

Minerva Access is the Institutional Repository of The University of Melbourne

Author/s:

Wong, Ngai Man Annie

Title:

Novel biomarkers for melanoma immunotherapy

Date:

2020

Persistent Link:

<https://hdl.handle.net/11343/252887>

Terms and Conditions:

Terms and Conditions: Copyright in works deposited in Minerva Access is retained by the copyright owner. The work may not be altered without permission from the copyright owner. Readers may only download, print and save electronic copies of whole works for their own personal non-commercial use. Any use that exceeds these limits requires permission from the copyright owner. Attribution is essential when quoting or paraphrasing from these works.

Novel Biomarkers for Melanoma Immunotherapy

Ngai Man Annie Wong

ORCID ID: 0000-0002-6037-8916

Submitted in the total fulfilment of the requirements of the degree of

Doctor of Philosophy

Sir Peter MacCallum Department of Oncology

Faculty of Medicine, Dentistry and Health Sciences

The University of Melbourne

May 2020

Abstract

Traditionally, metastatic melanoma had a dismal prognosis, but the recent advent of immune checkpoint inhibitors (ICI) has extended survival from months to years for some patients. There is an urgent need to identify prognostic and predictive biomarkers for melanoma patients treated with ICI, given that only a minority of patients respond, coupled with the potential treatment related toxicities. This thesis aimed to investigate clinical factors, functional PET imaging and tumour immune profiling as candidate biomarkers for ICI in patients with melanoma.

Firstly, Chapter 3 focused on baseline performance status as a biomarker for outcome following anti-PD-1. The hypothesis was that unlike cytotoxic chemotherapy, baseline performance status was not correlated with outcome following ICI, owing to its distinct mechanism of action. However, in the cohort of 91 patients treated with anti-PD-1 at Peter MacCallum Cancer Centre, poor performance status was correlated with poor survival and low response rate to anti-PD-1. Furthermore, patients with poor performance status were more likely to be hospitalised and more likely to die in hospital.

Patient characteristics and blood parameters were further examined in Chapter 4, but specific to a cohort of patients with melanoma brain metastases. Melanoma commonly metastasise to the central nervous system and this is associated with extremely poor survival (1, 2). Recently, combination ICI has resulted in intracranial responses and durable survival(3, 4). Most of the existing literature in biomarkers in melanoma brain metastases also predates the introduction of ICI (1, 2, 5-8), therefore investigation of biomarkers in patients with melanoma brain metastases treated with ICI is needed. A post-hoc analysis of patients with melanoma brain metastases as part of the phase II Anti-PD1 Brain Collaboration study was performed to identify possible predictors of clinical outcome or toxicity. In this study, patients were randomised to receive either nivolumab monotherapy or nivolumab in combination with ipilimumab. High C-reactive protein, a marker of systemic inflammation, was correlated with poor survival. Treatment with combination ICI, hypernatraemia and increased body mass index were

associated with higher likelihood of severe toxicity at 120 days, whereas CRP was not associated with higher toxicity.

The thesis then went on to examine the role of FDG PET functional imaging as a source of biomarkers for outcome following ICI in Chapter 5. Baseline pre-treatment tumoural FDG-PET avidity (measured by SUVmax or metabolic tumour volume) as well as FDG-avidity in the immune system (measured by spleen to liver ratio) were assessed in relation to survival outcomes. Interestingly, tumoural PET avidity was not correlated with survival, whereas high spleen to liver ratio was correlated with poor survival after ipilimumab. This was subsequently validated in a combined cohort of patients from two separate European centres. High spleen to liver ratio was correlated with low albumin in a multivariate analysis, thus suggesting a possible association with systemic inflammation. Early on-treatment PET (EOT PET) were assessed in a small subset of 16 patients, and several challenges were identified that may limit the use of FDG PET in this early juncture as a biomarker for outcome after ICI.

In-depth characterisation of tumoural immune landscape is crucial to improving the understanding of melanoma immuno-biology, with potential implications for biomarker development. Chapter 6 aimed to compare the immune profile of UV related skin cancers (melanoma, cutaneous squamous cell carcinoma and Merkel cell carcinoma) using orthogonal methods of bulk RNA-sequencing and multi-spectral immunohistochemistry. The three skin cancers showed distinct immune landscapes, with melanoma having a significantly higher intratumoural T cell infiltrate compared to Merkel cell carcinoma, whereas PD-L1 density was highly variable across three skin cancers. Transcriptomic analyses of melanoma samples with high PD-L1 density were associated with upregulation of genes related to leucocyte proliferation, migration and adaptive immune responses, in contrast to MCC samples with high PD-L1 density, where such a signature was not observed.

Lastly, an in-depth case study of six patients highlighted how multi-factorial biomarkers such as clinical factors, functional PET imaging, baseline blood parameters, and multi-spectral immunohistochemistry can be applied together.

In conclusion, this thesis evaluated multi-factorial biomarkers including clinical, functional imaging and tumoural immune profiling biomarkers. These studies add to the evolving literature on biomarkers associated with ICI treatment. It is envisaged that

with time, these complementary methods of understanding the patient and tumoural immune environment can aid rational selection of immune based therapies for patients with advanced melanoma.

Declaration

This is to certify that:

- (i) the thesis comprises only my original work towards the PhD except where indicated in the preface;
- (ii) due acknowledgement has been made in the text to all other material used; and
- (iii) the thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Signature:

Ngai Man Annie Wong

Preface

The work included in the thesis is my own with the following exceptions:

1. The article included in Chapter 3 of the thesis entitled, ‘Clinical and palliative care outcomes for patients with poor performance status treated with anti-programmed death-1 monoclonal antibodies for advanced melanoma’, was published in *Asia Pacific Journal of Clinical Oncology*, December 2017. For this article, I designed the study, collected clinical data, performed the survival analyses, made the tables and figures and wrote the manuscript; Dr Molly Williams collected data and performed some of the statistical analysis; Dr Donna Milnes, Dr Kortnye Morris, Dr Peter Lau Dr Fullerton and Dr Odette Spruyt collected data and contributed to the manuscript. Professor Grant McArthur helped conceptualise the study and contributed to the manuscript.
2. The post-hoc analysis of biomarkers in patients with brain metastases for Chapter 4 used individual patient data from the Anti-PD1 Brain Collaboration clinical trial (3). The clinical trial was conducted by the investigators as listed in the publication. I designed the study and performed the statistical analyses to investigate the candidate biomarkers. Dr Serigne Lo and Professor Georgina Long from the Melanoma Institute of Australia, Sydney, provided access to the data and assistance with interpretation of results. Professor Grant McArthur provided with assistance with interpretation of results.
3. The article included in Chapter 5, ‘18F-FDG PET/CT based spleen to liver ratio associates with clinical outcome to ipilimumab in patients with metastatic melanoma’, was published in *Cancer Imaging*, April 2020. For this study, I designed the study, performed the FDG PET analyses, performed some of the statistical analyses, made the tables and figures, wrote the manuscript; Dr Jason Callahan performed FDG PET analyses and contributed to the manuscript and figures; Dr Marleen Keyaerts and Professor Bart Neyns provided the clinical and imaging data for the validation cohort from UZ Brussels and contributed to the results interpretation and manuscript; Dr Johanna Mangana and Ms Susanne Aberle provided the clinical data for the validation cohort from University Hospital Zurich and contributed to the results interpretation and manuscript; Dr Alan Herschtal provided the majority of the statistical analyses, Dr Sonia Fullerton and Dr Donna Milne collected clinical data, Dr Amir Iravani provided contributions to the manuscript; Professor Grant McArthur and Professor Rodney J. Hicks conceptualised the study design and contributed to the manuscript.
4. The comparison of UV-related skin cancers study in Chapter 6 was conceptualised by Professor Grant McArthur, Dr Richard Tothill (Laboratory

Head, Rare Diseases Oncogenomics Laboratory, University of Melbourne) and myself. I performed the multi-spectral immunohistochemistry (mIHC) staining of melanoma samples, performed the mIHC and transcriptomic analyses on all 3 skin cancers and made the tables and figures. Melanoma samples were provided by the Melanoma Research Victoria (previously the Melbourne Melanoma Project) through Dr Sonia Mailer. The tumoural region of interest was marked by Dr Chris Angel, Anatomic pathologist, Peter MacCallum Cancer Centre. The tissue sectioning of the melanoma samples was performed by the Centre of Advanced Histology and Microscopy, Research Division, Peter MacCallum Cancer Centre. Histological slides of the Merkel cell carcinoma (MCC) samples were provided by Dr Richard Tothill, University of Melbourne, Professor Richard Scolyer and Dr James Wilmott from Melanoma Institute of Australia, and Dr Anthony Gill from Royal North Shore Hospital. Histological slides of cutaneous squamous cell carcinoma (cSCC) were provided by Professor Jürgen Becker, University Hospital Essen, Germany. The mIHC T cell characterisation protocol was optimised by Heloise Halse for melanoma, Dr Kelly Waldeck optimised the protocol for MCC and I optimised the staining protocol for cSCC. The mIHC staining of the MCC and cSCC samples was performed by Dr Kelly Waldeck. The RNA-sequencing was performed by the Molecular Genomics Core facilities at Peter MacCallum Cancer Centre. The tumour mutational burden was derived from formalin fixed and paraffin embedded samples using a variant calling bioinformatics technique created by Dr Andrew Pattison, Rare Diseases Oncogenomics Laboratory, University of Melbourne. I performed the other transcriptomic analyses including the principal components analysis, differential gene expression analysis and gene set enrichment analyses.

I received funding support for this thesis from the Royal Australasian College of Physicians and was the recipient of the Vincent Fairfax Foundation Research Entry Scholarship in 2016 and the New Zealand Fellows Research Entry Scholarship in 2017.

Acknowledgements

I would like to extend my deepest gratitude to:

My primary supervisor, Professor Grant McArthur, for the opportunity to work on such an exciting research topic in this rapidly changing field of melanoma immunoncology. Your unwavering enthusiasm inspired me to ask clinically relevant questions to improve patient care. I have learned so much from you over the years, and am grateful for the time you have taken to open my eyes to critical thinking and the powers of translational research.

My other supervisors, Associate Professor Carleen Cullinane and Professor Rod Hicks, for your ongoing support, encouragement and friendship. Thank you for your allowing me to join your research laboratory and teaching me so much in your respective art.

My mentor, Professor Sarah-Jane Dawson: Thank you for your guidance and sage advice. You are the perfect role model for a female oncologist. Your phone call to reassure me that it is possible to balance clinical medicine, research *and* family will be forever in my heart.

My ‘informal’ mentors, collaborators, fellow researchers and students: Research is tough! Everyone is short on time and resource. Yet everyone has always been exceptionally generous with their time to share their expertise and wisdom. I will remember this and pay it forward, always. It is often said that it takes a village to raise a child, and so it is with researchers too. I wish to express my deepest gratitude to my entire research village.

My patients at Peter MacCallum Cancer Centre. I learned so much from you during my time as a clinical fellow there. Your bravery and eternal optimism has kept me going every step of the way.

My family: this thesis is not possible without your continued support. You have always fostered my curiosity and intellectual pursuits in every possible way. Thank you to my parents who moved in with me to take on the childcare responsibilities for baby Sam, so to make it possible for me to finish the PhD from New Zealand. Thank you to Bronwyn, my mother-in-law, who tirelessly proof-read this thesis. Lastly, Tim, my long-suffering husband, you have always been so supportive of my career and I am forever grateful.

Tables

Table 1.1 Comparison of American Joint Committee on Cancer 7 th and 8 th Edition of Tumour Node Metastasis (TNM) staging system of melanoma	4
Table 1.2. Randomised phase III studies of immune checkpoint inhibitors in advanced melanoma	20
Table 1.3. Blood, tumoural and faecal biomarkers for anti-CTLA4 and anti-PD-1 treatments for advanced melanoma, adapted from Manson et al (235).....	54
Table 2.1 Reagents used for the melanoma or cSCC T cell characterisation mIHC panel	70
Table 2.2 Reagents used for the MCC T cell characterisation mIHC panel	71
Table 2.3 Reagents used for the Pan-immune characterisation mIHC panel	72
Table 4.1 Candidate biomarkers considered in the post-hoc analysis of Anti-PD1 Brain Collaboration study	100
Table 4.2 Patient demographics.....	101
Table 4.3 Continuous variables considered in survival and toxicity analyses	103
Table 4.4 Treatment and toxicity outcome.....	104
Table 4.5 Univariate and multivariate Cox regression analysis for overall survival for combined Cohort A & B.....	105
Table 5.1 Patient clinical and disease characteristics	132
Table 5.2 Baseline PET parameters of discovery cohort (all patients treated with immunotherapy, n =90)	133
Table 5.3 (A) Association between PET parameters and progression free survival, for ipilimumab and anti-PD-1 treatment, analysed as continuous variable and dichotomized at the median for the cohort.....	135
Table 5.4 (A) Multivariate analysis for progression free survival after ipilimumab....	142
Table 5.5 Early on-treatment PET parameters compared to baseline PET prior to ipilimumab or anti-PD-1	161
Table 7.1 Patient demographics, disease characteristics, treatment outcome and biomarkers	204

Figures

Figure 1.1. Modified schema of the mitogen-activated protein kinase (MAPK) pathway in melanoma	9
Figure 4.1 Simplified study schema of the Anti-PD-1 in Brain Collaboration clinical trial (3).....	106
Figure 4.2 Kaplan-Meier curve of overall survival stratified by C-reactive protein (CRP) and white cell count (WCC).....	107
Figure 5.1. Spleen to Liver Ratio (SLR) on positron emission tomography.....	129
Figure 5.2 (A). Kaplan Meier curves of FDG PET parameters and Progression free survival after ipilimumab; (B). Kaplan Meier curves of FDG-PET parameters and Progression free survival after anti-PD-1; (C). Kaplan Meier curves of FDG-PET parameters and overall survival from the start of first line immunotherapy; (D). Kaplan Meier curves of Spleen to Liver Ratio (SLR) and overall survival after ipilimumab and anti-PD-1 respectively	138
Figure 5.3 Overall survival curves stratified by metabolic tumour volume quintile....	140
Figure 5.4 SLR and overall survival after ipilimumab in the validation cohort.....	144
Figure 5.5 Kaplan-Meier survival curves for patients treated with either ipilimumab (n=16) or anti-PD-1 (n=16) and its association with EOT PET changes.	163
Figure 6.1 (A) Study schema and (B) methodology for multi-spectral immunohistochemistry (mIHC).....	184
Figure 6.2 (A) Comparison of intratumoural T cell subsets and PD-L1 ⁺ cells in 3 skin cancers by mIHC; (B) DNT is the predominant T cell subset in a subset of MCC samples; (C) PD-L1+density is not associated with predominant T cell subset; (D) PD-L1 is not associated with Merkel polyomavirus status in MCC or ulceration status in melanoma; (E) CD8 was higher in MCPyV+ MCC than MCPyV- MCC but did not differ with ulceration in melanoma; (F) CD3 was not associated with MCPyV in MCC or ulceration in melanoma; (G) CD4 was not associated with MCPyV in MCC or ulceration in melanoma; (H) FOXP3 was not associated with MCPyV in MCC or ulceration in melanoma	187
Figure 6.3 (A) Spearman correlation of T cell subsets and PD-L1 cells; (B) Spatial distance between cell types.....	190
Figure 6.4 (A) Principal components analysis of differentially expressed genes across 3 skin cancers; (B) Unsupervised clustering of immune related genes.....	192
Figure 6.5 (A) Correlation between PD-L1 staining by mIHC and PD-L1 expression by mRNA; (B) Correlation between PD-L1 density by tumour mutational burden	194
Figure 6.6 Differential gene expression analyses of melanoma (n=17) and Merkel cell carcinoma (MCC, n=19) samples.....	196
Figure 6.7 (A) Enriched GO Biological Processes associated with high-PDL1 melanoma; (B) Network analysis for genes associated with high PD-L1 melanoma; (C)	

Enriched GO Biological Processes associated with high-PDL1 melanoma; (D) Network analysis for genes associated with high PD-L1 melanoma 198

Figure 7.1 Modified Immunogram for immune checkpoint inhibitors for advanced melanoma 212

Figure 7.2 Modified immunograms for patients in Chapter 7 213

Publications

First author manuscripts resulting from the thesis:

Wong ANM, Williams M, Milne D, Morris K, Lau P, Spruyt O, Fullerton S and McArthur GA

Clinical and palliative care outcomes for patients of poor performance status treated with anti-programmed death1 for advanced melanoma Asia-Pacific Journal of Clinical Oncology. 2017; 13(6):385-390

Wong ANM, Hofman M, McArthur GA, Hicks RJ

The Advantages and Challenges of Using FDG PET/CT for Response Assessment in Melanoma in the Era of Targeted Agents and Immunotherapy European Journal of Nuclear Medicine and Molecular Imaging 2017; 44(Suppl 1):67-77.

Wong ANM, Billett A, Milne D

Balancing the Hype with Reality: What Do Patients with Advanced Melanoma Consider When Making the Decision to Have Immunotherapy? Oncologist. 2019;24(11):e1190–e1196.

Wong ANM, Callahan J, Keyaerts M, Neyns B, Mangana J, Aberle S, Herschtal A, Fullerton S, Milne D, Iravani A, McArthur GA and Hicks RJ

¹⁸F FDG PET/CT based spleen to liver ratio associates with clinical outcome to ipilimumab in patients with metastatic melanoma. Cancer Imaging, 2020; 20 (36): 1-12

Other manuscripts:

Parakh S, Park J, Mendis S, Rai R, Xu S, Lo S, Drummond M, Rowe C, **Wong ANM**, McArthur GA, Andrew H, Andrews M, Cebon J, Guminski A, Kefford R, Long G, Menzies A, Klein O, and Carlino M

Efficacy of anti-PD-1 therapy in patients with melanoma brain metastases
British Journal of Cancer 2017 Jun 6;116(12):1558-1563. doi: 10.1038/bjc.2017.142.

Menzies A, Johnson DB, Ramanujam S, Atkinson VG, **Wong ANM**, Park J, McQuade J, Shoushtari AN, Tsai KK, Eroglu Z, Klein O, Hassel JC, Sosman J, Guminski A, Sullivan RJ, Ribas A, Carlino MS, Davies MA, Sandhu SK, Long GV. Anti-PD-1 therapy in patients with advanced melanoma and pre-existing autoimmune disorders or major toxicity with ipilimumab
Ann Oncol (2017) 28 (2): 368-376.

Presentations:

Wong ANM, Callahan J, Beresford J, Herschtal A, Fullerton S, Milne D, Hicks RJ, McArthur GA

Spleen to liver ratio (SLR): Novel PET imaging biomarker for prediction of overall survival after ipilimumab and anti-PD-1 in patients with metastatic melanoma. Presented at ASCO ASM 2016 (Merit Award)

Wong ANM, Waldeck K, Neeson P, Halse H, Angel C, Colebatch C, Raleigh J, Hatzimihalis A, Gyorki D, Fox S, Sandhu S, Johnston M, Cullinane C, Scolyer R, Wilmott J, Gill A, Becker J, Hicks R, Tothill R and McArthur G

Comparison of primary Merkel cell carcinoma (MCC) and melanoma T-cell immuno-profile and PD-L1 expression by multi-spectral immunohistochemistry (mIHC)

Presented at World Congress of Melanoma ASM 2017

Abbreviations

%ID	Percentage injected dose
¹⁸ F FDG PET	2-deoxy-2-[fluorine-18] fluoro-D-glucose positron emission tomography
ABC [study]	Anti-PD-1 in Brain Collaboration [study]
AJCC	American Joint Committee of Cancer
Anti-CTLA4	Anti-CTL-associated antigen 4
APC	Antigen presenting cells
Anti-PD-1	Anti-Programmed Death-1
Anti-PD-L1	Anti-Programmed Death-Ligand1
AE	[drug related] Adverse events
BLR	Bone marrow-to-Liver SUVmax ratio
BTS	Baseline tumour size
CNS	Central nervous system
CTCAE	Common Terminology Criteria for Adverse Events
cSCC	Cutaneous squamous cell carcinoma
CRP	C-reactive protein
CT	Computerised tomography
ctDNA	Circulating tumour DNA
DNA	Deoxyribonucleic acid
DTIC	Dacarbazine
EAP	Expanded access program
ECOG	European Cooperative Oncology Group
EORTC	European Organisation for Research and Treatment of Cancer
EOT PET	Early on-treatment PET
FDG PET	Fluoro-D-glucose positron emission tomography

FFPE	Formalin fixed and paraffin embedded
FLT PET	Fluorothymidine positron emission tomography
GM-CSF	Granulocyte macrophage colony-stimulating factor
HR	Hazard ratio
ICI	Immune checkpoint inhibitors
IHC	Immunohistochemistry
IL2	Interleukin-2
Indel	Insertion/deletion
IQR	Interquartile range
IrAE	Immune-related adverse events
IrRC	Immune-related response criteria
LDH	Lactate dehydrogenase
L	Line [of therapy]
LS	Lymphocyte score
LUSC	Lung squamous cell carcinoma
MAPK	Mitogen activated protein kinase
MCPyV	Merkel Cell Polyoma Virus
MCC	Merkel cell carcinoma
MHC	Major histocompatibility complexes
MTV	Metabolic tumour volume
mIHC	Multi-spectral immunohistochemistry
MTIC	Methyl triazeno imidazole carboxamide
NMSC	Non-melanoma skin cancer
NLR	Neutrophil to Lymphocyte Ratio
NR	Not reported
ORR	Objective response rate
OS	Overall survival

PD-1	Programmed Death-1
PD-L1	Programmed Death-Ligand1
PD-L1 TPS	PD-L1 Tumour Proportion Score
PD-L2	Programmed Death-Ligand 2
PEG-IFN α -2 α	Pegylated interferon
PERCIST	PET Response Criteria in Solid Tumours
PET	Positron emission tomography
PFS	Progression-free survival
PMD	Progressive Metabolic Disease
PS	Performance status
RECIST	Response Evaluation Criteria in Solid Tumours
RNA	Ribonucleic acid
RNAseq	Ribonucleic acid sequencing
RPA	Radiation Therapy Oncology Group Recursive Partitioning Analysis
SLR	Spleen to liver ratio
SUL	SUVpeak normalised by lean body mass
SUV	Standardised uptake value
TBST	Tris Buffer Saline and Tween
TCGA	The Cancer Genome Atlas Network
TCR	T Cell Receptor
TIL	Tumour infiltrating lymphocytes
TLG	Total lesion glycolysis
TMB	Tumour mutational burden
TNM	Tumour, Node, Metastases [staging system]
Treg(s)	Regulatory T cells
TSA	Tyramide signal amplification

TVEC	Talimogene laherparepvec
ULN	Upper limit of normal
WES	Whole exome sequencing

Glossary

Antibody	A protein produced by some immune cells in response to foreign or tumoural antigens
Antigen	Any substance that triggers an immune response including foreign sources (such as bacteria, viruses) or abnormal proteins in cancer cells
Antigen-presenting cells (APCs)	Immune cells that digest antigens in a way that “presents” them to T-cells and B-cells to instruct B- and T-cells to destroy cells with the “presented” antigen.
Biomarker	A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. Biomarkers can be used to predict the prognosis of an illness (<i>prognostic biomarker</i>) or likelihood of response to a treatment (<i>predictive biomarker</i>).
Cytokines	Proteins secreted by certain immune cells so they can communicate with each other. Cytokines in the blood can be measured as a marker of an inflammatory response. Cytokines can also be manufactured and used as a immunotherapy treatment.
Immune cells	Cells in the immune system that help defend against infections or cancers.
Immune checkpoint inhibitors (ICI)	An immunotherapy that fights cancer by blocking receptors on immune cells. These can block inhibitory receptors such as ‘CTLA4’ or ‘PD-1’ and thereby ‘release the brakes’ on the immune cells to kill cancer. There are also ICI that can stimulate the immune system by ‘stepping on the accelerator’ e.g. OX40
Immune-related adverse events (IRAEs)	The immune system’s overreaction to immunotherapy resulting in often mild, but potentially life-threatening toxicity.
Lymphocyte	A type of immune cell (white blood cells) in lymphatic tissue and blood. The main types are T-lymphocytes (T-cells) and B-lymphocytes, which both help the immune system fight cancer.
Monoclonal antibodies (mAbs)	Laboratory-made proteins that target specific proteins or molecules on the surface of cells. Monotherapy that target immune checkpoints can be used as cancer treatment

PD-1 (programmed cell death-1)

An inhibitory receptor that help regulate the body's immune response. A type of checkpoint inhibitor blocks PD-1 receptors, in effect "releasing the brakes" on the immune system.

Receptors (immune receptors)

Surface molecules on immune cells that bind to the surface of other immune cells. This typically causes the cell to produce signals that regulate specific functions in the immune system.

T-cells

White blood cells (immune cells) that play a significant role in the immune system's fight against infection and disease.

Tumour-infiltrating lymphocyte (TIL)

A type of immune cell that invades a tumour mass or microenvironment. A type of immunotherapy removes TIL cells from a patient's tumour and re-engineers them in a laboratory to seek and destroy tumour-specific cancer cells.

Tumour microenvironment

The area that surrounds and sustains a tumour. It is made up of normal cells, blood vessels and extracellular matrix.

Table of Contents

Abstract	i
Declaration	iv
Preface	v
Acknowledgements	vii
Tables	viii
Figures	ix
Publications	xi
Abbreviations	xiii
1 Background	1
1.1 Melanoma: epidemiology, subtypes, staging, overview of treatment	1
1.1.1 Epidemiology	1
1.1.2 Melanoma pathogenesis and subtypes	1
1.1.3 Staging	2
1.1.4 Melanoma immunobiology	5
1.1.5 Overview of treatment	6
1.2 Immunotherapy	11
1.2.1 Immune Checkpoint Inhibitors (ICI)	13
1.2.2 Summary of clinical trials for ICI in advanced melanoma	16
1.2.3 ICI in non-melanoma skin cancers	28
1.2.4 ICI future directions	32
1.3 Biomarkers	34
1.3.1 Clinical biomarkers	35
1.3.2 Functional imaging biomarkers	41
1.3.3 Biological biomarkers	43
1.3.4 Application of biomarkers	56
1.4 Hypotheses and questions addressed in this thesis	58
2 Materials and Methods	61
2.1 Performance status as a biomarker for anti-PD-1	61
2.2 Biomarkers in brain metastases	62
2.3 Functional PET imaging	62
2.4 Comparison of UV related cutaneous malignancies	64

2.5	Patient case studies for the application of biomarkers.....	69
2.6	Tables	70
3	Performance status as a biomarker for anti-PD-1.....	73
3.1.1	Introduction	73
3.1.2	Aims	74
3.1.3	Summary of results.....	75
3.1.4	Discussion	75
3.1.5	Publication: ‘Clinical and palliative care outcomes for patients of poor performance status treated with antiprogrammed death-1 monoclonal antibodies for advanced melanoma.’	78
4	Clinical biomarkers in patients with brain metastases treated with ICI85	
4.1.1	Introduction	85
4.1.2	Aims	91
4.1.3	Results.....	91
4.1.4	Discussion	94
4.1.5	Conclusion	99
4.1.6	Tables.....	100
4.1.7	Figures	106
5	Functional FDG PET imaging biomarkers	108
5.1	Baseline PET/CT characteristics: Spleen to liver ratio	111
5.1.1	Aims	114
5.1.2	Summary of results.....	114
5.1.3	Results not presented in the publication	114
5.1.4	Discussion	115
5.1.5	Manuscript accepted and in-press	121
5.2	Early treatment PET/CT response as a biomarker	159
5.2.1	Aim.....	160
5.2.2	Summary of Results	161
5.2.3	Discussion	164
5.2.4	Conclusion	165
6	Comparison of immune profile of UV related cutaneous malignancies.....	167

6.1.1	Introduction	167
6.1.2	Aims	171
6.1.3	Results.....	171
6.1.4	Discussion	175
6.1.5	Figures	182
7	Application of biomarkers: case studies	199
7.1.1	Introduction	199
7.1.2	Aims	201
7.1.3	Results.....	201
7.1.4	Discussion	206
7.1.5	Conclusion	209
7.1.6	Figures	210
8	Conclusions	220
8.1	Discussion	220
8.2	Conclusion	225
	Appendix	227
A.1	Patient cohort treatment and biomarker summaries.....	227
9	References	234

1 Background

1.1 Melanoma: epidemiology, subtypes, staging, overview of treatment

1.1.1 Epidemiology

Australia has one of the highest incidence of cutaneous melanoma worldwide (49 cases per 100,000), with melanoma being the third most commonly diagnosed cancer in both genders in 2019 (9, 10). The incidence of melanoma has almost doubled from 27 cases per 100,000 in 1982 to 49 cases per 100,000 in 2016 (10). Over 90% of melanoma are diagnosed as early stage disease (Stage I and II) and the relative survival for early stage disease is 91% (9). However, mortality rises sharply for those who present with advanced stage of disease. The historic median survival for patients with metastatic melanoma (Stage IV) was less than one year and the 5-year survival rate was less than 10% (11). Recent advances in effective therapies with molecularly targeted treatments and immunotherapy have resulted in durable improvements in survival for a subset of patients.

1.1.2 Melanoma pathogenesis and subtypes

Melanomas arise from melanocytes, the melanin pigment-producing cells which are present along the skin basal epidermis, as well as in hair follicles. Melanin pigment provides some protection from ultra-violet radiation, and therefore cutaneous melanoma rates are higher in those with fair skin and low amounts of melanin. There is a large body of epidemiological and preclinical evidence that associates ultra-violet radiation with the development of cutaneous melanoma (12-17). Molecular characterisation of melanoma has also identified a predominant ultra-violet mutational signature (C>T nucleotide transition) within cutaneous melanoma, whereas other melanomas tend to have structural changes and mutations of unknown aetiology (18, 19). Approximately 10% of cutaneous melanomas develop in individuals who have one or more first degree relatives with melanoma (20). There are some uncommon inherited risk factors such as the presence of germline mutations in CDKN2A, or CDK4 (21, 22). There are also some inherited conditions with an increased risk of developing melanomas, such as

xeroderma pigmentosum, familial retinoblastoma, Lynch syndrome type II and Li-Fraumeni syndrome (20, 23).

There are four main subtypes of melanoma depending on the site of origin: cutaneous, acral (hands and feet), mucosal (internal mucosal membranes) and ocular (24). Cutaneous melanoma is the most common subtype in Caucasians, whereas acral and mucosal subtypes are more common in non-Caucasians. These melanoma subtypes have distinct clinical and biological characteristics owing to their different aetiology (18). This thesis focuses on cutaneous melanoma, as it is the most common subtype and the most studied in terms of immunotherapy development.

Cutaneous melanoma can also be classified by its molecular characterisation. The understanding of melanoma biology has evolved significantly over the past decade owing to the significant advances made available through high throughput sequencing of genomic data. The Cancer Genome Atlas Network (TCGA) is an exceptional effort in modern science to classify cancer using a multi-platform approach. In the landmark paper (19), 333 melanomas were studied using DNA, RNA, and protein based analyses. Four molecular subtypes were established based on the most frequently mutated genes: mutant BRAF, mutant RAS, mutant NF1 and triple wild-type. These molecular subtypes have direct therapeutic implications, which will be discussed later in this literature review.

1.1.3 Staging

Melanoma is staged using the Tumour, Node, Metastasis (TNM) system as per the American Joint Committee of Cancer (AJCC) clinical and pathological criteria (11, 25). AJCC Stage I and II melanomas are limited to the skin, whereas stage III involves locoregional nodal spread, and stage IV involves metastatic spread beyond the local regional lymph nodes (11). In stage I and II disease, tumoural characteristics such as Breslow thickness, ulceration and mitotic rate of the primary tumour are highly prognostic. In stage III disease, the number of lymph nodes involved correlates with prognosis (25, 26). The detection of lymph node involvement clinically or radiologically confers a worse prognosis compared to those with microscopic lymph node involvement detected by lymph node dissection alone.

Since the 1990s, sentinel lymph node biopsy has enabled accurate assessment of lymphatic involvement (27, 28). For intermediate Breslow thickness melanoma (1-4mm), the chance of locoregional spread is high, whereas the chance of metastatic spread is low (<20%) (29). Patients with locoregional nodal spread have a 50-60% chance of developing systemic relapse. Accurate staging and surveillance in these patients with high risk disease enables early detection of locoregional relapse which is still amenable to surgical intervention. Advances in diagnostic imaging technologies such as computerised tomography (CT) or functional imaging with positron emission tomography (PET) have enabled more accurate staging, with the detection of otherwise occult metastatic disease. Patients with metastatic disease can then receive systemic treatments, rather than surgery alone.

Advanced melanoma is further categorised into subgroups, depending on the sites of metastatic involvement (11). In the AJCC 7th edition (11, 30), stage IV melanoma was sub-classified into 3 groups depending on the sites of metastatic involvement: with M1a, involving cutaneous, subcutaneous or nodal spread; M1b, pulmonary metastases or M1c, visceral metastases outside of the lungs or if the serum lactate dehydrogenase (LDH) level is elevated. The site of metastatic involvement is important prognostically, with M1a being associated with the best prognosis among those with metastatic disease, followed by those with pulmonary involvement, then finally the worst prognosis is associated with involvement of extra-pulmonary involvement (11). LDH is well recognised to be a poor prognostic indicator in multiple cancers including melanoma (26, 31, 32). In the AJCC 7th edition, patients with elevated LDH at the time of diagnosis were assigned stage M1c, regardless of site of metastatic involvement (11). These staging criteria were updated in 2018 in the AJCC 8th edition (25), where the M1d substage was added to denote the presence of intracranial metastases. Elevated LDH has been modified so that it is no longer grouped together as M1c, but instead is added as a suffix of 1 to each of the substages to denote this additional factor of poor prognosis, i.e. M1a(1), M1b(1), M1c(1) and M1d(1).

Table 1.1 Comparison of American Joint Committee on Cancer 7th and 8th Edition of Tumour Node Metastasis (TNM) staging system of melanoma

	AJCC (7th Edition)
T	Primary Tumour (T)
Tis	NA
T1(a/b)	≤ 1.00mm (+/- ulceration or mitoses ≥ 1/mm ²)
T2(a/b)	1.01-2.00mm (+/- ulceration)
T3(a/b)	2.01-4.00mm (+/- ulceration)
T4(a/b)	> 4.00mm (+/- ulceration)
N	Regional Lymph Node (N)
N0	0
N1(a/b)	1 (micro/macrometastasis)
N2(a/b)	2-3 (micro/macrometastasis)
N2c	in-transit disease without nodes
N3	≥ 4 metastatic nodes, or matted nodes, or in-transit disease with nodes
M	Distant Metastasis (M)
M0	No distant metastasis
M1a	Distant skin, subcutaneous, or nodal metastasis only
M1b	Lung metastasis
M1c	All other visceral metastasis or any distant metastasis with elevated LDH

	AJCC (8th Edition)
T	Primary Tumour (T)
Tis	NA
T1a	< 0.8mm (- ulceration)
T1b	< 0.8mm (+ ulceration) 0.8-1.0mm (+/- ulceration)
T2(a/b)	1.01-2.00mm (+/- ulceration)
T3(a/b)	2.01-4.00mm (+/- ulceration)
T4(a/b)	> 4.00mm (+/- ulceration)
N	Regional Lymph Node (N)
N0	0
N1(a/b)	1 (micro/macrometastasis)
N1c	In-transit, satellite and/or micrometastases without nodes
N2(a/b)	2-3 (micro/macrometastasis)
N2c	in-transit, satellite and/or micrometastases with one node
N3	≥ 4 metastatic nodes, or matted nodes, or in-transit disease with nodes
M	Distant Metastasis (M)
M0	No distant metastasis
M1a (0/1)	Distant skin, subcutaneous, or nodal metastasis only (-/+ elevated LDH)
M1b (0/1)	Lung metastasis (-/+ elevated LDH)
M1c (0/1)	All other visceral metastasis or any distant metastasis with elevated LDH (-/+ elevated LDH)
M1d (0/1)	Central nervous system metastasis (-/+ elevated LDH)

Adapted from Balch et al (11) and Gershenwald et al (25)

For distant disease, the main changes include the new M1d category for disease involvement in the central nervous system; as well as the addition of the suffix 1, to denote an elevated lactate dehydrogenase (LDH) at diagnosis.

