augment NK cell activity [51,52]. Increased CD8+CD161+ T cells have been associated with improved prognosis [51,52]. Similar to other T cell populations, MAIT cells can become dysfunctional, as a result of chronic antigen stimulation [51,52].

1.2.3.2 B cells

B cells are positive for CD19 and negative for CD3 surface markers [11]. While T cell-mediated immunity has been extensively studied in cancer, investigations into the role of B cells remain relatively limited [35]. B cell roles in cancer immunity include antigen presentation and antibody production [53,54]. B cells function as antigen-presenting cells, facilitating the activation of T lymphocytes [53,54]. Following antigen recognition, B cells become activated and differentiate into plasmablasts (which are transient), as well as into plasma cells and memory B cells with prolonged lifespans [53,54]. The antibodies produced are highly specific to the initiating antigen and have anti-tumour properties [53,54]. However, their binding to tumour antigens can lead to the formation of circulating immune complexes (CICs), which may exhibit pro-tumour effects [53,54]. IgA producing B cells are a poor prognostic indicator [53,54].

1.2.4 Inflammatory Indexes

Inflammatory indexes incorporate two or three inflammatory cells [55]. Neutrophil-to-lymphocyte ratio (NLR) provides insight into the body's general immune status and inflammatory state [20]. NLR is utilised as a tool to predict prognosis in many solid cancers [35]. Platelet-to-lymphocyte ratio (PLR) and lymphocyte-to-monocyte ratio (LMR) have also been identified as predictive biomarkers with the literature largely utilising overall survival as the primary endpoint [55].

Systematic immune-inflammation index (SII) calculated using the formula (platelet x neutrophils / lymphocytes), was first used as a prognostic indicator in hepatocellular carcinoma [20]. SII incorporates platelets whose activation can influence immune responses by modulating cytotoxic immune cell activity [56,57]. Activated platelets can bind tumour cells leading to immune evasion [57]. Research has revealed that elevated SII leads to worse outcomes for solid tumours [18,55]. The Systemic Inflammation Response Index (SIRI) is another composite inflammatory marker that incorporates three immune cell

populations, calculated as (monocytes $\times$ neutrophils) / lymphocytes. [58]. A meta-analysis across diverse cancer populations demonstrated that, elevated SIRI levels are significantly associated with poor clinical outcomes [58]. SIRI demonstrated significant associations with sex, tumour stage including; size, and lymph node metastasis but not age [58]. The study also demonstrated the superior prognostic performance of SIRI compared to NLR, PLR, and LMR [58].

Combined nutritional and inflammatory markers, including the haemoglobin, albumin, lymphocyte, platelet (HALP) score (calculated as haemoglobin $\times$ albumin $\times$ lymphocyte / platelets) and the Prognostic Nutritional Index (PNI) incorporating albumin and lymphocyte levels have emerged as novel prognostic indicators in oncology [42,55,57]. Albumin serves as a nutritional marker and is often decreased as NCSLC progresses [56,57].

1.2.5 Cell Surface Proteins

1.2.5.1 Introduction

Cell surface proteins involved in cancer immunity include receptors that either activate (stimulatory) or suppress (inhibitory) immune responses [20]. By promoting survival, proliferation, and cytokine release, stimulatory receptors help promote anti-tumour immunity [40]. ICPs, or inhibitory receptor proteins, help suppress immune activity to maintain self-tolerance and prevent autoimmunity [14,15,58,59]. Tumour cells can hijack these immune-regulating proteins to avoid immune surveillance [14,15,20,58,59]. Members of the CD28 family, including; CD28, ICOS, CTLA4, PD1, and BTLA, play diverse roles in immune regulation, acting through both stimulatory and inhibitory pathways [20].

1.2.5.2 Stimulatory receptor proteins

CD28 and CD27 provides important secondary co-stimulatory signals for activated CD4⁺ and CD8⁺ T cells [25,38,59]. CD28 expression is elevated in central memory T cells relative to naïve T cells and is important for activation and expansion of T cells [37,40]. Following T cell activation, CTLA4 is upregulated and binds ligands B7-1 (CD80) and B7-2 (CD86) with stronger affinity than CD28, thereby limiting further T cell proliferation and preventing excessive immune activation [22]. CD28 is also reduced on surface of T cells in normal ageing process [59]. CD27 plays a key role in sustaining T cell function and

long-term immunity [37]. Reduction of both CD27 and CD28 occurs when T cells are chronically exposed to antigens and can be attributed to telomere shortening, dysfunction and T cell senescence and anergy [42]. Loss of CD27 occurs earlier than CD28 in CD4⁺ T cells, and CD4⁺CD28⁻ subsets are rarely observed in healthy young people [42]. In contrast, CD8⁺ T cells lose CD28 prior to losing CD27 [42]. The decrease is attributed to the loss of CD28⁺ T cell rather than the downregulation of the receptor [42].

Inducible co-stimulatory receptor (ICOS) is upregulated upon immune cell activation and promotes the production of IL-4 and IL-21, which are important for T cell differentiation [22]. Reduced ICOS on T cells has been associated with lymph node and tumour invasion [22]. CD127, the receptor for IL-7, plays a key role in the survival and expansion of differentiated T cells [42]. CD127 is highly expressed alongside CD28 but is absent on Tregs [42]. Natural Killer Group 2, member D (NKG2D) is expressed on CD4⁺ CD8⁺ T cells, NK cells and $\gamma\delta$ T cells and can be upregulated by IL15 [35,38]. NKG2D is involved in the activation of NK cells and co-stimulation of T cells [47,48]. NKG2D expression promotes NK cell cytotoxicity and cytokine secretion, alongside T cell differentiation [40,48]. Internalisation of NKG2D on immune cells mediated by ligands produced by tumour cells allows for immune escape [48].

CD69 is an early activation marker, predominately on lymphocytes and NK cells, expressed within 60 minutes of antigen stimulation and is absent on unstimulated immune cells [60-62]. The primary ligand for CD69 is Galectin-1 (Gal-1), facilitating cytokine production [61,62]. Other ligands include calprotectin, myosin light chains 9, 12a/b and oxidised low-density lipoprotein [61,62]. CD69 is a marker for memory T cells and helps retain these cells within peripheral tissues [61,62]. Persistent expression of CD69 on T cells has been linked to T cell exhaustion [61,62]. The presence of CD69-positive $\gamma\delta$ T cells is linked to reduced metastatic spread and better prognosis [61,62]. However, when CD69 is expressed on Tregs, tissue-resident memory cells, and tumour cells, it serves as a poor prognostic indicator [61,62].

1.2.5.3 Immune checkpoint proteins

ICPs are cell surface receptors elevated on activated immune cells, which are exploited by tumour cells to evade the immune system [59,25]. They are also known as inhibitory

receptors and have immunosuppressive effects [14]. As discussed in Section 1.1, immune checkpoint blockade represents a major breakthrough in cancer immunotherapy [9].

ICPs include Cytotoxic T cell antigen 4 (CTLA4), programmed death protein 1 (PD1), Lymphocyte activation gene 3 (LAG3), T cell immunoglobulin mucin-domain containing 3 (TIM3) and B and T lymphocyte associated attenuator (BTLA) [22,43,59,60,63-65]. Their expression increases in response to antigen stimulation, promoting immune cell activation, and is absent on naïve cells. [25,23]. Chronic antigen exposure leads to continued elevated expression, loss of immune cell function, then impaired ability to respond to activation [14,18,19,24,59]. These receptors also increase Treg function [14,18,19,24].

CTLA4 acts to reduce immune response and is expressed on T regs and effector T cells. It does so by limiting IL-2 production [15,11,22]. It competes with CD28 for binding, leading to inhibition of T cell cycle [11,20,22]. CTLA4 is the first immune checkpoint receptor upregulated upon T cell activation, followed by the expression of additional co-stimulatory and inhibitory checkpoint molecules, that collectively regulate T cell responses [22]. Cancer leads to continuous expression of CTLA4 on immune cells, which helps the tumour evade immune attack [22].

PD1 is an immune checkpoint protein upregulated on immune cells including activated T cells, NK and B cells and its ligand include PDL1 or PDL2 [19,43,59]. This pathway plays a physiological role in maintaining self-tolerance during inflammation and is critical for the differentiation and suppressive function of regulatory T cells [19,43,59]. PDL1 is broadly expressed on blood and tissue cells, while PDL2 is more selectively expressed on immune cells, particularly macrophages and dendritic cells [43,63]. Tumour cells hijack the PD1 / PDL1 pathway to evade immune surveillance [15,59,63]. Engagement of PD1 with its ligands, suppresses TCR/CD28 signalling and blocks effective T cell activation [20]. The presence of PD1 on T cell surfaces is a key marker indicating T cell exhaustion [63].

LAG3 is expressed on activated and exhausted immune cells and its upregulation is linked to poor clinical outcomes [15,23]. LAG3 can be expressed on Tregs as well as effector T cells [24]. LAG3 is induced by cytokines IL-2, IL-7, IL-12, and suppressed by IL-4 [21].

LAG3 has minimal functional activity on NK cells [23]. Considering TIM3, patients with CD4⁺ and CD8⁺ TIL and TIM3 expression, were shown to have poor survival and correlate with stage and nodal disease [14, 15, 54,65]. TIM3 and PD1 may work together to create a pro-tumour environment in the TME, through immune checkpoint invasion [64,65].

BTLA through interaction with its ligand herpes virus entry mediator (HVEM), inhibits the activation and proliferation of T and B cells, as well as the secretion of pro-inflammatory cytokines [25,60]. BTLA upregulation has been linked with poor outcomes [25,60]. BTLA also exists in soluble form (sBTLA), which has been shown to be a negative predictor of survival in cancers [60]. Not many studies discuss the relevance of soluble BTLA in cancers, however it has been correlated as having a negative impact on survival in different cancers [60].

1.2.5.4 Chemokine receptor proteins

Cytokines and chemokines are signaling proteins that facilitate communication between immune cells and help coordinate the immune response to cancer [27,28,19]. Chemokines are a subset of cytokines, with roles including chemotaxis and angiogenesis [24,28]. There are 50 known chemokines, which control the movement of cells by interacting with their specific receptors [27]. One such receptor, chemokine receptor 3 (CXCR3), also known as CD183 is mainly expressed on activated T, B and NK cells [27,66,67]. It has a bidirectional regulatory role [27,59]. CXCR3 is activated by its ligands CXCL9, CXCL10, and CXCL11, which are upregulated in response to interferon- γ (IFN- γ) and their ligands [66,67]. Tumour cells can alter the expression of these ligands to either promote or evade immune cell recruitment [66,67].

The CCR7 chemokine axis, along with its ligands CCL21 and CCL19, exerts an antagonistic effect in cancer immunity [68]. CCR7 expression is downmodulated as effector T cells become more differentiated [68]. CCR7 expression on tumour cells are linked to metastasis and poor survival [66]. CXCR3 and CCR7 are discussed in this study due to their potential as prognostic cancer biomarkers [66-68].

1.2.6 Soluble Plasma Proteins

Cytokines can be pro-inflammatory, anti-inflammatory, or both, with some exhibiting different roles depending on the cell type that produces them [26,28,73]. Both immune cells

(including senescent T cells) and tumour cells secrete cytokines that modify the TME and promote tumour progression [18,40]. Cytokine anti-tumour activity includes inhibiting cell proliferation, promoting apoptosis, and facilitating immune cell targeting of cancer cells [26]. An increase of pro-inflammatory cytokines due to prolonged antigen exposure, can lead to dysregulated cytokine production, resulting in chronic inflammation and systemic inflammatory response syndrome (SIRS) [18].

Interleukins and Interferons (IFN) activity are investigated for their role in different cancers [19]. IL-2 is involved in NK and T- cell proliferation, including Treg cells [24,26]. A decrease of Interferon (IFN)- γ which induces apoptosis of tumour cells, influences metastasis [11,18,38]. Pro-inflammatory interleukins that promote tumorigenesis are elevated in cancer and following surgery [17,18,19,66]. IL6 and IL10 facilitates tumour cell expansion while suppressing apoptotic pathways with excessive levels associated with poor prognosis [2,17,18,66,38].

Chemokines play a crucial role in directing leukocytes to sites of tissue damage [2]. There are four subfamilies with motifs including CXC, CC, C and CX3C [2]. The majority of chemokines produced by cancer cells, along with the co-expression of their receptors, facilitate tumour growth [27]. These include, CXCL12, CXCL19, CXCL21 and CXCL25 which are involved in metastasis [27,75]. CXCL10 on the other hand mediates the movement of CXCR3⁺ T cells to the TME, where higher levels are associated with improved survival [27]. CXCL10 therapies have been shown to suppress cancers in pre-clinical trials, especially when given alongside ICI's [27,75].

1.2.7 Summary

Cancer-related inflammation through the innate and adaptive immune system attempts to eradicate cancer, though tumour cells often evade this defence. Key processes like immune cell dysfunction, immune checkpoint expression, cytokine imbalances and hypercoagulation, facilitate tumour immune evasion leading to tumour progression. Circulating immune cells, cell surface proteins, cytokines, and coagulation factors are emerging as prognostic tools across cancer types. Understanding these immune mechanisms guides immunotherapeutic strategies and biomarker development.

1.3 IMMUNE BIOMARKERS IN LUNG CANCER

1.3.1 Introduction

Chronic lung inflammation from smoking and other risk factors drives immune dysfunction, contributing to lung cancer development [11,76]. Studies have demonstrated that immune alterations within the TME are evident even in early-stage hyperplasia which can lead to changes in circulating immune markers [11]. Immune marker expression can vary between histological subtypes of NSCLC, reflecting differences in tumour biology and immune evasion mechanisms [11,76]. This section reviews current literature on immune biomarkers in NSCLC.

1.3.2 Tumour Immune Microenvironment

The immune response in the TME is known as the tumour immune microenvironment (TIME) and is suppressed by hyperplastic and tumour cells [2]. The composition and functionality of the TIME influences prognosis in NSCLC [2]. Immune cells functional ability are changed, subsequently allowing immune evasion [39]. Immunosuppression occurs due to a self-protective process, so as not to allow immune related tissue damage [39]. In hyperplasia immune changes are observed in a myriad of ways including; an increase in pro-inflammatory cytokines [9,17]. Migration of CD4+, CD8+ Tcells, B cells, Treg cells, neutrophils, macrophages occur into the TME occur [15]. Lymphocytes that migrate to the TME are known as Tumour Infiltrating Lymphocytes (TILs) [11,15]. TILs can remain on periphery or infiltrate tumour where they are more effective [15]. Pre-clinical trials with TILs are promising [11].

During the early stages of tumour development, naïve immune cells become short-lived, and potent effector cells eliminate immunogenic cancer cells and can then differentiate into long-lived, resting memory cells [41]. Effector T cells play a key role in modulating the immune response within the TME [41]. Immunosuppressive Tregs negatively impact immunotherapy and can exceed 50% in the TME [42]. It is hypothesised that T cell anergy, exhaustion and senescence potentially start within the TME [38,41]. NK cells with or without CD16+ and CD69+ can activate immune cells in the TME [14].

1.3.3 Peripheral blood

Metastasis occurs when the tumour spreads systemically via lymph, blood or locally [41]. Immune cells have been shown to promote tumour growth and metastasis and can be

observed in peripheral blood of patients with NSCLC [41]. T cells have a major role to play in patient immunity to NSCLC [11,22,42], as discussed below. Studies have demonstrated a correlation between the cytotoxic function of TILs and peripheral blood T cells, highlighting the potential of circulating T cells as a biomarker for immune activity within the TME [70]

1.3.4 Cell populations

T cells are key mediators of both tumour development and therapeutic immunotherapy intervention in NSCLC [11]. CD8⁺ T cells are generally associated with a favourable prognosis in NSCLC; however, most studies assess their impact within the context of the TME [11]. Senescent T cells are thought to be elevated in the TME compared to peripheral blood, in patients with NSCLC, especially in later stages [11,38]. Patients with increased peripheral senescent T cells have a poorer prognosis [37].

Tregs are elevated in lung cancer patients, which is stage dependent, and are a poor prognostic indicator [11,37]. They have the potential to be used as a biomarker for size, recurrence and metastasis [11,37]. Anti-tumour $\gamma\delta$ T cells are reduced in patients with NSCLC compared to healthy controls, like due to downregulation of activation markers, a trend that is also observed in older individuals [46,47]. $\gamma\delta$ T cells and those activated by bisphosphonates are currently under investigation showing promising results in pre-clinical trials [11,46,47]. MAIT cells in NSCLC have been demonstrated to exhibit a pro-tumorigenic phenotype [51,52]. Elevated levels of MAIT cells in NSCLC are correlated with disease advancement and poorer clinical outcomes [51,52].

In a study by Saavedra et al., which included only Stage III and IV NSCLC patients, B cells were found to be reduced in the blood of lung cancer patients compared to healthy controls [63]. They promote anti-tumour immunity through antigen presentation to T cells in NSCLC, thereby supporting T cell function [63]. Elevated levels of NKT cells in NSCLC are associated with improved survival outcomes; however, their numbers are often reduced, especially in advanced stages of the disease [49,50].

Within the TME, NK cells predominantly exhibit a CD16 negative phenotype, characterized by reduced cytotoxicity and impaired production of IFN- γ , a key cytokine involved in immune cell activation [36]. NK cell impairment is similarly evident in smokers relative to non-smokers [36]. Novel therapies aimed at reversing NK cell exhaustion are emerging,

with the potential to restore NK cell functionality, including cytokines, antibody and CAR-NK cells [36].

Myeloid cells also play an important role in lung cancer immunity [11,32]. N1 neutrophils support the early activation and proliferation of CD4⁺ and CD8⁺ T cells, while N2 neutrophils inhibit the effector functions of CD8⁺ T cells in the lung TME [11,32]. Macrophages release cytokines allowing the recruitment of immune cells and conversely promote accumulation of Tregs in the TME [11,32].

1.3.4.1 Inflammatory Indexes

An increase in neutrophils and platelets and a corresponding reduction in lymphocytes occur as a result of lung cancer [57,58]. Similar to other cancers, inflammatory indexes including NLR, PLR, LMR, SIRI and SII can be used to predict prognosis in NSCLC [30,55-57,69]. NLR, PLR and SII were also associated as a marker for disease development [30,55-57,69]. Studies mostly incorporate patients with advanced-stage disease or those undergoing treatment; either chemoimmunotherapy or surgery [43,52]. Most of the research focuses on NLR, showing that elevated levels before surgery and immunotherapy are linked to poor survival [57,69]. Albumin, as a nutritional marker, may hold particular prognostic relevance in lung cancer patients, who often present with nutritional deficits due to comorbid COPD [57,37]. In addition, persistent nutritional deficiencies have been shown as a risk factor for postoperative complications and prolonged length of stay [57]. PNI has been investigated in patients with lung cancer, and lower PNI levels have been associated with poorer prognosis [57]. HALP score too is associated with higher stage and worse outcomes in patients with NSCLC [43].

1.3.5 Cell surface proteins

1.3.5.1 Stimulatory receptor proteins

Similar to other cancers, markers including CD27, CD28, CD127, ICOS, NKG2D, and CD69 contribute to the immune response in NSCLC, influencing T cell activation, survival, and cytotoxic function [37,41,61,62,69]. Increased expression of TIM3 has been shown to coincide with reduced CD28 in T cell senescence [39]. CD69-positive memory T cells are retained within the TME, where CD69 also plays a role in promoting T cell exhaustion [61,62]. Research on CD69 in NSCLC is limited, some studies have examined its role in

TILs within the TME [61,62]. Upregulation of ICOS is noted on surface of immune cells in NSCLC [17].

1.3.5.2 Immune checkpoint proteins

Upregulation of ICPs are observed on immune and tumour cells in NSCLC [17,19,20,23,63-65]. PD1 is upregulated on immune cells in NSCLC and is associated with poor prognosis, reflecting immune exhaustion within the TME [17,23]. PD1/PDL1 is a central immune checkpoint pathway in NSCLC and the main target of immune checkpoint blockade therapies [14,17,19,20]. As in other cancers, CTLA4 in NSCLC competes with the activation marker CD28 [23]. CTLA4 blockade is most effective when combined with anti-PD1/PDL1 inhibitors, demonstrating a complementary effect [23]. Increased LAG3 expression in NSCLC is linked to adverse clinicopathological features and poorer clinical outcomes [23]. LAG3 and PD1 are often co-expressed on immune cells and act synergistically to suppress immune responses [23].

Studies show increased BTLA and PD1 expression on T cells in NSCLC, leads to reduced effector function, particularly cytokine secretion [25,60]. BTLA expression rises in advanced stages and is linked to poor prognosis when found on tumour cells [25,60].

TIM3's involvement in NSCLC is not yet well characterized [65] According to Su et al., TIM3 expression on tumour cells and TILs is correlated with poor survival [65]. The worst prognosis was observed in patients with co-expression of TIM3 and PD1 on CD8⁺ TILs, while those with single expression had intermediate outcomes, and patients lacking both markers had the best survival rates [65]. PD1, CTLA4, and LAG3 are highly expressed on Tregs within the TME of NSCLC patients, leading to enhanced immunosuppressive activity and reduced anti-tumour immune responses [23].

1.3.5.3 Chemokine receptor proteins

CCR7 expression on lung cancer tumour cells is associated with improved prognosis, which contrasts with its role in other cancers. [28,68]. Binding of CCR7 with chemokines CCL19 and CCL21, lead to release of chemokines CXCL9 and CXCL10 ligands of CXCR3 [29]. This results in anti-tumour immunity through reduction of metastasis, in patients with NSCLC [28]. Different to other cancers, in NSCLC, CXCR3 expression is compartmentalized, with tumour cells lacking expression and immune cells in the TME exhibiting high levels [66,67]. CD8⁺ T cells expressing CXCR3 and their associated

chemokine ligands, recruit additional effector immune cells and exert anti-angiogenic effects, collectively contributing to tumour suppression [2,66,67].

1.3.6 Soluble Plasma Proteins

Interleukins are used as clinical biomarkers in the diagnosis of lung cancer, as they are closely linked with tumour initiation and progression [2]. IL-22 facilitates NSCLC cells resistance to immune targeted killing [11]. IL-4, IL-6 and IL8 promote proliferation of both immune and cancer cells in NSCLC [2,26]. IL-2 contributes to the functional recovery of exhausted T cells in NSCLC [11]. Interferons have anti-tumour activity via inhibition of angiogenesis, cell lysis and immune recognition of lung cancer cells [2]. These include IFN- α , IFN- β and IFN- γ [2].

Elevated levels of pro-inflammatory cytokines cause immune cell accumulation into the TME and are poor prognostic indicators [14,72]. Chemokines CXCL9, CXCL10 and CXCL11 are responsible for immune cell migration to the TME and through interaction with receptor CXCR3 facilitate in reducing lung cancer progression [15,68]. Reduced IL-21 is associated with BTLA expression and has anti-tumour properties, as does IFN- α [20,25]. CCL4 has a dual role in anti-tumour and pro-tumour immunity and is associated with reduced recurrence rates [28].

1.3.8 Summary

Chronic inflammation and immune dysfunction play a key role in the development and progression of NSCLC. The TME influences prognosis, with key immune cells like CD8⁺ T cells, Tregs, and NK cells contributing to either anti- or pro-tumour activity. Circulating immune cell populations, inflammatory indexes, cell surface receptors including ICPs and soluble proteins offer prognostic insights.

1.4 IMMUNE BIOMARKERS IN IMMUNOTHERAPY FOR LUNG CANCER

1.4.1 Introduction

Due to perioperative and adjuvant treatment protocols, some patients who have undergone lung cancer resection surgery, will require chemoimmunotherapy [14,15,17]. Biomarkers have been identified to help predict patient responses to immunotherapy, particularly as resistance and subsequent disease progression are common challenges in these patients

[14,15]. Tumour PDL1 expression is commonly used as a predictive biomarker for clinical outcomes in patients treated with anti-PD1/PDL1 inhibitors [20]. Immune biomarkers, offer potential for evaluating pre-treatment efficacy treatment response, however none are clinically validated to date [20]. In this section, baseline immune biomarkers will be examined and how they influence the efficacy of ICI therapy.

1.4.2 Cell populations

Studies have shown a relationship between the cytotoxic function of TILs and peripheral blood T cells, highlighting the potential of circulating T cells as a useful biomarker [67]. Studies have shown patients who respond to immunotherapy are more likely to have elevated CD4+, CD8+, NK cells post treatment [11,22,73]. In particular, patients with functional EM T cells demonstrate enhanced responses to ICI therapy, as these cells provide rapid and sustained immune surveillance against tumour cells, contributing to better treatment outcomes [22,74]. Elevated numbers of immunosuppressive cells including Tregs are associated with resistance [15,37]. MAIT cells can also be utilised as a predictive marker in ICI treatment [51,52]. $\gamma\delta$ T cells have primarily been investigated as a therapeutic option for NSCLC, with limited research exploring their potential as biomarkers [11,46].

NK cells are required for ICI response; pre-clinical data shows they are elevated in responders to PD1 therapy and act as a biomarker for increased overall survival [75,76]. Neutrophils facilitate immunosuppression and angiogenesis, thereby playing a significant role in mediating resistance to immunotherapy [20]. Reducing neutrophils in ICI therapy, improved treatment response [32]. However, neutrophils are also associated with anti-tumour activity and are important for tumour elimination post-ICI therapy [32].

1.4.2.1 Inflammatory Indexes

NLR and LMR are the most researched inflammatory indexes in patients receiving ICI therapy [14,20,40,74]. Low NLR at baseline and a reduction of NLR correlates with improved clinical outcomes [14,20,38,67]. In patients with PDL1 expression greater than 50%, elevated baseline PLR has been associated with poor prognosis [20]. Baseline elevation of SII is linked to poor survival in NSCLC patients receiving ICIs, though current evidence is limited by small sample sizes [20]. A meta-analysis evaluating SIRI in patients

receiving anti-PD1/PDL1 therapy concluded that it serves as a consistent prognostic indicator [58,74].

1.4.3 Cell surface proteins

1.4.3.1 Stimulatory receptor proteins

Limited studies exist reviewing stimulatory receptor proteins and ICI treatment. An increased frequency of CD8⁺ CD28⁺ T cells and, in patients with NSCLC, prior to ICI treatment, is correlated with unfavourable clinical outcomes [37,39]. Conversely patients with high levels of CD8⁺ T cells had a better prognosis [37,39]. Elevated levels of CD69⁺ T cells have been observed in patients who responded to immunotherapy, highlighting the importance of this activation marker in promoting immune activation [74].

1.4.3.2 Immune checkpoint proteins

As immune checkpoint proteins are involved in keeping the immune system in check, ICIs are associated with many immune related toxicities [24]. Other challenges include differences in patient response to treatment, alongside development of resistance [24]. Elevated levels of ICPs on the surface of immune cells have been observed in patients who respond to ICI therapy [74]. One study specifically noted higher levels of CD4⁺ PD1⁺ T cells and CD8⁺ PD1⁺ T cells in patients, exhibiting treatment efficacy compared to those that did not respond [74]. LAG3 and TIM3 expression in isolation has been linked to resistance to ICIs and poorer therapeutic outcomes [23,65]. Therapeutic resistance has been linked to TILs also, expressing multiple inhibitory receptors including LAG3, TIM3 and PD1 [15].

1.4.3.3 Chemokine receptor proteins

The presence of CXCR3⁺ T cells increase the efficacy of PD1 blockade and loss of CXCR3 causes reduced ICI effectiveness [66]. CXCR3⁺ deficiency on Tregs in pre-clinical trials promote CD8⁺T cells which have an anti-tumour response and reduction of tumour development [42,66]. CXCL9/10 therapy is synergistic with anti-PDI ICI via increase of CXCR3⁺ T cells in pre-clinical studies [40,66].

1.4.4 Soluble Plasma Proteins

Prognostic indicators for poor response to immunotherapy in NSCLC include raised pro-inflammatory cytokines (IL-6) [2,14]. Proinflammatory markers including IL-2 receptor

subunit α (IL-2RA), IL6, CCL7 and CCL19 were shown to be increased post immunotherapy [42]. Increased levels of IL-1 β , TNF- α , IFN- γ during immunotherapy, may reflect an effective therapeutic response, with TNF- α linked with improved survival outcomes [2,74]. Patients with higher baseline levels of IFN- α , IL-2, and IL-17 were associated with an increased risk of developing immune-related adverse side effects following immunotherapy [2]. CXCL19 and CXCL21 ligands of CCR7 are associated with post immunotherapy recurrence [61].

Soluble ICPs have also been investigated for treatment response to ICIs [60]. sBTLA has been used to show non-responders to immunotherapy in cancer patients and has been associated with lower survival rates [60]. Elevated levels of soluble PD-L1 (sPD-L1) are associated with poor prognosis in patients receiving ICI therapy [20].

1.4.6 Summary

Baseline immune biomarkers like elevated CD8+ T cells and inflammatory indexes help predict response to ICI therapy in NSCLC, while high Treg levels and soluble markers for example IL-6, sPDL1, indicate poor prognosis. Immune checkpoint proteins such as PD1 correlate with treatment efficacy or resistance. Despite promise, most biomarkers lack clinical validation.

1.5 IMMUNE BIOMARKERS IN CANCER SURGERY

1.5.1 Introduction

Surgery triggers systemic inflammation and immunosuppression the level of which depends on the extent of damage caused and type of surgical procedure performed [18,19,64,37]. Anaesthetic medications and blood transfusion can also impact immunosuppression [24,75]. Following surgery, the first defence mechanism is the innate immune response involving neutrophils, macrophages, and natural killer cells, followed by adaptive immune responses [18,19,74].

Postoperative immunosuppression provides a window for cancer cell proliferation and/or the awakening of dormant cancer cells, leading to rapid recurrences or metastases [18,19,46,37]. The onset of immune suppression can occur prior to wound closure and may persist for up to six months post-surgery [18]. Normal functioning of the immune system

is important in controlling perioperative circulatory tumour cells (CTCs), which can spread during surgical manipulation [18,78,79]. Some of these CTCs can remain dormant and undergo expansion in response to systemic inflammation [18].

1.5.2 Cell populations

Disease associated molecular patterns (DAMPs) released after tissue damage, trigger systemic inflammation [18,37,75]. Prolonged elevation of white blood cell counts after surgery has been linked to reduced survival rates [18,75]. Surgical intervention triggers the formation of neutrophil NET matrices, and the resulting activated neutrophils promote cell cycle acceleration in CTCs, facilitating cancer dissemination [18,75]. Increased post-surgical levels of NETs are associated with tissue damage and poor prognosis, and can interact with platelets to facilitate cancer dissemination [18].

The post-operative period is marked by an increase in immunosuppressive cell populations, including MDSCs, M-2 macrophages and Tregs [37,75]. Post-operatively, Treg levels were more elevated in patients with advanced-stage disease, as well as those who underwent more invasive procedures [18,37,75]. Post-operative reductions are observed in key immune cell populations, including CD4⁺T cells and CD8⁺ T cells lasting from two weeks to months [18,37,68]. NK cell responses post-surgery are bifunctional and context-dependent. A brief initial elevation may occur, driven by sympathetic triggers like pain, hypovolemia, and hypoxia, or pharmacological factors like opioid use [76,77]. However, this is typically superseded by a prolonged decrease in NK cell density and activity, mediated by the suppressive effects of anaesthesia and post-surgical inflammatory signalling [76,77]. Much of the current literature examines the functional activity of NK cells following surgery, whereas changes in NK-cell frequency is less extensively studied [76,77]. NK and T cells are important for killing CTCs [16,76,77] and are associated with prognosis [59,76]. The invasiveness of surgery influences inflammation, with minimally invasive procedures causing less disruption to CD4⁺ T cells and NK cells compared to open procedures [18,76,77].

1.5.2.1 Inflammatory Indexes

Elevated NLR, PLR and LMR pre-operatively are poor prognostic indicators, post cancer resection surgery [18,30,31]. PLR increases due to increase in platelets post operatively

and corresponding immunosuppression of lymphocytes [77]. Platelets aggregate with CTCs, facilitating metastatic spread and protection against immune cells [77]. Elevated SII and systemic SIRI have been associated with adverse clinical outcomes, including decreased overall survival and a higher incidence of postoperative complications, particularly infectious events [18,30,31].

1.5.3 Cell surface proteins

1.5.3.1 Stimulatory receptor proteins

Research on stimulatory and co-stimulatory receptors, such as CD27, CD28, ICOS, and CD69, has primarily focused on cancer immunity, with limited data available in the context of the post-resection setting [38,40,70]. Activated platelets downregulate NKG2D expression on NK, leading to impaired function through reducing IFN- γ secretion and tumour cell killing capacity [18,78].

1.5.3.2 Immune checkpoint proteins

Few studies have investigated the impact of surgical intervention on ICP expression in immune cells, as most research has focused on their expression in tumour cells. Available evidence indicates an increase in PD1/PDL1 expression on the surface of immune cells post-operatively [18,78]. One such study demonstrates increased levels of PD1/PD-L1 and CTLA4 on the surface of immune cells 5 days post operatively [79]. ICPs including TIM3, LAG3 and PD1 are also increased on the surface of Tregs post operatively, contributing to impaired T cell activation [18].

1.5.3.3 Chemokine receptor proteins

No published studies to date have investigated CCR7 expression on immune cells in peripheral blood before, during, or after surgery for solid tumours. Research investigating CXCR3 expression on immune cells in the perioperative setting also remains limited [77,81,82,83]. In a study of 27 patients undergoing gastrectomy for gastric cancer, peripheral blood lymphocytes exhibited elevated CXCR3 expression following surgery, with levels also significantly higher than those observed in healthy controls [81]. Analysis of TILs in resected renal cell carcinoma specimens, demonstrated high CXCR3 expression, which was identified as a prognostic marker associated with tumour grade, pathological stage, and likelihood of recurrence [83]. These findings suggest a potential role for CXCR3

as an immune biomarker in the postoperative immune response across multiple malignancies.

1.5.4 Soluble Plasma Proteins

Surgery triggers a surge in pro-inflammatory cytokines, chemokines, and growth factors, accompanied by a reduction in anti-inflammatory cytokines [18,78]. TGF- β is linked with wound healing, alongside cancer progression [18]. Patients with post-operative complications across various cancers exhibit elevated levels of pro-inflammatory cytokines, such as IL-6, IL-8, IL-18, TNF- α , and IL-1 β , alongside reduced levels of anti-inflammatory cytokines, including IFN- γ and IL-10 [12,18,37]. A rise in TGF- β and IL-6 has been shown to negatively impact NK cytotoxicity [37]. Notably, IL-1 and TNF- α have been linked to CTC adhesion and immune evasion [12,18]. Therefore, alongside post-operative complications, increased pro-inflammatory cytokines and decreased anti-inflammatory cytokines are associated with tumour progression and serve as poor prognostic indicators [12,18]. Inhibition of pro-inflammatory cytokines have the potential to improve prognosis post-surgical resection, as well as a therapy to fight cancer [18,80].

Chemokines, facilitate the movement of neutrophils to sites of injury; this alongside the increased numbers of immature neutrophils in the peripheral blood, creates opportunities for tumour cells to metastasise [18]. CCL2 for example, which increases post-operatively, is involved in macrophage recruitment [18]. Antibodies against CCL2 have been shown to have anti-tumour effects in pre-clinical trials [18].

1.5.6 Summary

Surgical resection in cancer patients triggers systemic inflammation and immunosuppression, influenced by factors such as surgical complexity, anaesthetics, and blood transfusions. This immune disruption facilitates cancer progression by impairing anti-tumour responses and enabling CTC survival. Postoperative changes include elevated pro-inflammatory cytokines, increased immunosuppressive cells (e.g., Tregs, MDSCs), and reduced effector cells (CD4⁺, CD8⁺ T cells). A range of immune-related biomarkers, including inflammatory cell populations, surface markers, plasma proteins and systemic indexes, have been investigated as prognostic indicators to predict postoperative outcomes and cancer progression, however few longitudinal studies exist.

1.6 IMMUNE BIOMARKERS IN LUNG CANCER SURGERY

1.6.1 Introduction

Minimally invasive procedures have been shown have less disruption on immune response, in the immediate post-operative period (POP) [4,16]. Postoperative complications, including POP and length of hospital stay, are reduced following minimally invasive procedures compared to open surgery [4]. Conflicting evidence exists regarding postoperative inflammation following minimally invasive procedures, with some studies reporting a heightened inflammatory response in VATS compared to RATS [4,82]. Patients who undergo lung cancer resection surgery with Enhanced Recovery After Surgery (ERAS) principles, are shown to reduce systemic inflammation post-operatively, thereby improving outcomes, as is the case with this study [14,18].

Much of the research into immune profiling of lung cancer involves advanced stages and /or tumour infiltrating lymphocytes (TILs) in the tumour immune microenvironment (TIME) [17,67]. Few research articles describe the immune profile of peripheral blood in perioperative, early stage NSCLC patients [18,19,63,82].

1.6.2. Cell populations

Three studies were found examining cell populations in peri-operative patients undergoing lung cancer resection [40,72,79]. Riemann *et al* analyse cell populations of pre-operative patients (staging not clarified) and healthy controls [79]. They discovered that T regulatory cells (Tregs), monocytes and naïve T cells indicate poor prognosis in NSCLC patients, with age and smoking history negatively impacting these immune markers [79]. Wang *et al* again analyses immune cell populations in peripheral blood, where Stage I-IV untreated patients are compared to healthy controls [40]. Results reveal that CD4+CD28+, CD8+CD28+ and naïve Tcells were lower in patients with NSCLC compared to healthy controls, with natural killer (NK) and lymphocyte ratios higher; this was stage dependent [40]. They concluded that disruption of the immune response (especially T cells) occurs in patients in NSCLC and this could be used as a predictor for screening [40].

Deboever *et al* looked at 35 different markers via flow cytometry at three timepoints; pre-operatively, 24 hours and 4 weeks [72]. This research focused on differences between cell populations in minimally invasive compared to open procedures for lung cancer resection

[72]. They concluded that peripheral blood mononuclear cell (PBMC) populations in peripheral blood showed no difference between operative procedures at 4 weeks [72].

1.6.2.1 Inflammatory Indexes

NLR, PLR, alongside LMR, SIRI and systematic SII have been evaluated in peri-operative lung cancer as survival indicators and predictors of post-operative complications [14,31]. NLR and LMR were predictors of post-operative complications [14]. Xiaowei et al. concluded that the SII, in combination with FEV1, could serve as a predictive tool for post-operative pulmonary complications [31]. Elevated SII has also been shown to be a negative predictor for OS and PFS [49]. A Chinese study of 1,486 patients identified 483, as the optimal preoperative SII cut-off for predicting POP, with higher SII levels associated with increased severity [13]. The study also demonstrated that SII had a superior predictive performance, compared to NLR and PLR [13]. Currently, there is limited evidence regarding the perioperative utility of SIRI; however, a study conducted in China suggests that it may serve as a prognostic marker following surgical resection [74]. No large scale clinical trials have been performed to accurately assess these indexes [26].

1.6.3 Cell surface proteins

1.6.3.1 Stimulatory receptor proteins

Two studies have investigated CD28 expression on the surface of peripheral T cells, following lung cancer resection surgery [63,84]. The aforementioned study by Wang *et al.* demonstrated a reduction in CD28 expression on the surface of T cells following lung cancer resection surgery [84]. Chen *et al.* analysed peripheral blood samples collected before surgery and up to three months postoperatively, demonstrating a reduction in CD28 negativity on CD8⁺ T cells following tumour resection [85].

No longitudinal perioperative lung cancer surgery studies were found that measure, cell surface expression of CD27, ICOS, NKG2D, or CD69 on peripheral immune cell subsets before vs after resection.

1.6.3.2 Immune checkpoint proteins

Research on ICPs has predominantly focused on their expression in tumour cells and advanced disease stages, with a notable scarcity of literature examining ICPs on peripheral immune cells during the perioperative period. Specifically, data on BTLA, LAG3, and

CTLA4 in this context are lacking. However, studies by Xu *et al.* have provided insights into perioperative changes in PD1 and TIM3 expression [18,86]. PD1 has shown to increase on surface of immune cells due to surgical stress [18,19]. Xu *et al.* discovered an increase in PD1/PDL1 immune cell expression, peaking on the 5th post-operative day [18], which may correlate with reduced T and NK cells and extent of resection, however oesophageal, pulmonary and thyroid surgery were included [18]. Open procedures with increased surgical stress showed an increased level of PD1 expression on immune cells [18]. Another study reported increased PD1 expression on TILs following surgical resection, with higher levels correlating with advanced tumour stage and grade, and an association with poorer overall prognosis [87]. Xu *et al.* demonstrated a postoperative reduction in TIM3 expression, in 21 patients, on peripheral NKT and NK cells, within 4 to 20 weeks following surgical resection, indicating that tumour removal influences TIM3 expression on immune cells [86].

1.6.3.3 Chemokine receptor proteins

No studies have specifically investigated CCR7 or CXCR3 expression on peripheral immune cells in patients undergoing lung cancer resection surgery across peri-operative timepoints.

1.6.4 Soluble Plasma Proteins

Peripheral levels of anti-inflammatory cytokines decrease and those of pro-inflammatory cytokines increase following lung cancer resection surgery [18]. IFN- γ , for example, decreases in the hours after surgery, this cytokine is involved in control of metastasis [18]. Elevated levels of pro-inflammatory cytokines, including TNF- α , IL-6 and IL-8 are associated with post-operative pulmonary complications [14,18]. Kaufmann *et al.* obtained blood samples at the time of wound closure and again 24 hours post-operatively, and evaluated post-operative complications occurring within 30 days [12]. Elevated levels of IL-6, IL-8, and IL-10 were detected at wound closure in patients who later developed post-operative complications, with surgical invasiveness identified as an additional contributing factor [12].

IL-2, IL-7, IL-15 and IL-21 and IFN- α influence PD-1 expression on peripheral T cells [18]. Xu *et al.* showed higher levels of IFN- α , IL-6 and TNF- α post-operatively at 24 hours

[18]. PD1 expression on CD4⁺ T cells on day 5 were shown to be influenced by IFN- α [18]. CCL2 plays a key role in driving the recruitment of Tregs into the TME, and blockade of CCL2 has been shown to reduce Treg accumulation [41].

Cytokine levels, particularly IL-6 and IL-8, have been reported to be significantly higher following open thoracotomy compared to VATS and RATS [72]. Various studies have reported increased IL-6 levels following VATS compared to RATS, while others have found no significant difference between the two approaches [72,79,80]. However, no validated or large-scale clinical trials currently exist to confirm these findings.

1.6.6 Summary

There is limited data on peri-operative changes in immune cell populations and cell surface markers such as CD28, CD27, or ICPs on peripheral immune cells. Most existing studies focus on tumour cells or advanced disease, highlighting a significant gap in early-stage, perioperative immune profiling.

1.7 IMMUNOPHENOTYPING OF PERIPHERAL BLOOD OF NSCLC PATIENTS

1.7.1 Peripheral Blood Mononuclear cells

This novel study investigates peripheral blood mononuclear cells (PBMCs) in patients undergoing surgical resection for NSCLC, incorporating analysis of immune cell populations and individual immune checkpoint markers. Building upon ongoing research with the All-Ireland Cancer Liquid Biopsies (CLuB) Consortium, this work aligns with the consortium's objective of developing viable alternatives to invasive surgical biopsy techniques [89]. Three timepoints were selected as discussed in Materials and Methods, Chapter 2. Flow cytometry was employed to enumerate immune cell populations and quantify surface expression of relevant immune markers. These immunological findings were then correlated with clinicopathological data and subjected to survival analysis.

1.7.2. Full Blood Count

A Full Blood Count (FBC) were collected from each patient at all three peri-operative timepoints. These measurements allowed for the calculation of various inflammatory indexes, which provided insights into the patients' systemic inflammatory and coagulation status throughout the surgical period. This comprehensive approach enabled the correlation

of inflammatory markers and coagulation activity with clinical outcomes and immune changes following lung cancer resection.

1.7.3. Summary

Monitoring PBMCs and FBC longitudinally allows for a better understanding of the inflammatory and immunological shifts that occur in response to surgical intervention. This approach holds significant potential for the identification of biomarkers that can predict postoperative outcomes, the likelihood of complications, and the risk of disease recurrence, thereby offering valuable tools to guide personalized patient management and improve long-term prognosis following lung cancer surgery.

CHAPTER 2

MATERIALS

&

METHODS

2.1 STUDY POPULATION AND DESIGN

2.1.1 Introduction

This longitudinal study focused on adult patients diagnosed with early-stage NSCLC who were deemed surgically operable following multidisciplinary team (MDT) evaluation. All participants included in this cohort had a histologically confirmed pre-operative tissue diagnosis of either adenocarcinoma or squamous cell carcinoma. Eligible patients underwent surgical resection for NSCLC at St. James's Hospital (SJH), Dublin, between November 2023 and August 2024. Detailed demographic and clinical characteristics of the study population are presented in Chapters 3–5.

Patient recruitment was conducted through St. James's Hospital and the Trinity Translational Medicine Institute (TTMI), Dublin, under ethical approval granted as outlined in Section 2.2. Recruitment criteria excluded individuals with a known history of autoimmune disease, a known history of cancer or those receiving systemic immunosuppressive therapy. In addition, none of the patients received neoadjuvant chemotherapy or immunotherapy prior to surgical intervention. For patients who were subsequently selected for adjuvant therapy, treatment initiation occurred only after completion of the final blood sampling, at the second follow-up (2FU) timepoint.

Three defined timepoints were established to facilitate longitudinal analysis of immune profiles, chosen to balance feasibility with the biological relevance of post-operative immune dynamics. These timepoints were as follows:

- **Pre-operative (Baseline):** Prior to induction of anaesthesia.
- **Early post-operative (1st Follow-up, 1FU):** Two to four days following surgical resection.
- **Late post-operative (2nd Follow-up, 2FU):** Six to nine weeks following surgery, coinciding with the routine outpatient follow-up visit.

At each timepoint, peripheral venous blood was obtained via venepuncture for immune profiling. PBMCs were isolated and processed according to the standardised protocol described in Section 1.4. All laboratory analyses and sample processing were conducted within the facilities of TTMI.

2.1.2 Patient samples inflammatory indexes

At each designated timepoint, peripheral venous blood was collected into ethylenediaminetetraacetic acid (EDTA) anticoagulated tubes for the purpose of performing an FBC. This analysis included quantification of haemoglobin concentration, and enumeration of neutrophil, lymphocyte, monocyte, and platelet populations. Additional blood samples were obtained in serum separator tubes, containing a clot activator and gel barrier, for the assessment of serum albumin as part of routine liver function testing. All haematological and biochemical analyses were performed in the accredited Haematology Laboratories of St. James's Hospital, Dublin. Laboratory results were subsequently retrieved from the institutional Electronic Patient Record (EPR) system for data collation and analysis.

2.1.2 Patient samples for flow cytometry

At each designated timepoint, peripheral venous blood was collected into EDTA anticoagulated tubes and processed on the same day in TTMI. Plasma was separated and cryopreserved for subsequent analysis, while PBMCs were isolated and cryopreserved according to the standardised protocols, outlined in Sections 2.4 and 2.5. Thawing of PBMCs was performed as per 2.4.3. Preparation and analysis via flow cytometry was performed for the three timepoints for each patient on the same day.

2.2 ETHICAL APPROVAL

2.2.1 Ethics and Governance

Ethical approval for this study (Project ID: 0723) was granted by the St. James's Hospital/Tallaght University Hospital Joint Research Ethics Committee under submission number 2918. The study protocol, participant information leaflet, and informed consent form were reviewed and approved as part of this ethical assessment process. Each participant was provided with a document titled "*Thoracic Biobank Information Leaflet*" in advance of recruitment to ensure full understanding of the study's aims, procedures, and data handling practices. Written informed consent was subsequently obtained by trained medical professionals using the approved "*Thoracic Biobank Consent Form*." All data collection, storage, and processing procedures were conducted in accordance with the principles of the Declaration of Helsinki and in compliance with the General Data Protection Regulation.

2.3 MATERIALS FOR FLOW CYTOMETRY

2.3.1 Equipment

Equipment used in this study and their manufacturing details are as per Table 1

Table 2.1: Equipment

Equipment	Model	Manufacturer
Centrifuge	Eppendorf 5415	ThermoFisher Scientific
Haemocytometer	Neubaur	ThermoFisher Scientific
CO2 Incubator	180i	ThermoFisher Scientific
Flow Cytometer	LSRFortessa	Becton Dickinson
Balance	Adventure Pro	Ohaus
Pipettes: p10, p20, p100, p200, p1000	Eppendorf	ThermoFisher Scientific
Vortex	Vortex Genie	Scientific Industries
Water Bath	WCW-010E	Germany Industries
Microscope	Inverted	Olympus Corporation

2.3.2 Consumables

Refer to Table 2 for general consumables used in this study

Table 2.2: General consumables

Reagent	Brand	Manufacturer
Vacutainer blood tubes	Becton Dickinson	
Phosphate Buffer Saline (PBS)	Gibco	Invitrogen
15mL / 50mL Falcon Tubes	ThermoFisher Scientific	
Lymphoprep	STEMCELL Technologies	ThermoFisher Scientific
Foetal Bovine Serum (FBS)	Gibco	ThermoFisher Scientific
Roswell Park Memorial Institute (RPMI) 1640 with Glutamax	Gibco	ThermoFisher Scientific
25mM HEPES	Gibco	Invitrogen
Penicillin-Streptomycin (Penstrep)	Gibco	Invitrogen
Trypan Blue	Gibco	ThermoFisher Scientific
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich	
Mr Frosty Freezing Container	Nalgene	ThermoFisher Scientific
Cryovials	Nunc,	ThermoFisher Scientific
T75 Culture Flask	Sarstedt,	ThermoFisher Scientific
5mL Flow cytometry tubes	Falcon, round bottom	ThermoFisher Scientific
30% Bovine Serum Albumin (BSA)	Sigma-Aldrich	
10% Sodium azide	ThermoFisher Scientific	
4% Paraformaldehyde	Santa Cruz	ThermoFisher Scientific
3.5mL Transfer Pipettes	Sarstedt	ThermoFisher Scientific
10mL / 50mL plastic pipettes		

2.3.3 Antibodies and flow cytometry reagents

Refer to Table 3 for antibodies and Table 4 for flow cytometry reagents

Table 2.3: Antibodies for flow cytometry

Fluorochrome	Surface Antibody	Company	Catalog No.	Clone
APC-Vio770	V81	Miltenyi Biotec	130-120-438	REA173 R9.12
Vio Bright 515	CD127	Miltenyi Biotec	130-120-027	REA614 A019D5
PerCP-Vio700	CD19	Miltenyi Biotec	130-113-648	REA675 SJ25-C1
VioBlue	V82 TCR	Miltenyi Biotec	130-111-015	REA771 123R3
VioGreen	CD8	Miltenyi Biotec	130-110-684	REA734 HIT8a
BV605	CD314/NKG2D	Biolegend	320831	ID11
BV650	CD45RA	Biolegend	304136	HI100
BV711	CD4	Biolegend	317440	OKT4
BUV737	CD3	BD Biosciences	612752	SK7
PE	CD56	Miltenyi Biotec	130-113-312	REA196 NCAM16.2
PE-Vio-615	CD16	Miltenyi Biotec	130-119-995	REA423 3G8
PE Vio 770	CCR7	Miltenyi Biotec	130-117-396	REA546 G043H7
Alexa Fluor 700	PD-1	Biolegend	329952	EH12.2H7
APC-Vio770	CD27	Miltenyi Biotec	130-114-163	EA499 LG.3A10
Vio Bright 515	BTLA / CD272	Miltenyi Biotec	130-127-112	REA1293 J168-540
PerCP-Vio700	CD19	Miltenyi Biotec	130-113-648	REA675 SJ25-C1
VioBlue	CD16	Miltenyi Biotec	130-113-958	REA423 3G8
BV605	ICOS	Biolegend	313538	C398.4A
BV650	CD28	Biolegend	302946	CD28.2
PE-Vio-615	CTLA4/ CD152	Miltenyi Biotec	130-130-454	REA1003 BNI3
PE Vio 770	CD366/ TIM3	Miltenyi Biotec	130-121-419	REA635 F38-2E2
Alexa Fluor 700	CD14	Biolegend	301822	M5E2
APC-Vio770	Valpha.7.2	Miltenyi Biotec	130-123-896	REA179 3C10
Vio Bright 515	CD161	Miltenyi Biotec	130-122-801	REA631 191B8
VioBlue	CD223/LAG-3	Miltenyi Biotec	130-126-631	REA351 17B4
BV605	CD 69	Biolegend	310938	FN50
PE Vio 770	CD183	Miltenyi Biotec	130-120-593	REA232 G025H7

Table 2.4. Reagents for flow cytometry

Reagent	Manufacturer	Material Number
MACS Comp Bead Kit, anti-REA	Miltenyi Biotec	130-104-693
One Comp eBeads	Invitrogen	01-1110-42
Zombie UV Fixable Viability Kit	Biolegend	423107
FcR Blocking Reagent	Miltenyi Biotec	130-059-901
5mL Falcon Round bottom flow cytometry Tubes	ThermoFisher Scientific	352052

2.4 METHODS FOR FLOW CYTOMETRY

2.4.1 Principles

Flow cytometry is an analytical technique that employs laser-based detection to characterise individual cells, as they pass in single file through a focused fluid stream [90,91]. As cells pass through the laser beam, fluorochrome-conjugated markers bound to specific cellular components emit fluorescence in response to absorbed light [90,91]. Flow cytometry can detect and analyse cells and particles ranging in size from approximately 0.5 μM to 100 μM [80,81]. Light scattering occurs through forward scatter (FSC) and side scatter (SSC) [80,81]. FSC provides an overall measure of cell size, while SSC reflects the internal granularity of the cell [90,91]. Flow cytometry detectors capture the fluorescence emitted by each cell, providing information on the frequency, intensity, and distribution patterns of marker expression [90,91]. The percentage frequency reflects the proportion of cells expressing that marker, which was used to quantify marker expression in this study [90,91].

The BD LSRFortessa operates through three integrated systems. The BD FACSDiva software is used for data acquisition, with files are subsequently exported for detailed analysis into FlowJo software. The fluidics system generates a single-cell stream for interrogation, while the optical system comprises five lasers—red, blue, violet, ultraviolet, and yellow. Multiple fluorochromes can be excited by a single laser and are separated into distinct wavelength ranges by the optical system, directing each signal to a specific detector. The BD LSRFortessa is capable of detecting up to 18 fluorochromes simultaneously.

2.4.2 Panel Design

Three antibody panels were developed during optimisation to minimise spectral spillover and to accurately characterise distinct cell populations, incorporating surface, stimulatory, and checkpoint markers. Panel compositions are detailed in Table 5, and the corresponding cell populations and specific markers are listed in Table 6. Cell populations are characterised as per Chapter 1.

Table 2.5: Three antibody panels utilised for patient samples

Laser	Band	Fluorochrome	Panel 1	Panel 2	Panel 3
Red 633 nm	730/45	Alexa Fluor 700,	CD25	PD-1	CD14
	780/60	APC-Vio770	$\gamma\delta$ T V δ 1	CD27	V α .7.2
Blue 488 nm	530/30	Vio Bright 515	CD127	BTLA	CD161
	710/50	PerCP-Vio700	CD19	CD19	CD19
Violet 406 nm	450/50	VioBlue	$\gamma\delta$ T V δ 2	CD16/ LAG-3	LAG-3
	525/50	VioGreen	CD8	CD8	CD8
	610/20	BV605	NKG2D	ICOS	CD69
	660/20	BV650	CD45RA	CD28	CD28
	710/50	BV711	CD4	CD4	CD4
Ultra-violet 355 nm	450/50	Live Dead stain	DCS	DCS	DCS
	740/35	BUV737	CD3	CD3	CD3
Yellow 561 nm	582/15	PE	CD56	CD56	CD56
	610/20	PE-Vio-615	CD16	CTLA-4	CD16
	780/60	PE Vio 770	CCR7	TIM3	CD183

Table 2.6: Specific cell populations and markers for each panel

Panel 1	Panel 2	Material Number
$\gamma\delta$ T cells	T Helper / Cytotoxic T cells	T Helper / Cytotoxic T cells
Naïve T Cells	B cells	B cells
T Helper / Cytotoxic T cells	NK cells	NK cells
T Reg cells	NKT cells	NKT cells
Memory T cells (CM, EM, TDEM)	Immune checkpoint proteins	MAIT cells
NK cells	Co-stimulatory proteins	Monocytes
CD56+T cells (NKT-like)		Cytokine receptor
B cells		Co-stimulatory proteins
Cytokine receptor		

2.4.3 Preparation and cryopreservation of PBMCs

Blood was diluted 1:1 with PBS medium containing FBS in a 50 mL falcon tubes. The diluted blood was layered onto 15 mL of lymphoprep in a separate 50 mL falcon tube using a transfer pipette. Centrifugation was performed for 25 min at 400 x g with the acceleration and brake off (minimum deceleration). The plasma was removed and frozen at -80°C. The buffy coat layer and lymphoprep layer was removed without disrupting the red cell pellet. This was added to 1% FBS and PBS and centrifuged for 8 minutes at 450 x g with the brake on. The supernatant was then discarded and pellet re-suspended in freezing mixture (90% FBS+10% DMSO). This was made in advance and allowed to cool, post exothermic reaction. This mixture was immediately placed in 4 cryovials which were frozen at -80 °C in a Mr Frosty container. The following day the vials were transferred to liquid nitrogen.

2.4.4 Cell Culture

Vials retrieved from liquid nitrogen storage and kept at -20 °C until ready to thaw. Hot water was used with constant shaking to disperse the heat. Cells were diluted drop-wise with 10 mL of complete RPMI medium (500 mL RPMI 1640 with Glutamax + 50 mL FBS + 12.5 mL HEPES + 5 mL Penstrep). The cells were then pelleted by centrifugation at 450 x g for 10 minutes with break on. The supernatants were discarded and cell pellets resuspended in 5 mL of complete RPMI. The cells were left overnight in a T75 flask in the CO2 incubator.

2.4.5 Staining

The follow day cells were centrifuged at 450 x g for 10 minutes with break on and the pellet re-suspended in 1 mL complete RPMI. Cells counts were performed using haemocytometer and trypan blue. Flow cytometry tubes were labelled for each antibody panel and an unstained sample as a control. 500,000 cells was added to each tube. 2 mL PBS was placed in each tube, vortexed and cells pelleted by centrifugation at 450 x g for 8 minutes. The supernatants were discarded and unstained tube was resuspended in 0.5 mL PBA buffer (580 mL PBS + 20 mL 30% BSA + 1.2 mL 10% sodium azide). To all the remaining tubes 100mL of dead cell stain as prepared in section 2.4.6 was included, cells were vortexed and incubated for 15 minutes in the dark. Then, 2 mL of PBA buffer was added to each tube, vortexed and spun at 450 x g for 8 minutes. Fc receptor blocking reagent was prepared as per manufacturer's instructions (3 µL of block and 47 µL PBA buffer for each tube). 50 µL of Fc receptor block solution was added to each tube, vortexed and incubated for 15 minutes

in the dark. 2 mL of PBS buffer was added to each tube, vortexed and spun at 450 x g for 8 minutes. Antibody cocktails were then prepared as per optimisation in Section 2.4.10 with 50 μ L added to each tube. Tubes were then vortexed and incubated for 20 minutes in the dark. 2 mL PBA buffer was then added to each tube, vortexed and spun at 450 x g for 8 minutes. The cells were then fixed with 250 μ L of 4% paraformaldehyde for 10 minutes. 2mL PBA buffer was then added to each tube, vortexed and spun at 450 x g for 8 minutes. Cells were resuspended in 0.3 mL PBA buffer. The tubes were kept overnight in the fridge for analysis by flow cytometry the following day.

2.4.6 Live Dead Stain

On the same day as sample staining was performed, cell viability stains were also prepared as per manufacturer's instructions. A 1:1000 zombie UV live dead stain was performed. 500,000 cells were killed using 60 °C on a heated plate for 5 minutes, followed by cooling for 5 minutes on ice. The same amount of live cells was then added to the dead cells. 100 μ L of the 1:1000 zombie UV live dead stain, diluted in PBS, was added to this mix and incubated for 15 minutes in the dark. 2 mL PBA buffer was then added to each tube, vortexed and spun at 450 x g for 8 minutes. Cells were resuspended in 0.3 mL PBA buffer. The tubes were kept overnight in the fridge for analysis by flow cytometry the following day.

2.4.7 Compensation

Fluorescence spillover may arise when multiple fluorochromes expressed on a single cell emit at similar wavelengths, causing detection by the same channel and potentially producing false-positive signals [80,81]. To address this, bead controls were used enabling the software to quantify spectral overlap and apply compensation adjustments to the measurements. As Miltenyi Biotec antibodies are anti-REA; MACS Comp Bead kit was used for Miltenyi Biotec antibodies. For BD and Biolegend antibodies One Comp eBeads were utilised. Compensation was carried out on the day of flow cytometry, prior to sample acquisition. During optimization, a 14-fluorochrome panel was selected, to minimize spectral spillover between channels.

2.4.8 Sample Acquisition

Prior to acquisition, all FACS tubes were vortexed to ensure a homogeneous single-cell suspension and minimize cell clumping. Following compensation, each sample was run

through the flow cytometer under standardized settings to acquire data on fluorescence intensity and marker expression. Data were collected for 100,000 events per sample to provide robust statistical representation of the cellular populations of interest.

2.4.9 Gating Strategy

Data was analysed using FlowJo v10 software (BD Life Sciences). Gating strategy was performed as per Figure 1. Fluorescence minus one (FMO) controls were used to set accurate gating boundaries, by including all fluorochromes except the one of interest. They helped distinguish true signal from background fluorescence and spillover, ensuring precise identification of cell populations and markers of interest, especially in complex multicolour panels [80,81].

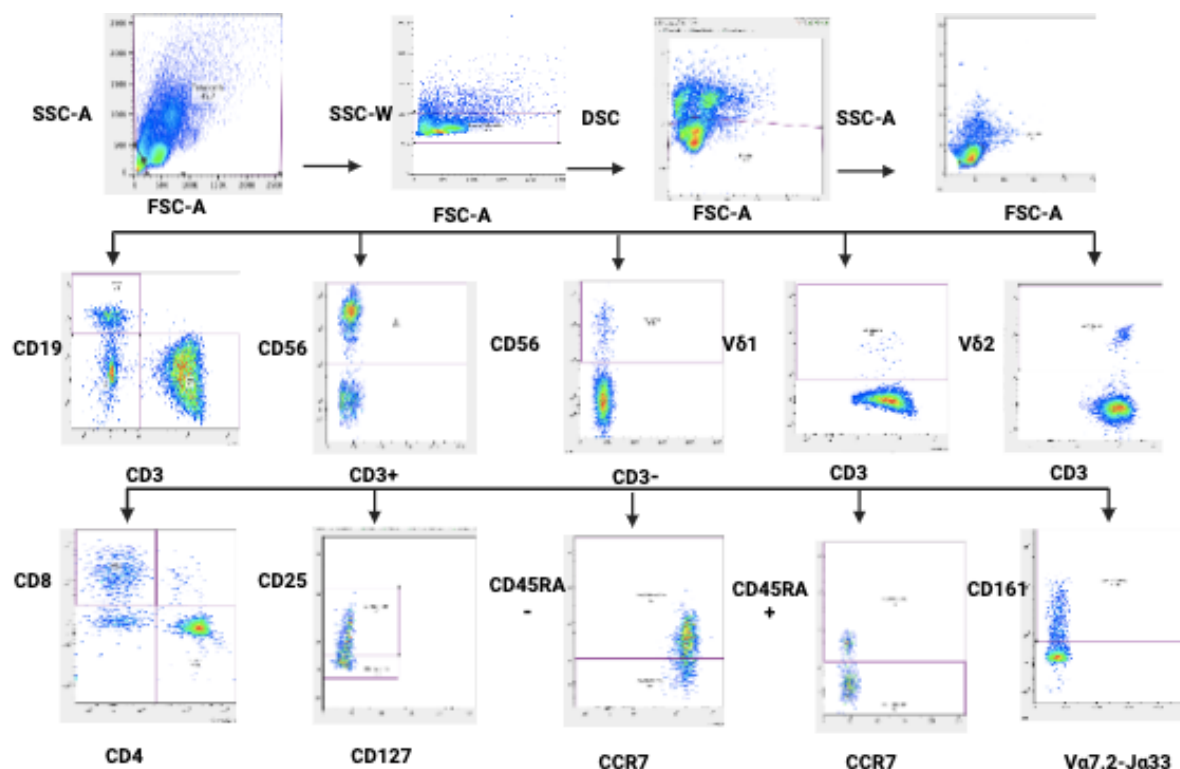


Figure 2.1 Gating strategy for the detection of T cells, B cells, NKT cells, NK cells, V δ 1 T cells, V δ 2 T cells, CD4+, CD8+ T cells, T regs, Naïve, CM, EM, TDEM T cells and MAIT cells by flow cytometry.