1.1.4 Melanoma immunobiology

The importance of the interaction between melanoma and the immune system has long been recognised, where tumour infiltrating lymphocytes have been associated with good prognosis in a number of cancer types including melanoma, colorectal cancer and triple negative breast cancer (33, 34). Specifically, the presence of tumour infiltrating lymphocytes in early stage melanoma has been associated with improved prognosis (35-37). Attempts to further define the immune subset were made in a study of 47 patients with Stage I and II melanoma (AJCC 7th edition) (38). In this study, the presence and density of CD8 lymphocytes as well as occurrence of HLA-DR cells were independently associated with overall survival; CD4 lymphocytes and CD68 cells, however, were not independently prognostic of survival (38). Unfortunately, this study was limited by the technology of the time and CD4 and CD68 macrophages not able to be enumerated. The density of peri-tumoural dendritic cells was also identified as significant for survival in a study of 82 cutaneous melanoma (39). There have also been conflicting studies reporting on the prognostic significance of FOXP3⁺ regulatory T cells, where some studies showed no association with prognosis(40), whereas others showed an association with poor prognosis (41, 42). Lastly, CD68 macrophages at the invasive margin have been found to be independently associated with poor prognosis in 227 AJCC 7th edition Stage I and II melanomas (43).

Functional studies of the immune system have also been performed using genomic profiling techniques. In the aforementioned TCGA study of melanoma, approximately 67 (20%) of the 333 cutaneous melanoma specimens were primary melanomas (19). Unsupervised hierarchical clustering analysis of 1500 genes in these samples revealed three signatures: immune, keratin and MITF-low. Good prognosis was observed in patients with regionally metastatic disease who exhibited the immune signature. A proposed lymphocyte score (LS), a semi-quantitative measure of lymphocytes in a specimen was also associated with improved survival. LCK and SYK, two immune related proteins involved in T cell and B cell signalling respectively were also examined using reverse-phase protein array. High LCK levels, but not SYK levels, were found to be positively associated with survival. There was considerable overlap between the immune signature, high LS and LCK expression.

The Lund study was another large study to report on the transcriptomic immune profile of melanoma(44). In this study, 57 stage IV melanoma samples into 4 molecular classes: high immune response, proliferative, pigmentation and normal-like subtype. The high immune response subtype was associated with the best prognosis, and was externally validated in 44 stage III/IV patients. The high immune response category was associated with high CD3 density and high expression of 30 immune related genes. These molecular categories were later simplified into 2 categories: low grade and high grade forms of melanoma and validated in early stage disease (45). In this study 210 cutaneous melanomas were studied, with the same 4 classes confirmed to be preserved in the early stages of disease. Given the good survival associated with the high immune and normal-like classes, these were grouped into a low grade- subgroup, whereas the proliferative and pigmentation subclasses were grouped into a high grade- subgroup. This low grade- and high grade- classification was subsequently validated in an independent study of 224 primaries and 76 secondaries, where this classification was confirmed to be prognostic independent of tumour thickness, ulceration status and mitotic rate (46).

These seminal studies emphasised the importance of an immune signature on melanoma prognosis, and signalled the immune system as a potential therapeutic target.

1.1.5 Overview of treatment

The introduction of effective systemic therapies over the past decade has resulted in unprecedented improvements in survival and quality of life for patients with metastatic melanoma. Melanoma is exemplary of the possibilities that can be achieved by rapid translation of basic science into the clinic. Equally, the expeditious entry of these novel therapeutics into the clinic has raised many unanswered questions, including how to identify the subset of patients who will derive durable benefits from these treatments.

The treatment of melanoma has paved the way for other cancer types and revolutionised therapeutic strategies for combating cancer, specifically by going beyond surgery, cytotoxic chemotherapies and radiation. Instead, the new cornerstones of melanoma treatment are based on inhibition of driver mutations within the tumour which enable its growth, as well as reinvigoration of the exhausted immune system for tumour killing. As testament to the scientific advances and perseverance of both patients and clinical trial investigators, the 5-year survival of metastatic melanoma has risen astoundingly

from less than 10% to over 50% in the most recent updates of immunotherapy studies (11, 47). The treatment of metastatic melanoma will be summarised below.

Before the advent of effective systemic treatments, surgery for resectable metastatic disease was a mainstay of treatment. Surgical series report durable survival in selected patients, and metastatectomy was offered to patients where it was technically feasible despite the lack of randomised studies (48-51). Palliative radiation treatment was also used for symptomatic disease. Since the introduction of systemic therapies with proven survival benefit, these local modalities are reserved for treatment of refractory disease, or are being evaluated in conjunction with immunotherapy in attempt to stimulate an abscopal response in the case of radiation (52-55).

Cytotoxic chemotherapies have not been shown to improve overall survival (OS) in randomised studies. Dacarbazine (DTIC), an alkylating chemotherapy, approved in the 1970s, was an option as a palliative treatment, with a treatment response rate (mainly partial response) of approximately 15% (56). However, DTIC has not been evaluated against best supportive care, and made little difference to overall survival in randomised studies compared with other agents (56-59). Variations in dosing regimens of DTIC or the addition of combination cytotoxic chemotherapy, hormonal or immunotherapy to DTIC failed to improve survival despite considerable toxicity (60-64).

Temozolomide is an oral preparation of another alkylating agent which, like DTIC, is a prodrug of the active methyl triazeno imidazole carboxamide, MTIC. The distinct advantage of temozolomide was its ability to penetrate the central nervous system (CNS), which is important as CNS metastases affect about one third of patients with metastatic melanoma (1, 65). Temozolomide was confirmed to have equal efficacy as DTIC in a phase III randomised study (66). Even though the progression-free survival (PFS) was longer with temozolomide compared to DTIC (1.9 vs. 1.5 months, $p=0.012$), this did not translate into an overall survival benefit: median OS 7.7 months with temozolomide compared with 6.4 months with DTIC (Hazard Ratio, HR 1.18, 95% CI 0.92-1.52). Other chemotherapy regimens such as fotemustine or carboplatin with paclitaxel also failed to demonstrate any survival benefit over DTIC (67, 68).

As a result of the lack of effective systemic treatments, enrolment into clinical trials remained the recommended standard of care treatment for metastatic melanoma even in the late 2000s (69, 70). Subsequently, there was a major breakthrough in the treatment

of melanoma, and indeed in the treatment of cancer in general, which involved the targeting of specific mutations within the tumour with small molecular inhibitors. In melanoma, this involves the use of BRAF and MEK inhibitors to target the Mitogen Activated Protein Kinase (MAPK) pathway, which controls cell cycling, response to growth stimuli, motility and cellular survival (71-73).

Approximately 35-50% of metastatic melanomas harbour a BRAF mutation, which results in constitutive activation of the MAPK pathway; this in turn drives cellular proliferation, melanoma growth and metastasis (74). Most BRAF mutations involve a substitution of valine with glutamic acid at the 600 codon (V600E) or valine with lysine (V600K), or less commonly, other mutations at V600 or surrounding amino acids. The V600 mutations can be targeted by oral BRAF inhibitors such as vemurafenib, dabrafenib or encorafenib. Other mutations in this gene are variably sensitive to these agents or MEK inhibitors such as cobimetinib, trametinib or binimetinib; whereas some mutations are entirely resistant (75-77). BRIM3, a phase III clinical trial in BRAF-V600E mutant melanoma, showed that vemurafenib significantly improved PFS and OS when compared to chemotherapy using DTIC (78). The BRAF inhibitor, dabrafenib, also significantly increased PFS compared to dacarbazine (79). Encorafenib is another BRAF inhibitor with a longer dissociation half-life than vemurafenib or dabrafenib, and hence was proposed to have more sustained target inhibition (80). BRAF inhibitor monotherapy is associated with response rates of approximately 50% (78, 79, 81).

Despite extremely rapid and dramatic responses to BRAF inhibitors, a series of secondary events including treatment driven mutations eventually lead to acquired resistance to therapy (82-84). The concurrent targeting of MEK can reduce or prevent resistance to BRAF inhibitors. MEK is immediately downstream of BRAF in the MAPK pathway and was identified as the gene target of several resistance mechanisms (82, 85). MEK inhibitors are routinely started in combination as a result of the following positive randomised studies. The COMBI-d study went on to show that the combination of dabrafenib and trametinib significantly improved both PFS and OS when compared to dabrafenib monotherapy (86). The phase III study of dabrafenib and trametinib compared to vemurafenib was stopped after a pre-planned interim analysis confirmed it had met its OS efficacy endpoint (87). A phase III study investigated vemurafenib in combination with cobimetinib or placebo and, again, confirmed an improvement in PFS with the combination (88). Encorafenib and binimetinib was the

latest combination to be evaluated against encorafenib monotherapy and vemurafenib monotherapy in a randomised phase III study (81). Dual inhibition by encorafenib and binimetinib showed improved PFS compared to encorafenib or vemurafenib monotherapy. Across the studies, the combination use of BRAF and MEK inhibitor was associated with a consistently higher response rate of approximately 70% compared to 50% with monotherapy with resultant improvements in PFS and OS.

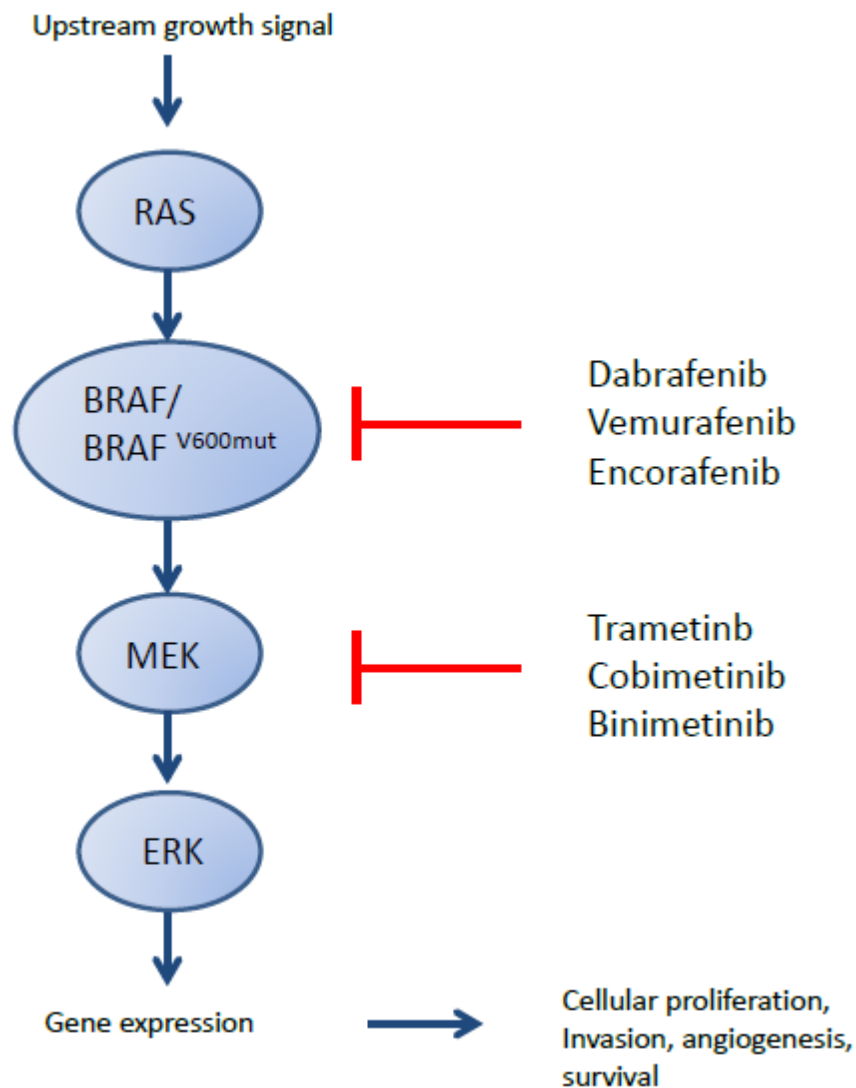


Figure 1.1. Modified schema of the mitogen-activated protein kinase (MAPK) pathway in melanoma

Aberrant MAPK signalling is present in 35-50% of melanoma, with constitutive activation of the RAS/RAF/MEK/ERK cascade. Approximately 80% of the mutations BRAF are valine to glutamic acid (V600E) substitution mutations, and approximately 16% are valine to lysine (V600K) substitutions. These mutations can be therapeutically blocked by combination BRAF and MEK inhibitors as shown.

Lastly, although BRAF inhibitor monotherapy is generally well tolerated, it can be associated with side effects which are dose limiting, including: fatigue, rash, arthralgia, photosensitivity or pyrexia. BRAF inhibition can cause paradoxical activation of the upstream RAS node, leading to the development of keratoacanthomas and squamous cell carcinomas (89, 90). Interestingly, the combination of BRAF and MEK inhibitors can reduce the incidence of these keratoacanthomas and squamous cell carcinomas (81, 89, 90). Dual targeting of BRAF and MEK is associated with reduced toxicity and improved efficacy compared to BRAF monotherapy and is therefore a standard of care treatment for patients with BRAF mutated melanoma. Despite a higher response rate to combination inhibition of BRAF and MEK compared to BRAF monotherapy, the median PFS is still only between 11-15 months with combination therapy across studies (81, 86-88). This highlights the need to identify resistance mechanisms and therapeutics that can induce more durable survival.

Molecular targeted therapies were developed simultaneously with immune checkpoint inhibitors (ICI), but these two classes of treatments have not yet been compared in randomised studies. Immunotherapy is an option for patients whose melanoma does not harbour a sensitive mutation or patients whose tumour has become resistant to BRAF and MEK inhibitors. Equally there is mounting evidence that MAPK targeted therapies modulate the tumour microenvironment with possible consequences for effectiveness of second line immunotherapy. The optimal sequencing of these treatments is an area of ongoing research and will not be explored here. There are ongoing clinical trials which aim to address this important issue: IMMU-TARGET (*NCT02902042*), EORTC 1612 (*NCT03235245*), and SECOMBIT (*NCT02631447*). Furthermore, there are studies evaluating the combination of targeted and immune therapies. Early studies of combined targeted therapy and immunotherapies were hampered by toxicity (91, 92), but potentially more tolerable combinations are being investigated (*TRILOGY*, *NCT02908672*; *COMBI-i*, *NCT02967692*). The details of the seminal clinical trials of immunotherapy will be reviewed below.

1.2 Immunotherapy

There was an early recognition of the importance of the immune system in melanoma. Spontaneous regression of metastatic melanoma was first described in 1866 and a literature review identified 75 additional reports of durable survival associated with this phenomenon (93). Furthermore, the risk of melanoma development is noted to be higher in the immunocompromised, such as patients with chronic lymphocytic leukaemia or HIV/AIDS or after organ transplantation (94-98). These observations led to attempts to modulate the immune system as a form of treatment for cancer. In 1898, Dr William Coley reported tumour regressions in a subset of patients with sarcoma whom he injected with bacterial vaccine (99). Since then, investigation into various methods of stimulating the immune system followed, with a number of studies evaluating the treatment of advanced melanoma with vaccines and cytokine therapies such as high dose interleukin 2 (IL2) and interferon. Consistently, there were reports of patients who had gained long term benefit from immunotherapy but this applied to only a very small subset of patients despite high toxicities.

In detail, high dose IL2 was evaluated in three randomised studies, and demonstrated an objective response rate (ORR) of 5-27% with a complete response rate of 0-11% (63, 100-102). Notably, in these studies, durable survival was observed in responders (ranging from 6-66 months, with a median OS of 27 months). A large number of non-comparative studies has since been conducted and showed an even more impressive survival in patients who obtained a complete response from the treatment (103). Exceptionally high rates of severe or life-threatening toxicity (Common Terminology Criteria for Adverse Events, CTCAE, grade 3 or 4) were seen throughout the studies, with nausea, vomiting, diarrhoea, cardiovascular shock, renal and hepatic impairment, as well as encephalopathy. Studies which reported rates of toxic deaths approximate this rate to be 4% with high dose IL2 (104). Given the substantial toxicity, high dose IL2 was a treatment option for only a very select group of patients with excellent performance status in specialised centres despite its tantalising durable responses.

Interferon alpha had an established role in the adjuvant setting but did not improve survival in the metastatic setting (105, 106). Response rate to single agent interferon-alpha was 22% but this did not translate to an improvement in survival (105). Several

types of toxicities were associated with interferon, namely flu-like illness (fatigue, pyrexia, myalgia, headache and arthralgia) as well as the more serious hepatitis, haematological toxicities, and debilitating fatigue or neuropsychiatric side effects. Pegylated interferon (PEG-IFN α -2a) showed better tolerability, with efficacy similar to that of interferon (106).

Early in the development of immune modulators, cancer vaccines appeared particularly exciting. Tumour specific vaccines were developed with tumoural responses observed, particularly in patients with an expanded clone of anti-tumour cytotoxic T cells (107, 108). Unfortunately, the utility of vaccines in clinical practice was limited by the inability to reproducibly elicit an anti-cancer immune response, as well as by the presence of inhibitory mechanisms within the tumour environment which were left unchecked (109). Other efforts to modulate the immune system to treat cancer include adoptive T cell transfer and injectable vaccines. Adoptive T cell transfer is the process by which genetically engineered autologous lymphocytes specific to tumour antigens are reinfused back into the patient, resulting in tumoural regression (110-113).

Injectable vaccines were also developed, such as subcutaneous treatments of BCG injected directly into melanotic lesions resulting in local control but not long term survival (114). More recently, cancer vaccines such as Talimogene laherparepvec (TVEC) have been evaluated. TVEC is a herpes simplex virus type 1 derived oncolytic immunotherapy designed to replicate within tumours and produce granulocyte macrophage colony-stimulating factor (GM-CSF). In the phase III OPTiM clinical study, TVEC injections resulted in an objective response rate of 26.4% with a durable response rate of 16.3% at 6 months for patients with unresectable stage III or stage IV melanoma(115). However, a limitation of percutaneous therapy is that it is applicable only to patients with cutaneous and subcutaneous metastases, which poses technical challenges in the metastatic setting but may be more appropriate as a neoadjuvant strategy.

While there are challenges in translating some of these therapies to the general patient with metastatic melanoma, this exciting approach of modulating the immune system as a cancer therapeutic set the scene for the development of ICI.

1.2.1 Immune Checkpoint Inhibitors (ICI)

The major paradigm shift in cancer immunotherapy was the advent of ICI, where the response rates were far higher and toxicity far more tolerable than with other immune based treatments. Since 2011, seven different ICI have been approved in 15 different malignancies (116). Understanding the mechanism of action of these agents could potentially enable the identification of those who are most likely to respond to treatment, as well as possible mechanisms of resistance to therapy.

Immune checkpoints are part of a normal physiological process whereby activated T cells are inhibited in order to regulate immune responses and prevent autoimmunity. Immune tolerance occurs both centrally at the thymus, where high affinity self-reactive clones are eliminated, as well as peripherally, mediated by regulatory T cells, T-cell anergy, cell-extrinsic tolerogenic signals and peripheral clonal deletion (117). During tumour progression, there is tumour immunoediting, where tumours selectively develop mechanisms to evade detection by the immune system (118). In the final stage of tumour escape, tumours can utilise complex mechanisms to evade the immune system, such as the upregulation of inhibitory checkpoints, recruitment of regulatory T cells or myeloid cellular subsets into the tumour microenvironment, as well as production of immunosuppressive cytokines such as TGF-beta and IL10 (119-121).

The crucial steps of T cell activation have been nicely summarised in the cancer-immunity cycle (122). Three major signals are required for the activation of T cells, namely: 1) Recognition of antigen between major histocompatibility complexes (MHC) class 1 on antigen presenting cells and T Cell Receptor (TCR) on T cells; 2) Co-stimulation signals and 3) Inflammatory cytokines. Cancer neoantigen specific T cells initially undergo clonal selection, with subsequent priming and activation following T cell recognition of corresponding antigens on MHC expressed by antigen presenting cells. For full activation, a costimulatory signal is required between antigen-presenting cells and T cells (between B7 and CD28). After activation and proliferation, T cells are trafficked to specific sites mediated by cytokines control. In order to achieve tumour killing, effector T cells release interferon gamma and other cytotoxic cytokines upon encountering cognate antigens on MHC. The durability of immune based therapy is thought to be attributed to the formation of memory T cells, which can remain quiescent until antigen re-exposure.

Upon T cell activation, negative feedback mechanisms (via checkpoints) are initiated in order to regulate the inflammatory response. CTLA4 was the first immune checkpoint to be characterised in 1987, and is upregulated upon T-cell engagement (123). CTLA4 was subsequently discovered to competitively bind to B7 on antigen presenting cells, thereby preventing the costimulatory effects of CD28 (which is mediated by PI3K and AKT) (124). CTLA4 primarily functions to regulate T cell activity at sites of T cell priming (e.g. secondary lymphoid organs) but it is also active in peripheral tissues (117). In addition to its modulatory activity on effector T cells, CTLA4 is also crucial to the function of regulatory T cells (Tregs) (125, 126). Specific loss of CTLA4 on Tregs can induce aberrant T cell activation, resulting in autoimmunity (127). CTLA4 can also limit the availability of B7 ligands through transendocytosis of B7 ligands from antigen presenting cells (APC) (128).

Programmed Death-1 (PD-1) and its ligands, Programmed Death-Ligand1 (PD-L1) and Programmed Death-Ligand 2 (PD-L2), were subsequently discovered (129). The main function of PD-1 is to maintain peripheral tolerance by inhibiting the effector phase of T cells. PD-1 is expressed on T and B cells upon activation (130). Its ligands PD-L1 and PD-L2 are expressed widely on peripheral tissues, and are inducible in response to inflammatory cytokines such as interferon (131, 132). Within the tumour microenvironment, tumour cells and immune cells can express PD-L1 which attenuate T cell responses by inhibiting proliferation and cytokine production, as well as promoting T-cell apoptosis; with resultant immune evasion by the tumour (132-135). The two main mechanisms that regulate PD-L1 expression on tumour cells are 'adaptive immune resistance' and 'innate immune resistance' (136). Adaptive immune resistance is the inflammation/interferon dependent pathway in which immune cells induce PD-L1 expression on tumour or surrounding immune cells (137, 138). Innate immune resistance describes the upregulation of PD-L1 by tumour which can result from constitutive PD-L1 (*CD274*) gene amplification or aberrant activation of oncogenic signalling pathways such as JAK/STAT, c-jun/STAT3 or PI3K pathways (139-142).

Crucial scientific discoveries pertaining to these immune checkpoints have enabled the development of immune checkpoint inhibitors which have been transformative in the treatment of cancer. At the time of writing, seven ICI have Food and Drug Administration approval in USA: one inhibitor of CTLA4 (ipilimumab), three inhibitors of PD-1 (pembrolizumab, nivolumab, and cemiplimab) and three inhibitors of PD-L1

(atezolizumab, durvalumab, and avelumab) (116). In the treatment of advanced melanoma, the anti-CTLA4 (ipilimumab) and the two anti-PD-1 agents (pembrolizumab or nivolumab) are available as monotherapy or in combination (ipilimumab and nivolumab combination). The key proposed mechanisms of action of anti-CTLA4 and anti-PD-1 will be reviewed below, followed by a summary of the seminal clinical trials for ICI in melanoma. An understanding of the underlying mechanisms of action for these novel treatments may help identify possible biomarkers to predict the subset of patients who are more likely to respond.

Essentially, the blockade of immune checkpoints allows for attenuation of the normal regulatory control mechanisms, thereby allowing an unrestrained enhancement of T cell activation and tumour killing. However, several additional mechanisms of action have also been proposed. Firstly, regarding anti-CTLA4, these work primarily within tumour-draining lymph nodes, and sterically inhibit CTLA4 in their competition for B7-1 and B7-2 costimulatory ligands, which enables unrestrained CD28-mediated co-stimulation of T cell activation (143). As a result, APCs are able to cross-present tumour antigens to prime T cells. Anti-CTLA4 also leads to an expansion of neoantigen-specific CD8 T cells as well as a PD-1⁺ICOS⁺TBET⁺ Th1- like CD4 effectors (similar to T follicular helper cells) within the tumour microenvironment (144, 145). This expansion of ICOS⁺ CD4 effectors has also been observed in patients treated with ipilimumab in a number of different cancer types including melanoma (146-148). Anti-CTLA4 also depletes Tregs within the tumour, which are known to be immunosuppressive (149-152). Antibody-dependent cellular cytotoxicity mediated depletion of Tregs has also been proposed, i.e. Fc mediated cell depletion of Tregs by monocytes, and this correlated with increased response to ipilimumab (153, 154). Lastly, remodelling and broadening of the peripheral TCR repertoire has been observed after anti-CTLA4, resulting in the increase of the functional reactivity of peripheral CD8 T cells for melanoma antigens (155, 156). Multiple distinct mechanisms contribute to the antitumour effects of anti-CTLA4, but questions remain about the relative contributions of these mechanisms and whether murine models, in which these mechanisms have been identified, are representative of what occurs in humans.

The exact mechanisms of action for PD-1 blockade are not well elucidated despite extensive research (117). Anti-PD-1 is thought to cause reinvigoration of CD8 T cells, with resultant increase in not only the number of CD8 T cells but also their functional

activity. Anti-PD-1 leads to inhibition of the PD-1 mediated attenuation of proximal TCR signalling, allowing for exhausted T cells to be restored. Correspondingly, responders to anti-PD-1 are observed to have a pre-existing population of CD8 effector cells within the tumour microenvironment (157, 158). However, the fact that responses are observed in PD-L1-negative tumours suggests that a pre-existing immune infiltrate is not absolutely necessary, and that new populations of effector T cells can be recruited. There is both clinical and preclinical evidence that points to an expansion of CD8 T cells following anti-PD-1; a novel T cell subset of CXCR5+ PD-1+CD8 T cells was also seen after anti-PD-1 in a chronic viral model (159, 160). More recently, intensive research has revealed the additional contribution of other immune cell types in anti-PD-1 treatment, such as CD4, B cells and myeloid subsets (161-165). While this is not surprising given the importance of these other cellular subsets in general immunity, this recent focus away from CD8 effector cells opens the door to additional mechanisms by which anti-PD-1 exerts its anti-tumour effects; particularly the mechanisms that underpin the CD8 T cell recruitment and generation of immune memory (163, 164). Furthermore, these observations may lead to the development of a suite of novel therapeutics targeting these immune cell types either as monotherapy or in combination with anti-PD-1 or anti-CTLA4. Similarly, detailed characterisation of the mechanisms of action for these agents also facilitates the identification of likely responders as well as mechanisms of resistance.

1.2.2 Summary of clinical trials for ICI in advanced melanoma

There are currently two immune checkpoints targeted for advanced melanoma, namely anti-CTLA4 and anti-programmed death 1 (anti-PD-1) monoclonal antibodies (166). The details of the key phase III studies are reviewed in Table 1.2.

Ipilimumab, a monoclonal antibody targeting CTLA4, was the first systemic treatment to demonstrate improved overall survival in a randomised study of patients with advanced melanoma (166). In this study, ipilimumab monotherapy and ipilimumab with gp100 peptide vaccine both showed a prolonged overall survival compared to the gp100 vaccine in previously treated patients. Notably, this survival benefit has been maintained with subsequent follow-up, with durable survival observed in 20% of patients. Two dosages of ipilimumab (10mg/kg vs. 3mg/kg) were then evaluated in a randomised study of treatment-naïve patients, with 10mg/kg being associated with an

improved OS compared to 3mg/kg (167). However, this higher dose was also associated with higher rates of toxicity (34% compared to 18% respectively). Ipilimumab is no longer used as monotherapy in the first line setting, but the 3mg/kg dose is used in standard practice (168).

In a pooled analysis of phase II and phase III studies of anti-CTLA4 (1861 patients in 10 prospective and two retrospective studies), the median OS was 11.4 months (95% CI, 10.7 to 12.1 months). The survival curve began to plateau around year 3, with follow-up of up to 10 years. Three-year survival rates were 22%, 26%, and 20% for all patients, treatment-naïve patients, and previously treated patients, respectively (169). This study highlights that while a durable benefit is observed in a subset of patients, the majority of patients did not respond.

Ipilimumab monotherapy is no longer the standard of care treatment, owing to the superior efficacy and reduced toxicity of anti-PD-1 agents (170). Two anti-PD-1 monoclonal antibodies, pembrolizumab and nivolumab, have been extensively studied in advanced melanoma. Both agents have shown durable responses of 30-40% in phase III clinical studies of both treatment-naïve and previously treated patients. The longest follow-up of survival data come from the phase 1 studies, with an impressive 5 year PFS of 21% and 5 year OS of 34% seen with pembrolizumab (171).

CHECKMATE-066, the phase III clinical trial, compared nivolumab to DTIC, and showed that 73% of patients were alive at one year with an objective response rate of 40% following nivolumab (172). This study showed an improvement in overall survival (median OS 38 months with nivolumab v 11 months with DTIC, HR 0.46) with 3 year survival of 51% v 22% respectively (173). KEYNOTE-006 was the seminal study to show an improved OS of pembrolizumab against ipilimumab monotherapy, and anti-PD-1 has replaced ipilimumab monotherapy as the standard of care treatment in the first line setting (174). The most recent update of this study reported an improvement of median OS of 32.7 months with pembrolizumab cohorts compared with 15.9 months in the ipilimumab cohort (HR 0.73) and 5 year survivals of 39% and 31% respectively (175). CHECKMATE-067 is a phase III study which compared combination nivolumab and ipilimumab with monotherapy of either agent, where the combination showed a further increase in response rate (ORR 58% combination, 44% nivolumab, 19% ipilimumab) (176). This has translated to an impressive 5 year OS of 54% in

patients treated with the combination, which is the highest of any agent evaluated in a phase III ICI study of metastatic melanoma to date (177).

These exciting responses seen with immunotherapy are tempered by immune related toxicity as well as significant financial cost (166, 178, 179). Generally ipilimumab is associated with severe (CTCAE grade 3-4) adverse events in 15-20% of patients, whereas anti-PD-1 have lower toxicity around 10-15% (172, 174). The combination ipilimumab and nivolumab has the highest toxicity yet, with grade 3-4 adverse events of 55% of patients treated with the combination compared with, 16% of patients treated with nivolumab and 27% of patients treated with ipilimumab (176). With longer follow up, the toxicity rates in the combination and anti-PD-1 arms are higher (59% in the combination, 23% in nivolumab and 28% in ipilimumab), given the longer duration that patients remain on these efficacious therapies (177). Of note, patients who enter into clinical trials are of good baseline performance status and the toxicity of these treatments, especially the combination ICI, may be higher in real world practice (180). One academic institution presented its experience of combination ipilimumab and nivolumab, where 91% of the 64 patients treated experienced a clinically relevant adverse event and 35% of patients required hospitalisation during their treatment course (180).

Lastly, the concept of financial toxicity is increasingly recognised with cancer care and estimated to cost \$173 billion by 2020 in USA (181). The cost of cancer care is also rising in Australia: the annual PBS expenditure on anticancer drugs increased on average by 19% per annum, from \$65 million in 1999-2000 to \$466 million in 2011-2012 (182). Through the Australian publicly funded Pharmaceutical Benefit Scheme (PBS), the publicly listed dispensed price for maximum amount (per dose) in 2020 for ipilimumab is \$45762.26, \$10053.46 for nivolumab and \$8717.46 for pembrolizumab (183). One treatment course of ipilimumab involves four doses, while nivolumab and pembrolizumab are administered every two and three weeks respectively for as long as they remain effective. There have been efforts to reduce the frequency of administration of anti-PD-1 agents, owing to their long half-life and proposed comparability of pharmacokinetic properties of less frequent doses based on in-silico modelling, but the optimal duration of treatment remains unknown (184, 185). Hence, patients may possibly remain on these treatments for years, resulting in a huge financial burden both to the patient and to the health care system. There is an urgent need to

differentiate patients who are likely to respond from the non-responders. Such differentiation becomes critical in order to avoid futile treatments, unnecessary toxicity and wasted health resources.

Table 1.2. Randomised phase III studies of immune checkpoint inhibitors in advanced melanoma

Study	Line of therapy	Experimental arm (No. of patients, n)	ORR (%)	PFS median (months)	PFS 12m (%)	PFS 24m (%)	OS median	OS 12m (%)	OS 24m (%)	All AE %pts	G3/4 AE %pts	Pre-specified subgroup (HR)	Notes
ANTI-CTLA4													
Ipilimumab v Ipi+gp100 v gp100 (166)	2L (prior chemotherapy or IL2)	3:1:1 Ipilimumab + gp100 vaccine (403), Ipilimumab + placebo (137), gp100 + placebo (136)	5.7, 10.9, 1.5	2.76, 2.86, 2.76	NR, NR, NR	NR, NR, NR	10.0 (HR 0.68)^*, 10.0 (HR 0.66)^*, 6.4	43.6, 45.6, 25.3	21.6, 23.5, 13.7	88.9, 80.2, 78.8	17.4, 22.9, 11.4	LDH: Ipi Combo (<ULN 0.70 v >ULN 0.71); Ipi mono (<ULN 0.56 v >ULN 0.76);	
Ipilimumab + Dacarbazine v Ipi (186)	1L	1:1 Ipilimumab + Dacarbazine (250), Ipilimumab (252)	33.2, 30.2	NR, NR HR 0.76,	NR, NR	NR, NR	11.2, 9.1 (HR 0.72)^*	47.3, 36.3	28.5, 17.9 20.8, 12.2 (3y OS)	98.8, 94.0	56.3, 27.5	Efficacy seen in all subgroups: Age, sex, ECOG performance status, baseline serum lactate dehydrogenase level, and	

												substage of metastatic disease	
Ipilimumab 10mg/kg v 3mg/kg (167)	1L	1:1 Ipilimumab 10mg/kg (365), Ipilimumab 3mg/kg (362)	15.3, 12.2	2.8, 2.8	NR, NR	NR, NR	15.7, 11.5^*	54.3, 47.6	38.5, 31.0 31.2, 23.2 (3y OS)	79, 63^	34, 18	BRAF: Wt HR 0.92 v Mut HR0.65; ECOG 0 HR 0.80 v ECOG 1 HR 1.00; LDH <2ULN HR 0.84 v >ULN HR 0.97	Both ipi had declined EORTC QLQ-C30 until end treatment;EQ- 5D & ED-5D VAS decline only in 10mg/kg

Study	Eligibility	Experimental arm (No. of patients, n)	ORR (%)	PFS median (months)	PFS 12m (%)	PFS 24m (%)	OS median	OS 12m (%)	OS 24m (%)	All AE %pts	G3/4 AE %pts	Pre-specified subgroup (HR)	Notes
ANTI-PD-1													
KEYNOTE-006 (170, 174)	1L	1:1:1 Pembrolizumab q2w (279), Pembrolizumab q3w (277), Ipilimumab (278)	33.7, 32.9, 11.9	5.6, 4.1, 2.8 (both pembro v ipi, HR 0.58)^*	39, 38, 19	31, 28, 14	NR (HR 0.68)^*, NR (HR 0.68)^*, 16m	74, 68, 59	55, 55, 43	80, 73, 73	13.3, 10.1, 19.9 (inc G5)	BRAF: Wt HR 0.57 q2w, HR 0.66 q3w; Mut HR 0.67 q2w, HR 0.84 q3w; ECOG 1 HR, 0.71 q2w; HR, 0.60 q3w, ECOG 0 HR, 0.55 q2w; HR, 0.75 q3w; PDL+ HR, 0.55 q2w; HR, 0.58 q3w, PDL- HR, 0.91 q2w; HR,	

												1.02 q3w	
KEYNOTE-006: Updated analysis, Pembrolizumab Cohorts combined(175)	1L	1:1:1 Pembrolizumab q2w or q3w (combined analysis 556), Ipilimumab (278)	42, 17	8.4, 3.4 (HR 0.57)^*	NR, NR	23, 7 (48 m PFS)	32.7, 15.9 (HR 0.73)^*	NR, NR	39, 31 (5y OS)	80, 74	17, 20		Treatment for 24m on pembro. Analysis of pts receiving 2nd course pembro (n=103)

CHECKMATE 037 (187, 188)	2L after ipi	2:1 Nivolumab (272), ICC (Dacarbazine or carboplatin, paclitaxel; 133)	27, 10	3.1, 3.7 (HR 1.0)^	NR, NR	20, 4	15.7, 14.4 (HR 0.95)^	NR, NR	39, 34	68, 79	14, 34	ECOG 0 v 1: HR, 0.64 0.49 to 0.83; Brain mets no v yes: HR, 0.61; 95% CI, 0.45 to 0.83; LDH<ULN v >ULN HR, 0.60; 95% CI, 0.46 to 0.78 Higher ORR in higher PD- L1 for Nivo but no optimal threshold identified	Higher rates of cross over post study to antiPD-1 in ICC group (41% v 11%) Higher bad prognostic features in nivolumab group: Brain mets (20% v 14%) and raised LDH 52% v 38%)
CHECKMATE 066 (172, 173)	1L	BRAF Wildtype 1: 1 Nivolumab (210), Dacarbazine (208)	40, 14	5.1, 2.2 (HR 0.43)*	NR, NR	32, 3 (3y PFS)	38, 11 (HR 0.46)^*	73, 42	51, 22 (3y OS)	73, 76	12, 18	Nivolumab arms: PD-L1+ HR 0.30, PD-L1- HR 0.48 ECOG 0: HR 0.32, ECOG 1: HR 0.64	Post study therapy: ICI 36% Nivo v 51% DTIC ORR to 2nd line ipilimumab in Nivolumab arm was 10.3% (n=68)

													LDH: ≤ULN: HR 0.35, >ULN HR 0.41	
--	--	--	--	--	--	--	--	--	--	--	--	--	--	--

Study	Eligibility	Experimental arm (No. of patients, n)	ORR (%)	PFS median (months)	PFS 12m (%)	PFS 24m (%)	OS median	OS 12m (%)	OS 24m (%)	All AE %pts	G3/4 AE %pts	Pre-specified subgroup (HR)
Anti-CTLA4 and AntiPD-1												
CHECKMATE 067(176, 177, 189, 190)	1L	1:1:1 Combination Nivolumab + Ipilimumab (314), Nivolumab (316), Ipilimumab (315)	58, 44, 19	11.5 (HR 0.42)*, 6.9 (HR 0.53)*, 2.9	49, 42, 18	46, 39, 14	Not reached at 60, 37, 20 HR 0.54	74, 67, NR	59, 45, 64	96, 86 86	59, 23, 28	<u>4 year OS</u> PD-L1 ≥ 1%: Combo HR vs nivo, 0.92, Nivo HR vs ipi, 0.45; PD-L1 < 1%: Combo HR vs nivo, 0.68, Nivo HR vs ipi, 0.60 PD-L1 ≥ 5%: Combo HR vs nivo, 0.86, Nivo HR vs ipi, 0.43; PD-L1 < 5%: Combo HR vs nivo, 0.78, Nivo, HR vs ipi, 0.54 PD-L1 ≥ 10%: Combo HR vs nivo, 0.67, Nivo HR vs ipi, 0.48; PD-L1 < 10%: Combo HR vs nivo, 0.83, Nivo HR vs ipi, 0.52 BRAF mut: Combo HR vs nivo, 0.62, Nivo HR vs ipi, 0.73; BRAF wt:

												Combo HR vs nivo, 0.89, Nivo HR vs ipi, 0.47 LDH ≤ ULN: Combo HR vs nivo, 0.77, Nivo, HR vs ipi, 0.51; > ULN: Combo HR vs nivo, 0.78, Nivo, HR vs ipi, 0.59; > 2 × ULN: Combo HR vs nivo, 0.70, Nivo HR vs ipi, 0.57
--	--	--	--	--	--	--	--	--	--	--	--	--

Adapted from J. Luke (191). The response, toxicity rates and survival data with the longest follow-up are presented in this table.

^primary endpoint, *statistically significant; ORR, objective response rate; PFS, progression free survival; OS, overall survival; AE, drug related adverse event; G3/4, Common Terminology Criteria for Adverse Events Grade 3 or 4; 12m, 12 months; 24m, 24 months; pts, patients; HR, hazard ratio; L, line of therapy; NR, not reported; LDH, Lactate Dehydrogenase; ULN, upper limit of normal; Pembro, pembrolizumab; BRAF Wt, BRAF wildtype; BRAF Mut, BRAF Mutation; Brain mets, brain metastases; ECOG European Cooperative Oncology Group Performance Status.

1.2.3 ICI in non-melanoma skin cancers

As discussed previously, ICI are thought to be particularly effective in cancers with a high mutational burden. Mutational burden can arise from mutagens such as UV radiation or virally driven processes. Accordingly, ICI has also been highly efficacious in other cutaneous skin cancers such as Merkel Cell Carcinoma (MCC) and cutaneous squamous carcinoma (cSCC), and response rates to ICI in these two cancers exceed even those of melanoma (192-195). A deeper understanding of the immune landscape of these non-melanoma skin cancers may highlight potential immune processes that are differentially active in these cancers, and therefore reveal vulnerabilities for therapeutic targets that are disease-specific.

Merkel cell carcinoma

Merkel cell carcinoma is a rare but aggressive cutaneous neuroendocrine malignancy with a 5-year mortality rate three times higher than that of melanoma (46% vs 15% respectively) (196). MCC has a high chance of loco-regional recurrence and propensity to spread to lymph nodes and distant sites. As with melanoma, Australia and New Zealand have the highest incidence of MCC internationally, approximating 4 per 100,000 in men and 1.5 per 100,000 in women (197-199).

Risk factors for MCC include increased UV exposure and advanced age, with rates as high as 20 per 100,000 in those over 80 years of age (198, 200). Immunosuppression is another risk factor for MCC, for example in patients who are immunocompromised or are on immune-suppressants after solid organ transplants (201, 202). The understanding of the pathophysiology underpinning MCC has deepened since the discovery of the Merkel cell Polyomavirus (MCPyV) in 2008 (203). Internationally, MCC is associated with MCPvY in approximately 80% of cases. However, only 20% of cases are associated with MCPvY in Australia (198). Instead, MCC in Australia is more likely to be MCPyV negative and more associated with UV damage (204). MCPyV negative tumours are more likely to have a high mutation load (10 mutations/Mb in one series) and exhibit a UV related signature of C > T transitions, whereas MCPyV positive tumours have a low mutational burden (0.4 mutations/Mb) and are not associated with the UV related signature (205, 206). Given the association of UV related high mutational burden or its viral aetiology, immunotherapy was a rational therapeutic strategy for MCC (206).

Indeed, multiple phase II studies evaluating ICI have shown impressive results in patients with metastatic MCC. Prior to the era of immunotherapy, advanced MCC was treated with cytotoxic platinum-based chemotherapy. Despite a high initial response rate of 55% in first line treatment, the median PFS was only three months and OS was nine months (207). Second line treatment had an even lower response rate ranging from 9-23% and a PFS of 2 months (207-209). Anti-PD-L1/PD-1 treatments were initially trialled in patients with chemo-refractory disease. The first study was a phase II trial of avelumab (JAVELIN200 Part A) in 88 patients with metastatic MCC (194). The ORR in this study was 32%, and 82% of the responses were ongoing at the time of analysis (median follow-up of 10.4 months). The median PFS was 2.7 months with a 6-month PFS of 40%. In terms of overall survival, the 6-month OS was 69% with a median OS of 11.3 months. Grade 3 or 4 adverse events occurred in 11% of patients. Anti-PD-1 in the second line setting was also evaluated in a phase II study of nivolumab in patients with virally associated cancers (195). There were 25 patients with MCC included in this study, and responses were observed in treatment-naïve (71%) as well as previously treated patients (63%); the median PFS and OS was not reached in almost one year of follow up. The results of this study must be interpreted with caution, given the small number of patients and that it has only been presented in abstract form. Nonetheless, these two studies highlight the potential of targeting the PD-L1/PD-1 axis in the treatment of MCC.

Efficacy of anti-PD-1 in MCC in the first line setting was evaluated in a phase II study of pembrolizumab in 26 patients with metastatic MCC (192). In this study, the ORR was 56%, where 4/26 patients experienced a complete response and 10/26 patients had partial response. The 6 month PFS was 67%. 17/26 (65%) had MCPyV+ tumours, and the response rate was 62% in patients with MCPyV+ tumours and 44% in those with MCPyV- tumours. Grade 3 or 4 treatment related adverse events occurred in 15% of patients. This study was then extended to include 24 additional patients with updated follow-up confirming durable clinical benefit, with ORR of 56%, CR of 24%, PR of 32%. The median PFS in this expanded cohort was 16.8 months, with a 24-month PFS rate of 48% and 24-month OS of 69%. The viral status and PD-L1 status did not correlate with ORR, PFS or OS (210). A study of avelumab in the first line setting (JAVELIN200 Part B) also showed similarly encouraging results (193). The pre-planned interim results of this study of 119 patients showed an ORR of 62%, with

durable response of 83% in responding patients (193). Grade 3 toxicity was observed in 20% of patients, with no grade 4 toxicity observed. Investigations are already underway to evaluate antiPD-1 agents earlier in the disease course, such as the adjuvant (*ClinicalTrials.gov Identifier NCT02196961*) and neo-adjuvant setting (211).

Cutaneous squamous cell carcinoma (cSCC)

The epidemiological data for non-melanoma skin cancer (NMSC) are limited as these diagnoses are not captured in many cancer registries. CSCC accounts for 20% of NMSC and is the second most common type of NMSC after basal cell carcinoma (10). The age-standardised incidence of cSCC was estimated to be 387 cases per 100,000 in Australia based on survey data (10), whereas the incidence in NZ was been reported as high as 425 per 100,000 in some regions (212). Risk factors for cSCC development includes advanced age, chronic sun exposure and immunosuppression (213). Most cases of NMSC present in early stage disease and are associated with excellent survival with surgical treatment alone. Nonetheless, NMSC remains a significant burden on the healthcare system and accounted for the most common cancer related hospitalisations (25%) in 2016-2017 in Australia (9).

Metastatic cSCC has a poor prognosis measured in months, with few systemic options. Cytotoxic chemotherapies have been studied in small, non-randomised studies with heterogeneous patient populations though some responses were noted (214, 215). Two prospective phase II studies evaluated retinoids in combination with IFN α in unresectable cSCC and noted a response rate of approximately 20% in patients with metastatic disease (216, 217). Cemiplimab, an anti-PD-1 monoclonal antibody was evaluated in a prospective phase I/II study in immunocompetent patients with locally advanced or metastatic cSCC (218). An objective response was observed in 13 of 26 (50%) patients in the phase I cohort, and 28 of 59 (47%) patients in the phase II cohort. The median duration of follow up was short at 7.9 months, and the median PFS and OS were not reached. The duration of response exceeded 6 months in the 57% of the 28 patients with metastatic disease who responded. Forty-two percent of patients experienced a grade 3 or greater toxicity during the study. There were 11 deaths noted, with 3 deaths related to adverse events. Other studies evaluating pembrolizumab and nivolumab in recurrent or metastatic cSCC are underway (*NCT03284424 and NCT03834233 respectively*).

Locally advanced cSCC is also associated with significant morbidity and poor survival (219). Patients are often treated with repeated surgical procedures associated with significant morbidity. Systemic options are also limited, with EGFR inhibitors being the most effective, albeit only with response rates of 10-30% and short duration of response of approximately 6 months in prospective studies (220-224). More recently the use of cemiplimab in locally advanced cSCC was evaluated in a phase II study (225). Complete responses were observed in 10 (13%) of 78 patients and partial responses were observed in 24 (31%) patients, with 58% of patients alive with no evidence of progression at 12 months. Grade 3-4 toxicity was experienced by 47% of patients with one case of treatment-related aspiration pneumonia reported. Approximately 60% of patients in this study had pre-treatment tissue available for exploratory biomarker analysis, and responses were observed regardless of PD-L1 Tumour Proportion Score (PD-L1 TPS) expression or tumour mutation burden (225).

As with melanoma and MCC, there is interest in evaluating the role of ICI in early high risk cSCC. Phase III clinical trials evaluating cemiplimab and pembrolizumab as an adjuvant treatment after surgery and radiation therapy in patients with high-risk cSCC are also being undertaken but have yet to be reported (*NCT03969004 and NCT03833167*).

Summary

Over the past decade, ICI have proven to be highly efficacious not only in metastatic melanoma, but also in other non-melanoma skin cancers. There is also emerging data about the efficacy of ICI in early stage disease, where adjuvant or neo-adjuvant therapies may result in improved cure rates in high risk loco-regional disease. Improving the understanding of the immune processes at play across skin cancers is crucial in the discovery of biomarkers that can aid rational therapeutic decision making for ICI.

1.2.4 ICI future directions

Combination ICI

Since 2006, there has been an explosion of immuno-oncology clinical trials with 2250 clinical trials registered up until 2018 (226). Seventy-six percent of these clinical trials are evaluating an anti-PD-1 or anti-PD-L1 based combination. There is great interest in the investigation of ICI combinations given that the majority of patients do not respond to ICI monotherapy. The studies combine anti-PD-1/anti-PD-L1 with several other agents, including inhibitors of other checkpoints, as well as chemotherapy, radiation, small molecular inhibitors and injectable vaccines. Although combination immunotherapy may improve response rates, this may come at the cost of higher toxicity. Improved understanding of predictors of response and mechanisms of resistance may help to personalise the type of immunotherapy that is required to gain durable benefits for individual patients, as well as avoiding futile and toxic treatments.

ICI in early stage melanoma

Given the success of ICI in metastatic melanoma, there is significant interest in introducing these agents earlier in the disease process, in an effort to improve the chance of cure. Stage III melanoma is associated with a recurrence rate of 40-60%, and historic treatments such as adjuvant interferon alfa-2b or adjuvant pegylated interferon were associated with only marginal benefits despite significant toxicity (227, 228). Ipilimumab 10mg/kg was the first ICI to be evaluated against placebo in the EORTC 18071 study (229). While this study showed an improvement in recurrence-free and overall survival (HR 0.72) with ipilimumab compared to placebo, the treatment was toxic (41.6% grade 3/4 immune related adverse event) and associated with a treatment related death rate of 1%, hence limiting its uptake in clinical practice (229). Two phase III clinical trials then evaluated anti-PD-1 agents in the adjuvant setting in 2017 (230). In the CHECKMATE 238 study, patients with Stage IIIB, IIIC or resected Stage IV melanoma were randomised to adjuvant nivolumab or ipilimumab (230). Nivolumab was associated with improved relapse free survival and lower toxicity compared to ipilimumab (HR 0.65 relapse or death, $p < 0.001$). Grade 3 or 4 adverse events were experienced by 14.4% of patients treated with nivolumab and 45.9% of patients treated with ipilimumab. In the KEYNOTE-054 study, pembrolizumab showed an improvement in relapse free survival compared to placebo in patients with Stage IIIB or

IIIC melanoma (HR 0.57 relapse or death, $p < 0.001$). Grade 3 to 5 adverse events were observed in 14.7% of patients treated with pembrolizumab, with one death related to pembrolizumab induced myositis (231). Longer follow up of these studies is eagerly awaited to understand whether an improvement in relapse-free survival will be translated to an improvement in overall survival. There are also other studies exploring the use of these therapies in stage II melanoma as well as the neo-adjuvant setting. Given the shift of therapy into adjuvant and neo-adjuvant setting, there is a need to further our understanding of the tumour immune microenvironment of early stage disease, particularly as ICI biomarker discovery to date has been focused largely on metastatic disease.

1.3 Biomarkers

There is a great need for biomarkers to identify likely responders, given that only a subset of patients with metastatic melanoma respond to ICI, coupled with the possibility of severe immune-related toxicity and financial burden. A biomarker can be defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (232). There are prognostic and predictive biomarkers, where prognostic biomarkers reflect the natural history of untreated patients with that characteristic, whereas predictive biomarkers identify the subpopulation of patients who will respond to a therapy (233). An example of a *prognostic* marker is elevated lactate dehydrogenase (LDH), where the prognosis of patients with an elevated LDH is worse than that of patients with a normal LDH. Hence the incorporation of LDH as part of the AJCC criteria for stage IV melanoma (25). In contrast, BRAF mutations are a *predictive* marker, as patients whose tumour harbours BRAFV600 are likely to respond to BRAF targeted therapies, whereas patients without the BRAFV600 mutation do not generally respond.

Despite intensive research over the past decade into possible predictive biomarkers for immunotherapy in advanced melanoma, very few have been adopted into clinical practice. Biomarker discovery can entail a number of challenges including the technical challenges of biomarker testing, validation of biomarkers as well as their implementation, resulting in the low translation rate (0.1%) of biomarkers into clinical practice (234). More comprehensive methods of characterising the patient’s immune status as well as the immune profile of the tumour microenvironment have been proposed but these biomarkers are yet to be incorporated into routine practice (Table 2) (235, 236). The lack of standardization of biomarker measurements, the invasive nature of tumoural biopsies and the technology required, are some of the limitations. Below is a literature review of biomarkers for melanoma immunotherapy. Since an immense amount of research has been published in the last decade, only seminal studies will be reviewed. Table 1.3 shows a summary of the key biomarkers for anti-CTLA4 and anti-PD-1.

1.3.1 Clinical biomarkers

Clinical biomarkers are patient characteristics or basic laboratory tests already available in clinical practice, that can be prognostic or predictive of response to ICI. Certain well recognised prognostic biomarkers are identified in patients with metastatic melanoma. The most important clinical factors are already incorporated into the AJCC staging criteria, including stage of disease at diagnosis with its division into metastatic substages (i.e. different sites of metastatic involvement), as well as presence of elevated baseline LDH (25). Some basic laboratory tests have also been associated with responses or improved responses to ICI. This first section on biomarkers will summarise the clinical characteristics and routine blood tests that are available in clinical practice.

Sites of metastatic involvement

As mentioned previously, the specific sites of metastatic involvement have different prognostic significance as described by the AJCC criteria (11, 25). Of the metastatic sites, cutaneous and nodal metastases have the best prognosis, followed by lung, then non-pulmonary metastases. Central nervous systemic metastases with involvement of brain or leptomeningeal have the worse prognosis.

Some studies have analysed the specific sites of metastatic involvement and their prognostic significance after ICI. Recently, a retrospective analysis of the 5 year survival data for the phase 1 study of nivolumab in patients with metastatic melanoma (107 patients, 39.6% of study), metastatic non-small cell lung cancer (129 patients, 47.8%), and metastatic renal cancer (34 patients, 12.6%) was published (237). This study found that patients with liver and bone involvement were both associated with a lower likelihood of 5 year survival: odds ratio (OR) 0.31, $p = 0.02$ and OR 0.31, $p = 0.04$ respectively, whereas good patient baseline performance status (ECOG 0) was associated with higher likelihood of survival, OR 2.74, $p = 0.003$. The presence of liver metastases in patients with melanoma was evaluated in further detail in another large retrospective analysis, where liver involvement was correlated with low tumoural CD8 and poorer PFS (238). This study included a discovery cohort of 223 patients treated in KEYNOTE-001 and a validation cohort of 113 patients treated in KEYNOTE-002, KEYNOTE-006 and expanded access programs (EAP), as well as a NSCLC comparison cohort. Within the melanoma cohort: female gender, elevated LDH, ECOG

> 0, and the presence of liver metastasis were significantly associated with outcome in multi-variate analysis. Liver metastasis was associated with reduced objective response rate and shortened PFS: ORR 30.6%; median PFS, 5.1 months in those with liver involvement, compared with patients without liver metastasis (ORR, 56.3%; median PFS, 20.1 months, $p \leq 0.0001$). This finding was confirmed in the validation cohort ($p = 0.0006$). The presence of liver metastasis significantly increased the likelihood of progression (OR, 1.852; $p < 0.0001$). In a subset of biopsied patients ($n = 62$), liver metastasis was associated with reduced CD8+ T-cell density at the invasive tumour margin (patients with liver metastases, 547 ± 164.8 ; patients without liver metastases, $1,441 \pm 250.7$; $p < 0.016$). Similar findings have been shown in patients with BRAF mutated melanoma, where liver involvement was also associated with shorter PFS in a pooled analysis of 1365 patients treated with dacarbazine or BRAF and MEK inhibitors (239). Therefore, liver involvement remains prognostic in the ICI and targeted therapies era, with its correlation with low tumoural CD8 providing a possible mechanism for low responses observed with ICI.

Patients with CNS involvement have poor survival and have been largely excluded from the landmark randomised studies that have informed standard of care treatment in metastatic melanoma (172, 174, 176, 187, 240). There have been phase II studies assessing anti-CTLA4 but response rates were low and survival was poor (241). The evidence for anti-PD-1 in brain metastases was limited but retrospective series indicated that intracranial responses were observed in about 20% of patients (242). More recently, specific studies addressing this challenging clinical scenario have been undertaken (3, 4). An Australian multi-site randomised phase II study of combination nivolumab and ipilimumab compared to nivolumab monotherapy showed an impressive durable intracranial response rate of approximately 46% in the combination arm (3, 243). Similarly high responses were observed in another single arm phase II study of combination nivolumab and ipilimumab with intracranial response rate of 56% observed in small asymptomatic brain metastases (4). In terms of targeted therapies, combination dabrafenib and trametinib has been evaluated in a multi-cohort study of both symptomatic and asymptomatic patients with BRAFV600 mutated brain metastases, with response rates of 44-59%; however, the response is less durable, approximating 4.5-8.3 months (244). Overall, melanoma brain metastases remain a

distinctly challenging clinical scenario and require specific investigation into additional biomarkers with ICI in CNS disease.

Lactate Dehydrogenase (LDH)

LDH is an established prognostic factor in melanoma and is incorporated into the AJCC staging criteria (11, 25, 245, 246). Studies have also evaluated the prognostic significance of LDH in the era of ICI, and it remains prognostic in retrospective studies of anti-CTLA4 (247, 248). More recently, a large retrospective pooled analysis of 359 patients in EAP, KEYNOTE-001, KEYNOTE-002, KEYNOTE-006 found that LDH remained independently prognostic of OS in a model of high relative lymphocyte count, high relative eosinophil count and those with lung metastases as the only site of metastatic involvement (248). In this study, a discovery cohort of 177 patients was used to optimise the cut-point for continuous variables. Significant negative correlations with OS were observed for a high LDH ratio compared to upper limit of normal (ULN) ($p < 0.001$), high leucocyte count ($p < 0.02$) and high absolute and relative neutrophil counts (both $p < 0.001$). Significant positive correlations with OS were observed for high absolute ($p < 0.02$) and relative ($p < 0.001$) eosinophil counts, high absolute ($p < 0.002$) and relative ($p < 0.001$) lymphocyte counts, and high relative basophil counts ($p < 0.001$). A confirmatory cohort of 182 patients showed 9 factors remained significant: LDH ratio ≤ 1.7 ULN versus > 1.7 ULN, and LDH ≤ 2.5 ULN versus > 2.5 ULN, absolute leucocyte count < 0.05 . However, patients with unresectable stage III disease, or metastases located to distant lymph nodes/soft tissue or lung had significantly longer OS as compared with patients with other visceral metastases in the discovery and confirmation cohorts (both $p < 0.001$). In a multivariate model, only 4 factors remained significant: LDH > 2.5 ULN (HR 2.5; $p < 0.001$), relative lymphocyte count 17.5% (HR 1.9; $p < 0.001$), relative eosinophil count 1.5% (HR 2.2; $p < 0.001$), and no involvement of visceral metastasis other than lung (HR 2.5; $p < 0.001$). These four factors were independently associated with longer OS.

Elevated LDH has also been associated with lower likelihood of response to MAPK targeted therapy. Pooled analyses of clinical characteristics of patients treated with dabrafenib and trametinib or vemurafenib and cobimetinib have found that elevated LDH is associated with short PFS and OS (249, 250). Again, LDH $> 2.5 \times \text{ULN}$ was associated with the worst survival after these therapies (249). LDH appears to remain strongly prognostic after ICI or MAPK targeted therapies.

Performance status

Performance status is a functional summation of a patient's fitness, and has long been recognised to be prognostic in patients with metastatic disease across many cancer types including melanoma (251, 252). Patients with poor performance status are generally excluded from the clinical trials that establish the standard of care treatments. Cytotoxic chemotherapy in patients with poor baseline performance status is associated with low response rate, high toxicity and short survival (251, 253). As a result, the American Society of Clinical Oncology has recognised avoidance of treatment of patients with poor performance status (Eastern Cooperative Oncology Group Performance Status, ECOG PS, ≥ 3 , defined as capable of only limited self-care, confined to bed or chair more than 50% of waking hours) (254) as a one of the top 5 priorities of their value directed 'choosing wisely' campaign (255). Molecularly targeted therapies are the exception to this recommendation, having a mechanism of action distinct from traditional cytotoxic therapies; they are able to induce tumour response and clinical benefit even in patients with poor performance status, such as the case in molecularly driven lung cancer (255, 256). A similar case could be argued for the use of MAPK targeted therapy, where the predictive mutation is known, and the likelihood of response is high and rapid (88, 257, 258). Despite this, there is recent data to suggest that patients with poorer performance status are less likely to respond, even to targeted therapy (250). In a pooled analysis of BRIM-2, BRIM-3, BRIM-7 and coBRIM clinical trials of vemurafenib and cobimetinib, ECOG performance status >0 , along with high baseline LDH (greater than twice the ULN), and sum of longest diameter of target lesions ($>44\text{mm}$) were associated with poorer overall survival.

It was not known whether performance status would still remain prognostic or predictive for new immunotherapies, given their novel mechanism of action compared with conventional cytotoxic therapies as well as their lower toxicity (187). Diem and colleagues showed that patient performance status, LDH and the number of organs involved remain independently prognostic, in their retrospective review of 134 patients treated with ipilimumab (247). Aforementioned recent pooled analyses of anti-PD-1 studies also showed poorer survival in patients with ECOG >0 , but these analyses were either performed in mixed cohorts with multiple cancer types or involved patients in clinical trials or expanded access programmes (237, 248). Patients treated in clinical practice are likely to have even lower performance status (i.e. ECOG ≥ 2); the palliative

care needs of these patients remain largely unexamined. Our published manuscript as part of this PhD addressed these issues and will be examined in detail in Chapter 3 (259).

Immune related adverse events (IrAE)

Approximately 15-20% of patients treated with anti-CTLA4 experience a grade 3 or 4 IrAE, whereas this affects approximately 10-15% of patients treated with anti-PD-1. There is limited data on predictors for the development of IrAE. Retrospective analysis of a mixed cohort of 78 patients with metastatic melanoma, lung, renal cancer treated with ipilimumab, pembrolizumab or nivolumab found that male sex, history of autoimmunity or IrAE with previous ipilimumab, stage 3-4 renal impairment, and lack of steroid use were associated with a higher likelihood of IrAE (260). Importantly for clinical practice, discontinuation for treatment toxicity in the clinical trials did not appear to reduce the effectiveness of the combination ICI treatment (261).

Some studies have associated the development of an IrAE with improved outcome following ICI. In the aforementioned post hoc analysis of the phase 1 study of patients with melanoma, lung and renal cancer treated with nivolumab (237): IrAE of any grade was associated with longer OS at 5 years (median OS 19.8 months) or grade 3 or more (median OS 20.3 months) compared with those without IrAE (median 5.8 months). There are also retrospective series that have reported improved PFS and OS after ICI associated with IrAE, particularly rash and vitiligo (262-264). Some of these retrospective studies are limited in their statistical methodology, since responding patients remain on treatment longer and therefore show a higher rate of IrAE when compared to non-responding patients, who stop the treatment after a short period due to disease progression or death. Another challenge of this clinical biomarker is that patients develop these immune related side effects after treatment initiation and therefore cannot be used as a predictor of treatment response a priori. However, given the uncertainty of the optimal duration of therapy, there may be merit in establishing on-therapy biomarkers of patients who are likely to derive long term benefit so that the duration of treatment can be minimised for likely responders.

Basic laboratory tests

In addition to LDH, several studies have evaluated methods of profiling the immune system using basic laboratory tests in clinical practice, including lymphocyte subsets.

In terms of anti-CTLA4, higher response and improved survival have been observed with high baseline lymphocyte count (265, 266), eosinophilia (267), low monocyte count (266). Poor response has been observed in patients with high neutrophil to lymphocyte (NLR) ratio, with $NLR > 5$ being prognostic in a number of studies (268-270). Some dynamic blood parameters have been associated with response to anti-CTLA4 and improved survival, such as reduction of neutrophil to lymphocyte ratio (271). In patients treated with anti-PD-1, relative lymphocyte count, relative eosinophil count and neutrophil-to-lymphocyte ratio and C-reactive protein (CRP) have been correlated with survival in retrospective analyses (270, 272, 273).

Disease burden

Pre-treatment tumour assessment in clinical practice is routinely performed using computerised tomography, and criteria have been established for tumour response assessment so that comparison can be used across clinical trials. This allows the quantification of a patient's disease burden, as well as the specific organ of metastatic involvement as mentioned previously. A retrospective analysis of patients treated with pembrolizumab as part of the phase 1 study KEYNOTE-001 showed that baseline tumour size (BTS) $> 10.2\text{cm}$ was independently associated negatively with OS (274). In this study, BTS was quantified as the sum of longest dimensions of all measurable baseline target lesions using RECIST version 1.1 (i.e. with maximum of 5 measurable lesions in total, and 2 lesions per organ). In univariate analyses, low BTS $< 10.2\text{cm}$ was associated with improved overall survival (HR 0.38, $p < 0.001$) and higher objective response rate (44% v 23%, $p < 0.001$). In multivariable analyses, BTS remained independently associated with overall survival, but not with objective response rate. High BTS was also associated with Eastern Cooperative Oncology Group performance status 1, elevated LDH, stage M1c disease, and liver metastases (all $p < 0.001$). Other studies have used different thresholds for continuous variables such as BTS, but higher disease burden has been consistently associated with poorer survival (159). Huang et al. went on to assess the relative ratio of exhausted T cells post therapy in relation to tumour burden and found this to be associated with survival, albeit in a small cohort (159). Other functional imaging such as positron emission tomography and its prognostic significance will be covered in detail in a Chapter 5.

High tumour burden on CT has been associated with poor survival after treatment with anti-PD-1. Baseline tumour size (BTS) was independently associated with overall

survival in 583 patients with metastatic melanoma treated in the phase 1 study of pembrolizumab (KEYNOTE001). In this study, BTS was measured by adding the sum of the longest dimensions of all measurable lesions on baseline target lesions CT scans. Imaging assessment of metastatic disease is paramount not only to document the site of metastatic involvement but also provide an assessment of tumour burden. The following section will review the role of functional imaging in advanced melanoma and the potential functional imaging biomarkers in relation to ICI treatments.

1.3.2 Functional imaging biomarkers

Functional imaging with modalities such as 2-deoxy-2-[fluorine-18] fluoro-D-glucose positron emission tomography (FDG PET) provides not only accurate staging but potentially a source of biomarkers for melanoma immunotherapy. Given the rise of PET as a standard clinical imaging modality in staging and surveillance of melanoma, it is important to evaluate PET as a possible source of biomarkers in advanced melanoma. Firstly the prognostic significance of PET parameters in melanoma will be explored, followed by an exploration of these PET parameters in relation to melanoma treatment.

Standardised Uptake Value (SUV) is a semi-quantitative measure of FDG uptake and, consequently, glucose utilisation within a specified region of interest. SUVmax, the maximum SUV within a tumour, has been shown to be highly prognostic in a number of cancer types, including melanoma, non-small cell lung cancer, cervical cancer, and renal carcinoma (275-277). However, the absolute SUVmax threshold for significance varied depending on the tumour type, and not all of the studies have been externally validated. High SUVmean was associated with shorter disease-free survival in patients with Stage IIIB melanoma (278). There are limited data about the prognostic value of SUVmax or SUVmean on baseline scans prior to melanoma treatment for metastatic disease. In a retrospective series presented in abstract form, 23 patients with tumoural SUVmax>9.44 on pre-treatment 18F FDG PET/CT was associated with tumour progression within six months after treatment with an anti-PD-1 monoclonal antibody (279).

As mentioned previously, high baseline tumour size as measured by CT is associated with poor OS in patients who received pembrolizumab in the KEYNOTE-001 study(274). It is hypothesised that metabolic tumour volume (MTV) on FDG PET/CT may be a more representative assessment of metabolically active burden of disease than

BTS as measured by CT. Measurable disease as quantified by CT using criteria such as RECIST 1.1 underestimates the burden of disease as it encompasses a maximum of only 5 measurable lesions at baseline (280). Furthermore, lymph nodes are measurable only if the short diameter is greater than 15mm and bone lesions are excluded in these criteria. Hence, FDG PET is more able to quantitate the full extent of disease burden when parameters such as total lesion glycolysis (TLG) and MTV are used (281). TLG is a composite measurement of FDG avidity multiplied by the entire volume of disease whereas MTV is only the volume of metabolic avid tumour above a set threshold (281, 282). Lastly, FDG PET/CT provides information about the glucose uptake, and therefore the metabolic and cellular activity of the tumour load can be evaluated, in addition to the assessment of disease volume.

MTV has been associated with prognosis in a number of studies across tumour types including melanoma (283, 284). In a pre-operative series of 41 patients with early stage disease, MTV was associated with short disease free survival and poor melanoma specific survival (277). MTV was evaluated in a study of 35 patients with metastatic melanoma treated with BRAF and MEK inhibition combination in the phase 1b clinical trial (BRIM7). Low MTV (<47.5ml) was associated with improved overall survival after BRAF targeted therapy (HR 0.14, p=0.03) (285).

Lastly, an often-overlooked use of this functional imaging modality is the use of FDG PET to evaluate the patient's immune status or immune response to the tumour. There are normal physiologically conserved SUV ratios within the lymphatic and endorecticular system, and these can be altered in an inflammatory state. For example, the spleen to liver SUV ratio is conserved, where the liver is normally of higher avidity than the spleen; however, the reverse is true in inflammatory states or after GCSF injections (286-289). The significance of spleen to liver ratio has also been studied in pancreatic and cholangiocarcinoma (286-288) but not in melanoma. High splenic avidity in pancreatic cancer and cholangiocarcinoma was found to be associated with poor survival and markers of inflammation.

In relation to melanoma immunotherapy, splenic avidity on PET was evaluated in 10 patients treated with tremelimumab, an anti-CTLA4 monoclonal antibody (290). Patients had matched ¹⁸F FDG and ¹⁸F fluorothymidine (FLT) PET scans at baseline and again after a median of 2 months after tremelimumab treatment. FLT is an

analogue of thymidine and ^{18}F FLT PET provides a functional assessment of cellular proliferation. Splenic avidity was low in baseline FDG PETs and did not change significantly post treatment. Interestingly, there was a significant increase in splenic FLT uptake (both splenic SUV_{mean} and splenic SUV_{max}). This increase in splenic avidity was not correlated with objective response to therapy. Tremelimumab is not used in clinical practice as monotherapy for melanoma as it failed to demonstrate an overall survival benefit in randomised studies (291). However, this interesting translational PET study showed the potential of non-invasive imaging of the immune system in relation to melanoma immunotherapy (290). Further evaluation of splenic avidity in treatments used in clinical practice, in relation to treatments such as ipilimumab or anti-PD-1 agents, is warranted because it may provide a surrogate for the host immune state and potential biomarker.

Understanding how PET imaging correlates with host immune factors and with the intratumoural microenvironment may also provide insights into the biology of immunotherapy and identify novel biomarkers predictive of clinical response.