PBMCs were stained with a dead cell stain (DCS), CD3, V δ 1 V δ 2, CD4, CD8, CD19, CD56, CD25, CD127, CD45RA, CCR7, CD161 and the Va7.2, T cell receptors and analysed by flow cytometry.

Upper panels, left to right: flow cytometry dot plots showing forward scatter area (FSC-A) plotted against side scatter area (SSC-A) with an electronic gate drawn around the total cells. Dot plot showing FSC-A plotted against FSC-width (FSC-W) for gated cells with a gate drawn around the single cells; Dot plot showing FSC-A plotted against the DCS for gated single cells with a gate drawn around the live cells. Flow cytometry dot plots showing forward scatter area (FSC-A) plotted against side scatter area (SSC-A) with an electronic gate drawn around the lymphocytes.

Centre panels, Left to Right: Five dot plots showing expression of CD3 (T cells) and either CD19 (B cells, CD56 and CD3- (NK cells), CD56 and CD3+ (NKT cells), V δ 1 and CD3 (V δ 1 cells) and V δ 2 and CD3 (V δ 2 cells).

Lower panels, Left and Right: Dot plot showing expression of CD4 and CD8 by gated T cells with gates drawn around the CD4+ T cells, CD8+ T cells. From both CD4+ and CD8+ cells Tregs were identified with CD25+ and CD127-. From both CD4+ and CD8+ cells naïve and TDEM T cells were identified with CD45RA+ and CCR7+/- From both CD4+ and CD8+ cells CM and EM were identified with CD45RA- and CCR7+/- From CD3+ cells CD161+ and Va7.2 and Ja7.33

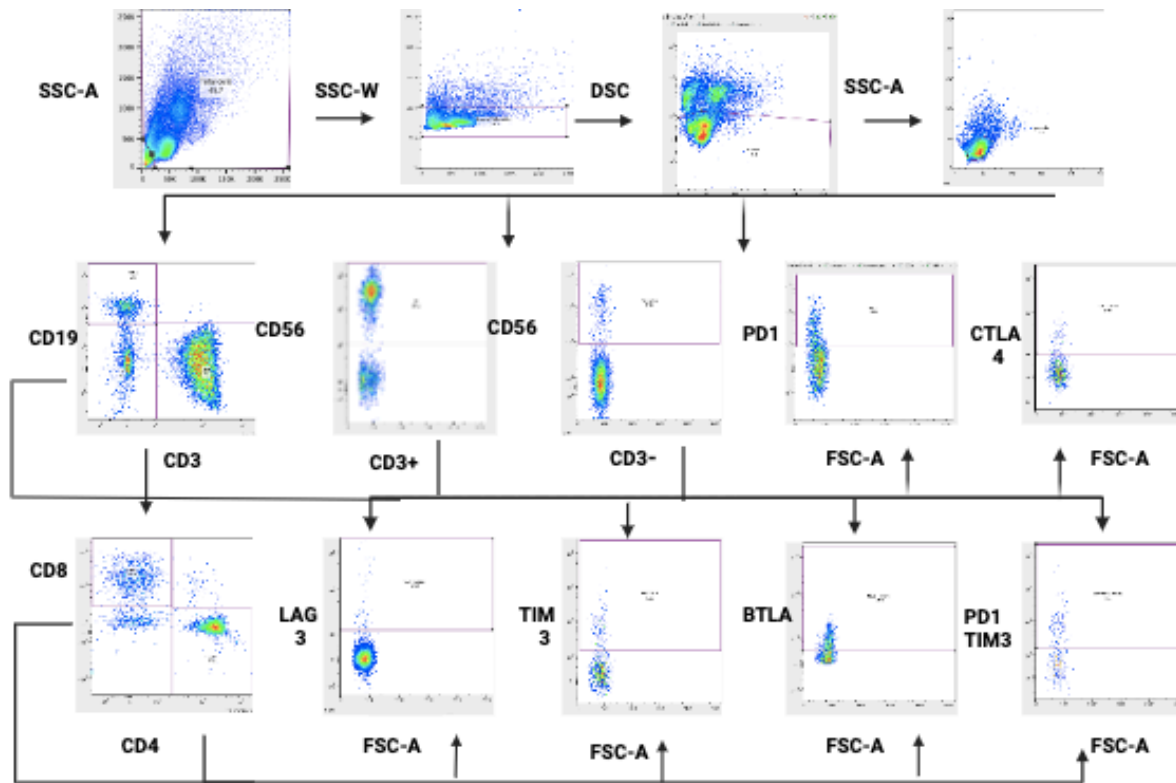


Figure 2.2 Gating strategy for the detection immune checkpoint inhibitors PD1, CTLA4, LAG3, TIM3, BTLA and co-expression of PD1 and TIM3 on CD4+, CD8+ T cells, B cells, NK cells and NKT cells by flow cytometry.

PBMCs were stained with a dead cell stain (DCS), CD3, CD4, CD8, CD19, CD56, PD1, LAG3, TIM3 and BTLA antibodies and analysed by flow cytometry.

Upper panels, left to right: flow cytometry dot plots showing forward scatter area (FSC-A) plotted against side scatter area (SSC-A) with an electronic gate drawn around the total cells. Dot plot showing FSC-A plotted against FSC-width (FSC-W) for gated cells with a gate drawn around the single cells; Dot plot showing FSC-A plotted against the DCS for gated single cells with a gate drawn around the live cells. Flow cytometry dot plots showing forward scatter area (FSC-A) plotted against side scatter area (SSC-A) with an electronic gate drawn around the lymphocytes.

Centre panels, Left to Right: Five dot plots showing expression of CD3 (T cells) and either CD19 (B cells), CD56 and CD3- (NK cells), CD56 and CD3+ (NKT cells) and PD1 and CTLA4 on CD4+, CD8+ T cells, B cells NK cells and NKT cells.

Lower panels, Left and Right: Dot plot showing expression of CD4 and CD8 by gated T cells with gates drawn around the CD4+ T cells, CD8+ T cells. Expression of LAG3, TIM3, BTLA and co-expression of PD1 and TIM3 on CD4+, CD8+ T cells, B cells, NK cells and NKT cells.

1.4.10 Optimization

For each antibody, a series of serial dilutions was performed to determine the optimal concentration for use in patient samples. These dilutions were subsequently analysed using flow cytometry to assess staining performance. To quantify the ability of each fluorochrome-conjugated antibody to discriminate between positive and negative cell populations, the stain index (SI) was employed. The stain index provides a robust measure of signal-to-noise ratio, allowing the identification of the antibody concentration that yields the clearest separation between specific signal and background fluorescence.

The stain index is calculated using the following formula:

$$\text{Stain Index (SI)} = \frac{\text{MFI (positive)} - \text{MFI (negative)}}{2 \times \text{SD (negative)}}$$

MFI (positive): Median fluorescence intensity of positive population

MFI (negative): Median fluorescence intensity of negative population

SD (negative): Standard deviation of the negative population

By calculating the SI for each dilution, the concentration that produced the highest stain index was selected as the optimal antibody concentration. This approach ensures maximum distinction between positive and negative populations, while minimizing nonspecific background staining, thereby improving the reliability of subsequent analyses of patient samples.

2.4.11 Statistical Analysis

Statistical analyses were conducted using STATA version 18.0 software. Data obtained from FlowJo v10.10.0 software, were expressed as percentage frequencies and subsequently transformed using the arcsine-square root transformation to meet the assumptions of parametric analysis, as detailed in Section 4.1.6 of Chapter 4. Statistical significance was defined as a p-value < 0.05. To evaluate changes across the three timepoints, linear mixed-effects models were applied, allowing for the consideration of both fixed and random effects in repeated measures data. For survival outcomes, Cox proportional hazards regression analysis was employed to assess the impact of relevant co-variables on patient survival. Detailed descriptions of the statistical procedures are provided in Section 4.1.6 of Chapter 4. The clinicopathological variables included in the analyses were age, gender, histological subtype, disease stage, surgical approach, and the presence

or absence of post-operative complications, enabling a comprehensive assessment of potential confounding factors and their influence on the observed outcomes. This study focused on characterizing immune cell populations and inhibitory checkpoint receptors. Analysis of co-stimulatory and cytokine receptors is planned as part of future investigations.

2.5 METHODS FOR INFLAMMATORY INDEXES

2.5.1 NLR Calculation

All immune cell population counts were extracted from EPR records of consented patients. NLR was calculated as follows:

$$\text{NLR} = \text{Neutrophil} / \text{Lymphocyte}$$

2.5.2 PLR Calculation

PLR was calculated as follows:

$$\text{PLR} = \text{Platelets} / \text{Lymphocyte}$$

2.5.3 LMR Calculation

LMR was calculated as follows:

$$\text{LMR} = \text{Lymphocyte} / \text{Monocyte}$$

2.5.4 SII Calculation

SII was calculated as follows:

$$\text{SII} = \frac{\text{Platelets} \times \text{Neutrophils}}{\text{Lymphocytes}}$$

2.4.5 SIRI Calculation

SIRI was calculated as follows:

$$\text{SIRI} = \frac{\text{Neutrophils} \times \text{Monocytes}}{\text{Lymphocytes}}$$

2.4.6 HALP Calculation

HALP was calculated as follows:

$$\text{HALP} = \frac{\text{Haemoglobin} \times \text{Albumin} \times \text{Lymphocytes}}{\text{Platelets}}$$

2.4.7 PNI Calculation

PNI was calculated as follows:

$$\text{PNI} = [10 \times \text{albumin}] + [0.005 \times \text{Lymphocyte count}]$$

2.4.8 Statistical Analysis

Receiver Operating Characteristic (ROC) curve analysis was employed to assess the suitability of the inflammatory index in predicting post-operative complications and prognosis [92]. The area under the curve (AUC) was employed as a threshold-independent metric to assess test performance, with values below 0.5 indicating unacceptable accuracy, values of 0.7 and above were considered acceptable, above 0.8 deemed good, and values exceeding 0.9 regarded as excellent [92]. The optimal cutoff values were identified using the Youden J Statistic (Youden Index (YI)) [92], which is calculated as the sum of the true positive rate (sensitivity) and the false negative rate ($1 - \text{specificity}$) [3]. Values close to 1 suggest the index is more effective at distinguishing between outcomes, whereas values near 0 indicate limited usefulness in clinical decision-making [92]. For more information refer to Section 3.1.7 of Chapter 3.

CHAPTER 3

RESULTS

Inflammatory Indexes

3.1 INTRODUCTION

An increase in neutrophils and platelets and a corresponding reduction in lymphocytes occur as a result of lung cancer [42]. Similar to other cancers, inflammatory indexes including NLR, PLR, LMR, SIRI and SII can be used to predict prognosis in NSCLC [28,49,52,57]. NLR, PLR and SII were also associated as markers for disease development [57]. Studies mostly incorporate patients with advanced-stage disease or those undergoing treatment; either chemoimmunotherapy or surgery [42,57]. Most of the research focuses on NLR, showing that elevated levels before surgery and immunotherapy are linked to poor survival [57]. Albumin, as a nutritional marker, may hold prognostic relevance in lung cancer patients, who often present with nutritional deficits due to comorbid COPD [57]. PNI (which incorporates albumin) has been investigated in patients with lung cancer, and lower PNI levels have been associated with poorer prognosis [57]. The HALP score is also associated with higher stage and worse outcomes in patients with NSCLC [57].

3.2 PATIENT CHARACTERISTICS

3.2.1 Patient Demographics

Patient clinicopathological characteristics are outlined in Table 3.1. Details of patient recruitment is discussed in Section 2.1.1. The patient cohort had a median age of 68.1 years, with a standard deviation (STD) of ± 17.1 years, indicating a relatively wide age distribution among the study population. Females comprised 63% (n=24) of the cohort, while 89% (n=34) of patients were identified as either current or former smokers, reflecting the well-established association between smoking and pulmonary disease. Pre-operative pulmonary function tests revealed a mean forced expiratory volume in one second (FEV1) of 93% predicted (STD ± 21.2), suggesting preserved ventilatory function in the majority of patients.

Table 3.1: Clinicopathological Characteristics for Inflammatory Indexes

Patient Characteristic	Total Cohort % (n=38)
Age (years +-STD)	68.1 $\pm$ 17.1
Gender	
Male	37 (14)
Female	63 (24)
Smoking History	89 (34)
FEV1	92.9 $\pm$ 21.2
Minimally Invasive Procedure	84 (32)
Pneumonectomy	4 (2)
Lobectomy	87 (33)
Bilobectomy	8 (3)
Size (cm +-STD)	3.3 $\pm$ 2.1
Stage	
I	56 (21)
II	22 (8)
III	22 (8)
Histology	
Adenocarcinoma	60 (23)
Squamous	40 (15)
Nodal Disease	26 (10)
Post-operative Complications	45 (17)
Post Operative Pneumonia	32 (12)

STD: Standard deviation

3.2.2 Surgical Approach

A total of 84% of patients (n = 32) underwent a minimally invasive surgical approach, utilizing either VATS or RATS, reflecting a preference for less invasive techniques in the management of this patient population. The majority of patients, 87% (n = 33), underwent lobectomy, which remains the gold standard surgical treatment for resectable NSCLC [6]. 4% of patients (n = 2) required a pneumonectomy due to the extent or location of disease, while 8% (n = 3) underwent a bi-lobectomy. These findings illustrate the predominance of lobectomy as the surgical procedure of choice, with more extensive resections reserved for select cases, where anatomical or oncological considerations necessitated a broader surgical intervention.

3.2.3 Histopathology

Histopathological analysis revealed that 40% of tumours were classified as squamous cell carcinoma, while the remaining 60% were identified as adenocarcinoma. This distribution

reflects the predominance of adenocarcinoma as the most common histological subtype of NSCLC, consistent with global epidemiological trends [2,5].

3.2.4 Staging

The median tumour size was 3.3 cm, (STD±2.1). Based on pathological staging (as per the 9th edition of the TNM staging for lung cancer), 56% of patients (n = 21) were found to have stage I disease, suggesting early detection in a majority of cases. 22% (n = 8) were diagnosed with either stage II or stage III disease. Lymph node metastasis was identified in 26% of patients (n = 10).

3.2.5 Post-operative Complications

Post-operative complications were observed in 45% (n=17) of patients. The most frequently encountered complication was POP, which affected 32% (n=12) of patients. Additional complications included cardiac arrhythmias, VTE, prolonged air leak (>5days), and wound infections. These findings underscore the critical need for reliable prognostic biomarkers to identify patients who are at the highest risk of experiencing adverse postoperative outcomes, thereby enabling tailored peri-operative management and improved clinical decision-making.

3.2.6 Survival statistics

Within the study cohort, three patients died from causes unrelated to NSCLC. Disease recurrence was observed in six patients (19%), including four cases with pulmonary nodules, one with lymph node metastasis, and one with vertebral metastasis. The median follow-up period was 14 months. There were three non-cancer-related deaths and two individuals lost to follow-up, therefore these patients were excluded from the disease-free survival calculations. Refer to Table 3.2 for details.

Table 3.2: Survival Statistics for Inflammatory Indexes

Patient Characteristic	Cohort % (n)
Recurrence	19 (6)
RIP	8 (3)
Median Follow up	14 months
Lost to follow up	2
Median Time to Recurrence	12.5 months

3.2.7 *Specific Analysis*

The false positive rate was plotted against the true positive rate to evaluate the diagnostic accuracy of the test, as outlined by standard ROC analysis methodology [92]. The diagonal line on the ROC plot represents the performance of a random classifier, indicating no discriminative power [92]. An ROC curve that lies above this line suggests that the test is capable of distinguishing between diseased and non-diseased individuals [92]. Diagnostic performance is considered strongest when the curve approaches the upper left corner of the plot, reflecting a high true positive rate and a low false positive rate [92]. AUC was utilised to measure test performance with values above 0.7 deemed acceptable.

Optimal cut-off points were determined using Youden's Index (YI), which identifies the threshold that maximizes the difference between the true positive rate and false positive rate—that is, the maximum vertical distance between the ROC curve and the line of no discrimination [92]. Conversely, when the curve lies on or below the diagonal, this indicates that the test performs at a level equivalent to or worse than random chance, rendering the results diagnostically uninformative or potentially misleading [92].

Analyses of post-operative complications, POP, and disease-free survival were conducted using inflammatory indexes; NLR, PLR, LMR, SII, SIRI, HALP and PNI. Both baseline and first follow-up values were evaluated for postoperative complications, whereas baseline values only were included in the survival analysis. While 1FU shows potential as a predictive timepoint for post-operative complications, subsequent immune recovery makes it unsuitable for evaluating DFS. Logistic regression analysis was performed to assess whether clinicopathological variables including age, gender, lung function, histological subtype, tumour stage and surgical approach were associated with inflammatory indexes, that met AUC and ROC criteria for cut-off determination. Surgical approach was defined based on, whether the patient underwent a minimally invasive or open procedure. Forced Expiratory Volume in 1 second (FEV1) is used in this study as a predictor of lung function. It measures the volume of air a person can forcibly exhale in the first second of a breath and serves as a key indicator of airway obstruction. Smoking history was not included as a co-variate in the analysis because the majority of patients (89%) reported a history of smoking, limiting its variability and utility as a predictor.

3.3 NEUTROPHIL-TO-LYMPHOCYTE RATIO

3.3.1 Baseline NLR to determine post-operative complications

Baseline NLR values were calculated for each patient within the study cohort. Clinical complications, as defined in Section 3.1.6, were systematically collated to assess their association with NLR values. In order to evaluate the diagnostic performance of baseline NLR in predicting complications, standard performance metrics were computed, including sensitivity, specificity, and false positive rates. These values were then used to construct a ROC curve.

The ROC curve (Figure 3.1) initially lies above the line of no discrimination, indicating some capacity of baseline NLR to differentiate between patients with and without post-operative complications at certain thresholds. However, the curve subsequently intersects and in parts runs below, the diagonal line. This pattern suggests inconsistent diagnostic performance across the range of threshold values. While there may be limited discriminative ability at specific cut-offs, the overall configuration of the ROC curve reflects a lack of reliable predictive value for baseline NLR in this context. The presence of segments below the random classifier line further indicates that, at some thresholds, the test performs worse than chance, potentially leading to misclassification. The AUC was 0.56. Taken together, these findings imply that baseline NLR is not a diagnostically informative marker for predicting complications in this patient population.

3.3.2 1FU NLR to determine post-operative complications

NLR values at the 1FU were calculated for each patient in the study cohort. Key diagnostic performance metrics for 1FU NLR, including sensitivity, specificity, and false positive rates, were computed and subsequently used to generate a ROC curve.

The ROC curve predominantly lies above the diagonal line of no discrimination, except at higher threshold values. This indicates that the test performs better than chance across most thresholds but exhibits reduced discriminative ability at higher cutoff points [85]. The area AUC was calculated to be 0.76, reflecting acceptable diagnostic accuracy.

With a Youden's Index of 0.53, an NLR cutoff value of 4.9 at first follow-up was determined to be the optimal threshold for predicting complications. This value provides the best balance between sensitivity and specificity, yielding a sensitivity of 76% and a

false positive rate of 24%. Overall, this cutoff demonstrates strong predictive performance, suggesting that an NLR at 1FU value of 4.9 is a clinically meaningful marker for identifying patients at higher risk of developing complications. Refer to Figure 3.1 for ROC curve.

3.3.3 Baseline NLR to determine POP

Baseline NLR values were calculated for each patient within the study cohort. The occurrence of POP was evaluated in relation to these NLR values to investigate potential associations. Sensitivity, specificity, and false positive rates were calculated, with the specificity and false positive rates subsequently used to construct the ROC curve (Figure 3.3).

Consistent with the baseline NLR-complications analysis, the ROC curve for predicting POP initially lies above the line of no discrimination but later intersects and falls below it. The calculated AUC was 0.54. The result of the ROC and AUC indicates that baseline NLR does not provide reliable predictive value for POP in this patient cohort.

3.3.4 1FU NLR to determine POP

NLR values at 1FU were calculated for each patient, and sensitivity, specificity, and false positive rates were used to generate the ROC curve (Figure 3.1). The resulting curve intersects the diagonal line at both higher and lower thresholds, suggesting that the test demonstrates limited discriminative capacity across the range of potential cut-off values. While predictive accuracy is compromised at the extremes, the test may retain diagnostic utility at selected thresholds. The AUC is 0.55, which is only marginally above the reference value of 0.50 that represents no discriminative ability. This suggests that the test performs only slightly better than chance in distinguishing between patients with POP and those without, highlighting its limited overall diagnostic performance.

3.3.5 Baseline NLR as a prognostic indicator

The baseline NLR showed limited prognostic value for disease-free survival, with an AUC of 0.49. The ROC curve runs along the diagonal line which reflects a lack of discriminative power. Therefore, NLR measured at baseline is not a reliable predictor of disease-free survival in this analysis. Refer to Figure 3.1 for ROC curve.

3.3.6 Review of acceptable cut-offs with clinicopathological characteristics

Regarding post-operative complications, NLR at the first follow-up with a cut-off of 4.9 was deemed suitable based on AUC and ROC curve analysis. Overall, 47% of patients had an NLR above this threshold at 1FU. Regression analysis including clinical co-variates, was used to assess post-operative complications for NLR above 4.9 at 1FU. NLR was significantly associated with higher odds of complications (coefficient = 3.32, $p = 0.016$), indicating a strong independent effect. Stage, histology, minimally invasive surgery, gender, age and lung function did not influence regression model. Overall, elevated NLR above 4.9 at 1FU emerged as an important predictor of post-operative complications. Refer to Table 3.3 for details.

Table 3.3: Multivariate logistic regression analysis for NLR at 1FU

Dependent Variable	Coefficient (β)	Std Err (SE)	p value	95% Confidence Interval
NLR above 4.9	3.32	1.39	0.016	0.62-6.01
Gender	-0.65	1.24	0.602	-3.08-1.79
Surgical approach	-2.16	1.78	0.225	-5.65-1.33
Stage 1	6.13	3.35	0.067	0.22-10.1
Stage 2	5.16	12.52	0.041	0.22-10.1
Stage 3	-6.14	3.35	0.067	-12.7-0.44
Histology	-1.02	1.16	0.377	-3.31-1.25
Age	0.012	0.009	0.209	0.009-0.032
FEV1	-0.005	0.003	0.130	-0.012-0.002

3.3.7 Summary

Baseline NLR showed no predictive value for post-operative complications, POP and DFS (AUC 0.49-0.56), with ROC curves indicating poor discriminative ability. In contrast, NLR measured at 1FU demonstrated better diagnostic performance, with an optimal cut-off of 4.9, determined by Youden's Index (sensitivity 76%, false positive rate 24%, AUC 0.76). Regression analysis revealed that NLR >4.9 at 1FU was significantly associated with higher odds of post-operative complications (coefficient = 3.32, $p = 0.016$). Overall, elevated 1FU NLR, emerged as key predictor of post-operative complications in this cohort, with Stage 2 disease showing statistical significance

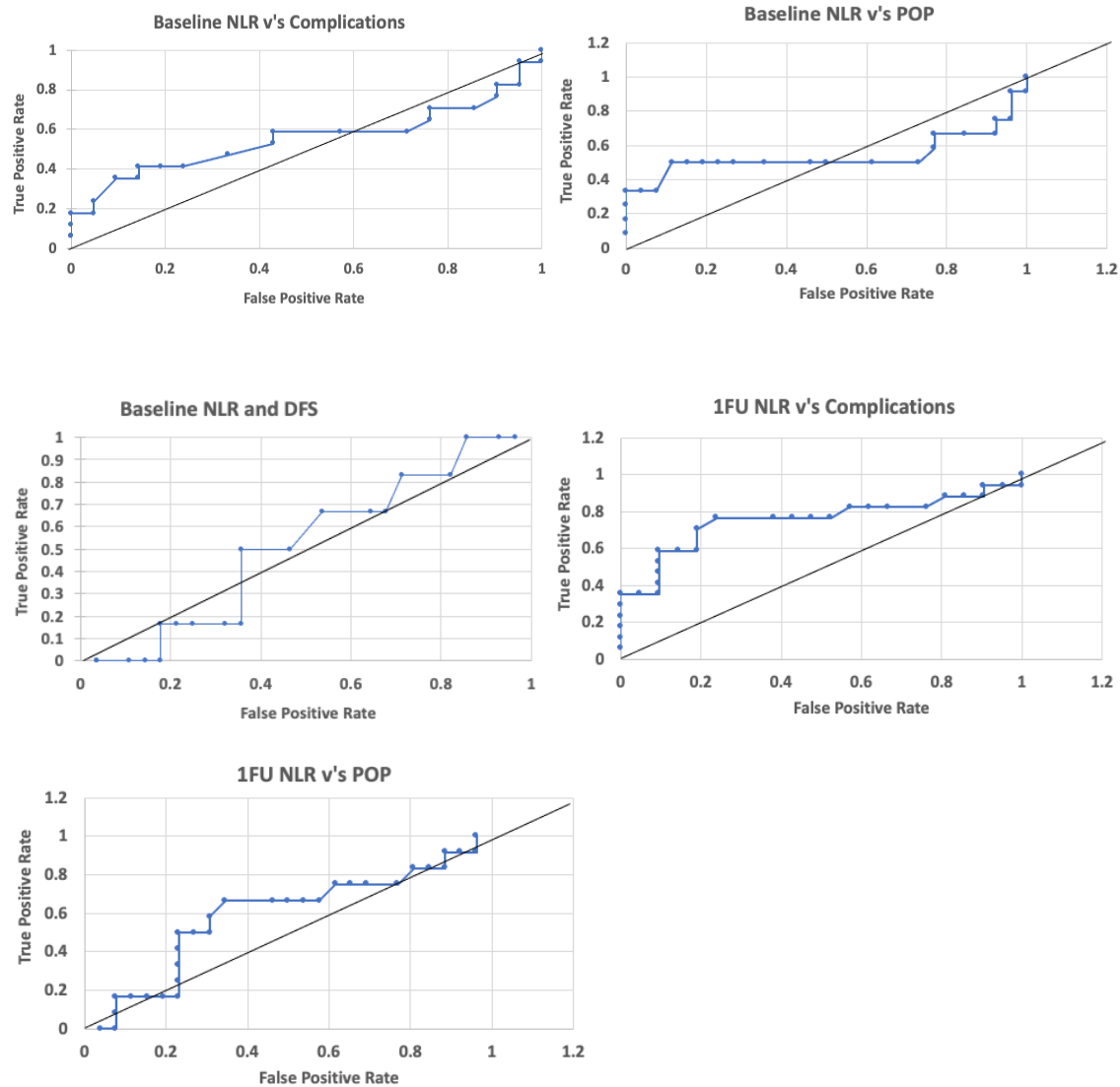


Figure 3.1 ROC analysis of NLR at baseline and 1FU for post-operative complications, post-operative pneumonia and disease-free survival. The false positive rate was plotted against the true positive rate to perform a ROC plot. The diagonal line on the ROC plot represents the performance of a random classifier, indicating no discriminative power. A ROC curve that lies above this line suggests that the test is capable of distinguishing between diseased and non-diseased individuals, with values above 0.7 deemed acceptable. If AUC was above 0.7, optimal cut-off points were determined using Youden's Index. Baseline NLR was not predictive of post-operative complications (AUC=0.56), POP (AUC=0.54) or DFS (AUC=0.49). NLR at 1FU was predictive of post-operative complications (AUC=0.76, YI = 0.53, optimal cutoff 4.9) but not POP (AUC=0.54).

3.4 PLATELET-TO-LYMPHOCYTE RATIO

3.4.1 Baseline PLR to determine post-operative complications

Baseline PLR values were calculated for each patient, with sensitivity, specificity, and false positive rates determined; sensitivity and false positive rates were then used to generate the ROC curve shown in Figure 3.2. The ROC curve for PLR in predicting post-operative complications initially lies above the diagonal but subsequently intersects and falls below it at higher thresholds. The AUC was 0.56. This pattern suggests that baseline PLR is not a reliable predictor of complications in this patient cohort.

3.4.2 1FU PLR to determine post-operative complications

PLR values at the 1FU were calculated. Key diagnostic performance metrics for 1FU PLR, including sensitivity, specificity, and false positive rates, were computed and subsequently plotted on a ROC curve in Figure 3.2. The ROC curve for PLR at 1FU lies above the diagonal line of no discrimination. The calculated AUC was 0.76, which reflects acceptable diagnostic accuracy according to established benchmarks [85]. With a Youden's Index of 0.41, an optimal PLR cut-off value of 190 at the first follow-up, was identified. At this threshold, the test demonstrated a sensitivity of 65% and a false positive rate of 24%, suggesting that PLR at 1FU may serve as a moderately effective predictor of complications in this patient population.

3.4.3 Baseline PLR to determine POP

An ROC curve (Figure 3.2) was generated by plotting the true positive rate against the false positive rate. The curve largely follows the diagonal line of no discrimination, intersects it, and subsequently falls below. The calculated AUC was 0.46, which is below the threshold of 0.50 and reflects performance worse than chance. This finding indicates that baseline PLR has no meaningful discriminatory capacity and is not a reliable predictor of POP in this patient population.

3.4.4 1FU PLR to determine POP

An ROC curve was generated from true positive and false positive rates at the first follow-up. The curve predominantly follows the diagonal line of no discrimination, intersecting and subsequently falling below it. The calculated AUC was 0.56, indicating that PLR at 1FU has limited discriminatory capacity and is not a reliable predictor of POP. See Figure 3.2 for ROC curve.

3.4.5 Baseline PLR as a prognostic indicator

Baseline PLR demonstrated poor prognostic ability for DFS, with an area under the ROC curve of 0.38. Additionally, the ROC curve lying largely below the diagonal line further confirms the poor predictive performance, implying that baseline PLR values are not associated with disease-free survival in this context and may even trend oppositely. Overall, baseline PLR does not serve as a reliable prognostic marker for DFS. See Figure 3.2 for ROC analysis.

3.4.6 Review of acceptable cut-offs with clinicopathological characteristics.

Multivariate regression analysis was performed to identify predictors of post-operative complications using PLR >190 at 1st follow up. It was associated with higher odds of complications (coefficient = 1.77, $p = 0.061$), indicating a trend toward increased risk, though not statistically significant. Clinical covariates were not significant. 39% of patients had an elevated PLR at 1FU. Refer to Table 3.4 for details.

Table 3.4: Logistic Regression Analysis for PLR 1FU to predict post-operative complications

Dependent Variable	Coefficient (β)	Std Err (SE)	p value	95% Confidence Interval
PLR above 190	1.77	0.94	0.061	-0.08-3.62
Gender	-0.91	0.92	0.323	-2.72-0.89
Surgical approach	-1.15	1.13	0.309	-3.36-1.07
Stage 1	3.6	1.94	0.062	-1.18-7.43
Stage 2	2.93	1.63	0.073	0.28-6.13
Stage 3	0.73	0.61	0.079	0.14-3.79
Histology	-0.92	0.92	0.314	-2.72 – 0.87
Age	0.019	0.011	0.070	-0.01-0.04
FEV1	-0.006	0.004	0.095	-0.012-0.004

3.4.7 Summary

Baseline PLR showed poor predictive performance for post-operative complications, POP and disease-free survival, with ROC AUCs of 0.38-0.56. At the first follow-up, PLR demonstrated moderate discriminatory ability for complications, with an AUC of 0.76, an optimal cut-off of 190, sensitivity of 65%, and a false positive rate of 24%. Multivariate analysis indicated a trend toward higher complication risk with PLR >190 at 1FU. Overall, baseline PLR is unreliable, while 1FU PLR may serve as a moderately effective predictor of post-operative complications.

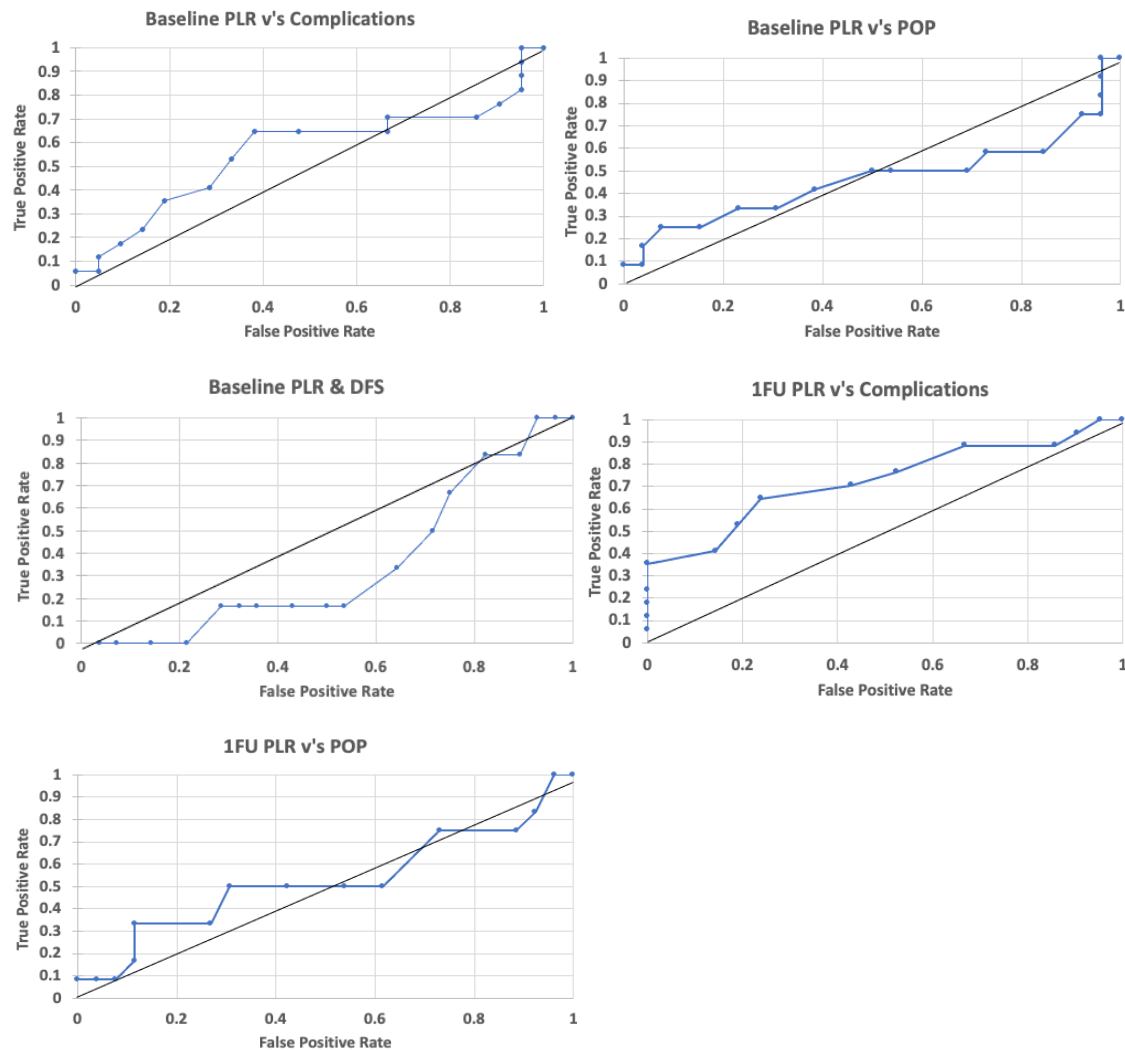


Figure 3.2. ROC analysis of PLR at baseline and 1FU for post-operative complications, post-operative pneumonia and disease-free survival. The false positive rate was plotted against the true positive rate to perform a ROC plot. The diagonal line on the ROC plot represents the performance of a random classifier, indicating no discriminative power. A ROC curve that lies above this line suggests that the test is capable of distinguishing between diseased and non-diseased individuals, with values above 0.7 deemed acceptable. If AUC was above 0.7, optimal cut-off points were determined using Youden's Index. Baseline PLR was not predictive of post-operative complications (AUC=0.56), POP (AUC=0.46) or DFS (AUC=0.38). PLR at 1FU was predictive of post-operative complications (AUC=0.76, YI = 0.53, optimal cutoff 190) but not POP (AUC=0.56).

3.5 LYMPHOCYTE-TO-MONOCYTE RATIO

3.5.1 Baseline LMR to determine post-operative complications

Baseline LMR values were calculated, and clinical complications were recorded to evaluate their association with LMR. Sensitivity, specificity, and false positive rates were derived and applied to generate an ROC curve. The ROC curve, seen in Figure 3.3, initially lies above the line of no discrimination but subsequently intersects, runs along, and in parts falls below the diagonal line representing random classification. The calculated AUC was 0.36, indicating poor discriminatory capacity of baseline LMR for predicting complications.

3.5.2 1FU LMR to determine post-operative complications

LMR values at the first follow-up were calculated, and their association with clinical complications was assessed. Sensitivity, specificity, and false positive rates were determined and used to generate an ROC curve, seen in Figure 3.3. The curve lies slightly above the line of no discrimination at the beginning but predominantly falls below it thereafter. The AUC was 0.39, indicating that 1FU LMR is not a reliable predictor of complications in this cohort.

3.5.3 Baseline LMR to determine POP

Baseline LMR values were calculated, and their association with POP was evaluated. Sensitivity, specificity, and false positive rates were determined and applied to generate an ROC curve. The curve lies just above the line of no discrimination initially, but soon intersects and runs below it at higher values. The AUC was 0.51, which is effectively equivalent to random classification, indicating that baseline LMR does not provide meaningful predictive value for POP. Refer to Figure 3.13 for ROC analysis.

3.5.4 1FU LMR to determine POP

LMR values at the first follow-up were calculated, and their association with POP was assessed. Sensitivity, specificity, and false positive rates were determined and used to generate an ROC curve, seen in Figure 3.3. The ROC curve for 1FU LMR runs along the line of no discrimination across all values, with an AUC of 0.39. This indicates that 1FU LMR has no predictive value for POP in this cohort.

3.5.5 Baseline LMR as a prognostic indicator

ROC analysis for baseline LMR in relation to disease-free survival yielded an area AUC of 0.43. The ROC curve running below the diagonal line, intersecting it, and then continuing along the diagonal further reflects inconsistent predictive performance—showing that LMR values at baseline do not effectively distinguish between patients who remained disease-free and those who experienced recurrence. Refer to Figure 3.3 for ROC curve.

3.5.6 Summary

Baseline and 1st follow-up LMR values could not distinguish between patients who developed post-operative complications or POP and those who did not in our cohort. Furthermore, LMR demonstrated limited prognostic value for disease-free survival. This suggests that, within our study population, LMR may not be a reliable biomarker for either short-term post-surgical outcomes or long-term oncological prognosis.

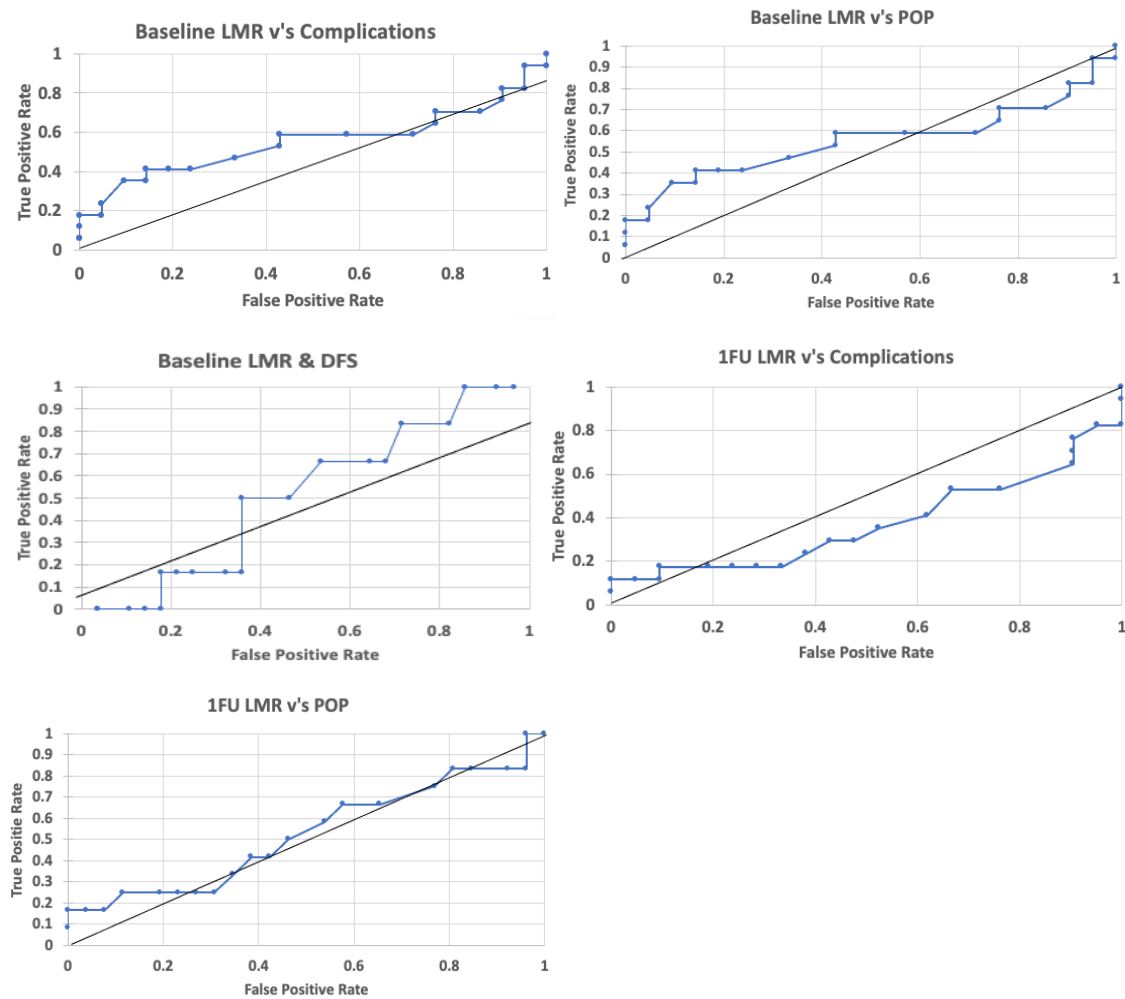


Figure 3.3. ROC analysis of LMR at baseline and 1FU for post-operative complications, post-operative pneumonia and disease-free survival. The false positive rate was plotted against the true positive rate to perform a ROC plot. The diagonal line on the ROC plot represents the performance of a random classifier, indicating no discriminative power. A ROC curve that lies above this line suggests that the test is capable of distinguishing between diseased and non-diseased individuals, with values above 0.7 deemed acceptable. If AUC was above 0.7, optimal cut-off points were determined using Youden's Index. Baseline LMR was not predictive of post-operative complications (AUC=0.36), POP (AUC=0.51) or DFS (AUC=0.43). LMR at 1FU was not predictive of post-operative complications (AUC=0.39) or POP (AUC=0.39).

3.6 SYSTEMIC IMMUNE INFLAMMATORY INDEX

3.6.1 Baseline SII to determine post-operative complications

SII values at baseline were calculated using neutrophil, platelet, and lymphocyte counts, and their association with post-operative complications was evaluated. Sensitivity, specificity, and false positive rates were determined and applied to generate an ROC curve, seen in Figure 3.4. The curve initially lies above the line of no discrimination, but subsequently intersects and runs predominantly below it. The AUC was 0.51, indicating no meaningful predictive value for post-operative complications.

3.6.2 1FU SII to determine post-operative complications

At the first follow-up, SII values were evaluated for their ability to predict post-operative complications using ROC curve analysis. The AUC was 0.74, indicating acceptable discriminatory capacity. Based on Youden's Index of 0.43, an optimal cut-off value of 1050 was identified. At this threshold, the test demonstrated a sensitivity of 76% and a false positive rate of 36%, suggesting that SII at 1FU may serve as a moderately effective predictor of post-operative complications. Refer to Figure 3.4 for ROC curve.

3.6.3 Baseline SII to determine POP

At baseline, SII values were assessed for their association with POP using ROC curve analysis, see Figure 3.18. The resulting curve intersects the line of no discrimination approximately midway and then runs predominantly below it. An AUC of 0.55, shows only slightly above chance, indicating poor overall discriminatory ability. This suggests that baseline SII has minimal predictive value for POP in our patient cohort.

3.6.4 1FU SII to determine POP

At the first follow-up, SII values were analysed for their predictive ability regarding POP using ROC curve analysis, seen in Figure 3.4. The curve runs largely along the diagonal line, intersects it at higher threshold values, and subsequently falls below. An AUC of 0.44 is below the reference value of 0.50, indicating performance worse than chance. This finding demonstrates that SII at 1FU lacks discriminatory capacity and is not a reliable predictor of POP in this cohort.

3.6.5 Baseline SII as a prognostic indicator

ROC analysis for baseline SII as a prognostic indicator of DFS yielded an AUC of 0.56. An AUC of 0.56 indicates weak discriminative power. The ROC curve, shown in Figure 3.4, runs below the diagonal line, intersects it at three points, and then continues along the line, suggesting inconsistent predictive performance across different threshold values. Overall, baseline SII shows limited ability to predict disease-free survival.

3.6.6 Review of acceptable cutoffs with clinicopathological characteristics

At the first follow-up, an SII value above 1050 was identified as a potential predictor of post-operative complications within our cohort. Overall, 47% of patients demonstrated an elevated SII at this threshold. Multivariate regression analysis revealed that an elevated SII (>1050) was significantly associated with the occurrence of post-operative complications (coefficient = 2.39, $p = 0.025$). Other examined variables did not demonstrate statistically significant associations with post-operative outcomes. Refer to Table 3.5 for details.

Table 3.5: Multivariate logistic regression analysis for SII at 1FU

Dependent Variable	Coefficient (β)	Std Err (SE)	p value	95% Confidence Interval
SII above 1050	2.39	1.06	0.025	0.31-4.48
Gender	-0.13	1.13	0.903	-2.35-2.08
Surgical approach	-1.52	1.45	0.290	-4.22-1.29
Stage 1	4.37	2.56	0.067	-0.32-9.73
Stage 2	3.24	1.85	0.079	-0.38-6.86
Stage 3	0.52	0.47	0.470	0.09-3.02
Histology	-1.12	1.02	0.275	-3.13-0.89
Age	0.013	0.012	0.266	-0.011-0.039
FEV1	-0.004	0.004	0.306	-0.013-0.004

3.6.7 Summary

Baseline SII showed no predictive value for post-operative complications, POP, or disease-free survival (AUCs 0.51–0.56). In contrast, SII at first follow-up demonstrated moderate discrimination (AUC = 0.74) with an optimal cut-off of 1050. Multivariate analysis

confirmed elevated SII (>1050) and nodal disease as independent predictors of complications ($p = 0.025$ and $p = 0.022$, respectively). Overall, only SII at first follow-up showed predictive potential in this cohort.

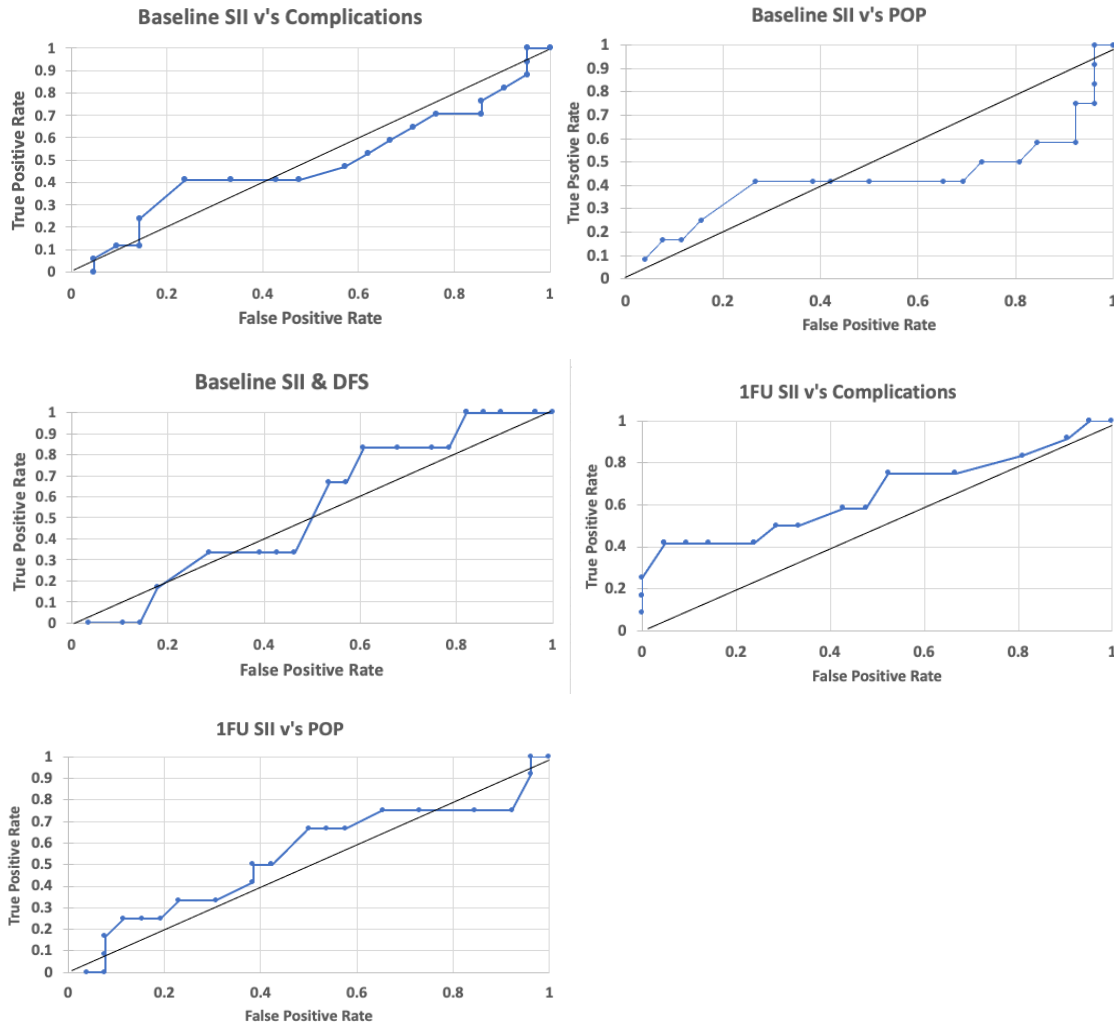


Figure 3.4. ROC analysis of SII at baseline and 1FU for post-operative complications, post-operative pneumonia and disease-free survival. The false positive rate was plotted against the true positive rate to perform a ROC plot. The diagonal line on the ROC plot represents the performance of a random classifier, indicating no discriminative power. A ROC curve that lies above this line suggests that the test is capable of distinguishing between diseased and non-diseased individuals, with values above 0.7 deemed acceptable. If AUC was above 0.7, optimal cut-off points were determined using Youden's Index. Baseline SII was not predictive of post-operative complications (AUC=0.51), POP (AUC=0.55) or DFS (AUC=0.56). SII at 1FU was predictive of post-operative complications (AUC=0.74, YI = 0.43, optimal cutoff 1050) but not POP (AUC=0.44).

3.7 SYSTEMIC INFLAMMATORY RESPONSE INDEX

3.7.1 Baseline SIRI to determine post-operative complications

Baseline SIRI values, calculated as the ratio of neutrophils and monocytes to lymphocytes, were determined for all patients. ROC curve analysis, as seen in Figure 3.5, was then performed to evaluate their predictive capacity for post-operative complications. The resulting curve intersected the line of no discrimination at approximately the midway point, ran alongside it, and fell below the line at higher threshold values. An AUC of 0.57 reflects performance only slightly above chance, indicating that baseline SIRI has limited discriminatory power for predicting complications.

3.7.2 1FU SIRI to determine post-operative complications

ROC curve analysis was conducted for SIRI values at the first follow-up to assess their predictive performance for post-operative complications. Refer to Figure 3.5. The curve remained predominantly above the line of no discrimination, intersecting it only at higher threshold values. AUC was 0.66, reflecting modest discriminatory ability. A cut-off value of 4 demonstrates a sensitivity of 65% and false positive rate of 29%. The calculated Youden's Index of 0.36 reflects a moderate balance between sensitivity and specificity, suggesting that this cut-off provides a reasonable trade-off between correctly detecting true cases and minimizing false alarms and could be a potential index for determining post-operative complications.

3.7.3 Baseline SIRI to determine POP

ROC analysis was performed to evaluate the predictive ability of baseline SIRI for the development of POP. The area under the curve (AUC) was 0.53, indicating that the discriminatory performance of baseline SIRI was close to chance level. The ROC curve largely runs along the diagonal reference line and intersects it at multiple points, further confirming the lack of discriminatory power. Refer to Figure 3.5 for ROC curve.

3.7.4 1FU SIRI to determine POP

ROC analysis was conducted to determine the predictive value of SIRI measured at first follow up for POP. The ROC curve ran above the reference line however, the calculated AUC was 0.46. This indicates that the discriminatory performance of 1FU SIRI was below chance level, and therefore it has no meaningful predictive utility for POP for these patients. See Figure 3.5 for ROC analysis.

3.7.5 Baseline SIRI as a prognostic indicator

ROC analysis demonstrated an AUC of 0.70, indicating moderate discriminative ability of the test in distinguishing between outcome groups. At threshold of 2.4, the model achieved a sensitivity of 67%, and a false positive rate of 46%. The corresponding Youden's Index of 0.21 reflects a relatively modest overall diagnostic performance, suggesting that its ability to effectively balance sensitivity and specificity remains limited. Refer to Figure 3.5 for ROC analysis.

3.7.6 Review of acceptable cut-offs with clinicopathological characteristics

3.7.1.1: Regression analysis for SIRI to predict post-operative complications

SIRI of >4 at 1FU has the potential to predict post-operative complications in our cohort. 42% of patients had a cutoff above the threshold. Regression analysis identified that SIRI >4 at first follow-up, Stage 1 and 3 disease were significant ($p = 0.032$, 0.018 , and 0.042 , respectively). Refer to Table 3.6 for details.

Table 3.6: Multivariate Logistic Regression Analysis for SIRI at 1FU

Dependent Variable	Coefficient (β)	Std Err (SE)	p value	95% Confidence Interval
SIRI above 4	1.79	0.84	0.032	0.16-3.43
Gender	-0.89	0.88	0.308	-0.26-0.82
Surgical approach	-0.99	1.15	0.389	-3.26-1.26
Stage 1	5.19	2.55	0.042	0.18-10.2
Stage 2	3.39	1.92	0.078	-0.37-7.15
Stage 3	-5.19	2.55	0.042	-10.2-0.18
Histology	-0.95	00.99	0.338	-2.89-0.99
Age	0.16	0.01	0.147	-0.006-0.034
FEV1	-0.006	0.003	0.098	-0.013-0.01

3.7.1.2: Regression analysis for SIRI to predict DFS

Pre-operative SIRI >2.4 was observed in 44% of patients and could be used to predict disease-free survival (DFS) with an AUC of 0.7 and Youden's index of 0.21 on ROC analysis. Regression analyses at first follow-up showed no significant associations. SIRI

did not significantly predict DFS (all $p > 0.19$), indicating limited prognostic value in this cohort. Refer to Table 3.7.

Table 3.7: Logistic Regression for SIRI to predict DFS

Dependent Variable	Coefficient (β)	Std Err (SE)	p value	95% Confidence Interval
SIRI above 2.4	1.01	0.88	0.250	-0.71-2.72
Gender	-0.85	1.41	0.547	-3.60-1.91
Stage 1	-2.42	2.23	0.276	-6.80-1.95
Stage 2	1.41	1.68	0.402	-1.89-4.71
Stage 3	0.73	0.61	0.709	0.14-3.79
Histology	2.10	1.39	0.194	-1.06 – 5.27
Age	0.01	0.01	0.885	-0.02-0.02
FEV1	-0.009	0.003	0.780	-0.007-0.005

3.7.7 Summary

Baseline SIRI showed limited predictive ability for post-operative complications and POP, with AUCs near chance levels (0.53,0.57). At first follow-up, SIRI demonstrated modest discrimination for complications (AUC 0.66, sensitivity 65%, false positive rate 29%, cut-off 4) but poor performance for POP (AUC 0.46). Regression analysis indicated that SIRI >4 at first follow-up, significantly associated with higher odds of complications, Stage 1 and 3 was also significant. Pre-operative SIRI >2.4 showed moderate predictive value for DFS (AUC 0.70, Youden's index 0.21), but did not significantly predict DFS in regression analysis. Overall, SIRI has limited prognostic utility but may offer moderate predictive value for complications at first follow-up.

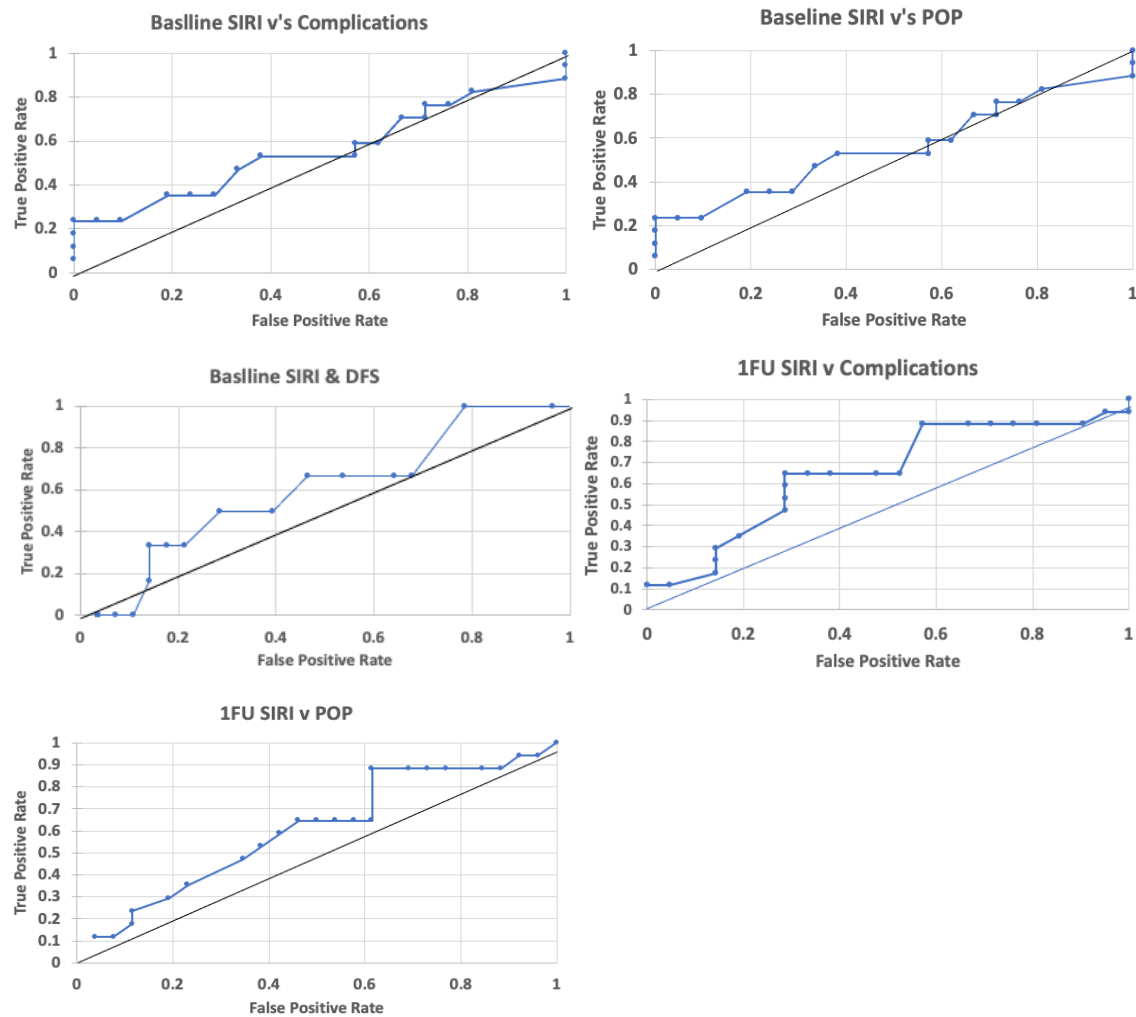


Figure 3.5. ROC analysis of SIRI at baseline and 1FU for post-operative complications, post-operative pneumonia and disease-free survival. The false positive rate was plotted against the true positive rate to perform a ROC plot. The diagonal line on the ROC plot represents the performance of a random classifier, indicating no discriminative power. A ROC curve that lies above this line suggests that the test is capable of distinguishing between diseased and non-diseased individuals, with values above 0.7 deemed acceptable. If AUC was above 0.7, optimal cut-off points were determined using Youden's Index. Baseline SIRI was not predictive of post-operative complications ($AUC=0.57$) and POP ($AUC=0.53$), however is potentially predictive of DFS ($AUC=0.70$, $YI = 0.21$, optimal cutoff 2.4). SIRI at 1FU was predictive of post-operative complications ($AUC=0.7$, $YI = 0.36$, optimal cutoff 4) but not POP ($AUC=0.46$) in this cohort.

3.8 HAEMOGLOBIN ALBUMIN LYMPHOCYTE PLATELET SCORE

3.8.1 Baseline HALP to determine complications

ROC analysis was performed to assess the predictive ability of the HALP index measured at baseline for post-operative complications. Haemoglobin, albumin, lymphocyte, and platelet counts were obtained for each patient to calculate the HALP score. The ROC curve largely followed and remained below the diagonal reference line, with an area AUC of 0.40. This result indicates that baseline HALP demonstrated poor discriminatory performance, falling below chance level and lacking meaningful predictive value for postoperative complications. Refer to Figure 3.6 for details.

3.8.2 1FU HALP to determine post-operative complications

ROC analysis was performed to assess the predictive ability of the HALP index measured at first follow up for post-operative complications. The ROC curve, seen in Figure 3.6, remained below the diagonal reference line, with an area AUC of 0.41. 1FU HALP provides no useful prediction of post-operative complications in our cohort.

3.8.3 Baseline HALP to determine POP

ROC analysis was performed to evaluate the predictive performance of the HALP index measured at first follow-up for POP. Refer to Figure 3.28. The ROC curve largely followed the diagonal reference line, yielding an AUC of 0.56. This suggests that the HALP index at first follow-up demonstrated only minimal discriminatory ability, performing close to chance level and providing limited predictive value for POP.

3.8.4 1FU HALP to determine POP

ROC analysis was conducted to assess the predictive ability of the HALP index measured at first follow-up for POP. The ROC curve remained predominantly below the line of discrimination with an AUC of 0.44. The findings indicate that baseline HALP lacked effective discriminatory power, performing worse than chance and failing to predict POP. Refer to Figure 3.6

3.8.5 Baseline HALP as a prognostic indicator

ROC analysis for baseline HALP in relation to DFS demonstrated an AUC of 0.70, indicating moderate predictive accuracy. At a cut-off value of 54, the model achieved a sensitivity of 67% and a false positive rate of 15%, resulting in a Youden's Index of 0.52.

In comparison, a lower cut-off of 35 yielded a higher sensitivity of 82% but also a substantially higher false positive rate of 43%, with a Youden's Index of 0.39. Overall, the cut-off of 54 is the more optimal threshold, demonstrating a stronger balance between sensitivity and specificity and reflecting better discriminatory performance for predicting disease-free survival based on baseline HALP. Refer to Figure 3.6 for ROC analysis.

3.8.6 Review of acceptable cut-offs with clinicopathological characteristics

Pre-operative HALP, using a threshold of 5.4, can be used as a prognostic marker to predict recurrence in this patient cohort, with 29% of patients demonstrating a baseline HALP above this cut-off. Regression analysis indicated that patients with HALP >54 had a significantly higher likelihood of improved disease-free survival (coefficient = 1.83, $p = 0.043$). Clinical and tumour characteristics were not significant. Refer to Table 3.8 for details.

Table 3.8: Logistic Regression for HALP to predict DFS

Dependent Variable	Coefficient (β)	Std Err (SE)	p value	95% Confidence Interval
HALP above 5.4	1.83	0.91	0.043	0.05-3.6
Gender	-0.86	1.40	0.539	-3.60-1.88
Stage 1	-3.04	2.24	0.175	-7.43-1.35
Stage 2	0.43	2.25	0.850	-3.97-4.83
Stage 3	3.03	2.24	0.175	-1.35-7.43
Histology	1.69	1.58	0.283	-1.39-4.80
Age	0.01	0.01	0.158	-0.01-0.03
FEV1	-0.01	0.01	0.481	-0.01-0.01

3.8.7 Summary

Baseline and HALP at 1FU are not suitable indexes to determine post-operative complications or POP in our cohort. Pre-operative HALP >54 was associated with improved disease-free survival, with 29% of patients above this threshold. Multivariate logistic regression showed HALP was a significant predictor of DFS (coefficient = 1.83, $p = 0.043$). These results suggest that higher pre-operative HALP may serve as an independent, cost-effective biomarker for lower recurrence risk.

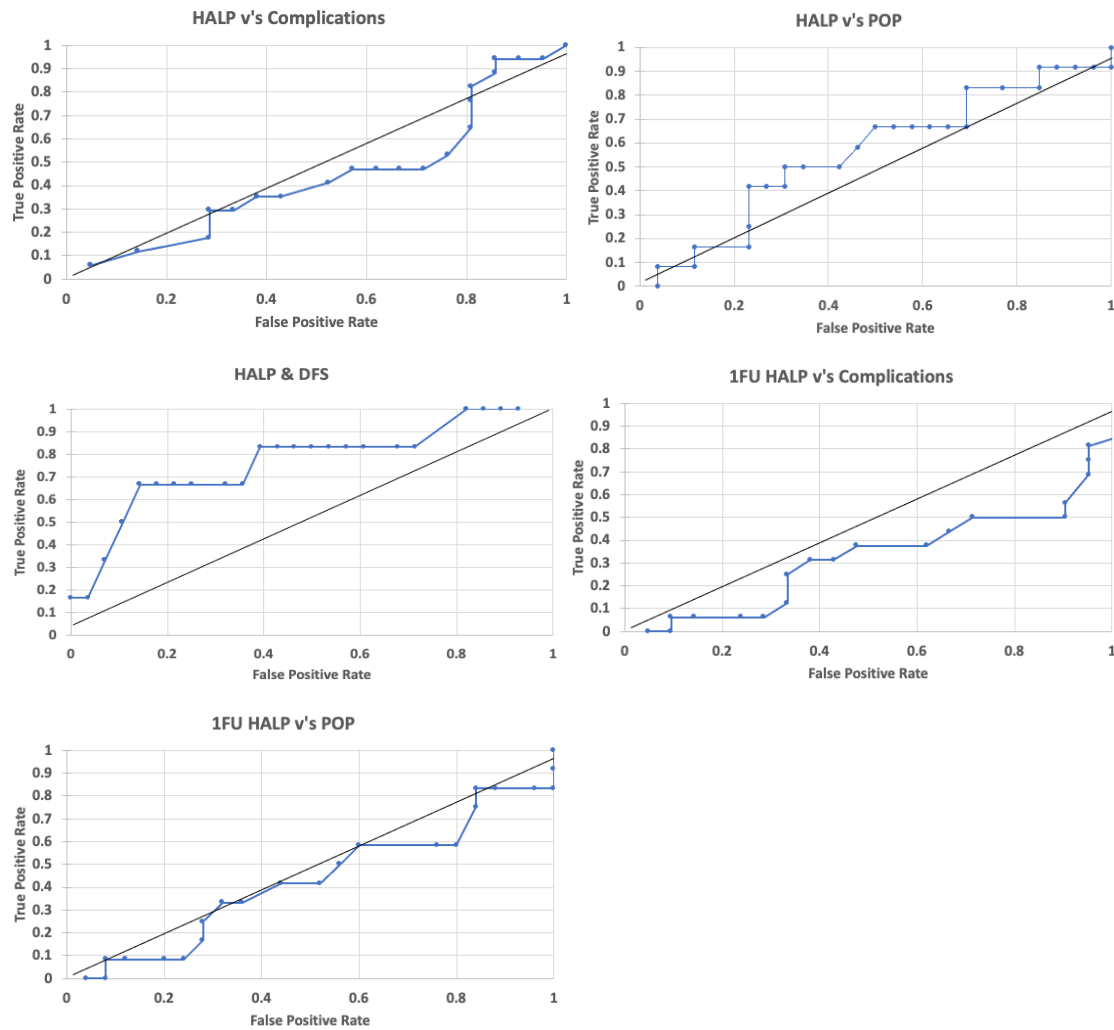


Figure 3.6. ROC analysis of HALP at baseline and 1FU for post-operative complications, post-operative pneumonia and disease-free survival. The false positive rate was plotted against the true positive rate to perform a ROC plot. The diagonal line on the ROC plot represents the performance of a random classifier, indicating no discriminative power. A ROC curve that lies above this line suggests that the test is capable of distinguishing between diseased and non-diseased individuals, with values above 0.7 deemed acceptable. If AUC was above 0.7, optimal cut-off points were determined using Youden's Index. Baseline HALP was not predictive of post-operative complications ($AUC=0.40$) and POP ($AUC=0.56$), however is potentially predictive of DFS ($AUC=0.70$, $YI = 0.52$, optimal cutoff 54). HALP at 1FU was not predictive of post-operative complications ($AUC=0.41$) or POP ($AUC=0.44$) in this cohort.

3.9 PROGNOSTIC NUTRITIONAL INDEX

3.9.1 Baseline PNI to determine post-operative complications

ROC analysis was conducted to examine the relationship between PNI measured at baseline and post-operative complications. PNI, derived from serum albumin concentration and lymphocyte count, was calculated for each patient. The ROC plot closely followed the diagonal reference line and predominantly ran underneath. The AUC of 0.36. These findings indicates that baseline PNI showed little distinction between patients with and without postoperative complications, suggesting limited clinical usefulness in this context. Refer to Figure 3.7 for details.

3.9.2 1FU PNI to determine post-operative complications

ROC analysis was performed to explore the association between the PNI measured at first follow-up and post-operative complications. The ROC curve lay predominantly below the diagonal reference line, with an AUC of 0.29. This outcome indicates that PNI at first follow-up offered minimal differentiation between patients who did and did not experience post-operative complications, highlighting its limited clinical relevance in this setting. Refer to Figure 3.7.

3.9.3 Baseline PNI to determine POP

ROC analysis was performed to assess baseline PNI in relation to POP. The ROC curve (seen in Figure 3.7) initially rose above the diagonal reference line, intersected it, and then fell below before crossing again at higher threshold values. The AUC was 0.54, indicating minimal separation between patients with and without POP and therefore limited ability for PNI to distinguish them.

3.9.4 1FU PNI to determine POP

ROC analysis was conducted to evaluate baseline PNI in relation to POP. The curve travelled largely below the diagonal reference line, with an area under the curve of 0.38. This pattern reflects limited discriminatory ability and minimal distinction between patients with and without POP. Refer to Figure 3.7 for ROC curve

3.9.5 Baseline PNI as a prognostic indicator

ROC analysis for baseline PNI in relation to DFS showed an AUC of 0.72, suggesting good discriminatory ability. See Figure 3.7. To identify optimal threshold, two cutoffs were

considered. Cutoff 56 had a sensitivity of 67%, a false positive rate of 18% and Youden's Index of 0.49. At a cutoff of 37, the sensitivity increased to 83%, but the false positive rate also rose to 61%, Youden's Index was 0.22. A higher Youden's Index indicates a more effective cutoff with a better trade-off between true positive and false positive results. Therefore, a cutoff value of 56 is the statistically superior choice, demonstrating better overall test performance for predicting disease-free survival based on baseline PNI.

3.9.6 Review of acceptable cutoffs with clinicopathological characteristics

Pre-operative PNI, using a cutoff of 56, may be used to predict recurrence in this patient cohort, with 27% of patients exceeding this threshold. Regression analysis demonstrated that PNI <56 was significantly associated with improved disease-free survival (coefficient = 2.04, $p = 0.027$). These findings indicate that PNI could serve as an independent prognostic marker for DFS in this population. Refer to Table 3.9 for details.

Table 3.9: Logistic Regression for PNI to predict DFS

Dependent Variable	Coefficient (β)	Std Err (SE)	p value	95% Confidence Interval
HALP above 5.4	2.04	0.92	0.027	0.24-3.82
Gender	-1.04	1.41	0.462	-3.80-1.72
Stage 1	-3.02	2.16	0.163	-7.25-1.22
Stage 2	0.43	2.19	0.982	-4.35-4.25
Stage 3	3.02	2.16	0.163	-1.22-7.26
Histology	1.69	1.58	0.283	-1.39-4.80
Age	0.01	0.01	0.197	-0.01-0.03
FEV1	-0.01	0.01	0.627	-0.01-0.02

3.9.7 Summary

ROC analyses revealed that baseline and follow-up PNI showed poor discrimination for post-operative complications and POP, with AUC values ranging from 0.29 to 0.54. In contrast, ROC analysis for baseline PNI in relation to DFS demonstrated good predictive ability (AUC = 0.72). A cutoff of 56 provided the best balance between sensitivity and specificity (Youden's Index = 0.49). Regression analysis confirmed that PNI <56 was

significantly associated with improved DFS ($p = 0.027$), suggesting PNI may serve as an independent prognostic marker for recurrence

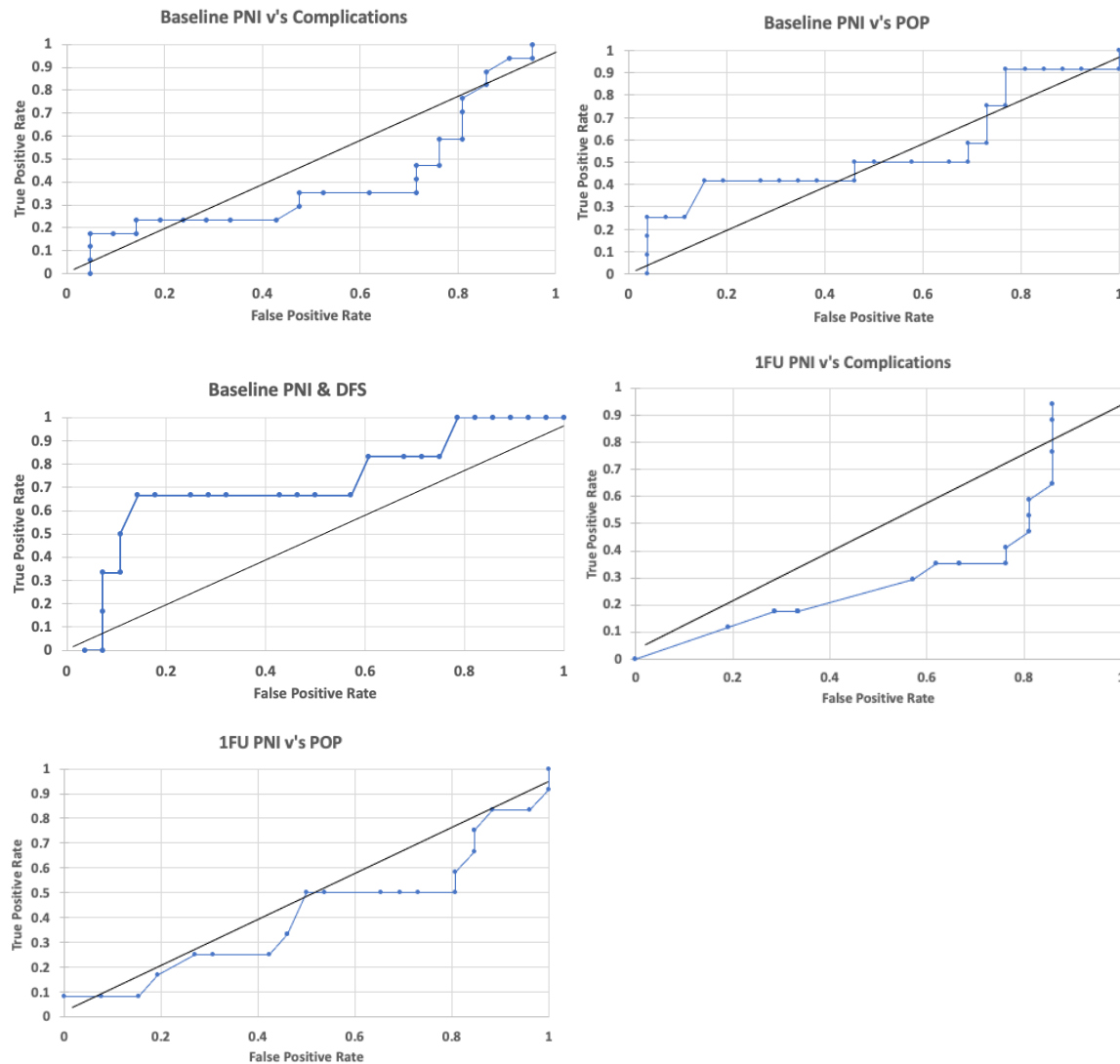


Figure 3.7. ROC analysis of PNI at baseline and 1FU for post-operative complications, post-operative pneumonia and disease-free survival. The false positive rate was plotted against the true positive rate to perform a ROC plot. The diagonal line on the ROC plot represents the performance of a random classifier, indicating no discriminative power. A ROC curve that lies above this line suggests that the test is capable of distinguishing between diseased and non-diseased individuals, with values above 0.7 deemed acceptable. If AUC was above 0.7, optimal cut-off points were determined using Youden's Index. Baseline PNI was not predictive of post-operative complications ($AUC=0.36$) and POP ($AUC=0.54$), however is potentially predictive of DFS ($AUC=0.72$, $YI = 0.49$, optimal cutoff 56). PNI at 1FU was not predictive of post-operative complications ($AUC=0.29$) or POP ($AUC=0.38$) in this cohort.

3.10 DISCUSSION

In this study, nearly half of the patients (45%) experienced post-operative complications following lung cancer surgery, including pulmonary, cardiac, thromboembolic, and wound-related events. Analysis of systemic inflammatory indices revealed that early post-operative measurements taken within 2–4 days after surgery were predictive of these complications, whereas baseline (pre-operative) values showed no prognostic value. Among these markers, NLR >4.9, SII >1050, and SIRI >4 were significantly associated with an increased risk of complications, while PLR demonstrated a trend toward significance, suggesting that early post-operative systemic inflammation, reflects a heightened risk of adverse outcomes. These results are consistent with limited existing literature, which has similarly indicated the potential prognostic value of early inflammatory changes, but lacks large-scale validation.

In contrast to their predictive role in post-operative morbidity, none of the above inflammatory indices were associated with DFS in this cohort. Instead, nutritional and immune composite scores—specifically baseline HALP (>54) and PNI (>56)—emerged as independent prognostic indicators for DFS, with higher values correlating with improved outcomes. Collectively, these findings emphasize that early post-operative assessment of systemic inflammatory indexes may serve as a clinically actionable approach for identifying patients at elevated risk of complications and optimizing perioperative management. The study further underscores the need for large, ethnically diverse randomized controlled trials to confirm optimal cutoff thresholds and to establish the broader prognostic relevance of these biomarkers across different patient populations.

CHAPTER 4

RESULTS

Immune Cell Populations

4.1 INTRODUCTION

Chronic inflammation and immune dysfunction play a key role in the development and progression of NSCLC. Surgical resection in cancer patients triggers systemic inflammation and immunosuppression. This immune disruption impairs anti-tumour responses with post-operative changes causing increased immunosuppressive cells including Tregs and reduced effector cells (CD4+, CD8+ T cells). Circulating immune cell populations therefore can offer predictive insights for post-operative complications and prognosis. Most existing studies focus on immune cells in advanced disease, with limited longitudinal studies, highlighting a significant gap in early-stage, peri-operative immune profiling. This chapter focuses on identification and analysis of immune cell populations in patients undergoing lung cancer surgery, incorporating three peri-operative timepoints. Patient demographics clinicopathological characteristics, surgical approach, and post-operative complications, statistical analysis and survival statistics are discussed in Section 4.2 Flow cytometry was utilised to identify percent frequency of immune cell populations, present in peripheral blood samples.

Analysis encompassed a comprehensive spectrum of immune cell populations, including both helper and cytotoxic T lymphocytes, along with their major differentiation states—naïve, CM, EM, and TDEM subsets. Tregs and $\gamma\delta$ T-cell subsets, specifically V δ 1 and V δ 2 populations, were also examined, together with NKT-like cells, MAIT cells, B lymphocytes, NK cells, and monocytes. Collectively, this selection captures the diverse array of immune effector and regulatory cell types, that contribute to the dynamic interplay of anti-tumour and pro-tumour immune responses, during the peri-operative period of NSCLC surgery.

4.2 PATIENT CHARACTERISTICS

4.2.1 Patient Demographics

Refer to Table 4.1 for clinicopathological characteristics. In our patient cohort the median age was 68.3 years, with a standard deviation (STD) of ± 7.6 years. Females comprised 62% (n=21) of the cohort, while 91% (n=31) of patients were identified as either current or former smokers. Pre-operative pulmonary function tests revealed a mean forced expiratory volume in one second (FEV1) of 93% predicted (STD ± 21.2), suggesting preserved ventilatory function in the majority of patients.

Table 4.1: Clinicopathological Characteristics for cell populations

Patient Characteristic	Total Cohort % (n=34)
Age (years $\pm$ -STD)	68.3 $\pm$ 7.6
Gender	
Male	38 (13)
Female	62 (21)
Smoking History	91 (31)
FEV1	92.9 $\pm$ 21.2
Minimally Invasive Procedure	79 (27)
Pneumonectomy	6 (2)
Lobectomy	88 (30)
Bilobectomy	6 (2)
Size (cm $\pm$ -STD)	3.6 $\pm$ 2.2
Stage	
I	56 (19)
II	21 (7)
III	23 (8)
Histology	
Adenocarcinoma	59 (20)
Squamous	41 (14)
Nodal Disease	29 (10)
Post-operative Complications	44 (15)
Post Operative Pneumonia	32 (11)

STD: Standard deviation

4.2.2 Surgical Approach

A total of 79% of patients (n = 27) underwent a minimally invasive surgical approach, utilizing either VATS or RATS. The majority of patients, 88% (n = 30), underwent lobectomy, which remains the gold standard surgical treatment for resectable NSCLC [6]. 6% of patients (n = 2) required a pneumonectomy and bi-lobectomy due to the extent or location of disease.

4.2.3 Histopathology

Histopathological analysis revealed that 41% (n=14) of tumours were classified as squamous cell carcinoma, while the remaining 59% (n=20) were identified as adenocarcinoma. This distribution reflects the predominance of adenocarcinoma as the most common histological subtype of NSCLC, consistent with global epidemiological trends [2,5].

4.2.4 Staging

The median tumour size was 3.6 cm, (STD $\pm$ 2.2). Based on pathological staging (9th staging edition), 56% of patients (n = 19) were found to have stage I disease, suggesting early detection in a majority of cases. 21% (n = 7) were diagnosed with stage II and 23% (n=8) diagnosed with stage III disease. Lymph node metastasis was identified in 29% of patients (n = 10).

4.2.5 Post-operative Complications

Post-operative complications were observed in 44% (n=15) of patients. The most frequently encountered complication was POP, which affected 32% (n=11) of patients. Additional complications included cardiac arrhythmias, VTE, prolonged air leak (>5days), and wound infections. These findings underscore the critical need for reliable prognostic biomarkers to identify patients who are at the highest risk of experiencing adverse postoperative outcomes, thereby enabling tailored peri-operative management and improved clinical decision-making.

4.2.6 Survival Statistics

Within the study cohort, three patients died from causes unrelated to NSCLC. Disease recurrence was observed in six patients (18%), including four cases with pulmonary nodules, one with lymph node metastasis, and one with vertebral metastasis. The median follow-up period was 14 months. There were three non-cancer-related deaths and two individuals lost to follow-up, therefore these patients were excluded from the disease-free survival calculations. Refer to Table 4.2 for details. Using Kaplan–Meier and life table methods, estimated DFS rates were 88% (SE:0.06, CI: 0.68-0.96) at 12 months and 65% (SE:0.13, CI:0.68-0.96) at 18 months (Figure 4.1).

Table 4.2: Survival Statistics for cell populations

Patient Characteristic	Cohort % (n)
Recurrence	18 (6)
RIP	8 (3)
Median Follow up	14 months
Lost to follow up	2
Median Time to Recurrence	12.5 months

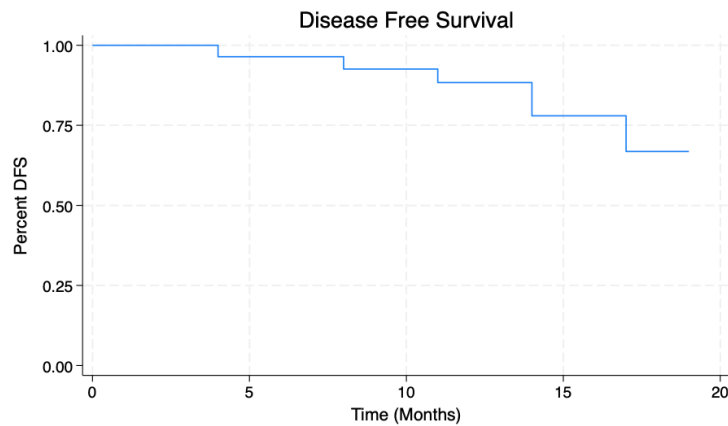


Figure 4.1: Kaplan–Meier graph for the patient cohort. DFS was 88% at 12 months and 65% at 18 months.

4.2.7 Specific Statistical Analysis

For longitudinal data, linear mixed-effects models represent the most appropriate statistical approach, as they account for repeated measurements within subjects and the inherent correlations in the data. [93,94]. Unlike traditional ANOVA or paired tests, mixed models allow for unbalanced datasets and missing observations, thereby improving statistical power [93,94]. The inclusion of both fixed and random effects accommodates individual variability, which is critical for capturing differences in immune responses [93,94]. Additionally, mixed models are more robust to violations of normality assumptions, often encountered in biological data [93,94]. To further ensure any violation of normality is accounted for, arcsine square root transformation was performed. The arcsine square-root transformation stabilizes variance in proportion data [95]. The arcsine-square-root transformation linearizes and stabilizes proportion data for analysis, expanding values near 0 and 1 to reduce skew [95]. Clinicopathological characteristics including age, gender, stage, histology, surgical approach and complications were included in mixed-effects model.

For the survival analysis, DFS was considered only, as no deaths were attributed to NSCLC during the median follow-up period of 14 months. DFS was initially evaluated using Kaplan–Meier and life table methods. Cox proportional hazards regression was then applied to assess the association between the longitudinal slope of each cell population and DFS. Slope, which calculated for each patient, summarises the linear change over three timepoints, for each patient. This approach reduces multiple correlated measurements to a single continuous variable, capturing the dynamic trajectory of the population. The slope

was then included as a covariate in Cox proportional hazards regression to assess whether the rate of change predicted recurrence, while avoiding multicollinearity and preserving model stability. A positive slope indicates increasing cell frequency, while a negative slope reflects a decline. Baseline was used as the reference time point for comparisons across all three time points, as well as individually against 1FU and 2FU. Clinicopathological characteristics including age, gender, stage, histology and post-operative complications were considered. Surgical approach was not considered as DFS events are limited and including surgical approach overfit the model, weakening inference about immune cell effects.

4.3 HELPER T CELLS

4.3.1 CD4+ T cell population

Flow cytometry gating strategy was conducted to accurately identify lymphocyte populations, following the protocol illustrated in Figure 2.1. Initially, lymphocytes were gated based on their characteristic forward and side scatter profiles to exclude debris and non-lymphoid cells. Subsequently, antibodies specific for CD3 and CD4 surface markers were employed to delineate the helper T cell subset within the lymphocyte population. The CD3+ gate was used to define total T cells, while the CD4+ subset within this population represented the CD4+ helper T cell population.

4.3.1.1 Longitudinal analysis of CD4+ T cells

A mixed-effects linear regression model with random intercepts for sample was fitted to examine changes in CD4+ T cells across time points. The overall effect of time was not significant ($\chi^2(2) = 2.6$, $p = 0.06$). Compared with baseline, CD4+ T cells did not differ significantly at 1FU ($\beta = 0.005$, 95% CI: -0.039-0.050, $p = 0.824$) or 2FU ($\beta = -0.043$, 95% CI: -0.087-0.001, $p = 0.056$). Refer to Figure 4.2 for details.

4.3.2: Impact of CD4+ T cell changes on DFS

The slope of CD4+ T cells over three timepoints was not associated with recurrence (HR 0.99, 95% CI 0.87–1.13, $p=0.932$). Median slopes were slightly lower in patients who recurred (−1.25) compared with those who did not (−2.45). Clinical factors including histology (HR 7.82, 95% CI 1.75–35.02, $p=0.007$) and stage (HR 5.78, 95% CI 1.89–17.63, $p=0.002$) were independently associated with recurrence, while age, gender, and post-operative complications were not significant. Overall, the model was highly significant (LR

$\chi^2=30.57$, $p<0.001$), indicating that CD4+ T cell slope did not predict recurrence, whereas key clinical variables (histology and stage) were predictive. See Figure 4.4 and 4.5 for DFS and representative flow cytometry plots.

4.3.2 CD4+ Naïve T cells

Flow cytometry gating was performed to identify the naïve CD4+ T cell subset as per Figure 2.1. Briefly, lymphocytes were first gated based on forward and side scatter, followed by selection of CD3+CD4+ T cells. Within this population, naïve T cells were defined as CD45RA+CCR7+ and expressed as a percentage of total CD4+ T cells.

4.3.2.1 Longitudinal analysis of CD4+ Naïve T Cells

A mixed-effects linear regression model with random intercepts for sample was fitted to examine changes in CD4+ naïve T cells across time points. The overall effect of time was not significant ($\chi^2(2) = 5.48$, $p = 0.2634$). Compared with baseline, CD4+ naïve T cells did not differ significantly at 1FU ($\beta = -0.002$, 95% CI: -0.042-0.039, $p = 0.932$) or 2FU ($\beta = -0.030$, 95%CI: -0.707-0.010, $p = 0.144$). There was a trend toward significance when comparing baseline with 1FU and Stage III ($\beta = 0.87$, 95% CI: -0.010-0.183, $p = 0.081$). A random intercept for participant ID was included to account for baseline differences in CD4+ naïve T cell levels between individuals, capturing meaningful inter-individual variability beyond the effect of time (variance: 0.013, 95% CI: 0.007–0.023). All further cell populations also showed this inter-individual variability. See Figure 4.2.

4.3.2.2: Impact of CD4+ Naïve T Cell Changes on DFS

The slope of CD4 naïve T cells over the three timepoints was not associated with recurrence (HR 0.998, 95% CI 0.93–1.08, $p=0.957$). Median slopes were slightly lower in patients who recurred (−4.28) compared with those who did not (−2.25). Clinical factors including histology (HR 7.72, 95% CI 1.84–32.44, $p=0.005$) and stage (HR 5.72, 95% CI 1.95–16.76, $p=0.001$) were independent predictors. Age, gender, and complications were not significant, and the overall model was highly significant ($\chi^2=30.57$, $p<0.001$). See Figure 4.4 and 4.5 for DFS and representative flow cytometry plots.

4.3.3 CD4+ Central Memory T cells

Flow cytometry gating was performed to identify the CD4+ CM T cell subset as per Figure 2.1. Briefly, lymphocytes were first gated based on forward and side scatter, followed by

selection of CD3+CD4+ T cells. Within this population, CM T cells were defined as CD45RA-CCR7+ and expressed as a percentage of total CD4+ T cells.

4.3.3.1: Longitudinal analysis of CD4+ CM T Cells

A mixed-effects linear regression model with random intercepts for sample was fitted to examine changes in CD4+ CM T cells across time points. The overall effect of time was not significant ($\chi^2(2) = 5.48$, $p = 0.2634$). Compared with baseline, CD4+ CM T cells were significantly different at 1FU ($\beta = 0.034$, 95% CI: 0.001-0.068, $p = 0.048$) but not at 2FU ($\beta = 0.025$, 95% CI: -0.008-0.059, $p = 0.142$). There was a trend toward significance when comparing baseline with 2FU and Stage III ($\beta = -0.67$, 95% CI: -0.145-0.011, $p = 0.094$).

Even though the overall model test is not significant, patients with post-operative complications have lower CD4+ CM T cells at baseline ($\beta = -0.139$, 95% CI: 0.020-0.285, $p = 0.022$), but change over time is similar to those without complications. Refer to Figures 4.2 and 4.3.

4.3.3.2: Impact of CD4+ CM T Cell Changes on DFS

Higher slope of CD4+ CM T cells over three timepoints was associated with increased risk of recurrence (HR 1.19, 95% CI 1.02–1.40, $p=0.032$). Patients who developed recurrence had slightly higher slopes compared with those who did not (median 1.35 vs. 0.37). Clinical factors including histology (HR 8.08, 95% CI 2.05–31.80, $p=0.003$), stage (HR 9.62, 95% CI 2.48–37.36, $p=0.001$), and complications (HR 8.15, 95% CI 1.50–44.17, $p=0.015$) were also independently predictive, whereas age and gender were not significant. The overall model was highly significant ($\chi^2=35.19$, $p<0.001$). See Figure 4.4 and 4.5 for DFS and representative flow cytometry plots.

4.3.4 CD4+ Effector Memory T cells

Flow cytometry gating was performed to identify the EM CD4+ T cell subset as per Figure 2.1. Briefly, lymphocytes were first gated based on forward and side scatter, followed by selection of CD3+CD4+ T cells. Within this population, EM T cells were defined as CD45RA-CCR7- and expressed as a percentage of total CD4+ T cells.

4.3.4.1: Longitudinal analysis of CD4+ EM T Cells

A mixed-effects linear regression model with random intercepts for sample was fitted to examine changes in CD4+ EM T cells across time points. The overall effect of time was not significant ($\chi^2(2) = 3.39$, $p = 0.1834$). Compared with baseline, CD4+ EM T cells were not significantly different at 1FU ($\beta = -0.021$, 95% CI: -0.064-0.021, $p = 0.320$) or at 2FU ($\beta = 0.018$, 95% CI: -0.023-0.059, $p = 0.392$). See Figure 4.2.

There was a statistical significance with CD4+EM cells for stage II ($\beta = 0.218$, 95% CI: 0.732-0.3651, $p = 0.003$) and stage III ($\beta = 0.194$, 95% CI: 0.194-0.073, $p = 0.009$), especially when considering CD4+EM cells in stage II and 1FU ($\beta = -0.124$, 95% CI: -0.226-0.022, $p = 0.017$).

4.3.4.2: Impact of CD4+ EM T Cell Changes on DFS

The slope of CD4+ EM T cells over three timepoints was not associated with recurrence (HR 1.00, 95% CI 0.82–1.22, $p=0.997$), with slightly lower median slopes in patients who recurred (-0.39 vs. 1.36). Clinical factors including histology (HR 7.61, 95% CI 1.50–38.69, $p=0.014$) and stage (HR 5.66, 95% CI 1.93–16.61, $p=0.002$) were independent predictors, while age, gender, and post-operative complications were not significant. Overall model fit was highly significant ($\chi^2=30.56$, $p<0.001$). See Figure 4.4 and 4.5 for DFS and representative flow cytometry plots.

4.3.5 CD4+ Terminally Differentiated Effector Memory T cells

Flow cytometry gating was performed to identify the CD4+ TDEM T cell subset as per Figure 2.1. Briefly, lymphocytes were first gated based on forward and side scatter, followed by selection of CD3+CD4+ T cells. Within this population, TDEM T cells were defined as CD45RA+CCR7- and expressed as a percentage of total CD4+ T cells.

4.3.5.1 Longitudinal analysis of CD4+ TDEM T Cells

A mixed-effects linear regression model with random intercepts for sample was fitted to examine changes in CD4+ TDEM T cells across time points. The overall effect of time was not significant ($\chi^2(2) = 1.52$, $p = 0.4680$). Compared with baseline, CD4+ TDEM T cells were not significantly different at 1FU ($\beta = -0.004$, 95% CI: -0.034-0.025, $p = 0.778$) or at 2FU ($\beta = 0.013$, 95% CI: 0.018-0.252, $p = 0.367$). Refer to Figure 4.2

4.3.5.2: Impact of CD4+ TDEM T Cell Changes on DFS

The slope of CD4+ TDEM cells over the three timepoints was not associated with recurrence (HR 0.98, 95% CI 0.83–1.16, $p=0.856$), with slightly lower median slopes in patients who recurred (-0.06) compared with those who did not (0.18). Clinical factors including histology (HR 7.54, 95% CI 1.98–28.67, $p=0.003$) and stage (HR 5.59, 95% CI 2.03–15.35, $p=0.001$) were independent predictors, while age, gender, and complications were not significant. Overall, the model was highly significant ($\chi^2=30.60$, $p<0.001$), indicating that CD4 TDEM slope did not predict recurrence, whereas clinical variables were predictive. See Figure 4.4 and 4.5 for DFS and representative flow cytometry plots.

4.3.6 CD4+ Regulatory T cells

Flow cytometry gating was performed to identify the CD4+ Treg subset as per Figure 2.1. Total cells, single cells and live cells were identified and lymphocytes were gated based on forward and side scatter, followed by selection of CD3+CD4+ T cells. Within this population, Tregs were defined as CD25+CD127- and expressed as a percentage of total CD3+ T cells.

4.3.6.1 Longitudinal analysis of CD4+ Tregs

A mixed-effects linear regression model with random intercepts for sample was fitted to examine changes in CD4+ Tregs across time points. The overall effect of time was significant ($\chi^2(2) = 11.79$, $p = 0.0027$). Compared with baseline, CD4+ Tregs increased significantly at 1FU ($\beta = 0.025$, 95% CI: 0.001-0.049, $p = 0.041$) and also at 2FU ($\beta = 0.042$, 95% CI: 0.017-0.065, $p = 0.001$).

Baseline CD4+ Tregs cells were higher in Stage III patients compared to Stage I ($\beta = 0.070$, 95% CI: 0.007-0.119, $p = 0.031$). Older individuals show small increases or decreases over time ($\beta = -0.004$, 95% CI: -0.007- -0.001, $p = 0.008$), younger ages have minimal or negative change. See figure 30. Baseline CD4+ Tregs do not vary strongly with age. There is a trend toward significance for gender ($\beta = 0.046$, 95% CI: -0.105- -0.001, $p = 0.060$) with women having slightly higher numbers of CD4+ Tregs comparing to males. Histology, complications and surgical approach do not significantly affect % CD4 Tregs over time. Refer to Figure 4.2-4.3.

4.3.6.2: Impact of CD4+ Treg changes on DFS

Higher slope of CD4+ Treg cells was associated with a trend toward reduced recurrence risk (HR 0.67, 95% CI 0.42–1.07, $p = 0.096$). Patients without recurrence showed higher median slope values (0.7) compared to those with recurrence (0.43). Histology and stage remained strong independent predictors of recurrence ($p \leq 0.002$), with an overall significant model fit (LR $\chi^2 = 34.13$, $p < 0.001$). See Figure 4.4 and 4.5 for DFS and representative flow cytometry plots.

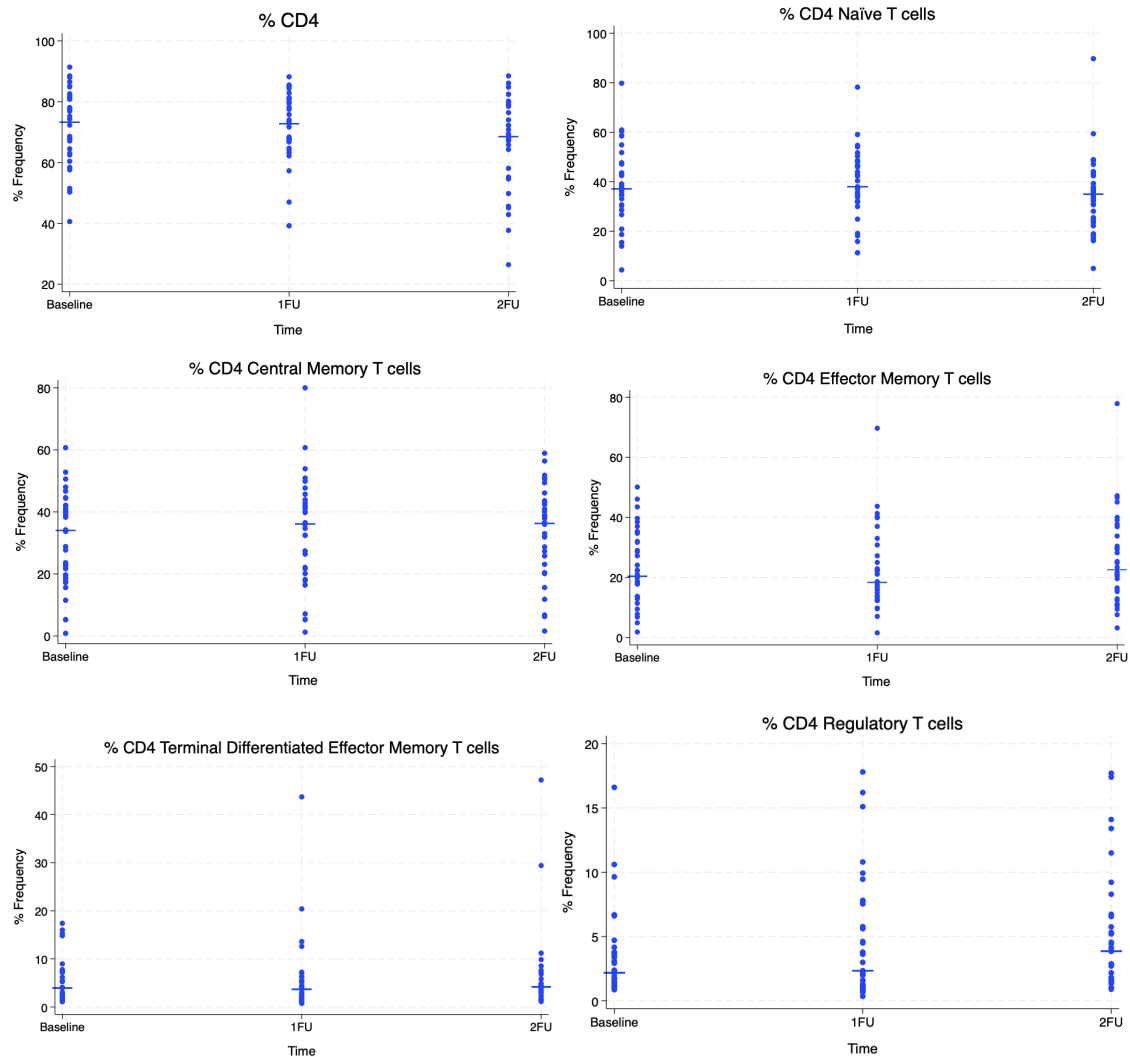


Figure 4.2. Dot plot showing % frequency of CD4+ T cells subsets at each timepoint. For all subsets lymphocytes were gated based on forward and side scatter, followed by identification of CD3+ T cells ($n=34$). Within the CD3+ gate, CD4+ cells were quantified and expressed as a percentage of total CD3+ T cells (%frequency). This was performed for each timepoint. For each subset within the CD3+ gate, CD4+ naïve T cells (CD45RA+CCR7+), CD4+ EM T cells (CD45RA-CCR7-), CD4+ TDEM T cells (CD45RA+CCR7-) and CD4+ Tregs (CD25+CD127-) were quantified and expressed as a percentage of total CD3+ T cells (%frequency). Mixed-effects linear regression revealed a significant difference in frequency over time for CD4+ Tregs ($p=0.0027$) and no statistical difference observed for CD4+ ($p = 0.06$), CD4+ naïve ($p=0.2634$), CD4+ CM ($p=0.1209$), CD4+ EM ($p=0.1571$) and CD4+ TDEM ($p=0.4680$). Dots representing each patient and horizontal line at median value.

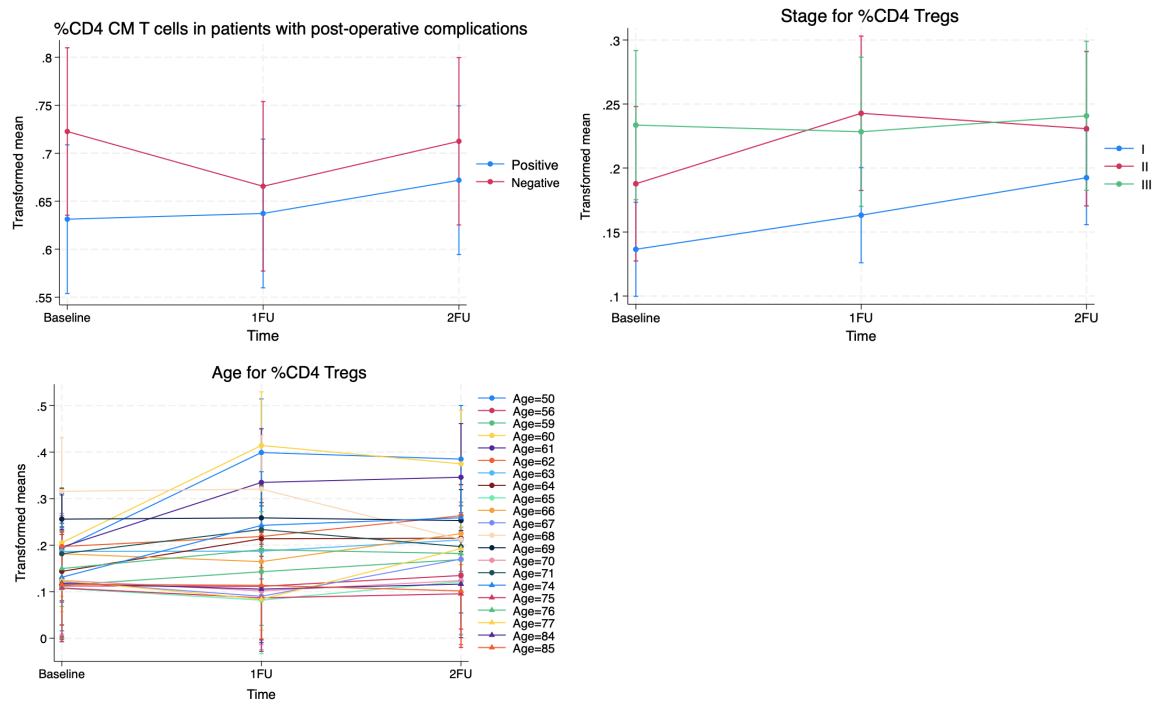


Figure 4.3. Predicted means plot showing differences over time of CD4+ subsets in association with clinicopathological characteristics. Data obtained from FlowJo v10.10.0 software, were expressed as percentage frequencies and subsequently transformed using the arcsine-square root transformation to meet the assumptions of parametric analysis. Plotted values in this figure represent transformed means. To evaluate changes across the three timepoints, linear mixed-effects models were applied in association with age, gender, histological subtype, disease stage, surgical approach, and the presence or absence of post-operative complications. Patients with post-operative complications have lower CD4+ CM T cells at baseline ($p = 0.022$). Baseline CD4+ Tregs cells were higher in Stage III patients compared to Stage I ($p = 0.031$). Older individuals show small increases or decreases over time ($p = 0.008$), younger ages have minimal or negative change.

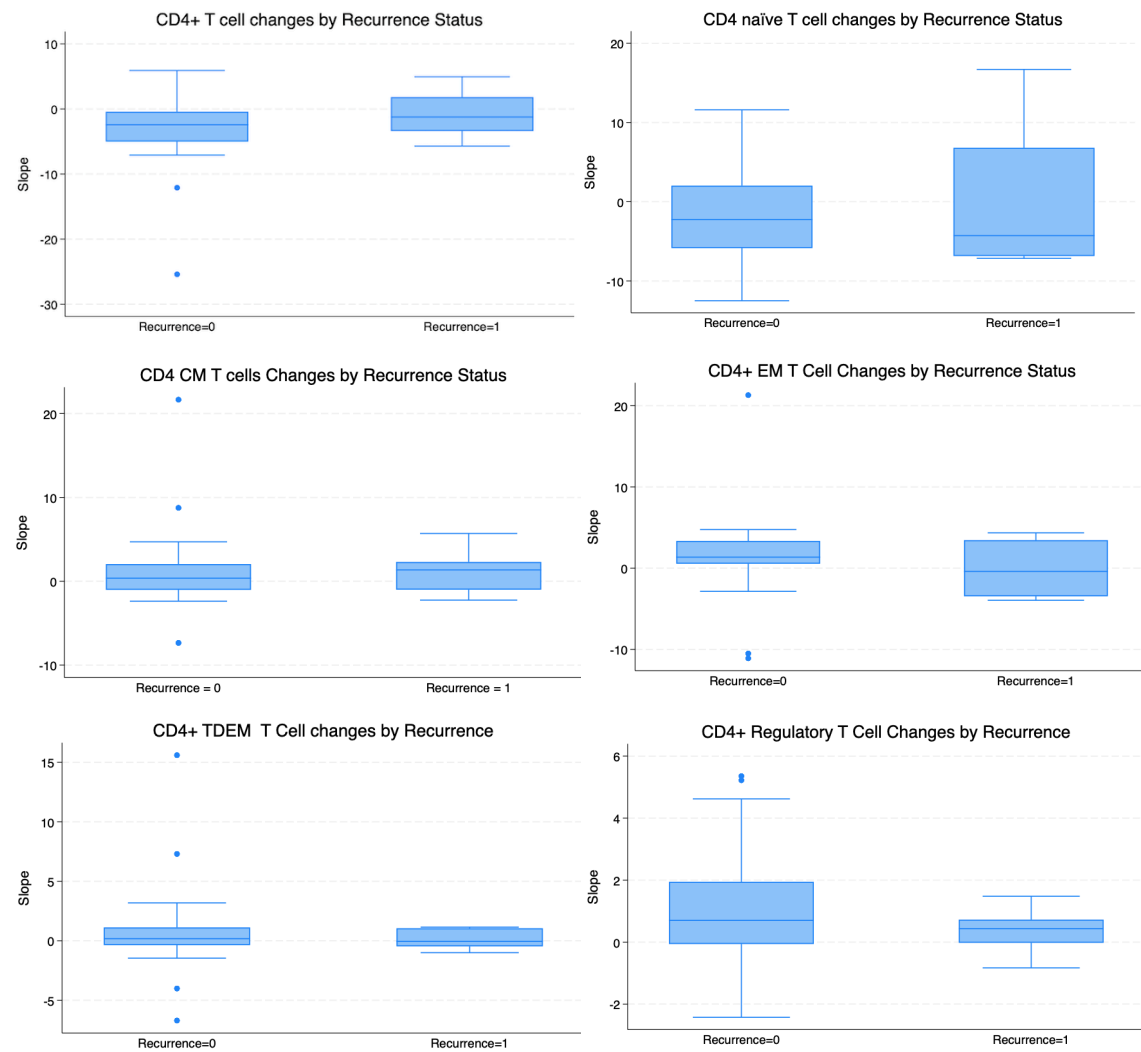


Figure 4.4. Boxplots of CD4+ T cell subsets changes (slopes) in patients with and without recurrence The longitudinal slope summarises the linear change over three timepoints in patients ($n=34$) for CD4+, CD4+ naïve, CD4+ CM, CD4+ EM, CD4+ TDEM and CD4+ Regulatory T cells. Cox proportional hazards regression was utilised to assess the association between the slope and DFS. A positive slope indicates increasing cell frequency, while a negative slope reflects a decline. Baseline was used as the reference time point for comparisons across all three time points, as well as individually against 1FU and 2FU. The slope of CD4+ ($p=0.932$), CD4+ naïve ($p=0.957$), CD4+ EM ($p=0.997$), CD4+ TDEM ($p=0.856$) and CD4+ regulatory ($p=0.096$) T cells over the three timepoints were not associated with recurrence. Higher slope of CD4+ CM T cells was associated with increased risk of recurrence ($p=0.032$). Patients who developed recurrence had slightly higher slopes compared with those who did not (median 1.35 vs. 0.37).

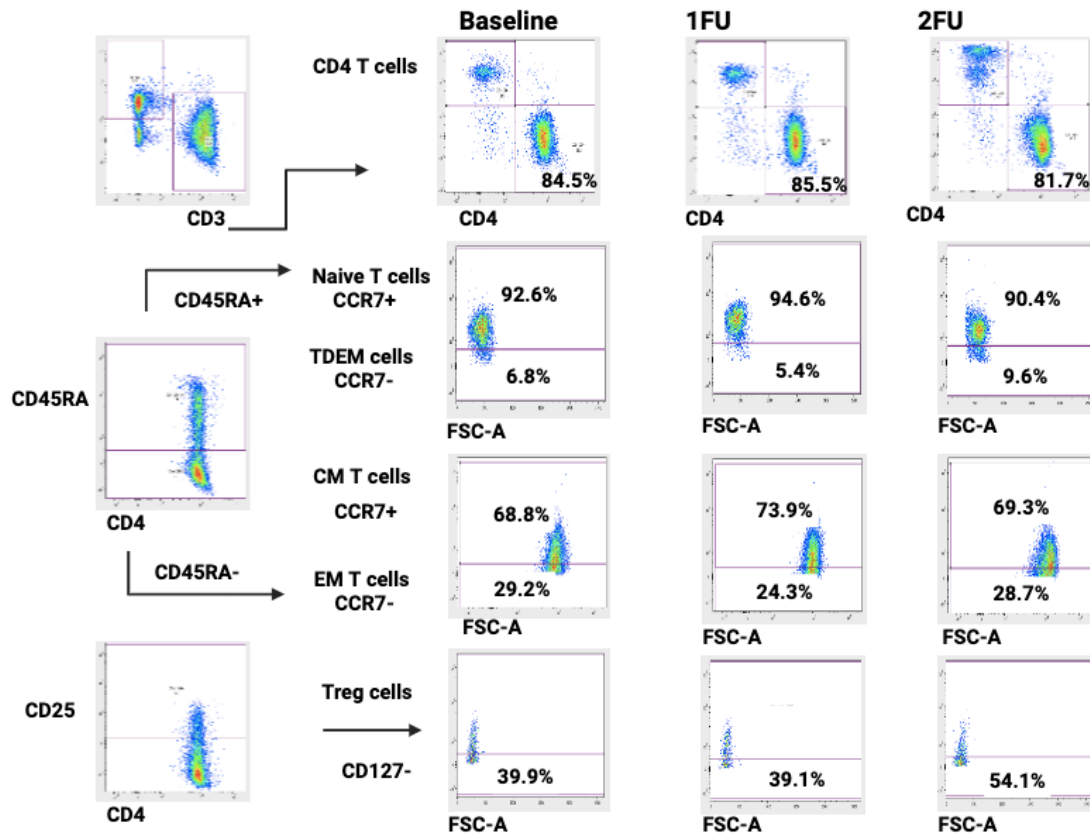


Figure 4.5. Representative flow cytometry plots for CD4+ T cell subsets at different timepoints. PBMCs were stained with a dead cell stain (DCS), CD3, CD4, CD56, CD25, CD127, CD45RA and CCR7 antibodies and analysed by flow cytometry. Forward scatter area (FSC-A) and scatter area (SSC-A) was used to determine total cells, following this FSC-A and FSC-width (FSC-W) was used to determine single cells, then FSC-A and DCS was used to determine live cells. Finally, FSC-A and SSC-A was used to determine lymphocytes. This was performed at baseline, 1FU and 2FU. Percentage frequency is shown on dot plot for each cell type.

Upper panels, left to right: First dot plot showing expression of CD3 (T cells) and following this; representative dot plots of CD4+ T cells at baseline, 1FU and 2FU.

Centre panels, Left to Right: First dot plot shows CD4 plotted against CD45RA. CD45RA+ was then plotted against CCR7 to determine CD4+ naïve T cells (CD4+CD45RA+CCR7+) and CD4+ terminally differentiated effector memory cells (TDEM) (CD4+CD45RA+CCR7-). CD45RA- was then plotted against CCR7 to determine CD4+ central memory T cells (CD4+CD45RA-CCR7-) and CD4+ effector memory cells (TDEM) (CD4+CD45RA-CCR7-). Representative dot plots shown here at baseline, 1FU and 2FU.

Lower panels, Left and Right: First dot plot shows CD4 plotted against CD25. CD25+ was then plotted against CD127- to determine CD4+ Treg cells. Representative plots shown here at baseline, 1FU and 2FU.

Table 4.3: CD4+ populations showing % frequency at the three timepoints

Cell population / Timepoint	% Baseline	% 1FU	% 2FU
CD4+	73.3	73.3	68.6
CD4 Naïve	37.2	39.9	35
CD4 CM	35	36	36.25
CD4 EM	20.5	18.6	22.6
CD4 TDEM	4	3.7	3.9
CD4+ Treg	2.32	2.67	3.9

Table 4.4: Mixed-effects linear regression comparing CD4+ baseline with 1FU and 2FU

Time	Coefficient (β)	Std Err (SE)	p value	95% Confidence Interval (CI)
CD4+ 1FU	0.005	0.022	0.824	-0.039-0.050
CD4+ 2FU	-0.043	0.226	0.056	-0.087-0.001
CD4 Naïve 1FU	-0.002	0.209	0.932	-0.042-0.039
CD4 Naïve 2FU	-0.030	0.207	0.144	-0.707-0.010
CD4 CM 1FU	0.034	0.017	0.048	0.001-0.068
CD4 CM 2FU	0.025	0.017	0.142	-0.008-0.059
CD4 EM 1FU	-0.021	0.022	0.320	-0.064-0.021
CD4 EM 2FU	0.018	0.021	0.392	-0.023-0.059
CD4 TDEM 1FU	-0.004	0.015	0.778	-0.034-0.025
CD4 TDEM 2FU	0.013	0.019	0.367	0.018-0.252
CD4+ Tregs1FU	0.025	0.012	0.041	0.001-0.049
CD4+ Treg 2FU	0.042	0.012	0.001	0.017-0.065

Table 4.5: Mixed-effects linear regression with clinicopathological characteristics for CD4+ T cells

Statistical Analysis	Chi2 (χ^2) (2)	Overall p value	Stage II p value	Stage III p value	Age p value	Gender p value	Histology p value
CD4+	5.48	0.0646	0.808	0.725	0.317	0.391	0.188
CD4+ Naïve	2.6	0.2634	0.960	0.340	0.500	0.957	0.420
CD4+ CM	4.23	0.1209	0.950	0.565	0.881	0.805	0.325
CD4+ EM	3.70	0.1571	0.170	0.133	0.193	0.997	0.093
CD4+ TDEM	1.53	0.4680	0.727	0.766	0.715	0.327	0.798
CD4+ Treg	11.79	0.0027	0.082	0.031	0.008	0.060	0.113

Table 4.6: Mixed-effects linear regression incorporating complications and surgical approach for CD4+ T cells

Statistical Analysis	Complications p value	Surgical approach p value
CD4+	0.142	0.293
CD4+ Naïve	0.698	0.576
CD4+ CM	0.022	0.194
CD4+ EM	0.125	0.283
CD4+ TDEM	0.078	0.501
CD4+ Treg	0.692	0.366

Table 4.7: Descriptive summary statistics incorporating median slope and interquartile range (IQR) for CD4+ T cell populations

Cell Population	Median Slope Recurrence = 0	IQR Recurrence = 0	Median Slope Recurrence = 1	IQR Recurrence = 1
CD4+	-2.45	-5- -0.45	-1.25	-3.4-1.8
CD4+ Naïve	-2.25	-5.85-2.03	-4.28	-6.85-6.8
CD4+ CM	0.365	-1.03-2.05	1.35	-1-2.3
CD4+ EM	1.36	0.55- 3.35	-0.039	-3.45- - 3.45
CD4+ TDEM	0.18	-0.37-1.11	-0.58	-0.47-1.05
CD4+ Treg	0.7	-0.06- 1.94	0.43	-0.02-0.73

Table 4.8: Cox Regression analysis for CD4+ T cells adjusted for clinicopathological characteristics

Cell Population	Hazard Ratio	95% CI	P value	Age	Gender	Histology	Stage	Complications
CD4+	0.994	0.87-1.13	0.932	0.283	0.352	0.007	0.002	0.070
CD4+ Naïve	0.958	0.93-1.08	0.957	0.280	0.368	0.005	0.001	0.084
CD4+ CM	1.19	1.01-1.4	0.032	0.127	0.419	0.003	0.001	0.015
CD4+ EM	1.00	0.82-1.22	0.997	0.305	0.382	0.014	0.002	0.063
CD4+ TDEM	0.98	0.83-1.16	0.856	0.201	0.340	0.003	0.001	0.070
CD4+ Treg	0.672	0.42-1.07	0.096	0.187	0.686	0.002	0.000	0.105

4.4 CYTOTOXIC T CELLS

4.4.1 CD8+ T cell population

Flow cytometry gating strategy was conducted to accurately identify lymphocyte populations, following the protocol illustrated in Figure 2.1. Initially, lymphocytes were gated based on their characteristic forward and side scatter profiles to exclude debris and non-lymphoid cells. Subsequently, antibodies specific for CD3 and CD8 surface markers were employed to delineate the cytotoxic T cell subset within the lymphocyte population. The CD3+ gate was used to define total T cells, while the CD8+ subset within this population represented the CD8+ cytotoxic T cell population.

4.4.1.1 Longitudinal analysis of CD8+ T Cells

A mixed-effects linear regression model with random intercepts for sample was fitted to examine changes in CD8+ across time points. The overall effect of time was not significant ($\chi^2(2) = 3.06$, $p = 0.2171$). Compared with baseline, CD8+ did not differ significantly at 1FU ($\beta = -0.011$, 95% CI: $-0.056-0.033$, $p = 0.616$) or 2FU ($\beta = 0.027$, 95% CI: $-0.027-0.071$, $p = 0.227$). Refer to Figure 4.6.

4.4.3.1: Impact of CD8+ T Cell Changes on DFS

The slope of CD8+ T cells over three timepoints was not associated with recurrence (HR 1.04, 95% CI 0.92–1.18, $p=0.529$). Median slopes were slightly higher in patients who recurred (0.99) compared with those who did not (0.55). Histology (HR 9.81, 95% CI 1.96–49.15, $p=0.005$) and stage (HR 6.50, 95% CI 2.11–20.04, $p=0.001$) were independent predictors. Age, gender, and post-operative complications were not significant, and the overall model was highly significant (LR $\chi^2=30.95$, $p<0.001$). See Figure 4.8 and 4.9 for DFS and representative flow cytometry plots.

4.4.2 CD8+ Naïve T cells

Flow cytometry gating was performed to identify the naïve CD8+ cytotoxic T cell subset as per Figure 2.1. Briefly, total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD3+CD8+ T cells. Within this population, naïve T cells were defined as CD45RA+CCR7+ and expressed as a percentage of total CD8+ T cells.

4.4.2.1 Longitudinal analysis of CD8+ naïve T Cells

A mixed-effects linear regression model with random intercepts for sample was fitted to examine changes in CD8+ naïve T cells across time points. The overall effect of time was significant ($\chi^2(2) = 22.02$, $p < 0.001$). Compared with baseline, CD8+ naïve T cells did not differ significantly at 1FU ($\beta = 0.007$, 95% CI: -0.033-0.048, $p = 0.716$) however there was a statistically significant difference at 2FU ($\beta = -0.081$, 95% CI: -0.121-0.039, $p < 0.001$).

At baseline patients with post-operative complications had lower CD8+ naïve T cells ($\beta = -0.133$, 95% CI: -0.243- -0.023, $p = 0.018$) compared to those without complications. Over the two follow ups naïve CD8 T cells decline significantly by 2FU ($\beta = -0.104$, 95% CI: -0.158- -0.005, $p = 0.000$), this is similar regardless of complications. See Figure 6.

Patients who underwent open procedures had lower CD8+ naïve T cells at baseline, compared to those who underwent minimally invasive procedures ($\beta = -0.213$, 95% CI: -0.339- -0.087, $p = 0.001$). CD8+ naïve T cell populations decline significantly by 2FU ($\beta = -0.089$, 95% CI: -0.134- -0.043, $p < 0.001$) however change over time is not significantly difference between groups. See Figure 4.7.

4.4.2.2: Impact of CD8+ Naïve T Cell Changes on DFS

A higher slope of CD8+ naïve T cells over three timepoints was associated with a reduced risk of recurrence (HR 0.71, 95% CI 0.58–0.87, $p = 0.001$), with patients who recurred showing more negative median slopes (-4.10) compared with those who did not (-2.55). Histology (HR 42.65, 95% CI 5.86–310.47, $p < 0.001$), stage (HR 38.69, 95% CI 5.77–259.35, $p < 0.001$), and patients with post-operative complications (HR 11.17, 95% CI 2.16–57.68, $p = 0.004$) were independent predictors. Age and gender were not significant. The overall model was highly significant (LR $\chi^2 = 46.32$, $p < 0.001$). See Figure 4.8 and 4.9 for DFS and representative flow cytometry plots.

4.4.3 CD8+ Central Memory T cells

Flow cytometry gating was performed to identify the CM CD8+ cytotoxic T cell subset as per Figure 2.1. Briefly, total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD3+CD8+ T cells. Within this population, CM T cells were defined as CD45RA-CCR7+ and expressed as a percentage of total CD8+ T cells.

4.4.3.1 Longitudinal analysis of CD8+ CM T Cells

A mixed-effects linear regression model with random intercepts for sample was fitted to examine changes in CD8+ CM T cells across time points. The overall effect of time was not significant ($\chi^2(2) = 3.57$, $p = 0.1677$). Compared with baseline, CD8+ CM T cells did not differ significantly at 1FU, however there was a trend toward significance ($\beta = 0.037$, 95% CI: -0.004-0.078, $p = 0.083$) or 2FU ($\beta = 0.032$, 95% CI: -0.009-0.072, $p = 0.130$). Refer to Figure 4.6.

4.4.3.2: Impact of CD8+ CM T Cell Changes on DFS

The slope of CD8+ CM T cells over three timepoints was not associated with recurrence (HR 0.97, 95% CI 0.79–1.20, $p=0.799$). Median slopes were slightly higher in patients who recurred (1.82) compared with those who did not (0.54). Histology (HR 8.47, 95% CI 1.76–40.86, $p=0.008$) and stage (HR 5.83, 95% CI 2.08–16.38, $p=0.001$) were independent predictors. Age, gender, and complications were not significant, and the overall model was highly significant (LR $\chi^2=30.63$, $p<0.001$). See Figure 4.8 and 4.9 for DFS and representative flow cytometry plots.

4.4.4 CD8+ Effector Memory T cells

Flow cytometry gating was performed to identify the EM CD8+ cytotoxic T cell subset as per Figure 2.1. Briefly, total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD3+CD8+ T cells. Within this population, EM T cells were defined as CD45RA-CCR7- and expressed as a percentage of total CD8+ T cells.

4.4.4.1 Longitudinal analysis of CD8+ EM T Cells

A mixed-effects linear regression model with random intercepts for sample was fitted to examine changes in CD8+ EM T cells across time points. The overall effect of time was not significant ($\chi^2(2) = 6.74$, $p = 0.0344$). Compared with baseline, CD8+ EM T cells did not differ significantly at 1FU, ($\beta = -0.021$, 95% CI: -0.064-0.021, $p = 0.320$) or 2FU ($\beta = 0.018$, 95% CI: -0.023-0.059, $p = 0.392$). See Figure 4.6.