1.3.3 Biological biomarkers

Identification of responders from poor responders to immunotherapy has to date been partially limited by the inability to fully characterize the immune landscape of the tumour microenvironment. Novel techniques have gone beyond clinical characteristic and basic laboratory tests, not only identifying likely responders, but also going on to describe some of the resistance mechanisms for ICI. Comprehensive examination of the tumour and the patient's blood or stool can provide biomarkers complementary to clinical characteristics and functional imaging as previously reviewed.

Tumoural immune profiling

Several studies have shown that immune infiltrate into the tumour is prognostic of survival in the metastatic setting (33, 292). The presence of intratumoural T cells in pre-treatment biopsies are associated with response to anti-CTLA4 (293). Dynamic changes within the tumour are also observed with anti-CTLA4 responsiveness, including increase in TILs, increased CD4 T cells/Treg ratio(145, 294), as well as increase in tumoural Granzyme B⁺ lymphocyte, high FOXP3 and high IDO expression

after treatment (293). Immunosuppressive immune subsets such as macrophages have also been associated with poor outcome following anti-PD-1 treatment (295).

In a landmark paper, Tumeh and colleagues showed that the presence of cytotoxic T cells in the invasive margin and within the tumour were predictive of tumour response to anti-PD-1 in melanoma (296). However, this study did not explore the presence or spatial relationships of inhibitory immune cells such as macrophages, T regulatory cells, myeloid derived suppressor cells or B cells in relation to effector T cells. Some classifications have proposed to using PD-L1 expression and presence of T cells as a framework for classifying tumours in a more nuanced fashion, beyond the commonly used terms of ‘hot’/‘inflamed’ tumour, in reference to a tumour with pre-existing T cells and high PD-L1 expression; this as opposed to referring to tumours that do not have a pre-existing population of T cells as ‘cold’ (297, 298). While these frameworks provide a more rational stratification of cancers, greater in-depth knowledge of the underlying mechanisms of immunosuppression is required to provide rational therapeutic strategies.

Furthermore, subsequent studies have suggested that on-treatment biopsies are more predictive of response than are pre-treatment biopsies. A longitudinal study of patients with melanoma treated with anti-CTLA4 or anti-PD-1 found that while baseline CD3 and CD8 levels were slightly higher in responders, levels of CD3, CD4, CD8, and T-cell activation markers such as PD-1, PD-L1 and LAG3 were significantly higher after 2-3 doses of anti-PD-1 in responders compared to non-responders (299). These authors did not find any difference in levels of immunosuppressive CD68+ myeloid subsets in non-responders compared to non-responders for anti-CTLA4 or anti-PD-1. Other studies have also found a change in the phenotype of T cells in responders to nivolumab monotherapy (n=107) or combination nivolumab and ipilimumab (n=86) (300), with higher levels of HLA-DR, and activation markers such as ICOS and/or Ki67 expressed on CD4 and CD8 cells.

Tumoural PD-L1 expression

Given that anti-PD-1 and anti-PD-L1 monoclonal antibodies work by inhibiting these immune checkpoints, tumoural PD-L1 expression on immunohistochemistry (IHC) assays is a logical candidate biomarker to explore. Early studies showed that tumours

expressing the immune-suppressive molecule of B7-H1 (PD-L1) were highly associated with tumour infiltrating lymphocytes and IFN- γ , indicating a host response to inflammation within the tumour microenvironment(138). This study also found that tumoural PD-L1 expression in patients with metastatic melanoma (n=56) was significantly associated with improved overall survival compared to those with PD-L1 negative tumours, whereas this relationship was not found in primary melanoma (n=43).

PD-L1 expression by IHC has been examined in multiple clinical trials of patients treated with anti-PD-1 or anti-PD-L1 ICI, but the association of PD-L1 expression with outcome has been variable. Forty-two of the 296 patients treated in the phase 1 study of nivolumab had tumoural samples evaluable for PD-L1 staining (301). PD-L1 positivity was defined as greater than 5% of tumour cells expressing the staining using the murine PD-L1 monoclonal antibody 5H1 (301). Thirty-six percent of the 25 patients with a PD-L1 positive tumour experienced an objective response to nivolumab. None of the 17 patients with PD-L1 negative tumours had a response. Multiple clinical trials across a range of cancer types have also shown that PD-L1 expression can enrich for responders to anti-PD-1 and anti-PD-L1 monoclonal antibodies, however, tumour responses have been observed in patients with PD-L1 negative tumour (302-306). Some studies have not shown a correlation between PD-L1 and anti-PD-1 response, particularly in two renal cancer studies (307-309).

One of the many challenges of implementing PD-L1 as a predictive biomarker in clinical practice is that the IHC assays used, and the thresholds for significance have varied between the different clinical trials. Different companies used different assays for their drug development: clone 22C3 was used for pembrolizumab, 28-8 for nivolumab, SP142 for atezolizumab and SP263 for durvalumab. Furthermore, assessment of SP142 has also included PD-L1 expression on *immune cells*, in addition to *tumoural* PD-L1 expression as was the case for the other three assays. The different studies also used different thresholds for significance, depending on the percentage of cells expressing PD-L1. The Blueprint PD-L1 IHC Assay Comparison Study compared these four assays in 30 lung cancer cases treated in a phase 1 trial (310). This study showed that three assays revealed comparable staining: 22C3, 28-8 and SP263, whereas SP142 was an outlier with lower staining in tumour cells. In this study, about a third of cases would be classified differently depending on the different assays used. Separate studies have confirmed SP142 as an outlier compared to other assays in lung cancer

particularly with lower expression on tumour cells, but not immune cells(311). In a customised, laboratory-developed study of 34 melanoma samples, 5 assays were assessed: 5H1, 22C3, 28-8, SP263 and SP142. Interestingly, this study found all 5 assays were concordant, and the few discordant cases were due to focal heterogeneity of PD-L1 expression across the tumour section(138, 312). This raises the importance of technical aspects of the assay optimisation including factors such as primary antigen retrieval, primary antibody concentration, antigen retrieval, amplification, or signal development (312).

PD-L1 expression has been examined in the seminal phase III studies for anti-PD-1 in advanced melanoma. In CHECKMATE-037 of previously treated patients, PD-L1 testing was positive in 49.7% of patients using the cut-off of 5% of tumour cells positively stained with the 28-8 assay (187). Pre-planned subgroup analyses in this study showed that PD-L1 positive tumours were associated with a higher response rate compared with PD-L1 negative tumours when treated with second line nivolumab(ORR 43.6% vs 20.3% respectively). In the CHECKMATE-066 study of first line nivolumab, PD-L1 was positive in 35.4% of patients (172). However, Nivolumab was superior to DTIC chemotherapy regardless of PD-L1 expression. The studies with pembrolizumab in melanoma used a different assay and a different cut off, with PD-L1 positivity defined as PD-L1 staining in $\geq 1\%$ of tumour cells using the 22C3 antibody (174). In KEYNOTE-006, 80.5% patients had PD-L1 positive samples using this threshold. In this study, pembrolizumab was superior to ipilimumab regardless of PD-L1 expression. Both of these studies showed that response rates were higher in PD-L1 positive tumours compared with PD-L1 negative tumours, but responses were still observed in PD-L1 negative tumours, which is in contrast to the original findings of the phase 1 study of nivolumab (301). Hence, patients cannot be excluded from treatment due to tumoural PD-L1 negativity, as responses are still observed, albeit at a lower likelihood.

The CHECKMATE-067 study of combination nivolumab and ipilimumab attempted to explore the optimal threshold for PD-L1 staining, with pre-specified analyses to assess efficacy at 1%, 5% and 10% (176). Using the 28-8 assay, 23.6% of the study population was deemed PD-L1 positive, using a cut-off at 5% IHC expression on tumour cells. In this study, combination of nivolumab and ipilimumab was superior to either monotherapy within the study population (176). The most discriminatory PD-L1 threshold appeared to be 1%, where the combination had improved OS in the low PD-

L1 group (combination vs nivolumab HR 0.68 in patients with PD-L1<1%), whereas the combination was associated with a similar OS to nivolumab monotherapy in PD-L1>1% (HR 0.98) (177). The efficacy did not differ in the combination or monotherapy groups when the PD-L1 5% or PD-L1 10% thresholds were used.

There are several other technical challenges with the use of PD-L1 IHC. Firstly, there is spatial and temporal change of PD-L1 expression. Multiple studies have demonstrated a discordance of PD-L1 staining in metastases within patients (313-315). Longitudinal studies of matched primary and metastatic melanoma have also shown marked heterogeneity, with implications for use of archival tissue for PD-L1 testing for entry into clinical trials or treatment decision making (315).

There is also an increasing recognition that PD-L1 expression is contextual and that whether the PD-L1 is expressed on tumour cells or on immune cells may be important for prognosis. PD-L1 expression on non-tumour cells is well documented, with expression seen on lymphocytes as well as macrophages and dendritic cells (138, 157). In one study of patients treated with anti-PD-1 or anti-CTLA4 ICI, PD-L1 within the stromal compartment were found to be more highly correlated with improved survival compared to PD-L1 staining within intratumoural compartment (316). Not surprisingly, PD-L1 was higher in both intratumoural and stromal compartments of responders (CR, PR) versus (SD, PD). The importance of PD-L1 expression on immune cells more specifically has been highlighted in different cancers and appears crucial to its mechanism of action (157, 304, 316-318). Recent studies have also explored the different mechanisms by which PD-L1 on different cells can induce a tumour response in lung cancers (319). Again this study showed that PD-L1 expression was more prevalent on immune cells and was associated with IFN induced adaptive regulation with higher T cells and effector T cells; whereas PD-L1 expression on tumour cells was associated with epigenetic dysregulation and was associated with poor immune infiltration, sclerotic or desmoplastic stroma, and mesenchymal features (319). More precise characterisation of PD-L1 staining, and which immune cell types are co-expressors of PD-L1 may lead to more rational therapeutic strategies.

So far the proposed classification of tumour immune landscape involves stratification of tumours into ‘hot’/‘inflamed’ and ‘cold’/‘non-inflamed’ tumours depending on the presence of tumour infiltrating lymphocytes (TIL) and PD-L1 expression (297). This is

an over-simplified stratification which also makes assumptions regarding the possible inhibitory cells or processes that might be present. It therefore does not lend itself to rational selection of combination immunotherapy for those harbouring a ‘non-inflamed’ tumour.

Developments in genomic studies have also given further insights into the functional status of the immune system within the tumour. More recently, mass cytometry has further characterised immune subsets within the tumour. Mass cytometry builds upon the success of flow cytometry and enables over 40 simultaneous parameters to be quantified by replacing fluorophores with mass tags (320). It is thus possible to discern the identity and behaviour of numerous cell types from a single experiment (161, 320). Mass cytometry profiling of pre-treatment samples to combination ICI has also identified a memory T cell phenotype (EOMES⁺CD69⁺CD45RO⁺) in 19 patients (321). Even though this technique is extremely powerful in identifying immune subsets with in-depth characterisation of its functional states, orthogonal applications of immunohistochemistry are important so that the spatial relationships between immune cells are retained.

Immunogenomics

The activation of the immune system requires recognition of the tumour proteins by antigen presenting cells. Therefore, the presentation of tumoural protein is crucial to the initiation of immune mediated killing and the effectiveness of ICI. There are two broad categories of tumour proteins that can be recognised by the immune system: 1) non-mutated self-antigens and 2) MHC class I restricted and MHC class II restricted neoantigens, which arise as a result of non-synonymous somatic mutations (322, 323). Non mutated self-antigens are proteins that are expressed on trophoblasts or male gametes, for instance cancer-testis antigens such as MAGEA and NYESO-1. The MAGEA cancer antigen was recently shown to be associated with a poor response to anti-CTLA4 but not to anti-PD-1 (324). Furthermore, the MAGE-A antigen was not associated with poor survival using the TCGA data; it is therefore a predictive biomarker but not a prognostic one.

The primary targets of immunotherapy are tumour-specific neoantigen peptides that have arisen from somatic mutations within the cancer genome (325, 326). Accordingly, some of the highest tumoural responses to ICI have been observed in cancers with a

high mutation burden such as melanoma and lung cancer (301, 327). Tumour mutational burden (TMB) as a predictive biomarker has been evaluated in a number of different cancer types, and a meta-analysis has confirmed that high TMB is associated with higher rates of ICI response across 27 cancer types (328).

In melanoma, high TMB is associated with anti-CTLA response (329, 330). Snyder and colleagues were the first to propose a neoantigen tetra-peptide and high tumour burden as being associated with clinical benefit (i.e. response for greater than 6 months) to ipilimumab treatment in advanced melanoma (329). An overall survival benefit was also seen in patients with TMB>100mutations. Although the finding of the neoantigen tetra-peptide could not be replicated in other sample sets, the association between high TMB and response to anti-CTLA4 was again demonstrated in a second study (330). Interestingly, a study of matched pre/on-treatment samples in 68 patients with advanced melanoma treated with anti-PD-1 did not find an association between high TMB and response to anti-PD-1 in previously treated patients (331). Rather, pre-treatment TMB was associated with OS only in ICI-naïve patients, whereas early on-treatment (4 weeks) reduction in TMB was significantly associated with anti-PD-1 response and OS in patients regardless of prior ICI. Although these findings are of interest, this dynamic biomarker requires on-treatment samples, rather than being discriminatory for response prior to treatment. Furthermore, this study also highlights the importance of the influence of previous therapy on the tumour microenvironment, with significant implications for biomarker interpretation (331).

The molecular subtype of melanoma does not appear to be associated with responsiveness to anti-PD-1 (331). However, there are some specific mutations that have been found to be associated with innate or acquired resistance to ICI. PTEN loss has been associated with reduced T cell infiltration and reduced response to ICI in murine models and a cohort of 39 patients treated with anti-PD-1 (332). On the other hand, recurrent mutations of *SERPINB3* and *SERPINB4*, which encode protease inhibitors, appear to be predictive of prolonged survival after anti-CTLA4 therapy, but were not prognostic in the TCGA dataset (333). This indicates that there are some mutations which are predictive of response to specific treatments, rather than prognostic in themselves.

Mutations within the interferon signalling machinery or pathways (JAK-STAT) have been identified as primary or acquired resistance mechanisms for both anti-CTLA4 and anti-PD-1 (334-336). B2M mutations were also identified with resultant impairment of antigen presentation via MHC I (335). HLA heterozygosity has also recently been recognised as having a significant role in the responsiveness to ICI, in that HLA-B44 are associated with good survival after ICI, whereas HLA-B62 are associated with reduced responsiveness (337). These studies identified not only potential biomarkers but also highlight the mechanism of resistance that need to be overcome in order to achieve an adequate anti-tumour response.

The use of publicly available genomic datasets across tumour types such as the TCGA has enabled the implementation of correlative studies which attempt to understand the contribution of insertion/deletion (Indel) mutations and somatic copy number alterations (SCNA) in relation to responsiveness to ICI across cancer types (338, 339). Indel burden was associated with responsiveness to ICI but not survival (338). Interestingly, SCNA was associated with lower response to anti-CTLA4 and poor survival (339) and has been validated in a separate study of patients treated with sequential anti-CTLA4 and anti-PD-1 therapy (340).

Transcriptomic studies using RNA sequencing have echoed the importance of the interferon pathway, with the IFN- γ related inflammatory Nanostring profile being associated with response to anti-PD-1 in clinical trials (341). This study was piloted on 19 patients with melanoma treated with anti-PD-1 and thereafter validated on 220 patients with other cancer types. This 'T-cell inflamed' gene expression signature included genes related to antigen presentation, chemokine expression and cytotoxicity as well as adaptive immune resistance (341). Gene expression profiling of paired pre- and post-treatment samples in a longitudinal study of patients treated with anti-CTLA4 and anti-PD-1 also found that responders to anti-PD-1 had higher expression of genes related to cytolytic activity, antigen presentation and interferon signalling, with lower levels of *VEGFA* (299). Other groups have again evaluated transcriptomes in patients treated with anti-CTLA4 and found that responsive tumours tend to have a higher expression of memory T cells and proliferation gene expression (342), in addition to the IFN- γ and Th1 gene signatures (343) and expression of the CTLA-4 and PD-L2 (330).

Transcriptomic studies have also provided insights into some possible mechanisms of resistance to ICI (344). Hugo and colleagues described an IPRES (innate resistance to PD-1) signature on bulk RNA sequencing data of 28 patients, which included genes associated with angiogenesis and mesenchymal transition. However, these findings have not been able to be replicated in larger cohorts (331). Most recently, transcriptomic studies performed on single cells, presented the ability to dissect the gene expression of particular cell types within the tumour microenvironment (i.e. separating out immune cells from tumour and stromal cells). Two groups performed single cell transcriptomic studies on melanoma cohorts and found that resistant tumours had a T cell exclusion program which was reversible by CDK4/6 inhibitors in murine models, whereas responsive tumours had a TCF⁺CD8⁺ immune population (345, 346).

Orthogonal study of the tumour samples using multiple novel technologies could reveal the genomic landscape of immune related genes, in terms of its activation, trafficking, antigen presentation and metabolism. These immune-profiling techniques can also be used to characterise immune cellular subsets within melanoma and potentially identify immunological biomarkers for immunotherapy.

Peripheral blood biomarkers

A major limitation of tumoural immunoprofiling is the need for invasive biopsies, which can be painful and cause complications which add to patient morbidity. Hence peripheral blood based biomarker testing is a relatively non-invasive procedure that may be more appropriate for repeated sampling, especially if on-treatment samples are more predictive of response (331).

Comprehensive profiling of immune subsets within peripheral blood has revealed some immune cellular subsets associated with response to ICI. For anti-CTLA4, several studies have shown that low baseline or a decrease in myeloid derived suppressor cells have been associated with benefit after ipilimumab (153, 266, 347-349). The evidence on regulatory T cells (Treg) is more conflicting, with some studies reporting a high peripheral Treg subset or an increase being associated with benefit after neoadjuvant ipilimumab (266, 347). ICOS is a co-stimulatory receptor expressed on the surface of T cells after activation; a persistent increase of peripheral CD4⁺ICOS^{hi} T cells has been associated with survival after ipilimumab (146). For anti-PD-1, an increase in

Ki67⁺PD-1⁺CD8 cells has been associated with response, especially relative to a reduction in tumour burden (159).

Blood based genomic analyses have also gained traction over the past few years as a predictive biomarker. Groups have assessed the sequencing of blood-derived cell free DNA to determine relative TMB with a targeted next generation sequencing gene panel, or have followed known single mutations such as BRAF, NRAS or TERT longitudinally in the case of cutaneous melanoma(350, 351). Retrospective analysis of 2 large randomised studies of anti-PD-L1 in lung cancer has correlated TMB as assessed in cfDNA with overall survival (350).

Several studies have evaluated the role of tracking circulating tumour DNA (ctDNA) in the setting of targeted therapy in metastatic melanoma (352-354). These studies show that an early fall in ctDNA levels is prognostic of long-term response, and that ctDNA can be used to monitor for acquired resistance. The role of ctDNA monitoring has also been evaluated in patients treated with anti-PD-1 (355-357). One study of 76 patients found that low baseline or decreased ctDNA levels after therapy were associated with anti-PD-1 response when compared to patients with persistently high levels of ctDNA (ORR 72%, 77% and 6% respectively) (355). CtDNA response was also independently associated with overall survival in a multivariable model considering LDH, tumour stage, performance status and disease volume. The utility of ctDNA in identifying patients with pseudo-progression after anti-PD-1 was also examined in 129 patients, 9 of whom experienced pseudo-progression (356). In patients with pseudo-progression, ctDNA levels reduced despite progression by CT assessment. The sensitivity of ctDNA at 12 weeks for predicting pseudo-progression was 90% and specificity was 100%. However, the application of these results is limited at the present time, as ctDNA level was detectable in only 70% of patients with a known mutation; indicating that there is still a need for complementary methods of biomarker monitoring (355, 356).

There has been limited success with the use of cytokines in the peripheral blood as a predictive biomarker. Clinical benefit with anti-PD-1 in one study was associated with lower pre-treatment levels of IFN γ (p=0.04), IL-1 β (p<0.01), Fractalkine (p=0.02) (159) but these findings have not been consistently reported(358). Instead, this second study found no correlation between 65 cytokines and outcome after ICI, but a predictive score using cytokines was identified. The CYTOX score of 11 cytokines was found to be

upregulated in patients treated with anti-PD-1 monotherapy or combination anti-CTLA4 and anti-PD-1 (358).

Faecal biomarkers

The significance of the microbiome to prognosis and prediction of response to ICI is increasingly recognised. Preclinical models have shown that tumour responses after anti-PD-L1 are enhanced by *Bifidobacterium* spp and that anti-CTLA4 responses were enhanced by *Bacteroides* spp (359, 360). Baseline faecal samples in patients have also confirmed the importance of faecal microbiota diversity and composition (147). In the first study, 26 patients' faecal samples were analysed longitudinally, showing that baseline *Faecalibacterium* and other Firmicutes are associated with improved PFS after ipilimumab, but also higher frequency of ipilimumab-induced colitis (147). More comprehensive profiling of baseline faecal samples also showed that responders had more *Bifidobacterium longum*, *Collinsella aerofaciens*, and *Enterococcus faecium* than did non-responders. Interestingly, this study went on to show that oral administration of faecal matter from responding patients into germ-free mice was able to improve anti-PD-1 treatment with enhanced T cell killing (361). The third study showed that responders to anti-PD-1 had a more diverse microbiome with more Ruminococcaceae family, leading to an immune-reactive environment and lower levels of proinflammatory cytokines (362). These three studies used different profiling and analysis methods, which may have led to the identification of different bacterial species in relation to response. Nonetheless, the importance of examining the microbiome in the context of ICI has been highlighted. Correspondingly, multiple retrospective studies have also correlated poor response and poor survival in patients receiving antibiotics after ICI (363-365). Given all of these intriguing observations, there are now clinical studies evaluating dietary modifications and their impact on ICI across multiple cancer types, as well as faecal transplant studies for patients with melanoma who are resistant to therapy (NCT03817125, NCT03772899, NCT03353402).

Table 1.3. Blood, tumoural and faecal biomarkers for anti-CTLA4 and anti-PD-1 treatments for advanced melanoma, adapted from Manson et al (235)

		Good Response	Poor response	Toxicity
Anti-CTLA4	Blood	- High baseline lymphocytes* (266, 366) and increase after treatment(265, 367) - Decreased neutrophil: lymphocyte ratio on treatment*(271) - Low baseline Eosinophil* and increase after treatment (contradictory data)(367, 368); high eosinophil good survival(266) - High baseline CD16+ monocytes - Increase blood/tumour(153) CD4+ICOS^{hi} T cells*(146, 148) - Presence of NY-ESO-1 Ab(369) - Presence NY-ESO-1 specific CD8*(369) - Decrease in circulating Tregs (contradictory data)(153), increase associated with benefit after neoadjuvant ipilimumab(347) - High Tregs*(266) - Low baseline and decrease in MDSC*(266, 347, 348) - Decrease in monocytic MDSC(349) - Maintenance of high frequency TCR clonotypes with high avidity TCR(156, 370)	- High baseline LDH(247, 266, 366)* - High baseline neutrophil: lymphocyte ratio(268, 371)* - High baseline soluble CD25(372)	- High baseline and post treatment eosinophils (368) - Increased IL17 after treatment correlate with colitis - Increased CD177 and CEACAM1 after treatment correlate with colitis - Increase TCR repertoire(155)
	Tumour	- T cell (memory) proliferation gene expression(342) - Presence of intratumoural lymphocyte and on treatment increase in TILs(293) - Increased CD4 T cells/Treg ratio(145, 294) - Increase in tumoural Granzyme B lymphocyte after treatment(293) - High FOXP3 and high IDO expression(293) - Recurrent <i>SERPINB3</i> and <i>SERPINB4</i> mutations (333) - High mutational load(329, 330) - Transcriptomic signature of immune activation (IFN-γ and Th1 genes)(373) - TCF+CD8+ population identified by scRNA(374)	- Gene expression of MAGE-A cancer germline antigens(324) - High somatic copy number alterations (aneuploidy)(339) - Loss of IFN-γ pathway genes(334)	- Infiltration of neutrophils into the gut lamina propria correlate with colitis
	Faecal	- Increased faecal <i>Faecalibacterium</i> and <i>Firmicutes</i>*(147)		- Increased faecal <i>Faecalibacterium</i> and <i>Firmicutes</i> associated with colitis*(147)

		Good Response	Poor response	Toxicity
Anti-PD-1	Blood	-Transient increase CD8+HLADR+Ki67+lymphocytes - Increased levels of IFN-γ, IL-18, ITAC - Decreased IL-6, LDH - Increase in Ki67⁺PD-1⁺CD8 cells: tumour burden(159)	- Increased neutrophil:lymphocyte ratio(270) - High LDH(248)	-CYTOX score of 11 cytokines (358)
	Tumour	- Presence of peri- and intratumoural CD8 T cells(296) - High intratumoural PD-L1 expression(136, 301) - High intratumoural PD-L1 expression on TILs for anti-PD-L1 mab(157) - Increase in granzyme B CD8+ T cells(158) - High baseline clonal TCR repertoire(158) - Increased T cell clonal expansion(158) - High plasmablast and B cell signatures*(162) - High CXCR3 and CXCL9 and CXCL10 *(164) - Grade 3/4 toxicity(237)* - Reduction in TMB 4 weeks post treatment(331)* - Gene expression of cytolysis and NK proliferation(342) - Gene expression PD-1, 12 signatures CD4, CD8 T-cell activation, NK cells, and IFN activation(321, 375) - Gene expression of immunosuppressive checkpoint genes e.g. <i>PDCD1</i>, <i>TIGIT</i>, <i>TNFRSF9</i> (CD137), <i>IDO</i>, and <i>LAG3</i>(321) - Increased insertion or deletion mutations(338)	- Transcriptomic signature of innate anti-PD-1 resistance(344) - Loss of PTEN (332) - JAK 1/2 mutation in primary(336) or acquired resistance(335) - T cell exclusion programme on scRNA(345) - Increased gene expression of <i>CA9</i> (hypoxic marker), <i>WNT3</i>, <i>LAMB4</i>, <i>ITIH5</i>, <i>GRIA2</i>, <i>GRIA4</i>, <i>GRIK3</i>(321)	
	Faecal	- High baseline <i>Bifidobacterium longum</i>, <i>Collinsella aerofaciens</i>, and <i>Enterococcus faecium</i>(359) - High baseline microbiome diversity and higher Ruminococcaceae(362)		

*increase in survival after treatment

1.3.4 Application of biomarkers

The above literature review summarised some of the major developments in biomarker discovery, including possible patient characteristics as well as blood, tumoural or faecal biomarkers. A vast array of biomarkers is being explored and many of these studies have yet to be validated. Deeper understanding of host and tumoural immune interaction can also help to reveal resistance mechanisms and identify possible strategies to overcome this resistance. However the challenge remains how to incorporate these biomarkers into routine clinical use.

A combination of biomarkers is likely needed in order to predict response from ICI, given the complex processes involved in initiating and maintaining an anti-cancer response in an individual patient. Blank and colleagues proposed a ‘cancer immunogram’ as a framework to explore the various determinants that are integral to the host and tumoural immune interaction, including general immune status, immune cell infiltration, presence of [immune] checkpoints or soluble inhibitors, or inhibitory tumour metabolism, as well as tumoural sensitivity to immune effectors and tumoural foreignness (376). Such a framework is helpful in furthering the understanding of the determinants of response to cancer immunotherapeutics and resistance mechanisms, but is not easily applicable to an individual patient given the lack of prioritisation among the various biomarkers. Importantly, many biomarkers are able to enrich for likely responders but do not fully discriminate between responders and non-responders, and therefore cannot be used to exclude patients from treatment. Research is also needed into how firstly to prioritise the different biomarkers; and then how best to utilise these biomarkers to facilitate treatment decision making.

Furthermore, there is also considerable variation in the definition of ‘clinical benefit’ within the biomarker discovery literature, with challenges in comparing biomarkers across studies, as well as implications for reproducibility of particular biomarkers. Some studies have used CT reassessment of absence of disease, stable disease or decreased tumour volume for longer than 6 months without specifying the frequency of imaging (299, 329). Others have employed RECIST v1.1 commonly used in clinical trials and categorised the patients accordingly (331, 351). Some investigators incorporated the use of immune related RECIST Criteria (296, 377) whereas others did not specify whether these imaging criteria were used or not. Some studies have

included stable disease as part of the 'clinical benefit' cohort, whereas others have listed this group of patients separately.

Nonetheless, research into biomarkers for cancer immunotherapy improve our understanding of the mechanisms of response and begin to clarify patient groups that are more likely to receive benefit from ICI.

1.4 Hypotheses and questions addressed in this thesis

There is an urgent need to identify prognostic and predictive biomarkers for ICI in patients with melanoma, given that only a minority of patients respond, coupled with the potential toxicities of treatment. This thesis was initiated during the infancy of biomarker discovery for ICI in 2014. The overarching aim of the thesis is to identify novel biomarkers for ICI in patients with melanoma, using a combination of clinical factors, functional PET imaging, and comprehensive immune profiling of the tumour micro-environment.

The aims of the thesis is:

1. To assess patient performance status as a biomarker for tumour response, toxicity and survival outcome after anti-PD-1 treatment for advanced melanoma.
2. To identify baseline clinical characteristics and blood parameters that are associated with outcome after ICI treatment for melanoma brain metastases.
3. To assess functional FDG-PET imaging parameters as biomarkers for outcome after anti-CTLA4 or anti-PD-1 treatment for advanced melanoma
4. To compare the immune landscape of UV-related cutaneous malignancies (melanoma, Merkel cell carcinoma and squamous cell carcinoma)

1.4.1 Hypotheses and questions addressed in the thesis

The first two results chapters (Chapter 3 and 4 respectively) focused on the evaluation of patient clinical factors as potential biomarkers for outcome following ICI. Firstly, patient baseline performance status was assessed as a biomarker for treatment response and survival following anti-PD-1 in Chapter 3. The hypothesis was that, unlike cytotoxic chemotherapy, baseline performance status was not correlated with outcome following ICI, owing to its distinct mechanism of action. In our published manuscript (259), we compared response rates and survival after anti-PD-1 in patients with good baseline performance status to patients with poor performance status. We also evaluated whether baseline performance was associated with important palliative care measures, such as treatment and hospitalisation within last month of life, as well as the place of death.

Secondly, clinical biomarkers were evaluated in patients receiving ICI treatment for melanoma brain metastases in Chapter 4. Patients with melanoma brain metastases have very poor survival, with distinct clinical challenges previously owing to the lack of effective treatments for intracranial disease. Previous studies evaluated biomarkers for brain metastases prior to the introduction of ICI treatment (1, 2, 5-8). The hypothesis is that additional clinical features may be prognostic in patients treated with ICI for melanoma brain metastases. Chapter 4 was a post-hoc analysis of data from the Anti-PD-1 Brain Collaboration clinical trial, a multi-centre phase II study of combination ipilimumab and nivolumab or nivolumab in patients with melanoma brain metastases (3). Clinical factors and laboratory tests were evaluated as potential biomarkers for outcome using logistic regression models for treatment response and toxicity. Survival analyses were performed to test clinical characteristics and blood parameters as univariate and multi-variate variables.

In Chapter 5, the thesis then goes on to examine the role of FDG PET functional imaging as a biomarker for outcome following ICI. Some studies have reported an association between large tumour burden seen on CT with poor survival after anti-PD-1, but routinely used CT criteria do not fully evaluate the total burden of disease (274, 280). Baseline tumoural PET avidity (as measured by SUVmax or metabolic tumour volume) as well as immune avidity (measured by spleen to liver ratio) were evaluated as biomarkers for survival in patients treated with anti-CTLA4 and anti-PD-1 for advanced melanoma. The hypothesis is that PET assessment of not only disease volume but tumoural glycolytic activity or FDG-PET avidity within the immune system (e.g. high spleen to liver ratio) may be highly correlated with outcome. In our manuscript, we showed that high spleen to liver ratio was correlated with poor survival after ipilimumab (anti-CTLA4), so the study went on to investigate the clinicopathological correlates of high spleen to liver ratio. Additionally, we also attempted to evaluate early on-treatment PET (EOT PET) response was assessed as a predictor of treatment response at 6 months following anti-CTLA4 or anti-PD-1 for advanced melanoma.

The understanding of tumour immunobiology of skin cancer has been limited by the immune profiling techniques such as traditional immunohistochemistry. Nonetheless, the presence of tumour infiltrating lymphocytes and PD-L1 staining cells are both recognised as key features of immune activation in cancers. Comprehensive characterisation of T cell subsets and correlates of PD-L1 staining cells, in terms of

density and cellular spatial relationships as well as transcriptomic profile, will improve the understanding of the immune landscape of early stage skin cancers. Chapter 6 aimed to compare the immune profile of high-risk UV related skin cancers (melanoma, cutaneous squamous cell carcinoma and Merkel cell carcinoma) using orthogonal methods of bulk RNAseq and multi-spectral immunohistochemistry (m-IHC). The hypothesis is that these orthogonal methods of tumoural immune profiling may identify specific T cell subsets or transcriptomic profiles associated with PD-L1 infiltration that are present in melanoma but distinct from other cutaneous cancers. The frequency and spatial relationships of intra-tumoural T cell subsets and PD-L1 cellular infiltration were compared for the three skin cancers using m-IHC; and the transcriptomic profiles of high PD-L1 infiltrated melanoma were compared with high PD-L1 infiltrated Merkel cell carcinoma.

Lastly, the use of multi-factorial biomarkers for six patients with advanced melanoma treated with ICI is explored. These cases highlight the potential use of a combination of biomarkers including clinical factors, PET imaging, baseline blood parameters, as well as multi-spectral immunohistochemistry.

Overall, this thesis aims to provide an exploration of clinical, imaging and tumoural biomarkers intended to improve patient selection for ICI in patients with melanoma.

2 Materials and Methods

2.1 Performance status as a biomarker for anti-PD-1

Methods are as summarised in the published manuscript, ‘Clinical and palliative care outcomes for patients of poor performance status treated with anti-programmed death1 for advanced melanoma Asia-Pacific Journal of Clinical Oncology. 2017; 13(6):385-390’ (259). All patients with advanced melanoma (unresectable stage IIIC or IV) who received at least one dose of anti-PD1 antibody at Peter MacCallum Cancer Centre were included in the study. Retrospective review of medical records was undertaken. Patient demographics, stage of melanoma, brain or leptomeningeal involvement, baseline lactate dehydrogenase (LDH), response rates, treatment related toxicities, and survival data were recorded. Tumour responses were performed on a range of modalities including computerised tomography (CT), positron emission tomography (PET/CT) and magnetic resonance imaging (MRI) and radiologists’ assessment of response were recorded. Tumour response includes any radiological tumour reduction, progressive disease includes all enlarging lesions or new lesions and stable disease denotes neither tumour response nor progression. Toxicities were graded using the Common Terminology Criteria for Adverse Events (CTCAE version 4.0). Immune related adverse events were defined as treatment related diarrhoea, colitis, endocrinopathy, hepatitis, pneumonitis, rash and other toxicities deemed by the treating oncologist.

European Cooperative Oncology Group Performance Status (ECOG PS) scale was used to grade each patient’s performance status prior to treatment(254). For patients in the cohort who died, additional palliative care data were collected, including treatment or hospitalisation within one month of death, admission to an intensive care unit, and place of death. Chi-square tests were used to compare differences in the proportion of patients receiving each measure according to ECOG PS status (ECOG PS 0-1 vs ECOG PS 2-3). Kaplan-Meier estimates of OS were calculated separately for patients grouped by performance status and compared using a log-rank test, where P-values ≤ 0.05 were considered to be statistically significant. The median follow-up time was estimated from the inverse Kaplan-Meier method. Statistical analyses were performed using Graphpad Prism Version 6.0 5.

2.2 Biomarkers in brain metastases

A post-hoc analysis of Anti-PD-1 in Brain Collaboration (ABC) clinical trial (3) was performed to investigate clinical factors and laboratory parameters as biomarkers for outcome following ICI for melanoma brain metastases. The ABC clinical trial is multi-centre phase II study of combination ipilimumab and nivolumab or nivolumab in patients with melanoma brain metastases. Patients with asymptomatic brain metastases were treated in cohort A (combination nivolumab and ipilimumab), and cohort B (nivolumab monotherapy) were included. Cohort C from the original study was excluded, as this was a mixed cohort of patients with leptomeningeal disease, with symptomatic metastases despite previous local therapies.