There was a statistical significant higher numbers of CD8+EM cells in stage II ($\beta = 0.218$, 95% CI: 0.732-0.3651, $p = 0.003$) and stage III ($\beta = 0.194$, 95% CI: 0.194-0.073, $p =$

0.009) patients at baseline compared to Stage I. Refer to Figure 4.7. Patients with adenocarcinoma histology had elevated levels of CD8+EM cells compared to squamous cells at baseline ($\beta = -0.124$, 95% CI: 0.149-0.232, $p = 0.0026$), changes over time were not statistically significant.

4.4.4.2: Impact of CD8+ EM T Cell Changes on DFS

The slope of CD8 EM T cells was strongly associated with recurrence (HR 2.04, 95% CI 1.28–3.27, $p = 0.003$). Median slopes were higher in patients who recurred (3.15) than those who did not (1.2). Histology (HR: 14,145, $p = 0.002$), stage (HR:2,022, $p = 0.001$), and post-operative complications (HR:65.6, $p = 0.003$) were also significant predictors. Age and gender were not significant ($p > 0.3$), and the overall model fit was strong (LR $\chi^2 = 58.24$, $p < 0.001$). See Figure 4.8 and 4.9 for DFS and representative flow cytometry plots.

4.4.5 CD8+ Terminally Differentiated Effector Memory T cells

Flow cytometry gating was performed to identify the TDEM CD8+ cytotoxic T cell subset as per Figure 2.1. Briefly, total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD3+CD8+ T cells. Within this population, TDEM T cells were defined as CD45RA+CCR7- and expressed as a percentage of total CD8+ T cells.

4.4.5.1 Longitudinal analysis of CD8+ TDEM T Cells

A mixed-effects linear regression model with random intercepts for sample was fitted to examine changes in CD8+ TDEM T cells across time points. The overall effect of time was significant ($\chi^2(2) = 6.74$, $p = 0.0344$). Compared with baseline, CD8+ TDEM T cells differed significantly at 1FU ($\beta = -0.048$, 95% CI: 0.087-0.009, $p = 0.016$) but not at 2FU ($\beta = -0.048$, 95% CI: -0.046-0.032, $p = 0.717$). Refer to Figure 4.6.

There was a statistical significance with CD8+ TDEM cells for stage II ($\beta = -0.265$, 95% CI: -0.408- -0.120, $p = 0.000$) and stage III ($\beta = -1.55$, 95% CI: -295- -0.011, $p = 0.035$) compared to Stage I at baseline, however there is no change over time. Patients with adenocarcinoma had lower % frequency of CD8+ TDEM at baseline ($\beta = -0.056$, 95% CI: -0.16- 0.004, $p < 0.001$) compared to squamous histology. See Figure 6 for details. There is a trend toward decrease at 1FU, but it is not statistically significant ($\beta = -0.202$, 95% CI: -0.310- -0.094, $p = 0.060$). Refer to Figure 4.7.

4.4.5.2: Impact of CD8+ TDEM T Cell Changes on DFS

Cox regression showed no significant association between slope of CD8+ TDEM T cells and recurrence (HR = 0.97, 95% CI 0.87–1.08, $p = 0.57$). Histology (HR = 6.47, $p = 0.01$) and stage (HR = 5.14, $p = 0.003$) were significant predictors, while complications showed a trend ($p = 0.069$). Median slopes were slightly lower in the recurrence group (median = -0.30) compared to the non-recurrence group (median = -1.0), indicating a modestly flatter or declining CD8+ TDEM T cell trajectory over time, among those who recurred. The overall model fit was significant (LR $\chi^2 = 30.88$, $p < 0.001$). See Figure 4.8 and 4.9 for DFS and representative flow cytometry plots.

4.4.6 CD8+ Regulatory T cells

Flow cytometry gating was performed to identify the CD8+ Treg cell subset as per Figure 2.1. Briefly total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD3+CD8+ T cells. Within this population, CD8+ Treg cells were defined as CD25+CD127- and expressed as a percentage of total CD3+ T cells.

4.4.6.1 Longitudinal analysis of CD8+ Tregs

A mixed-effects linear regression model revealed the overall effect of time was significant ($\chi^2(2) = 13.51$, $p = 0.0012$). Compared with baseline, CD8+ Tregs differed significantly at 1FU ($\beta = 0.027$, 95% CI: 0.002-0.051, $p = 0.032$) and also at 2FU ($\beta = 0.045$, 95% CI: 0.021-0.068, $p < 0.001$). Age showed a weak trend toward lower CD8+Tregs in older patients ($\beta = -0.004$, 95% CI: 0.012-0.123, $p = 0.068$). Stage, histology, gender, post-operative complications and surgical approach are not significant predictors. Refer to Figure 4.6.

4.4.6.2: Impact of CD8+ Regulatory T Cell Changes on DFS

Higher CD8+ Treg slopes were not significantly associated with recurrence risk (HR 0.95, 95% CI 0.69–1.30, $p = 0.735$). Patients without recurrence had higher mean slope values (1.10) compared to those with recurrence (0.52), suggesting greater expansion of CD8+ Tregs in non-recurrent cases. Histology and stage were strong independent predictors ($p \leq 0.004$), and the overall model fit was significant (LR $\chi^2 = 30.68$, $p < 0.001$). See Figure 4.8 and 4.9 for DFS and representative flow cytometry plots.

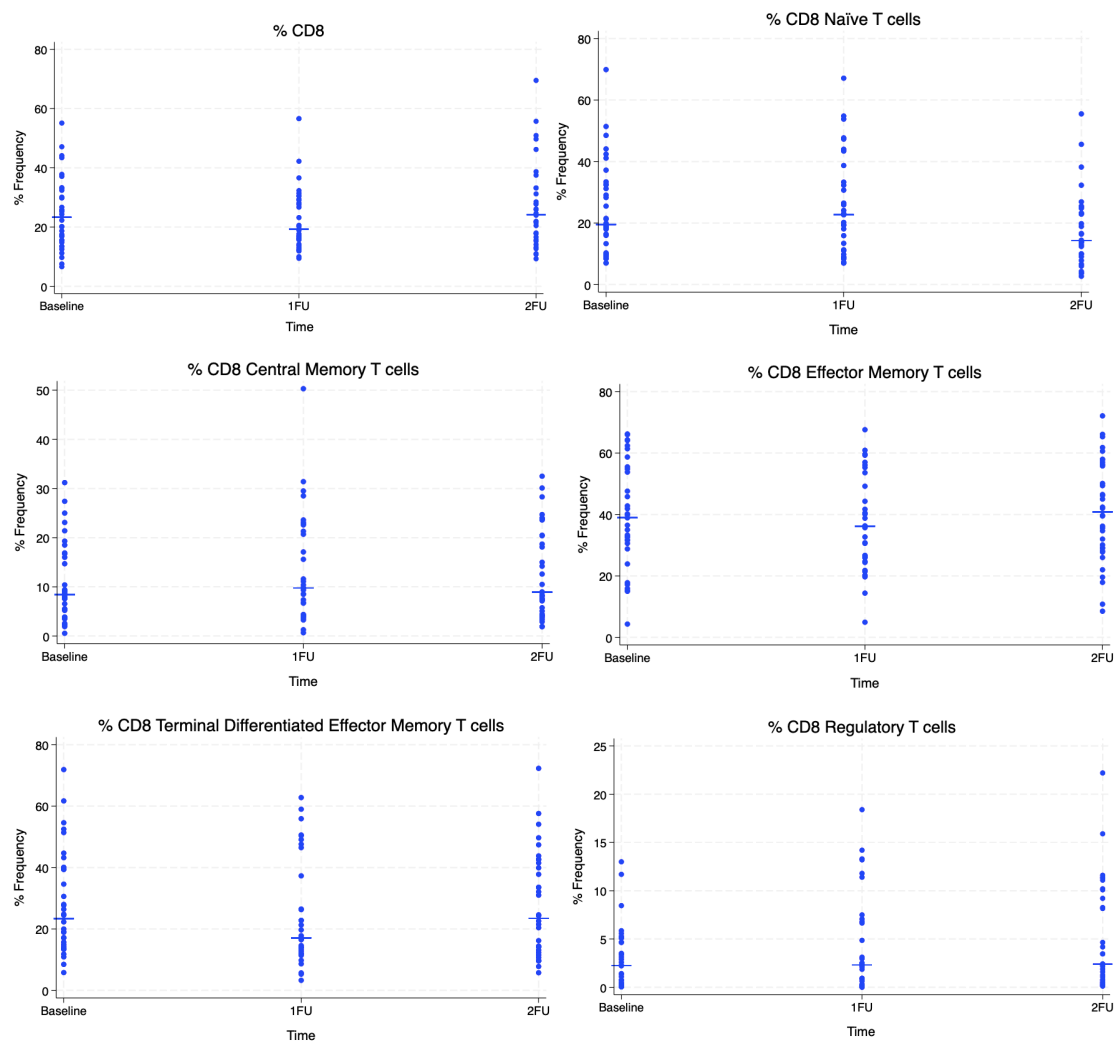


Figure 4.6. Dot plot showing % frequency of CD8+ T cells subsets at each timepoint. For all subsets lymphocytes were gated based on forward and side scatter, followed by identification of CD3+ T cells ($n=34$). Within the CD3+ gate, CD8+ cells were quantified and expressed as a percentage of total CD3+ T cells (%frequency). This was performed for each timepoint. For each subset within the CD3+ gate, CD8+ naïve T cells (CD45RA+CCR7+), CD8+ EM T cells (CD45RA-CCR7-), CD8+ TDEM T cells (CD45RA+CCR7-) and CD8+ Tregs (CD25+CD127-) were quantified and expressed as a percentage of total CD3+ T cells (%frequency). Mixed-effects linear regression revealed a significant difference in frequency over time for CD8+ naïve cells ($p<0.001$), CD8+ TDEM (0.0344) and CD8+ Tregs ($p=0.0012$). No statistical difference was observed for CD8+ ($p = 0.2171$), CD8+ CM ($p=0.1677$) and CD8+ EM ($p=0.1834$). Dots representing each patient and horizontal line at median value.

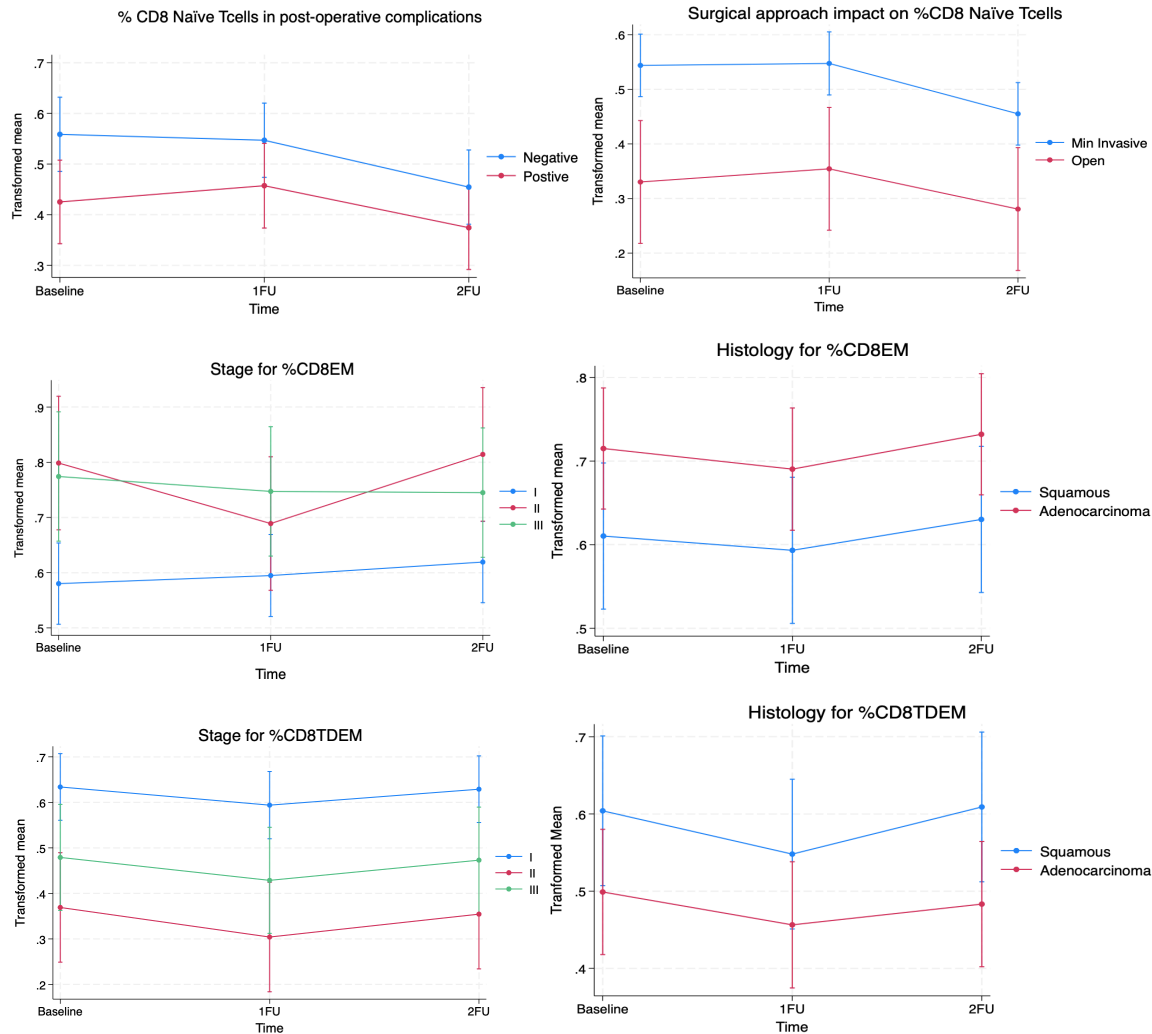


Figure 4.7. Predicted means plot showing differences over time of CD8+ subsets in association with clinicopathological characteristics. Data obtained from FlowJo v10.10.0 software, were expressed as percentage frequencies and subsequently transformed using the arcsine-square root transformation to meet the assumptions of parametric analysis. Plotted values in this figure represent transformed means. To evaluate changes across the three timepoints, linear mixed-effects models were applied in association with age, gender, histological subtype, disease stage, surgical approach, and the presence or absence of post-operative complications. Patients with post-operative complications have lower CD8+ naïve T cells at baseline ($p = 0.018$). Patients who underwent open procedures had lower CD8+ naïve T cells at baseline, compared to those who underwent minimally invasive procedures ($p = 0.001$). There was a statistical significant difference in CD8+ EM and TDEM cells in stage II ($p = 0.003$, <0.001) and stage III ($p = 0.009$, 0.035) patients at baseline compared to Stage I. Patients with adenocarcinoma histology had higher CD8+ EM ($p = 0.0026$) and lower of CD8+ TDEM ($p = <0.001$) percent frequencies compared to squamous cells.

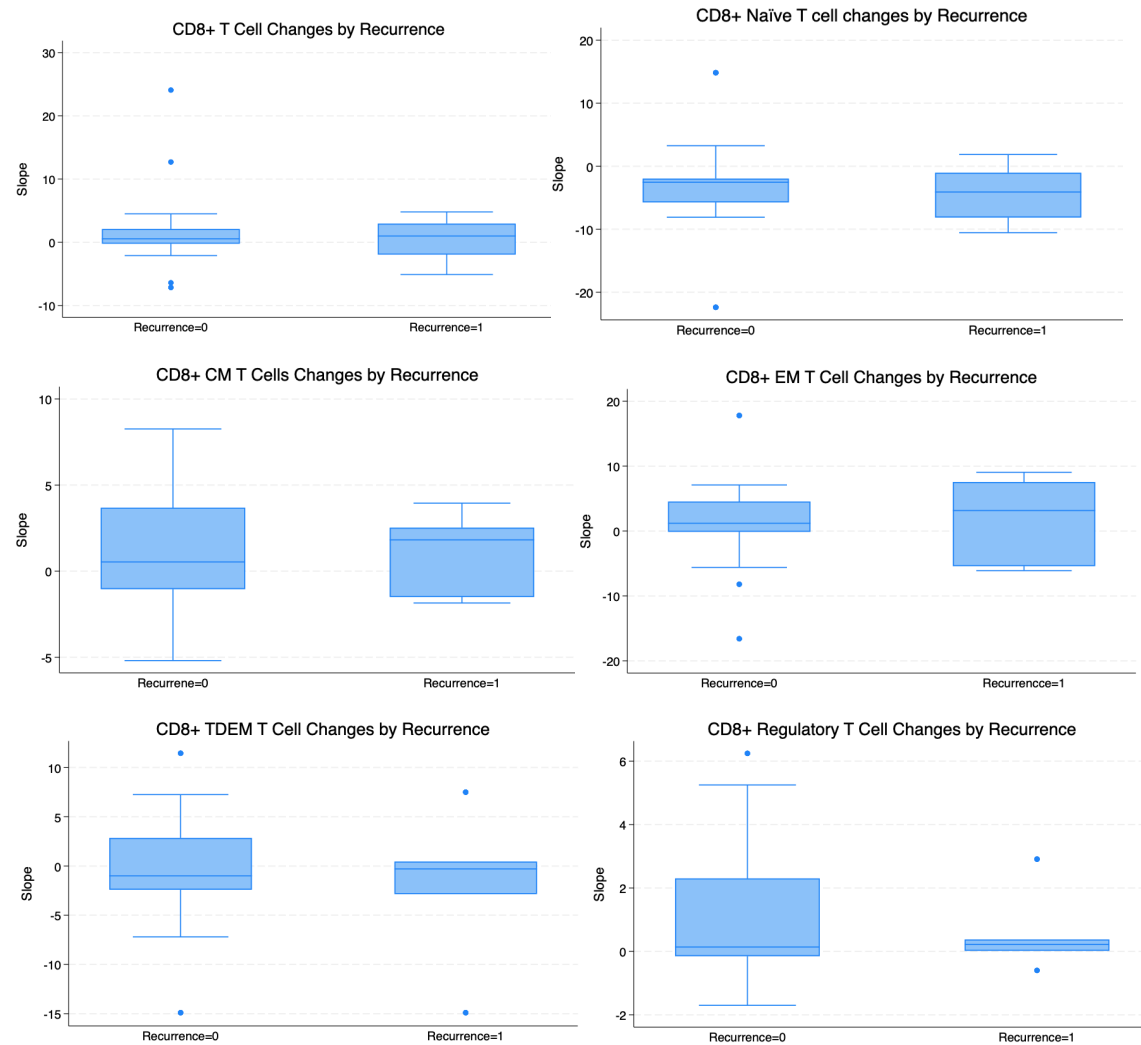


Figure 4.8. Boxplots of CD8+ T cell subsets changes (slopes) in patients with and without recurrence. The longitudinal slope summarises the linear change over three timepoints in patients ($n=34$) for CD8+, CD8+ naïve, CD8+ CM, CD8+ EM, CD8+ TDEM and CD8+ Regulatory T cells. Cox proportional hazards regression was utilised to assess the association between the slope and DFS. A positive slope indicates increasing cell frequency, while a negative slope reflects a decline. Baseline was used as the reference time point for comparisons across all three time points, as well as individually against 1FU and 2FU. The slope of CD8+ ($p=0.529$), CD8+ CM ($p=0.799$), CD8+ TDEM ($p=0.57$) and CD8+ regulatory ($p=0.735$) T cells over the three timepoints were not associated with recurrence. Higher slope of CD8+ naïve ($p=0.001$), CD8+ EM ($p=0.003$) were associated with increased risk of recurrence. Patients who developed recurrence had slightly higher slopes compared with those who did not.

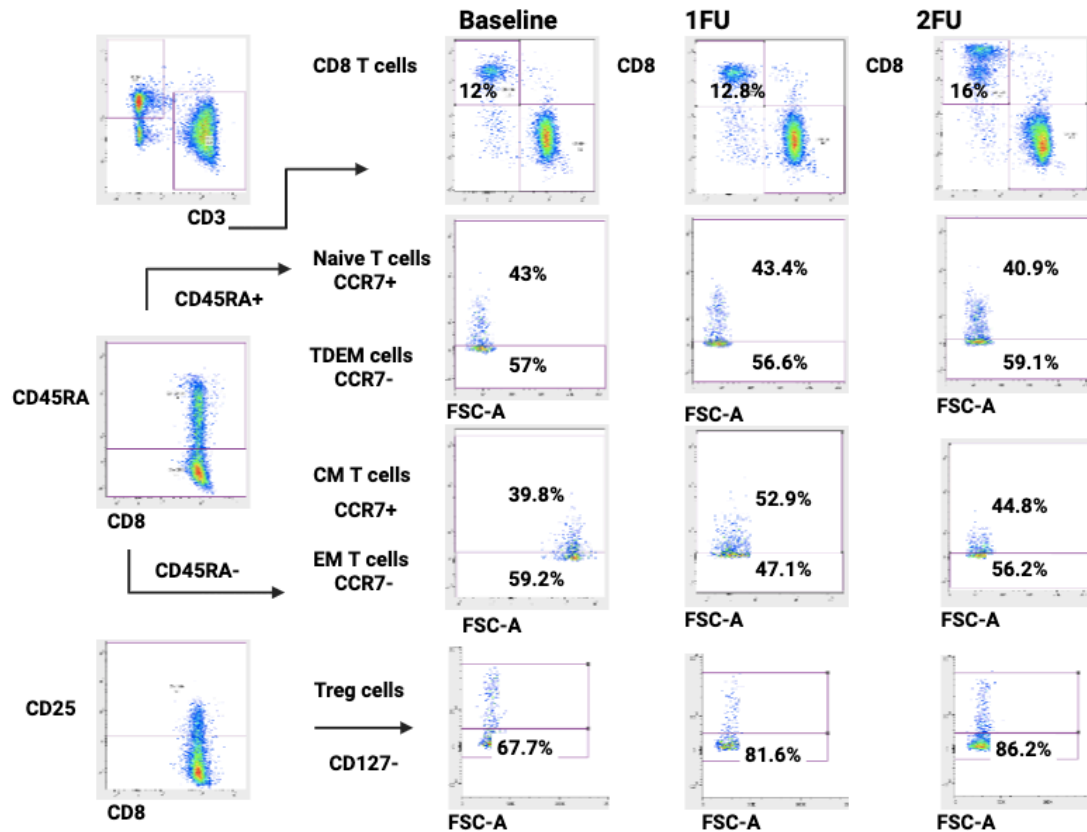


Figure 4.9. Representative flow cytometry plots for CD8+ T cell subsets at different timepoints. PBMCs were stained with a dead cell stain (DCS), CD3, CD8, CD56, CD25, CD127, CD45RA and CCR7 antibodies and analysed by flow cytometry. Forward scatter area (FSC-A) and scatter area (SSC-A) was used to determine total cells, following this FSC-A and FSC-width (FSC-W) was used to determine single cells, then FSC-A and DCS was used to determine live cells. Finally, FSC-A and SSC-A was used to determine lymphocytes. This was performed at baseline, 1FU and 2FU. Percentage frequency is shown on dot plot for each cell type.

Upper panels, Left to Right: First dot plot showing expression of CD3 (T cells) and following this; representative dot plots of CD8+ T cells at baseline, 1FU and 2FU.

Centre panels, Left to Right: First dot plot shows CD8 plotted against CD45RA. CD45RA+ was then plotted against CCR7 to determine CD8+ naïve T cells (CD8+CD45RA+CCR7+) and CD8+ terminally differentiated effector memory cells (TDEM) (CD8+CD45RA+CCR7-). CD45RA- was then plotted against CCR7 to determine CD8+ central memory T cells (CD8+CD45RA-CCR7-) and CD4+ effector memory cells (TDEM) (CD8+CD45RA-CCR7-). Representative dot plots shown here at baseline, 1FU and 2FU.

Lower panels, Left and Right: First dot plot shows CD8 plotted against CD25. CD25+ was then plotted against CD127- to determine CD8+ Treg cells. Representative plots shown here at baseline, 1FU and 2FU.

Table 4.9: CD8+ T cell populations showing % frequency

Cell Population	% Baseline	% 1FU	% 2FU
CD8+	23.3	19.3	24.2
CD8 Naïve	20.6	22.7	14.3
CD8 CM	8.4	9.8	11.6
CD8 EM	39	36.2	40.9
CD8 TDEM	23.4	17.1	23.5
CD8+ Treg	2.3	2.4	2.4

Table 4.10: Mixed-effects linear regression comparing baseline with 1FU and 2FU for CD8+ T cells

Cell population / Timepoint	Coefficient (β)	Std Err (SE)	p value	95% Confidence Interval
CD8+ 1FU	-0.114	0.023	0.616	-0.056-0.033
CD8+ 2FU	0.027	0.225	0.227	-0.027-0.071
CD8+ Naïve 1FU	0.007	0.021	0.716	-0.033-0.048
CD8+ Naïve 2FU	-0.081	0.021	<0.001	-0.121-0.039
CD8+ CM 1FU	0.037	0.021	0.083	-0.004-0.078
CD8+ CM 2FU	0.032	0.021	0.130	-0.009-0.072
CD8+ EM 1FU	-0.021	0.022	0.320	-0.064-0.021
CD8+ EM 2FU	0.018	0.021	0.392	-0.023-0.059
CD8+ TDEM 1FU	-0.048	0.020	0.060	-0.087-0.009
CD8+ TDEM 2FU	-0.007	0.019	0.717	-0.046-0.032
CD8 Treg 1FU	0.027	0.012	0.032	0.002-0.051
CD8 Tregs2FU	0.045	0.012	0.000	0.021-0.068

Table 4.11: Mixed-effects linear regression with clinicopathological characteristics for CD8+ T cells

Statistical Analysis	Chi2 (χ^2) (2)	Overall p value	Stage II p value	Stage III p value	Age p value	Gender p value	Histology p value
CD8+	3.06	0.2171	0.914	0.931	0.656	0.220	0.183
CD8+ Naïve	22.01	<0.001	0.538	0.073	0.570	0.113	0.0179
CD8+ CM	3.47	0.1677	0.146	0.474	0.847	0.636	0.824
CD8+ EM	3.39	0.1834	0.003	0.009	0.403	0.702	0.026
CD8+ TDEM	6.74	0.0344	<0.001	0.035	0.582	0.473	<0.001
CD8+ Treg	13.51	0.0012	0.254	0.795	0.068	0.106	0.759

Table 4.12: Mixed-effects linear regression incorporating complications and surgical approach for CD8+ T cells

Statistical Analysis	Complications P value	Surgical approach p value
CD8+	0.207	0.340
CD8+ Naïve	0.018	0.001
CD8+ CM	0.454	0.831
CD8+ EM	0.125	0.973
CD8+ TDEM	0.971	0.076
CD8+ Treg	0.254	0.109

Table 4.13: Descriptive summary statistics incorporating median slope and interquartile range (IQR) for CD8+ T cell populations

Cell Population	Median Slope Recurrence = 0	IQR Recurrence = 0	Median Slope Recurrence = 1	IQR Recurrence = 1
CD8+	-0.55	-0.23- 2.1	-0.99	-1.95- 2.95
CD8+ Naïve	-2.55	-5.75- -1.98	-4.1	-8.15- - 1.05
CD8+ CM	0.799	0.79-1.20	1.35	-1-2.3
CD8+ EM	1.2	-0.13- 4.55	3.15	-5.4- 7.55
CD8+ TDEM	-1	-7.2-7.25	-0.30	-2.85-0.45
CD8+ Treg	0.139	-0.15-2.30	0.22	0.02-0.38

Table 4.14: Cox Regression analysis for CD8+ T cells adjusted for clinicopathological characteristics

Cell Population	Hazard Ratio	95% CI	P value	Age	Gender	Histology	Stage	Complications
CD8+	1.04	0.92-1.18	0.529	0.248	0.360	0.005	0.001	0.088
CD8+ Naïve	0.71	0.57-0.87	0.001	0.475	0.803	0.000	0.000	0.004
CD8+ CM	0.97	0.79-1.20	0.799	0.310	0.378	0.008	0.001	0.076
CD8+ EM	2.04	1.27-3.27	0.003	0.373	0.818	0.002	0.001	0.003
CD8+ TDEM	0.97	0.86-1.08	0.566	0.309	0.389	0.010	0.003	0.069
CD8+ Treg	0.946	0.69-1.30	0.735	0.280	0.452	0.004	0.001	0.060

4.6 $\gamma\delta$ T CELLS

4.6.1 V δ 1 $\gamma\delta$ T cells

Flow cytometry gating was performed to identify the $\gamma\delta$ T cell subsets, as depicted in Figure 2.1. Total cells, single cells and live cells were identified and lymphocytes were gated based on forward and side scatter, followed by selection of CD3⁺ T cells. Within this population, $\gamma\delta$ T cells were identified as TCR $\gamma\delta$ +V δ 1⁺ T cells, which were expressed as a percentage of total CD3⁺ T cells.

4.6.1.1 Longitudinal analysis of V δ 1 $\gamma\delta$ T cells

A mixed-effects linear regression model revealed the overall effect of time was not significant ($\chi^2(2) = 3.08$, $p = 0.2140$). Compared with baseline, V δ 1 $\gamma\delta$ T cells did not differ significantly at 1FU, ($\beta = -0.004$, 95% CI: -0.075-0.066, $p = 0.896$) or 2FU ($\beta = 0.052$, 95% CI: -0.018-0.123, $p = 0.145$). V δ 1 $\gamma\delta$ T cells numbers decline with increasing age ($\beta = -0.007$, 95% CI: -0.011- -0.003, $p = 0.001$), no change was observed over the timepoints. Refer to Figure 4.10 and 4.11.

4.6.1.2: Impact of V δ 1 $\gamma\delta$ T cells T Cell Changes on DFS

Variation in V δ 1 $\gamma\delta$ T slopes showed no significant association with recurrence (HR 0.79, 95% CI 0.01–85.81, $p = 0.922$). Mean slopes were slightly higher in non-recurrent patients (0.08) than in recurrent ones (0.00), suggesting minimal dynamic differences over time. Histology and stage remained significant predictors ($p \leq 0.004$), and the overall model was robust (LR $\chi^2 = 30.57$, $p < 0.001$). Refer to Figure 4.12.

4.6.2 V δ 2 $\gamma\delta$ T cells

Flow cytometry gating was performed to identify the $\gamma\delta$ T cell subsets as per Figure 2.1. Total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD3⁺ T cells. Within this population, $\gamma\delta$ T cells were identified as TCR $\gamma\delta$ +V δ 2⁺ T cells, which were expressed as a percentage of total CD3⁺ T cells.

4.6.2.1 Longitudinal analysis of V δ 2 $\gamma\delta$ T cells

On analysis by mixed-effects linear regression, the overall effect of time was not significant ($\chi^2(2) = 2.4$, $p = 0.3008$). Compared with baseline, V δ 2 $\gamma\delta$ T cells did not differ significantly

at 1FU, ($\beta = -0.012$, 95%CI: -0.032-0.006, $p = 0.207$) or 2FU ($\beta = 0.002$, 95%CI: -0.018-0.021, $p = 0.876$).

Patients who underwent an open procedure had significantly lower V δ 2 $\gamma\delta$ T cells ($\beta = -0.060$, 95%CI: -0.111- -0.009, $p = 0.021$). Surgical procedure type and not temporal changes drives differences in V δ 2 $\gamma\delta$ T cells. Refer to Figure 4.10 and 4.11.

4.6.2.2. Impact of V δ 2 $\gamma\delta$ T Cell Changes on DFS

Changes in V δ 2 $\gamma\delta$ T slopes were not significantly associated with recurrence (HR 0.90, 95% CI 0.61–1.33, $p = 0.598$). Mean slopes were slightly negative in non-recurrent patients (−0.25) and near zero in recurrent ones (0.02), indicating limited temporal variation. Histology and stage remained strong independent predictors ($p \leq 0.003$), and the overall model was significant (LR $\chi^2 = 30.81$, $p < 0.001$). Refer to Figure 4.12.

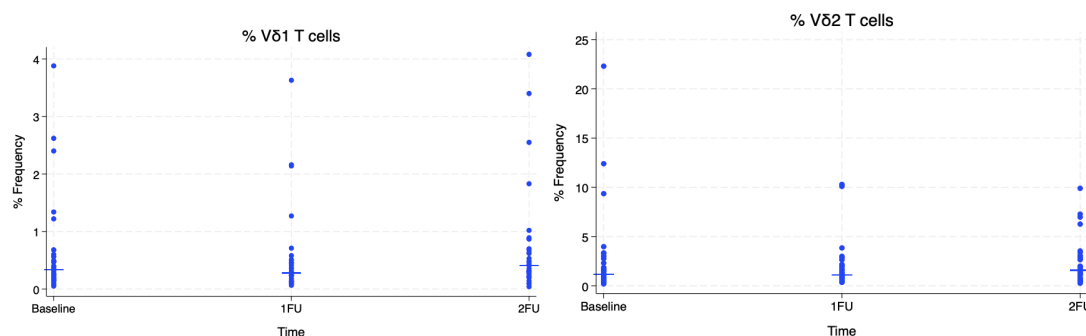


Figure 4.10 Dot plot showing % frequency of $\gamma\delta$ T cells at each timepoint.

For both subsets lymphocytes were gated based on forward and side scatter, followed by identification of CD3+ T cells ($n=34$). Within the CD3+ gate, $\gamma\delta$ T cells were quantified and expressed as a percentage of total CD3+ T cells (% frequency). This was performed for each timepoint. For each subset within the CD3+ gate, V δ 1 $\gamma\delta$ T cells (TCR $\gamma\delta$ +V δ 1+) and V δ 2 $\gamma\delta$ T cells (TCR $\gamma\delta$ +V δ 2+) were then quantified and expressed as a percentage of total CD3+ T cells (% frequency). Mixed-effects linear regression revealed no statistical difference for V δ 1 $\gamma\delta$ T cells ($p = 0.2140$) or V δ 2 $\gamma\delta$ T cells ($p = 0.3008$) over time. Dots representing each patient and horizontal line at median value

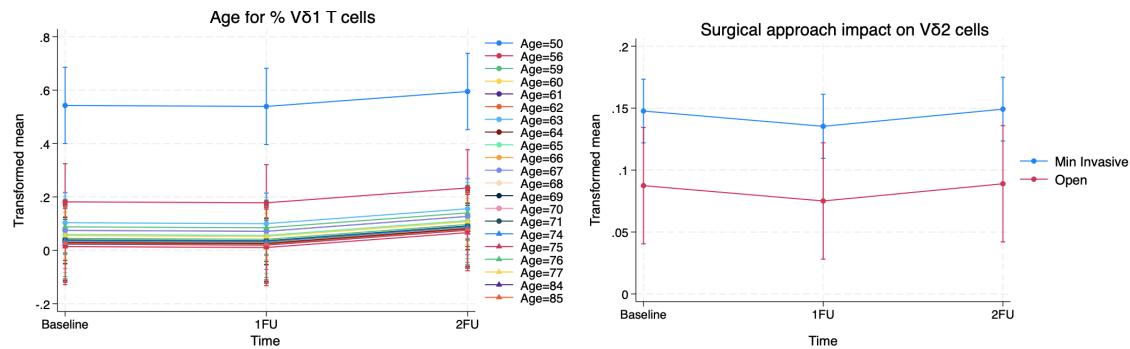


Figure 4.11. Predicted means plot showing differences over time of $\gamma\delta$ T cells in association with clinicopathological characteristics. Data obtained from FlowJo v10.10.0 software, were expressed as percentage frequencies and subsequently transformed using the arcsine-square root transformation to meet the assumptions of parametric analysis. Plotted values in this figure represent transformed means. To evaluate changes across the three timepoints, linear mixed-effects models were applied in association with age, gender, histological subtype, disease stage, surgical approach, and the presence or absence of post-operative complications. $V\delta 1$ $\gamma\delta$ T cells numbers decline with increasing age ($p = 0.001$). Patients who underwent an open procedure had significantly lower $V\delta 2$ $\gamma\delta$ T cells ($p = 0.021$).

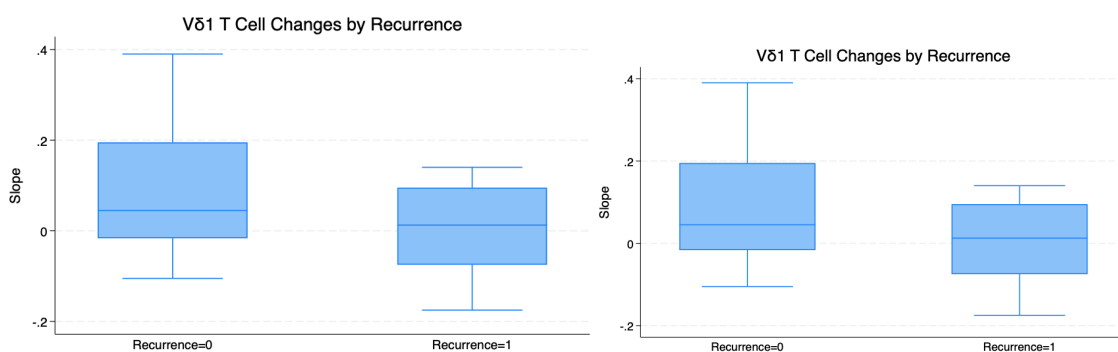


Figure 4.12. Boxplots of $\gamma\delta$ T cells subsets changes (slopes) in patients with and without recurrence. The longitudinal slope summarises the linear change over three timepoints in patients ($n=34$) for $V\delta 1$ $\gamma\delta$ T cells and $V\delta 2$ $\gamma\delta$ T cells. Cox proportional hazards regression was utilised to assess the association between the slope and DFS. A positive slope indicates increasing cell frequency, while a negative slope reflects a decline. Baseline was used as the reference time point for comparisons across all three time points, as well as individually against 1FU and 2FU. The slope of $V\delta 1$ $\gamma\delta$ T cells ($p = 0.922$), and $V\delta 2$ $\gamma\delta$ T cells ($p = 0.598$) over the three timepoints were not associated with recurrence.

Table 4.21: Percent frequency of $\gamma\delta T$ cells

Cell Population	% Baseline	% 1FU	% 2FU
V δ 1 $\gamma\delta T$	0.34	0.28	0.41
V δ 2 $\gamma\delta T$	1.34	1.11	1.79

Table 4.22: Mixed-effects linear regression comparing baseline with 1FU and 2FU for $\gamma\delta T$ cells

Cell population / Timepoint	Coefficient (β)	Std Err (SE)	p value	95% Confidence Interval
V δ 1 $\gamma\delta T$ 1FU	-0.004	0.036	0.896	-0.075-0.066
V δ 1 $\gamma\delta T$ 2FU	0.052	0.034	0.145	-0.018-0.123
V δ 2 $\gamma\delta T$ 1FU	-0.012	0.009	0.207	-0.032-0.006
V δ 2 $\gamma\delta T$ 2FU	0.002	0.013	0.876	-0.018-0.021

Table 4.23: Mixed-effects linear regression incorporating complications and surgical approach for $\gamma\delta T$ cells

Statistical Analysis	Complications P value	Surgical approach p value
V δ 1 $\gamma\delta T$	0.510	0.722
V δ 2 $\gamma\delta T$	0.932	0.021

Table 4.24: Mixed-effects linear regression with clinicopathological characteristics for $\gamma\delta T$ cells

Cell Population	Chi2 (χ^2) (2)	p value	Stage II p value	Stage III p value	Age p value	Gender p value	Histology p value
V δ 1 $\gamma\delta T$	3.08	0.2140	0.190	0.750	0.001	0.829	0.740
V δ 2 $\gamma\delta T$	2.4	0.3008	0.180	0.959	0.995	0.122	0.988

Table 4.25: Descriptive summary statistics incorporating median slope and interquartile range (IQR) for $\gamma\delta$ T cell populations

Cell Population	Median Slope Recurrence = 0	IQR Recurrence = 0	Median Slope Recurrence = 1	IQR Recurrence = 1
V δ 1 $\gamma\delta$ T	0.45	-0.17- 0.20	0.13	-0.75-0.09
V δ 2 $\gamma\delta$ T	0.21	-0.08-0.70	0.14	-0.11-0.22

Table 4.26: Cox Regression analysis for $\gamma\delta$ T T cells adjusted for clinicopathological characteristics

Cell Population	Hazard Ratio	95% CI	P value	Age	Gender	Histology	Stage	Complications
V δ 1 $\gamma\delta$ T	0.791	0.01-85.8	0.922	0.290	0.363	0.003	0.004	0.082
V δ 2 $\gamma\delta$ T	0.901	0.62-1.32	0.598	0.289	0.345	0.003	0.001	0.117

4.7 NKT-like CELLS

4.7.1 NKT-like (CD3+CD56+) cell populations

Flow cytometry gating was performed to identify the NKT-like cell subset as per Figure 2.1. Briefly total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD3+ T cells. Within this population, NKT-like cells were defined as CD56+ and expressed as a percentage of total CD3+ T cells.

4.7.1.1 Longitudinal analysis of NKT-like Cells

A mixed-effects linear regression model showed the overall effect of time was significant ($\chi^2(2) = 6.4$, $p = 0.0409$). Compared with baseline, NKT-like cells did not differ significantly at 1FU, ($\beta = 0.014$, 95% CI: -0.020-0.049, $p = 0.422$), however 2FU showed statistical significance ($\beta = 0.043$, 95% CI: 0.009-0.078, $p = 0.013$). Stage, age are gender are not statistically significant. Patients with adenocarcinoma tend to have slightly lower NKT-like cells ($\beta = -0.051$, 95%CI: 0.031-0.0639, $p = 0.097$), however this was not statistically significant. Refer to Figure 4.13.

4.6.1.2: Impact of NKT-like Cell Changes on DFS

Changes in NKT-like cell slopes were not significantly associated with recurrence (HR 0.70, 95% CI 0.33–1.47, $p = 0.346$). Mean slopes were lower in recurrent patients (0.17) compared with non-recurrent ones (0.69), suggesting reduced temporal increase. Age showed a protective effect ($p = 0.016$), while histology, stage, and complications were strong independent predictors ($p \leq 0.017$). The overall model was highly significant (LR $\chi^2 = 31.43$, $p < 0.001$). Refer to Figure 4.14.

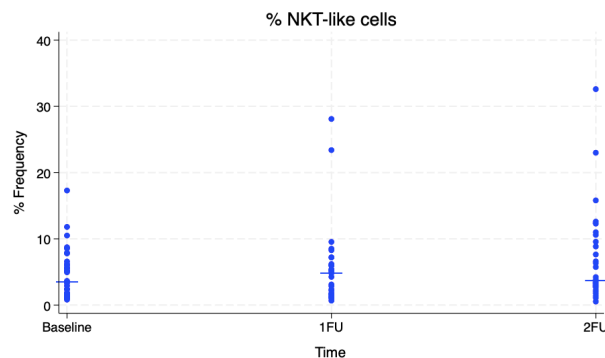


Figure 4.13. Dot plot showing % frequency of NKT-like cells at each timepoint.

Lymphocytes were gated based on forward and side scatter, followed by identification of CD3+ T cells ($n=34$). Within the CD3+ gate, NKT-like cells were identified as CD56+ and quantified as a percentage of total CD3+ T cells (% frequency). Mixed-effects linear regression showed a statistical difference over time was observed for NKT-like cells ($p = 0.0409$). Dots representing each patient and horizontal line at median value

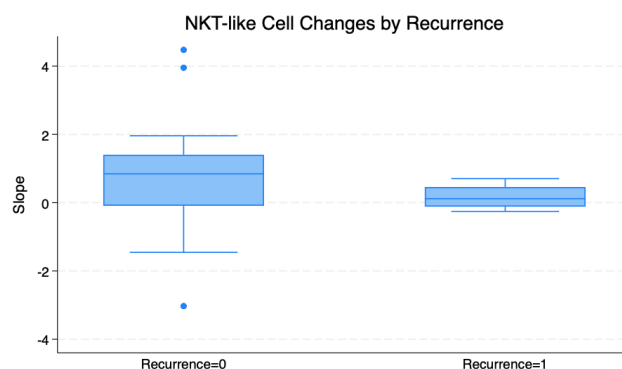


Figure 4.14. Boxplots of NKT-like cells changes (slopes) in patients with and without recurrence.

The longitudinal slope summarises the linear change over three timepoints in patients ($n=34$) for NKT-like cells. Cox proportional hazards regression was utilised to assess the association between the slope and DFS. A positive slope indicates increasing cell frequency, while a negative slope reflects a decline. Baseline was used as the reference time point for comparisons across all three time points, as well as individually against 1FU and 2FU. The slope of V δ 1 $\gamma\delta$ T cells ($p = 0.346$) over the three timepoints, was not associated with recurrence.

Table 4.27: Percent frequency of NKT-like cells

Cell Population	% Baseline	% 1FU	% 2FU
NKT-like	3.28	4.81	3.7

Table 4.28: Mixed-effects linear regression comparing baseline with 1FU and 2FU for NKT-like cells

Cell Population /Timepoint	Coefficient (β)	Std Err (SE)	p value	95% Confidence Interval
NKT-like 1FU	0.014	0.018	-0.422	-0.020-0.049
NKT-like 2FU	0.043	0.017	0.013	0.009-0.078

Table 4.29: Mixed-effects linear regression with clinicopathological characteristics for NKT-like cells

Cell Population	Chi2 (χ^2) (2)	p value	Stage II p value	Stage III p value	Age p value	Gender p value	Histology p value
NKT-like	6.4	0.0409	0.197	0.150	0.434	0.533	0.097

Table 4.30: Mixed-effects linear regression incorporating complications and surgical approach for NKT-like cells

Cell Population	Complications P value	Surgical approach p value
NKT-like	0.136	0.152

Table 4.31: Descriptive summary statistics incorporating median slope and interquartile range (IQR) for NKT-like cell populations

Cell Population	Median Slope Recurrence = 0	IQR Recurrence = 0	Median Slope Recurrence = 1	IQR Recurrence = 1
NKT-like	0.85	-0.09- 1.4	0.12	-0.15-0.46

Table 4.32: Cox Regression analysis for NKT-like cells adjusted for clinicopathological characteristics

Cell Population	Hazard Ratio	95% CI	P value	Age	Gender	Histology	Stage	Comp-lications
NKT-like	0.346	0.33-1.47	0.346	0.016	0.542	0.003	0.001	0.017

4.8 CD161+ associated T cells

4.8.1. MAIT cells

Flow cytometry gating was performed to identify the MAIT cell subset, as per Figure 2.1. Total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD3+ T cells. Within this population, MAIT cells were defined as V α 7.2+CD161+ and expressed as a percentage of total CD3+ T cells.

4.8.1.1 Longitudinal analysis of MAIT Cells

A mixed-effects linear regression model revealed the overall effect of time was significant ($\chi^2(2) = 15.75$, $p = 0.004$). Compared with baseline, MAIT cells did not differ significantly at 1FU, ($\beta = -0.006$, 95% CI: -0.015-0.002, $p = 0.184$), however 2FU showed statistical significance ($\beta = 0.012$, 95% CI: 0.002-0.021, $p = 0.009$).

Patients with Stage III disease exhibited higher levels of MAIT cells ($\beta = 0.079$, 95%CI: 0.026-0.133, $p = 0.003$) compared to Stage I, while Stage II was not significant. Refer to Figure 4.3. Patients with complications were associated with a significant lower numbers of MAIT cells at baseline ($\beta = -0.042$, 95%CI: 0.079- -0.004, $p = 0.030$). See Figure 4.15 and 4.16.

4.8.1.2: Impact of MAIT Cell Changes on DFS

MAIT-cell slopes showed a non-significant trend toward lower values in recurrent patients (mean -0.024) versus non-recurrent patients (mean 0.26). Age trended protective ($p = 0.089$), while histology, stage, and complications were significant risk factors ($p \leq 0.052$). The overall model was highly significant (LR $\chi^2 = 28.64$, $p = 0.0001$). Variability in MAIT slopes was much higher in non-recurrent patients, indicating more heterogeneity in their temporal changes. See Figure 4.17

4.8.2 CD4+ CD161+ T cells

Flow cytometry gating was performed to identify the CD4+CD161+ cell subset as per Figure 2.1. Total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD3+ T cells. Within this population, CD4+CD161+ cells were expressed as a percentage of total CD3+ T cells.

4.7.2.1 Longitudinal analysis of CD4+CD161+ T cells

A mixed-effects linear regression model revealed overall effect of time was significant ($\chi^2(2) = 6.44$, $p = 0.040$). Compared with baseline, CD4+ CD161 cells did not differ significantly at 1FU, ($\beta = 0.015$, 95% CI:-0.017-0.047, $p = 0.363$), however 2FU showed statistical significance ($\beta = 0.041$, 95% CI: 0.009-0.073, $p = 0.012$). Refer to Figure 4.15.

4.8.2.2: Impact of CD4+CD161+ T cell changes on DFS

Cox analysis shows CD4+CD161+ T cells have a non-significant trend toward protection (HR 0.905, $p = 0.163$), while older age also trends protective (HR 0.933, $p = 0.144$). Histology and stage remain strong risk factors for recurrence, with high hazard ratios and significant p-values. Median CD4+CD161+ T cells is slightly higher in recurrence cases (2.65 vs. 1.47), but variability is large. See Figure 4.17.

4.8.3 CD8+CD161+ T cells

Flow cytometry gating was performed to identify the CD8+CD161+ cell subset as depicted in Figure 2.1. Total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD3+ T cells. Within this population, CD8+CD161+ cells were expressed as a percentage of total CD3+ T cells.

4.8.3.1 Longitudinal analysis of CD8+ CD161+ T cells

A mixed-effects linear regression model revealed the overall effect of time was not significant ($\chi^2(2) = 1.34$, $p = 0.5110$). Compared with baseline, CD8+CD161+ cells did not differ significantly at 1FU, ($\beta = -0.007$, 95% CI: -0.042-0.027, $p = 0.670$) or 2FU ($\beta = 0.013$, 95% CI: -0.022-0.047, $p = 0.468$).

Stage II is associated with a statistically significant decrease in CD8+CD161+ T cells at baseline ($\beta = -0.153$, 95%CI: -0.247-0.060, $p = 0.001$) compared to Stage I, while Stage III is not significant. Refer to Figure 4.15 and 4.16.

4.8.3.2 Impact of CD8+ CD161+ T Cell Changes on DFS

Cox regression shows CD8+CD161+ T cells have a non-significant trend toward higher risk (HR 1.14, $p = 0.121$), while older age trends slightly protective (HR 0.947, $p = 0.266$). Histology, stage, and complications remain significant predictors of recurrence.

Median CD8+CD161+ T cells are slightly higher in recurrence cases (0.59 vs. 0.35), but with large variability. See Figure 4.17.

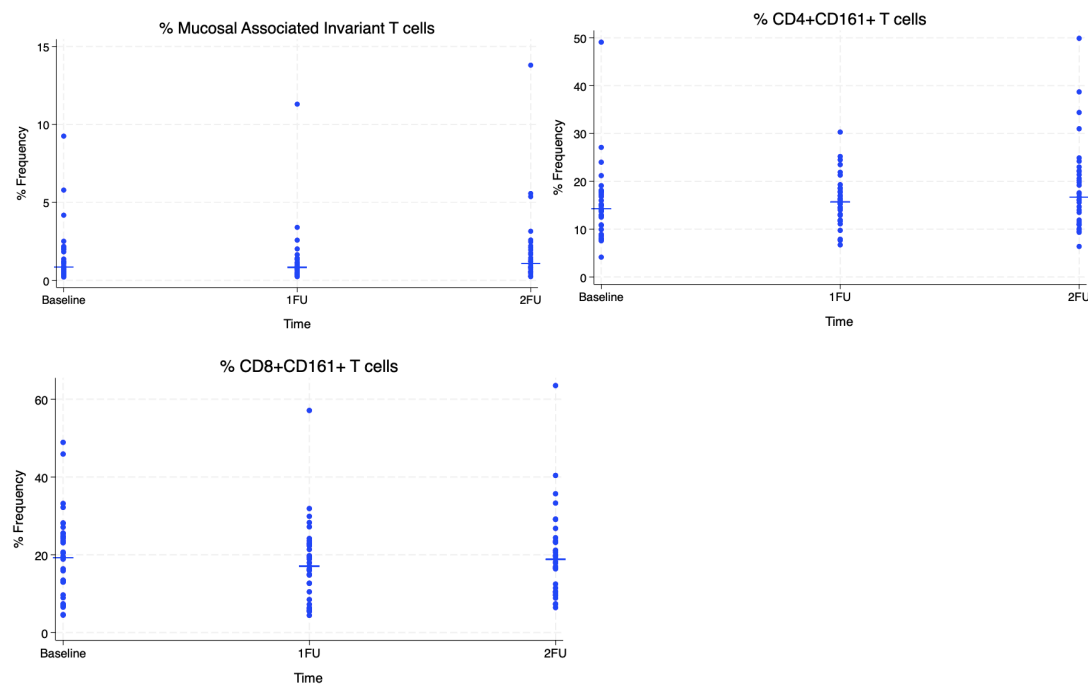


Figure 4.15. Dot plot showing % frequency of CD161+ associated T cells at each timepoint. Lymphocytes were gated based on forward and side scatter, followed by identification of CD3+ T cells ($n=34$). MAIT cells ($Va7.2+CD161+$), $CD4+CD161+$ and $CD8+CD161+$ T cells were identified within the CD3+ population and expressed as a percentage of total CD3+ T cells (% frequency). Mixed-effects linear regression showed a statistical difference over time was observed for MAIT cells ($p = 0.004$) and $CD4+CD161+$ T cells ($p = 0.040$), however there no significant difference for $CD8+CD161+$ T cells ($p = 0.5110$). Dots representing each patient and horizontal line at median value

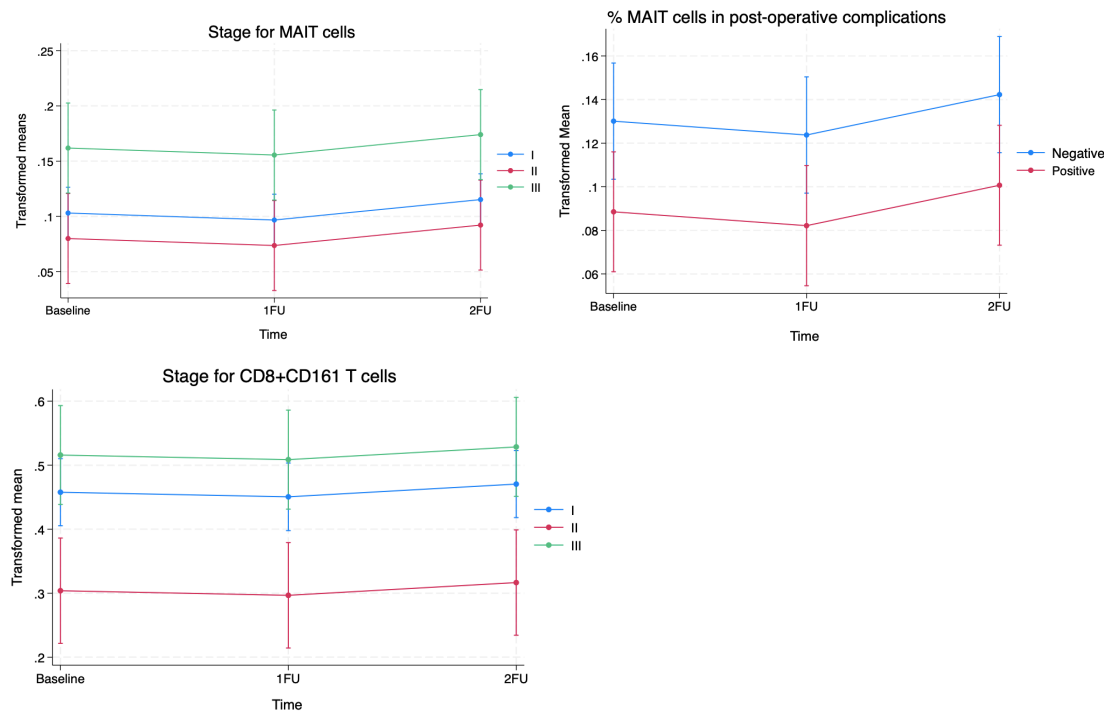


Figure 4.16. Predicted means plot showing differences over time of CD161 associated T cells in association with clinicopathological characteristics. Data obtained from FlowJo v10.10.0 software, were expressed as percentage frequencies and subsequently transformed using the arcsine-square root transformation to meet the assumptions of parametric analysis. Plotted values in this figure represent transformed means. To evaluate changes across the three timepoints, linear mixed-effects models were applied in association with age, gender, histological subtype, disease stage, surgical approach, and the presence or absence of post-operative complications. Patients with Stage III disease exhibited higher levels of MAIT cells ($p = 0.003$) compared to Stage I, while Stage II was not significant. Patients with complications were associated with a significant lower numbers of MAIT cells at baseline ($p = 0.030$). Stage II is associated with a statistically significant decrease in CD8+CD161+ T cells at baseline ($p = 0.001$) compared to Stage I, while Stage III is not significant.

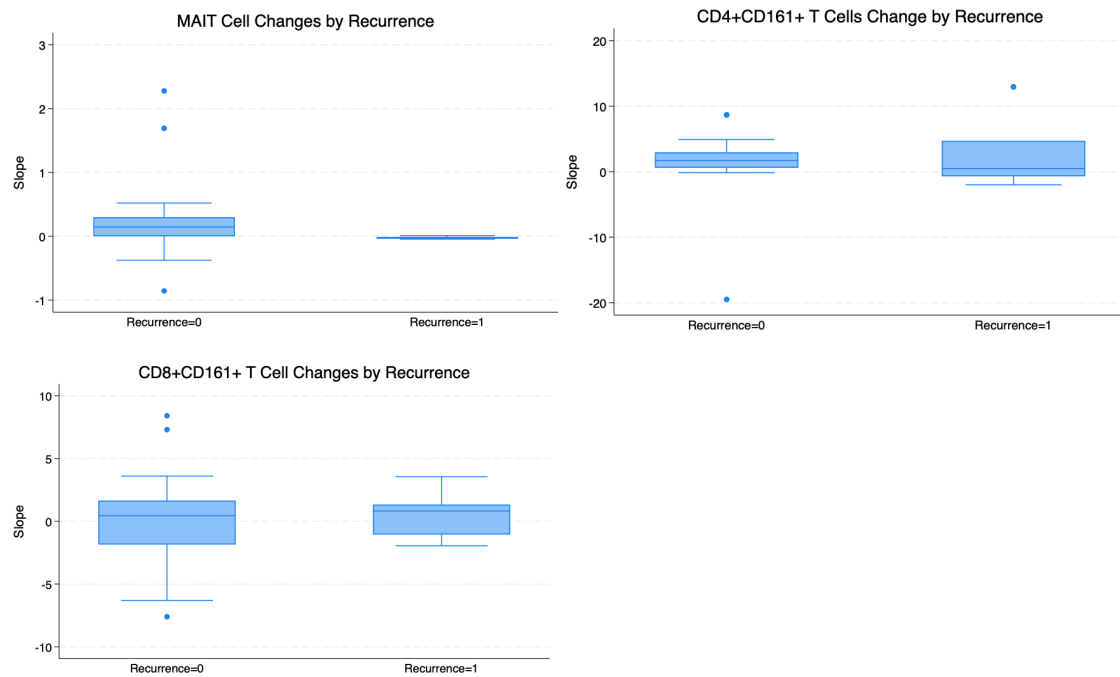


Figure 4.17. Boxplots of CD161+ associated T cells changes (slopes) in patients with and without recurrence. The longitudinal slope summarises the linear change over three timepoints in patients ($n=34$) for MAIT, CD4+CD161+ and CD8+ CD161+ T cells. Cox proportional hazards regression was utilised to assess the association between the slope and DFS. A positive slope indicates increasing cell frequency, while a negative slope reflects a decline. Baseline was used as the reference time point for comparisons across all three time points, as well as individually against 1FU and 2FU. The slope of MAIT ($p = 0.504$), and CD4+ CD161+ T cells ($p = 0.163$) and CD8+ CD161+ T cells ($p = 0.121$) over the three timepoints were not associated with recurrence.

Table 4.33: Percent frequency of MAIT and CD161+ cells

Cell Population	% Baseline	% 1FU	% 2FU
MAIT 1FU	0.88	0.88	1.1
CD4+ CD161+	14.3	15.9	16.7
CD8+ CD161+	19.6	17.1	19.5

Table 4.34: Mixed-effects linear regression comparing baseline with 1FU and 2FU for MAIT and CD161+ T cells

Time	Coefficient (β)	Std Err (SE)	p value	95% Confidence Interval
MAIT 1FU	-0.006	0.005	0.184	-0.015-0.002
MAIT 2FU	0.012	0.004	0.009	0.002-0.021
CD4+ CD161+ 1FU	0.015	0.016	0.363	-0.017-0.047
CD4+ CD161+ 2FU	0.041	0.016	0.012	0.009-0.073
CD8+ CD161+ 1FU	-0.007	0.017	0.670	-0.042-0.027
CD8+ CD161+ 2FU	0.013	0.018	0.468	-0.022-0.047

Table 4.35: Mixed-effects linear regression with clinicopathological characteristics for MAIT and CD161+ T cells

Cell populations	Chi2 (χ^2) (2)	p value	Stage II p value	Stage III p value	Age p value	Gender p value	Histology p value
MAIT cells	15.75	0.0004	0.223	0.003	0.185	0.375	0.772
CD4+ CD161+	6.44	0.0400	0.983	0.559	0.301	0.597	0.460
CD8+ CD161+	1.34	0.5110	0.001	0.213	0.560	0.315	0.314

Table 4.36: Mixed-effects linear regression incorporating complications and surgical approach for MAIT and CD161+ T cells

Cell populations	Complications P value	Surgical approach p value
MAIT	0.030	0.931
CD4+ CD161+	0.106	0.733
CD8+ CD161+	0.232	0.951

Table 4.37: Descriptive summary statistics incorporating median slope and interquartile range (IQR) for MAIT and CD161+ T cells

Cell Population	Median Slope Recurrence = 0	IQR Recurrence = 0	Median Slope Recurrence = 1	IQR Recurrence = 1
MAIT	0.15	0- 0.30	-0.03	-0.04--0.01
CD4+ CD161+	1.7	0.60-2.95	0.48	-0.70-4.7
CD8+ CD161+	0.45	-1.85-1.65	0.82	-1.05-1.33

Table 4.38: Cox Regression analysis for MAIT and CD161+ T cells adjusted for clinicopathological characteristics

Cell Population	Haz Ratio	95% CI	P value	Age	Gender	Histology	Stage	Comp- lications
MAIT	0.401	0.03- 5.83	0.504	0.089	0.88	0.019	0.004	0.052
CD4+ CD161+	0.161	0.79- 1.04	0.163	0.144	0.284	0.002	0.001	0.161
CD8+ CD161+	1.14	0.97- 1.35	0.121	0.266	0.323	0.001	0.001	0.029

4.9 B CELLS

4.9.1 B cell population

Flow cytometry gating was performed to identify the B cells, as per Figure 2.1. Total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD19+ cells.

4.9.1.1 Longitudinal analysis of B cells

A mixed-effects linear regression model showed the overall effect of time was not significant ($\chi^2(2) = 0.68$, $p = 0.7123$). Compared with baseline, B cells did not differ significantly at 1FU, ($\beta = 0.004$, 95% CI: -0.022-0.029, $p = 0.775$) or 2FU ($\beta = -0.007$, 95%CI: 0.033-0.0187, $p = 0.596$). Time, stage, gender, age, and histology showed no statistically significant effects at the 0.05 level, although stage II ($p = 0.072$) and gender ($p = 0.084$) showed marginal trends. See Figure 4.18.

4.9.1.2: Impact of B Cell Changes on DFS

Cox regression shows higher B cell slope is protective against recurrence (HR 0.65, $p = 0.048$), with a trend for younger age slightly increasing risk (HR 0.90, $p = 0.075$). Histology and stage remain strong, significant predictors of recurrence. Median B cell slope values are lower in recurrence cases (-0.49 vs. 0.52). See Figure 4.19.

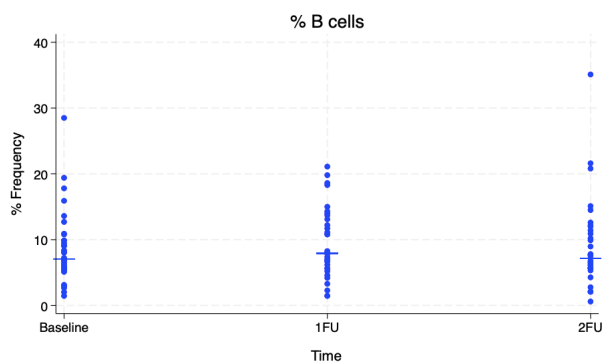


Figure 4.18. Dot plot showing % frequency of B cells at each timepoint. Total cells were first filtered to isolate single, viable cells. Lymphocytes were then selected using forward- and side-scatter properties, followed by gating of CD19+ cells (% frequency of lymphocytes). Mixed-effects linear regression showed no statistical difference over time observed for B cells ($p = 0.7123$). Dots representing each patient and horizontal line at median value

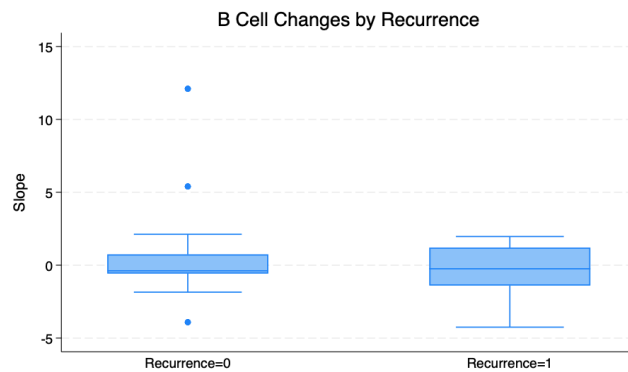


Figure 4.29. Boxplots of B cells changes (slopes) in patients with and without recurrence. The longitudinal slope summarises the linear change over three timepoints in patients ($n=34$) for B cells. Cox proportional hazards regression was utilised to assess the association between the slope and DFS. A positive slope indicates increasing cell frequency, while a negative slope reflects a decline. Baseline was used as the reference time point for comparisons across all three time points, as well as individually against 1FU and 2FU. The slope of B cells ($p = 0.504$) over the three timepoints was associated with recurrence.

Table 4.39: Percent frequency and descriptive statistics of B cells

Cell Population	% Baseline	% 1FU	% 2FU
B cells	7.07	7.92	7.2

Table 4.40: Mixed-effects linear regression comparing baseline with 1FU and 2FU for B cells

Time	Coefficient (β)	Std Err (SE)	p value	95% Confidence Interval
B cells 1FU	0.004	0.013	0.775	-0.022-0.029
B cells 2FU	-0.007	0.013	0.596	0.033-0.0187

Table 4.41: Mixed-effects linear regression with clinicopathological characteristics for B cells

Statistical Analysis	Chi2 (χ^2) (2)	p value	Stage II p value	Stage III p value	Age p value	Gender p value	Histology p value
B cells	0.68	0.7123	0.072	0.541	0.390	0.084	0.229

Table 4.42: Mixed-effects linear regression incorporating complications and surgical approach for B cells

Cell populations	Complications P value	Surgical approach p value
B cells	0.226	0.716

Table 4.43: Descriptive summary statistics incorporating median slope and interquartile range (IQR) for B cell populations

Cell Population	Median Slope Recurrence = 0	IQR Recurrence = 0	Median Slope Recurrence = 1	IQR Recurrence = 1
B cells	-0.39	-0.58- 0.74	-0.25	-1.4-1.2

Table 4.44: Cox Regression analysis for B cells adjusted for clinicopathological characteristics

Cell Population	Hazard Ratio	95% CI	P value	Age	Gender	Histology	Stage	Complications
B cells	0.14	0.42-0.99	0.048	0.075	0.881	0.001	0.001	0.088

4.10 NATURAL KILLER CELLS

4.10.1 NK cell populations

Flow cytometry gating was performed to identify NK cells as per Figure 2.1. Briefly, lymphocytes were first gated based on forward and side scatter, followed by selection of CD3- CD56+ cells

4.10.1.1 Longitudinal analysis of NK cells

On analysis using mixed-effects linear regression model, the overall effect of time was significant ($\chi^2(2) = 16.54$, $p = 0.0003$). Compared with baseline, NK cells did not differ significantly at 1FU, ($\beta = 0.014$, 95% CI: -0.029-0.057, $p = 0.537$), however 2FU showed statistical significance ($\beta = 0.083$, 95% CI: 0.040-0.125, $p < 0.001$). Adenocarcinoma histology had higher NK cell populations at baseline compared to squamous ($\beta = 0.067$, 95% CI: 0.004-0.130, $p = 0.036$) with a similar increase over time. Refer to Figure 4.20 and 4.21.

4.10.1.2: Impact of NK Cell Changes on DFS

Higher NK cell slope shows a trend toward increased recurrence risk (HR 1.14, $p = 0.103$), while younger age slightly increases risk (HR 0.91, $p = 0.095$). Histology and stage remain strong, significant predictors of recurrence. Median NK values are higher in recurrence cases (4.09 vs. 2.06). Overall, NK elevation trends with recurrence, but tumour characteristics dominate risk prediction. Refer to Figure 4.22.

4.10.2 NK CD16+ cell populations

Flow cytometry gating was performed to identify NK cells as per Figure 2.1. Briefly, total cells, single cells, live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD3+ CD56- cells, following this NK CD16+ cells were identified using CD16 gate.

4.10.2.1 Longitudinal analysis of NK CD16+ cells

A mixed-effects linear regression model showed the overall effect of time was significant ($\chi^2(2) = 15.67$, $p = 0.0004$). Compared with baseline, NKCD16+ significantly differed at 1FU ($\beta = -0.148$, 95% CI: -0.026- -0.036, $p = 0.010$), and 2FU ($\beta = -0.221$, 95% CI: -0.332- -0.109, $p = 0.000$).

In the mixed-effects model for NK CD16+ cells, Stage III was significantly associated with lower NKCD16 levels compared to Stage I ($\beta = -0.282$, CI: -0.545- -0.018 $p = 0.036$), while Stage II showed a non-significant trend toward lower levels ($\beta = -0.176$, 95% CI: -0.437- 0.086, $p = 0.189$). Refer to Figure 4.20 and 4.21.

4.10.2.2: Impact of NK CD16+ cell changes on DFS

Higher NK CD16+ slope is significantly associated with increased recurrence risk (HR 1.075, 95% CI 1.016–1.138, $p = 0.012$), while age shows a non-significant trend (HR 0.920, 95% CI 0.816–1.037). Histology (HR 7.374, 95% CI 1.963–27.693, $p = X$) and stage (HR 11.611, 95% CI 2.952–45.663, $p = Y$) remain strong, independent predictors of recurrence. Median NK CD16 slopes are higher in recurrence cases (-3.39 vs. -8.42). Overall, elevated NK CD16+ activity trends with recurrence, suggesting its potential as a prognostic immune biomarker. See Figure 4.22.

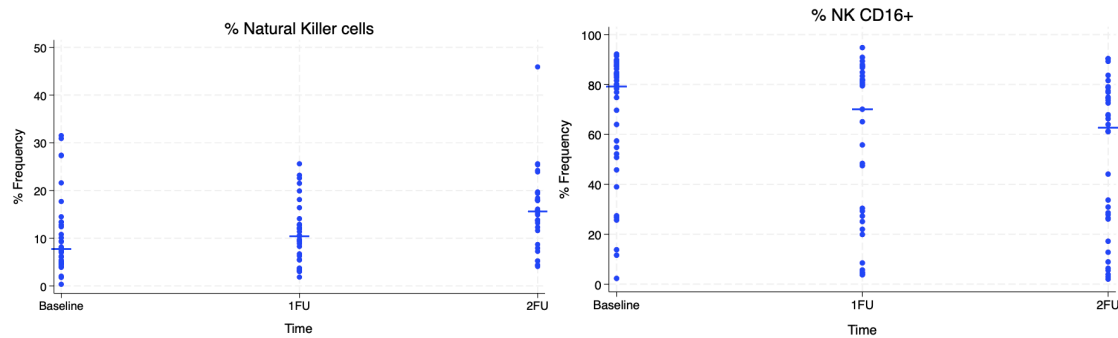


Figure 4.20. Dot plot showing % frequency of NK cells at each timepoint. Total cells, single, cells, live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by identification of CD3- and CD56+ cells (NK cells) and then CD16+ cells (NK CD16+ cells) and expressed as a total percent frequency of lymphocytes. Mixed-effects linear regression showed statistical significant difference over time for NK cells ($p = 0.0003$) and NK CD16+ cells ($p = 0.0004$.) Dots representing each patient and horizontal line at median value

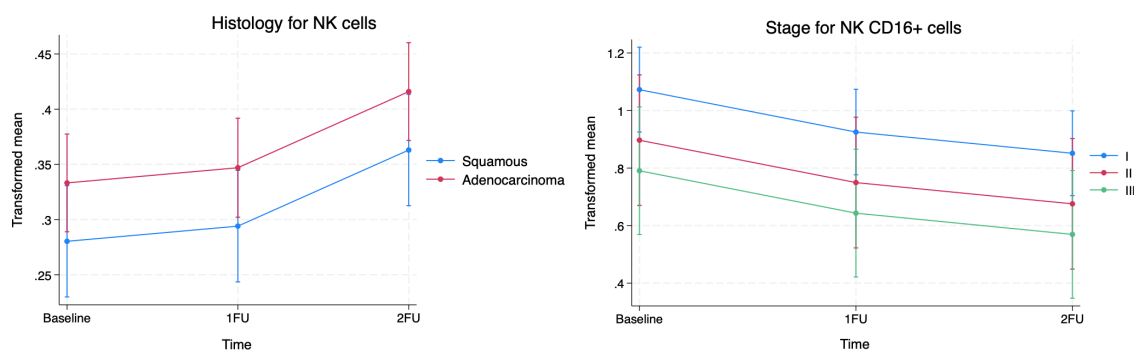


Figure 4.21. Predicted means plot showing differences over time of NK cells in association with clinicopathological characteristics. Data obtained from FlowJo v10.10.0 software, were expressed as percentage frequencies and subsequently transformed using the arcsine-square root transformation to meet the assumptions of parametric analysis. Plotted values in this figure represent transformed means. To evaluate changes across the three timepoints, linear mixed-effects models were applied in association with age, gender, histological subtype, disease stage, surgical approach, and the presence or absence of post-operative complications. Adenocarcinoma histology had higher NK cell populations at baseline compared to squamous ($p = 0.036$) with a similar increase over time. Stage III was significantly associated with lower NK CD16+ levels compared to Stage I ($p = 0.036$), while Stage II was not significant.

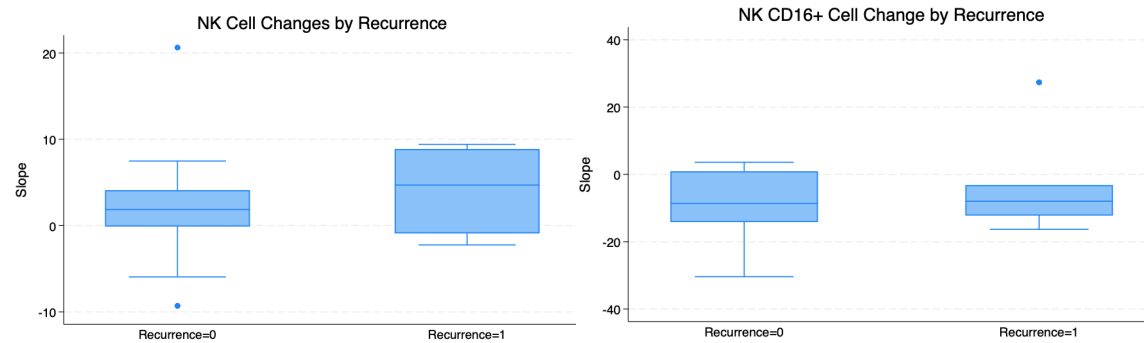


Figure 4.22. Boxplots of NK cells subsets changes (slopes) in patients with and without recurrence. The longitudinal slope summarises the linear change over three timepoints in patients ($n=34$) for NK and NK CD16+ cells. Cox proportional hazards regression was utilised to assess the association between the slope and DFS. A positive slope indicates increasing cell frequency, while a negative slope reflects a decline. Baseline was used as the reference time point for comparisons across all three time points, as well as individually against 1FU and 2FU. The slope of NK cells ($p = 0.103$) over the three timepoints was not associated with recurrence, however a higher slope was associated with recurrence for NK CD16+ cells ($p = 0.012$).

Table 4.45: Percent frequency and descriptive statistics of NK cells

Cell Population	% Baseline	% 1FU	% 2FU
NK	8.1	10.4	15.6
NK CD16+	79	70.1	62.7

Table 4.46: Mixed-effects linear regression comparing baseline with 1FU and 2FU for NK cells

Cell population / Timepoint	Coefficient (β)	Std Err (SE)	p value	95% Confidence Interval
NK 1FU	0.014	0.022	0.537	-0.029-0.057
NK 2FU	0.083	0.022	<0.001	0.040-0.125
NK CD16+ 1FU	-0.149	0.057	0.010	-0.262- -0.036
NK CD16+ 2FU	-0.221	0.057	<0.001	-0.332 - -0.109

Table 4.47: Mixed-effects linear regression with clinicopathological characteristics for NK cells

Statistical Analysis	Chi2 (χ^2) (2)	p value	Stage II p value	Stage III p value	Age p value	Gender p value	Histology p value
NK	16.54	0.0003	0.773	0.295	0.493	0.671	0.036
NK CD16+	15.67	0.0004	0.189	0.036	0.202	0.723	0.742

Table 4.48: Mixed-effects linear regression incorporating complications and surgical approach for NK cells

Cell populations	Complications P value	Surgical approach p value
NK	0.373	0.175
NK CD16+	0.721	0.653

Table 4.49: Descriptive summary statistics incorporating median slope and interquartile range (IQR) for NK cell populations

Cell Population	Median Slope Recurrence = 0	IQR Recurrence = 0	Median Slope Recurrence = 1	IQR Recurrence = 1
NK	1.85	-0.11- 4.09	4.69	-0.9-8.88
NK CD16+	-8.65	-14.15-0.90	-7.08	-12.25-3.2

Table 4.50: Cox Regression analysis for NK cells adjusted for clinicopathological characteristics

Cell Population	Hazard Ratio	95% CI	P value	Age	Gender	Histology	Stage	Complications
NK	0.103	0.97-1.34	0.103	0.095	0.161	0.005	0.001	0.133
NK CD16+	1.07	1.02-1.14	0.012	0.174	0.290	0.003	<0.001	0.137

4.11 MONOCYTES

4.11.1 Monocyte cell populations

Flow cytometry gating was performed to identify NK cells as per Figure 2.1. Briefly, total cells, single cells and live cells were identified, followed by selection of CD14⁺ CD16⁺ cells.

4.11.1.1 Longitudinal analysis of monocytes

A mixed-effects linear regression model revealed overall effect of time was not significant ($\chi^2(2) = 2.87$, $p = 0.2382$). Compared with baseline, monocytes cells did not differ significantly at 1FU, ($\beta = -0.021$, 95% CI: -0.049-0.070, $p = 0.141$) or 2FU ($\beta = -0.000$, 95% CI: -0.027-0.028, $p = 0.999$).

In the mixed-effects model for monocyte expression, Stage II was significantly associated with lower monocyte levels compared to Stage I ($\beta = -0.120$, 95% CI: -0.203-0.036, $p = 0.005$), while Stage III showed a non-significant trend toward lower levels ($\beta = -0.054$, 95% CI: -0.137-0.030, $p = 0.209$). Refer to Figure 4.23 and 4.24.

4.11.1.2: Impact of Monocyte changes on DFS

Higher monocyte slope trends toward increased recurrence risk (HR 1.537, 95% CI 0.928–2.545, $p=0.095$), though not statistically significant. Age and gender show no clear effect, while histology and stage strongly predict recurrence (HR 4.137, 95% CI 0.979–17.483; HR 7.681, 95% CI 2.443–24.154). Median monocyte slope is slightly negative in non-recurrence (-0.61) but positive in recurrence (0.74). Refer to Figure 4.25.

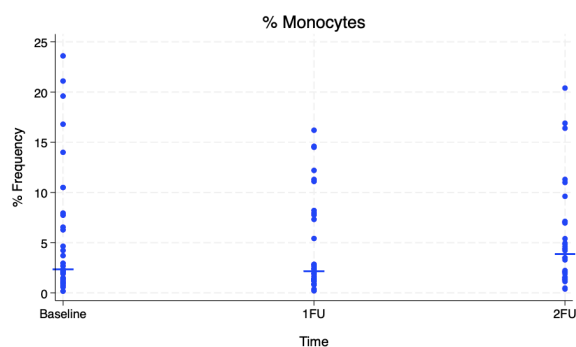


Figure 4.23. Dot plot showing % frequency of monocytes at each timepoint. Total cells were first filtered to isolate single, viable cells, followed by gating of CD14⁺ and CD16⁺ cells (% frequency of total cells). Mixed-effects linear regression showed no statistical difference over time observed for monocytes ($p = 0.2382$). Dots representing each patient and horizontal line at median value.

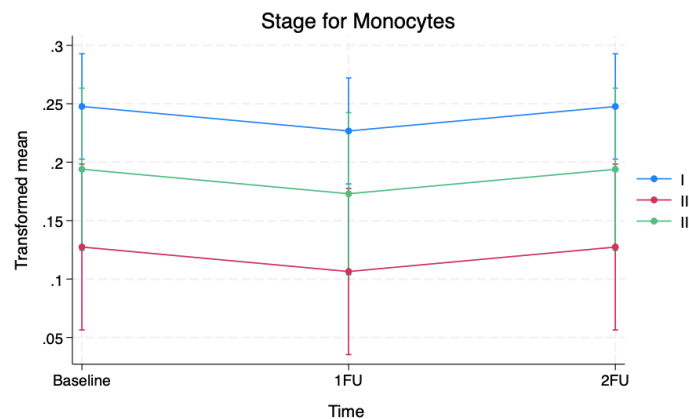


Figure 4.24. Predicted means plot showing differences over time of monocytes in association with clinicopathological characteristics. Data obtained from FlowJo v10.10.0 software, were expressed as percentage frequencies and subsequently transformed using the arcsine-square root transformation to meet the assumptions of parametric analysis. Plotted values in this figure represent transformed means. To evaluate changes across the three timepoints, linear mixed-effects models were applied in association with age, gender, histological subtype, disease stage, surgical approach, and the presence or absence of post-operative complications. Stage II was significantly associated with lower monocyte levels compared to Stage I ($p = 0.005$), while Stage III showed a non-significant trend toward lower levels ($p = 0.209$).

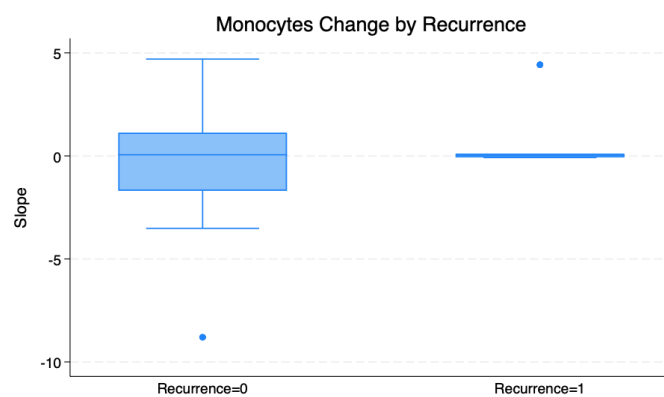


Figure 4.25. Boxplots of monocytes changes (slopes) in patients with and without recurrence. The longitudinal slope summarises the linear change over three timepoints in patients ($n=34$) for monocytes. Cox proportional hazards regression was utilised to assess the association between the slope and DFS. A positive slope indicates increasing cell frequency, while a negative slope reflects a decline. Baseline was used as the reference time point for comparisons across all three time points, as well as individually against 1FU and 2FU. The slope of monocytes ($p = 0.095$) over the three timepoints was not associated with recurrence.

Table 4.51: Percent frequency and descriptive statistics of monocytes

Cell Population	% Baseline	% 1FU	% 2FU
Monocytes	2.4	2.2	3.9

Table 4.52: Mixed-effects linear regression comparing baseline with 1FU and 2FU for monocytes

Time	Coefficient (β)	Std Err (SE)	p value	95% Confidence Interval
Monocytes 1FU	-0.021	0.014	0.141	-0.049-0.070
Monocytes 2FU	-0.000	0.014	0.999	-0.027-0.028

Table 4.53: Mixed-effects linear regression with clinicopathological characteristics for monocytes

Cell Population	Chi2 (χ^2) (2)	p value	Stage II p value	Stage III p value	Age p value	Gender p value	Histology p value
Monocytes	2.87	0.2382	0.005	0.209	0.462	0.591	0.181

Table 4.54: Mixed-effects linear regression incorporating complications and surgical approach for monocytes

Cell populations	Complications P value	Surgical approach p value
Monocytes	0.193	0.141

Table 4.55: Descriptive summary statistics incorporating median slope and interquartile range (IQR) for monocytes

Cell Population	Median Slope Recurrence = 0	IQR Recurrence = 0	Median Slope Recurrence = 1	IQR Recurrence = 1
Monocytes	0.55	-1.69- 1.13	0.35	-0.07-0.11

Table 4.56: Cox Regression analysis for monocytes adjusted for clinicopathological characteristics

Cell Population	Hazard Ratio	95% CI	P value	Age	Gender	Histology	Stage	Complications
Monocytes	1.54	0.93-2.55	0.095	0.587	0.391	0.053	0.000	0.404

4.12 DISCUSSION

4.12.1. Mixed-Effects Modelling of Immune Dynamics

Longitudinal analysis showed CD4⁺ T cell populations remained stable, whereas CD8⁺ T cells were dynamic. Naïve CD8⁺ T cells decreased from a median of 20.3% to 14.3% ($\chi^2(2) = 22.02$, $p < 0.001$) at 2FU and did not recover by 6–9 weeks postoperatively. This likely reflects activation and redistribution to tissues following surgical stress [18,19]. CD8⁺ EM cells were stable overall, but increased in advanced-stage disease or adenocarcinoma ($p \leq 0.003$), with Stage II highest. This suggests chronic antigen exposure drives expansion and maintenance of memory-like effector cells [37,41] in Stage II with diminished responses observed in Stage III. CD8⁺ TDEM T cells transiently decreased at 1FU (17.1% vs. 23.4% baseline) and were lower in advanced disease ($p < 0.035$) and adenocarcinoma ($p < 0.001$). TDEM T cells retain rapid cytotoxic potential but exhibit limited proliferative capacity [37,41]. Stage II disease had lower numbers compared to Stage III or Stage I. This pattern likely reflects a robust response in Stage I, followed by redistribution into the TME in Stage II, and subsequent TME-mediated suppression along with systemic immune inhibition in Stage III [37,41]. Adenocarcinoma is potentially associated with higher mutational burden and neoantigen diversity [96], which may drive stronger antigen-specific CD8⁺ memory responses. This is particularly evident in adenocarcinoma among smokers, which is especially pertinent to our cohort, as individuals with a history of smoking, constitute 91% of the study population. Chronic stimulation is known to lead to functional exhaustion, resulting in high peripheral counts, without full cytotoxic competence cells [37,41].

CD4⁺ Tregs increased from 2.32% at baseline to 2.67% at 1FU and 3.9% at 2FU ($\chi^2(2) = 11.79$, $p = 0.0027$). Stage III patients had higher CD4⁺ Tregs ($p = 0.031$), indicating pre-existing immunosuppressive activity in advanced disease. Age-related differences suggest immunosenescence, with older patients showing small increases or decreases, whereas

younger patients have minimal or negative changes ($p = 0.008$). CD8⁺ Tregs remained stable (2.3–2.4%). The postoperative rise in CD4⁺ Tregs likely reflects a homeostatic response to surgical stress and tissue injury [85], aiming to limit excessive inflammation, with CD8⁺ Tregs less sensitive to surgical stress or tumour driven signals.

V δ 1 $\gamma\delta$ T cells were stable over time but declined with age ($p = 0.001$). Open surgery patients had lower V δ 2 $\gamma\delta$ T cells ($p = 0.021$). These findings highlight that age and type of surgery impact $\gamma\delta$ T cell populations, potentially affecting anti-tumour immune competence in the perioperative period [46,47]. NKT-like cells increased over time ($\chi^2(2) = 6.4$, $p = 0.041$), particularly at 2FU. MAIT cells increased ($\chi^2(2) = 15.75$, $p = 0.004$), highest in Stage III ($p = 0.003$); Stage II had lower CD8⁺CD161⁺ T cells ($p = 0.001$). MAIT cells are recognized for their dual, paradoxical roles in cancer immunity, contributing to both chronic inflammation and to anti-tumour responses [51,52]. This pattern may reflect Stage II-associated migration of MAIT cells into tissues, with progression to Stage III promoting chronic inflammation and systemic recruitment of these cells [51,52]. Postoperative complications were associated with lower MAIT cells ($p = 0.030$).

NK cells increased ($\chi^2(2) = 16.54$, $p = 0.0003$), while the proportion of CD16⁺ NK cells decreased significantly ($\chi^2(2) = 15.7$, $p = 0.0004$). Previous studies have primarily described transient postoperative suppression of NK-cell function rather than changes in NK-cell abundance [37,77]. Restoration of NK-cell populations after tumour resection may occur through the alleviation of tumour-induced immune suppression and reduced expression of exhaustion markers [97]. The divergent trajectories of total NK cells and the CD16⁺ subset suggest dynamic postoperative remodelling of the NK-cell compartment, potentially reflecting alterations in NK-cell phenotype and functional state during immune recovery [37,76,77].

Monocytes were stable, lowest in Stage II ($p = 0.005$), suggesting a stage-specific modulation of the myeloid compartment. This is likely caused by differentiation and recruitment of monocytes as TAMs in the TME in Stage II, with systemic inflammation leading to their suppression in later stages [11,32]. B cells remained stable over the perioperative period and were not influenced by clinical or tumour characteristics,

suggesting that this lymphocyte subset is largely unaffected by surgical stress or disease variables.

In summary, CD8⁺ T cells, CD4⁺ Tregs, NK, NKT-like, and MAIT cells exhibit perioperative changes, while CD4⁺ T cells, B cells, monocytes, and $\gamma\delta$ T cells remain largely stable. Patterns are influenced by age, surgery type, disease stage, and tumour type. Advanced disease is linked to dynamic CD8⁺ T cell responses, increased Tregs, MAIT cell expansion, and reduced monocytes, reflecting perioperative immunoregulation and immune exhaustion.

4.12.2. Associations with Disease-Free Survival

Slope differences in immune subsets were associated with DFS. Negative slopes, representing declining cell frequencies over time, were observed in CD8⁺ naïve T cells and CD16⁺ NK cells in patients who experienced recurrence. In contrast, positive slopes, reflecting increasing cell frequencies, were seen in B cells in non-recurrent patients, however CD4⁺ CM T cells and CD8⁺ EM T cells cell slopes were elevated in recurrent cases.

Increased CD8⁺ EM T cells over time were seen in patients with recurrence (median slope 3.15 vs. 1.20, $p = 0.001$), alongside a more pronounced decline in CD8⁺ naïve T cells (median slope -4.10 vs. -2.55 , $p = 0.003$). These findings are consistent with persistent antigen exposure driving differentiation from naïve to antigen-experienced T-cell populations and have been associated with reduced immune repertoire diversity and T-cell dysfunction in cancer [34,37, 98,99]. In addition, a higher rate of change in CD4⁺ CM T cells was associated with an increased risk of recurrence (median slope 1.35 vs. 0.37, $p = 0.032$). Given the role of central memory T cells in maintaining antigen-specific immune responses, this may reflect ongoing immune stimulation by residual tumour antigen following surgery [100,101].

Patients who developed recurrence demonstrated a greater decline in CD16⁺ NK cells over time compared with those who remained disease-free (median slope -8.65 vs. -7.08 , $p = 0.012$). As CD16⁺ NK cells represent the most cytotoxic NK-cell subset, their reduction may reflect impaired innate anti-tumour immunity [102]. Similar alterations have been reported within the NSCLC tumour microenvironment, where NK cells exhibit reduced

CD16 expression and diminished cytotoxic function, suggesting that persistent tumour-associated immune suppression may contribute to recurrence [34,37,102,103].

In contrast B cells were protective with median slopes significantly lower in patients who experienced recurrence (HR 0.65, $p = 0.048$). B cells can mediate tumour suppression through multiple mechanisms, including the production of tumour-specific antibodies, antigen presentation to T cells, and the formation of tertiary lymphoid structures, which have been linked to improved outcomes in various cancers [32,54].

These findings suggest that the dynamic shifts of memory, naïve T cell and NK populations may reflect an immunologic milieu favouring relapse, with histology, stage, and post-operative complications further modulating outcomes, highlighting the prognostic relevance of longitudinal immune monitoring.

CHAPTER 5

RESULTS

Immune Checkpoint Proteins

5.1 INTRODUCTION

ICPs are inhibitory receptors on activated immune cells, including T cells, NK cells, and B cells, exploited by tumours to evade immune responses [62,25]. Key ICPs—PD1, CTLA4, LAG3, TIM3, and BTLA—regulate T cell activation, enhance Treg function, and under chronic antigen exposure drive immune exhaustion [17,19,20,23,63-65]. In NSCLC, these ICPs are upregulated on TILs and peripheral immune cells, contributing to immunosuppression, with co-expression of PD1 and TIM3 on CD8⁺ TILs correlating with poor prognosis [23,64,65]. Most research focuses on tumour cells or advanced disease, with limited data on peripheral ICPs during the peri-operative period, particularly BTLA, LAG3, and CTLA4 [23,24,60,101]. Studies by Xu et al. report peri-operative increases in PD1 on immune cells peaking around day 5, influenced by surgical stress and procedure type, while TIM3 on NK and NKT cells decreases postoperatively [18,65].

This study examines peri-operative expression of PD1, CTLA4, LAG3, TIM3, BTLA, and PD1/TIM3 co-expression, chosen for their roles in immune suppression, T cell exhaustion, prognostic relevance in NSCLC, and post-operative modulation. Investigating these markers provides critical insights into dynamic immune checkpoint regulation and potential windows for peri-operative immunotherapeutic intervention. Future work will extend this approach to assess more co-expression patterns across additional ICPs.

5.2 PATIENT CHARACTERISTICS

Patient demographics clinicopathological characteristics, surgical approach, and post-operative complications are as described in chapter 4. An arcsine square-root transformation was applied prior to performing linear mixed-effects models for the analysis of longitudinal data, using the same clinicopathological variables outlined in chapter 4. A random intercept for participant ID was included in all models to account for baseline differences between individuals, ensuring that observed time effects reflect true within-person change rather than between-person variability. For survival analysis, only DFS was considered due to no NSCLC related mortality in this cohort, with median follow of 14 months. Kaplan–Meier and life table analyses were conducted as detailed in Chapter 4. Cox proportional hazards regression was employed to evaluate recurrence statistics; incorporating the same clinicopathological variables described in chapter 4.

5.3 PD1

5.3.1 PD1 on CD4+ T cells

Flow cytometry gating was performed to identify CD4+ PD1+ T cells as per Figure 2.2. Briefly, total cells, single cells and live cells were identified and then lymphocytes gated based on forward and side scatter, followed by selection of CD3+CD4+ T cells. Within this population PD1+ was expressed as a percentage of total CD4+ T cells.

5.3.1.1 Longitudinal analysis of CD4+PD1+ T cells

A mixed-effects linear regression model with random intercepts for participant was fitted to examine changes in CD4+ PD1+ T cell expression across time points. The overall effect of time was significant ($\chi^2(2) = 23.72$, $p < 0.001$). Compared with baseline, PD1 expression on CD4+ T cells was significantly higher at 1FU ($\beta = 0.119$, 95% CI: 0.046–0.192, $p = 0.001$) and at 2FU ($\beta = 0.176$, 95% CI: 0.104–0.248, $p < 0.001$).

Stage II ($\beta = 0.187$, 95% CI: 0.022–0.351, $p = 0.026$) and Stage III ($\beta = 0.203$, 95% CI: 0.040–0.366, $p = 0.014$) exhibited higher CD4+ PD1+ expression relative to Stage I at baseline, Gender was associated with increased CD4+ PD1+ expression ($\beta = 0.226$, 95% CI: 0.122–0.330, $p < 0.001$), indicating higher expression in females. Histology, surgical approach and post-operative complications were not significant ($p > 0.2$). Refer to Figure 5.1 and 5.2 and Table 5.1-5.4 for details.

5.3.1.2: Impact of CD4+ PD1+ T cell changes on DFS

PD1+ expression on CD4+ T cells increased over time in both recurrence and non-recurrence groups, with a higher median slope in those who recurred (11.1) compared to those that didn't (2.1). However, this temporal change was not associated with recurrence (HR 1.05, 95% CI 0.96–1.14, $p = 0.31$). Histology (HR 6.39, 95% CI 1.61–25.40) and stage (HR 4.48, 95% CI 1.51–13.30) remained strong independent predictors of recurrence. Overall, CD4+PD1+ T cells slope did not demonstrate prognostic significance in this cohort. Refer to Figure 5.3 for DFS and Figure 5.4 for representative flow cytometry plots and Tables 5.5 & 5.6.

5.3.2 PD1+ on CD8+ T cells

Flow cytometry gating was performed to identify the CD8+ PD1+ T cells as per Figure 2.2. . Briefly, total cells, single cells and live cells were identified and then lymphocytes gated

based on forward and side scatter, followed by selection of CD3+CD8+ T cells. Within this population PD1+ was expressed as a percentage of total CD8+ T cells.

5.3.2.1 Longitudinal analysis of CD8+PD1+ T Cells

Mixed regression model revealed overall effect of time was significant ($\chi^2(2) = 26.68$, $p < 0.001$). Compared with baseline, CD8+ PD1+ expression was significantly higher at 1FU ($\beta = 0.110$, 95% CI: 0.044–0.176, $p = 0.001$) and 2FU ($\beta = 0.170$, 95% CI: 0.105–0.236, $p < 0.001$).

Gender was associated with higher PD1+ expression on CD8+ T cells at baseline ($\beta = 0.126$, 95% CI: 0.058–0.194, $p < 0.001$), this difference is maintained over time. Age, histology, stage, surgical approach and post-operative complications were not significant ($p > 0.75$). Refer to Figure 5.1 & 5.2 and Table 5.1-5.4 for details..

5.3.2.2: Impact of CD8+PD1+ T Cell Changes on DFS

PD1+ slope on CD8+ T cells showed no association with recurrence (HR $\approx$ 1.00, 95% CI 0.91–1.09, $p=0.992$), indicating that the rate of change in CD8+PD1+ T cell expression over time was not prognostic. Median slopes were 2.50 in the non-recurrence group vs 6.34 in those who recurred, showing a numerically steeper increase in recurrence but without statistical significance. In contrast, histology (HR 7.61, 95% CI 1.94–29.86, $p=0.004$) and stage (HR 5.67, 95% CI 1.94–16.60, $p=0.002$) were strong independent predictors of recurrence. Refer to Figure 5.3 for DFS and Figure 5.4 for representative flow cytometry plots and Tables 5.5 & 5.6. and Tables 5.5 & 5.6 for details.

5.3.3 PD1+ on NKT-like cells

Flow cytometry gating was performed to identify PD1 expression on surface NKT-like cells as per Figure 2.2. Briefly total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD3+ CD56+ NKT-like cells. Following this NKT-like PD1+ cells were identified.

5.3.3.1: Longitudinal analysis of NKT-like PD1+ Cells

A mixed-effects model revealed the overall effect of time was significant ($\chi^2(2) = 23.41$, $p < 0.001$). Compared with baseline, NKT-like PD1+ expression was significantly higher at

1FU ($\beta = 0.137$, 95% CI: 0.063–0.211, $p < 0.001$) and at 2FU ($\beta = 0.171$, 95% CI: 0.097–0.244, $p < 0.001$).

Stage II ($\beta = 0.343$, 95% CI: 0.151–0.535, $p < 0.001$) and Stage III ($\beta = 0.180$, 95% CI: –0.010–0.369, $p = 0.063$) exhibited higher NKT-like PD1+ expression at baseline relative to Stage I. Females showed higher NKT-like PD1+ expression than males across all time points ($\beta = 0.153$, 95% CI: 0.036–0.271, $p = 0.010$), and adenocarcinoma was also associated with higher expression ($\beta = 0.141$, 95% CI: 0.015–0.267, $p = 0.029$). Age, post-operative complications and open surgery was not significant ($p > 0.60$). Refer to Figure 5.1-5.2 and Table 5.1-5.4 for details.

5.3.3.2: Impact of NKT-like PD1+ Cell Changes on DFS

The slope of PD1 expression on NKT-like cells showed a trend toward association with recurrence (HR = 0.93, 95% CI 0.87–1.00, $p = 0.062$), suggesting a greater decline in NKT-like PD1+ cells over time may be linked to increased recurrence risk. Median slopes were 3.6 in the non-recurrence group vs 3.2 in those who recurred. Histology (HR = 10.31, 95% CI 2.66–40.03, $p = 0.001$) and stage (HR = 8.12, 95% CI 2.42–27.18, $p = 0.001$) remained strong independent predictors of recurrence. Overall, NKT-like cell PD1+ slope suggests a biologically meaningful trend, but did not reach statistical significance in this cohort. Refer to Figure 5.3 for DFS and Figure 5.4 for representative flow cytometry plots and Tables 5.5 & 5.6.

5.3.4 PD1+ on NK cells

Flow cytometry gating was performed to identify PD1 expression on surface NK cells as per Figure 2.2. Briefly total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD3-CD56+ cells. Following this NK PD1+ cells were identified.

5.3.4.1: Longitudinal analysis of NK PD1+ Cells

A mixed-model showed overall effect of time was significant ($\chi^2(2) = 29.28$, $p < 0.001$). Compared with baseline, NK PD1+ expression increased significantly at 1FU ($\beta = 0.098$, 95% CI: 0.012–0.184, $p = 0.026$) and 2FU ($\beta = 0.211$, 95% CI: 0.108–0.313, $p < 0.001$).

Stage III ($\beta = 0.170$, 95% CI: 0.022–0.318, $p = 0.025$) exhibited higher NK PD1+ expression relative to Stage I at baseline, while Stage II and stage-by-time interactions were not significant (all $p > 0.13$). Gender was associated with higher NK PD1+ expression ($\beta = 0.136$, 95% CI: 0.029–0.244, $p = 0.013$), indicating higher expression in females at baseline. Age showed a trend toward reduced expression with increasing age ($\beta = -0.007$, 95% CI: -0.014–0.001, $p = 0.079$). Refer to Figure 5.1-5.2 and Table 5.1-5.4 for details.

5.3.4.2: Impact of NK PD1+ Cell Changes on DFS

Patients with recurrence had slightly lower median slope values (6.6) and greater variability compared with those without recurrence (7.65), indicating more heterogeneous NK PD1+ expression in this group. However, PD1+ expression on NK cells was not significantly associated with recurrence in Cox regression analysis. Histology (HR = 8.13, 95% CI: 2.17–30.54, $p = 0.002$) and stage (HR = 7.36, 95% CI: 2.27–23.85, $p = 0.001$) were significant predictors of recurrence, while other factors showed no significant association. Refer to Figure 5.3 for DFS and Figure 5.4 for representative flow cytometry plots and Tables 5.5 & 5.6.

5.3.5 PD1+ on CD19+ (B) cells

Flow cytometry gating was performed to identify PD1 expression on B cells as per Figure 2.2. Total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD19+ cells. Following this CD19+PD1+ cells were identified.

5.3.5.1 Longitudinal analysis of CD19+ PD1+ Cells

A mixed model revealed overall effect of time was significant ($\chi^2(2) = 30.70$, $p < 0.001$). Compared with baseline, PD1 expression on B cells showed a trend toward increase at 1FU ($\beta = 0.079$, 95% CI: -0.004–0.163, $p = 0.063$) and was significantly higher at 2FU ($\beta = 0.230$, 95% CI: 0.148–0.313, $p < 0.001$). Gender was associated with higher PD1 expression in B cells ($\beta = 0.119$, 95% CI: 0.014–0.223, $p = 0.026$), with greater expression in females across time points. Refer to Figure 5.1 and 5.2 and Table 5.1-5.4 for details.

5.3.5.2: Impact of CD19+ PD1 Cell Changes on DFS

Patients with recurrence had lower median slope values (2.77) and less variability compared with those without recurrence (5.55). PD1+ on B cells was significantly

associated with recurrence (HR = 0.94, 95% CI: 0.89–0.997, $p = 0.039$). Histology (HR = 5.37, 95% CI: 1.44–20.02, $p = 0.012$) and stage (HR = 9.07, 95% CI: 2.51–32.74, $p = 0.001$) were also significant predictors. Overall, patients who recur have B cells with lower, less dynamic PD1 expression. Refer to Figure 5.3 for DFS and Figure 5.4 for representative flow cytometry plots and Tables 5.5 & 5.6. and Tables 5.5 and 5.6 for details.

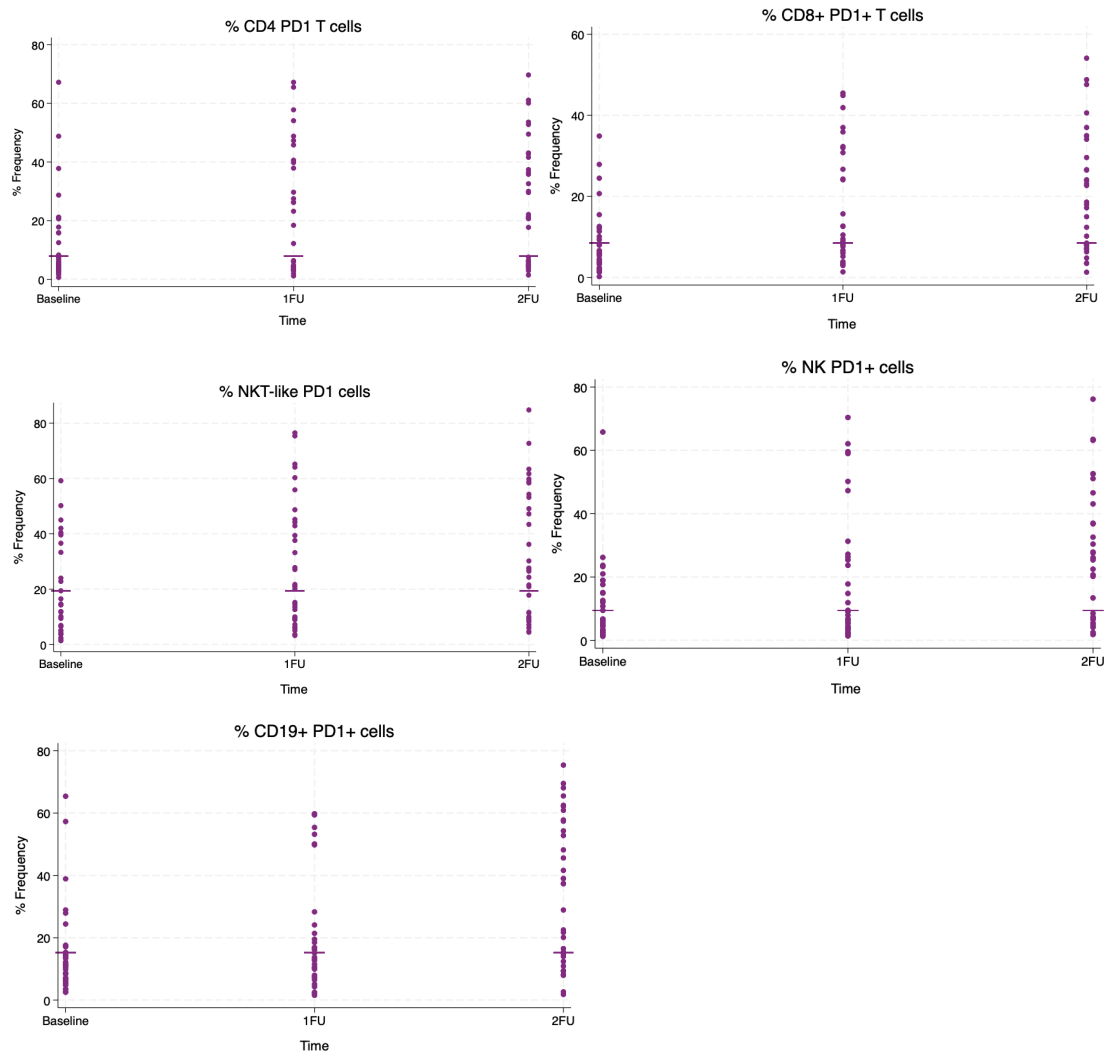


Figure 5.1. Dot plots showing % frequency of PD1 expression on immune cells at each timepoint. Total cells were first filtered to isolate single and viable cells. Lymphocytes were then selected using forward- and side-scatter properties. Gating of CD3+ was utilised to identify T cell subsets; CD4+ cells and CD8+ cells, alongside NKT-like cells (CD56+). Gating of CD3- and CD56+ was utilised to identify NK cells. Gating of CD19+ was utilised to identify B cells. PD1 positivity was then defined as a percentage frequency of CD4+ T cells, CD8+ T cells, NKT-like cells, NK cells and B cells. Mixed-effects linear regression revealed a significant difference in frequency over time for PD1 expression on all immune cells analysed ($p < 0.001$). Dots representing each patient and horizontal line at median value.

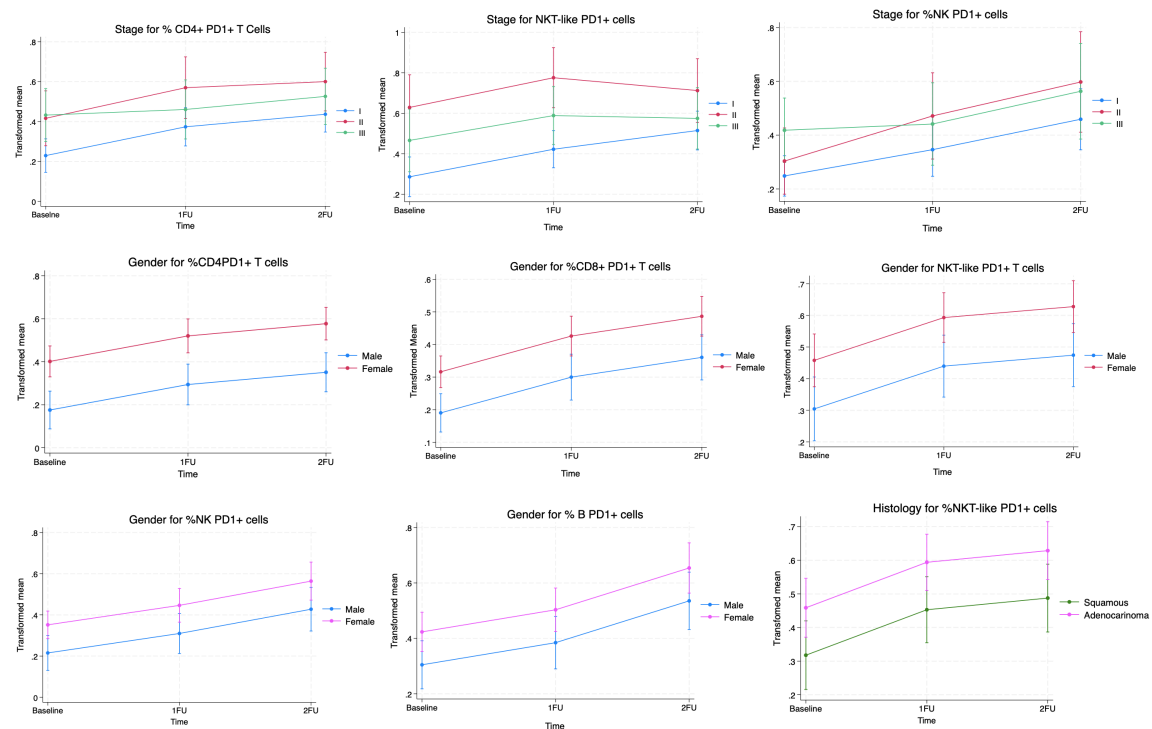


Figure 5.2. Predicted means plot showing differences over time of PD1 expression on immune cells in association with clinicopathological characteristics. Data obtained from FlowJo v10.10.0 software, were expressed as percentage frequencies and subsequently transformed using the arcsine-square root transformation to meet the assumptions of parametric analysis. Plotted values in this figure represent transformed means. To evaluate changes across the three timepoints, linear mixed-effects models were applied in association with age, gender, histological subtype, disease stage, surgical approach, and the presence or absence of post-operative complications. Expression on CD4+ T cells, CD8 + T cells, NKT cells, NK cells and B cells was analysed. PD1 expression on CD4+ cells ($p=0.026$, $p=0.014$), NKT-like cells ($p<0.001$) and NK cells ($p=0.025$) was higher in Stage II and/or Stage III patient respectively, compared to Stage I. Female gender was associated with increased PD1 expression in CD4+ T cells (<0.001), CD8+ T cells ($p<0.001$), NKT-like cells ($p=0.010$), NK cells ($p=0.013$) and B cells ($p=0.014$). Adenocarcinoma histology was associated with increased PD1 expression in NKT-like cells only ($p=0.029$).

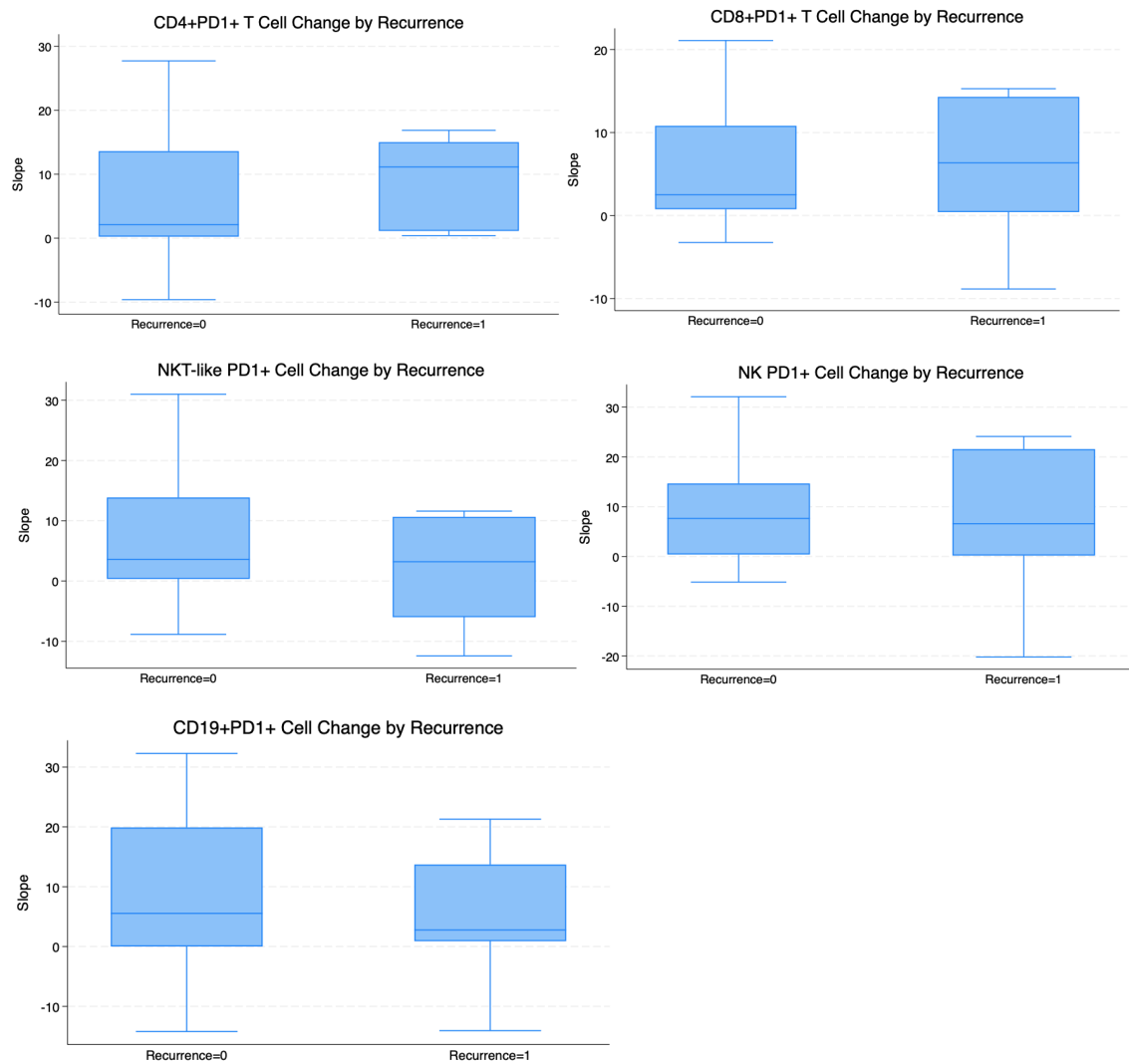


Figure 5.3. Boxplots of PD1 expression on immune cells and changes over time (slopes) in patients with and without recurrence. The longitudinal slope summarises the linear change over three timepoints in patients ($n=34$) for PD1 expression on CD4+ T cells, CD8 + T cells, NKT cells, NK cells and B cells. Cox proportional hazards regression was utilised to assess the association between the slope and DFS. A positive slope indicates increasing cell frequency, while a negative slope reflects a decline. Baseline was used as the reference time point for comparisons across all three time points, as well as individually against 1FU and 2FU. The slope of PD1 expression on CD4+T cells ($p=0.312$), CD8+ T cells ($p=0.992$), NKT-like ($p=0.062$) and NK ($p=0.298$) cells over the three timepoints were not associated with recurrence. Higher slope of B cells was associated with increased risk of recurrence ($p=0.039$). Patients with recurrence had lower median slope values (2.77) and less variability compared with those without recurrence (5.55)

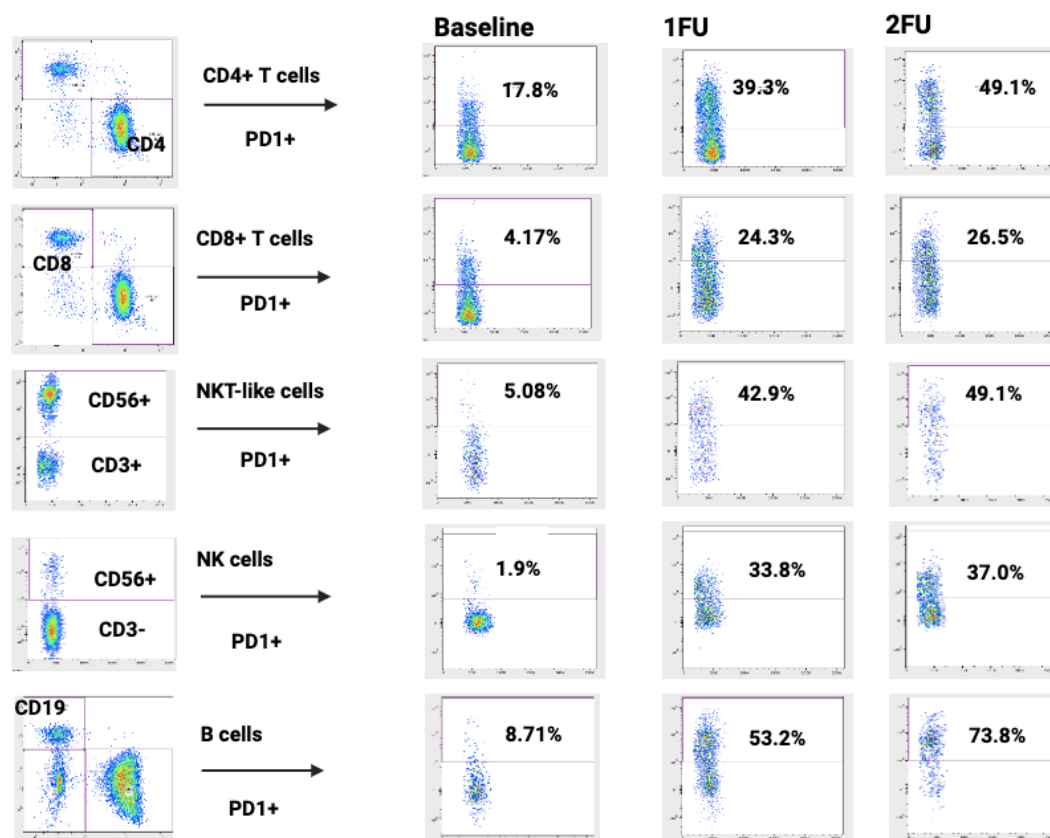


Figure 5.4. Representative flow cytometry plots for PD1+ on immune cells at different timepoints. PBMCs were stained with a dead cell stain (DCS), CD3, CD4, CD8, CD56, CD19 and PD1 antibodies and analysed by flow cytometry. Forward scatter area (FSC-A) and scatter area (SSC-A) was used to determine total cells, following this FSC-A and FSC-width (FSC-W) was used to determine single cells, then FSC-A and DCS was used to determine live cells. Finally, FSC-A and SSC-A was used to determine lymphocytes. CD3 +CD56 was utilised to identify T cells, NKT-like cells and NK cells. CD19 was utilised to identify B cells. This was performed at baseline, 1FU and 2FU. Percentage frequency is shown for PD1 positive cells.

Upper panel, left to right: First dot plot shows expression of CD4+ T cells, following this; representative dot plots of PD1+CD4+ T cells at baseline, 1FU and 2FU.

First centre panel, Left to Right: First dot plot shows expression of CD8+ T cells, following this; representative dot plots of PD1+CD8+ T cells at baseline, 1FU and 2FU.

Second centre panel, Left to Right: First dot plot shows expression of NKT-like cells (CD3+CD56+), following this; representative dot plots of PD1+ NKT-like cells at baseline, 1FU and 2FU.

Third centre panel, Left to Right: First dot plot shows expression of NK cells (CD3-CD56+), following this; representative dot plots of PD1+ NK cells at baseline, 1FU and 2FU.

Lower panel, Left and Right: First dot plot shows expression of B cells (CD19+) following this; representative dot plots of PD1+ B cells at baseline, 1FU and 2FU.

Table 5.1: Percent Frequency for PD1+ cells

Cell population / Timepoint	% Baseline	% 1FU	% 2FU
CD4+ PD1+	4.9	6.37	20.7
CD8+ PD1+	6.39	8.54	9.35
NKT-like PD1+	13.1	20	22.9
NK PD1+	5.68	6.76	25.7
CD19+ PD1+	10.75	10.4	21.3

Table 5.2: Mixed-effects linear regression comparing PD1+ cells at baseline with 1FU and 2FU

Time	Coefficient (β)	Std Err (SE)	p value	95% Confidence Interval (CI)
CD4+PD1+ 1FU	0.144	0.050	0.004	0.046-0.242
CD4+PD1+ 2FU	0.207	0.046	<0.001	0.115-0.299
CD8+ PD1+1FU	0.106	0.044	0.015	0.020-0.193
CD8+PD1+ 2FU	0.169	0.043	0.000	0.084-0.254
NKT PD1+ 1FU	0.136	0.047	0.004	0.042-0.229
NKT-like PD1+ 2FU	0.229	0.049	<0.001	0.132-0.326
NK PD1+ 1FU	0.097	0.044	0.026	0.011-0.183
NK PD1+ 2FU	0.211	0.052	<0.001	0.108-0.313
CD19 PD1+ 1FU	0.079	0.051	0.119	-0.020-0.178
CD19 PD1+ 2FU	0.213	0.059	<0.001	0.099-0.328

Table 5.3: Mixed-effects linear regression with clinicopathological characteristics for PD1+ cells

Statistical Analysis	Chi2 (χ^2) (2)	Overall p value	Stage II p value	Stage III p value	Age p value	Gender p value	Histology p value
CD4+ PD1+	23.72	<0.001	0.026	0.014	0.098	<0.001	0.748
CD8+ PD1+	26.68	<0.001	0.342	0.170	0.894	<0.001	0.758
NKT-like PD1+	23.41	<0.001	<0.001	0.063	0.643	0.010	0.029
NK PD1+	29.28	<0.001	0.465	0.025	0.079	0.013	0.741
CD19+ PD1+	30.70	<0.001	0.953	0.594	0.123	0.014	0.302

Table 5.4: Mixed-effects linear regression incorporating complications and surgical approach for PD1+ cells

Statistical Analysis	Complications P value	Surgical approach p value
CD4+ PD1+	0.210	0.281
CD8+ PD1+	0.554	0.702
NKT-like PD1+	0.848	0.603
NK PD1+	0.675	0.819
CD19+ PD1+	0.312	0.527

Table 5.5: Descriptive summary statistics incorporating median slope and interquartile range (IQR) for PD1+ cell populations

Cell Population	Median Slope Recurrence = 0	IQR Recurrence = 0	Median Slope Recurrence = 1	IQR Recurrence = 1
CD4+ PD1+	2.1	0.25- 13.6	11.1	1.14-15
CD8+ PD1+	2.5	0.77-10.08	6.34	0.42-14.3
NKT-like PD1+	3.6	0.37-13.85	3.2	-6-10.65
NK PD1+	7.65	0.42- 14.03	6.59	0.20.-21.57
CD19+ PD1+	5.55	0-7.19.9	2.77	0.09-13.7

Table 5.6: Cox Regression analysis for PD1+ cells adjusted for clinicopathological characteristics

Cell Population	Haz Ratio	95% CI	P value	Age	Gender	Histology	Stage	Complications
CD4+ PD1+	1.05	0.96-1.13	0.312	0.614	0.238	0.008	0.007	0.055
CD8+ PD1+	1.00	0.91-1.09	0.992	0.295	0.381	0.004	0.002	0.079
NKT-like PD1+	0.93	0.87-1.00	0.062	0.171	0.955	0.001	0.001	0.072
NK PD1+	0.97	0.92-1.03	0.298	0.204	0.487	0.002	0.001	0.109
CD19+ PD1+	0.94	0.89-2.00	0.039	0.189	0.284	0.012	0.001	0.052

5.4 CTLA4

5.4.1 CTLA4 + on CD4+ T cells

Flow cytometry gating was performed to identify CD4+ CTLA+ T cells as per Figure 2.2. Briefly, total cells, single cells and live cells were identified and then lymphocytes gated based on forward and side scatter, followed by selection of CD3+CD4+ T cells. Within this population CTLA+ was expressed as a percentage of total CD4+ T cells.

5.4.1.1 Longitudinal analysis of CD4+CTLA4+ T Cells

A mixed model analysis revealed overall effect of time was significant ($\chi^2(2) = 16.31$, $p = 0.0003$). Compared with baseline, CTLA4 expression on CD4+ T cells was significantly higher at 1FU ($\beta = 0.022$, 95% CI: 0.004–0.040, $p = 0.014$) and 2FU ($\beta = 0.036$, 95% CI: 0.018–0.054, $p < 0.001$). Stage II was associated with higher baseline CTLA4 expression compared with stage I ($\beta = 0.044$, 95% CI: 0.012–0.076, $p = 0.007$), whereas other factors analysed were not significant. Refer to Figures 5.5 & 5.6 and Tables 5.7-5.10 for details

5.4.3.1: Impact of CD4+CTLA-4+ T Cell Changes on DFS

The Cox model shows that a lower slope trends toward a higher recurrence risk however this was not statistically significant (HR = 0.35, 95% CI: 0.12–1.04, $p = 0.06$). Patients

without recurrence had a higher median slope (0.74) compared to those with recurrence (0.37). Histology (HR = 8.57, 95% CI: 2.28–32.25, $p = 0.001$) and stage (HR = 7.59, 95% CI: 2.43–23.66, $p < 0.001$) remain strong independent predictors of recurrence. Refer to Figure 5.7 for DFS and Figure 5.8 for representative flow cytometry plots and Tables 5.11 and 5.12.

5.4.2 CTLA4+ on CD8+ T cells

Flow cytometry gating was performed to identify CD8+ CTLA+ T cells as per Figure 2.2. Briefly, total cells, single cells and live cells were identified and then lymphocytes gated based on forward and side scatter, followed by selection of CD3+CD8+ T cells. Within this population CTLA+ was expressed as a percentage of total CD8+ T cells.