The following statistical analyses were performed on R version 3.5.1. Survival analyses were performed in R using the Survival and Survminer packages. Patient characteristics and blood parameters were used as variables to generate predictive models for treatment response and survival. The variables were analysed as continuous variables, thresholded at the median of the cohort as 'high' or 'low', or thresholded at a clinically relevant level (i.e. upper limit of normal). Logistic regression models for response were generated. Response was defined as complete response or partial response as the best overall response using RECIST 1.1. Univariate and multivariable Cox regression models were generated for PFS and OS for the entire cohort, then for each of the treatment cohorts separately. Kaplan Meier curves, univariate and multivariable Cox regression models were generated for landmark treatment toxicity at 120 days for the entire cohort, then for each of the treatment cohorts separately.

2.3 Functional PET imaging

Baseline PET biomarkers

Baseline FDG-PET parameters were examined as prognostic biomarkers in patients treated with ipilimumab or anti-PD-1 at Peter MacCallum Cancer Centre. The detailed methodology of the study is described in the manuscript, '18F FDG PET/CT based spleen to liver ratio (SLR) associates with clinical outcome to ipilimumab in patients with metastatic melanoma'(378). In brief, all patients with unresectable stage III or stage IV melanoma who received monotherapy with ipilimumab or

anti-PD1 (either pembrolizumab or nivolumab) and had completed a pre-treatment FDG-PET scan at Peter MacCallum Cancer Centre (PMCC) between 1st July 2010 and 30th June 2015 were included. Medical records were retrospectively reviewed for patient demographics, disease characteristics, blood parameters, treatment and clinical outcome. Treatment response was determined by local treating oncologist based on available clinical and imaging data. We then sought to validate the association between $SLR > 1.1$ and OS after ipilimumab in an additional cohort. Eligible patients treated at UZ Brussel, Brussels and University Hospital Zurich, Zurich were combined into a single validation cohort. Patients treated with at least one dose of ipilimumab were included and patient demographics, date of death or last contact were recorded.

FDG PET/CT parameters (tumoural SUVmax, MTV and SLR) were tested for association with PFS and OS after ipilimumab and anti-PD1, with treatment line and metastasis substage considered as confounding variables(11, 379). PFS and OS were tested for association with PET/CT parameters using Cox proportional hazards regression. The median tumoural SUVmax and MTV were calculated using all pre-treatment scans in the discovery cohort (n=110). These median values were used to dichotomize the cohort to compare high tumoural SUVmax versus low tumoural SUVmax, as well as high MTV versus low MTV. Tumoural SUVmax and MTV were also analysed as continuous variables. The SLR normally varies within a tight range of 0.9-1.0; consequently $SLR > 1.1$ was considered abnormally high and used as the threshold for survival analyses. PFS was assessed and modelled separately for ipilimumab and anti-PD1 and was measured from the start of immunotherapy to the date of progression or death; patients were censored at date of last contact. Multivariate analyses for overall survival considered LDH, metastasis substage (AJCC 7th Edition)(11), MTV, ALC and SLR. Multi-variable model for SLR considered neutrophil:lymphocyte ratio, haemoglobin, LDH, ALC $\times 10^9$, albumin, lymphocyte: monocyte ratio and patient performance status. Statistical analyses were performed using the base package and the survival package (R version 3.4.2). Kaplan-Meier survival curves were used to present survival outcome for SUV and MTV as dichotomized at the median for the treatment cohort, or at the abnormal threshold of

SLR>1.1.

A subset of patients in the discovery cohort were also enrolled in a separate biomarker study where their sera were stored at multiple time points. Pre-treatment samples were taken at baseline, prior to treatment at week 3 and prior to week 6. We sought to evaluate baseline and dynamic changes in the cytokine profile of patients with high SLR compared to those with normal SLR. Stored serum samples were tested using the BD Cytometric Bead Array Enhanced Sensitivity kit for IL1 β , IL2, IL4, IL6, IL10, IL12p70, IL17A, TNF, IFN γ as per the manufacturer's protocol. Experiments were performed in duplicate. Samples were analysed using the BD FACSVerse analyser and results summarised using Graphpad Prism.

Early on-treatment PET biomarkers

Early on-treatment (EOT) scans were performed 2-3 weeks after the commencement of treatment. PET parameters of SUVmax, MTV and SLR on EOT PET scans were compared to baseline PET parameters and analysed as a predictor of clinical benefit at 6 months. Patients were defined as deriving clinical benefit if the treating clinician deemed the patient to have complete response or partial response or stable disease at 6 months as assessed by radiological or clinical assessment. No formal radiological treatment assessment criteria were used. Patients who died without radiological assessment were defined to have progressive disease. Waterfall plots are presented to show the absolute change of each PET parameter on EOT PET scans compared to baseline PET scans.

Overall survival was calculated as date of death from date of first treatment or censored at the date of last contact. For each baseline PET parameter, log-rank survival analyses were performed to assess 1) any absolute reduction on EOT PET scans compared to baseline PET and 2) > 30% reduction from baseline PET. Survival analyses were performed using log-rank test in GraphPad Prism Version 8.0.2.

2.4 Comparison of UV related cutaneous malignancies

Orthogonal immunoprofiling techniques were used to interrogate the immune profile of primary skin cancer samples including melanoma, Merkel cell carcinoma (Merkel or

MCC), and cutaneous squamous cell carcinoma (cSCC). Novel tyramide signal amplification (TSA) multiplex immunohistochemistry technique was used, where 7 antibody markers were stained simultaneously to explore the density of T cell populations, PD-L1 staining cells and their spatial relationships with tumour cells. Bulk ribonucleic acid sequencing (RNAseq) was then performed on serial sections from the same samples to identify differentially expressed genes.

Multispectral immunohistochemistry (mIHC)

Samples of melanoma were collected through Melanoma Research Victoria. Samples of Merkel cell carcinoma were collected at 3 Australian institutions (Peter MacCallum Cancer Centre, Melanoma Institute of Australia and Royal North Shore Hospital). Samples of squamous cell carcinoma were collected from University of Essen, Germany.

The Opal mIHC staining method for T cell characterisation in melanoma was optimised by Heloise Halse (380). This method was subsequently modified by Dr Kelly Waldeck for MCC and myself for SCC. 4µm formalin fixed and paraffin embedded (FFPE) sections of tumour were mounted on Superfrost Plus slides (Thermo-Scientific). Slides were dewaxed, washed in water for 2min, followed by wash in Tris Buffer Saline and Tween (TBST) twice for 2 min. For the T cell characterisation panel, each antibody marker was applied in turn: CD3, CD4, CD8, FOXP3, PD-L1, tumour marker (SOX10 for melanoma, synaptophysin for MCC and p40 for SCC) and DAPI. See Table 2.1 and Table 2.2 for details. Tonsillar tissue was used as positive control for CD3, CD4, CD8, FOXP3, DAPI and placental tissue was used as positive control for PD-L1. Primary antibodies were applied to the slides and incubated at room temperature for 30 min and washed with TBST 3 times for 5 min each time. Secondary antibodies were applied and incubated for 10min at room temperature. Slides were washed 3 times with TBST for 5 min. TSA fluorescent dyes were applied and incubated at room temperature for 10 min. Slides were washed three times in TBST for 5 min. Slides were microwaved in antigen retrieval buffer for 1min at 100% power, 15 min at 10% power, then cooled for 30 min before the next antibody was applied. After all 6 antibodies had been applied in sequence, DAPI was applied for nuclei counterstain and incubated for 2min at room temperature. Vectrashield medium was applied and covered with coverslip.

All slides were scanned on Vectra® 3.0 Automated Quantitative Pathology Imaging System, 200 slide (Perkin Elmer, MA) at 10X magnification, with subsequent high-powered fields of view (HPF) taken at 20X (resolution of 0.5um per pixel) throughout the tissue section to obtain a representation of tumour, stroma and tumour margin. Images were analysed on inForm® Software v2.2 (Perkin Elmer, MA) to demarcate tissue compartments (i.e. tumour, stroma or tumour margin), and cellular phenotypes were identified based on the fluorescence intensity of each antibody. Tumour was defined as area with tumour positive cells (SOX10 for melanoma, synaptophysin for MCC and P40 for SCC), stroma was defined as tumour negative areas distant from the tumour; and tumour margin was the region immediately adjacent to the tumour perimeter. The InForm software was trained to recognise each cellular phenotype based on 20 representative cells, with subsequent batch analysis of all high powered images for each specimen sample, with 1.338/mm² in each HPF. Cellular density was calculated for each sample and expressed as cells/mm². For the melanoma samples, PD-L1 staining cells were further phenotyped dependent on co-staining of CD3 or SOX10 to examine whether the PD-L1 expression was on T cells or tumour cells respectively. Spatial relationships between cells were measured using the HALO software program (381). Composite images from the Vectra microscope were used and cells were phenotyped. The average distance between PD-L1 cells to T cell subsets and the average distance between tumour cells to T cells subsets were calculated, and compared between the three cancers.

Statistical analysis for mIHC

R version 3.3.1 and Graphpad Prism 7 were used to analyse the data generated from mIHC. Kruskal-Wallis test was used to compare cellular density for the 3 different skin cancers. Samples were then categorised by their predominant T cell subtype, where the T cell subset (either CD3, CD4, CD8 or FOXP3) represented greater than 50% of the intratumoural T cells. Associations of PD-L1 density were studied in more detail as follows. Mann-Whitney test was used to compare PD-L1 density in ulcerated and non-ulcerated melanoma. Mann-Whitney test was also used to compare PD-L1 density in Merkel Cell Polyoma Virus (MCPyV) positive and MCPyV negative MCC samples. Spearman correlation was used to test the correlation between density of T cell subsets (CD3, CD4, CD8, FOXP3) as well as PD-L1+ cells. Only significant results are

represented in the figures. The average distances between T cell subsets and PD-L1+ cells were compared for the 3 skin cancers using the Kruskal-Wallis test.

RNA-sequencing (RNAseq)

A matched subset of samples studied by mIHC went on to be profiled by bulk RNA sequencing (RNAseq): 17 melanoma, 19 MCC and 5 SCC. Serial FFPE slides used for mIHC were used for RNAseq. RNA was isolated from macro-dissected FFPE tissue sections using the AllPrep DNA/RNA FFPE Kit (Qiagen, Germany) according to the manufacturer's instructions. RNA extraction was done using the RNeasy FFPE RNA extraction kit according to manufacturer protocols (Qiagen, Germany). RNA-seq library preparation and hybridisation capture enrichment were done using either Illumina RNA-Access (Illumina, USA) or Agilent RNA-Direct (Agilent, USA) kits according to manufacturers' protocols. Total RNA-seq libraries were prepared using the NEBNext Ultra™ II Directional RNA Library Prep Kit for Illumina (rRNA depletion workflow)(NEB, USA). Sequencing was done using Illumina HiSeq 2000, NextSeq and Nova-Seq 6000 (Illumina, USA). Gene expression analyses were performed using R version 3.5.0. Gene symbols were converted to Entrez ID using the annotations in the Org.Hs.eg.db package. Of the 57,905 genes, 38,895 genes were discarded for this analysis. All counts per million (CPM) and log₂CPM values were normalised and filtered using the *EdgeR* package (382). Only genes with counts of CPM greater than 1.0 in five or more samples were considered expressed (as the smallest group, squamous cell carcinoma, consisted of 5 samples). This filtering resulted in a list of 25,582 genes.

Multi-dimensional scaling plots were used as a visualisation of principle components analysis. This identifies the greatest determinant of variation for the gene expression data. Unsupervised clustering of samples was performed on a customised list of 1270 genes related to immune processes and immune cell types, previously published by Galon and colleagues (383) and Nanostring PanCancer Immune panel (384). Gene expression of *PDCDI* was correlated with PD-L1 density using Spearman correlation.

Tumour mutational burden was correlated with PD-L1 using Spearman correlation. Variant calling from RNA-Seq was performed to generate the tumour mutation burden. Variants were called using a modified version of the GATK best practices for variant calling from RNA-Seq (<https://gatkforums.broadinstitute.org/gatk/discussion/3892/the->

gatk-best-practices-for-variant-calling-on-rnaseq-in-full-detail), with the exception that GATK version 4 rather than 3 was used (GATK version 4.1.0.0) (385, 386). Variants were also called using VarScan (version 2.4.3)(387) with a minimum of 8 bases of coverage and 3 bases supporting each variant call. To be considered a true call, the variant had to be called by both the GATK HaploypeCaller and VarScan. To filter out germline variants, all variants present in the dbSNP (version 151) were removed and additionally any variant present in more than 20% of the cohort was removed. To remove mutations caused by RNA-editing, any sites present in the RADAR RNA-editing database (388) were also removed. The method was originally developed using 167 lung squamous cell carcinoma (LUSC) RNA-Seq samples from the TCGA with matched whole exome sequencing (WES) data. The method was able to call variants with only 6% recall, but 74% precision. The TMB from RNA-Seq variant calling was also validated against an external cohort made up of 11 WGS samples comprising of 9 cancer of unknown primary (CUP) samples and 2 MCC samples with a Pearson's correlation of 0.98. Despite the low recall rate, UV mutational signatures were still able to be identified and were dominant in the viral negative samples. TMB was calculated for a given sample by counting the total number of exon bases that had a coverage of 8 or more using Mosdepth (version 0.2.5) (389), dividing this by the total number of detected coding mutations and multiplying by 1 million (mutations/megabase).

Differential expression analysis was performed on the melanoma and MCC samples with the *limma-voom* package (390) with the design 0 + condition. Melanoma and MCC samples were dichotomized at the median of the group for PD-L1 density to define high PD-L1 subgroup and low PD-L1 subgroup. Volcano plots were used to visualise genes that were expressed differentially between 1) high and low PD-L1 expressing melanoma, 2) high and low PD-L1 expressing MCC and 3) high PD-L1 expressing melanoma compared to high PD-L1 expressing MCC. For the purpose of the Volcano plots, $\log(p\text{-value})$ was presented graphically with significant values presented as $p\text{-value} < 0.01$. Genes with an adjusted $P\text{-value}$ of < 0.01 (based on the moderated $t\text{-statistic}$ using the Benjamini-Hochberg (BH) method to control the false discovery rate and a $|\log_2FC| > 2$ were considered significantly differentially expressed to identify the number of genes upregulated in a) high PD-L1 staining melanoma compared to high PD-L1 staining MCC. Gene set enrichment and network analyses were performed on

the significantly upregulated and down-regulated genes in melanoma compared to MCC using the Metascape gene set enrichment analyses program (391).

2.5 Patient case studies for the application of biomarkers

Patients with advanced melanoma who were treated at Peter MacCallum Cancer Centre were offered the option of participating in a voluntary biomarker research study, whereby their clinical, imaging, blood and tissue samples were stored for research. Patients' demographics, disease characteristics, treatment timeline and outcomes (best response to each line of treatment) are presented. Pre-treatment FDG PET scans performed in the 6 weeks prior to commencing treatment were analysed for metabolic tumour volume (MTV) and spleen to liver ratio (SLR). Biospecimens were taken at diagnosis, pre-treatment and throughout treatment course. Routine pre-treatment laboratory tests were performed, including LDH, neutrophil: lymphocyte count (NLR), albumin.

Pre-treatment tumoural samples were evaluated using multi-spectral immunohistochemistry. The mIHC T cell panel was used to study the density and distribution of SOX10 (melanoma), CD3, CD4, CD8, FOXP3, PD-L1+CD3 and PD-L1+SOX10. The Pan-Immune panel was used to study the density and distribution of CD3 (T cells), CD11c (dendritic cells), CD20 (B cells), CD68 (macrophages) and SOX10 (melanoma). The primary antibody reagents are described in Table 2.1 Reagents used for the melanoma or cSCC T cell characterisation mIHC panel and Table 2.3 Reagents used for the Pan-immune characterisation mIHC panel. The staining protocol, data acquisition and analysis methodology for the Pan-immune panel was the same as for the T cell panel.

2.6 Tables

Table 2.1 Reagents used for the melanoma or cSCC T cell characterisation mIHC panel

T cell characterisation panel	Primary antibody	Secondary antibody	TSA fluorescent dye
CD3	Rabbit monoclonal CD3, Spring Bioscience, Clone SP7, concentration 1:1000	Rb (1:1000)	520 (1:50)
CD4	Rabbit monoclonal CD4, Spring Bioscience, Clone SP35, concentration 1:100	Rb (1:500)	650 (1:50)
CD8	Mouse monoclonal CD8, Thermofisher, clone 4B11, concentration 1:500	Ms (1:1000)	690 (1:50)
FOXP3	Rabbit polyclonal FOXP3, BioSB, concentration 1:100	Rb (1:1000)	570 (1:50)
PD-L1	Rabbit monoclonal PD-L1, Spring Bioscience, clone SP142, concentration 1:1000	Rb (1:1000)	540 (1:50)
SOX10 (Melanoma tumour stain)	Mouse monoclonal SOX10, Biocare Medical, clone BC34, concentration 1:500	Ms (1:1000)	620 (1:50)
P40 (cSCC tumour stain)	Mouse monoclonal P40, Biocare Medical, clone BC28, 1:1000	Ms (1:1000)	620 (1:50)

The tumour stain for melanoma (SOX10) can be swapped directly with the tumour stain for cSCC (P40), while the other T cell subsets and PD-L1 reagents remain the same.

Table 2.2 Reagents used for the MCC T cell characterisation mIHC panel

T cell characterisation panel	Primary antibody	Secondary antibody	TSA fluorescent dye
CD3	Rabbit monoclonal CD3, Spring Bioscience, Clone SP7, concentration 1:1000	Rb (1:1000)	520 (1:50)
CD4	Rabbit monoclonal CD4, Spring Bioscience, Clone SP35, concentration 1:100	Rb (1:500)	650 (1:50)
CD8	Mouse monoclonal CD8, Thermofisher, clone 4B11, concentration 1:500	Ms (1:1000)	620 (1:50)
FOXP3	Rabbit polyclonal FOXP3, BioSB, concentration 1:100	Rb (1:1000)	570 (1:50)
PD-L1	Rabbit monoclonal PD-L1, Spring Bioscience, clone SP142, concentration 1:1000	Rb (1:1000)	540 (1:50)
Synaptophysin (MCC tumour stain)	Mouse monoclonal Synaptophysin, Leica Biosystems, clone 27912, Concentration 1:100	Ms (1:1000)	690 (1:50)

Table 2.3 Reagents used for the Pan-immune characterisation mIHC panel

Pan-immune characterisation panel	Primary antibody	Secondary antibody	TSA fluorescent dye
CD3	Rabbit monoclonal CD3, Spring Bioscience, Clone SP7, concentration 1:1000	Rb (1:1000)	520 (1:50)
CD11c	Rabbit monoclonal CD11c, BioSB, clone EP157, concentration 1/2000	Rb (1:500)	570 (1:50)
CD20	Mouse monoclonal CD20, DAKO, clone L26, concentration 1/500	Ms (1:1000)	690 (1:50)
CD68	Mouse monoclonal CD68, Thermofisher, clone 514H12, concentration 1/200)	Rb (1:1000)	540 (1:50)
PD-L1	Rabbit monoclonal PD-L1, Spring Bioscience, clone SP142, concentration 1:1000	Rb (1:1000)	650 (1:50)
SOX10 (Melanoma tumour stain)	Mouse monoclonal SOX10, Biocare Medical, clone BC34, concentration 1:500	Ms (1:1000)	620 (1:50)

3 Performance status as a biomarker for anti-PD-1

3.1.1 Introduction

Baseline performance status has been associated with prognosis in multiple cancer types including melanoma. Performance status is commonly defined using the European Cooperative Oncology Group (ECOG PS) grading, where ECOG PS 0, asymptomatic; ECOG PS 1, restricted in activity but ambulatory; ECOG PS 2, ambulatory, capable of all self-care and up and about more than 50% waking hours; ECOG PS 3, capable of limited self-care but confined to bed or chair more than 50%, ECOG PS 4, completely disabled and cannot carry out any self-care, ECOG PS 5, dead (254). The landmark randomised clinical trials of anti-CTLA4 or anti-PD-1 or their combination in melanoma have routinely excluded patients with poor performance status, i.e. patients with ECOG PS more than 1(166, 172, 174, 176, 187). Treatment with traditional cytotoxic chemotherapy in patients with poor baseline PS is associated with poor survival and reduced quality of life (255, 392). Given the distinct mechanism of action of ICI, it was not known whether patients with poor baseline PS would be able to derive tumour response to it. Furthermore, the unprecedented success of ICI in treatment resistant cancers, coupled with the favourable toxicity profile compared to chemotherapy, has resulted in significant interest from both clinicians and patients to trial ICI even in patients with poor PS (240, 393). This problem is exacerbated by the lack of robust predictive biomarkers that differentiate likely responders from non-responders, resulting in challenging clinical scenarios where patients are faced with potential hope of durable response even though the majority of patients will be treatment resistant (394, 395).

At the time of starting this thesis, there was a paucity of literature on outcomes of patients with poor baseline performance status prior to ICI, so our retrospective study was one of the first published studies to report on this challenging group of patients. Our publication entitled, ‘Clinical and palliative care outcomes for patients of poor performance status treated with antiprogrammed death-1 monoclonal antibodies for advanced melanoma.’ is presented here as the results of this thesis chapter.

3.1.2 Aims

The aims of the study were:

1. To compare the survival and tumour response rate of anti-PD-1 in patients with good baseline performance status (ECOG PS 0-1), with that of patients with poor baseline performance status (ECOG PS 2-3).
2. To compare the rates of treatment within last month of life, hospitalisation and place of death in patients with good baseline performance status (ECOG PS 0-1), with those patients with poor baseline performance status (ECOG PS 2-3).
3. To compare the toxicity rate of anti-PD-1 in patients with good baseline performance status (ECOG PS 0-1), with that of patients with poor baseline performance status (ECOG PS 2-3).

3.1.3 Summary of results

1. Patients with poor baseline PS have poor survival (median OS for ECOG PS 0-1 was 19.5 months compared to 1.8 months for ECOG 2-3 with HR 5.5; 95% CI 9.1-50.3, $p=0.0001$).
2. Patients with poor baseline PS have lower tumour response rate, with 23/58 (39%) in ECOG PS 0-1 and 2/16 (12%) in ECOG PS 2 and 0/8 in ECOG PS 3.
3. ECOG PS 2–3 patients were more likely to be treated and hospitalised within the last month of life compared to ECOGPS 0–1 patients, RR 1.75 (95% CI, 1.04–2.56, $P = 0.019$) and RR 1.73 (95% CI, 1.10–2.16, $P = 0.009$), respectively.
4. Patients with poor PS are more likely to die in hospital RR 2.68 (95% CI, 1.17–6.51, $P = 0.016$).
5. Toxicity did not differ depending on baseline performance status.

3.1.4 Discussion

This small retrospective study reported on the outcomes of 91 patients treated with anti-PD-1 at Peter MacCallum Cancer Centre. Patients with poor performance status (ECOG PS 2 and 3) had poor survival and lower likelihood of tumour response compared to those with good performance status. Patients with poor performance status were more likely to be treated and hospitalised within their last month of life, and were more likely to die in hospital. To our knowledge, our study was the first to publish on efficacy and toxicity outcomes in patients with poor performance status in advanced melanoma (259). These findings are in keeping with existing data about poor outcomes in patients who are treated with cytotoxic chemotherapy. Despite reports of exceptional and rapid response with immunotherapy (396), this was not observed in patients with poor performance status. It is noted that a minority of patients (12%) with poor performance status did derive a tumour response, despite its association with a dismal median survival of 1.8 months.

Recently a prospective phase II study evaluated nivolumab in challenging populations, such as those with central nervous system metastases, poor performance status, and patients with underlying auto-immune conditions or previous immune toxicities from ipilimumab (397). Sixty-six of the total one thousand and eight patients in the study had ECOG PS 2. The median survival for the patients with poor PS was less than 3

months compared to 21.4 months in the total population. Interestingly there was a subset of patients with poor PS who had durable survival, where 12 of 66 patients (18.8%) with ECOG PS2 were alive at 18 months. Patients with poor performance status also showed other characteristics associated with poor prognosis, for instance: 27.3% had M1c disease with brain metastases, 13.6% had mucosal melanoma, 78.8% had raised LDH greater than upper limit of normal. This study is the first prospective study of anti-PD-1 in patients with poor PS; the findings are in keeping with the findings of our retrospective study.

Another point of interest is that prospective studies of patients with good performance status (ECOG PS 0 and 1) also show trends for improved survival in patients who are asymptomatic (237, 248). This observation would be supportive of more intensive screening of patients at high risk of systemic relapse in order to detect patients while they are asymptomatic. Furthermore, it also supports the treatment by means of adjuvant therapy, where patients are yet to have visible metastatic disease and are more likely to be at maximal performance status.

Toxicity in the phase II study of nivolumab in challenging populations was reported as comparable to previous clinical trials of anti-PD-1 (397). 18.2% and 13.6% of the total study population and ECOG PS 2 subgroup experienced grade 3 or 4-treatment related toxicity respectively. Our retrospective study had similar findings where 13% of patients with ECOG PS 2 or 3 experienced a grade 3 or 4 toxicity (259). However, both studies may be underestimating the toxicities experienced by patients with poor PS as their duration of treatment is shorter, and yet the rate of toxicities is similar to patients with good PS where the treatment is longer, thus giving a longer period of time to accrue toxicities. Therefore, toxicities at short landmark intervals such as 3 month and 6 months may be more informative.

Randomised, prospective studies of patients with poor performance status (such as ECOG PS2) are needed to differentiate whether performance status is a predictive biomarker for ICI as well as a prognostic biomarker. Data on the palliative care needs of patients with poor performance status are also lacking. Our group and other authors have also expressed challenges over how to balance patients' hopes in the face of uncertainty, given that only the minority of patients will achieve these durable responses (394, 398). Some of the potential problems of ICI in this patient cohort relate to the

lack of palliative care involvement if patients perceive themselves to be having treatment with durable response, and the associated opportunity costs that come with remaining on futile treatment. There are also significant financial implications of trialling these expensive treatments, either as a personal cost to the patient or to the health system. Lastly, there is concern about the use of steroids in patients receiving immunotherapy, as it potentially reduces the efficacy of treatment (399). This results in a reluctance to use steroids by both clinicians and patients. The lack of steroid use potentially worsens patients' quality of life as steroids have been demonstrated to relieve various cancer related symptoms (400). Prospective studies may also be able to better assess toxicity using landmark time points, with particular consideration of financial toxicity. A more comprehensive exploration of the quality of life domains using validated tools may be able to highlight some of the palliative care needs of patients with poor performance status.

3.1.5 Publication: 'Clinical and palliative care outcomes for patients of poor performance status treated with antiprogrammed death-1 monoclonal antibodies for advanced melanoma.'

Clinical and palliative care outcomes for patients of poor performance status treated with anti-programmed death-1 monoclonal antibodies for advanced melanoma

Annie Wong^{1,2}  Molly Williams³ Donna Milne⁴  Kortnye Morris¹
 Peter Lau^{1,2} Odette Spruyt^{2,3} Sonia Fullerton³ Grant McArthur^{1,2}

¹Department of Cancer Medicine, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia

²Sir Peter MacCallum Department of Oncology, University of Melbourne, Melbourne, Victoria, Australia

³Department of Palliative Care, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia

⁴Department of Cancer Experiences Research, Peter MacCallum Cancer Centre, Melbourne, Victoria, Australia

Correspondence

Annie Wong, Medical Oncologist, Department of Cancer Medicine, Peter MacCallum Cancer Centre, Victorian Comprehensive Cancer Centre, 305 Grattan St, Melbourne, VIC 3000, Australia.
 Email: Annie.wong@petermac.org

Abstract

Background: Anti-programmed death-1 antibodies (anti-PD1) have response rates of 40% in metastatic melanoma. Patients with poor performance status (PS) were excluded from clinical trials, yet use of anti-PD1 is widespread in clinical practice. Literature regarding clinical and palliative care outcomes in patients with poor PS treated with anti-PD1 is lacking.

Methods: Retrospective review of outcomes for all patients with advanced melanoma treated with anti-PD1 between 2012 and June 2015 at Peter MacCallum Cancer Centre, a tertiary specialist cancer center in Australia.

Results: Between 2012 and 2015, 91 patients received anti-PD1: median age 63, 65% males, 77% elevated LDH > 1xULN (37/48 patients). Fifty-eight patients had baseline ECOG PS of 0–1 (64%), 24 patients ECOG PS 2–3 (26%) and ECOG PS was not recorded in nine patients (10%). Median overall survival (OS) for the ECOG PS 0–1 group was 19.5 months and 1.8 months for ECOG PS 2–3 (HR 5.5; 95% CI, 9.1–50.3; $P = 0.0001$). Tumor response was 23/58 (39%) in ECOG PS 0–1, 2/16 (12%) in ECOG PS 2 and 0/8 in ECOG PS 3. Toxicity did not differ between different groups. ECOG PS 2–3 patients were more likely to be treated and hospitalized within the last month of life compared to ECOG PS 0–1 patients, RR 1.75 (95% CI, 1.04–2.56, $P = 0.019$) and RR 1.73 (95% CI, 1.10–2.16, $P = 0.009$), respectively. ECOG PS 2–3 patients were more likely to die in an acute hospital RR 2.68 (95% CI, 1.17–6.51, $P = 0.016$).

Conclusions: Patients with poor baseline PS have a significantly lower OS and reduced response to anti-PD1. Further quality of life and palliative care research is needed.

KEYWORDS

anti-programmed death-1, immunotherapy, melanoma, palliative care

1 INTRODUCTION

Immune checkpoint inhibitors such as anti-programmed death one antibodies (anti-PD1) have rapidly entered clinical practice for many tumor types including melanoma,^{1–3} non-small cell lung cancer,^{4,5} renal cancer⁶ and Hodgkins lymphoma.⁷ Biomarkers which predict response to these treatments are under intensive investigation, although few have translated into clinical practice at the same pace as the treatments themselves. Traditional clinical biomarkers such as performance status (PS) have yet to be formally assessed, as patients with poor PS were excluded from the landmark clinical trials. Unlike cytotoxic chemotherapy, anti-PD1 antibodies are well tolerated and offer the

prospect of durable benefit. As such, clinicians may be tempted to use these agents in patients with poor PS, with the hope they will salvage aggressive disease.

Traditional cytotoxic chemotherapy in patients with poor PS are associated with shorter survival and worse quality of life in some cancer types,^{8,9} hence the American Society of Clinical Oncology have recommended against the use of chemotherapy in patients with European Cooperative Oncology Group (performance status (ECOG PS) score of three or more.¹⁰ However, anti-PD1 antibodies have a distinct mechanism of action and toxicity profile compared to chemotherapy. Therefore, it is important to review the efficacy and toxicity rates in patients with poor PS treated with anti-PD1 antibodies to determine if these

agents are a therapeutic benefit in this subset of patients. Furthermore, there are some palliative care outcomes such as anti-PD1 treatment patterns in the last month of life and the place of death which may inform the palliative care needs for those with advanced malignancies. We sought to investigate the real-world outcomes of anti-PD1 treatment in melanoma patients of both good and poor performance status.

1 METHOD

All patients with advanced melanoma (unresectable stage IIIC or IV) who received at least one dose of anti-PD1 antibody at Peter MacCallum Cancer Centre, a specialist cancer center in Australia, were included in the study. Retrospective review of medical records was undertaken. Patient demographics, stage of melanoma, brain or leptomeningeal involvement, baseline lactate dehydrogenase (LDH), response rates, treatment related toxicities and survival data were recorded. Tumor responses were performed on a range of modalities including computerized tomography (CT), positron emission tomography (PET/CT) and magnetic resonance imaging (MRI) and radiologists' assessment of response were recorded. Tumor response includes any radiological tumor reduction, progressive disease includes all enlarging lesions or new lesions and stable disease denotes neither tumor response nor progression. Toxicities were graded using the Common Terminology Criteria for Adverse Events (CTCAE version 4.0). Immune-related adverse events were defined as treatment related diarrhea, colitis, endocrinopathy, hepatitis, pneumonitis, rash and other toxicities deemed by the treating oncologist.

ECOG PS scale was used to grade each patient's performance status prior to treatment.¹¹ ECOG PS for patients in the cohort who died, additional palliative care data were collected, including treatment or hospitalization within one month of death, admission to an intensive care unit and place of death. Chi-square tests were used to compare differences in the proportion of patients receiving each measure according to ECOG PS status (ECOG PS 0–1 vs ECOG PS 2–3). Kaplan–Meier estimates of OS were calculated separately for patients grouped by performance status and compared using a log-rank test, where $P \leq 0.05$ were considered to be statistically significant. The median follow-up time was estimated from the inverse Kaplan–Meier method. Statistical analyses were performed using Graphpad Prism Version 6.0.5. This study was approved by the institutional research ethics committee.

2 RESULTS

Between 2012 and 2015, 91 patients were treated with anti-PD1 antibodies for advanced melanoma. Fifty-eight (67%) patients had baseline ECOG PS of 0–1, 16 (13%) patients had ECOG PS 2 and 8 (9%) had ECOG PS 3. Nine (10%) patients without ECOG PS recorded were excluded from analysis. The baseline characteristics of patients are described in Table 1. The median follow-up from the start of anti-PD1 treatment for all patients was 16.8 months. Tumor responses were assessed using positron emission tomography in 34 patients (41%),

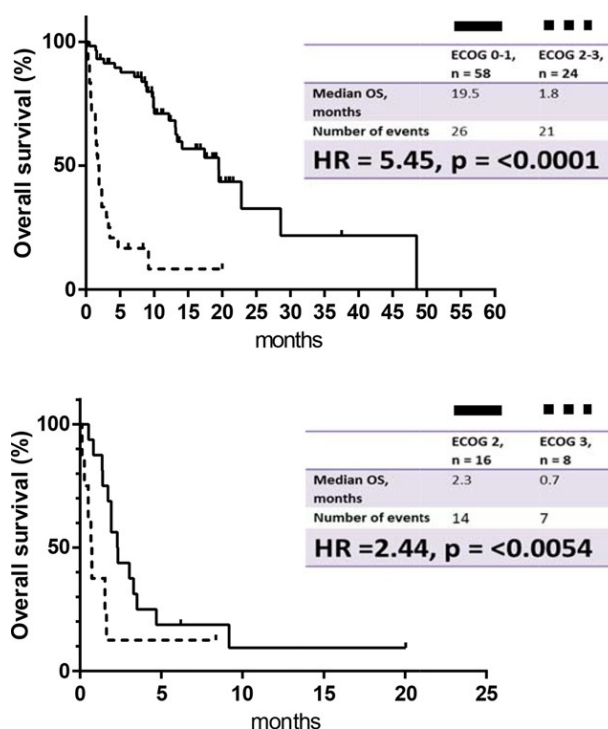


FIGURE 1 Overall survival after anti-PD1 for patients with metastatic melanoma. Overall survival of patients with advanced melanoma with ECOG PS 0–1 compared to ECOG PS 2–3 (top); overall survival of patients with advanced melanoma divided into subgroups of ECOG PS 2 compared to ECOG PS 3 (bottom) [Colour figure can be viewed at wileyonlinelibrary.com]

computerized tomography in 28 patients (34%), MRI in 3 patients (4%) and clinical progression (not imaged) in 17 patients (21%; Table 2).

Median overall survival (OS) for ECOG PS 0–1 group was 19.5 months versus 1.8 months for ECOG PS 2–3 group (HR 5.5; 95% CI, 9.1–50.3; $P = 0.0001$; Figure 1, top). When the poor performance status group was subdivided into ECOG PS 2 versus ECOG PS 3, the median OS for ECOG PS 2 and ECOG PS 3 was 2.3 months versus 0.7 months, respectively (HR 2.4; 95% CI, 1.1–10.6; $P = 0.005$; Figure 1, bottom).

3 TOXICITY

The incidence of severe, life-threatening or fatal (CTCAE version 4 grade 3–5) treatment-related adverse events or immune-related adverse events did not differ significantly between the two groups (RR 0.30, $P = 0.58$ and RR 2.04, $P = 0.15$, respectively; Table 3).

There was one patient in the ECOG PS 3 group where a treatment-related death could not be excluded: a 62-year-old woman with metastatic melanoma to lungs, bone, liver and lymph nodes. She presented with breathlessness and hypoxia after two doses of anti-PD1 and was found to have an increase in pulmonary infiltrates, which did not respond to high-dose corticosteroids or antibiotics. Although she had bilateral pulmonary metastases, an immune-related pneumonitis could not be excluded. Of note, there were no other grade 2–4 immune-related adverse events in the poor performance group.

TABLE 1 Baseline demographic and disease characteristics

Characteristics	ECOG PS 0–1 (n = 58)	(%)	ECOG PS 2 (n = 16)	(%)	ECOG PS 3 (n = 8)	(%)
Median age (range) (year)	60 (30–88)		73.5 (36–88)		54.5 (36–79)	
Male sex, no. (%)	36	(62)	13	(81)	4	(50)
Elevated LDH > 1xULN	22/33	(67)	10/10	(100)	5/5	(100)
LDH Not recorded	25		6		3	
Metastasis stage, no. (%)						
IIIC	2	(3)	0	(0)	0	(0)
M1a	4	(7)	1	(6)	0	(0)
M1b	14	(24)	2	(13)	1	(13)
M1c	38	(66)	13	(81)	7	(88)
Central nervous system Metastases, no. (%)						
Brain	9	(16)	6	(38)	0	(0)
Leptomeningeal	2	(3)	1	(6)	0	(0)
Subtype						
Cutaneous	37	(64)	11	(68)	6	(75)
Mucosal	7	(12)	2	(13)	0	(0)
Acral	1	(2)	0	(0)	1	(13)
Ocular	5	(9)	0	(0)	0	(0)
Unknown primary	8	(14)	3	(19)	1	(13)
Line of previous systemic therapy						
0	7	(12)	1	(6)	1	(13)
1	26	(45)	8	(50)	2	(25)
2	12	(21)	3	(31)	5	(63)
3 or more	13	(22)	4	(25)	0	(0)

TABLE 2 Tumor responses after anti-PD1 treatment

	ECOG PS 0–1 (n = 58)	(%)	ECOG PS 2 (n = 16)	(%)	ECOG PS 3 (n = 8)	(%)
Tumor Response	23	(40)	2	(12)	0	(0)
Stable disease	7	(12)	0	(0)	1	(12)
Progressive disease	28	(48)	14	(88)	7	(88)
Total	58		16		8	

1 PATTERNS OF HEALTH CARE IN THE LAST MONTH OF LIFE

Fifty-two patients (57%) of the total cohort died. Of these, 26 had a baseline ECOG PS of 0–1, 21 were ECOG PS 2–3 and five had no baseline ECOG PS recorded and were excluded from analysis. Eighty-one percent of the ECOG PS 2–3 group received anti-PD1 antibody in the last month of life, compared with 46% of the ECOG PS 0–1 group (RR 1.75, $P = 0.019$; Table 4). Similarly, 90% of the patients of ECOG PS 2–3 were admitted to hospital in the last month of life, whereas only 52% of the ECOG PS 0–1 patients had hospital admissions (RR 1.73, $P = 0.009$). All hospitalization within the last month of life was for management of disease progression. The only apparent admission for treatment related toxicity was the aforementioned case of the patient with pulmonary infiltrates post anti-PD1. Patients of poor baseline performance status ECOG PS 2–3 were more likely to die in

an acute setting compared to those with good baseline performance status (62% vs 23% respectively; RR 2.68; $P = 0.016$). There was no significant difference in the proportion of patients who had an intensive care unit (ICU) stay in their last month of life between the ECOG PS 0–1 and ECOG PS 2–3 groups, with two patients in each group admitted to ICU in their last month of life.

2 DISCUSSION

We conducted a retrospective study into the real-world outcomes and end of life health care utilization patterns of melanoma patients treated with anti-PD1 antibodies. The ECOG PS 0–1 cohort included both patients naïve to systemic treatment and those heavily pre-treated but exhibited outcomes broadly similar to that of phase III trials with response rates of approximately 40%.^{2,3,12} However, our

TABLE 3 Maximum grade of treatment related adverse event and immune-related adverse event

CTCAE v4 adverse event	ECOG PS 0–1 (n = 58)	(%)	ECOG PS 2 (n = 16)	(%)	ECOG PS 3 (n = 8)	(%)	CTCAE immune-related adverse event	ECOG PS 0–1 (n = 58)	(%)	ECOG PS 2 (n = 16)	(%)	ECOG PS 3 (n = 8)	(%)
Grade 0	30	(52)	10	(63)	8	(100)	Grade 0	37	(61)	15	(94)	7	(88)
Grade 1	19	(33)	3	(19)	0	(0)	Grade 1	12	(20)	1	(6)	0	(0)
Grade 2	6	(10)	1	(6)	0	(0)	Grade 2	2	(3)	0	(0)	0	(0)
Grade 3	3	(5)	2	(13)	0	(0)	Grade 3	9	(15)	0	(0)	0	(0)
Grade 4	0	(0)	0	(0)	0	(0)	Grade 4	0	(0)	0	(0)	0	(0)
Grade 5	0	(0)	0	(0)	0	(0)	Grade 5	0	(0)	0	(0)	1	(13)
Total	58		16		8		Total	58		16		8	

TABLE 4 Treatment and hospitalization within one month of life; place of death

	Patients, no. (%)		RR (95% CI)	P value
	ECOG PS0–1 (n = 26)	ECOG PS2–3 (n = 21)		
Treatment in the last month of life				
Yes	12 (46)	17 (81)	1.75 (1.04–2.56)	0.019
No	14 (54)	4 (19)		
Hospital admission in the last month of life				
Yes	13 (52)	18 (90)	1.73 (1.10–2.16)	0.009
No	12 (48)	2 (10)		
Place of death				
Acute hospital	6 (23)	13 (62)	2.68 (1.17–6.51)	0.016
Not acute hospital	20 (77)	8 (38)		

Determined using the Chi-square test for the difference in proportions between ECOG PS 0–1 and ECOG PS 2–3 patients.

outcomes for patients with baseline performance status of ECOG PS 2–3 were poor compared to those of ECOG PS 0–1. In particular, ECOG PS 3 patients did not exhibit any radiological tumor response and had a median OS of only 0.7 months. The historic survival data for melanoma patients of poor performance status is lacking, as these patients are routinely excluded from clinical trials. In general, untreated advanced melanoma is associated with a prognosis of 6–10 months.^{13–15} Pooled survival data from EORTC melanoma trials prior to the era of anti-PD1 antibodies reported that patients with ECOG PS ≥ 1 with liver or gastrointestinal metastases had a median survival of 2.9 months.¹⁶ Our dataset suggests that ECOG PS performance status remains a useful predictor of clinical outcome in the era of anti-PD1 treatment, in keeping with previous studies with chemotherapy. The paucity of responses in ECOG PS 2–3 patients might be reflective of the aggressiveness of their disease and the majority of this cohort possessed both M1c staging and elevated LDH which conferred a worse outcome. Our findings show that patients of ECOG PS 3 had a very short survival after treatment with anti-PD1 antibodies and this is consistent with ASCO guidelines that recommend against the use of chemotherapy for patients with ECOG PS 3.

Overall, this study provides some real-world data to enable clinicians to have a more realistic discussion with their patients about the use of these agents in patients with poor performance status. This in turn may facilitate advanced care planning and discussions about end of life care, given the context of short survival and low response rate. In our cohort, the rate of severe or life-threatening toxicities

did not differ greatly between ECOG PS 0–1 and ECOG PS 2–3. It is important to note that the toxicity rate is low in the poor performance group, although this could potentially be explained by the short duration on treatment and short survival. There is a perception that immunotherapy agents have lower toxicity rates than chemotherapy, and hence less likely to do harm even if the response rate is low. However, commencing immunotherapy may restrict the use of supportive medications such as corticosteroids because of concerns of reducing the efficacy of immunotherapy. This may pose a dilemma in management of cancer-related symptoms such as anorexia, nausea or lethargy, which are likely to benefit from corticosteroids. Importantly, corticosteroids can also rapidly palliate symptoms from brain metastases, which can present in up to 75% of advanced melanoma patients. Withholding steroids potentially impairs symptomatic management in these patients. Corticosteroid use patterns in the last month of life was not feasible in this study as patient received their end of life care in different locations (home, hospice or inpatient), however this would be an important parameter to consider in future studies.

Furthermore, the atypical immune-related responses seen with immunotherapy agents compound the complexity of providing palliative care to patients with advanced cancers.^{17,18} Clinicians may feel compelled to continue immunotherapy and intensive supportive treatment despite such radiological progression even though only a minority of patients will have a delayed immune response. This poses challenges in decision making as to the appropriate level of aggressive

supportive medical management that should be offered given a minority of patients may indeed derive a durable response. However our data suggests that patients with ECOG PS 3 are unlikely to derive clinical benefit from anti-PD1 antibodies, and therefore may provide some framework for establishing the ceiling of care prior to embarking on such treatments.

Given the short survival of patients with ECOG PS 2–3, we then sought to examine end of life care outcomes. Patients with ECOG PS 2–3 experienced a higher rate of hospitalizations in the last month preceding death and were more likely to die in an acute hospital setting. Part of the palliative care and hospice movement's concern around the use of chemotherapies at the end of life is that they may interfere with achieving a "good death."^{19,20} Consensus themes about good death raised three important indicators namely details of the death scene and preparation for death, pain-free status and emotional wellbeing. Given the poor survival with immunotherapy in ECOG PS 2–3 patients, clinicians may be encouraged to discuss end of life care preferences in these patients. However, this remains as always, a sensitive exploration of patient and family wishes, understanding and goals of care.

Our study is limited by its single site retrospective design, heterogeneous imaging modality for tumor assessment and small patient numbers particularly in the ECOG PS 2 and 3 groups. However this study, to our knowledge, is the first to report on outcome, toxicity and health resource utilization in patients with poor performance status treated with anti-PD1 antibodies. This study highlights the need for ongoing research in this patient group as these exciting agents enter into routine oncology practice.

1 CONCLUSION

Patients with poor baseline performance status have a significantly lower OS and reduced chance of response to anti-PD1 antibodies compared to patients with good performance status. The findings from this study provide real-world data to enable clinicians to have realistic discussions with their patients about the use of these agents in patients with poor performance status. This in turn may facilitate advanced care planning and discussions about end of life care, given the context of short survival and low response rate. Further studies of the quality of life outcomes and palliative care needs of patients with poor performance status undergoing immunotherapy compared to those who opt for symptom management alone are required.

ACKNOWLEDGEMENTS

Thank you to Associate Professor Linda Mileshekin and Professor Mark Rosenthal for editorial advice on the manuscript.

DISCLOSURES

Annie Wong: nil
Molly Williams: nil

Donna Milne: Advisory role for Merck, Novartis. Honoraria from Merck, Novartis and Bristol Myers Squibb

Kortnye Morris: nil

Peter Lau: Honoraria from Bristol Myers Squibb

Odette Spruyt: nil

Sonia Fullerton: nil

Grant McArthur: Research funding from Celgene, Genetech-Roche, Bristol Myer Squibb, Amgen, MSD, Array, Novartis. Advisory role for Provectus

FUNDING

Annie Wong receives PhD research stipend support from Royal Australasian College of Physicians.

REFERENCES

- Robert C, Long GV, Brady B, et al. Nivolumab in previously untreated melanoma without BRAF mutation. *N Engl J Med*. 2015;372(4):320–330.
- Robert C, Schachter J, Long GV, et al. Pembrolizumab versus ipilimumab in advanced melanoma. *N Engl J Med*. 2015;372(26):2521–2532.
- Larkin J, Chiarion-Sileni V, Gonzalez R, et al. Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. *N Engl J Med*. 2015;373(1):23–34.
- Brahmer J, Reckamp KL, Baas P, et al. Nivolumab versus docetaxel in advanced squamous-cell non-small-cell lung cancer. *N Engl J Med*. 2015;373(2):123–135.
- Borghaei H, Paz-Ares L, Horn L, et al. Nivolumab versus docetaxel in advanced nonsquamous non-small-cell lung cancer. *N Engl J Med*. 2015;373(17):1627–1639.
- Motzer RJ, Escudier B, McDermott DF, et al. Nivolumab versus everolimus in advanced renal-cell carcinoma. *N Engl J Med*. 2015;373(19):1803–1813.
- Ansell SM, Lesokhin AM, Borrello I, et al. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. *N Engl J Med*. 2015;372(4):311–319.
- Stanley KE. Prognostic factors for survival in patients with inoperable lung cancer. *J Nat Cancer Inst*. 1980;65(1):25–32.
- Pater JL, Loeb M. Nonanatomic prognostic factors in carcinoma of the lung. A multivariate analysis. *Cancer*. 1982;50(2):326–331.
- Schnipper LE, Smith TJ, Raghavan D, et al. American society of clinical oncology identifies five key opportunities to improve care and reduce costs: the top five list for oncology. *J Clin Oncol*. 2012;30(14):1715–1724.
- Oken MM, Creech RH, Tormey DC, et al. Toxicity and response criteria of the Eastern Cooperative Oncology Group. *Am J Clin Oncol*. 1982;5(6):649–656.
- Weber JS, D'Angelo SP, Minor D, et al. Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomized, controlled, open-label, phase 3 trial. *The Lancet Oncol*. 2015;16(4):375–384.
- Tsao H, Atkins MB, Sober AJ. Management of cutaneous melanoma. *N Engl J Med*. 2004;351(10):998–1012.
- Balch CM, Soong SJ, Gershenwald JE, et al. Prognostic factors analysis of 17,600 melanoma patients: validation of the American

- Joint Committee on Cancer melanoma staging system. *J Clin Oncol*. 2001;19(16):3622–3634.
15. Balch CM, Gershenwald JE, Soong SJ, et al. Final version of 2009 AJCC melanoma staging and classification. *J Clin Oncol*. 2009;27(36):6199–6206.
 16. Manola J, Atkins M, Ibrahim J, Kirkwood J. Prognostic factors in metastatic melanoma: a pooled analysis of Eastern Cooperative Oncology Group trials. *J Clin Oncol*. 2000;18(22):3782–3793.
 17. Chiou VL, Burotto M. Pseudoprogression and immune-related response in solid tumors. *J Clin Oncol*. 2015; 33(31):3541–3543.
 18. Wong A, Fullerton S, Spruyt O, Brady B, McArthur G, Sandhu S. Integration of immuno-oncology and palliative care. *J Clin Oncol*. 2016;34(13):1561–1562.
 19. Prigerson HG, Bao Y, Shah MA, et al. Chemotherapy use, performance status, and quality of life at the end of life. *JAMA Oncol*. 2015;1(6):778–784.
 20. Earle CC, Park ER, Lai B, Weeks JC, Ayanian JZ, Block S. Identifying potential indicators of the quality of end-of-life cancer care from administrative data. *J Clin Oncol*. 2003;21(6):1133–1138.

How to cite this article: Wong ANM, Williams M, Milne D, et al. Clinical and palliative care outcomes for patients of poor performance status treated with anti-programmed death 1 monoclonal antibodies for advanced melanoma. *Asia-Pac J Clin Oncol*. 2017;13:385–390. <https://doi.org/10.1111/ajco.12702>

4 Clinical biomarkers in patients with brain metastases treated with ICI

4.1.1 Introduction

Central nervous system (CNS) metastases are a common complication of melanoma, affecting approximately 30% of patients; and up to 70% of patients were found to have brain metastases in an autopsy series (1, 401). Metastatic involvement of brain, spinal cord, leptomeninges are associated with significant morbidity and short survival approximating 4-5 months (1, 7).

Recent advances have been made in the treatment of melanoma brain metastases, with improvements in overall survival in patients treated with ICI and targeted therapies (3, 4, 244). Prognostic biomarkers in melanoma brain metastases were identified prior to the development of effective systemic treatment; it is therefore not known whether these biomarkers will remain significant in the setting of ICI treatment (1, 2, 5-8). This chapter will firstly review the traditional biomarkers in brain metastases, the evolving treatment paradigm for brain metastases and the potential prognostic candidates in the setting of ICI treatment.

Traditional biomarkers in melanoma CNS metastases prior to introduction of ICI

In recognition of the poor outcome associated with CNS involvement, the latest AJCC 8th edition staging manual has denoted CNS involvement as a separate subgroup of Stage IV disease, as M1d (25). Additionally, raised LDH is recognised as an independent marker of poor prognosis, even in patients with CNS involvement, and therefore is designated with the additional suffix of (1) (8, 25).

Several large retrospective studies have reported on prognostic factors in patients with CNS metastases (1, 2, 5-8). These studies have highlighted age, performance status, number of metastases, symptomatic metastases, status of extracranial disease as important prognostic factors.

The Radiation Therapy Oncology Group Recursive Partitioning Analysis (RPA) is a validated prognostic score based on 3 factors: age < or ≥65, Karnofsky Performance

status < or ≥ 70 , and status of extracranial disease (controlled or uncontrolled) (5, 6). A retrospective survival analysis of 686 patients from the Sydney Melanoma Unit cohort (between 1985 to 2000) also showed that younger age was important for survival, in addition to those who had surgical treatment, lack of extracranial involvement, and a long time between melanoma diagnosis and development of cerebral metastasis (7).

Unsurprisingly, multiple studies have shown that the higher the number of metastases, the worse the prognosis (1, 2). 743 patients treated at M.D. Anderson Cancer Centre between 1986-2004 found that increased number of parenchymal lesions and >3 intracranial lesions or leptomeningeal involvement was prognostic for poor overall survival; this was in addition to diagnosis before 1996 and development of extracranial disease after receiving systemic treatment (1). Memorial Sloan Kettering Cancer Centre also reported on their retrospective cohort of 355 patients treated between 1991 and 2003 (2). They found that ≥ 4 brain metastases, age >65 , extracranial metastases, presence of neurological symptoms, were each independently significant in a multivariate analysis. Another study of 265 patients treated at a German academic centre between 1986-2003 showed that a solitary metastasis was associated with good survival, independent of raised LDH, presence of bone metastases or local neurosurgical therapies (8). Overall, these studies highlight that patients with fewer metastases have a better outcome.

It must be acknowledged that the diagnostic modalities and treatment modalities available for brain metastases have changed over the course of these retrospective cohorts. Patients with oligometastatic disease were previously managed with surgery (1, 2, 7). Since the 1990s, stereotactic radiation has supplemented surgery as a treatment modality for oligometastatic disease. Patients with multiple metastases were otherwise treated with whole brain radiation or systemic treatments (mainly investigational agents and cytotoxic chemotherapies that had minimal survival benefit). Patients who were not candidates for whole brain radiation due to poor performance status or comorbidities were managed symptomatically with steroids and best supportive care. Importantly, the systemic treatments such as ICI and molecularly targeted therapies that have drastically improved survival of metastatic melanoma, became available from the early to mid-2000s (169, 170, 257). Despite these active systemic agents, brain and CNS metastases remain a challenging site with a lower rate

of treatment response compared to extracranial sites. The literature regarding contemporary systemic treatment of brain metastases will be summarised below.

Treatment for CNS metastases

Traditionally the central nervous system was considered a sanctuary site to systemic cytotoxic therapies. Temozolomide is able to cross the blood brain barrier but despite this, it was associated with low response rate and had minimal impact on survival (402). In a multi-centre phase II clinical trial of 151 patients treated with Temozolomide, only 7% of patients had a partial response and 29% had stable disease (402). The median overall survival was 3.4 months in this study.

Molecularly targeted therapy has shown significant activity for CNS metastases. Firstly, dabrafenib monotherapy was evaluated in a phase II study, BREAK-MB. In 139 patients with BRAFV600E mutated melanoma brain metastases, dabrafenib monotherapy had an intracranial response rate of 39% and a 6-month overall survival of 61%. Combination of BRAF and MEK inhibition had a higher response than BRAF monotherapy in metastatic melanoma without brain metastases; consequently, therapy was a rational treatment for intracranial disease (86). This combination was evaluated in the COMBI-MB study, where combination dabrafenib and trametinib were assessed in a multi-centre, phase II trial for melanoma brain metastases. Patients were treated within 4 cohorts: A) BRAFV600E mutated asymptomatic brain metastases with no previous local treatments, B) BRAFV600E asymptomatic brain metastases with previous local therapy, C) BRAFV600D/K/R asymptomatic brain metastases and D) patients with poor performance status regardless (ECOG 0, 1 or 2) of BRAFV600 mutation subtype (E/D/K or R) and regardless of local therapy. Intracranial responses observed were 44/76 (58%), 9/16 (56%), 7/16 (44%), 10/17 (59%) in each cohort respectively. 6-month PFS was 44%, 71%, 31% and 46% and 6-month OS was 81%, 69%, 88% and 79% respectively in each cohort. 12-month PFS of 19%, 47%, 16% and 8% and 12-month OS was 46%, 69%, 44%, 44% respectively. This study showed a high intracranial response rate, albeit short duration of disease control.

Durable responses have been observed with ICI for patients with metastatic melanoma but these studies have routinely excluded patients with active intracranial metastases (169). A number of studies have attempted to evaluate ICI for patients with melanoma brain metastases (403). Ipilimumab (10mg/kg) was evaluated in a phase II study in

melanoma brain metastases of 0.5-3cm (2 or more 0.3cm lesions on MRI were permissible) (241). In this study, previous whole brain radiation needed to be completed 14 days prior to study entry, and stereotactic radiation was permissible but not to the index lesion. Intracranial response rates were 24% in asymptomatic patients and 10% in symptomatic patients. Median intracranial and overall PFS was under three months in both symptomatic and asymptomatic patients. Median overall survival was 7.0 months in asymptomatic patients and 3.7 months in symptomatic patients. For asymptomatic patients, 6-month and 12-month overall survival was 55% and 31% respectively. Diarrhoea was the most common treatment toxicity (12% grade 3 in asymptomatic patients).

Limited data were available about the use of single agent anti-PD-1 in the treatment of brain metastases. One phase II study of pembrolizumab 10mg/kg in untreated brain metastases included 18 patients with untreated melanoma. Pembrolizumab was associated with an objective response in 4 (22%) of 18 patients (404). Following this small prospective study, two larger phase II studies subsequently reported on the use of combination anti-CTLA4 and anti-PD-1 specifically in melanoma brain metastases.

The Anti-PD1 Brain Collaboration (ABC) clinical trial is a multi-centre phase II randomised study of combination nivolumab and ipilimumab versus nivolumab monotherapy in patients with melanoma brain metastases (3). The study had 3 cohorts: A) untreated asymptomatic patients randomised to combination nivolumab and ipilimumab; B) untreated asymptomatic patients randomised to nivolumab monotherapy and C) patients whose disease had progressed on previous local therapy or patients who were symptomatic and were randomised to nivolumab monotherapy. The primary endpoint of the study was intracranial response at 12 weeks. 16 (46%) of 35 patients in cohort A had an objective response, compared to 5 (20%) out of 25% in cohort B and 1 (6%) of 16 patients in cohort C. At a median follow-up of 17 months, the median intracranial PFS was not reached in cohort A; 2.5 months in cohort B and 2.3 months in cohort C. The median overall survival was not reached in cohort A; 18.5 months in cohort B and 5.1 months in cohort C. Overall survival at 6 months was 78% for cohort A, 68% for cohort B and 44% for cohort C. Grade 3 treatment related adverse events were reported in 19 (54%) patients in cohort A, 4 (16%) patients in cohort B and 2(13%) patients in cohort C. Three (9%) grade 4 treatment related events occurred in cohort A whereas there were none in cohorts B & C. This study was the first to

demonstrate the high intracranial response rate from combined ICI. Even though the study was not powered for comparative survival statistics, this study established combination ICI as the standard of care treatment for untreated melanoma brain metastases.

CHECKMATE204 is the second phase II study to report on combined ICI in melanoma brain metastases. This study is a multi-centre, phase II trial of ipilimumab with nivolumab in patients with non-irradiated asymptomatic brain metastases of 0.5cm-3cm on MRI (4). Patients received ipilimumab 3mg/kg in combination with nivolumab every three weeks for four doses (induction), followed by nivolumab 3mg/kg fortnightly (maintenance treatment). Primary endpoint was the rate of the intracranial clinical benefit rate at 6 months (including stable disease, partial response and complete response). 94 patients had intracranial response rate of 57% (95% CI 47-68), the rate of complete response was 26%, the rate of partial response was 30% and the rate of stable disease was 2%. Twenty additional symptomatic patients were enrolled but the median follow up of this group of patients is less than six months and therefore not included in the published analysis. Sixteen (17%) of patients received BRAF inhibitors, MEK inhibitors or both. Eight (9%) patients received stereotactic radiation before study enrolment. Interestingly, the rate of clinical benefit was higher in patients with raised lactate dehydrogenase (67% vs. 51%), and higher in patients with high tumoural PD-L1 expression $\geq 5\%$ (76% vs. 48%). Reported survival analyses were 6-month and 9-month progression free survival of 64.2% and 59.5% for intracranial assessments, and 61.1% and 56.6% for global assessments. 6-month and 9-month survival rates were 92.3% and 82.8% respectively. In terms of toxicity, grade 3 to 4 treatment related adverse events occurred in 55% of patients, including one death due to immune mediated myocarditis. Nineteen (20%) patients discontinued treatment due to toxicity. Neurological adverse events were present in 34 (36%) patients, 7% of whom experienced grade 3 to 4 in severity. The most commonly treated related adverse event was headache (22%), while the other grade 3 to 4 CNS treatment adverse events include 2% brain oedema, 1% intracranial haemorrhage and 1% peripheral motor neuropathy. The study provided further supportive evidence for combined ICI for untreated melanoma brain metastases.

The optimal sequence of ICI and targeted therapy in melanoma brain metastases is not known. As with systemic melanoma, there is no randomised study to directly compare molecularly targeted treatment versus ICI for the treatment of BRAF mutated brain

metastases. Acknowledging the possible selection bias with different inclusion criteria for the different studies, it appears that targeted therapy of brain metastases has a comparable response rate of approximately 50%, but the duration of response is short. Furthermore, the rates of response to ICI were numerically lower in patients who have received previous BRAF/MEK inhibitors. Improving the understanding of biomarkers for each of the treatments in patients with brain metastases may establish an optimal treatment sequence.

Clinical biomarkers for patients with melanoma brain metastases treated with ICI

Data on biomarkers for patients with melanoma brain metastases treated in the era of immune checkpoint inhibitors are limited. It is not known whether the prognostic biomarkers identified in the aforementioned studies prior to the introduction of ICI would remain significant. A more contemporary study of 79 patients treated at the California Pacific Medical Center and San Francisco Oncology Associates between 2011 and 2015 was performed, where the median overall survival was 12.8 months. Male sex, cerebellar metastasis, higher number of brain lesions, and treatment with whole-brain radiation therapy were identified as associated with reduced survival (405). The authors also reported that treatment with craniotomy, stereotactic radiosurgery, or with anti-PD-1 antibody was associated with improved survival. However, only 8 (10%) of 79 patients in this study received combination ipilimumab and nivolumab as systemic treatment for their brain metastases, despite it being a standard of care treatment. Lastly, there are no known factors associated with toxicity from systemic treatments for brain metastases.

This chapter is an exploratory post-hoc analysis of the ABC clinical trial and attempts to identify prognostic factors for response, survival and toxicity for patients with melanoma brain metastases treated with ICI. The hypothesis is that traditional biomarkers such as age, performance status, high intracranial or high extracranial disease burden will remain important for outcome after ICI. An additional hypothesis is that other clinical factors associated with outcome after ICI for non-CNS melanoma as discussed in the literature review for the thesis will remain significant for outcome after ICI for intracranial disease. These factors include LDH, site of metastatic involvement (subcutaneous, lung, liver, bone, adrenal or splenic involvement), baseline markers of inflammation (anaemia, raised white cell count, raised platelet count, low albumin, high

CRP, high neutrophil: lymphocyte ratio >5) and increased body mass index (BMI).

Lastly, there is a paucity of data for clinical factors associated with toxicity in ICI in this population of patients, so it would be important to study whether these prognostic factors are also associated with treatment toxicity.

4.1.2 Aims

The aims of the study were:

1. To identify prognostic factors associated with clinical outcome (response rate, PFS, OS) in patients treated with ICI for asymptomatic melanoma brain metastases
2. To identify factors associated with treatment toxicity in patients treated with ICI for asymptomatic melanoma brain metastases

4.1.3 Results

In the ABC clinical trial, 35 patients in Cohort A received combination ipilimumab and nivolumab treatment arm, and 25 patients in Cohort B received nivolumab monotherapy (3). The study schema is shown in Figure 4.1 Simplified study schema of the Anti-PD-1 in Brain Collaboration clinical trial (1) and only the asymptomatic patients were included in this post-hoc analysis (i.e. Cohort A & B). A summary of the patient demographics, treatment outcomes and toxicities at 120 days are presented in Table 4.2 and Table 4.3 respectively.

For the purpose of the post-hoc biomarker analysis, 50 clinical factors and blood parameter variables considered as potential biomarker candidates, are listed in Table 4.1. The three main categories of biomarkers explored included 1) traditional prognostic factors in melanoma brain metastases; 2) patient, disease and treatment factors as well as 3) pre-treatment blood tests. Continuous candidate variables were explored continuous variables, dichotomised at the median of the cohort into ‘low’ or ‘high’ groups or split by clinically relevant thresholds. For continuous variables examined for the whole cohort, the mean, median, range and IQR are presented in Table 4.3.

Clinical and tumour characteristics

The candidate factors explored include traditional prognostic factors in brain metastases including age, performance status, intracranial and extracranial disease. The median age for the cohort was 63 years (range 29-86 years). There were 7 (20%) patients in the combination group, and 9 (36%) patients in the anti-PD1 monotherapy group with performance status ECOG 1 or more. The baseline intracranial burden was assessed either by the number of intracranial metastases (≥ 3 intracranial lesions) or as target sum of diameter (SOD) of intracranial disease as measured by RECIST criteria (mm). There were 21 (60%) patients in the combination group and 12 (48%) patients in the anti-PD-1 monotherapy group with 3 or more brain metastases. The median SOD of intracranial disease in the whole cohort was 27mm (range 5-90mm), 19mm (range 5-81mm) in the combination cohort, and 17mm (range 5-90mm) in the monotherapy cohort. The median SOD of the extracranial disease was 82mm (range 10-255mm), 89mm (range 10-255mm) in the combination cohort, 46mm (range 17-192mm) in the monotherapy cohort.

Pre-treatment laboratory tests

The pre-treatment laboratory tests include some known prognostic factors such as LDH, neutrophil: lymphocyte ratio or C-reactive protein, which have been proposed as being prognostic in ICI treated patients. LDH was elevated in 18 (51%) patients in the combination cohort and 14 (56%) patients in the anti-PD-1 monotherapy cohort. The NLR was abnormal (>5) in 3 (10%) patients in the combination cohort and 2 (10%) in the anti-PD-1 monotherapy cohort. CRP >5 was present in 24 (69%) of the combination cohort, and 15 (60%) of the anti-PD-1 monotherapy cohort. The other laboratory tests for the whole cohort (combination and monotherapy cohorts together) are described in Table 4.3.

Tumour response

At data-cut off (28th August 2017) (3), with a median follow up of 17 months (IQR 8-25), intracranial response was achieved by 16 (46%) of 35 patients in Cohort A, 5 (20%) of 25 in Cohort B. CRP as a continuous variable was the only factor associated with low response in the combination cohort (odds ratio 0.93, 95% CI 0.86-0.99, $p=0.04$) but not in the monotherapy cohort. No factors were statistically significant in association with response in the monotherapy cohort.

Survival analyses

The median intra-cranial PFS was 5.7 months in Cohort A, and 2.6 months in Cohort B, while the median OS was not reached in Cohort A, and was 18.5 months in Cohort B. Treatment-related adverse events were experienced by 34 (97%) of 35 patients in Cohort A, and 17 (68%) of 25 patients in Cohort B. For PFS in the combination cohort, CRP as a continuous variable (HR 1.1, 95% CI 1-1.1, $p<0.01$), ALP as a continuous variable (HR 1, 95% CI 1-1.1, $p=0.02$) and high ALP $> 88\text{U/L}$ (median of cohort) (HR 2.7, 95% CI 1.1-6.7) were associated with poor PFS in the univariate analysis. Only CRP remained significant in the multivariable analysis (HR 1.07, 95% CI 1.01-1.11, $p=0.006$).

For PFS in the monotherapy cohort, the following factors were significant in the univariate analysis but none of these factors remained significant in the multivariable analysis: CRP (HR 1.1, 95% CI 1-1.1, $p=0.006$), high CRP $>26\text{mg/L}$ (median of the cohort) (HR 6.5, 95% CI 1.6-27, $p=0.009$), extracranial disease $> 71\text{mm}$ (median target SOD by RECIST for cohort) (HR 4, 95% CI 1.4-12, $p=0.012$), LDH $>$ upper limit of normal (HR 3.2, 95% CI 1.3-9.8, $p=0.012$), neutrophil count as a continuous variable (HR 1.2, 95% CI 1-1.5, $p=0.039$), white cell count as a continuous variable (HR 1.2, 95% CI 1-1.4, $p=0.043$).

For PFS in the whole cohort, CRP as a continuous variable (HR 1, 95% CI 1-1.1, $p<0.001$), CRP $>$ median (HR 2.6, 95% CI 1.2-5.3, $p=0.011$) and ALP $>$ median (HR 2, 95% CI 1-3.7, $p=0.038$) were statistically significant in the univariate analysis. Only CRP remained significant in the multivariable analysis (HR 1.07, 95% CI 1.01-1.12, $p=0.01$).

For OS in the combination cohort, CRP (HR 1.1, 95% CI 1-1.1, $p=0.0043$), CRP $>$ median (HR 4.6, 95% CI 1.2-17, $p=0.024$), ALP $>$ median (HR 3.5, 95% CI 1.1-11, $p=0.032$) were statistically significant in the univariate analysis. Only CRP remained significant in the multivariable analysis (HR 1.08, 95% CI 1.02-1.14, $p=0.01$). For OS in the monotherapy cohort, white cell count $>$ median (HR 4.8, 95% CI 1.2-19, $p=0.023$), Hb $<100\text{g/L}$ (HR 11, 95% CI 1-130, $p=0.046$), and lymphocyte $>$ median (HR 3.5, 95% CI 1-12, $p=0.048$) were associated with poor OS in the univariate analysis. Only Hb $<100\text{g/L}$ was associated with poor OS in the multivariable analysis (HR 39, 95% CI 2.59-609.30, $p=0.008$).

For the OS in the whole cohort, the following factors were statistically significant in the univariate analysis: ALP (HR 1, 1-1.1, $p=0.002$), CRP (HR 1.1, 95% CI 1-1.1, $p=0.002$), LDH>median (HR 3.2, 95% CI 1.4-7.4, $p=0.006$), ALP>median (HR 2.9, HR 1.3-6.4, $p=0.010$), LDH>upper limit of normal (HR 3.1, 95% CI 1.3-7.5, $p=0.012$), CRP>median (HR 3.4, 95% CI 1.3-9.2, $p=0.014$), Hb<100g/L (HR 6.5, 95% CI 1.4-31, $p=0.018$), LDH (HR 1, 95% CI 1.1, $p=0.02$), white cell count>median (HR 2.4, 95% CI 1.1-5.4, $p=0.034$). Only CRP (HR 1.13, 95% CI 1.01-1.25, $p=0.026$) and raised white cell count >median (HR 3.07, 95% CI 1.01- 9.38, $p=0.049$) remained significant in the multivariable analysis. See Table 4.4 for details. The two significant variables of CRP and white cell count>median can be used to stratify the cohort into three prognostic groups. Figure 4.2 Kaplan-Meier curve of overall survival stratified by C-reactive protein (CRP) and white cell count (WCC) shows the Kaplan-Meier survival curve for the entire cohort when stratified by these two variables.