5.4.2.1 Longitudinal analysis of CD8+CTLA4+ T Cells

A mixed model analysis showed overall effect of time was not statistically significant. Adenocarcinoma and older age were significantly associated with higher CD8+ CTLA4+ expression ($\beta_{\text{histology}} = 0.084$, 95% CI: 0.043–0.125, $p = 0.000$; $\beta_{\text{age}} = 0.003$, 95% CI: 0.000–0.006, $p = 0.026$), whereas other covariates analysed and interactions with time were not. Refer to Figure 5.5-5.6 and Table 5.7-5.10 for details.

5.4.2.2: Impact of CD8+CTLA4+ T Cell Changes on DFS

The Cox proportional hazards analysis indicated that slope was significantly associated with recurrence risk (HR = 5.98, 95% CI: 1.65–21.70, $p = 0.007$), although the median slope was lower in patients who recurred (0.073) compared to those without recurrence (0.25), reflecting a skewed distribution in the recurrence group. Histology (HR = 44.01, 95% CI: 4.61–420.53, $p = 0.001$) and clinical stage (HR = 10.24, 95% CI: 2.62–40.05, $p = 0.001$) were strong independent predictors of recurrence. Post-treatment complications was also a significant predictor (HR = 21.05, 95% CI: 2.57–172.11, $p = 0.004$). Refer to Figure 5.7 for DFS and Figure 5.8 for representative flow cytometry plots and Tables 5.11 and 5.12.

5.4.3 CTLA4+ on NKT-like Cells

Flow cytometry gating was performed to identify CTLA4 expression on surface NKT-like cells as per Figure 2.2. Briefly total cells, single cells and live cells were identified and then

lymphocytes were gated based on forward and side scatter, followed by selection of CD3+ CD56+ cells. Following this NKT-like CTLA4+ cells were identified.

5.4.3.1 Longitudinal analysis of NKT-like CTLA4+ Cells

A mixed-effects analysis revealed the overall effect of time was marginally significant in the unadjusted model ($\chi^2(2) = 5.35$, $p = 0.069$). In the adjusted model with clinical and tumour characteristics, NKT-like CTLA4 expression was significantly higher at 1FU ($\beta = 0.042$, 95% CI: 0.001–0.083, $p = 0.046$) and 2FU ($\beta = 0.049$, 95% CI: 0.010–0.088, $p = 0.013$) compared with baseline. Stage II was associated with higher NKT-like CTLA4 expression compared with stage I ($\beta = 0.115$, 95% CI: 0.014–0.217, $p = 0.026$), and other covariates analysed were not significantly associated with expression. No significant interactions between time and stage were observed. Refer to Figure 5.5 & 5.6 and Tables 5.7-5.10 for details.

5.4.3.2: Impact of NKT-like CTLA4+ Cell Changes on DFS

The Cox proportional hazards analysis indicated that a lower slope was significantly associated with an increased risk of recurrence (HR = 0.86, 95% CI: 0.74–0.99, $p = 0.039$). Patients who experienced recurrence exhibited a lower median slope (0.048) compared to those without recurrence (0.46), suggesting reduced NKT-like cell CTLA4-related activity in recurrent cases. Histology (HR = 11.46, 95% CI: 2.80–46.95, $p = 0.001$), clinical stage (HR = 6.53, 95% CI: 2.14–19.97, $p = 0.001$), and post-treatment complications (HR = 5.51, 95% CI: 1.25–24.25, $p = 0.024$) were identified as strong independent predictors of recurrence. Refer to Figure 5.7 for DFS and Figure 5.8 for representative flow cytometry plots and Tables 5.11 and 5.12.

5.4.4 CTLA4+ on NK Cells

Flow cytometry gating was performed to identify PD1 expression on surface NK cells as per Figure 2.2. Briefly total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD3- CD56+ cells. Following this NK PD1+ cells were identified.

5.4.4.1 Longitudinal analysis of NK CTLA4+ Cells

A mixed-effects analysis showed overall effect of time was not significant ($\chi^2(2) = 2.09$, $p = 0.352$). Clinical and tumour factors did not significantly associated with NK CTLA-4

expression, and time-by-stage interactions were not significant. Refer to Figure 5.5 and Tables 5.7-5.10 for details.

5.4.4.2: Impact of NK CTLA4+ Cell Changes on DFS

The Cox proportional hazards analysis indicated that slope was significantly associated with recurrence risk (HR = 0.59, 95% CI: 0.35–0.98, $p = 0.042$), with more negative slopes protective. Patients who experienced recurrence exhibited a slightly higher (less negative) median slope (–0.048) compared with non-recurrence patients (–0.105), suggesting that reduced inhibitory regulation via NK cell CTLA4 may contribute to tumour recurrence. Advanced clinical stage (HR = 5.35, 95% CI: 1.88–15.21, $p = 0.002$) and post-treatment complications (HR = 10.15, 95% CI: 1.51–68.26, $p = 0.017$) were also strong independent predictors of recurrence. Refer to Figure 5.7 for DFS and Figure 5.8 for representative flow cytometry plots and Tables 5.11 and 5.12.

5.4.5 CTLA4+ on CD19+ (B) Cells

Flow cytometry gating was performed to identify CTLA4 expression on B cells as per Figure 2.2. Total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD19+ cells. Following this CD19+CTLA4+ cells were identified.

5.4.5.1 Longitudinal analysis of CD19+CTLA-4+ Cells

A mixed-effects analysis showed overall effect of time was significant ($\chi^2(2) = 26.19$, $p < 0.001$). Compared with baseline, CTLA4 expression on B cells did not significantly change at 1FU ($\beta = 0.020$, 95% CI: –0.031 to 0.071, $p = 0.443$), but was significantly higher at 2FU ($\beta = 0.123$, 95% CI: 0.073–0.174, $p < 0.001$). Baseline clinical characteristics were not significant and did not modify the time-related change. Refer to Figure 5.5 and Tables 5.7-5.10 for details.

5.4.5.2: Impact of CD19+CTLA4+ Cell Changes on DFS

Cox proportional hazards analysis demonstrated that a lower slope was significantly associated with increased recurrence risk (HR = 0.90, 95% CI: 0.82–0.98, $p = 0.012$). Patients who experienced recurrence had a slightly higher median slope (1.59) compared with non-recurrence patients (1.33), but overall the recurrence group exhibited less variability, suggesting CTLA4 expression changes on B cells over time. Histology (HR =

4.15, 95% CI: 1.11–15.54, $p = 0.035$), clinical stage (HR = 9.18, 95% CI: 2.65–31.84, $p < 0.001$), and post-treatment complications (HR = 5.42, 95% CI: 1.31–22.49, $p = 0.020$) were also strong independent predictors of recurrence. Refer to Figure 5.7 for DFS and Figure 5.8 for representative flow cytometry plots and Tables 5.11 and 5.12.

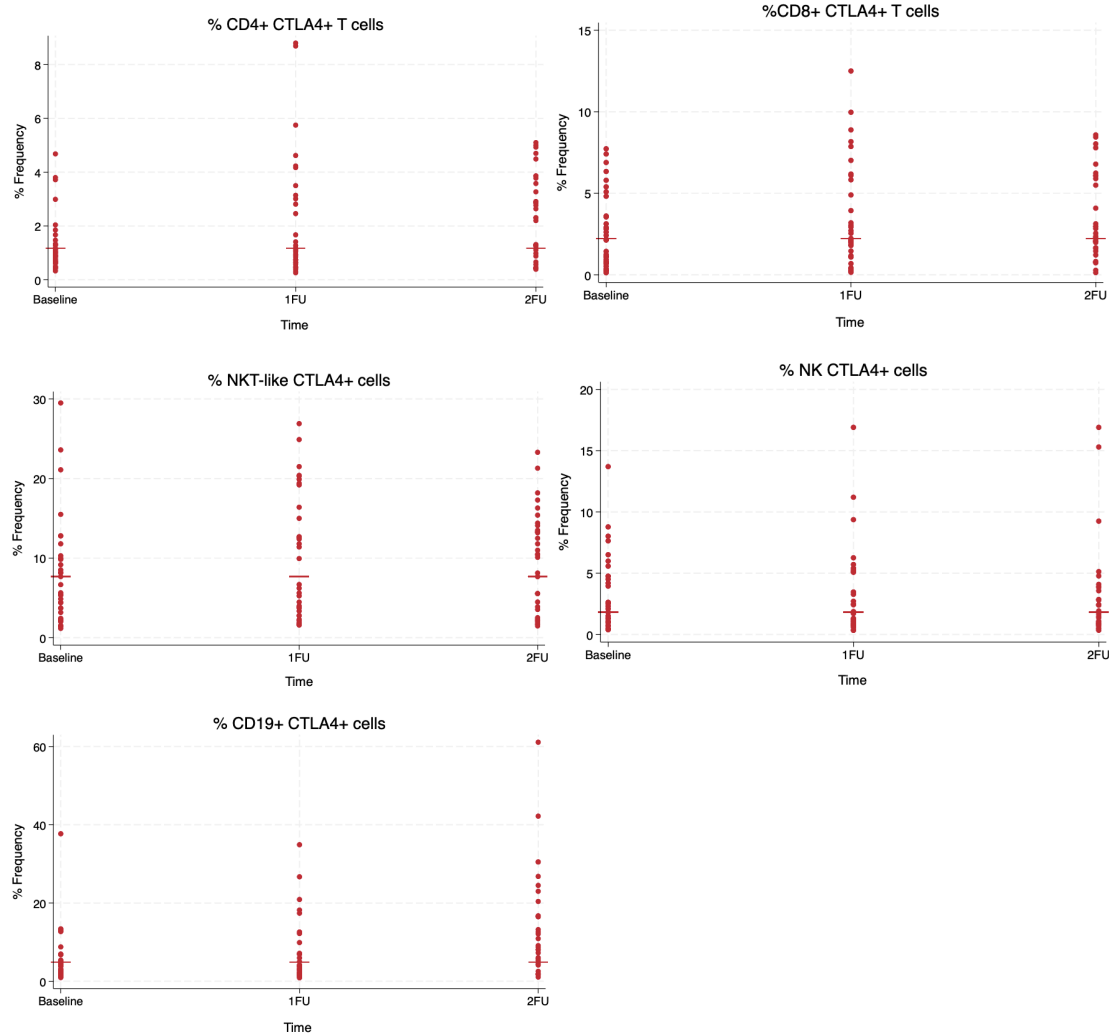


Figure 5.5. Dot plots showing % frequency of CTLA4 expression on immune cells at each timepoint. Total cells were first filtered to isolate single and viable cells. Lymphocytes were then selected using forward- and side-scatter properties. Gating of CD3⁺ was utilised to identify T cell subsets; CD4⁺ cells and CD8⁺ cells, alongside NKT-like cells (CD56⁺). Gating of CD3⁻ and CD56⁺ was utilised to identify NK cells. Gating of CD19⁺ was utilised to identify B cells. CTLA4 positivity was then defined as a percentage frequency of CD4⁺ T cells, CD8⁺ T cells, NKT-like cells, NK cells and B cells. Mixed-effects linear regression revealed a significant difference in frequency over time for CTLA4 expression on CD4⁺ T cells ($p=0.0003$), CD8⁺ T cells analysed ($p=0.193$) and B cells ($p<0.001$). For NKT-like cells ($p=0.689$) and NK cells ($p=0.3519$), there was no significant difference over time. Dots representing each patient and horizontal line at median value.

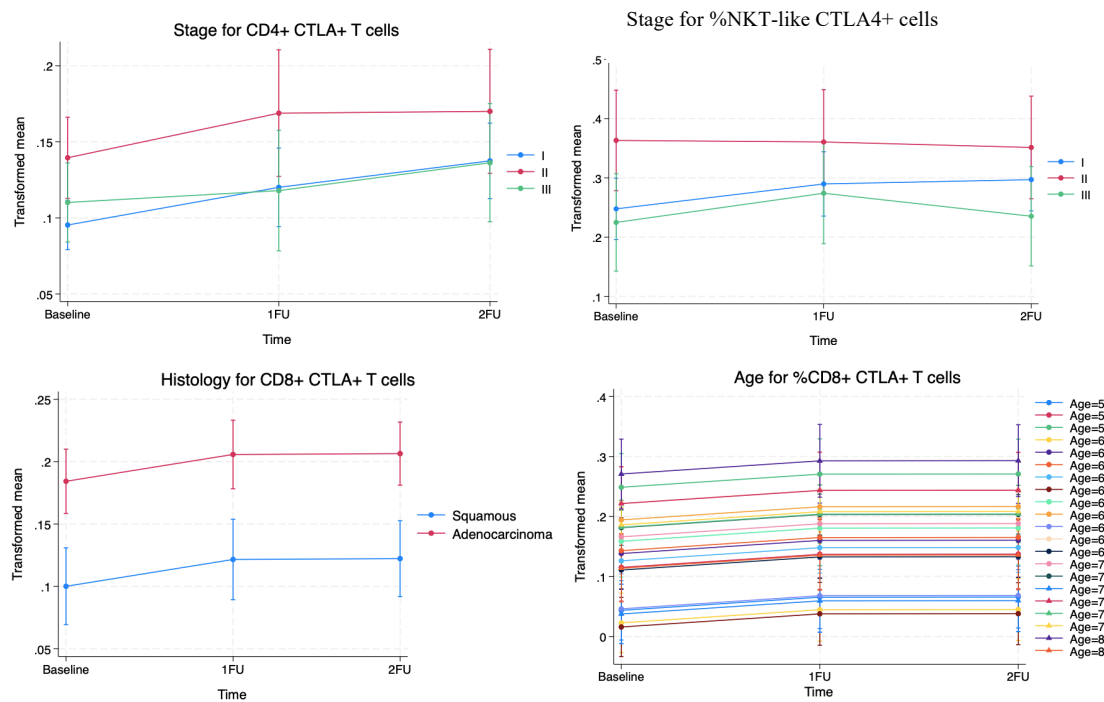


Figure 5.6. Predicted means plot showing differences over time of CTLA4 expression on immune cells in association with clinicopathological characteristics. Data obtained from FlowJo v10.10.0 software, were expressed as percentage frequencies and subsequently transformed using the arcsine-square root transformation to meet the assumptions of parametric analysis. Plotted values in this figure represent transformed means. To evaluate changes across the three timepoints, linear mixed-effects models were applied in association with age, gender, histological subtype, disease stage, surgical approach, and the presence or absence of post-operative complications. Expression on CD4+ T cells, CD8 + T cells, NKT cells, NK cells and B cells was analysed. CTLA4 expression on CD4+ cells ($p=0.007$) and NKT-like cells ($p=0.026$) was higher in Stage II compared to Stage I or Stage II. Adenocarcinoma histology was associated with increased CTLA4 expression in CD8+ T cells only ($p=0.026$). Older age was associated with increased CTLA4 expression in CD8+ T cells ($p=0.026$).

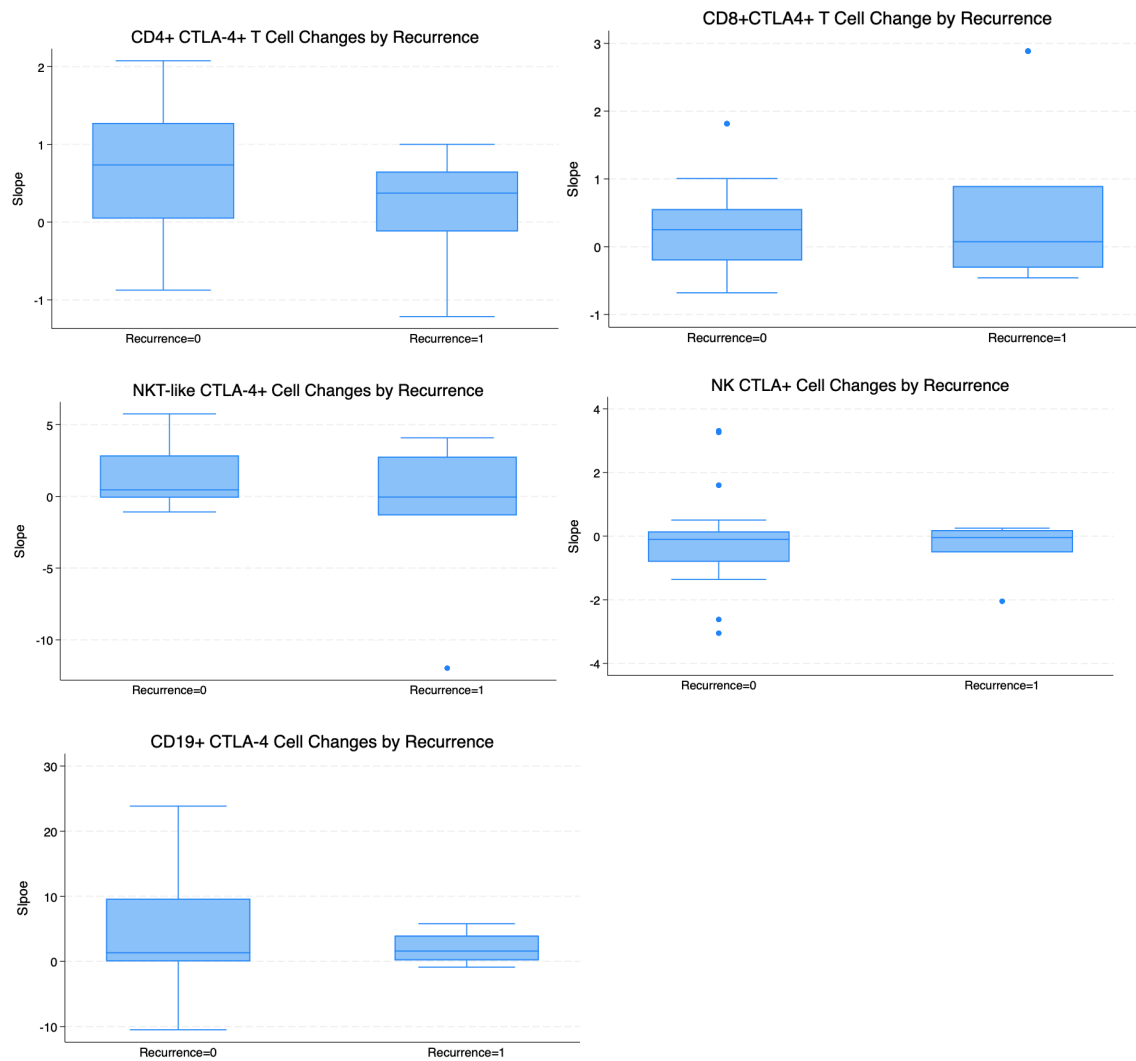


Figure 5.7. Boxplots of CTLA4 expression on immune cells and changes over time (slopes) in patients with and without recurrence. The longitudinal slope summarises the linear change over three timepoints in patients ($n=34$) for CTLA4 expression on CD4+ T cells, CD8 + T cells, NKT cells, NK cells and B cells. Cox proportional hazards regression was utilised to assess the association between the slope and DFS. A positive slope indicates increasing cell frequency, while a negative slope reflects a decline. Baseline was used as the reference time point for comparisons across all three time points, as well as individually against 1FU and 2FU. The slope of CTLA4 expression on CD8+ T cells ($p=0.007$), NKT-like cells ($p=0.039$), NK cells ($p=0.042$) and B cells ($p=0.012$) over the three timepoints were significantly associated with recurrence. The slope of CTLA4 expression on CD4+ T cells ($p=0.060$) were not associated with recurrence.

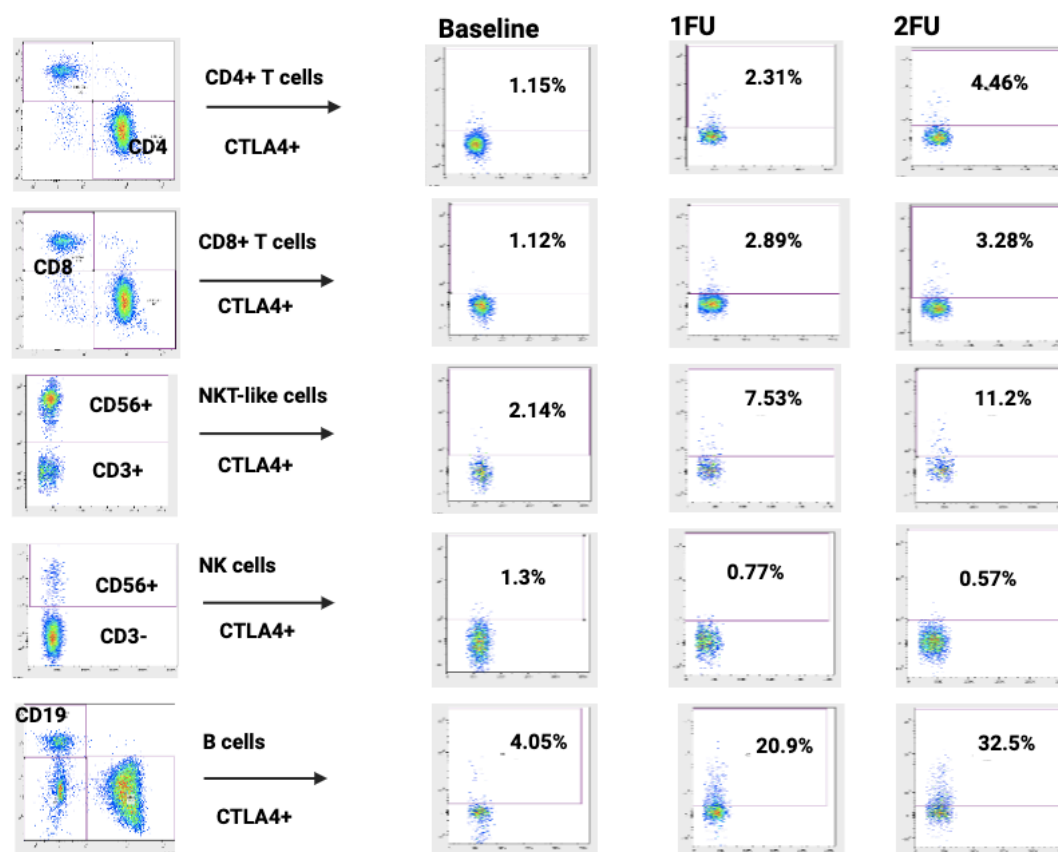


Figure 5.8. Representative flow cytometry plots for CTLA4⁺ on immune cells at different timepoints. PBMCs were stained with a dead cell stain (DCS), CD3, CD4, CD8, CD56, CD19 and CTLA4 antibodies and analysed by flow cytometry. Forward scatter area (FSC-A) and scatter area (SSC-A) was used to determine total cells, following this FSC-A and FSC-width (FSC-W) was used to determine single cells, then FSC-A and DCS was used to determine live cells. Finally, FSC-A and SSC-A was used to determine lymphocytes. CD3 was utilised to identify T cells, NKT-like cells and NK cells. This was performed at baseline, 1FU and 2FU. Percentage frequency is shown for CTLA4 positive cells.

Upper panel, left to right: First dot plot shows expression of CD4⁺T cells, following this; representative dot plots of CTLA4⁺CD4⁺ T cells at baseline, 1FU and 2FU.

First centre panel, Left to Right: First dot plot shows expression of CD8⁺ T cells, following this; representative dot plots of CTLA4⁺ CD8⁺ T cells at baseline, 1FU and 2FU.

Second centre panel, Left to Right: First dot plot shows expression of NKT-like cells (CD3⁺CD56⁺), following this; representative dot plots of CTLA4⁺ NKT-like cells at baseline, 1FU and 2FU.

Third centre panel, Left to Right: First dot plot shows expression of NK cells (CD3⁻CD56⁺), following this; representative dot plots of CTLA4⁺ NK cells at baseline, 1FU and 2FU.

Lower panel, Left and Right: First dot plot shows expression of B cells (CD19⁺) following this; representative dot plots of CTLA4⁺ B cells at baseline, 1FU and 2FU.

Table 5.7: Percent Frequency of CTLA4+ cells

Cell Population	% Baseline	% 1FU	% 2FU
CD4+CTLA4+	0.99	1.07	1.31
CD8+CTLA4+	2.17	2.16	2.12
NKT-like CTLA4+	6.46	9.95	10.25
NK+CTLA4+	2.21	1.31	1.43
CD19+CTLA4+	4.51	3.85	11.5

Table 5.8: Mixed-effects linear regression comparing baseline with 1FU and 2FU for CTLA4 cells

Cell population / Timepoint	Coefficient (β)	Std Err (SE)	p value	95% Confidence Interval
CD4+CTLA4+ 1FU	0.024	0.017	0.033	0.002-0.047
CD4+CTLA4+ 2FU	0.042	0.011	<0.001	0.020-0.063
CD8+CTLA4 +1FU	0.017	0.012	0.149	-0.006-0.042
CD8+CTLA4+ 2FU	0.006	0.009	0.501	-0.123-0.025
NKT-like CTLA4+ 1FU	0.042	0.021	0.046	0.000-0.083
NKT-like CTLA4+ 2FU	0.049	0.019	0.013	0.010-0.088
NK CTLA4+ 1FU	-0.011	0.009	0.210	-0.029-0.006
NK CTLA4+ 2FU	-0.022	0.012	0.072	-0.045-0.002
CD19+CTLA4+ 1FU	0.009	0.023	0.685	-0.037-0.037
CD19+CTLA4+ 2FU	0.105	0.037	<0.001	-0.031-0.179

Table 5.9: Mixed-effects linear regression with clinicopathological characteristics for CTLA4+ cells

Statistical Analysis	Chi2 (χ^2) (2)	Un-adjusted p value	Stage II p value	Stage III p value	Age p value	Gender p value	Histology p value
CD4+CTLA4+	16.31	0.0003	0.007	0.367	0.814	0.115	0.954
CD8+CTLA4+	7.9	0.0193	0.331	0.447	0.026	0.308	0.000
NKT-like CTLA4+	5.35	0.0689	0.026	0.659	0.922	0.834	0.620
NK CTLA4+	2.09	0.3519	0.341	0.871	0.343	0.369	0.602
CD19+CTLA4+	26.19	<0.001	0.761	0.698	0.752	0.133	0.953

Table 5.10: Mixed-effects linear regression incorporating complications and surgical approach for CTLA4+ cells

Statistical Analysis	Complications P value	Surgical approach p value
CD4+CTLA4+	0.840	0.426
CD8+CTLA4+	0.951	0.526
NKT-like CTLA4+	0.716	0.725
NK CTLA4+	0.675	0.819
CD19+CTLA4+	0.726	0.080

Table 5.11: Descriptive summary statistics incorporating median slope and interquartile range (IQR) for CTLA4+ cell populations

Cell Population	Median Slope Recurrence = 0	IQR Recurrence = 0	Median Slope Recurrence = 1	IQR Recurrence = 1
CD4+CTLA4+	0.735	0.45- -1.28	0.373	-0.12- 0.65
CD8+CTLA4+	0.25	-0.21- 0.55	0.073	-0.31- 0.90
NKT-like CTLA4+	0.46	-0.09-2.86	-0.05	-1.33-2.77
NK+CTLA4+	-0.11	-0.81-0.15	-0.05	-0.51-0.19
CD19+CTLA4+	1.33	0-9.64	1.59	0.16-3.97

Table 5.12: Cox Regression analysis for CTLA4+ cells adjusted for clinicopathological characteristics

Cell Population	Hazard Ratio	95% CI	P value	Age	Gender	Histology	Stage	Complications
CD4+CTLA4+	0.35	0.11-1.05	0.060	0.306	0.444	0.001	<0.001	0.262
CD8+CTLA4+	5.98	1.64-31.69	0.007	0.311	0.747	0.001	0.001	0.004
NKT-like CTLA4+	0.86	0.74-0.99	0.039	0.389	0.667	0.001	0.001	0.024
NK CTLA4+	0.59	0.35-0.98	0.042	0.239	0.100	0.223	0.002	0.017
CD19+ CTLA4+	0.90	0.82-0.97	0.012	0.205	0.114	0.035	<0.001	0.020

5.5 LAG3

5.5.1 LAG3+ on CD4+ T cells

Flow cytometry gating was performed to identify CD4+ LAG3+ T cells as per Figure 2.2. Briefly, total cells, single cells and live cells were identified and then lymphocytes gated based on forward and side scatter, followed by selection of CD3+CD4+ T cells. Within this population LAG3+ was expressed as a percentage of total CD4+ T cells.

5.5.1.1 Longitudinal analysis of CD4+LAG3+ T cells

A mixed-effects analysis revealed overall effect of time was significant ($\chi^2(2) = 21.45$, $p < 0.001$). The increase at 2FU was significant ($\beta = 0.031$, 95% CI: 0.003–0.058, $p = 0.028$), whereas the increase at 1FU was not. Stage III was associated with higher overall LAG-3 expression compared with stage I ($\beta = 0.097$, 95% CI: 0.039–0.156, $p = 0.001$). Refer to Figure 5.9 & 5.10 and Table 5.13-5.16.

5.5.1.2. Impact of CD4+LAG3+ T cell changes on DFS

Cox proportional hazards analysis demonstrated that a higher slope was significantly associated with increased recurrence risk (HR = 1.80, 95% CI: 1.26–2.57, $p = 0.001$). Patients who experienced recurrence had a slightly lower median slope (0.09) compared

with non-recurrence patients (0.21), but a greater spread in extreme values (higher variance), suggesting that some patients with sharply increasing LAG3+ expression on CD4+ T cells over time were at particularly high risk. Histology (HR = 25.55, 95% CI: 4.19–155.69, $p < 0.001$), clinical stage (HR = 9.88, 95% CI: 2.58–37.81, $p = 0.001$), and post-treatment complications (HR = 26.00, 95% CI: 2.52–267.95, $p = 0.006$) were also strong independent predictors of recurrence. Refer to Figure 5.11 for DFS and Figure 5.12 for representative flow cytometry plots and. Table 5.17 and 5.18.

5.5.2 LAG3+ on CD8+ T cells

Flow cytometry gating was performed to identify CD8+LAG3+ T cells as per Figure 2.2. Briefly, total cells, single cells and live cells were identified and then lymphocytes gated based on forward and side scatter, followed by selection of CD3+CD8+ T cells. Within this population LAG3+ was expressed as a percentage of total CD8+ T cells.

5.5.2.1 Longitudinal analysis of CD8+LAG3+ T cells

CD8+ T cells did not show significant changes in LAG3 expression at 1FU ($\beta = -0.094$, 95% CI: -0.222 to 0.034 , $p = 0.152$) or 2FU ($\beta = -0.085$, 95% CI: -0.202 to 0.033 , $p = 0.157$) compared with baseline. Adjusted models including clinicopathological factors also showed no significant time effects, interactions, or associations with baseline characteristics. Refer to Figure 5.39 and Table 5.13-5.16.

5.5.2.2. Impact of CD8+LAG3+ T Cell Changes on DFS

Cox proportional hazards analysis indicated that a higher slope was marginally associated with increased recurrence risk, however this was not statistically significant (HR = 1.13, 95% CI: 0.99–1.30, $p = 0.071$). Patients who experienced recurrence exhibited a higher median slope (0.42) compared with non-recurrence patients (0.09), suggesting that CD8+ T cells in recurrent cases showed greater upregulation of LAG-3 over time. Tumour-intrinsic and clinical factors, including histology (HR = 5.81, 95% CI: 1.54–21.98, $p = 0.009$), clinical stage (HR = 4.17, 95% CI: 1.22–14.32, $p = 0.023$), and post-treatment complications (HR = 6.86, 95% CI: 1.50–31.48, $p = 0.013$), were also independently predictive of recurrence. Refer to Figure 5.11 for DFS and Figure 5.12 for representative flow cytometry plots and. Table 5.17 and 5.18.

5.5.3 *LAG3 + on NKT-like cells*

Flow cytometry gating was performed to identify LAG3 expression on surface NKT-like cells as per Figure 2.2. Briefly total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD3+ CD56+ cells. Following this NKT-like LAG3+ cells were identified.

5.5.3.1 Longitudinal analysis of NKT-like LAG3+ cells

NKT-like cells did not show significant changes in LAG3 expression. Presence of complications was associated with higher overall NKT-like LAG3 expression ($\beta = 0.093$, 95% CI: 0.003–0.183, $p = 0.042$), but there were no significant interactions between complications and time. Refer to Figure 5.9 and 5.10 and Table 5.13-5.16 for details.

5.5.3.2: *Impact of NKT-like LAG3+ changes on DFS*

Lower LAG3+ slopes on NKT-like cells were significantly associated with higher recurrence risk (HR = 0.61, 95% CI: 0.39–0.94, $p = 0.025$). Patients with recurrence had a median slope of –0.51 compared with 0.14 in non-recurrence cases, indicating reduced dynamic LAG-3 expression. Histology, stage, and post-treatment complications were also independent predictors of recurrence. Refer to Figure 5.11 for DFS and Figure 5.12 for representative flow cytometry plots and. Table 5.17 and 5.18.

5.5.4 *LAG3+ on NK cells*

Flow cytometry gating was performed to identify LAG3 expression on surface NK cells as per Figure 2.2. Briefly total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD3- CD56+ cells. Following this NK LAG3+ cells were identified.

5.5.4.1 Longitudinal analysis of NK LAG3+ cells

NK LAG3 expression did not change significantly over time. Histology was strongly associated with NK LAG-3+ levels, with lower expression in adenocarcinoma compared with squamous across all time points (margins at 2FU: 0.111 vs. 0.219, both $p < 0.001$). No significant interactions were observed between time and stage or other covariates. Refer to Figure 5.9 & 5.10 and Tables 5.13-5.16 for details.

5.5.4.2 Impact of NK LAG3+ T Cell Changes on DFS

Cox regression analysis of LAG-3+ on T cells showed that slopes were slightly higher in patients with recurrence (0.17) compared with non-recurrence (0.23), but this association was not statistically significant (HR = 1.14, 95% CI: 0.84–1.56, $p = 0.398$). Despite the lack of significance, recurrence patients exhibited less variability in NK LAG-3 slopes, suggesting more consistent but modest expression changes over time. Histology, stage, and post-treatment complications remained significant predictors of recurrence. Refer to Figure 5.11 for DFS and Figure 5.12 for representative flow cytometry plots and. Table 5.17 and 5.18.

5.5.5 LAG3+ on CD19+(B) cells

Flow cytometry gating was performed to identify LAG3 expression on surface B cells as per Figure 2.2. Briefly total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD19+ cells. Following this CD19+LAG3+ cells were identified.

5.5.5.1 Longitudinal analysis of LAG3+ on B cells

LAG3 expression on B cells showed a significant overall change over time ($\chi^2 = 10.80$, $p = 0.0045$), with a trend toward higher levels at 2FU ($\beta = 0.037$, 95% CI: -0.015 to 0.088 , $p = 0.160$). Clinical covariates were not significantly associated with B cell LAG3 expression, while histology showed a trend toward lower expression in adenocarcinoma ($\beta = -0.071$, 95% CI: -0.145 to 0.004 , $p = 0.063$). Refer to Figure 5.9 and Tables 5.13-5.16.

5.5.5.2: Impact of CD19+ LAG3+ T Cell Changes on DFS

Cox regression analysis of LAG3+ on B cells showed that patients with recurrence had lower and less variable median slopes (0.0025) compared with non-recurrence patients (0.165), although the association was not statistically significant (HR = 0.80, 95% CI: 0.57–1.12, $p = 0.189$). These results suggest that B cells in recurrent cases exhibit more stable, modest LAG3 expression changes over time. Histology, stage, and post-treatment complications remained significant predictors of recurrence. Refer to Figure 5.11 for DFS and Figure 5.12 for representative flow cytometry plots and. Table 5.17 and 5.18.

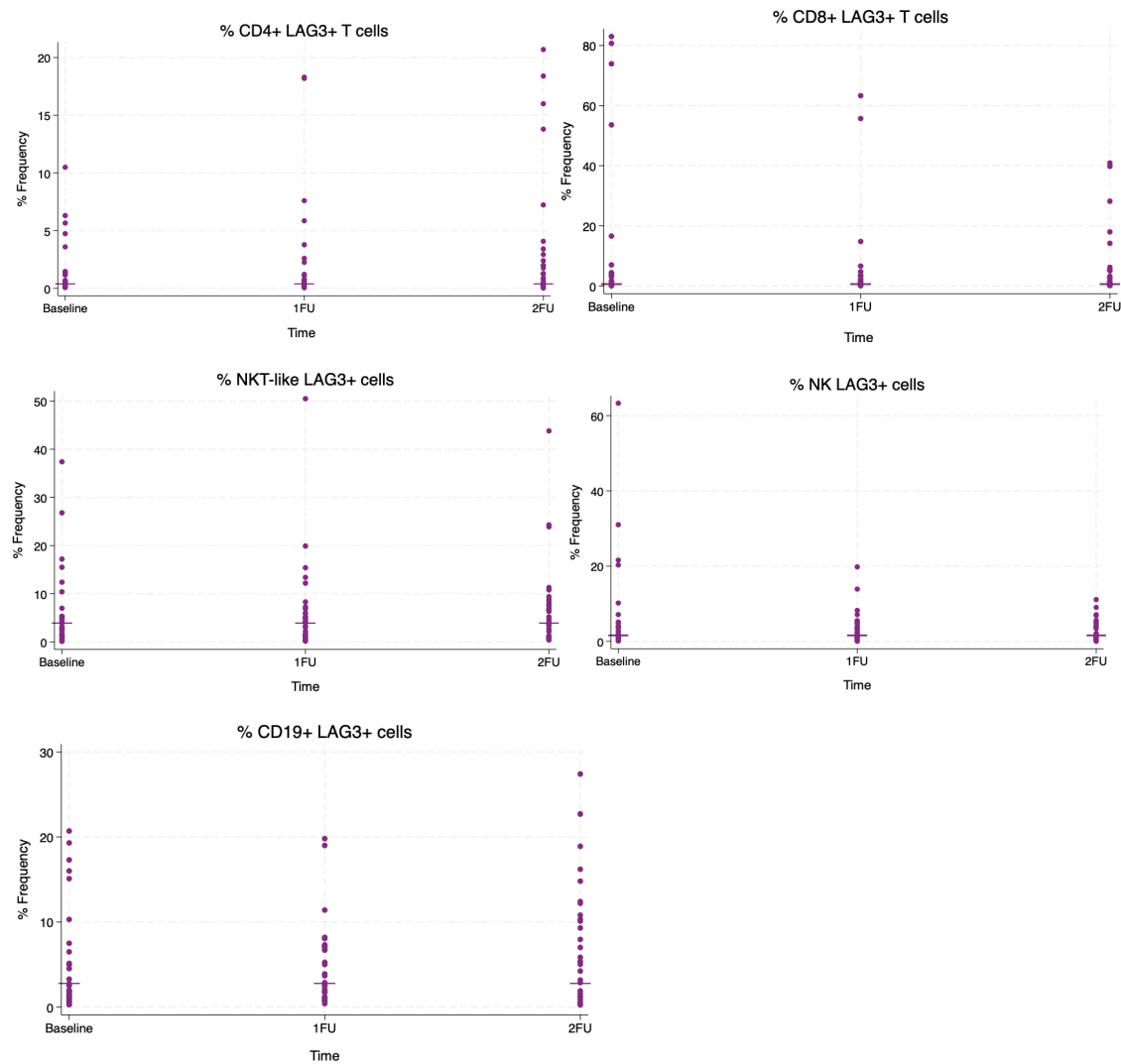


Figure 5.9. Dot plots showing % frequency of LAG3 expression on immune cells at each timepoint. Total cells were first filtered to isolate single and viable cells. Lymphocytes were then selected using forward- and side-scatter properties. Gating of CD3⁺ was utilised to identify T cell subsets; CD4⁺ cells and CD8⁺ cells, alongside NKT-like cells (CD56⁺). Gating of CD3⁻ and CD56⁺ was utilised to identify NK cells. Gating of CD19⁺ was utilised to identify B cells. LAG3 positivity was then defined as a percentage frequency of CD4⁺ T cells, CD8⁺ T cells, NKT-like cells, NK cells and B cells. Mixed-effects linear regression revealed a significant difference in frequency over time for LAG3 expression on CD4⁺ T cells ($p < 0.001$), and B cells ($p = 0.0045$). For CD8⁺ T cells ($p = 0.3525$), NKT-like cells ($p = 0.4288$) and NK cells ($p = 0.4651$), there was no significant difference over time. Dots representing each patient and horizontal line at median value.

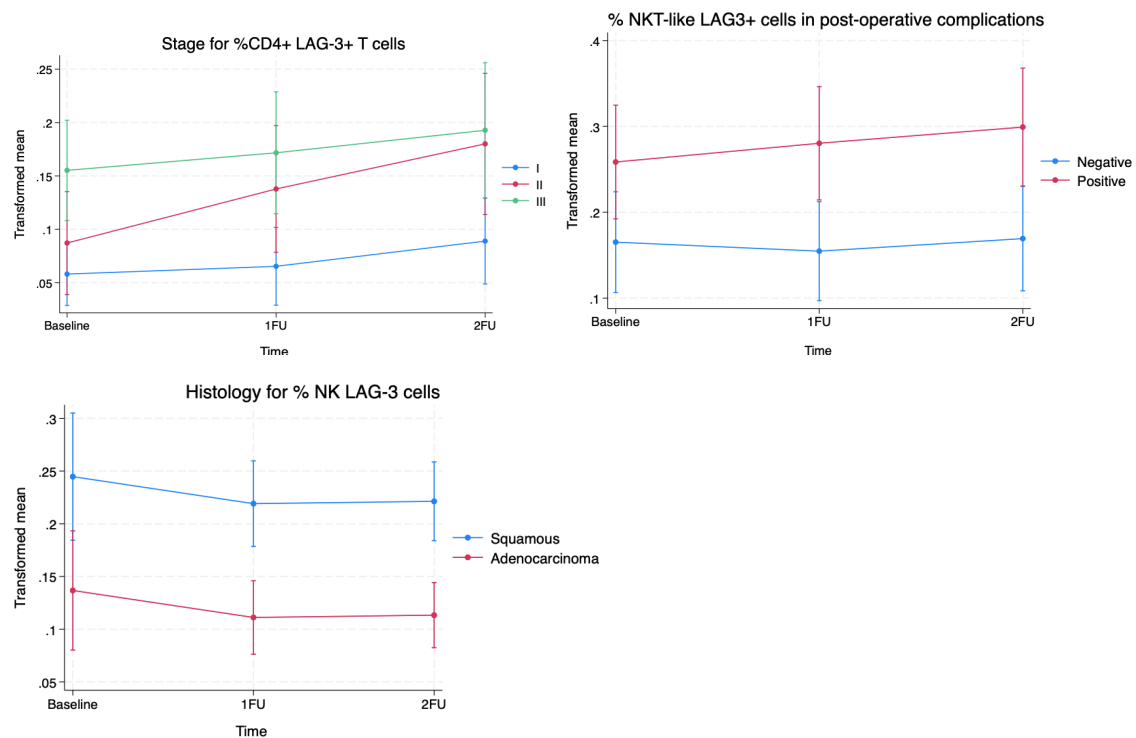


Figure 5.10. Predicted means plot showing differences over time of LAG3 expression on immune cells in association with clinicopathological characteristics. Data obtained from FlowJo v10.10.0 software, were expressed as percentage frequencies and subsequently transformed using the arcsine-square root transformation to meet the assumptions of parametric analysis. Plotted values in this figure represent transformed means. To evaluate changes across the three timepoints, linear mixed-effects models were applied in association with age, gender, histological subtype, disease stage, surgical approach, and the presence or absence of post-operative complications. Expression on CD4+ T cells, CD8 + T cells, NKT cells, NK cells and B cells was analysed. LAG3 expression on CD4+ cells ($p=0.001$) was higher in Stage III compared to Stage I or Stage II. Presence of complications was associated with higher overall LAG3 expression on NKT-like cells ($p = 0.042$). Histology was strongly associated with lower LAG-3+ levels, in adenocarcinoma compared with squamous histology on NK cells ($p < 0.001$).

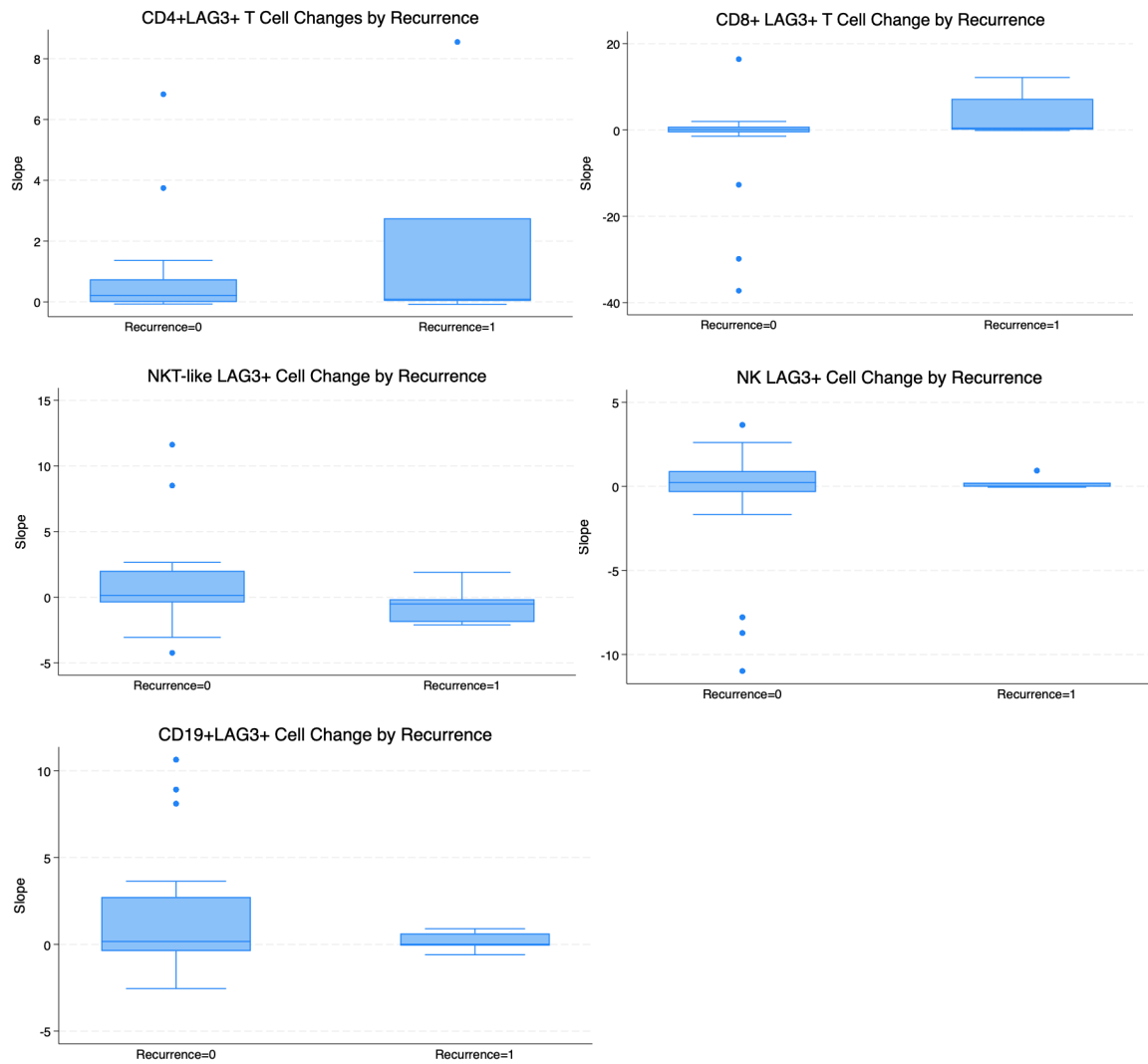


Figure 5.11. Boxplots of LAG3 expression on immune cells and changes over time (slopes) in patients with and without recurrence. The longitudinal slope summarises the linear change over three timepoints in patients ($n=34$) for LAG3 expression on CD4+ T cells, CD8 + T cells, NKT cells, NK cells and B cells. Cox proportional hazards regression was utilised to assess the association between the slope and DFS. A positive slope indicates increasing cell frequency, while a negative slope reflects a decline. Baseline was used as the reference time point for comparisons across all three time points, as well as individually against 1FU and 2FU. The slopes of LAG3 expression on CD4+ T cells ($p=0.001$) and NKT-like cells ($p=0.025$) over the three timepoints were significantly associated with recurrence. The slopes of LAG3 expression on CD8+ T cells ($p=0.071$), NK cells ($p=0.398$) and B cells ($p=0.189$) were not associated with recurrence.

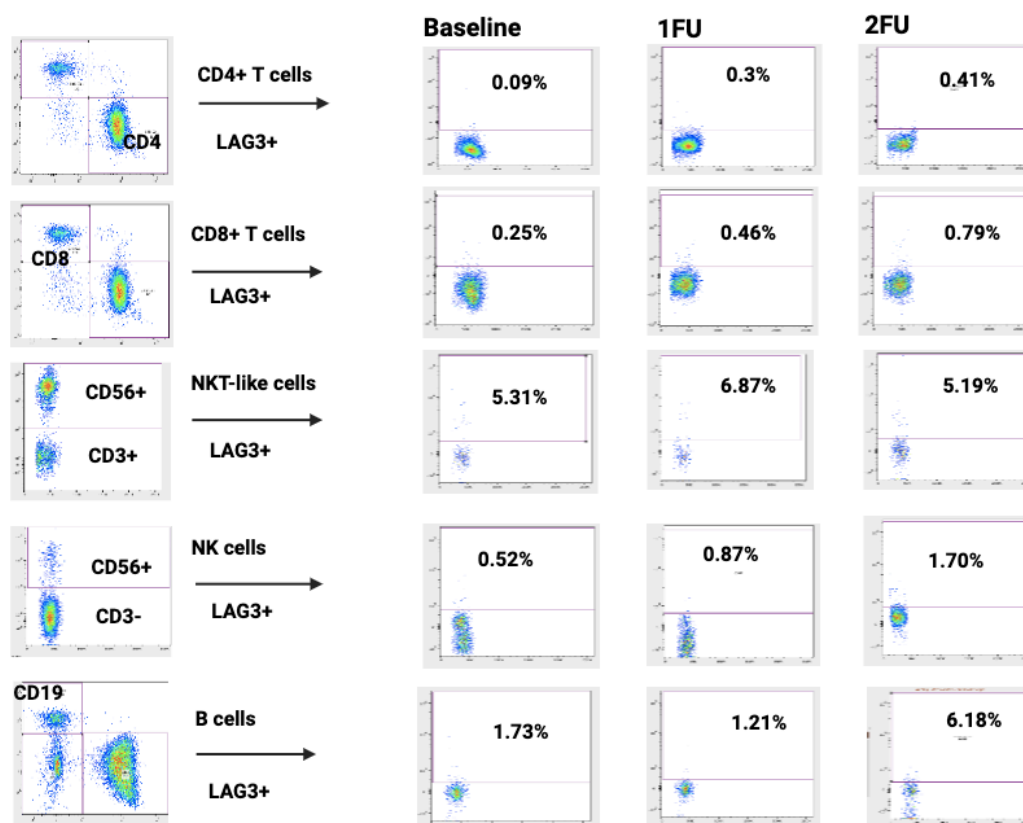


Figure 5.12. Representative flow cytometry plots for LAG3+ on immune cells at different timepoints. PBMCs were stained with a dead cell stain (DCS), CD3, CD4, CD8, CD56, CD19 and LAG3 antibodies and analysed by flow cytometry. Forward scatter area (FSC-A) and scatter area (SSC-A) was used to determine total cells, following this FSC-A and FSC-width (FSC-W) was used to determine single cells, then FSC-A and DCS was used to determine live cells. Finally, FSC-A and SSC-A was used to determine lymphocytes. CD3 was utilised to identify T cells, NKT-like cells and NK cells. This was performed at baseline, 1FU and 2FU. Percentage frequency is shown for LAG3 positive cells.

Upper panel, left to right: First dot plot shows expression of CD4+ T cells, following this; representative dot plots of LAG3+ CD4+ T cells at baseline, 1FU and 2FU.

First centre panel, Left to Right: First dot plot shows expression of CD8+ T cells, following this; representative dot plots of LAG3+ CD8+ T cells at baseline, 1FU and 2FU.

Second centre panel, Left to Right: First dot plot shows expression of NKT-like cells (CD3+CD56+), following this; representative dot plots of LAG3+ NKT-like cells at baseline, 1FU and 2FU.

Third centre panel, Left to Right: First dot plot shows expression of NK cells (CD3-CD56+), following this; representative dot plots of LAG3+ NK cells at baseline, 1FU and 2FU.

Lower panel, Left and Right: First dot plot shows expression of B cells (CD19+) following this; representative dot plots of LAG3+ B cells at baseline, 1FU and 2FU.

Table 5.13: Percent frequency for LAG3+ cells

Cell Population	% Baseline	% 1FU	% 2FU
CD4+ LAG3+	0.37	0.39	0.85
CD8+ LAG3+	0.4	0.62	1.09
NKT-like LAG3+	3.51	4.03	4.44
NK LAG3+	1.51	1.66	2.03
CD19+ LAG3+	1.86	2.77	5.19

Table 5.14: Mixed-effects linear regression comparing baseline with 1FU and 2FU for LAG3+ cells

Cell population / Timepoint	Coefficient (β)	Std Err (SE)	p value	95% Confidence Interval
CD4+LAG3+ 1FU	0.007	0.012	0.510	-0.014-0.028
CD4+ LAG3+ 2FU	0.031	0.014	0.028	0.003-0.058
CD8+ LAG3+ 1FU	-0.093	0.065	0.152	-0.222-0.034
CD8+ LAG3+ 2FU	-0.084	0.059	0.157	-0.202-0.032
NKT-like LAG3+ 1FU	0.001	0.021	0.995	-0.041-0.041
NKT-like LAG3+ 2FU	0.041	0.022	0.066	-0.002-0.083
NK LAG3+ 1FU	-0.026	0.036	0.469	-0.096-0.045
NK LAG3+ 2FU	-0.042	0.034	0.219	-0.109-0.02
CD19+ LAG3+ 1FU	-0.011	0.020	0.574	-0.050-0.028
CD19+ LAG3+ 2FU	0.027	0.026	0.160	-0.015-0.087

Table 5.15: Mixed-effects linear regression with clinicopathological characteristics for LAG3+ cells

Statistical Analysis	Chi2 (χ^2) (2)	p value Univariate	Stage II p value	Stage III p value	Age p value	Gender p value	Histology p value
CD4+LAG3+	21.45	<0.0001	0.326	0.001	0.383	0.561	0.625
CD8+ LAG3+	2.09	0.3525	0.870	0.706	0.496	0.543	0.550
NKT-like LAG3+	1.69	0.4288	0.741	0.161	0.479	0.182	0.408
NK LAG3+	1.53	0.4651	0.895	0.195	0.316	0.854	<0.001
CD19+LAG3+	10.8	0.0045	0.711	0.669	0.391	0.534	0.063

Table 5.16: Mixed-effects linear regression incorporating complications and surgical approach for LAG3+ T cells

Statistical Analysis	Complications P value	Surgical approach p value
CD4+ LAG3+	0.559	0.997
CD8+ LAG3+	0.254	0.109
NKT-like LAG3+	0.042	0.650
NK LAG3+	0.184	0.236
CD19+ LAG3+	0.555	0.618

Table 5.17: Descriptive summary statistics incorporating median slope and interquartile range (IQR) for LAG3+ cell populations

Cell Population	Median Slope Recurrence = 0	IQR Recurrence = 0	Median Slope Recurrence = 1	IQR Recurrence = 1
CD4+LAG3+	0.21	-0.005- 0.74	0.42	0.06-7.2
CD8+ LAG3+	0.09	-0.57-0.76	0.93	-6.22-3.75
NKT like LAG3+	0.14	-0.40-2.01	-0.51	-1.88--0.15
NK LAG3+	0.23	-8.73-3.61	0.17	-0.02-0.22
CD19+LAG3+	0.167	-0.37-2.73	0.003	-0.75-0.63

Table 5.18: Cox Regression analysis for LAG3+ cells adjusted for clinicopathological characteristics

Cell Population	Hazard Ratio	95% CI	P value	Age	Gender	Histology	Stage	Complications
CD4+LAG3+	1.80	1.26-2.57	0.001	0.129	0.603	0.000	0.001	0.006
CD8+ LAG3+	1.13	0.99-1.30	0.071	0.326	0.583	0.009	0.023	0.013
NKT-like LAG3+	0.61	0.39-0.94	0.025	0.597	0.243	0.002	0.000	0.048
NK LAG3+	1.14	0.83-1.55	0.398	0.209	0.538	0.003	0.001	0.046
CD19+LAG3+	0.88	0.57-1.12	0.189	0.471	0.367	0.005	0.001	0.047

5.6 TIM3

5.6.1 TIM3+ on CD4+ T cells

Flow cytometry gating was performed to identify CD4+ TIM3+ T cells as per Figure 2.2. Briefly, total cells, single cells and live cells were identified and then lymphocytes gated based on forward and side scatter, followed by selection of CD3+CD4+ T cells. Within this population TIM3+ was expressed as a percentage of total CD4+ T cells.

5.6.1.1 Longitudinal analysis of CD4+TIM3+ T cells

CD4+ T cell TIM3 expression showed a significant overall change over time ($\chi^2 = 16.89$, $p = 0.0002$), with higher expression observed at 1FU ($\beta = 0.024$, 95% CI: 0.004 to 0.044, $p = 0.020$) and 2FU ($\beta = 0.039$, 95% CI: 0.016 to 0.061, $p = 0.001$) compared with baseline. Clinical and tumour factors, were not significantly associated with TIM3 expression, and did not modify its change over time. Refer to Figure 5.13 and Tables 5.19-5.22 for details.

5.6.1.2: Impact of CD4+TIM3+ T Cell Changes on DFS

TIM3 expression on CD4+ T cells showed a slightly lower median slope in patients with recurrence (0.12) compared with those without recurrence (0.15), indicating less increase in TIM-3 over time in the recurrence group. Cox regression analysis indicates the slope

was associated with recurrence with a hazard ratio of 0.026 (95% CI 0.0013–0.502, $p = 0.016$), suggesting that a steeper increase in TIM3 over time is linked to lower recurrence risk. This may reflect a biological role of TIM3 on CD4+ T cells in maintaining effective T cell regulation and antitumour immunity, where insufficient upregulation could contribute to recurrence. Refer to Figure 5.15 for DFS and Figure 5.16 for representative flow cytometry plots and Tables 5.23 and 5.24 for details.

5.6.2 TIM-3+ on CD8+ T cells

Flow cytometry gating was performed to identify CD8+ TIM3+ T cells as per Figure 2.2. Briefly, total cells, single cells and live cells were identified and then lymphocytes gated based on forward and side scatter, followed by selection of CD3+CD8+ T cells. Within this population TIM3+ was expressed as a percentage of total CD8+ T cells.

5.6.2.1 Longitudinal analysis of CD8+TIM3+T cells

CD8+ T cell TIM3 expression showed a significant overall change over time ($\chi^2 = 10.00$, $p = 0.0067$), with a significant increase at 2FU compared with baseline ($\beta = 0.0218$, 95% CI: 0.0025 to 0.0411, $p = 0.027$) only. Increased age was associated with lower CD8+ TIM3 expression ($\beta = -0.0021$, 95% CI: -0.0040 – -0.00017 , $p = 0.033$), and adenocarcinoma histology was associated with reduced expression ($\beta = -0.0363$, 95% CI: -0.0669 to -0.0057 , $p = 0.020$), while other factors were not significant. Refer to Figure 5.13-5.14 and Tables 5.19-5.22 for details.

5.6.2.2. Impact of CD8+TIM-3+ Cell Changes on DFS

CD8+ TIM3 T cell expression slopes showed a median of 0.085 in patients with recurrence versus 0.028 in non-recurrence. The association with recurrence was not significant (HR 0.435, 95% CI 0.052–3.61, $p = 0.441$), indicating no clear impact of CD8+ TIM3+ cell dynamics on recurrence in this cohort. Stage (HR 12.943, 95% CI 1.94–86.2, $p = 0.008$) and post-operative complications (HR 5.194, 95% CI 1.09–24.7, $p = 0.038$) were predictive of recurrence. Refer to Figure 5.15 for DFS and Figure 5.16 for representative flow cytometry plots and Tables 5.23 and 5.24 for details.

5.6.3 TIM3+ on NKT-like cells

Flow cytometry gating was performed to identify TIM3 expression on surface NKT-like cells as per Figure 2.2. Briefly total cells, single cells and live cells were identified and then

lymphocytes were gated based on forward and side scatter, followed by selection of CD3+ CD56+ cells. Following this NKT-like TIM3+ cells were identified.

5.6.3.1 Longitudinal analysis of NKT-like TIM-3+T cells

TIM3 expression on NKT-like cells showed a non-significant overall change over time ($\chi^2 = 2.72$, $p = 0.257$), with a trend toward higher levels at 2FU ($\beta = 0.051$, 95% CI: -0.005 to 0.107 , $p = 0.073$). Clinical and tumour covariates, were not significantly associated with NKT-like TIM3+ expression. Refer to Figure 5.13 and Tables 5.19-5.22 for details.

5.6.3.2: Impact of NKT-like TIM-3+ Cell Changes on DFS

Patients with recurrence had slightly higher median slope values (1.25) compared with those without recurrence (0.95). TIM3+ expression on NKT-like cells was significantly associated with recurrence, showing a protective effect (HR = 0.59, 95% CI: 0.39–0.89, $p = 0.013$). Among clinical and tumour factors, histology (HR = 177.7, 95% CI: 3.45–9153.5, $p = 0.010$), stage (HR = 177.3, 95% CI: 5.28–5956.2, $p = 0.004$), and patients with post-operative complications (HR = 388.2, 95% CI: 4.08–36925.4, $p = 0.010$) were also significant predictors of recurrence, while age and gender were not. Refer to Figure 5.15 for DFS and Figure 5.16 for representative flow cytometry plots and Tables 5.23 and 5.24 for details.

5.6.4 TIM3+ on NK cells

Flow cytometry gating was performed to identify TIM3 expression on surface NK cells as per Figure 2.2. Briefly total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD3- CD56+ cells. Following this NK TIM3+ cells were identified.

5.6.4.1 Longitudinal analysis of NK TIM3+T cells

TIM3 expression on NK cells showed a trend toward a decrease over time ($\chi^2 = 4.61$, $p = 0.0997$), with the largest reduction observed at 2FU ($\beta = -0.054$, 95% CI: -0.125 to 0.018 , $p = 0.142$). Age and tumour histology were significantly associated with NK TIM-3 expression, with lower levels in older patients ($\beta = -0.006$, 95% CI: -0.012 to -0.001 , $p = 0.033$) and in adenocarcinoma histology ($\beta = -0.113$, 95% CI: -0.201 to -0.025 , $p = 0.012$). Refer to Figure 5.13-5.14 and Tables 5.19-5.22 for details.

5.6.4.2: Impact of NK TIM3+ Cell Changes on DFS

Patients with recurrence had higher median slope values (0.125) compared with non-recurrence patients (-0.08), indicating elevated and more heterogeneous TIM3 expression in the recurrence group. In Cox regression, TIM3+ expression on NK cells was significantly associated with recurrence, (HR = 1.22, 95% CI: 1.09–1.37, $p = 0.001$) indicating that higher TIM3+ slope in NK cells increases recurrence risk. Among tumour and clinical factors, Histology (HR = 17.76, 95% CI: 2.98–105.71, $p = 0.002$) and stage (HR = 11.12, 95% CI: 2.62–47.24, $p = 0.001$) were also significant predictors. Refer to Figure 5.15 for DFS and Figure 5.16 for representative flow cytometry plots and Tables 5.23 and 5.24 for details. Overall, both elevated TIM3+ expression on NK cells and key tumour characteristics were associated with higher recurrence risk.

5.6.5 TIM3+ on CD19+ cells

Flow cytometry gating was performed to identify TIM3 expression on surface B cells as per Figure 2.2. Briefly total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD19++ cells. Following this CD19+ LAG3+ cells were identified.