Toxicity analyses

For landmark toxicity at 120 days in the whole cohort, higher toxicity grade 1-3 was associated with combination therapy. Previous BRAF inhibitor was associated with lower risk of grade 1 toxicity (OR 0.17, 95% CI 0.05-0.67, $p=0.01$). Higher risk of grade 3 toxicity was associated with hypernatraemia (OR 1.27, 95% CI 1.03-1.64, $p=0.04$) and BMI>30 (OR 3.33, 95% 1.03-11.26, $p=0.05$).

4.1.4 Discussion

This exploratory retrospective analysis has identified CRP as a possible prognostic biomarker for OS in patients treated with ICI for brain metastases in the ABC study(3). Although hypothesis generating, this analysis is limited by its small sample size and requires external validation. Furthermore, a large number of candidate factors were investigated in the statistical modelling, which may have led to a high false discovery rate.

C-reactive protein

In this exploratory analysis, raised CRP was associated with poor overall survival in the whole cohort (both in the combination and the monotherapy cohort). CRP was also associated with low response rate and short PFS in the combination cohort but not the monotherapy cohort. When considering all patients within the study, CRP and raised

white cell count were able to stratify patients into 3 prognostic groups of good, intermediate and poor prognosis. However, these findings are limited as CRP was not available in approximately one third of patients (26% of patients in the combination cohort and 32% of the patients in the monotherapy cohort). Furthermore, there were only 4 patients with normal CRP and these patients had excellent outcome, which could heavily bias the analysis. CRP has long been recognised to be associated with poor prognosis in a variety of cancer types, including melanoma (406-411). Fang et al (410) showed that plasma CRP 10 mg/L or greater was predictive of poorer survival among patients with Stage I/II or stage III/IV melanoma, and of an elevated rate of recurrence among those with early-stage disease. A second study in melanoma used serum CRP cut-off of 3mg/L to further stratify patients with AJCC 7th edition Stage IV melanoma into good and poor prognostic groups (11, 412). These previous studies did not specify the systemic treatments used but these studies were reported prior to the widespread availability of ICI. Thus it is not known whether CRP would remain prognostic in patients treated with ICI, nor how it may apply to patients with melanoma brain metastases (410, 412).

Some retrospective studies have evaluated the role of peripheral blood inflammatory markers and its association with outcome after ICI. High neutrophil to lymphocyte ratio, regarded as a marker of generalised inflammation, has been associated with poor prognosis in the ICI setting (270, 413). A more recent retrospective analysis by Weber and colleagues (273) evaluated CRP and serum IL6 levels in patients with metastatic melanoma treated in prospective clinical trials of Checkmate 064, Checkmate 066 and Checkmate 067 (172, 189, 273, 379). Checkmate 064 is a phase II randomised study of sequential ipilimumab and nivolumab with a pre-specified switch; Checkmate 066 is a randomised phase III study of nivolumab versus dacarbazine, and Checkmate 067 is a randomised phase III study of nivolumab and ipilimumab versus nivolumab. Raised CRP and IL6 levels at baseline prior to ICI were associated with low response and poor survival. The number of patients included in each analysis was not specified in the presentation abstract. The authors reported that serum CRP>median was associated with short survival in the nivolumab cohort of Checkmate 064 ($p=0.0001$), as well as short survival in the dacarbazine cohort in Checkmate 066 ($p=0.001$) but not the nivolumab cohort($p=0.131$). CRP>median was associated with short survival in the ipilimumab and combination cohort ($p<0.0001$ and $p=0.0034$) but was non-significant

in the nivolumab cohort ($p < 0.11$). In summary, high CRP was associated with survival in combination (ipilimumab and nivolumab) treated patients; but rather curiously, high CRP was associated with poor outcome in anti-PD-1 treated patients in one of the three studies. Baseline IL6 was associated with low response ($p = 0.03$) and short OS in Checkmate 064 ($p = 0.003$). Raised IL6 at 12 weeks was associated with low response in the nivolumab ($p = 0.004$) and ipilimumab ($p = 0.006$) arms. Week-12 IL6 levels greater than the median were associated with short OS in the nivolumab ($p = 0.003$) and ipilimumab ($p = 0.0001$) arms. Baseline IL6 was significantly associated with CRP ($\rho = 0.71$, $p = 0.001$). Authors also reported that high CRP $> 10\text{mg/L}$ was associated with T cell suppression and reduced calcium flux within T cells in in vitro studies. This study has not yet been published in a peer review journal. All of these studies (Checkmate064, Checkmate 066 and Checkmate 067) excluded patients with active brain metastases. Given these intriguing results, CRP is worthy of further investigation in the context of patients with brain metastases.

CRP is an acute phase inflammatory protein which is produced in hepatocytes in response to cytokines, mainly IL6 as part of the innate immune response (414, 415). IL6 is produced both by T cells, monocytes or macrophages, fibroblasts, endothelial cells or by the tumour itself (415-419). Given the poor prognosis associated with elevated levels of CRP and IL6, attempting to neutralise or inhibit these blood cytokines/acute phase reactant is a rational therapeutic strategy in melanoma. IL6 can be targeted with other ICI such as tocilizumab; indeed, at the time of writing, there is an actively recruiting phase II study of tocilizumab in combination with ipilimumab and nivolumab in patients with untreated metastatic melanoma (*ClinicalTrials.gov Identifier: NCT03999749*).

CRP has not yet been evaluated in patients treated with BRAF and MEK inhibitors. Given the poor outcome observed with elevated CRP and IL6 in patients treated with ICI, it would be important to evaluate whether this is also seen in patients treated with molecularly targeted treatments. If these blood parameters prove not to be prognostic in molecularly treated patients, then it may be possible to use CRP as a test to guide the sequence of treatments, i.e. use targeted therapy first in patients with high CRP. This treatment strategy would need to be tested in a prospective clinical trial.

Traditional prognostic biomarkers in melanoma brain metastases

In our analysis, the traditional prognostic factors for melanoma CNS metastases such as age > 65, intracranial disease burden and performance status were not associated with outcome after ICI treatment. In terms of age as a prognostic factor, our findings are consistent with a retrospective series of 254 patients treated at two academic centres, where ICI remained efficacious regardless of age and no difference in survival was observed in older patients (420).

Performance status has been associated with reduced treatment response and shorter survival in retrospective and prospective studies of patients treated with anti-PD-1 for melanoma (259, 397). Importantly, patients in cohort C of the ABC study (i.e. patients where local therapies have failed or who are symptomatic or who have leptomeningeal disease) were excluded from our exploratory analysis. It is likely that patients with symptomatic or leptomeningeal disease had lower baseline performance status than the patients included in cohort A & B. Therefore, the assessment of performance status was only limited to a comparison of PS 0 compared to PS 1 in this retrospective analysis, and that was not associated with outcome after ICI. Patients with symptomatic CNS disease remain a challenging subgroup in clinical practice and presenting an area of need for novel therapeutic options.

Lastly, the number of brain metastases was not significantly associated with PFS or OS in contrast to retrospective series of patients prior to the immunotherapy era (1, 7, 8). The number of metastases may have lost its prognostic significance as patients with isolated metastases may have been treated with local therapies rather than enrolled into a study of systemic treatments. Furthermore, contemporary diagnosis of brain metastases is likely to involve routine MRI imaging which would detect tumours when they are of small volume, so the number of metastases may be high (>3 intracranial lesions) but remain small volume and asymptomatic. This again raises the potential benefit of routine surveillance for CNS involvement in order to detect patients with small volume metastases so that therapy can be commenced prior to the development of symptoms.

Biomarkers for ICI toxicity in patients with brain metastases treated with ICI

Unsurprisingly, treatment with combination nivolumab and ipilimumab was associated with higher risk of toxicity in this analysis, as has been demonstrated in multiple prospective studies (177, 190, 421). In this exploratory analysis, increased BMI was

associated with higher risk of grade 3 toxicity in the combination cohort. Increased BMI has been associated with higher response rate and better outcome in melanoma treated with ICI, but higher risk of toxicity has not been previously reported (422). It is possible that those who respond would remain on treatment for a longer duration and therefore have a greater chance of developing toxicity. However increased BMI was not associated with higher response rate or improved survival in the analysis of patients treated in the ABC study. Hyponatraemia was also associated with higher risk of developing grade three toxicity in the combination cohort. This has not previously been recognised as a risk factor in the literature. Sodium derangements have been observed in association with other immune related endocrinopathies, such as cortical insufficiency or dehydration from immune related colitis but again baseline sodium abnormalities have not been reported previously (423, 424). In this analysis, high CRP was not associated with higher risk of developing treatment toxicity. Overall, CRP was associated with response and survival but not with toxicity.

When analysing the factors associated with toxicity in each treatment cohort, raised white cell count (as a continuous variable as well as WCC greater than the median of the cohort) and high intracranial disease burden (baseline sum of longest intracranial metastases > median of the cohort) was associated with a lower risk of grade 3 toxicities in the combination arm. Even though raised white cell count has been shown to be associated with increased response, it has not been previously reported as a marker of toxicity (425). Similarly, ALP greater than 88 IU/L (median of the cohort) and previous BRAF inhibitor use were both associated with lower risk of grade 1 toxicity in the monotherapy cohort. Small retrospective series have reported on reduced responsiveness to ICI after previous BRAF targeted therapy, so this may potentially result in patients remaining on ICI therapy for a shorter duration (thus with subsequent lower rates of toxicity) (426, 427). ALP has not been previously reported in association with toxicity to anti-PD-1 therapy. Again, one must be cautious in the interpretation of these findings, given the high number of candidate factors assessed in relation to toxicity outcomes and the potential for false discovery. These findings are hypothesis generating and require validation.

4.1.5 Conclusion

In this exploratory analysis, CRP was associated with low response, poor PFS, poor OS in patients with asymptomatic melanoma brain metastases. Raised white cell count was also associated with poor OS in the whole cohort. Raised CRP and high white cell count are two factors that can be used to stratify patients with melanoma brain metastases into good, intermediate and poor prognosis groups. CRP was not associated with toxicity, whereas treatment with combination ICI, increased BMI>30 and hypernatraemia were associated with grade 3 toxicity at 120 days post treatment. This exploratory analysis is limited due to its retrospective, post-hoc nature and small sample size. These findings need to be validated in an independent cohort, but potentially these readily available clinical parameters could be used to stratify patients in prospective trials if validated.

4.1.6 Tables

Table 4.1 Candidate biomarkers considered in the post-hoc analysis of Anti-PD1 Brain Collaboration study

Traditional prognostic factors in melanoma brain metastases				
Age>65 years	Age>median of cohort (=63 years)	ECOG PS>0		
Target Intracranial RECIST sum of diameters (mm)	Target extracranial RECIST sum of diameters (mm)	Target Intracranial RECIST sum of diameters (mm)>median	Target extracranial RECIST sum of diameters (mm)>median	Number of intracranial metastases = 3 or more
Patient, disease and treatment factors				
Gender	BMI	BMI>30	BRAF mutation	Previous BRAF treatment
Lung involvement	Liver involvement	Adrenal involvement	Lymph node involvement	Spleen involvement
Bone involvement	Subcutaneous involvement			
Pretreatment laboratory tests				
Hb (continuous)	Hb<100	Hb>median		
Platelets (continuous)	Plt>median			
White cell count (continuous)	White cell count>median	Neutrophils (continuous)	Neutrophils>median	
Absolute Lymphocyte Count (continuous)	ALC>median	Lymphopenia (ALC<1.0)	NLR (continuous)	High NLR (>5)
Sodium (continuous)	Hyponatraemia (Na<135)	ALP (continuous)	AST (continuous)	TSH>median
Total protein (continuous)	TP>median	Albumin (continuous)	Albumin>median	Albumin<35
LDH (continuous)	Elevated LDH	LDH>median		
CRP (continuous)	CRP>median	High CRP (>5)		

ECOG PS, European Cooperative Oncology Group Performance Status; BMI, Body Mass Index; Intracranial RECIST, sum of measurable intracranial disease by RECIST criteria version 1.1; Extracranial RECIST, sum of measurable extra-cranial disease by RECIST criteria version 1.1; Hb, Haemoglobin; Plt, platelets; ALC, Absolute Lymphocyte Count; ALP; Alkaline phosphatase; AST; Aspartate transaminase; TP, Total Protein; TSH, Thyroid stimulating hormone; LDH, Lactate Dehydrogenase; CRP, C Reactive Protein; NLR Neutrophil to Lymphocyte Ratio

Table 4.2 Patient demographics

	Cohort A (ipi/nivo) n=35	%		Cohort B (nivo) n=25	%
Demographics					
Age (median)	59			63	
Sex (male)	29	83		19	76
BRAF mutation	19	54		14	56
Previous BRAF treatment	8	23		6	24
ECOG>0	7	20		9	36
BMI					
BMI>30	20	57		8	32
LDH					
LDH>ULN	18	51		14	56

Number of brain metastases					
1	11	31		6	24
2	3	9		7	28
≥3	21	60		12	48
Median Target Intracranial RECIST sum of diameters (mm)	19			17	
Median Target Extracranial RECIST sum of diameters (mm)	89			46	
Sites of metastasis					
Lung	21	60		10	40
Liver	18	51		7	28
Bone	2	6		1	4

Median White cell count (WCC)	8			7	
CRP					
Median CRP	27			23	
High CRP (>5)	24	69		15	60
Missing CRP	9	26		8	32

Table 4.3 Continuous variables considered in survival and toxicity analyses

Continuous variables	Mean	Median	Range	IQR
Age	60	63	[29, 86]	[53, 71]
BMI	27	27	[17, 35]	[25, 31]
Target Intracranial RECIST sum of diameters (mm)	27	19	[5, 90]	[13, 32]
Target extracranial RECIST sum of diameters (mm)	82	71	[10, 255]	[33, 118]
Haemoglobin	142	141	[107, 173]	[130, 149]
Platelet	259	258	[124, 504]	[211, 299]
White cell count	7.3	7.0	[3.0, 17.0]	[5.0, 9.0]
Neutrophil count	4.5	4.0	[1.0, 15.0]	[3.0, 5.0]
Absolute lymphocyte count (ALC)	1.2	1.0	[0.0, 3.0]	[1.0, 2.0]
Neutrophil: Lymphocyte Ratio (NLR)	3.6	3.0	[1.0, 15.0]	[2.0, 4.0]
Thyroid stimulating hormone (TSH)	1.7	1.4	[0.1, 11.2]	[0.9, 2.0]
Sodium (Na)	138	139	[129, 144]	[137, 140]
Alkaline phosphatase (ALP)	98	88	[52,294]	[70,112]
Aspartate aminotransferase (AST)	25	22	[8,87]	[17,30]
Total Protein (TP)	70	71	[58.0, 80.0]	[67.0, 73.0]
Albumin	41	41	[40.0 , 48.0]	[40.0, 44.0]
Lactate dehydrogenase (LDH)	313	253	[150, 2108]	[201, 303]
C-reactive protein (CRP)	25	26	[2, 49]	[12, 38]

Table 4.4 Treatment and toxicity outcome

	Cohort A (ipi/nivo)	%		Cohort B (nivo)	%
Best overall response:					
CR	1	3		2	8
PR	17	49		3	12
SD	2	6		2	8
PD	13	37		18	72
Not evaluable	2	6			
Survival					
PFS (months)	5.7			2.6	
OS (months)	NR			18.5	
Toxicity by 120 days (CTCAE)					
Grade 1	32	91		14	56
Grade 2	28	80		8	32
Grade 3	18	51		1	4
Grade 4	3	9		0	0

CR, Complete Response; PR, Partial Response; SD Stable disease; PD, Progressive Disease; PFS Progression-Free Survival; OS, Overall Survival; G1, NR, Not reached

Table 4.5 Univariate and multivariate Cox regression analysis for overall survival for combined Cohort A & B

	Univariate analysis			Multivariate analysis	
Variable	HR (95% CI)	p.value		HR (95% CI)	p.value
ALP	1 (1-1)	0.0022			
CRP	1.1 (1-1.1)	0.0023		1.13 (1.01,1.25)	0.026
LDH>median	3.2 (1.4-7.4)	0.0055			
ALP>median	2.9 (1.3-6.4)	0.0097			
Elevated LDH	3.1 (1.3-7.5)	0.012			
CRP>median	3.4 (1.3-9.2)	0.014			
Anaemia	6.5 (1.4-31)	0.018			
LDH	1 (1-1)	0.02			
WCC>median	2.4 (1.1-5.4)	0.034		3.07 (1.01,9.38)	0.049

The univariate cox regression survival analysis for overall survival for the entire cohort (i.e. Cohort A& B for the ABC clinical trial) identified a number of significantly associated variables including: ALP (both as a continuous variable and ALP>median), LDH (both as a continuous variable, LDH>median and elevated LDH>upper limit of normal), CRP (both as a continuous variable and CRP>median), anaemia, and White cell count (WCC)> median. In a multivariate analysis in the entire cohort, CRP as a continuous variable and WCC>median remained significantly associated with overall survival.

4.1.7 Figures

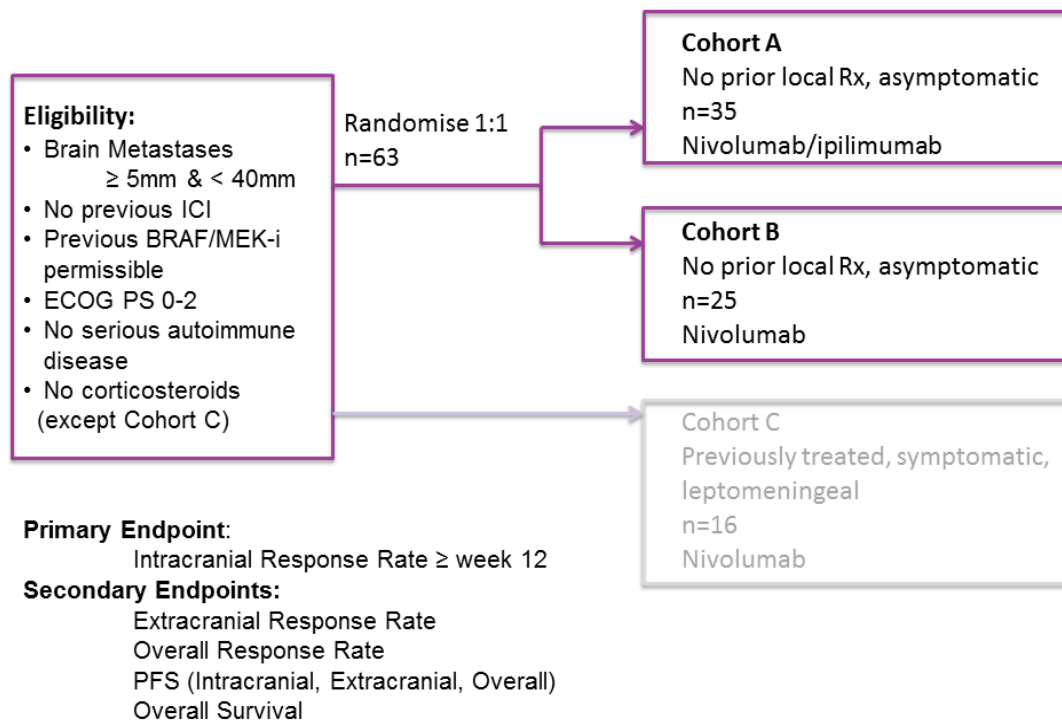


Figure 4.1 Simplified study schema of the Anti-PD-1 in Brain Collaboration clinical trial (3)

Patients with asymptomatic brain metastases who have not received prior local brain therapy were randomised to Cohort A (combination ipilimumab and nivolumab) or Cohort B (nivolumab monotherapy). Cohort C from the original study was excluded in the post-hoc analysis for candidate biomarkers as part of this thesis. The primary endpoints and secondary endpoints of the ABC study are as listed.

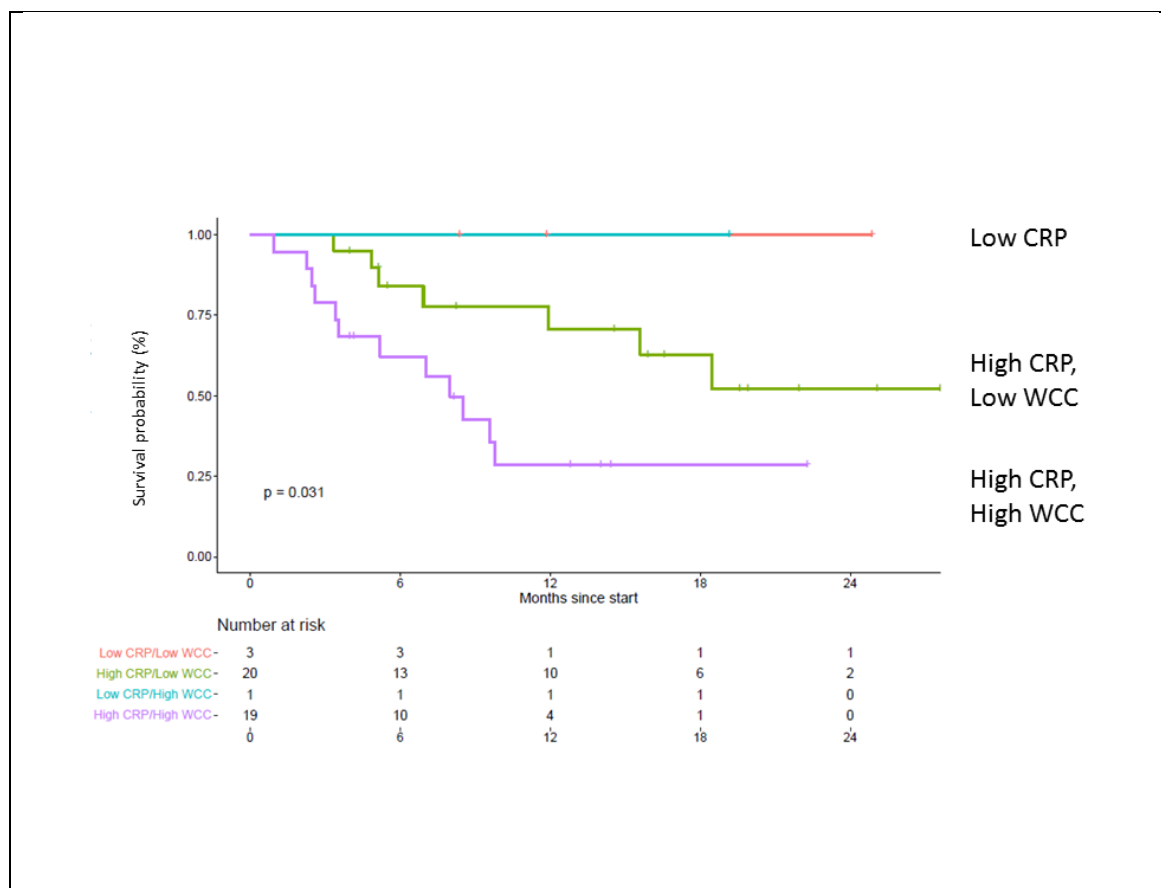


Figure 4.2 Kaplan-Meier curve of overall survival stratified by C-reactive protein (CRP) and white cell count (WCC)

Using the CRP and WCC, the two variables significantly associated with overall survival (OS) for the entire cohort of the post-hoc analysis, the cohort can be stratified into three prognostic groups. Low CRP is associated with excellent OS; high CRP but low WCC is associated with intermediate OS; whereas high CRP and high WCC are associated with poor OS.

5 Functional FDG PET imaging biomarkers

In this chapter, FDG PET biomarkers for ICI will be explored with a review of the existing literature, followed by two subchapters: Chapter 5.1 will investigate baseline FDG PET biomarkers, with the inclusion of the published manuscript, '18F-FDG PET/CT based spleen to liver ratio associates with clinical outcome to ipilimumab in patients with metastatic melanoma'. Chapter 5.2 will investigate the use of early-on-treatment FDG PET biomarkers in ICI for melanoma immunotherapy.

FDG PET is a non-invasive, semi-quantitative and tomographic assay of metabolic activity using a positron-emitting radioisotope-labelled glucose analogue(281, 428). FDG PET exploits cancer cells' preference for glycolysis regardless of oxygen conditions, known as the Warburg effect (429-431). Hence, FDG PET is able to detect small deposits of cancer as areas of high avidity compared to normal background tissue (431). Melanoma, in particular, is intensely FDG avid, which makes FDG PET/CT an optimal imaging modality for staging and surveillance of melanoma(432, 433).

Traditionally, conventional imaging with computerised tomography (CT) provides a diagnosis of cancer based on abnormal anatomic appearances, also highlighting sites of metastatic involvement. Due to low cost and widespread availability, CT remains a common imaging modality for metastatic disease in clinical practice. Functional imaging techniques such as (PET) combined with contrast CT (PET/CT) have a much higher sensitivity to detect advanced disease than CT alone in a number of different cancers including melanoma, lung cancer, head and neck cancer, breast cancer, prostate cancer (276, 433-436). One study also highlighted the improved sensitivity of FDG PET to detect melanoma bone and subcutaneous metastases compared to CT (434).

In a meta-analysis of 74 studies of 10,528 patients, PET combined with a 'low dose' CT was shown to have the highest sensitivity and specificity to stage metastatic melanoma (80% sensitivity, 95% CI 53-93% and 87% specificity, 95% CI 54-97%) when compared to ultrasound, CT or PET alone(433). Similarly, PET/CT also had the highest sensitivity and specificity to detect metastatic disease during surveillance of nodal disease (86% sensitivity, 95% CI 76-93% and 91% specificity, 95% CI 79-97%). Therefore, in the setting of high risk early stage disease, FDG PET has replaced CT as the gold standard imaging modality to exclude systemic spread prior to curative intent

treatment (278, 437). It should be noted though, that ultrasound remains the optimal imaging modality to detect nodal disease, owing to its high sensitivity in detecting low volume disease (433).

In a study of 49 patients, PET/CT was able to detect additional metastatic involvement in 27 patients (55%) and subsequently altered the clinical management in 49% of the patients (438). Another meta-analysis estimated that PET/CT changed the treatment decision in 15-64% of the cases (439).

There is also emerging evidence to support PET/CT in routine surveillance of high risk melanoma (278). In one study, one hundred and seventy patients with stage III melanoma were surveyed with a sub-stage adapted PET protocol post-operatively (432). Sixty-five (38%) patients relapsed, of which 69% were asymptomatic. The positive predictive values of individual scans were 56-83% in this cohort, whereas the negative predictive values were 89-96%. However, the authors recognise that the surveillance of melanoma needs to be stage adapted, so that more frequent imaging is offered to patients with the highest likelihood of relapse. A stage adapted surveillance schedule can reduce the possible harm of routine imaging to those with very low risk of relapse; such harm can include exposure to radiation, anxiety related to routine scans, so termed 'scanxiety', as well as minimising cost to the health system (440, 441).

Beyond its role in diagnosing and staging of cancer (428), FDG PET also has an established role in treatment assessment after therapy. Treatment response on FDG PET is correlated with clinical benefit following cytotoxic therapies in various cancers including non-small cell lung cancer, lymphoma and oesophageal cancer (442-447). Conventional treatment response assessment is performed on CT, with a reduction in the summation of tumour size being correlated with clinical benefit (280, 448). Currently the Response Evaluation Criteria in Solid Tumours (RECIST) version 1.1 is widely used across clinical trials as a way of standardising treatment response assessment and enabling a comparison of treatment efficacy across trials. Subsequent to RECIST 1.1, new criteria for treatment response assessment on PET were proposed, in an effort to overcome some of the limitations of anatomical assessment, particularly in cancers where stability of disease correlated with clinical benefit (281). Tumour response on PET can be measured quantitatively, where the reduction in tumoural avidity after therapy can be used as a surrogate of clinical benefit (281). Two main

criteria have been proposed to guide the assessment of treatment response using FDG PET, namely the European Organisation for Research and Treatment of Cancer (EORTC) PET criteria published in 1999 (449) and PET Response Criteria in Solid Tumours (PERCIST) published in 2009 (281). While PET treatment response is well established in other cancers following cytotoxic chemotherapy, only a small number of studies have evaluated PET treatment response assessment in advanced melanoma (258, 450, 451).

FDG PET was compared to CT and S100-B, a serum tumour marker for melanoma, as treatment assessment modalities following chemotherapy for metastatic melanoma in a retrospective study (450). In this study, 25 treatment-naïve patients were treated with 3 cycles of chemotherapy (9 patients received temozolomide monotherapy, 9 patients received dacarbazine monotherapy, 7 patients received temozolomide combination). Treatment response by PET or CT was defined as a greater than 30% decrease in the size or SUVmax in the known metastases. Concordant responses were observed in PET and CT in all patients. There was a trend towards improved overall survival in responders compared to non-responders (median OS of 18 months v 11 months, $p=0.072$) with an improved one-year overall survival (80% v 40%, $p=0.048$). S100B was normal in 8/25 patients at baseline, and not measured in 3 patients. S100B response did not correlate with survival. While chemotherapy is no longer a standard treatment for metastatic melanoma, this study posed the potential of response assessment on FDG PET as a surrogate marker of clinical benefit to therapy in melanoma.

A retrospective study used the PERCIST criteria on PET to assess treatment response to ipilimumab in 28 patients with stage III and stage IV melanoma (451). In this study, FDG PET scans were performed at baseline and after 2-4 cycles of treatment. Progressive Metabolic Disease (PMD), as defined by 30% increase in the SUVpeak normalised by lean body mass (SUL), was detected in 14 of the 21 patients with clinical progressive disease. Patients with PMD had poorer overall survival ($p=0.046$), poorer one-year survival (58% vs 100% in non-PMD) and poorer two-year survival (31% versus 73%).

One of the other challenges which has arisen in the era of immunotherapy is that of treatment response assessment. Conventional treatment response assessment used in

clinical trials can underestimate the benefit of immunotherapy due to the phenomenon of ‘pseudo-progression’ (452). Pseudo-progression is where tumours can exhibit a transient increase in size before subsequent reduction, and these atypical responses are associated with clinical benefit(453). Biopsies of tumours which exhibit this characteristic showed that these progressive lesions are in fact infiltrated by cytotoxic T cells and necrosis, as opposed to tumour cells in the case of genuine disease progression. These delayed responses are also correlated with clinical responses to ICI. The incidence of pseudo-progression is approximately 10% for anti-CTLA4 therapy and 7% for anti-PD-1 treatment (453, 454). Patients on combination immunotherapy may also experience the same phenomenon but the incidence is unknown.

Immune related response criteria (IrRC) are modified treatment response criteria which were proposed as a way to capture the novel tumour responses seen after immunotherapy treatment (452). The key differences of IrRC compared to WHO criteria are that new measurable lesions are incorporated into the sum of tumoural burden; new unmeasurable lesions do not constitute progressive disease; and progressive disease needs to be confirmed in subsequent imaging at least 4 weeks apart. IrRECIST and IRECIST criteria were subsequently proposed to simplify treatment response by using unidimensional assessment as opposed to multi-dimensional assessments in IrRC, as well as reducing the number of lesions assessed to 2 lesions per organ and 5 lesions in total (453, 455, 456).

In summary, assessment of tumour response to conventional cytotoxic therapies by PET imaging is well established, but response assessment to immunotherapies on PET requires further examination given their novel mechanism of action (457). Furthermore, it is not known whether early response on PET is a predictor of clinical benefit or whether FDG PET would also be hampered by the challenges of pseudo-progression.

5.1 Baseline PET/CT characteristics: Spleen to liver ratio

Functional imaging biomarkers present an attractive source of novel biomarkers for melanoma immunotherapy. This technology is widely used in clinical practice for the staging and surveillance of melanoma. Furthermore, it is a non-invasive quantitation of metabolic activity of both tumour and the body’s response to the tumour.

Baseline FDG PET imaging prior to immunotherapy may serve as a prognostic marker in two ways. Firstly, it can provide an accurate quantitation of metabolically active disease above what is appreciable on standard CT imaging by assessing the metabolic tumour volume as well as the tumoural SUVmax. Secondly, baseline scans can be used as a functional assessment of the patient's immune response to the cancer. Evaluation of immune organs such as the spleen or draining lymph nodes can be a functional evaluation of the immune response to the tumour.

Two recent studies have also attempted to evaluate whether baseline PET parameters can be a source of biomarkers after ICI. The first is a retrospective study of 142 patients who received ipilimumab monotherapy for advanced melanoma at Memorial Sloan Kettering Cancer Centre(458). This study evaluated SULmax (maximum SUV corrected by lean body mass), SULpeak (sum of peak SUV normalised by lean body mass in five target lesions/maximum two per organ), wMTV (whole body metabolic tumour volume) and wTLG (whole body Total Lesion Glycolysis). The PET parameters were dichotomized at the median of the cohort for their analysis: $SUL_{max} \geq 9.11$, $SUL_{peak} \geq 13.75$, $wMTV \geq 26.85 \text{ cm}^3$, and $wTLG \geq 78.74$ for survival analyses. In univariate analyses, there was a statistically significant relationship between MTV and overall survival (HR 1.93, $p=0.002$), as well as TLG and overall survival (HR1.64, $p=0.016$). Only wMTV, however, remained significantly associated with OS (HR2.02, $p=0.002$) when other clinical factors were considered, for instance, the presence of brain metastases, age ≥ 75 years, high LDH, prior chemotherapy. There was no PET evaluation of immune activation as a predictor of outcome in this study.

The second study is a retrospective study of 55 patients treated with antiPD-1 at two institutions(459). Baseline FDG PET scans were analysed for SUVmax, SUVmean, TMTV (total metabolic tumor volume), TLG (total lesion glycolysis), BLR (Bone marrow-to-Liver SUVmax ratio), and SLR (Spleen-to-Liver SUVmax ratio). PET parameters were dichotomized at the median of the cohort for statistical analyses: $SUV_{max} > 19.0$, $SUV_{mean} > 7.4$, $TMTV > 25 \text{ cm}^3$, $TLG > 258$, $BLR > 0.79$ and $SLR > 0.77$. Univariate analyses showed that shorter survival was correlated with high TMTV ($> 25 \text{ cm}^3$ (OS HR 2.9, $p=0.042$ and PFS HR 2.8, $p=0.021$) and BLR (> 0.79 , OS HR 2.6, $p=0.048$ and PFS HR 2.8, $p=0.042$). In multivariable analyses, survival was significantly associated with TMTV (OS HR 2.5, $p=0.02$, and PFS HR 2.2, $p=0.024$) and BLR (OS HR 2.3, $p=0.04$). Low TMTV and TLG correlated with best overall

response ($p = 0.03$). This study highlighted the potential of evaluating the immune system on PET imaging as a predictor of outcome to anti-PD-1 therapy.

The first part of this chapter explores the prognostic significance of PET parameters of SLR, SUVmax and MTV in patients treated with immune checkpoint inhibitors for advanced melanoma treated at Peter MacCallum Cancer Centre, Melbourne, Australia. These results have been submitted for publication.

5.1.1 Aims

The aims of this chapter were:

1. To assess baseline ^{18}F FDG PET/CT characteristics of tumoural SUVmax, MTV and Spleen to Liver Ratio as a predictor for PFS and OS after ipilimumab for advanced melanoma
2. To assess baseline ^{18}F FDG PET/CT characteristics of tumoural SUVmax, MTV and Spleen to Liver Ratio as a predictor for PFS and OS after antiPD-1 for advanced melanoma
3. To investigate clinicopathological correlates of high spleen to liver ratio on pre-treatment ^{18}F FDG PET/CT

5.1.2 Summary of results

1. High SLR>1.1 was associated with poor PFS and OS after ipilimumab, but not after anti-PD-1. This was validated in an independent cohort of European patients treated in Brussels and Zurich.
2. High SLR was an independent predictor of OS in univariate and multivariate survival analyses. Low albumin was significantly associated with high SLR.
3. Tumoural SUVmax was not associated with PFS or OS after ipilimumab or anti-PD-1.
4. MTV was not associated with PFS or OS after anti-PD-1. MTV was not associated with OS after ipilimumab when dichotomized at the median of the cohort, but it was associated with OS when analysed as a continuous variable.