5.6.5.1 Longitudinal analysis of CD19+ TIM3+T cells

TIM3 expression on B cells showed a significant increase over time in ($\chi^2 = 26.85$, $p < 0.001$), with the largest rise at 2FU ($\beta = 0.087$, 95% CI: 0.010–0.164, $p = 0.026$). Patients with complications had higher values compared with those without complications (coefficient = 0.1338, 95% CI: 0.0437–0.224, $p = 0.004$), while no other covariates were significantly associated with CD19+ TIM3 expression. Refer to Figure 5.13 & 5.14 and Tables 5.19-5.22 for details. This indicates a robust time-dependent increase in B cell TIM3 expression over the study period, with post-operative complications a strong predictor.

5.6.5.2: Impact of CD19+ TIM3+ Cell Changes on DFS

Patients with recurrence had the same median slope (0.58) compared with non-recurrence patients (0.58). In multivariable Cox regression, TIM3+ expression on T cells was not significantly associated with recurrence (HR = 0.94, 95% CI: 0.86–1.02, $p = 0.148$). Histology (HR = 6.68, 95% CI: 1.02–43.94, $p = 0.048$), stage (HR = 18.64, 95% CI: 2.62–132.61, $p = 0.003$), and patients with post-operative complications (HR = 27.39, 95% CI: 2.07–362.25, $p = 0.012$) were significant predictors of recurrence. Refer to Figure 5.15 for

DFS and Figure 5.16 for representative flow cytometry plots and Tables 5.23 and 5.24 for details.

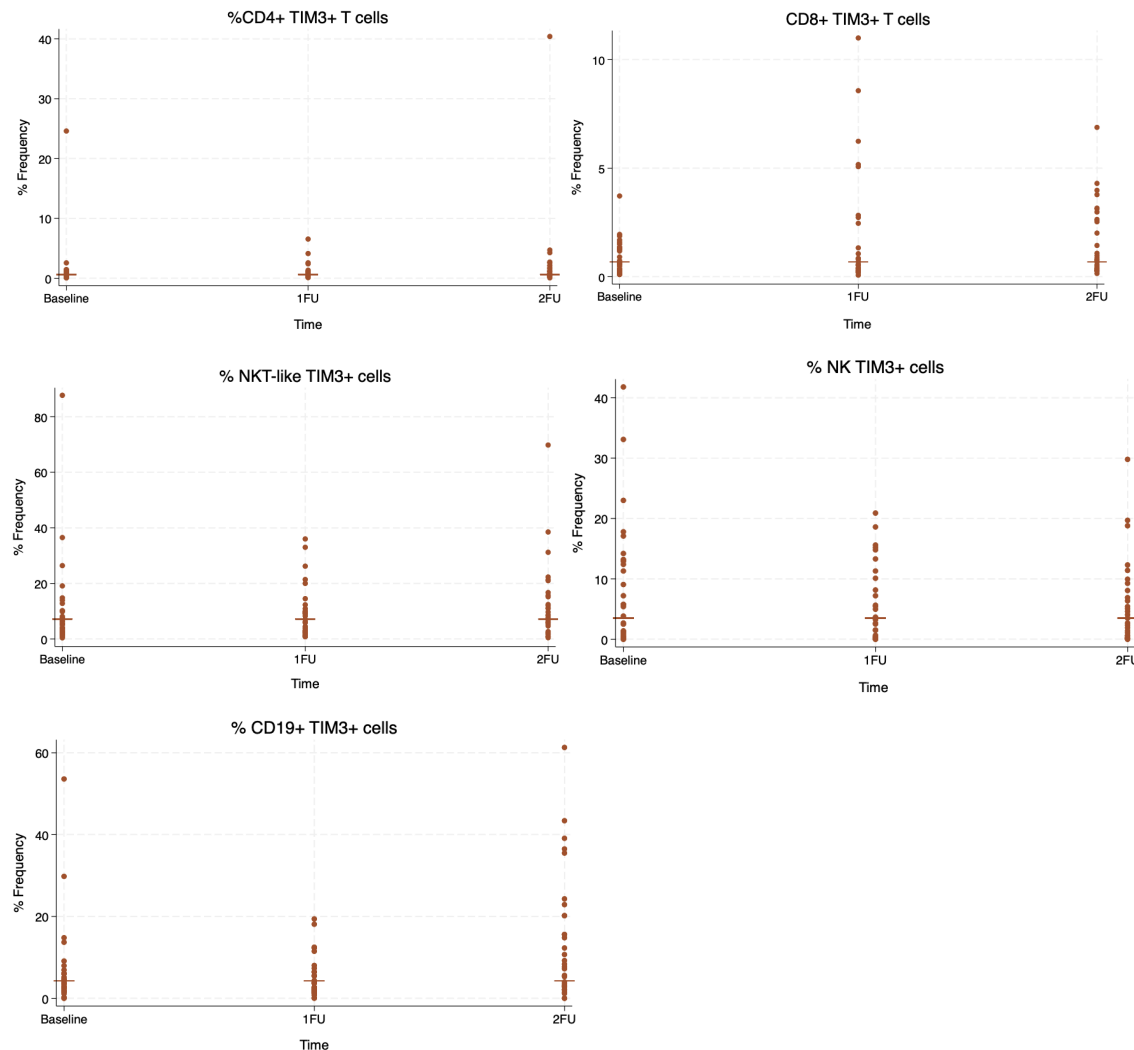


Figure 5.13. Dot plots showing % frequency of TIM3 expression on immune cells at each timepoint. Total cells were first filtered to isolate single and viable cells. Lymphocytes were then selected using forward- and side-scatter properties. Gating of CD3+ was utilised to identify T cell subsets; CD4+ cells and CD8+ cells, alongside NKT-like cells (CD56+). Gating of CD3- and CD56+ was utilised to identify NK cells. Gating of CD19+ was utilised to identify B cells. TIM3 positivity was then defined as a percentage frequency of CD4+ T cells, CD8+ T cells, NKT-like cells, NK cells and B cells. Mixed-effects linear regression revealed a significant difference in frequency over time for LAG3 expression on CD4+ T cells ($p=0.0002$), CD8+ T cells ($p=0.0067$), and B cells ($p=0.001$). For NKT-like cells ($p=0.42571$) and NK cells ($p=0.0997$), there was no significant difference over time. Dots representing each patient and horizontal line at median value.

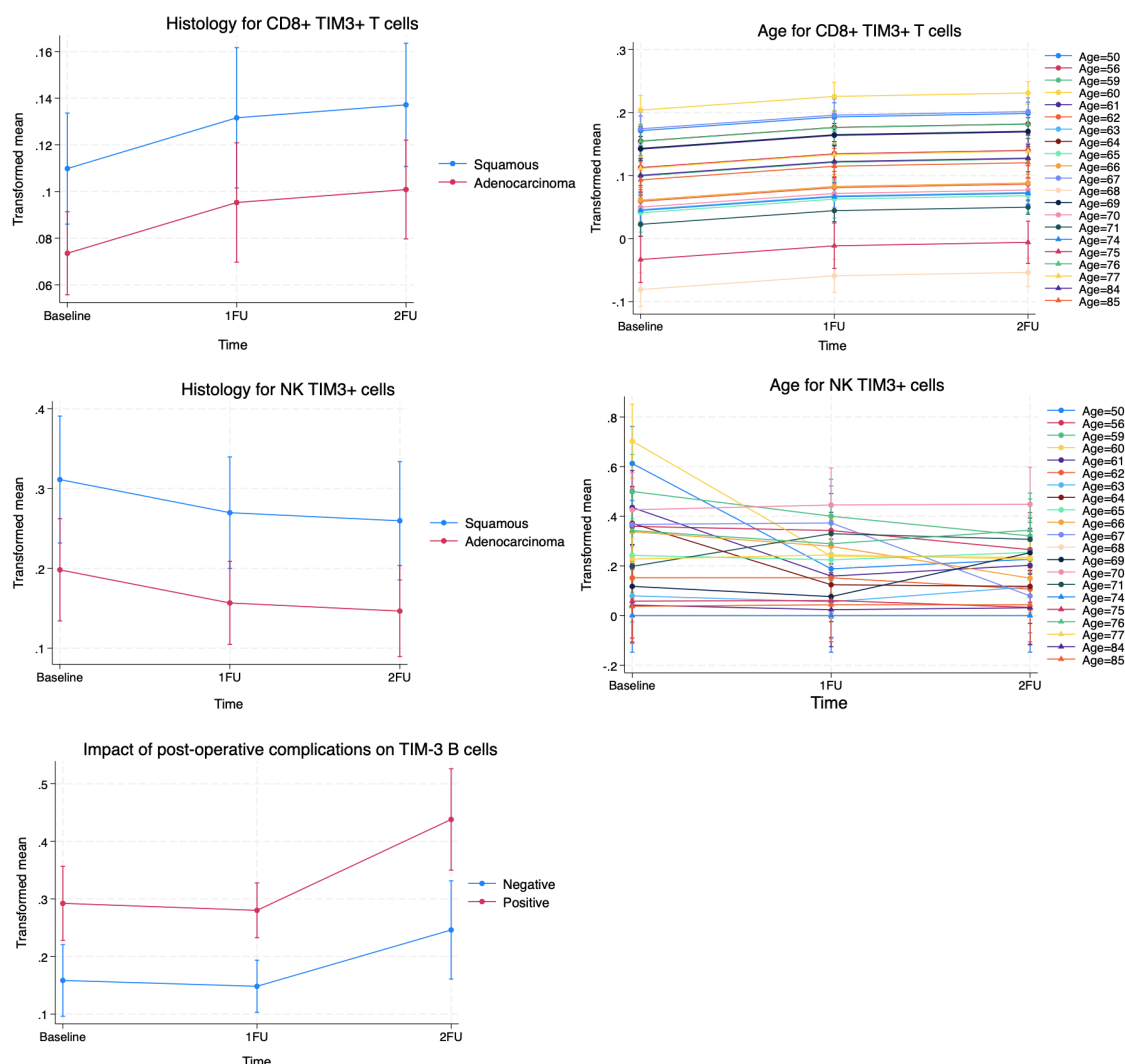


Figure 5.14. Predicted means plot showing differences over time of TIM3 expression on immune cells in association with clinicopathological characteristics. Data obtained from FlowJo v10.10.0 software, were expressed as percentage frequencies and subsequently transformed using the arcsine-square root transformation to meet the assumptions of parametric analysis. Plotted values in this figure represent transformed means. To evaluate changes across the three timepoints, linear mixed-effects models were applied in association with age, gender, histological subtype, disease stage, surgical approach, and the presence or absence of post-operative complications. Expression on CD4+ T cells, CD8 + T cells, NKT cells, NK cells and B cells was analysed. Histology was strongly associated with TIM3 positivity on CD8+ cells ($p=0.020$) and NK cells ($p=0.012$), with lower expression in adenocarcinoma compared with squamous histology. Increased age was associated with lower TIM3 expression on CD8+ T cells ($p = 0.033$) and NK cells ($p=0.033$). Patients with complications had higher TIM3 expression on B cells ($p = 0.004$).

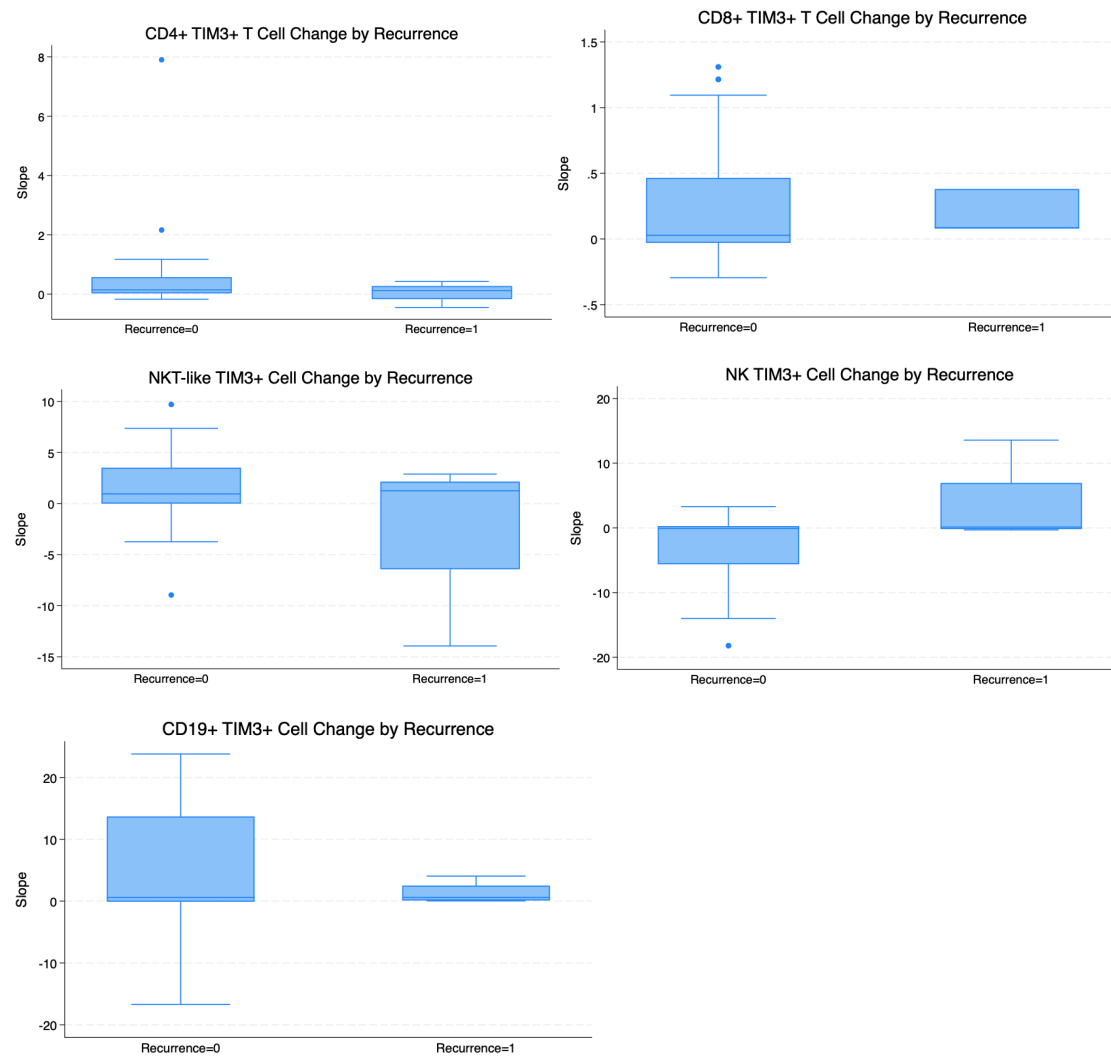


Figure 5.15. Boxplots of TIM3 expression on immune cells and changes over time (slopes) in patients with and without recurrence. The longitudinal slope summarises the linear change over three timepoints in patients ($n=34$) for TIM3 expression on CD4+ T cells, CD8 + T cells, NKT cells, NK cells and B cells. Cox proportional hazards regression was utilised to assess the association between the slope and DFS. A positive slope indicates increasing cell frequency, while a negative slope reflects a decline. Baseline was used as the reference time point for comparisons across all three time points, as well as individually against 1FU and 2FU. The slopes of TIM3 expression on CD4+ T cells ($p=0.016$), NKT-like cells ($p=0.013$) and NK cells ($p=0.001$) over the three timepoints were significantly associated with recurrence. The slopes of TIM3 expression on CD8+ T cells ($p=0.441$), and B cells ($p=0.148$) were not associated with recurrence.

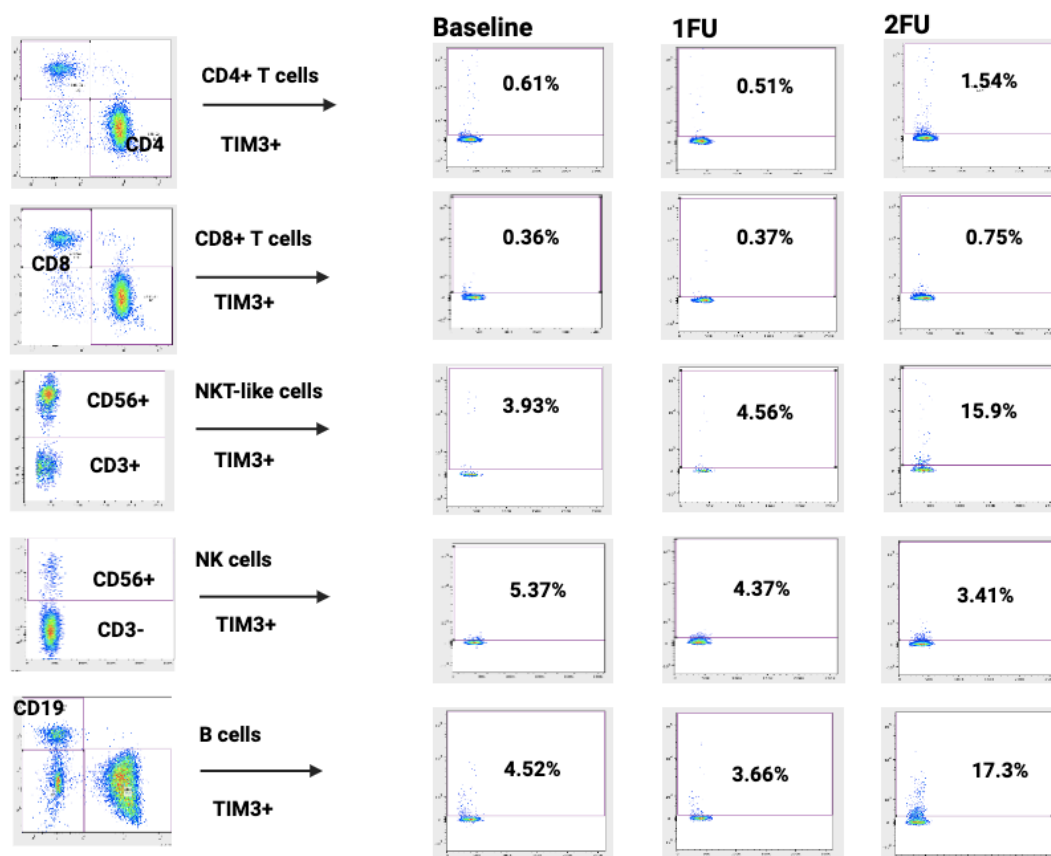


Figure 5.16. Representative flow cytometry plots for TIM3+ on immune cells at different timepoints. PBMCs were stained with a dead cell stain (DCS), CD3, CD4, CD8, CD56, CD19 and TIM3 antibodies and analysed by flow cytometry. Forward scatter area (FSC-A) and scatter area (SSC-A) was used to determine total cells, following this FSC-A and FSC-width (FSC-W) was used to determine single cells, then FSC-A and DCS was used to determine live cells. Finally, FSC-A and SSC-A was used to determine lymphocytes. CD3 was utilised to identify T cells, NKT-like cells and NK cells. This was performed at baseline, 1FU and 2FU. Percentage frequency is shown for TIM3 positive cells.

Upper panel, left to right: First dot plot shows expression of CD4+T cells, following this; representative dot plots of TIM3+CD4+ T cells at baseline, 1FU and 2FU.

First centre panel, Left to Right: First dot plot shows expression of CD8+ T cells, following this; representative dot plots of TIM3+ CD8+ T cells at baseline, 1FU and 2FU.

Second centre panel, Left to Right: First dot plot shows expression of NKT-like cells (CD3+CD56+), following this; representative dot plots of TIM3+ NKT-like cells at baseline, 1FU and 2FU.

Third centre panel, Left to Right: First dot plot shows expression of NK cells (CD3-CD56+), following this; representative dot plots of TIM3+ NK cells at baseline, 1FU and 2FU.

Lower panel, Left and Right: First dot plot shows expression of B cells (CD19+) following this; representative dot plots of TIM3+ B cells at baseline, 1FU and 2FU

Table 5.19: Percent frequency of TIM3+ cells

Cell Population	% Baseline	% 1FU	% 2FU
CD4+TIM3+	0.49	0.53	1.04
CD8+TIM3+	0.51	0.40	0.76
NKT-like TIM3+	5.46	8.11	9.68
NK TIM3+	5.4	3.59	3.48
CD19+TIM3+	3.72	3.74	8.35

Table 5.20: Mixed-effects linear regression comparing baseline with 1FU and 2FU for TIM3+ cells

Cell population / Timepoint	Coefficient (β)	Std Err (SE)	p value	95% Confidence Interval
CD4+ TIM3+ 1FU	0.023	0.010	0.020	0.002-0.043
CD4+ TIM3+ 2FU	0.039	0.012	0.001	0.015-0.061
CD8+ TIM3+ 1FU	0.018	0.014	0.187	-0.008-0.044
CD8+ TIM3+ 2FU	0.022	0.009	0.027	-0.002-0.041
NKT-like TIM3+ 1FU	0.033	0.025	0.199	-0.018-0.084
NKT-like TIM3+ 2FU	0.051	0.029	0.071	-0.005-0.107
NK TIM3+ 1FU	-0.043	0.033	0.194	-0.108-0.021
NK TIM3+ 2FU	-0.053	0.037	0.142	-0.125-0.018
CD19+ TIM3+ 1FU	-0.023	0.019	0.239	-0.062-0.016
CD19+ TIM3+ 2FU	0.087	0.039	0.026	0.010-0.1644

Table 5.21: Mixed-effects linear regression incorporating complications and surgical approach for TIM3+ cells

Statistical Analysis	Complications P value	Surgical approach p value
CD4+TIM3+	0.173	0.443
CD8+TIM3+	0.167	0.982
NKT-like TIM3+	0.057	0.070
NK TIM3+	0.793	0.444
CD19+TIM3+	0.004	0.427

Table 5.22: Mixed-effects linear regression with clinicopathological characteristics for TIM3+ cells

Cell Population	Chi2 (χ^2) (2)	p value	Stage II p value	Stage III p value	Age p value	Gender p value	Histology p value
CD4+TIM3+	16.89	0.0002	0.825	0.915	0.149	0.144	0.951
CD8+TIM3+	10.00	0.0067	0.318	0.350	0.033	0.451	0.020
NKT-like TIM3+	2.72	0.2571	0.628	0.691	0.528	0.149	0.926
NK TIM3+	4.61	0.0997	0.119	0.275	0.033	0.174	0.012
CD19+TIM3+	26.65	0.001	0.307	0.825	0.533	0.599	0.569

Table 5.23: Descriptive summary statistics incorporating median slope and interquartile range (IQR) for TIM3+ cell populations

Cell Population	Median Slope Recurrence = 0	IQR Recurrence = 0	Median Slope Recurrence = 1	IQR Recurrence = 1
CD4+TIM3+	0.15	0.03- 0.58	0.12	-0.17-0.27
CD8+TIM3+	-0.065	-2.11-0.26	-0.005	-1.34-2.05
NKT-like TIM3+	0.95	0-3.51	1.25	-6.42-2.14
NK TIM3+	-0.08	-5.61-0.28	0.13	-0.18-6.93
CD19+TIM3+	0.58	-0.085-13.70	0.58	0.11-2.5

Table 5.24: Cox Regression analysis for TIM3+ cells adjusted for clinicopathological characteristics

Cell Population	Haz Ratio	95% CI	P value	Age	Gender	Histology	Stage	Complications
CD4+TIM3+	0.03	0.001-0.50	0.016	0.099	0.360	0.009	0.002	0.017
CD8+TIM3+	0.43	0.05-3.6	0.441	0.199	0.627	0.549	0.008	0.038
NKT-like TIM3+	0.59	0.39-0.89	0.013	0.399	0.094	0.010	0.004	0.010
NK TIM3+	1.22	1.09-1.37	0.001	0.545	0.053	0.002	0.001	0.438
CD19+TIM3+	0.94	0.86-1.02	0.148	0.080	0.773	0.048	0.003	0.012

5.7 BTLA

5.7.1 BTLA on CD4+ T cells

Flow cytometry gating was performed to identify CD4+ BTLA+ T cells as per Figure 2.2. Briefly, total cells, single cells and live cells were identified and then lymphocytes gated based on forward and side scatter, followed by selection of CD3+CD4+ T cells. Within this population BTLA+ was expressed as a percentage of total CD4+ T cells.

5.7.1.1 Longitudinal analysis of CD4+BTLA+ T Cells

For CD4+ BTLA expression, mixed-effects modelling revealed a significant decrease over time. 2FU reduction compared to baseline was significant ($\beta = -0.129$, 95% CI: -0.247 to -0.012 , $p = 0.031$), whereas 1FU showed a non-significant reduction ($\beta = -0.080$, 95% CI: -0.188 to 0.027 , $p = 0.143$). Refer to Figure 5.17 and Tables 5.25-5.28 for details.

5.7.1.2: Impact of CD4+BTLA+ Cell Changes on DFS

There was no independent association between the rate of change in CD4+ BTLA+ cell proportions and recurrence (HR 1.02, 95% CI 0.96–1.07, $p = 0.57$). Median slope values were higher in the non-recurrence (-2.35) compared to recurrence patients (-10.12). Histology (HR 7.60, 95% CI 2.02–28.61, $p = 0.003$) and tumour stage (HR 6.51, 95% CI 2.07–20.52, $p = 0.001$) remained significant predictors of recurrence, while post-operative complications showed a borderline effect (HR 3.49, 95% CI 0.93–13.18, $p = 0.065$). Refer to Figure 5.19 for DFS and Figure 5.20 for representative flow cytometry plots and Tables 5.29 & 5.30.

5.7.2 BTLA+ on CD8+ T cells

Flow cytometry gating was performed to identify CD8+ BTLA+ T cells as per Figure 2.2. Briefly, total cells, single cells and live cells were identified and then lymphocytes gated based on forward and side scatter, followed by selection of CD3+CD8+ T cells. Within this population BTLA+ was expressed as a percentage of total CD8+ T cells.

5.7.2.1 Longitudinal analysis of CD8+BTLA+ T Cells

For CD8+ BTLA expression, mixed-effects analysis indicated a significant decrease over time. At the second follow-up (2FU), expression was significantly reduced compared to baseline ($\beta = -0.127$, 95% CI: -0.199 to -0.056 , $p < 0.001$), while the first follow-up (1FU) showed a non-significant reduction ($\beta = -0.033$, 95% CI: -0.097 to 0.032 , $p = 0.320$). Age

was positively associated with CD8+ BTLA expression ($\beta = 0.009$, 95% CI: 0.001 to 0.016, $p = 0.020$). Refer to Figure 5.17 & 5.18 and Tables 5.25-5.28 for details.

5.7.2.2: Impact of CD8+BTLA+ Cell Changes on DFS

There was no association between the rate of change in CD8+ BTLA+ cell proportions and recurrence (HR 0.99, 95% CI 0.89–1.10, $p = 0.84$). Median slopes were similar between recurrence and non-recurrence groups (-6.05 vs. -4.89), indicating no biologically meaningful difference in temporal BTLA expression on CD8+ T cells across outcomes. Again, histology (HR 7.55, 95% CI 1.96–29.02, $p = 0.003$) and tumour stage (HR 5.53, 95% CI 1.98–15.42, $p = 0.001$) remained strong independent predictors of recurrence, while postoperative complications showed a borderline association (HR 3.63, 95% CI 0.91–14.54, $p = 0.068$). Refer to Figure 5.19 for DFS and Figure 5.20 for representative flow cytometry plots and Tables 5.29 & 5.30.

5.7.3 BTLA+ on NKT-like cells

Flow cytometry gating was performed to identify BTLA expression on surface NKT-like cells as per Figure 2.2. Briefly total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD3+ CD56+ cells. Following this NKT-like BTLA+ cells were identified.

5.7.3.1 Longitudinal analysis of NKT-like BTLA+ T Cells

For NKT-like BTLA expression, mixed-effects analysis showed that expression decreased over time. At the 2FU, levels were significantly reduced compared to baseline ($\beta = -0.122$, 95% CI: -0.195 to -0.049 , $p = 0.001$), while the reduction at 1FU did not reach significance ($\beta = -0.053$, 95% CI: -0.120 to 0.014 , $p = 0.118$). Clinical and tumour characteristics did not influence NKT-like BTLA+ expression in this patient cohort. Refer to Figure 5.19 for DFS and Figure 5.20 for representative flow cytometry plots and Tables 5.29 & 5.30.

5.7.3.2: Impact of NKT-like BTLA+ Cell Changes on DFS

In the multivariable Cox regression model the NKT-like cell BTLA slope was not significantly associated with recurrence risk (HR = 0.99, 95% CI: 0.90–1.08, $p = 0.77$). Histology (HR = 7.53, 95% CI: 1.96–28.97, $p = 0.003$) and stage (HR = 5.36, 95% CI: 1.84–15.63, $p = 0.002$) remained strong independent predictors of recurrence. Refer to Figure 5.17 and Tables 5.29 & 5.30 for details.

5.7.4 *BTLA+ on NK cells*

Flow cytometry gating was performed to identify BTLA expression on surface NK cells as per Figure 2.2. Briefly total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD3-CD56+ cells. Following this NK BTLA+ cells were identified.

5.7.4.1 Longitudinal analysis of NK BTLA+ T Cells

BTLA expression on NK cells showed a significant increase over time in ($\chi^2 = 8.92$, $p = 0.012$). After adjusting for clinical covariates, the association between time and NK BTLA expression was attenuated. The increase at 1FU was not significant ($\beta = +0.017$, 95% CI: -0.026 to $+0.059$, $p = 0.436$), and the increase at 2FU showed only a borderline trend ($\beta = +0.028$, 95% CI: -0.002 to $+0.058$, $p = 0.066$). Notably, open surgery was associated with higher NK BTLA expression ($\beta = +0.088$, 95% CI: $+0.025$ to $+0.151$, $p = 0.006$). Refer to Figure 5.17 and 5.18 and Tables 5.25-5.28 for details.

5.7.4.2: Impact of NK BTLA+ Cell Changes on DFS

In the multivariable Cox model, the NK BTLA slope was not significantly associated with recurrence (HR = 1.22, 95% CI: 0.74–2.01, $p = 0.43$). Histology (HR = 11.69, 95% CI: 1.93–70.68, $p = 0.007$) and stage (HR = 6.02, 95% CI: 2.17–16.69, $p = 0.001$) remained strong predictors of recurrence, while complications showed a nonsignificant trend (HR = 3.38, $p = 0.08$). Median NK-BTLA slopes were slightly positive in both groups (Recurrence: 0.05 vs. No recurrence: 0.18), indicating no meaningful difference in longitudinal NK BTLA expression between outcomes. Refer to Figure 5.19 for DFS and Figure 5.20 for representative flow cytometry plots and Tables 5.29 & 5.30.

5.7.5 *BTLA+ on CD19+ cells*

Flow cytometry gating was performed to identify BTLA expression on surface B cells as per Figure 2.2. Briefly total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD19+ cells. Following this CD19+ BTLA+ cells were identified.

5.7.5.1 Longitudinal analysis of CD19+ BTLA+ T Cells

For BTLA expression on B cells, mixed-effects analysis indicated a significant decrease over time ($\chi^2 = 14.47$, $p = 0.007$). At the 2FU, expression was significantly lower compared

to baseline ($\beta = -0.225$, 95% CI: -0.388 to -0.061 , $p = 0.007$), while at 1FU a non-significant reduction ($\beta = -0.075$, 95% CI: -0.228 to 0.079 , $p = 0.341$) occurred. Higher disease stage (Stage III) and male gender were associated with lower expression (Stage III: $\beta = -0.370$, $p = 0.028$; male: $\beta = -0.294$, $p = 0.008$), whereas age showed a borderline positive association. Refer to Figure 5.17-5.18 and Tables 5.25-5.28 for details .

5.7.5.2 Impact of CD19+ BTLA+ Cell Changes on DFS

The CD19+ BTLA slope was significantly associated with recurrence (HR = 1.05, 95% CI: 1.01–1.10, $p = 0.026$), suggesting that patients with increasing BTLA expression on B cells over time had higher recurrence risk. Median slopes were similar between recurrence and non-recurrence groups (-3.05 vs. -3.0), indicating high variability but a trend toward sustained BTLA upregulation in some patients. Histology (HR = 10.02, 95% CI: 2.54–39.55, $p = 0.001$) and stage (HR = 10.90, 95% CI: 2.96–40.13, $p < 0.001$) remained dominant clinical predictors of recurrence. Refer to Figure 5.19 for DFS and Figure 5.20 for representative flow cytometry plots and Tables 5.29 & 5.30. Overall increasing BTLA expression on B cells alongside clinical and tumour factors contribute to higher recurrence risk.

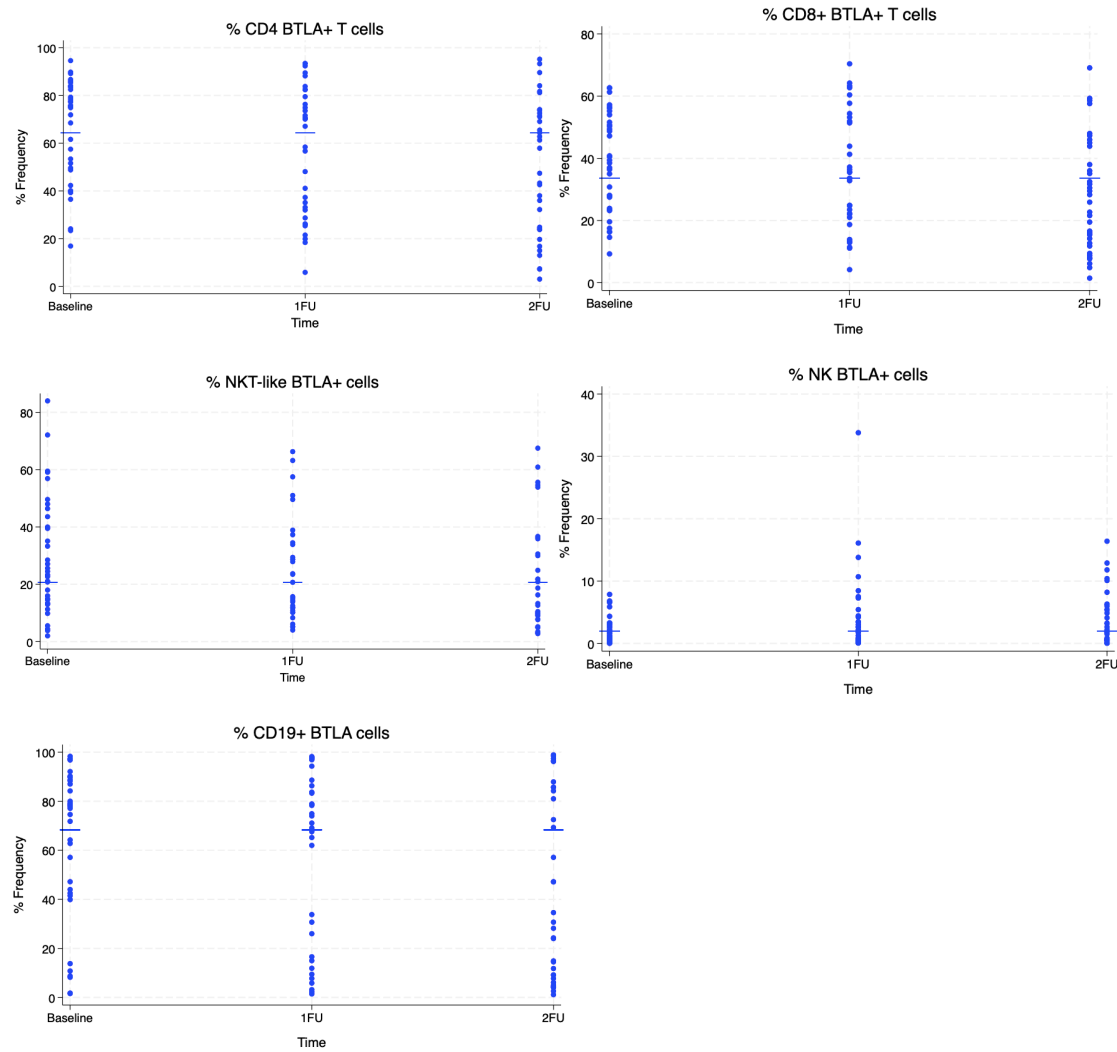


Figure 5.17. Dot plots showing % frequency of BTLA expression on immune cells at each timepoint. Total cells were first filtered to isolate single and viable cells. Lymphocytes were then selected using forward- and side-scatter properties. Gating of CD3+ was utilised to identify T cell subsets; CD4+ cells and CD8+ cells, alongside NKT-like cells (CD56+). Gating of CD3- and CD56+ was utilised to identify NK cells. Gating of CD19+ was utilised to identify B cells. BTLA positivity was then defined as a percentage frequency of CD4+ T cells, CD8+ T cells, NKT-like cells, NK cells and B cells. Mixed-effects linear regression revealed a significant difference in frequency over time for BTLA expression on all immune cells analysed; CD4+ T cells ($p=0.0004$), CD8+ T cells ($p=0.0001$), NKT-like cells ($p=0.0003$) and NK cells ($p=0.0116$), and B cells ($p=0.007$). Dots representing each patient and horizontal line at median value.

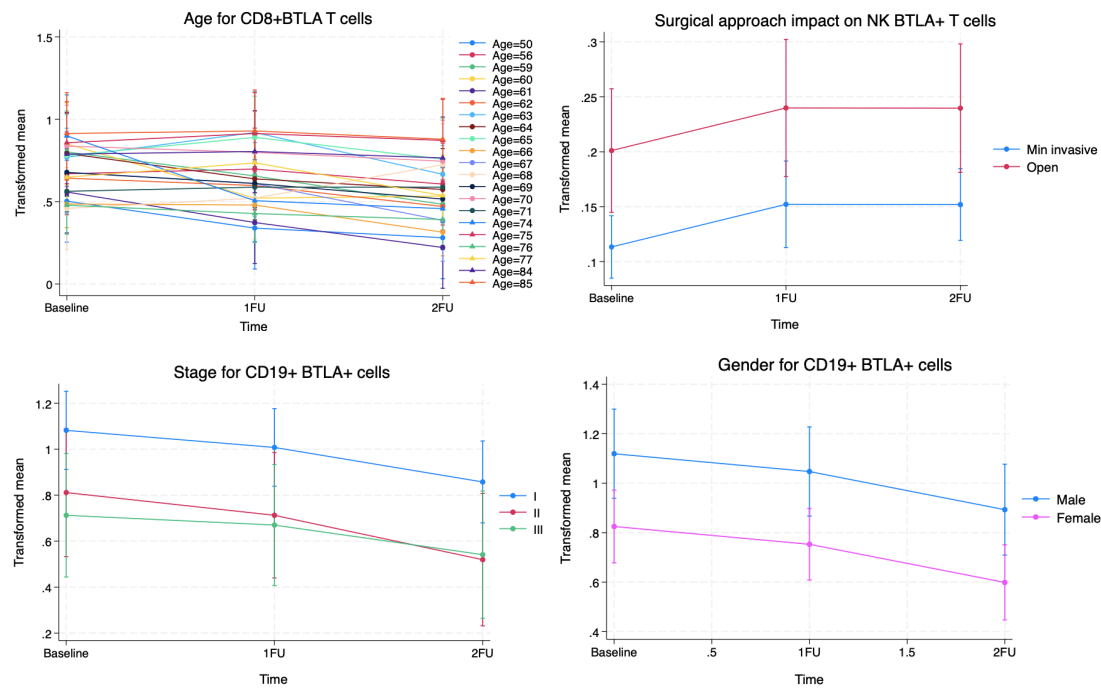


Figure 5.18. Predicted means plot showing differences over time of BTLA expression on immune cells in association with clinicopathological characteristics. Data obtained from FlowJo v10.10.0 software, were expressed as percentage frequencies and subsequently transformed using the arcsine-square root transformation to meet the assumptions of parametric analysis. Plotted values in this figure represent transformed means. To evaluate changes across the three timepoints, linear mixed-effects models were applied in association with age, gender, histological subtype, disease stage, surgical approach, and the presence or absence of post-operative complications. Expression on CD4+ T cells, CD8 + T cells, NKT cells, NK cells and B cells was analysed. Age was associated with lower TIM3 expression on CD8+ T cells ($p = 0.020$). Open surgery was associated with higher BTLA expression on NK cells ($p = 0.006$). Higher disease stage (Stage III) and male gender were associated with lower expression (Stage III: $p = 0.028$; male: $p = 0.008$) on B cells.

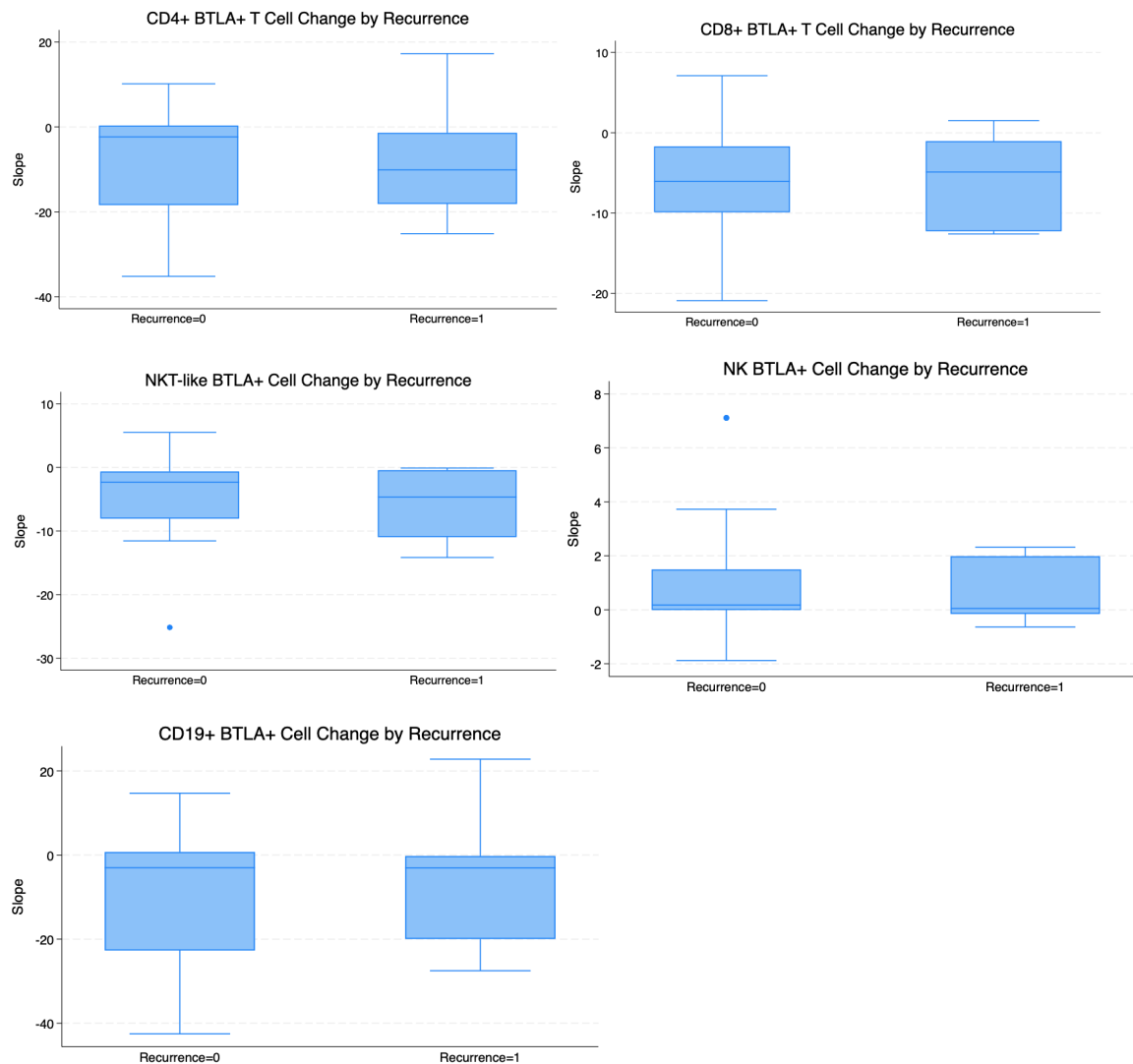


Figure 5.19. Boxplots of BTLA expression on immune cells and changes over time (slopes) in patients with and without recurrence. The longitudinal slope summarises the linear change over three timepoints in patients ($n=34$) for BTLA expression on CD4+ T cells, CD8 + T cells, NKT cells, NK cells and B cells. Cox proportional hazards regression was utilised to assess the association between the slope and DFS. A positive slope indicates increasing cell frequency, while a negative slope reflects a decline. Baseline was used as the reference time point for comparisons across all three time points, as well as individually against 1FU and 2FU. The slopes of BTLA expression on B cells ($p=0.026$) over the three timepoints were significantly associated with recurrence. The slopes of BTLA expression on CD4+ T cells ($p=0.556$), CD8+ T cells ($p=0.842$), NKT-like cells ($p=0.324$) and NK cells ($p=0.429$) and were not associated with recurrence.

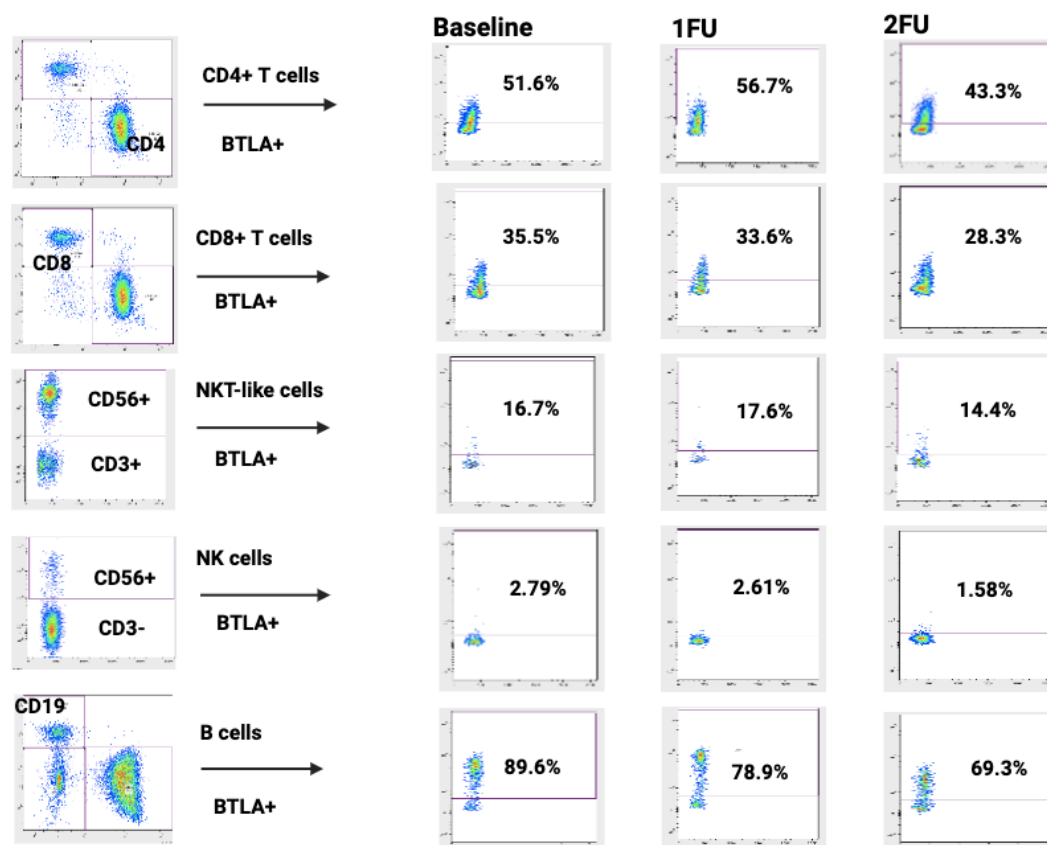


Figure 5.20. Representative flow cytometry plots for BTLA+ on immune cells at different timepoints. PBMCs were stained with a dead cell stain (DCS), CD3, CD4, CD8, CD56, CD19 and BTLA antibodies and analysed by flow cytometry. Forward scatter area (FSC-A) and scatter area (SSC-A) was used to determine total cells, following this FSC-A and FSC-width (FSC-W) was used to determine single cells, then FSC-A and DCS was used to determine live cells. Finally, FSC-A and SSC-A was used to determine lymphocytes. CD3 was utilised to identify T cells, NKT-like cells and NK cells. This was performed at baseline, 1FU and 2FU. Percentage frequency is shown for BTLA positive cells.

Upper panel, left to right: First dot plot shows expression of CD4+T cells, following this; representative dot plots of BTLA +CD4+ T cells at baseline, 1FU and 2FU.

First centre panel, Left to Right: First dot plot shows expression of CD8+ T cells, following this; representative dot plots of BTLA + CD8+ T cells at baseline, 1FU and 2FU.

Second centre panel, Left to Right: First dot plot shows expression of NKT-like cells (CD3+CD56+), following this; representative dot plots of BTLA + NKT-like cells at baseline, 1FU and 2FU.

Third centre panel, Left to Right: First dot plot shows expression of NK cells (CD3-CD56+), following this; representative dot plots of BTLA + NK cells at baseline, 1FU and 2FU.

Lower panel, Left and Right: First dot plot shows expression of B cells (CD19+) following this; representative dot plots of BTLA + B cells at baseline, 1FU and 2FU.

Table 5.25: Percent frequency and descriptive statistics of BTLA+ cells

Cell Population	% Baseline	% 1FU	% 2FU
CD4+BTLA+	74.9	68.6	59.6
CD8+BTLA+	39.4	34.6	28.9
NKT-like BTLA+	23.9	15.7	13
NK BTLA+	1.23	1.85	1.99
CD19+BTLA+	77.8	74	63.2

Table 5.26: Mixed-effects linear regression comparing baseline with 1FU and 2FU for BTLA+ cells

Cell Population /Timepoint	Coefficient (β)	Std Err (SE)	p value	95% Confidence Interval
CD4+ BTLA+ 1FU	-0.132	0.054	0.143	-0.187-0.027
CD4+ BTLA+ 2FU	-0.129	0.060	0.031	-0.246--0.015
CD8+ BTLA+ 1FU	-0.032	0.032	0.320	-0.096-0.031
CD8+ BTLA+ 2FU	0.127	0.036	<0.001	0.199- -0.056
NKT-like BTLA+ 1FU	-0.021	0.034	0.533	-0.088-0.046
NKT-like BTLA+ 2FU	-0.977	0.037	0.009	-0.171- -0.024
NK BTLA+ 1FU	0.017	0.022	0.436	-0.025-0.059
NK BTLA+ 2FU	0.028	0.015	0.066	-0.001-0.058
CD19+ BTLA+ 1FU	-0.075	0.078	0.341	-0.228-0.079
CD19+ BTLA+ 2FU	-0.225	0.083	0.007	-0.388-0.062

Table 5.27: Mixed-effects linear regression with clinicopathological characteristics for BTLA+ cells

Cell Population	Chi2 (χ ²) (2)	p value	Stage II p value	Stage III p value	Age p value	Gender p value	Histology p value
CD4+BTLA+	15.77	0.0004	0.294	0.332	0.053	0.358	0.940
CD8+BTLA+	23.9	0.0001	0.158	0.081	0.020	0.050	0.500
NKT-like BTLA+	16.2	0.0003	0.815	0.356	0.725	0.059	0.684
NK BTLA+	8.92	0.0116	0.311	0.108	0.196	0.799	0.846
CD19+BTLA+	14.47	0.0007	0.111	0.028	0.053	0.008	0.446

Table 5.28: Mixed-effects linear regression incorporating complications and surgical approach for BTLA+ cells

Cell Population	Complications P value	Surgical approach p value
CD4+BTLA+	0.396	0.513
CD8+BTLA+	0.420	0.455
NKT-like BTLA+	0.707	0.183
NK BTLA+	0.333	0.006
CD19+BTLA+	0.533	0.514

Table 5.29: Descriptive summary statistics incorporating median slope and interquartile range (IQR) for BTLA+ cells

Cell Population	Median Slope Recurrence = 0	IQR Recurrence = 0	Median Slope Recurrence = 1	IQR Recurrence = 1
CD4+BTLA+	-2.35	-18.4- 0.03	-10.12	-18.15-1.4
CD8+BTLA+	-6.05	-9.9--1.7	-4.89	-12.25--1.05
NKT-like BTLA+	-2.32	-8.0- -0.65	-4.65	-10.95- -0.45
NK BTLA+	0.18	-0.008-1.50	0.05	-0.15-1.99
CD19+BTLA+	-3	-22.7-0.73	-3.05	-19.94- -0.23

Table 5.30: Cox Regression analysis for BTLA+ cells adjusted for clinicopathological characteristics

Cell Population	Haz Ratio	95% CI	P value	Age	Gender	Histology	Stage	Complications
CD4+BTLA+	1.02	0.96- 1.07	0.556	0.248	0.434	0.003	0.001	0.065
CD8+BTLA+	0.99	0.88-1.04	0.842	0.305	0.364	0.003	0.001	0.068
NKT-like BTLA+	0.99	0.90-1.08	0.774	0.324	0.379	0.003	0.003	0.063
NK BTLA+	1.22	0.74-2.00	0.429	0.245	0.299	0.007	0.001	0.078
CD19+BTLA+	1.05	1.01-1.10	0.026	0.166	0.355	0.001	<0.001	0.238

5.8 PD1 and TIM3

5.8.1 PD1+ TIM3+ on CD4+ T cells

Flow cytometry gating was performed to identify co-expression of PD1 and TIM3 on CD4+ T cells as per Figure 2.2. Briefly, total cells, single cells and live cells were identified and then lymphocytes gated based on forward and side scatter, followed by selection of CD3+CD4+ T cells. Within this PD1 and TIM3 was expressed as a percentage of total CD4+ T cells.

5.8.1.1 Longitudinal analysis of CD4+PD-1+TIM-3+ T cells

Mixed-effects analysis showed a trend toward an overall increase over time ($\chi^2 = 5.32$, $p = 0.0699$). Examination of individual time points revealed significant increases compared with baseline at the first follow-up (1FU: $\beta = 0.064$, 95% CI: 0.002–0.127, $p = 0.043$) and second follow-up (2FU: $\beta = 0.060$, 95% CI: 0.022–0.098, $p = 0.002$), while clinical and tumour characteristics, did not significantly influence expression. Refer to Figure 5.21 and Tables 5.31-5.34.

5.8.1.2: Impact of CD4+ PD1+TIM3+ T Cell Changes on DFS

For CD4+ PD1+ TIM3+ cells, higher slope values were strongly protective against recurrence, with a hazard ratio of 0.046 (95% CI 0.006–0.34, $P = 0.003$). Median slopes were higher in non-recurrent cases (0.38) versus recurrent cases (0.135), supporting this protective effect. Among clinical and tumour factors, histology (HR 50.8, 95% CI 3.27–788.5, $P = 0.005$), stage (HR 62.6, 95% CI 4.62–848.5, $P = 0.002$) and post-operative complications were associated with increased risk (HR 4.63, 95% CI 1.20–17.83, $P = 0.026$) while age and gender were not significant. Refer to Figure 5.23 for DFS and Figure 5.24 for representative flow cytometry plots and Tables 5.35 and 5.36.

5.8.2 PD1+ TIM3+ on CD8+ T cells

Flow cytometry gating was performed to identify co-expression of PD1 and TIM3 on CD8+ T cells as per Figure 2.2. Briefly, total cells, single cells and live cells were identified and then lymphocytes gated based on forward and side scatter, followed by selection of CD3+CD8+ T cells. Within this PD1 and TIM3 was expressed as a percentage of total CD8+ T cells.

5.8.2.1 Longitudinal analysis of CD8+ PD1+TIM3+ T cells

CD8+ T cell PD1+TIM3+ expression showed a significant overall change over time ($\chi^2 = 14.94$, $p = 0.0006$). Compared with baseline, expression was non-significantly increased at 1FU ($\beta = +0.020$, 95% CI: -0.006 to $+0.047$, $p = 0.125$) and significantly higher at 2FU ($\beta = +0.046$, 95% CI: $+0.007$ to $+0.085$, $p = 0.021$). Other clinical and tumour factors were not significant. Refer to Figure 5.21 and Tables 5.31-5.34.

5.8.2.2: Impact of CD8+ PD1+TIM3+ Cell Changes on DFS

In multivariable Cox analysis, CD8+ PD1+ TIM3+ T cells were not significantly associated with recurrence (HR 0.93, 95% CI 0.51–1.68, $P = 0.797$), while higher histology risk (HR 14.10, 95% CI 2.40–82.89, $P = 0.003$), advanced stage (HR 12.12, 95% CI 2.32–63.20, $P = 0.003$), and postoperative complications (HR 26.87, 95% CI 1.79–402.36, $P = 0.017$) were significant. Median slopes were slightly higher in recurrence (0.23 vs 0.11), suggesting CD8+ PD1+ TIM3+ cells may not play a strong protective or pathogenic role in this cohort. Refer to Figure 5.23 for DFS and Figure 5.24 for representative flow cytometry plots and Tables 5.35 and 5.36.

5.8.3 PD1+ TIM3+ on NKT-like cells

Flow cytometry gating was performed to identify PD1 and TIM3 co-expression on surface NKT-like cells as per Figure 2.2. Briefly total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD3+ CD56+ cells. Following this NKT-like PD1+TIM3+ cells were identified.

5.8.3.1 Longitudinal analysis of NKT-like PD1+TIM3+ T cells

NKT cell PD1+TIM3+ co-expression showed a significant overall change over time ($\chi^2 = 14.51$, $p = 0.0007$). Mixed-effects analysis indicated increases at both follow-ups compared with baseline (1FU: $\beta = +0.048$, 95% CI: 0.005-0.091, $p = 0.028$; 2FU: $\beta = +0.122$, 95% CI: 0.062-0.182, $p < 0.001$). Stage II was associated with higher PD1+ TIM3+ expression overall ($\beta = +0.116$, 95% CI: $+0.010$ to $+0.221$, $p = 0.032$) on NKT-like cells. Open surgical procedures were associated with lower overall NKT-like PD1+TIM3+ co-expression ($\beta = -0.088$, 95% CI: -0.174 to -0.003 , $p = 0.043$), independent of time and complications. Refer to Figure 5.21-5.22 and Tables 5.31-5.34.

5.8.3.2: Impact of NKT-like PD1+TIM3+ Cell Changes on DFS

In multivariable Cox analysis, NKT-like PD1+ TIM3+ cells were significantly associated with lower recurrence risk (HR 0.66, 95% CI 0.50–0.87, $P = 0.003$), while higher age (HR 0.81, 95% CI 0.68–0.96, $P = 0.013$), histology (HR 25.28, 95% CI 2.49–257.00, $P = 0.006$), advanced stage (HR 91.04, 95% CI 4.48–1850.47, $P = 0.003$), and postoperative complications (HR 119.03, 95% CI 3.20–4426.86, $P = 0.010$) were also significant predictors. Median slope of NKT-like PD1+ TIM3+ cells was higher in non-recurrence (1.11 vs –0.18), also suggesting a protective role of these cells against recurrence. Refer to Figure 5.23 for DFS and Figure 5.24 for representative flow cytometry plots and Tables 5.35 and 5.36.

5.8.4 PD1+ TIM3+ on NK cells

Flow cytometry gating was performed to identify PD1 and TIM3 co-expression on surface NK cells as per Figure 2.2. Briefly total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD3- CD56+ cells. Following this NK PD1+TIM3+ cells were identified.

5.8.4.1 Longitudinal analysis of NK+ PD1+TIM3+ T cells

NK cell PD1 and TIM3 co-expression showed a significant overall change over time ($\chi^2 = 23.61$, $p < 0.001$), with a significant increase at 2FU compared with baseline ($\beta = +0.054$, 95% CI: 0.024–0.085, $p = 0.001$), while the 1FU increase was non-significant ($\beta = +0.012$, 95% CI: –0.008 to +0.033, $p = 0.236$). Histology showed a trend toward lower expression ($\beta = -0.045$, 95% CI: –0.094 to +0.003, $p = 0.065$), whereas other covariates were not significantly associated. Refer to Figure 5.21 and Tables 5.31–5.34.

5.8.4.2: Impact of NK PD1+TIM3+ Cell Changes on DFS

In this analysis, higher slopes of NK PD1+ TIM3+ cells were observed in patients without recurrence (median 0.15 vs. 0.26 in recurrence), but the association with recurrence was not statistically significant (HR = 0.40, 95% CI 0.12–1.32, $P = 0.131$). Histology (HR = 3.24, 95% CI 0.33–31.89, $P = 0.313$), stage (HR = 12.33, 95% CI 2.06–73.76, $P = 0.006$), and postoperative complications (HR = 57.73, 95% CI 3.10–1075.21, $P = 0.007$) remained significant predictors of recurrence. Refer to Figure 5.23 for DFS and Figure 5.24 for representative flow cytometry plots and Tables 5.35 and 5.36.

5.8.5 PD1+ TIM3+ on CD19+ cells

Flow cytometry gating was performed to identify PD1 and TIM3 co-expression on surface B cells as per Figure 2.2. Briefly total cells, single cells and live cells were identified and then lymphocytes were gated based on forward and side scatter, followed by selection of CD19+ cells. Following this CD19+ PD1+TIM3+ cells were identified.

5.8.5.1 Longitudinal analysis of CD19+ PD1+TIM3+ T cells

B cell PD1+TIM-3+ expression showed a significant overall change over time ($\chi^2 = 26.57$, $p < 0.001$), with a significant increase at 2FU compared with baseline ($\beta = +0.106$, 95% CI: 0.024-0.189, $p = 0.011$), while the 1FU change was non-significant ($\beta = -0.016$, 95% CI: -0.056-0.025, $p = 0.449$). Other covariates were not significantly associated with expression. Refer to Figure 5.21 and Tables 5.31-5.34.

5.8.5.2: Impact of CD19+ PD-1+TIM-3+ Cell Changes on DFS

Cox regression analysis revealed, CD19+ PD1+ TIM3+ cell slopes were slightly higher in patients without recurrence (median 0.98 vs. 0.59), but the association with recurrence was not statistically significant (HR = 0.95, 95% CI 0.88–1.02, $P = 0.162$). Histology (HR = 7.10, 95% CI 1.10–45.95, $P = 0.040$), stage (HR = 18.09, 95% CI 2.58–127.01, $P = 0.004$), and postoperative complications (HR = 26.03, 95% CI 1.99–340.27, $P = 0.013$) were significant predictors of recurrence. Refer to Figure 5.23 for DFS and Figure 5.24 for representative flow cytometry plots and Tables 5.35 and 5.36.. Overall, B cells co-expressing PD1 and TIM3 may have a limited protective role.

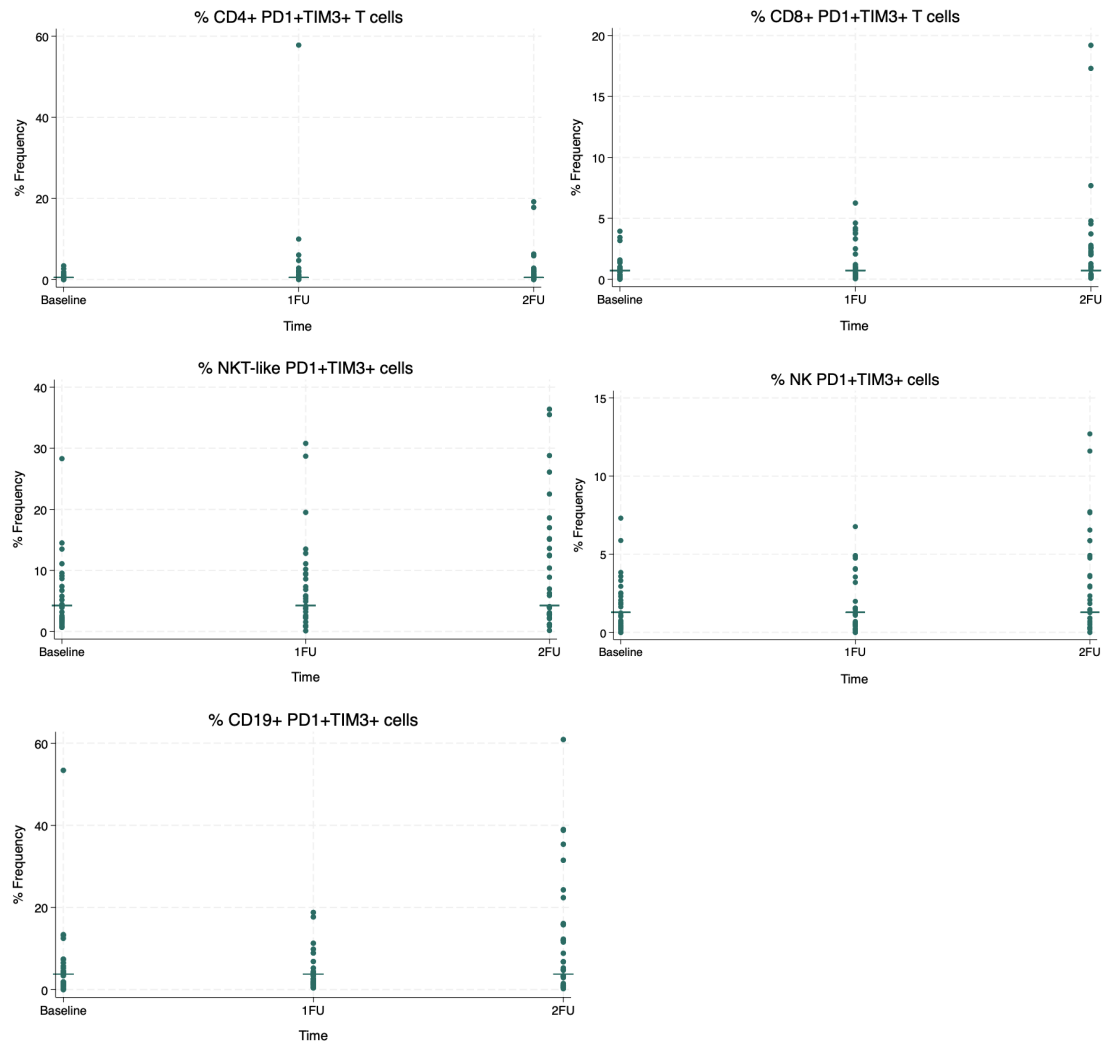


Figure 5.21. Dot plots showing % frequency of PD1 and TIM3 co-expression on immune cells at each timepoint. Total cells were first filtered to isolate single and viable cells. Lymphocytes were then selected using forward- and side-scatter properties. Gating of CD3+ was utilised to identify T cell subsets; CD4+ cells and CD8+ cells, alongside NKT-like cells (CD56+). Gating of CD3- and CD56+ was utilised to identify NK cells. Gating of CD19+ was utilised to identify B cells. PD1 and TIM3 positivity was then defined as a percentage frequency of CD4+ T cells, CD8+ T cells, NKT-like cells, NK cells and B cells. Mixed-effects linear regression revealed a significant difference in frequency over time for PD1 and TIM3 co-expression on all immune cells analysed; CD8+ T cells ($p=0.0006$), NKT-like cells ($p=0.0007$) and NK cells ($p=0.001$) and B cells ($p<0.0001$). For CD4+ T cells ($p=0.0669$), there was no significant difference over time. Dots representing each patient and horizontal line at median value.

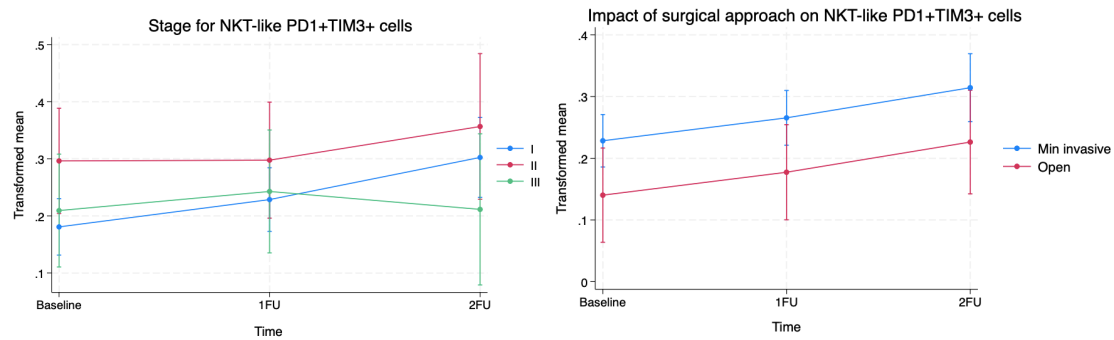


Figure 5.22. Predicted means plot showing differences over time of PD1 and TIM3 co-expression on immune cells in association with clinicopathological characteristics. Data obtained from FlowJo v10.10.0 software, were expressed as percentage frequencies and subsequently transformed using the arcsine-square root transformation to meet the assumptions of parametric analysis. Plotted values in this figure represent transformed means. To evaluate changes across the three timepoints, linear mixed-effects models were applied in association with age, gender, histological subtype, disease stage, surgical approach, and the presence or absence of post-operative complications. Stage II was associated with higher PD1 and TIM3 co-expression on NKT-like cells ($p = 0.032$). Open surgical procedures were associated with lower overall PD1 TIM3 co-expression on NKT-like cells ($p = 0.043$).

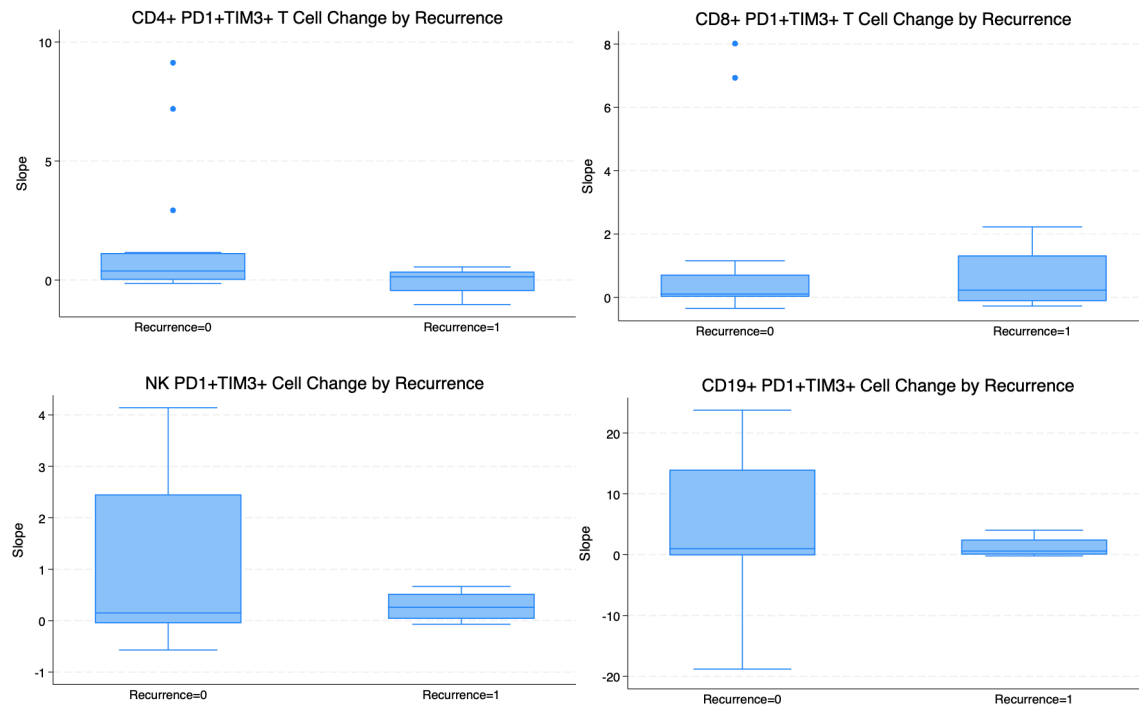


Figure 5.23. Boxplots of PD1 and TIM3 co-expression on immune cells and changes over time (slopes) in patients with and without recurrence. The longitudinal slope summarises the linear change over three timepoints in patients ($n=34$) for PD1 and TIM3 co-expression on CD4+ T cells, CD8 + T cells, NKT cells, NK cells and B cells. Cox proportional hazards regression was utilised to assess the association between the slope and DFS. A positive slope indicates increasing cell frequency, while a negative slope reflects a decline. Baseline was used as the reference time point for comparisons across all three time points, as well as individually against 1FU and 2FU. The slopes of PD1 and TIM3 co-expression on CD4+ T cells ($p=0.003$) and NKT-like cells ($p=0.003$) over the three timepoints were significantly associated with recurrence. The slopes of PD1 and TIM3 co-expression on CD8+ T cells ($p=0.797$), NK cells ($p=0.131$) and B cells ($p=0.162$) and were not associated with recurrence.

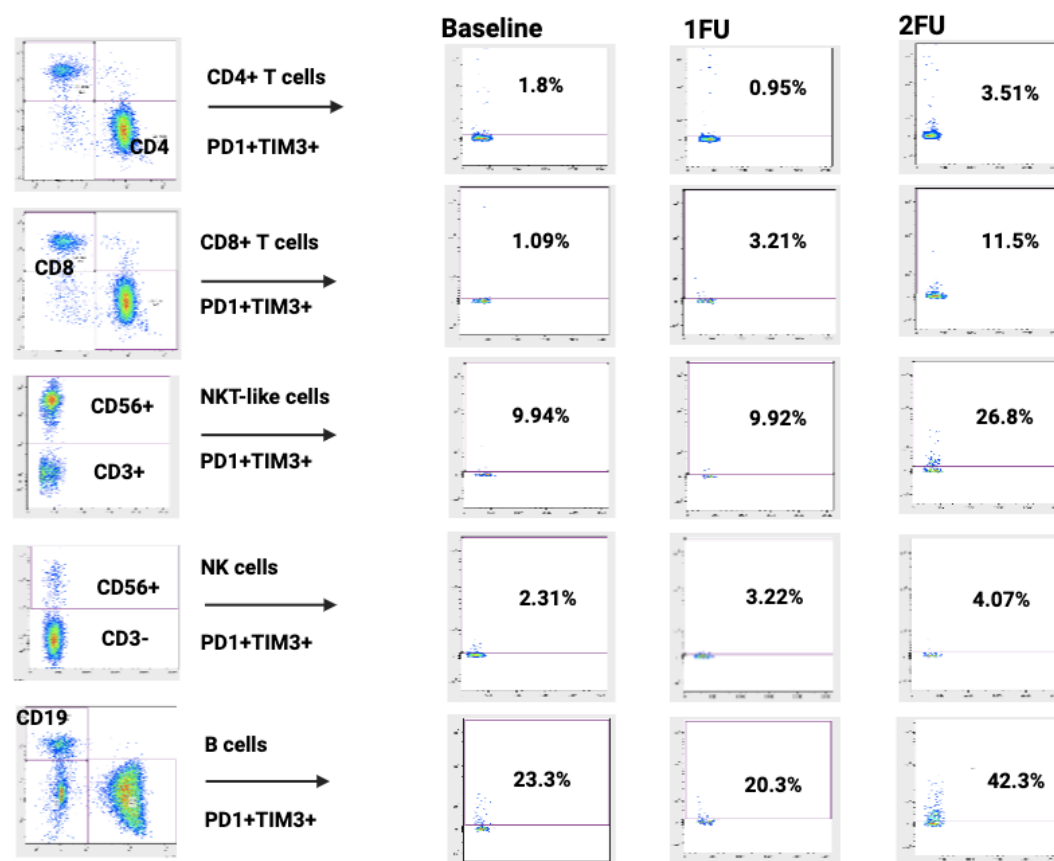


Figure 5.24. Representative flow cytometry plots for co-expression of PD1 and TIM3 on immune cells at different timepoints. PBMCs were stained with a dead cell stain (DCS), CD3, CD4, CD8, CD56, CD19, PD1 and TIM3 antibodies and analysed by flow cytometry. Forward scatter area (FSC-A) and scatter area (SSC-A) was used to determine total cells, following this FSC-A and FSC-width (FSC-W) was used to determine single cells, then FSC-A and DCS was used to determine live cells. Finally, FSC-A and SSC-A was used to determine lymphocytes. CD3 was utilised to identify T cells, NKT-like cells and NK cells. This was performed at baseline, 1FU and 2FU. Percentage frequency is shown for co-expression of PD1 and TIM3 positive cells.

Upper panel, left to right: First dot plot shows expression of CD4+ T cells, following this; representative dot plots of PD1+TIM3+CD4+ T cells at baseline, 1FU and 2FU.

First centre panel, Left to Right: First dot plot shows expression of CD8+ T cells, following this; representative dot plots of PD1+TIM3+ CD8+ T cells at baseline, 1FU and 2FU.

Second centre panel, Left to Right: First dot plot shows expression of NKT-like cells (CD3+CD56+), following this; representative dot plots of PD1+TIM3+ NKT-like cells at baseline, 1FU and 2FU.

Third centre panel, Left to Right: First dot plot shows expression of NK cells (CD3-CD56+), following this; representative dot plots of PD1+TIM3+ NK cells at baseline, 1FU and 2FU.

Lower panel, Left and Right: First dot plot shows expression of B cells (CD19+) following this; representative dot plots of PD1+TIM3+ B cells at baseline, 1FU and 2FU.

Table 5.21: Percent frequency of PD1+ TIM3+ cells

Cell Population	% Baseline	% 1FU	% 2FU
CD4+ PD1+ TIM3+	0.51	0.44	1.24
CD8+ PD1+ TIM3+	0.59	0.65	1.08
NKT-like PD1+ TIM3+	3.93	5.15	5.9
NK PD1+ TIM3+	1.02	1.21	2.08
CD19+ PD1+ TIM3+	3.77	2.64	6.8

Table 5.31: Mixed-effects linear regression comparing baseline with 1FU and 2FU for PD1+ TIM3+ cells

Cell population / Timepoint	Coefficient (β)	Std Err (SE)	p value	95% Confidence Interval
CD4+PD1+TIM3+ 1FU	0.064	0.032	0.043	0.002-0.127
CD4+PD1+TIM3+ 2FU	0.006	0.019	0.002	0.022-0.098
CD8+PD1+TIM3+ 1FU	0.020	0.013	0.125	-0.005-0.046
CD8+PD1+TIM3+ 2FU	0.045	0.019	0.021	0.007-0.085
NKT-like PD1+TIM3+ 1FU	0.041	0.022	0.028	0.005-0.091
NKT-like PD1+TIM3+ 2FU	0.121	0.021	0.000	0.062-0.182
NK PD1+TIM3+ 1FU	0.012	0.010	0.236	-0.008-0.032
NK PD1+TIM3+ 2FU	0.054	0.016	0.001	0.024-0.085
CD19+PD1+TIM3+ 1FU	-0.016	0.021	0.449	-0.056-0.028
CD19+ PD1+TIM3+ 2FU	0.106	0.041	0.011	0.024-0.189

Table 5.32: Mixed-effects linear regression with clinicopathological characteristics for PD1+ TIM3+ cells

Cell Population	Chi2 (χ^2) (2)	p value	Stage II p value	Stage III p value	Age p value	Gender p value	Histology p value
CD4+ PD1+ TIM3+	5.32	0.0669	0.933	0.691	0.624	0.946	0.691
CD8+ PD1+ TIM3+	14.94	0.0006	0.533	0.827	0.081	0.379	0.655
NKT-like PD1+ TIM3+	14.51	0.0007	0.032	0.625	0.812	0.982	0.824
NK PD1+ TIM3+	23.61	0.001	0.718	0.129	0.254	0.920	0.065
CD19+ PD1+ TIM3+	26.57	<0.0001	0.452	0.386	0.322	0.821	0.322

Table 5.33: Mixed-effects linear regression incorporating complications and surgical approach for PD1+ TIM3+ cells

Statistical Analysis	Complications P value	Surgical approach p value
CD4+ PD1+ TIM3+	0.055	0.226
CD8+ PD1+ TIM3+	0.193	0.465
NKT-like PD1+ TIM3+	0.357	0.043
NK PD1+ TIM3+	0.316	0.660
CD19+ PD1+ TIM3+	0.066	0.914

Table 5.34: Descriptive summary statistics incorporating median slope and interquartile range (IQR) for PD1+ TIM3+ cell populations

Cell Population	Median Slope Recurrence = 0	IQR Recurrence = 0	Median Slope Recurrence = 1	IQR Recurrence = 1
CD4+ PD1+TIM3+	-0.38	0.008- 1.14	0.14	-0.46-0.36
CD8+ PD1+TIM3+	0.105	0.14-0.72	0.23	-0.12-1.32
NKT-like PD1+ TIM3+	1.1	0.38-7.02	-0.18	-3.83-2.17
NK PD1+ TIM3+	0.15	-0.05-2.45	0.26	0.035-0.53
CD19+ PD1+ TIM3+	0.98	-0.11-13.98	0.59	0.18-2.49

Table 5.35: Cox Regression analysis for PD1+ TIM3+ cells adjusted for clinicopathological characteristics

Cell Population	Hazard Ratio	95% CI	P value	Age	Gender	Histology	Stage	Complications
CD4+ PD1+ TIM3+	0.045	0.006 - 0.33	0.003	0.074	0.289	0.005	0.002	0.026
CD8+ PD1+ TIM3+	0.93	0.51- 1.67	0.797	0.083	0.725	0.003	0.003	0.017
NKT-like PD1+ TIM3+	0.66	0.50- 0.87	0.003	0.013	0.153	0.006	0.003	0.010
NK PD1+ TIM3+	0.39	0.12- 1.32	0.131	0.093	0.826	0.313	0.006	0.007
CD19+ PD1+ TIM3+	0.95	0.88- 1.02	0.162	0.078	0.778	0.040	0.004	0.013

5.9 DISCUSSION

5.9.1. Mixed-Effects Modelling of Immune Dynamics

PD1 and CTLA4 expression increased significantly over time on CD4+ and CD8+ T cells, NKT-like cells, NK cells, and B cells ($p < 0.001$ – 0.019), with rises evident as early as 2–4 days post-operatively and persisting through 6–9 weeks. These early and sustained elevations likely reflect a combination of surgical stress, tissue injury, and systemic inflammation, which transiently activate inhibitory pathways to maintain immune homeostasis [18,63]. Higher baseline expression of PD1 and CTLA4 in advanced-stage disease ($p = 0.001$ – 0.026), along with associations with tumour histology and host factors, suggests that pre-existing tumour-induced immune exhaustion and patient-specific characteristics influence checkpoint levels even prior to surgery. Stage II exhibits the highest numbers of these immune checkpoints, likely reflecting active immune engagement, which subsequently declines in Stage III due to chronic activation and immune exhaustion [11,41].

LAG3 exhibited cell type-specific and heterogeneous dynamics across perioperative NSCLC patients. Expression was notably upregulated on CD4+ T cells and B cells ($p < 0.005$), while changes on other immune populations were less pronounced. Tumour histology exerted a modulatory effect, with adenocarcinoma patients exhibiting lower LAG3 frequencies on select cell subsets, a distribution pattern closely paralleling that of TIM3. Disease stage further modulated LAG3 levels, with the highest frequencies detected in Stage III, suggesting that advanced tumour burden and prolonged antigen exposure drive greater checkpoint upregulation.

TIM3 expression increased over time on CD4+ and CD8+ T cells and B cells, with the largest rises observed at the second follow-up, while NKT-like cells showed a non-significant trend toward higher expression. NK cells exhibited a trend toward decreased TIM3 expression over time. Age, tumour histology, and postoperative complications influenced expression in specific populations, but overall TIM3 upregulation was most consistent in T and B cells. BTLA expression showed cell type-specific dynamics over time: CD4+ and CD8+ T cells, NKT-like cells, and B cells all exhibited declines, with the most pronounced reductions at the second follow-up. NK BTLA expression, in contrast, tended to increase over time, particularly in patients undergoing open surgery. Age and

male gender influenced baseline BTLA expression in certain populations, but overall, inhibitory signalling decreases over time across most lymphocyte subsets.

Co-expression of PD1 and TIM3 on CD8⁺ T cells, NKT-like cells, NK cells, and B cells increased progressively over the post-operative period, particularly by 6–9 weeks, indicating a shift toward a more functionally exhausted or inhibitory immune phenotype ($p < 0.001$). This co-expression likely reflects a combination of surgical stress–induced immune modulation and the persistent effects of tumour-driven immune suppression, suggesting that adaptive and innate immune compartments are both affected [18,66]. Analysis by disease stage revealed that Stage II patients exhibited higher levels of PD1 and TIM3 co-expression on NKT-like cells ($p = 0.032$), whereas Stage III patients showed a relative downregulation, particularly at 2FU, though this trend did not reach statistical significance. The pronounced upregulation in Stage II may be attributable to chronic antigen stimulation, which drives simultaneous upregulation of multiple inhibitory checkpoint proteins as part of an adaptive feedback mechanism to prevent excessive immune activation [11,41]. In contrast, the attenuated co-expression observed in Stage III could reflect progressive immune exhaustion [11,41].

Overall, these findings suggest that lung cancer surgery triggers coordinated upregulation of multiple inhibitory pathways across immune cell subsets, resulting in a persistently immunosuppressed post-operative environment up to 6–9 weeks after surgery.

5.9.2. Associations with Disease-Free Survival

Lower slopes of PD1⁺ expression on NK and B cells were observed in patients with recurrence, with B cells ($p = 0.026$) showing a significant association and NKT-like cells ($p = 0.062$) a non-significant trend. Also with CTLA4, lower expression/slopes were statistically significant on CD8⁺ T cells, NK, NKT-like cells and B cells ($p = 0.007–0.042$), in patients with recurrence. This suggests that insufficient inhibitory checkpoint regulation may impair effective anti-tumour immunity. In contrast, strong CTLA4 activity could be protective on CD8⁺ T and NK cells, highlighting the role of balanced immune regulation in controlling tumour recurrence.

Rapid or excessive LAG3⁺ expression on CD4⁺ T cells was associated with recurrence ($p = 0.001$) with a trend toward significance on CD8⁺ T cells ($p = 0.071$), reflecting potential adaptive immune exhaustion in effector populations. In contrast, lower LAG3 slopes on

NKT-like cells were linked to higher recurrence risk ($p=0.025$), suggesting that insufficient checkpoint induction in innate-like lymphocytes may impair immune regulation and anti-tumour surveillance. B and NK LAG3 dynamics were modest and non-significant, underscoring T cell-specific roles in LAG3-mediated immune regulation.

On CD4⁺ T cells, steeper increases in TIM3 over time were associated with lower recurrence ($p=0.016$), suggesting that upregulation may support effective regulatory feedback and maintain functional T cell responses, whereas insufficient induction could permit tumour escape. Similarly, NKT-like cells exhibited a protective effect when TIM3 slopes increased ($p=0.013$), reinforcing the role of innate-like lymphocytes in maintaining immune homeostasis. Higher NK TIM3 slopes were linked to increased recurrence ($p=0.001$), potentially reflecting over-inhibition of cytotoxic activity, limiting tumour clearance. CD8⁺ T cell TIM3 dynamics were not associated with recurrence, highlighting cell-type specificity.

Increased BTLA expression on B cells was significantly associated with higher risk of tumour recurrence ($p = 0.026$), suggesting that sustained inhibitory signalling within the humoral immune compartment may contribute to impaired anti-tumour immunity and facilitate tumour escape. In contrast, BTLA expression on other immune cell populations did not demonstrate a significant relationship with recurrence in this cohort.

CD4⁺ T cells with co-expression of PD1 and TIM3 displayed a strong protective effect ($p=0.003$), with higher slopes (-0.38 non-recurrence v 0.15 recurrence) and was associated with markedly lower recurrence risk supporting a role for these cells in maintaining robust adaptive immune surveillance. NKT-like PD1+TIM3⁺ cells similarly exhibited a protective effect suggesting that innate-like lymphocytes contribute to tumour control when dynamic checkpoint co-expression is appropriately modulated [11,23,25,52]. By contrast, slopes of CD8⁺, NK, and B cells with PD1 and TIM3 co-expression were not significantly associated with recurrence, indicating that the protective effect may be most pronounced in helper T cell and NKT-like compartments, while cytotoxic and B cell populations may play a more context-dependent or indirect role.

In summary, for CD4⁺ T cells; LAG3 expression favours recurrence, with increased PD1+TIM3⁺ and TIM3 protective. For CD8⁺ T cells, CTLA4 shows mixed effects on recurrence and trend of increased LAG3 expression leading to recurrence. In NKT-like

cells, reduced ICPs (CTLA4, LAG3, TIM3) was associated with recurrence whereas dual PD1+TIM3+ was protective. PD1 on NK cells is protective, while TIM3 promotes tumour growth and CTLA4 can be both pro- and anti-tumour. In B cells, lower CTLA4 and LAG3 and higher BTLA promote recurrence.

CHAPTER 6

DISCUSSION

6.1 INTRODUCTION

The perioperative period represents a uniquely dynamic phase in the management of NSCLC, during which surgical resection, both removes the primary tumour and simultaneously induces profound systemic immunological changes. By integrating inflammatory indexes, comprehensive profiling of adaptive, innate, and innate-like lymphocyte subsets, and key immune checkpoint proteins, this study offers new insight into how surgery-induced immune disruption may influence oncological outcomes.

This longitudinal analysis, incorporating three perioperative timepoints—preoperatively, 2–4 days postoperatively, and 6–9 weeks postoperatively—characterises immune alterations in patients undergoing lung cancer resection. This discussion examines surgery as a driver of both inflammatory activation and immunosuppression, and the implications for oncological risk. It further evaluates the prognostic and predictive utility of inflammatory indices, immune cell populations, and ICP expression, including associations with post-operative complications and disease-free survival. Finally, implications for immunotherapy, adjuvant treatment strategies, and peri-operative management in NSCLC are considered.

6.2 NSCLC SURGICAL INDUCED IMMUNE MODULATION

6.2.1 Cell populations

Surgical resection, while the cornerstone of curative-intent treatment, is known to exert systemic effects that can transiently impair anti-tumour immunity[18,19,74]. The shifts observed across T-cell differentiation states in this study, likely reflect this post-operative disruption. TDEM CD8⁺ T cells are characterised by CD45RA⁺ CCR7⁻ in this study, which are known established markers for senescence [37]. Changes in naïve and TDEM CD8⁺ T cells may compromise the capacity for rapid cytotoxic responses at a time when circulating tumour cells or micro metastatic deposits may be present. Adenocarcinoma, with its comparatively higher mutational burden and broader neoantigen landscape [9], appears to elicit stronger antigen-specific CD8⁺ memory responses in this cohort. CD4⁺ T cells and their subsets remained largely stable, whereas CD8⁺ T cells showed post-operative susceptibility to functional decline, a vulnerability linked partly to their lower mitochondrial biogenesis [35,38].

The post-operative increase in Tregs, driven predominantly by CD4⁺ Tregs, is particularly noteworthy. This rise likely reflects a homeostatic response to surgical stress and tissue injury [85], aimed at constraining excessive inflammation, with CD8⁺ Tregs appearing less responsive to these cues. Elevated Treg levels may create a transiently immunosuppressive milieu that facilitates tumour-cell survival, seeding, or immune escape, consistent with their established association with adverse prognostic outcomes [11]. These findings align with reports across multiple solid tumour types describing postoperative augmentation of Treg activity, coupled with impaired effector responses [11]. The $\gamma\delta$ T-cell compartment—particularly V δ 1 and V δ 2 populations—also appears sensitive to surgical stress. Given their rapid cytotoxic potential, stress-surveillance functions, and MHC-independent recognition of malignant cells, changes in $\gamma\delta$ T-cell frequencies may blunt early innate-like anti-tumour defence [11,45]. MAIT cells, known for their paradoxical contributions to both chronic inflammation and anti-tumour immunity [51,52], and NKT-like cells, which support tumour immunosurveillance through direct cytotoxicity and by promoting T-cell activation [49,50], increased significantly over time in this study.

Alterations too in monocyte subsets, may influence post-operative inflammation, potentially fostering conditions permissive to metastatic outgrowth—particularly through expansion of immunosuppressive or pro-angiogenic monocyte populations [11,32]. B-cell frequencies remained largely unchanged throughout the longitudinal follow-up.

The present study demonstrated a significant increase in the proportion of circulating CD3-CD56⁺ NK cells following NSCLC resection, rising from 8.1% at baseline to 15.6% at 6–9 weeks postoperatively, while the proportion of CD16⁺ NK cells decreased significantly over the same period. Although these findings differ from the traditional description of postoperative NK-cell suppression, much of the existing literature focuses on NK-cell function rather than NK-cell quantity, with reductions in cytotoxic activity commonly reported during the immediate postoperative period [37,75,77]. The later follow-up period employed in the present study may reflect a phase of immune recovery. Although relatively few studies have examined long-term postoperative immune changes, Zhang et al. reported increasing NK-cell levels during postoperative recovery, suggesting that NK-cell expansion may occur over time rather than persistent suppression [37,75,76]. NSCLC is known to impair NK-cell function through mechanisms within the TME; consequently, tumour resection may facilitate restoration of the NK-cell compartment, which could be

mediated by reduction of exhaustion markers on surface of NK cells [97]. The concurrent increase in total NK-cell frequency and decrease in CD16⁺ NK cells, suggests postoperative remodelling of the NK-cell population [37,75,76].

Of the available studies examining peri-operative immune profiling in NSCLC surgery, only one has incorporated longitudinal analysis stratified by surgical approach; the remainder have relied primarily on comparisons with healthy controls [39,75,79]. Prior work has focused predominantly on CD4⁺, CD8⁺, naïve T cells, Tregs, and NK cells. This study provides a novel, more comprehensive characterization of the peri-operative immune landscape, including detailed profiling of T-cell differentiation states (naïve, CM, EM, TDEM), B cells, $\gamma\delta$ T cells, MAIT cells, NKT-like cells, and monocyte subsets. The emerging pattern highlights pro-tumour activity driven by CD4⁺ Tregs and MAIT cells, post-operative dysfunction among CD8⁺ T-cell subsets, and compensatory anti-tumour activity reflected in increases in MAIT (despite their dual role), NKT-like, and NK cells.

Overall, these findings demonstrate that surgical resection induces post-operative systemic inflammation and immunosuppression across multiple immune compartments. Characterising these immune shifts through peri-operative immune profiling provides a valuable framework for identifying patients at higher risk, guiding the development of targeted interventions, to preserve effector immunity and optimize oncological outcomes in the surgical setting

6.2.2 Immune Checkpoint Proteins

This study demonstrates that lung cancer surgery induces a widespread and sustained upregulation of multiple inhibitory immune checkpoints across both adaptive and innate immune compartments. Among the analysed cell populations—including CD4⁺ and CD8⁺ T cells, NKT-like cells, NK cells, and B cells—PD1 expression increased progressively over time. CTLA4 exhibited cell type-specific upregulation on CD4⁺ T cells, NKT-like cells, and B cells, while LAG-3 positivity increased selectively on CD4⁺ T cells and B cells. These early and sustained elevations likely reflect the combined effects of surgical stress, tissue injury, and systemic inflammation, transiently activating inhibitory pathways to maintain immune homeostasis [18,66]. In contrast, expression of BTLA over time decreased on T and B cells, with a trend toward increased expression on NK cells. This differential regulation suggests that reductions in adaptive BTLA may facilitate post-

operative anti-tumour immunity, whereas upregulation on innate cells such as NK cells may serve as compensatory mechanisms to limit excessive inflammatory responses [25].

TIM3 exhibited statistically significant upregulation on CD4⁺ and CD8⁺ T cells as well as B cells. TIM3 expression on tumour-infiltrating lymphocytes has previously been associated with poor prognosis in NSCLC [13,15,67]. Peri-operative TIM3 upregulation likely reflects adaptive immune activation, with TIM3 functioning as a critical regulator to balance effective anti-tumour responses while preventing excessive T- and B-cell activation [14,15,66]. The pronounced upregulation of both PD1 and TIM3 co-expression across multiple immune populations underscores a system-wide shift in immune checkpoint regulation during the peri-operative period.

Stage II disease, female sex, and adenocarcinoma histology were associated with higher PD1 and CTLA4 expression across immune subtypes, suggesting a more pronounced adaptive immune exhaustion phenotype. Adenocarcinomas often display higher mutational burden and neoantigen diversity, driving chronic T-cell stimulation and inhibitory checkpoint upregulation [101]. Conversely, squamous cell carcinoma was characterized by elevated LAG3 and TIM3 expression, indicating that distinct exhaustion pathways are preferentially activated depending on tumour histology. Squamous tumours, which typically elicit inflammation-driven, non-PD1-dominant exhaustion, appear to engage alternative inhibitory circuits such as LAG3 and TIM3.

Interestingly, female patients exhibited significantly higher PD1 expression across all cell types and timepoints in this study. This may reflect stronger baseline immune activation in females, potentially predisposing T cells to earlier or more pronounced exhaustion signalling [104,105]. Sex-specific differences in responses to PD1-targeted therapies have also been reported, with female patients demonstrating poorer outcomes following PD1 monotherapy than males, although the mechanisms underlying this disparity remain incompletely understood [105]. Evidence investigating sex differences in PD1 expression is currently limited, with one such study of 108 patients by Gu et al. reporting significantly higher levels of soluble PD1 and PD1⁺CD4⁺ T cells in females compared with males [104]. The authors proposed that elevated testosterone levels in males may contribute to this difference through downregulation of PD1 expression on T cells, a mechanism that has been supported by other studies [104,105]. However, the observed sex-related differences

in PD1 expression are likely multifactorial, reflecting the combined influence of hormonal, genetic, and immunological factors [105].

Research on ICPs has largely focused on their expression in tumour cells and advanced disease stages, with limited data on their dynamics in peripheral immune cells during the perioperative period. In particular, there is a paucity of literature regarding BTLA, LAG3, and CTLA4 in this context. While few studies have examined the impact of surgical intervention on ICP expression in immune cells, prior work has shown increased PD1/PDL1 and CTLA4 levels on immune cells five days postoperatively [79], and PD1 upregulation has been linked to surgical stress and disease stage [18,19]. Post operative reductions in TIM3 expression on peripheral NKT and NK cells has been shown previously, consistent with our observation of a trend toward decreased TIM3 on NK cells [86].

As ICIs are increasingly integrated into peri-operative regimens, understanding ICP dynamics on immune cells is critical, as peri-operative alterations may influence ICI efficacy. PD1 expression on T cells has been associated with improved ICI responses [77]; however, LAG3 and TIM3, particularly when co-expressed with PD1, alongside increased regulatory T cells, can mediate resistance [15]. While PD1/PDL1 expression on resected tumour cells informs immunotherapy strategies [2,3,14–19], evaluating these checkpoints on peripheral immune cells could address outstanding questions regarding treatment tolerance and resistance.

Collectively, these findings suggest that tumour-driven immune exhaustion—modulated by histology, stage, and patient-intrinsic factors—shapes checkpoint expression patterns even prior to surgical intervention. This emphasizes that the pre-operative immune landscape is biologically stratified, with potential implications for peri-operative immune vulnerability and responsiveness to immunomodulatory therapies.

6.3 IMMUNE RESPONSE TO PREDICT POST-OPERATIVE COMPLICATIONS

6.3.1 Inflammatory indexes

Currently, there are no meta-analyses in the literature that specifically evaluate the use of systemic inflammatory indexes as prognostic indicators for post-operative complications following lung cancer surgery. Existing evidence is limited to single-institution studies,

which have suggested that NLR, PLR, SII, and SIRI may have potential predictive value for post-operative complications [13-15,31]. Some studies have focused exclusively on baseline measurements for predicting POP [13,106], but data regarding early post-operative measurements remain scarce. In our cohort, none of the inflammatory indices measured at baseline were predictive of POP.

Regarding PLR, the literature is heterogeneous. Some studies have reported that baseline PLR is predictive of post-operative complications [107], while others have found no predictive value at baseline or post-operative day four [14]. In our cohort, a PLR cutoff of 190 at 1FU was a modest predictor of post-operative complications. For SII, one study described pre-operative SII as a prognostic indicator for pulmonary pneumonia occurring within seven days of surgery [107]. In our cohort, pre-operative SII was not predictive; however, an SII cutoff of 1050 at 1FU demonstrated predictive potential for post operative complications, with a sensitivity of 76% and a false-positive rate of 36%. Regression analysis confirmed statistical significance ($p = 0.025$).

Data regarding SIRI as a prognostic indicator for post-operative complications are extremely limited. One study suggested a baseline cutoff of 0.9 [82]. In our cohort, a SIRI cutoff of 4 at 1FU yielded a sensitivity of 65% and a false-positive rate of 29%, which was statistically significant on cox regression analysis ($p = 0.032$). Notably, baseline measurements of all inflammatory indexes, as well as 1FU measurements for LMR, were not predictive of complications in our cohort.

Most prior studies have been conducted in Chinese cohorts and have largely focused on pre-operative inflammatory indexes [9,10,13,15]. This study is unique in demonstrating that NLR, PLR, SII, and SIRI measured at 2–4 days post-operatively have potential as prognostic indicators for post-operative complications in lung cancer patients. Flow cytometry data presented in Chapters 4 and 5 further suggest that post-operative complications may influence recurrence, through alterations in specific immune cell populations, underscoring the interplay between systemic inflammation, immune response, and clinical outcomes. By identifying potential early post-operative biomarkers, this study provides critical insight into predicting complications and guiding patient management, underscoring its clinical and translational relevance.

Taken together, our findings highlight the potential utility of early post-operative inflammatory indices as prognostic tools for post-operative complications and emphasize the need for large, high-volume randomized controlled trials to validate these observations. Importantly, in our cohort, NLR, PLR, SII, and SIRI measured at 2–4 days post-operatively were successful in predicting post-operative complications, suggesting that early post-operative assessment of systemic inflammation, may provide clinically actionable information.

6.3.2. Immune cell populations

Post-operative complications were associated with distinct baseline immune profiles, including elevated frequencies of CD4⁺ CM T cells ($p = 0.022$), reduced proportions of CD8⁺ naïve T cells ($p = 0.018$), and decreased MAIT cells ($p = 0.030$). Although the longitudinal trajectories of immune cell populations were broadly similar between patients with and without complications, these elevated differences persisted across all follow-up timepoints, indicating that patients who developed post-operative complications maintained altered immune cell levels. These observations suggest that such patients possess an immune landscape that is modulated prior to surgery.

Moreover, given that steeper increases in CD4⁺ CM and CD8⁺ EM T cells were associated with heightened recurrence risk ($p < 0.02$), whereas greater slopes in CD8⁺ naïve T cells were protective ($p = 0.004$), it is plausible that patients experiencing post-operative complications, enter the post-operative period with an immune milieu that is less favourable for effective tumour control. To date, no studies have utilized flow cytometry to predict peri-operative changes in immune cell populations, in patients undergoing NSCLC resection. While prior research in other cancer types has examined peri-operative immune cell dynamics and their relationship to prognosis or surgical approaches, these studies have not specifically addressed patients, who subsequently develop post-operative complications [18,19,74,75].

These findings indicate that post-operative complications reflect pre-existing disruption in immune status, rather than being the primary driver of longitudinal T cell changes. Baseline immune dysregulation may therefore represent both a marker of susceptibility to post-operative complications and an unfavourable immunological context for tumour control,

highlighting the importance of peri-operative immune profiling in NSCLC patients to better stratify risk and potentially guide individualized peri-operative management strategies.

6.3.3. Immune checkpoint proteins

Post-operative complications were rarely associated with altered immune checkpoint activity. Patients with post-operative complications exhibited higher expression of inhibitory markers, including TIM3 on B cells and LAG3 on NKT cells ($p < 0.04$). These elevations likely reflect a homeostatic response to surgical trauma, where heightened inhibitory signalling serves to limit excessive inflammation and tissue damage [31]. The observation that these changes persisted through 6–9 weeks post-operatively, highlights that complications can induce prolonged immune modulation rather than transient effects limited to the immediate post-surgical period.

Clinically, post-operative complications emerged as among the strongest independent predictors of tumour recurrence ($p < 0.02$) across multiple immune checkpoint pathways and immune cell subsets, with hazard ratios frequently exceeding those associated with age or sex (HR range: 0.9–10 vs 5–26), though still lower than those for histology (HR 4 – 44). These results suggest that post-operative complications extend beyond surgical morbidity, exerting functional effects on immune surveillance and potentially creating a permissive environment for residual tumour cells to persist and proliferate. Collectively, these findings underscore the complex interplay between surgical stress, immune regulation, and checkpoint-mediated inhibitory signalling, highlighting the critical importance of minimizing post-operative complications. Furthermore, they emphasize the clinical relevance of the biomarkers for post-operative complications identified in this study, as preserving anti-tumour immunity, may be pivotal for improving long-term outcomes in patients with lung cancer.

6.4 POTENTIAL FOR IMMUNE CHANGES TO PREDICT PROGNOSIS

6.4.1 Inflammatory Indexes

Systemic inflammatory indexes reflecting the dynamic balance between systemic inflammation and immune response and have emerged as potential prognostic biomarkers in NSCLC [20]. In our cohort, NLR, PLR, LMR, SII, and SIRI did not demonstrate any significant prognostic value for DFS. Most studies in the existing literature have primarily evaluated these indexes with OS as the primary endpoint [17, 55, 56]. Regarding SII, two

meta-analyses assessing its relationship with DFS report conflicting results: Chen *et al.* (2024) found no significant association [56], whereas Huang *et al.* (2022) observed a positive correlation [55]. Similarly, a meta-analysis evaluating SIRI across various malignancies indicated that elevated SIRI was associated with poorer DFS; however, only one study contributed lung-specific data, comprising 307 patients. [58]. In a more focused study, Mi *et al.* reviewed 116 patients with SCLC and NSCLC, demonstrating that NLR >6.6, PLR >249, and PNI <43 were predictive of DFS [57].

In contrast, baseline HALP score was prognostic for DFS in our patient cohort, with a HALP score greater than 54, exhibited a higher likelihood of DFS (coefficient = 1.83, $p = 0.043$). Among the 14 studies reviewed in a recent meta-analysis of HALP in lung cancer [108], only two evaluated DFS and reported no significant association. One of these studies included patients undergoing adjuvant therapy [109], and the other focused exclusively on the rare adenosquamous carcinoma subtype [110]. Reported HALP cutoffs in the literature range from 23 to 48, with the majority of studies evaluating OS as the primary endpoint [111,112]. While our cutoff is slightly higher, it is appropriate given our focus on DFS, and the observed FP rate of 15% supports its clinical reliability.

Baseline PNI was also predictive of DFS in our cohort, with a cutoff of 56, sensitivity of 67%, FP rate of 18%, and higher survival likelihood (coefficient = 2.04, $p = 0.027$). Previously reported PNI cutoffs for lung cancer range from 40 to 50 [60,113]. Our slightly higher cutoff remains justifiable, given the acceptable FP rate, and highlights the possibility that optimal PNI thresholds may differ depending on whether DFS or OS is the primary endpoint.

Similar to data on post-operative complications the majority of studies and meta-analyses evaluating inflammatory indexes have been conducted in Chinese populations. Given that many current studies also do not assess DFS in NSCLC, RCTs involving larger and more ethnically diverse cohorts, are required to determine universally applicable cutoffs and to clarify the prognostic value of DFS in NSCLC patients.

6.4.2 Cell populations

The extent of immune-cell changes observed, highlights the considerable potential of peri-operative immune monitoring, as a source of clinically relevant biomarkers. Baseline

immune status is already known to correlate with immunotherapy outcomes in NSCLC [11,37,38,77], and extending this principle to the peri-operative setting may provide new predictive or prognostic tools.

CD8⁺ T cells play a central role in tumour cell elimination through cytotoxic activity and cytokine production [11,14,36]. They are generally associated with favourable prognosis in NSCLC, although most studies evaluate their impact within the TME [11]. Our findings indicate that peri-operative dynamics of peripheral CD8⁺ T cell subsets influence recurrence risk following NSCLC resection. Among CD8⁺ T-cell subsets, only EM and naïve populations were significantly associated with recurrence. Patients who developed recurrence exhibited a greater increase in CD8⁺ EM T cells over time than those who remained disease-free (median slope 3.15 vs. 1.20, $p = 0.001$), alongside a more pronounced decline in CD8⁺ naïve T cells (median slope -4.10 vs. -2.55 , $p = 0.003$).

Chronic tumour antigen exposure is known to drive differentiation of naïve T cells into effector and memory populations while progressively promoting T-cell dysfunction and exhaustion [40, 98-100]. Consequently, the increase in CD8⁺ EM cells observed in patients who subsequently recurred may represent a peripheral immune response to persistent tumour burden rather than effective tumour control [40,98-100]. This interpretation is supported by studies demonstrating accumulation of differentiated effector/memory CD8⁺ T-cell populations in patients with lung cancer and under conditions of chronic antigen stimulation [40,98-100]. Concurrent depletion of the naïve CD8⁺ pool may indicate reduced T-cell repertoire diversity and impaired immune competence, both of which have been associated with poorer outcomes in cancer [37, 79, 114]. Similar shifts from naïve to differentiated memory phenotypes have been reported in NSCLC and other solid tumours, supporting the hypothesis that expansion of CD8⁺ EM cells coupled with loss of naïve CD8⁺ T cells may represent a peripheral blood signature of recurrence risk following lung cancer resection [98-100,114].

A higher rate of change in CD4⁺ CM T cells was associated with an increased risk of recurrence (median slope 1.35 vs. 0.37, $p = 0.032$). As central memory T cells are maintained following antigen exposure and undergo rapid expansion upon antigen re-encounter, this finding may reflect persistent stimulation by residual tumour antigen following resection [39, 40, 98-100]. Although CD4⁺ CM cells play an important role in

sustaining anti-tumour immune responses, their expansion does not necessarily indicate effective tumour control, as chronic antigen exposure can promote the accumulation of memory populations in the setting of ongoing disease [39, 40, 98-100]. Therefore, the observed increase in CD4⁺ CM cells may represent a marker of continued tumour-immune interaction associated with occult residual disease and subsequent recurrence. Other populations, of CD4⁺ T cells, CD8⁺ T cells, Tregs, $\gamma\delta$ T cells, and MAIT cells, showed no clear impact on disease-free survival in our cohort.

Longitudinal changes in B cells, and NK CD16⁺ subsets also demonstrated distinct associations with recurrence risk. Higher B cell slopes appeared protective (median slope -0.25 vs. -0.4 , $p = 0.048$), supporting anti-tumour immunity, consistent with their role as antigen-presenting cells that facilitate activation of T lymphocytes [57]. Investigations of B cells in the peri-operative setting are limited, and this study represents the first longitudinal evaluation in NSCLC patients undergoing surgery.

Patients who developed recurrence exhibited a significantly greater decline in CD16⁺ NK cells over time than those who remained disease-free (median slope -8.65 vs. -7.08 , $p = 0.012$). CD16⁺ NK cells constitute the most mature and cytotoxic NK-cell subset and play a central role in direct tumour cell killing and antibody-dependent cellular cytotoxicity [109]. Consequently, a reduction in this population may indicate impaired innate anti-tumour immunity in patients with residual disease [98-100]. Within the NSCLC tumour microenvironment, NK cells are frequently characterised by reduced CD16 expression, diminished cytotoxic activity, and increased expression of inhibitory receptors, reflecting tumour-mediated immune suppression [36,37]. Persistent exposure to tumour-derived factors, including TGF- β and other immunosuppressive mediators, has been shown to promote NK-cell dysfunction and phenotypic shifts towards less cytotoxic CD16 negative populations [34, 75-77]. Therefore, the greater decline in circulating CD16⁺ NK cells observed among patients who recurred may reflect ongoing tumour-driven remodelling of the NK-cell compartment in response to occult residual disease following resection [75-77]. These findings suggest that longitudinal changes in CD16⁺ NK-cell frequencies may provide insight into postoperative immune surveillance and recurrence risk in NSCLC [36,37].

Increases in monocytes, trended toward elevated recurrence risk, potentially reflecting compensatory or exhausted innate immune responses. Monocytes differentiate into macrophages in the circulation, and their role in NSCLC is context-dependent, with subtypes capable of exerting dual paradoxical effects [11,32]. Previous studies have shown that monocyte expansion peri-operatively correlates with poor prognosis [79], and our findings provide additional insight, by demonstrating that increased monocyte frequencies throughout the peri-operative period are associated with higher recurrence risk.

Importantly, despite these immunological associations, post-operative complications, histology, and stage were consistently among the strongest predictors of recurrence across nearly all analyses. These results emphasize that while peri-operative immune dynamics provide important insights into recurrence risk, they operate within the broader context of tumour-intrinsic biology, highlighting the need to integrate both immunological and clinical parameters for optimal peri-operative risk stratification. These findings underscore the potential of peri-operative immune monitoring to inform prognostication and guide personalized post-operative management.

6.4.3 Immune Checkpoint Proteins

There is a complex interplay ICPs and disease-free survival. ICPs may serve dual functions; mitigating surgical stress and potentially targeting CTCs, which can exploit immune checkpoint pathways to evade immune surveillance [25,62]. While ICPs are primarily recognized for their immunosuppressive effects, when expressed on tumour cells [14], our findings indicate that their expression on certain immune cell subsets can exert both pro- and anti-tumour responses. Sustained expression of ICPs on immune cells has been associated with cellular exhaustion and impaired responsiveness to activation, a phenomenon largely documented in T cells [14,18,19,24,63,104,105]. Studies examining ICP expression on immune cells in peri-operative NSCLC are limited, particularly for ICPs beyond PD1 and T-cell populations [18,104,105]. In this study, PD1-positive NK cells were associated with a protective effect on disease-free survival, contrasting with previous reports showing that PD1 expression on TIL's following surgical resection of NSCLC correlates with poor prognosis [87]. While prior research on ICP upregulation has primarily focused on T cells—where persistent expression leads to exhaustion—our findings suggest that PD1-expressing NK cells may retain or even enhance their cytotoxic activity, resist exhaustion [37], and thus contribute to a protective anti-tumour response.

A particularly notable finding of this study is the role of CTLA4 expressed on the surface of CD8⁺ T cells. In the recurrence group, a skewed distribution characterised by insufficient activation and excessive checkpoint inhibition of CD8⁺ T cells was observed, which may be associated with disease recurrence. CTLA4 expression on immune cells has been predominantly characterized in T cells [22,101], where it competitively binds the CD28 receptor, thereby modulating and restraining T cell activation [101]. Its expression on B cells has been suggested to play a role in limiting isotype switching of antibodies [101], with supporting evidence primarily derived from in vitro experiments or preclinical mouse models indicating a potentially inhibitory function [101]. To our knowledge, this study is the first to report CTLA4 expression on B cells in a clinical context, demonstrating that the loss of this inhibitory receptor on B cells, as well as NK and NKT-like cells, is associated with tumour recurrence.

LAG3 has previously been identified as a potential biomarker due to its association with adverse clinical outcomes [23]. Consistent with these reports, the present study demonstrates that LAG3 expression on both CD4⁺ and CD8⁺ T lymphocytes is associated with an increased risk of reduced DFS. This effect likely reflects its role as an inhibitory receptor on these cells, promoting T-cell exhaustion and impairing effective anti-tumour immunity. Conversely, analogous to TIM3, decreased LAG3 expression on NKT-like and B cells was correlated with tumour recurrence. This reduction may indicate a loss of regulatory balance within these subsets, resulting in dysfunctional responses that are insufficient to sustain long-term anti-tumour activity. Collectively, as observed with other immune checkpoints in this study, the functional consequences of LAG3 expression appear to be highly cell-type specific and context-dependent.

BTLA predominantly exerts its pro-tumorigenic effects on B cells, where it has been shown to inhibit activation and proliferation [25]. Although BTLA has previously been reported as a poor prognostic indicator when expressed on tumour cells, its expression on immune cells has not been well characterized [63]. In the present study, BTLA was highly expressed on B cells, with median levels ranging from 63–77%, whereas its expression on other immune subsets was lower [25]. Notably, BTLA was the only immune checkpoint receptor to exhibit a progressive decrease over the time course of analysis, in contrast to other checkpoints, which generally increased. The dynamic reduction of BTLA may reflect

context-dependent regulation, potentially due to diminished need for inhibitory signalling as B-cell populations are altered during anti-tumour immune responses, or selective modulation within the tumour microenvironment that differentiates it from checkpoints primarily expressed on T cells or NK cells.

The role of TIM3 in NSCLC remains incompletely characterized. Previous studies have demonstrated that TIM3 expression on TILs is associated with poor prognosis, with the worst outcomes observed when TIM3 is co-expressed with PD1, particularly on CD8+ T cells [64, 65]. Although one study reported a reduction of TIM3 expression on NK cells, its impact on survival was not evaluated [86]. The differential effects of TIM3 on distinct immune cell subsets in circulating blood, observed in this study likely reflect context-dependent signalling and the functional specialisation of these cells. On CD4+ T cells and NKT-like cells, TIM3 may function as a regulatory checkpoint that limits excessive activation while permitting effective anti-tumour responses. This may facilitate proliferation, cytokine production, or memory formation, thereby contributing to tumour control. In contrast, TIM3 engagement on NK cells predominantly transduces inhibitory signals that suppress cytotoxic activity, degranulation, and interferon-gamma production, thereby diminishing their capacity to eradicate residual tumour cells. When co-expressed with PD1, these inhibitory pathways may act synergistically in NK cells to further attenuate anti-tumour activity. In CD4+ or NKT-like cells, TIM3 signalling may exert a more balanced or even protective effect, likely due to differences in downstream signalling cascades and the immunological context.

The evaluation of recurrence on ICPs demonstrated a strong independent predictive value of histology, stage, and post-operative complications, which emphasizes that tumour biology and peri-operative stress synergise with checkpoint dynamics, to determine recurrence risk. Overall, these findings underscore the biological principle that both adaptive and innate immune checkpoints, contribute to anti-tumour surveillance, and that perioperative shifts—whether excessive or insufficient—can tip the balance toward recurrence. They support the idea that longitudinal monitoring of multiple cell types and checkpoints may provide mechanistic insights into immune competence, beyond static baseline measurements, and could inform strategies to optimize perioperative immunomodulation in lung cancer patients.

6.5 IMPLICATIONS FOR PERI-OPERATIVE MANAGEMENT

6.5.1 Introduction

Testing peripheral blood samples for diagnosis, screening, and surveillance, represents a minimally invasive approach to monitor patients with NSCLC. The use of liquid biopsy to assess peri-operative immune responses, offers a cost-effective strategy to track patient outcomes throughout the treatment pathway [89]. Currently, the CLuB project focuses on extracellular vesicles, CTCs, and ctDNA as biomarkers for diagnosis, screening, and treatment surveillance [89]. This study builds upon that work, by extending the investigation to immune profiling during peri-operative management of NSCLC.

In Ireland and indeed worldwide, peri-operative management is evolving, with ongoing efforts to detect NSCLC at earlier stages, as exemplified by the ‘Lung Health Check’ pilot study and anticipated future screening program [8]. Concurrently, immunotherapy is increasingly becoming incorporated into both neoadjuvant and peri-operative treatment settings [1,4,6,19]. The investigation of peri-operative immune responses, as undertaken in this study, is of the utmost importance, and is particularly significant given that lung resection surgery remains the gold standard for early-stage NSCLC and offers patients the best chance of cure [6,12]. Moreover, this study highlights the value of longitudinal immune monitoring, demonstrating that temporal changes, rather than single time-point measurements, more accurately reflect a patient’s immune status and potential risk profile.

6.5.2 Post-operative Complications

Up to 45 % of patients undergoing resection for early-stage lung cancer experience a range of minor or major postoperative complications [12]. In our cohort, 44–45 % of patients experienced complications, with POP the most frequently observed at 32 %. These patients generally had good pre-operative lung function (median 92.9 % $\pm$ 21.2 SD), predominantly underwent minimally invasive surgical techniques (79 %) and ERAS protocol. This underscores an underlying process, contributing to post-operative complications. While immune dysfunction is known to occur in lung cancer, it has been relatively understudied in the peri-operative period, highlighting the potential value of peri-operative immune profiling to identify patients at greatest risk [11,18,19,72]. Respiratory complications following surgery have previously been associated with an increased risk of disease relapse [18]. This study not only confirms that post-operative complications, correlate with poorer prognosis but also identifies biomarker signatures, predictive of such complications. Peri-

operative immune profiling therefore holds promise, not only for reducing morbidity and preserving quality of life, but also for potentially modulating the risk of disease recurrence through early identification of vulnerable patients.

Early post-operative inflammatory indexes, including NLR, PLR, SII, and SIRI, have demonstrated potential as predictors of post-operative complications in NSCLC, when measured 2–4 days after surgery (1FU), a timepoint that is largely underexplored [13,31,75]. Prior studies have focused on baseline pre-operative measurements. Meta-analyses do not exist evaluating systemic inflammatory indexes, as prognostic markers for post-operative complications. Furthermore, distinct and novel baseline immune cell profiles—such as elevated CD4⁺ central memory T cells, reduced CD8⁺ naïve T cells, and decreased MAIT cells—were associated with subsequent complications and persisted longitudinally, suggesting that pre-existing immune dysregulation may serve as a marker of susceptibility. Post-operative complications were also linked to increased expression of inhibitory immune checkpoint proteins, including TIM3 on B cells and LAG3 on NKT-like cells, which persisted for several weeks and may contribute to impaired immune surveillance.

These findings indicate that both immune cell populations and checkpoint protein expression can reveal immune dysregulation prior to lung cancer resection, and that this dysregulation, alongside early post-operative inflammatory indexes, may help identify patients at elevated risk, which is important not only for morbidity but recurrence risk. Identifying patients through risk stratification using these immune biomarkers, could highlight those who may benefit from targeted interventions including; pre-operative prehabilitation programs and potential immune modulation via neoadjuvant immunotherapy regimens, as well as enhanced, stricter ERAS protocols with intensified postoperative monitoring and early intervention.

6.5.2 Peri-operative immunotherapy

Longitudinal immune profiling, as demonstrated in this study, provides critical insights that can inform patient stratification and guide peri-operative management in NSCLC, including surgical resection and immunotherapy. Current peri-operative immunotherapy strategies predominantly involve neoadjuvant, adjuvant, or combination regimens with ICI. Neoadjuvant the only reimbursed immunotherapy, currently under the HSE [1,6,9,19]. Response to ICIs is highly variable, with some studies reporting efficacy rates below 40%

and limited understanding of the factors driving differential patient outcomes [20]. Understanding peri-operative immune dynamics may therefore enable the identification, of predictive immune signatures, that can be readily implemented in clinical practice to guide personalised treatment strategies.

Peri-operative immune profiling reveals that both baseline status and early post-operative changes are informative for recurrence risk. Among cell populations, naïve CD8⁺ T cells and B cells were associated with protective immunity, whereas rapid post-operative expansion of EM CD8⁺ T cells correlated with a drive toward terminal differentiation, exhaustion, and higher recurrence risk. Monitoring these subsets could inform peri-operative immunomodulation, timing of immunotherapy, or enhanced surveillance. Additionally, peri-operative increases in monocytes and expansion of CD4⁺ Tregs, may create an immunosuppressive milieu. Therefore identifying these patients, who could benefit from interventions to restore effector function or mitigate immunosuppressive pathways.

Evaluation of ICP expression reveals complex, cell type-specific patterns with important implications for peri-operative immunotherapy. PD1 expression on NK cells was associated with preserved cytotoxicity and improved DFS, indicating that PD1⁺ NK cells represent a functionally competent subset that could be therapeutically leveraged. In contrast, overexpression of CTLA4 on CD8⁺ T cells correlated with impaired activation and increased recurrence risk, consistent with its established role as a negative regulator of T-cell priming and effector function [11,15,20]. Notably, insufficient CTLA4 activation on B cells, NK, and NKT-like cells, as well as on CD8⁺ T cells, was also associated with recurrence. These findings suggest that CTLA4 functions in a cell type- and context-dependent manner. Therapeutic strategies should therefore consider CTLA4 biology across immune lineages, including tailored CTLA4 modulators or combination regimens that enhance both adaptive and innate immunity without inducing excessive suppression. Considering that CTLA4 is the first immune checkpoint to be expressed on immune cells following activation, targeting this pathway may be a promising strategy, to mitigate early immune dysfunction [22].

Beyond PD1 and CTLA4, elevated LAG3 expression on CD4⁺ and CD8⁺ T cells, as well as on NKT-like and B cells, was linked to reduced DFS and recurrence, consistent with

their role in T-cell exhaustion and broader immune inhibition [21,23,24]. BTLA primarily suppressed B-cell activation and antigen presentation, whereas TIM3 exhibited cell type-specific effects: modulatory on CD4⁺ T and NKT-like cells but inhibitory on NK cells, reducing cytotoxicity and interferon- γ production, particularly when co-expressed with PD1. Patients entering ICI therapy with elevated ICPs such as LAG3 and TIM3—previously associated with resistance in tumour-infiltrating lymphocytes—tend to experience poorer clinical outcomes [23,68]. These results support tailored peri-operative immunotherapy approaches, such as PD1/LAG3 blockade, TIM3 inhibition with NK-cell activation, or BTLA modulation to restore B-cell function. Longitudinal monitoring of ICP expression on both immune and tumour cells may identify high-risk subsets and guide personalised peri-operative interventions.

6.5.3 Post-operative surveillance

The dynamic peri-operative changes in inflammatory indexes, immune cell populations, and checkpoint expression observed in this study underscore the potential utility of longitudinal immune monitoring in patients undergoing surveillance, after lung resection. Non-invasive tracking of peripheral CD8⁺ T cell subsets, B cells, NK cells, and NK CD16⁺ subsets could facilitate early identification of patients at higher risk of recurrence, complementing standard imaging such as CT scans, and inform individualised follow-up strategies [1,4,6].

Longitudinal assessment of immune checkpoint proteins further emphasizes the value of peri-operative immune surveillance. By integrating ICP monitoring with cell population dynamics and established indexes such as HALP and PNI, clinicians could obtain a comprehensive immunological profile, improving prognostic accuracy and supporting tailored surveillance protocols for post-resection patients, even in the absence of immunotherapy.

6.6 STUDY LIMITATIONS

Limitations of this study include the relatively small cohort, with 38 patients evaluated for inflammatory indexes and 34 patients assessed via flow cytometry. The antibody panels were constrained by laser availability and spectral spillover, limiting simultaneous assessment of multiple immune checkpoint proteins across all immune cell subsets; future studies incorporating expanded panels, including detailed T cell subset profiling, would be valuable. Assessment of smoking status was not feasible, as 89% of participants reported a history of smoking. While studies in patients without a smoking history could provide additional insights, such cohorts are challenging to recruit, given that smoking remains the predominant risk factor for NSCLC [1,2,4,5].

6.7 CONCLUSION

This study highlights the profound immunological shifts that occur during the peri-operative period in NSCLC and their critical influence on disease outcomes. Longitudinal immune monitoring reveals that dynamic changes in systemic immunity—spanning both adaptive and innate compartments—can provide actionable insights into patient risk, guiding more precise prognostication and informed clinical decision-making. Monitoring of inflammatory indexes, cell populations and ICP monitoring peri-operatively and also during immunotherapy and surveillance should be considered. This offers a minimally invasive strategy to identify high-risk patients, optimise surveillance, and tailor interventions to individual immune trajectories. These findings underscore the transformative potential of peri-operative immunological assessment, to refine patient care and improve long-term outcomes in lung cancer.

7.0 FUTURE PLANS

- Stimulatory receptor, cytokine receptor proteins and co-expression of ICPs included in the current antibody panels will be analysed and reported in future publications.
- Co-expression patterns of immune checkpoint proteins will be evaluated to further elucidate immune regulatory networks
- Overall survival analyses will be performed once sufficient follow-up data become available, complementing the current disease-free survival findings.
- Plasma proteomics, incorporating cytokines, chemokines, and immune checkpoint proteins, is underway to elucidate dynamic peri-operative immune and tumour-related changes. This approach aims to provide deeper insights into immune cell function.
- Findings could also be confirmed through functional assays to assess if T cells and NK cells are approaching exhaustion, senescence and/or anergy.
- Integration of immune profiling with other liquid biopsy constituents—including circulating tumour cells, ctDNA, and extracellular vesicles—from the same patient cohort (CLuB project), will enable the development of multi-component signatures for improved cancer diagnosis, prognosis, and treatment stratification.
- If changes in perioperative immune response are shown to have predictive, prognostic and/or therapeutic value in the future, whole blood phenotyping would be a quicker and more inexpensive alternative to PMBC preparations.

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