5.1.3 Results not presented in the publication

1. The rate of liver metastases in patients with high SLR was 35% whereas the rate of liver metastases was 37% in patients with normal SLR, so the rate of liver metastases did not appear different between the two groups.
2. Liver function tests, including serum bilirubin, alanine transferase (ALT), alanine phosphatase and gamma transferase, did not differ between patients with high SLR and patients with normal SLR. One patient had elevated (3x upper limit of normal) ALP in the high SLR group, whereas two patients had elevated (3xULN) ALP in the normal SLR group. Furthermore, ALP is not a liver

specific marker, and can also be raised in patients with increased bone turnover/bony metastases. In total, liver function tests were not too dissimilar in patients with high SLR and those with normal SLR.

5.1.4 Discussion

Our study evaluated baseline FDG PET parameters and their association with clinical outcome after immune checkpoint inhibitors. Baseline SUVmax and MTV were not associated with outcome after ipilimumab or antiPD-1. High SLR>1.1 was associated with poor PFS and OS after ipilimumab. High SLR>1.1 and its association with poor OS was then validated in an external European cohort from two different European centres. High SLR was associated with low albumin in a multivariable model but the biological mechanism of this PET parameter remains unknown.

SUVmax has not been shown to be prognostic in our study or in the two aforementioned studies of patients treated with either ipilimumab (458) or anti-PD-1 monotherapy for advanced melanoma (459). The studies where SUV uptake has been shown to be correlated with survival were in early stage melanoma (277, 283). The threshold for significance for SUVmax is above 2.2 and SUVmean>6.49 in these two studies. SUVmax in the studies of advanced disease included patients whose tumours have a much higher SUVmax: the medians for our study was 18, whereas the ipilimumab study median SUVmax was 9.11 (458), and the antiPD-1 study median SUVmax was 19 (459). Advanced disease is more likely to have disease where the metabolic pathways are more dysregulated and so are highly FDG avid. It may be that the threshold used in the studies of advanced disease was too high or, more likely, that SUVmax becomes a poor predictor of survival in advanced disease. The threshold at which SUVmax loses its clinical significance is not known. The two aforementioned studies highlight that MTV (which is a composite value of both volume of tumour burden as well as tumoural FDG uptake) is a more important biomarker for prognosis after immunotherapy for melanoma (458, 459).

Baseline MTV did not correlate with survival in our study of ipilimumab or antiPD-1 treated patients when it was dichotomized by the median of each treatment (i.e. comparing patients with high MTV versus low MTV). MTV was significantly associated with overall survival when analysed as a continuous variable, which suggested a non-linear relationship between MTV and OS. It must be noted that the

thresholds used to dichotomize the cohort were higher in our cohort compared to those in other studies. In our cohort, Ipilimumab treated patients were dichotomized at the MTV of 41mL, and antiPD-1 patients were dichotomized at 140mL. Other studies used a threshold of 26.85mL for patients treated with ipilimumab (458), while 25mL was used in a study of antiPD-1 treated patients (459). Both these studies found a statistically significant relationship between high MTV and short overall survival. When our cohort was split into 5 MTV quantiles, the group with the lowest MTV (<14mL) was associated with excellent OS.

The failure to identify a significant relationship between our MTV and OS within our cohort highlights the potential challenges of using the median as an exploratory threshold for biomarker discovery. One method of identifying an ‘optimal’ threshold for a biomarker is the use of a receiver operating curve (ROC) (460, 461). This statistical modelling identifies a cut-off where the biomarker is the most discriminating for the desired outcome. However, this method is still exploratory and runs the risk of overfitting the predictive model to the discovery cohort. Hence it is of paramount importance to validate the findings of a discovery cohort in an independent external cohort.

Low baseline tumour burden is associated with good clinical outcome. This has been reported in a number of studies including those assessing tumour burden on CT imaging or PET imaging (274, 458, 459). This observation is supportive of the strategy to treat high risk melanoma in the adjuvant setting, where metastatic deposits are only microscopic. Accordingly, clinical trials have demonstrated a significant improvement in OS for patients treated with adjuvant immunotherapy strategies such as ipilimumab and interferon alpha (104, 229). Recent clinical trials also showed a significant improvement in disease free interval for patients treated with adjuvant anti-PD-1, though the impact of adjuvant anti-PD-1 treatment has yet to be reported (230, 231).

Interestingly, high spleen to liver ratio was associated with poor survival after ipilimumab in our cohort. This observation was validated in an independent cohort in Europe. The proportion of patients who exhibited this PET signature was small (approximately 10% of each cohort) but it highlighted a group of patients in which the prognosis is very poor. Overall survival measured from the time of first immunotherapy (either ipilimumab or anti-PD-1) was significantly lower in those with

high SLR>1.1 with median OS of 1 month vs 14 months in those with normal SLR, HR 3.92, $p=0.003$. High SLR was associated with poor PFS after ipilimumab, but not after anti-PD-1 treatment. High SLR pre-treatment as an imaging biomarker has not been previously explored in patients with metastatic melanoma treated with ipilimumab. High SLR has also been associated with poor prognosis in other tumour types, including cholangiocarcinoma and rectal cancer (288, 462). In these studies of cholangiocarcinoma and rectal cancer, a significant threshold for poor survival was set at 1 and 0.731 respectively, whereas in our cohort SLR>1.1 was considered to be high.

More recently, other investigators have evaluated baseline PET biomarkers for patients treated with anti-PD-1 in the aforementioned study (459). Seban *et al.* did not reveal any relationship between high SLR (>0.77) and survival after anti-PD-1, which is consistent with the findings in the Peter MacCallum Cancer Centre cohort. Interestingly, the authors did observe that high bone to liver ratio (>0.79) was associated with poor PFS and OS after anti-PD-1. This raises the possibility that there may be FDG avid immune cells present within the bone marrow prior to anti-PD-1 treatment which may inhibit the action of anti-PD-1. The different prognostic significance of high SLR with ipilimumab compared to anti-PD-1 may highlight the different mechanism of action between the two treatments or perhaps inhibitory cell types that may be present in the spleen that are more relevant for ipilimumab therapy compared to anti-PD-1.

The mechanism of high SLR remains unknown. The liver is normally of higher avidity than the spleen in physiological conditions (463, 464). The converse (i.e. where splenic avidity is higher than that of the liver) has been observed in patients with inflammatory conditions, in cancers where inflammation is part of the clinical presentation, or post GCSF or anti-CTLA4 treatment (286, 464, 465). The spleen is the largest lymphoid organ in the body and consists of red pulp and white pulp (466). The red pulp is a complex vascular network of vessels where blood is filtered and splenic macrophages can recycle iron and remove bacteria from the bloodstream. The white pulp consists of T and B cell compartments which surround the branching arterial vessels. Here, immune cells are able to interact under strict orchestration by cytokines and chemokines. Within the T-cell zone, immune cells such as T cells, dendritic cells and B cells interact; whereas B cells undergo clonal expansion within the B-cell zone. Humans also have an inner and outer marginal zone which surrounds the white pulp, which is lacking in rodents. This marginal zone is a transitional area where

haemopoietic cells from the blood can enter the lymphoid compartment, which allows both innate and adaptive immune responses to be mounted efficiently. Such innate response involves resident immune cells such as marginal zone macrophages and marginal zone metallophilic macrophages. There are also marginal-zone B cells which travel out of the marginal zone into the white pulp, become antigen presenting cells and activate CD4 T cells and a subset of dendritic cells. It is not known which cells within the spleen are responsible for the increase in glucose uptake.

Previous studies have attempted to evaluate the biological correlates of splenic avidity (286, 287, 467). The second study evaluated the peripheral cytokine profile of 40 patients with upper gastrointestinal malignancies in relation to SLR. Within this study, 19 of the 40 patients had a high SLR. Patients with high SLR had high IL-1 β , IL-1RA, IL-4, IL-6, IL-7 and IL-13 compared to patients with normal SLR (286). High SLR was also associated with raised white cell count and raised C-reactive protein, which has been previously demonstrated in a study of 31 patients with high SLR (467). These studies suggest that a systemic acute inflammatory response or humoral cytokine response may be in keeping with this PET signature. We also studied 14 patients' cytokine profiles, but comparative statistics were not possible as only one patient exhibited high SLR. The descriptive statistics are presented within the supplementary results of the publication. Although the study was too small for comparative statistics, it was noted that the cytokine levels for the single patient with high SLR were within the range of patients with normal SLR. Studies of serum cytokines have yet to identify any predictive profile in relation to ICI response (468). However, a more targeted study of cytokines in the context of other inflammatory signatures such as high SLR may be more revealing of the cytokine profiles of patients with a very poor outcome following ipilimumab.

It is also important to consider the hepatic SUV uptake in the context of this SLR signature. In our study, patients with raised SLR had a mean and median liver SUV of 2.0 with an interquartile range (IQR) of 0.5, which is slightly lower than the normal reference range (lower limit of normal for liver SUV was 2.3) (469). In contrast, the normal SLR group had a mean and median liver SUV of 2.4, IQR 0.6; which is within the normal reference range. Within the high SLR group, the mean and median splenic SUV was 2.4, with IQR of 0.4; in contrast with normal SLR group, where the mean and median splenic SUV was 2.1, IQR 0.5. This raises the possibility that the high SLR

may be partially caused by low hepatic SUV uptake. When the liver was examined in more detail, there did not appear to be any difference in hepatic function between those with high SLR compared to those with normal SLR. Furthermore, albumin, which was highly correlated with SLR, is not correlated to other liver function tests such as bilirubin and ALT. Thus, it is more likely that albumin is low as part of an acute inflammatory process and not representative of generalised liver dysfunction in patients with high SLR. Lastly, the rates of intrahepatic metastases are also similar between patients with high SLR and normal SLR (rates of 35% and 37% respectively). This re-emphasises that the lower hepatic SUV uptake in patients with SLR is likely due to a functional process rather than a structural abnormality within the liver. Larger patient cohorts with high SLR are required to clarify the relative contributions of hepatic and splenic changes and their relevance on the immune system.

In the study of anti-PD-1 treated patients (459), baseline PET parameters were correlated with tumoural transcriptomes for 17 patients using NanoString nCounter Gene Expression Assay across 770 genes in the Pan Cancer Immune Pathways panel. Eleven predefined clusters totalling 42 genes were evaluated and High BLR was associated with upregulation of genes within the dendritic cells, regulatory T cells and memory T cell clusters. Of note, high SLR was also associated with upregulation of genes within the regulatory T cells as well as exhausted/HS CD8⁺ T cells clusters.

Lastly, can we hypothesise which immune cells have become FDG avid within the spleen, that would correlate with poor survival after anti-CTLA4 and not anti-PD-1? A number of immune cells preferentially utilise glucose for their metabolism, including activated T cells, myeloid derived suppression cells (470). Activated T cells switch from OXPHOS to aerobic glycolytic state, in order to generate ATP which is mediated by HIF and GLUT1. One possibility of highly avid spleen being a poor prognostic sign is the presence of activated T cells sequestered within the spleen and not able to travel to peripheral tumoural sites. This would be particularly true if the process by which T cells are released from the spleen were inhibited by anti-PD-1 and not anti-CTLA4. Therefore, treatment with anti-CTLA4 does not alleviate the problem and is associated with poor prognosis, whereas treatment with anti-PD-1 overcomes this problem and patient outcome is not affected despite the presence of high SLR. Furthermore, could there be glucose dependent inhibitory immune cells which counteract only the actions of anti-CTLA4 but have no effect on anti-PD-1? In terms of inhibitor immune cells,

there are myeloid cellular subtypes including tumour associated macrophages and myeloid derived suppressor cells both of which associate with poor response to ICI (470, 471). Activated M1 myeloid cells (associated with IL1, IL6, IL12, reactive nitrogen or oxygen species) utilise glycolysis whereas M2 macrophages, which are associated with IL4 and IL10 and are inhibitory to T cell activation, preferentially use OXPHOS and fatty acid oxidation. It may be that high populations of M1 are particularly relevant to ipilimumab treatment compared to anti-PD-1. These are possible reasons why high SLR is associated with poor prognosis but not anti-PD-1 (459). Understanding how high SLR is correlated with poor outcome with ipilimumab and not with anti-PD-1 would be very helpful in furthering the understanding of cellular populations within the spleen that contribute to the functions of different ICI.

One of the challenges of elucidating the biological mechanism of high SLR is the lack of a biological model system to replicate this PET finding. Within this PhD study, unsuccessful attempts were made to generate a murine model by transplantation of MC38, an immunogenic colorectal cancer cell line, into C57BL/6 mice. Five mice were imaged by FDG PET but splenic uptake was normal. GCSF was injected into one of the five mice to induce splenic avidity as described in human patients (289). However, this was also unsuccessful; consequently, the planned experiments to compare immune compartments within the spleen, bone marrow and peripheral blood did not proceed. Further mechanistic understanding of this PET parameter may assist the understanding of differential mechanisms of resistance to anti-CTLA4 treatments, as compared to anti-PD-1.

5.1.5 Manuscript accepted and in-press

This manuscript entitled, “¹⁸F-FDG PET/CT based spleen to liver ratio associates with clinical outcome to ipilimumab in patients with metastatic melanoma” was submitted to Cancer Imaging in February 2020 and has been accepted for publication. The references for the manuscript have been incorporated into the references for the PhD thesis.

Title Page

¹⁸F-FDG PET/CT based spleen to liver ratio associates with clinical outcome to ipilimumab in patients with metastatic melanoma.

*Annie Wong^{1,2+}, Jason Callahan³, Marleen Keyaerts^{4,5}, Bart Neyns⁶, Johanna Mangana⁷, Susanne Aberle⁸, Alan Herschtal⁹, Sonia Fullerton¹⁰, Donna Milne¹¹, Amir Iravani³, Grant A. McArthur^{1,2} * and Rodney J. Hicks^{1,2,3} **

1. Research Division, Peter MacCallum Cancer Centre, 305 Grattan St., Melbourne, VIC 3000, Australia

2. Sir Peter MacCallum Department of Oncology, University of Melbourne, VIC 3010, Australia

3. Department of Cancer Imaging, Peter MacCallum Cancer Centre, 305 Grattan St., Melbourne, VIC 3000, Australia

4. Nuclear Medicine Department, UZ Brussel, Laarbeeklaan 101, 1090 Brussels, Belgium

5. *In Vivo Cellular and Molecular Imaging Laboratory, Vrije Universiteit Brussel, Laarbeeklaan 103, 1090 Brussels, Belgium*
6. *Department of Medical Oncology, UZ Brussel, Laarbeeklaan 101, 1090 Brussels, Belgium*
7. *Department of Dermatology University Hospital Zurich, Switzerland*
8. *Department of Nuclear Medicine, University Hospital Zurich, Zurich Switzerland*
9. *Centre for Biostatistics and Clinical Trials, Peter MacCallum Cancer Centre, 305 Grattan St., Melbourne, VIC 3000, Australia*
10. *Department of Palliative Care, Peter MacCallum Cancer Centre, Melbourne, Australia*
11. *Department of Cancer Experiences Research, Peter MacCallum Cancer Centre, Melbourne, Australia*

**Equal contribution*

+Corresponding author

Emails:

Annie.Wong@petermac.org; Jason.Callahan@petermac.org;

Marleen.keyaerts@vub.ac.be; Bart.Neyns@uzbrussel.be; Johanna.Mangana@usz.ch;

Susanne.Aberle@usb.ch; Alan.Herschtal@petermac.org;

Sonia.Fullerton@petermac.org; Donna.Milne@petermac.org;

Amir.Iravani@petermac.org; Rod.Hicks@petermac.org; Grant.mcarthur@petermac.org

Abstract

Background:

Immune checkpoint blockade such as ipilimumab and anti-PD-1 monoclonal antibodies have significantly improved survival in advanced melanoma. Biomarkers are urgently needed as a majority of patients do not respond, despite treatment-related toxicities. We analysed pre-treatment ^{18}F -fluorodeoxyglucose positron emission tomography/computerised tomography (FDG PET/CT) parameters to assess its correlation with patient outcome.

Methods:

This retrospective study evaluated pre-treatment FDG PET/CT scans in a discovery cohort of patients with advanced melanoma treated with ipilimumab or anti-PD-1. Pre-treatment scans were assessed for maximum tumoural standardised uptake value (SUVmax), metabolic tumour volume (MTV) and spleen to liver ratio (SLR). Progression-free survival (PFS) and overall survival (OS) were characterised and modelled using univariable and multivariable analyses. Correlation of SLR and OS was validated in an independent cohort. Blood parameters and stored sera of patients from the discovery cohort was analysed to investigate biological correlates with SLR.

Results:

Of the 90 evaluable patients in the discovery cohort: 50 received ipilimumab monotherapy, 20 received anti-PD-1 monotherapy, and 20 patients received ipilimumab followed by anti-PD-1 upon disease progression. High SLR > 1.1 was associated with poor PFS (median 1 vs 3 months; HR 3.14, $p=0.008$) for patients treated with

ipilimumab. High SLR was associated with poor OS after ipilimumab (median 1 vs 21 months; HR 5.83, $p=0.0001$); as well as poor OS after first line immunotherapy of either ipilimumab or anti-PD-1 (median 1 vs 14 months; HR 3.92, $p=0.003$). The association of high SLR and poor OS after ipilimumab was validated in an independent cohort of 110 patients (median 2.3 months versus 11.9 months, HR 3.74). SLR was associated with poor OS in a multi-variable model independent of stage, LDH, absolute lymphocyte count and MTV.

Conclusions:

Pre-treatment Spleen to liver ratio (SLR) >1.1 was associated with poor outcome after ipilimumab in advanced melanoma. This parameter warrants prospective evaluation.

Keywords:

Melanoma, Immune Checkpoint Blockade, Biomarker, Positron Emission Tomography, Spleen to Liver Ratio

Article

Background:

The advent of immune checkpoint blockade has resulted in unprecedented improvements in survival for patients with metastatic melanoma. However, response rates of approximately 10% and 40% respectively for anti-CTLA4 (ipilimumab)(166, 169) and anti-PD-1 monoclonal antibodies(172, 174, 240), indicate that the majority of patients do not respond. In 2015, the combination ipilimumab and nivolumab demonstrated improved response rates and progression-free survival compared to single agent ipilimumab(176, 189). However, this combination resulted in significantly higher treatment-related toxicity than monotherapy (55% vs 16-27% patients experienced common toxicity criteria for adverse events grade 3 and 4 toxicities respectively). Even then, 42% of patients still failed to achieve an objective response. Hence, biomarkers that can predict clinical outcomes are urgently needed.

There are numerous candidate predictive biomarkers including expression of tumoural PD-L1 (296, 472) and tumoural mutational load(329), but these have not entered clinical practice due to their inability to clearly prospectively identify responders from non-responders. It is now recognised that a combination of different biomarkers may be needed to predict response given the complexity of the immune interaction with tumour(376). Blank et al suggested a ‘cancer immunogram’ wherein a combination of biomarkers such as tumoural mutational load, presence of T-cell checkpoints, soluble cytokines, metabolic factors and host immune factors should be considered. Here we explore how existing non-invasive functional imaging might provide a readily translatable source of novel biomarkers, given the current increase of ^{18}F -

fluorodeoxyglucose positron emission tomography/computerised tomography (FDG PET/CT) in the surveillance of high-risk melanoma(432) or at suspected relapse(437).

¹⁸F-fluoro-deoxy-glucose positron emission tomography (FDG PET) has long been the preferred functional imaging technique in melanoma in our facility(473). Our identification of malignancy is largely qualitative based on a combination of PET and CT appearances(474) since the use of the semi-quantitative measure of uptake intensity termed the standardised uptake value (SUV) can have limitations with regards to cancer evaluation due to the large number of technical factors that can influence its measurement(475). Nevertheless, the measurement of SUVmax has been shown to have some prognostic significance in a number of malignancies, including melanoma(283). Metabolic tumour volume (MTV) is a measure of metabolically active disease and, reflecting the burden of disease, might be more likely to correlate with clinical outcome.

In addition to studying PET-derived parameters of the tumour, there may be merit in examining the patients' tissues, such as the spleen or draining lymph nodes, as a way of characterising immune status. The SUV of normal organs, particularly the liver and spleen, generally have a rather narrow range(463, 476). For example, the liver normally exhibits homogenous uptake of FDG (normal SUVmax of liver in men range from 2.3-5.0 and 2.3-3.8 in women)(469), unless there is obvious disease infiltration or sarcoidosis. The spleen is the largest lymphoid organ in the body, and normally has less FDG uptake compared to the liver (normal SUVmax of spleen in men range from 1.6-4.1 and 1.6-3.2 in women), with an expected Spleen to Liver Ratio (SLR) of approximately 0.9(287, 288, 469). High splenic uptake or high SLR has been observed

in patients with infections as well as patients with cancers associated with an inflammatory state, such as Hodgkin's disease(477) or cholangiocarcinoma(286-288). There have also been reports of immune activation as a result of interferon alpha 2b or anti-CTLA4 (290). Hence PET evaluation of immune organs of patients receiving immunotherapy may be of clinical value. In this article, we analysed baseline FDG PET/CT performed on patients prior to immunotherapy and its correlation with clinical outcome to identify novel PET imaging biomarkers.

Methods:

Patients

All patients with unresectable stage III or stage IV melanoma (with progressive disease) who received monotherapy with ipilimumab or anti-PD-1 (either pembrolizumab or nivolumab) and had completed a pre-treatment FDG PET scan at Peter MacCallum Cancer Centre (PMCC) between 1st July 2010 and 30th June 2015 were included. Prior treatment with BRAF targeted therapies was permissible but patients treated with BRAF/MEK inhibitors post progression on ipilimumab or anti-PD-1 were excluded. Ipilimumab was given at 3mg/kg intravenously every three weeks for four doses. Pembrolizumab was given at 2mg/kg intravenously every three weeks, and nivolumab was given at 3mg/kg intravenously every two weeks until disease progression. Patients received anti-PD-1 either as monotherapy or upon progression after ipilimumab treatment (i.e. concurrent ipilimumab and nivolumab treatment was not included). Medical records were retrospectively reviewed for patient demographics, disease characteristics, blood parameters, treatment and clinical outcome. Treatment response was determined by local treating oncologist based on available clinical and imaging data.

We then sought to validate the association between SLR>1.1 and OS in an additional cohort. Eligible patients treated at UZ Brussel, Brussels and University Hospital Zurich, Zurich were combined into a single validation cohort. Patients treated with at least one dose of ipilimumab were included and patient demographics, date of death or last contact were recorded. Pre-treatment PET scans were assessed for SLR by the local nuclear medicine expert. The final statistical analysis was performed at PMCC. This study was approved by human research ethics committees and the requirement for informed consent was waived at the PMCC, the UZ Brussel, and the University Hospital Zurich.

FDG-PET/CT Imaging Analysis

Pre-treatment PET scans were performed within 6 weeks before commencing ipilimumab or anti-PD-1. At PMCC, the analysis was performed by using MIM software, which provided an automated volume-of-interest analysis on the basis of user-defined search regions. Parameters evaluated included the SUVmax for all tumour lesions, MTV and SLR. Metabolic tumour volume (mL), was calculated by multiplying the number of abnormal voxels identified within the volume of interest by the known voxel volume by adapting the PERCIST recommendations for contouring FDG-avid disease(281). All FDG-avid disease was contoured using an SUV threshold, which was applied to the whole body. The SUV threshold used was 1.5 times the mean SUV of the liver plus 2 standard deviations. The SLR is calculated by placing 2cm spherical volumes of interest in the liver and spleen (Figure 5.1).

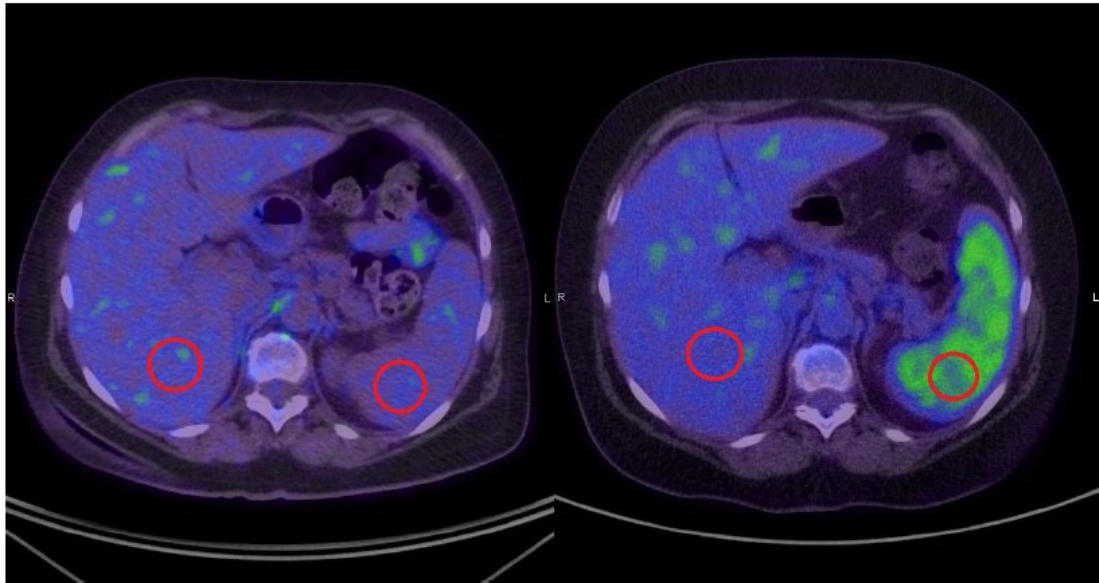


Figure 5.1. Spleen to Liver Ratio (SLR) on positron emission tomography

Example of normal splenic FDG tracer uptake (left) compared to abnormally high tracer uptake within the spleen (right). The SUV is calculated by placing 2cm spherical volumes of interest in the liver and spleen respectively (as depicted by red circles). The spleen to liver ratio (SLR) is calculated by dividing the SUVmean of the spleen by the SUVmean of the liver. $SLR > 1.1$ is considered abnormally high.

The ratio of the SUVmean of the spleen to the SUVmean of the liver is then calculated. A SLR of greater than 1.1 was considered abnormal. If the patient had metastases in either the liver or the spleen, the volumes of interest would be placed in the adjacent normal organ tissue to avoid areas of metastases. The validation sites were only required to evaluate the SLR. Detailed PET acquisition methodology is included in *supplementary materials (S1)*.

Statistical analyses

FDG PET/CT parameters (tumoural SUVmax, MTV and SLR) were tested for association with PFS and OS after ipilimumab and anti-PD-1, with treatment line and metastasis substage considered as confounding variables(11, 379). PFS and OS were

tested for association with PET/CT parameters using Cox proportional hazards regression. The median tumoural SUVmax and MTV were calculated using all pre-treatment scans in the discovery cohort (n=110). These median values were used to dichotomize the cohort to compare high tumoural SUVmax versus low tumoural SUVmax, as well as high MTV versus low MTV. Tumoural SUVmax and MTV were also analysed as continuous variables. The SLR normally varies within a tight range of 0.9-1.0; consequently SLR >1.1 was considered abnormally high and used as the threshold for survival analyses. PFS was assessed and modelled separately for ipilimumab and anti-PD-1 and was measured from the start of immunotherapy to the date of progression or death; patients were censored at date of last contact. Progression was assessed either clinically (if patient had died or was too unwell for imaging), or clinical imaging with either PET, CT or MRI. OS was assessed and modelled separately for a) ipilimumab, b) anti-PD-1 and c) first line immunotherapy (either ipilimumab or anti-PD-1, whichever was received first). OS was measured from start of treatment until death or censored at date of last contact. Other analyses for survival after first line immunotherapy considered LDH, metastasis substage (AJCC 7th Edition)(11), MTV, ALC and SLR. Statistical analyses were performed using the base package and the survival package (R version 3.4.2). Kaplan-Meier survival curves were used to present survival outcome for SUV and MTV as dichotomized at the median for the treatment cohort, or at the abnormal threshold of SLR>1.1. The additional cohorts (UZH and UHZ) were used to validate the association of SLR with OS after ipilimumab.

Biological analyses on discovery cohort

To explore the relationship between SLR and inflammatory and haematological parameters, a multi-variable linear regression model was built to explore predictor variables including: neutrophil:lymphocyte ratio, haemoglobin, LDH, ALC $\times 10^9$, albumin, lymphocyte: monocyte ratio and patient performance status (Supplementary materials S2).

A subset of patients in the discovery cohort were also enrolled in a separate biomarker study where their sera were stored at multiple time points. Pre-treatment samples were taken at baseline, prior to treatment at week 3 and prior to week 6. We sought to evaluate baseline and dynamic changes in the cytokine profile of patients with high SLR compared to those with normal SLR (Supplementary materials S3). Stored serum samples were tested using the BD Cytometric Bead Array Enhanced Sensitivity kit for IL1 β , IL2, IL4, IL6, IL10, IL12p70, IL17A, TNF, IFN γ as per the manufacturer's protocol. Experiments were performed in duplicate. Samples were analysed using the BD FACSVerse analyser and results summarised using Graphpad Prism.

Results:

Patient and disease characteristics for discovery cohort

Ninety-one patients received ipilimumab or anti-PD-1 with a pre-treatment FDG PET scan in the study period. One patient had metabolic tumour volume of zero and was excluded from all analyses. Of the 90 evaluable patients: 50 patients received ipilimumab only, 20 patients received anti-PD-1 only and 20 patients received ipilimumab followed by anti-PD-1. The cohort of patients is summarised in Table 5.1.

The median age of patients was 61 years, 67% were men, and 67% had visceral M1c disease. One patient was excluded from the analyses for SLR as she had previously undergone a splenectomy.

Table 5.1 Patient clinical and disease characteristics

Patient characteristic	n = 90
Age	
Mean	60.6
Range	30 - 87
Sex	
Female	30 (33%)
Male	60 (67%)
Stage	
IIIC	5 (6%)
M1a	7 (8%)
M1b	17 (19%)
M1c	61 (68%)
Mutation	
BRAFV600E	16 (18%)
BRAFV600K	2 (2%)
BRAF other	5 (6%)
RAS	9 (10%)
Other	1 (1%)
No mutation	54 (60%)
Not reported	3 (3%)
Lactate dehydrogenase (n=78)	
≤1xULN	26 (33%)
>1xULN	52 (66%)
Previous lines of therapy	
0	52 (58%)
1	30 (33%)
2	8 (9%)

Pre-treatment FDG PET and Patient Survival in discovery cohort

The 90 patients had a median tumoural SUVmax of 16.6 (Range 2.2-78.2, IQR 12.1-23.7), median MTV of 60.2ml (Range 0.03-2693.7, IQR 13.5-212.7) and median SLR of 0.9 (Range 0.7-1.4, IQR 0.8-1.0). See Table 5.2 for full details.

Table 5.2 Baseline PET parameters of discovery cohort (all patients treated with immunotherapy, n =90)

PET parameter	Mean	Median	Range	IQR
Tumoural SUV max	20.3	16.6	[2.2, 78.21]	12.12 - 23.74
SUV mean	6.9	6.1	[1.7, 18.92]	5.33 - 7.54
MTV (mL)	197.6	60.2	[0.03, 2694]	13.47 - 212.74
SLR	0.9	0.9	[0.669, 1.364]	0.81 - 0.97

PET, positron emission tomography; IQR, interquarter range; SUVmax, maximum Standardised Uptake Value; SUVmean, mean Standardised Uptake Value; MTV, Metabolic Tumour Volume; SLR, Spleen to Liver Ratio.

Baseline PET parameters of discovery cohort (patients treated with ipilimumab, n=70)

PET parameter	Mean	Median	Range	IQR
Tumoural SUV max	19.3	16.1	[2.2,71.8]	[11.6,21.5]
SUV mean	6.8	6.1	[1.7, 18.9]	[5.1 - 7.5]
MTV (mL)	175.2	36.3	[0.03,2693.7]	[12.5,167.1]
SLR	0.9	0.9	[0.669, 1.364]	0.81 - 0.97

PET, positron emission tomography; IQR, interquarter range; SUVmax, maximum Standardised Uptake Value; SUVmean, mean Standardised Uptake Value; MTV, Metabolic Tumour Volume; SLR, Spleen to Liver Ratio.

Baseline PET parameters of discovery cohort (patients treated with antiPD1, n=40)

PET parameter	Mean	Median	Range	IQR
Tumoural SUV max	20.3	16.6	[2.2, 78.21]	12.12 - 23.74
SUV mean	6.9	6.1	[1.7, 18.92]	5.33 - 7.54
MTV (mL)	197.6	60.2	[0.03, 2694]	13.47 - 212.74
SLR	0.9	0.9	[0.669, 1.364]	0.81 - 0.97

PET, positron emission tomography; IQR, interquarter range; SUVmax, maximum Standardised Uptake Value; SUVmean, mean Standardised Uptake Value; MTV, Metabolic Tumour Volume; SLR, Spleen to Liver Ratio.

Nine out of 69 (13%) ipilimumab-treated patients had a high SLR, and 5 of 40 (13%) anti-PD-1 patients had a high SLR. Tumoural SUVmax was not significantly associated with PFS after ipilimumab or antiPD1 when dichotomized at the median of the cohort (HR 0.78, $p = 0.348$ and HR 0.81, $p = 0.560$ respectively). See Table 5.3(A) for details. Tumoural SUVmax was also not significantly associated with PFS when analysed as a continuous variable, HR 1.00 for ipilimumab with $p=0.74$ and HR 0.98 for anti-PD-1 with $p=0.23$. MTV was not statistically significant in its association with PFS when dichotomized at the median of the cohort for ipilimumab (MTV> 41ml, HR 1.45 with $p=0.23$) or for anti-PD-1 (MTV>140ml, HR 0.94 with $p=0.86$). MTV was also not significantly associated with PFS when analysed as a continuous variable, HR 1.07 for ipilimumab with $p=0.07$ and HR=1.00 for anti-PD-1 with $p=0.86$. However, strikingly, high SLR > 1.1 was associated with poor PFS after ipilimumab compared to patients with normal SLR (median PFS 1.0 vs 3.0 months, HR 3.14 , $p = 0.008$), but was not associated with PFS after anti-PD-1 (median 3.0 vs 3.0 months, HR 0.56, $p = 0.324$). See Figure 5.2 for Kaplan-Meier survival curves for pre-treatment PET biomarkers.

Table 5.3 (A) Association between PET parameters and progression free survival, for ipilimumab and anti-PD-1 treatment, analysed as continuous variable and dichotomized at the median for the cohort

	Ipilimumab (n=70)					Anti-PD-1 (n=40)				
PET parameter	Level	n	HR	HR 95% CI	P-value	Level	n	HR	HR 95% CI	P-value
Tumoural SUVmax			0.997	[0.978, 1.02]	0.74			0.984	[0.958, 1.01]	0.23
MTV (mL)			1.07	[1.01, 1.14]	0.066			0.993	[0.924, 1.07]	0.86
SLR			8.24	[1.08, 63.1]	0.05			0.904	[0.0623, 13.1]	0.94
Tumoural SUVmax	≤18	39	1	-	0.348	≤18	16	1	-	0.56
	>18	31	0.78	[0.46, 1.31]		>18	24	0.81	[0.39, 1.66]	
MTV (mL)	≤41	34	1	-	0.231	≤140	20	1	-	0.86
	>41	36	1.45	[0.79, 2.68]		>140	20	0.94	[0.46, 1.90]	
SLR	≤1.1	60	1	-	0.008	≤1.1	35	1	-	0.32
	>1.1	9	3.14	[1.46, 6.75]		>1.1	5	0.56	[0.16, 1.92]	

Anti-PD-1, Anti-programmed death 1 monoclonal antibody; PET, positron emission tomography; HR, hazard ratio; CI, confidence interval; SUVmax, maximum Standardised Uptake Value; SUVmean, mean Standardised Uptake Value; MTV, Metabolic Tumour Volume

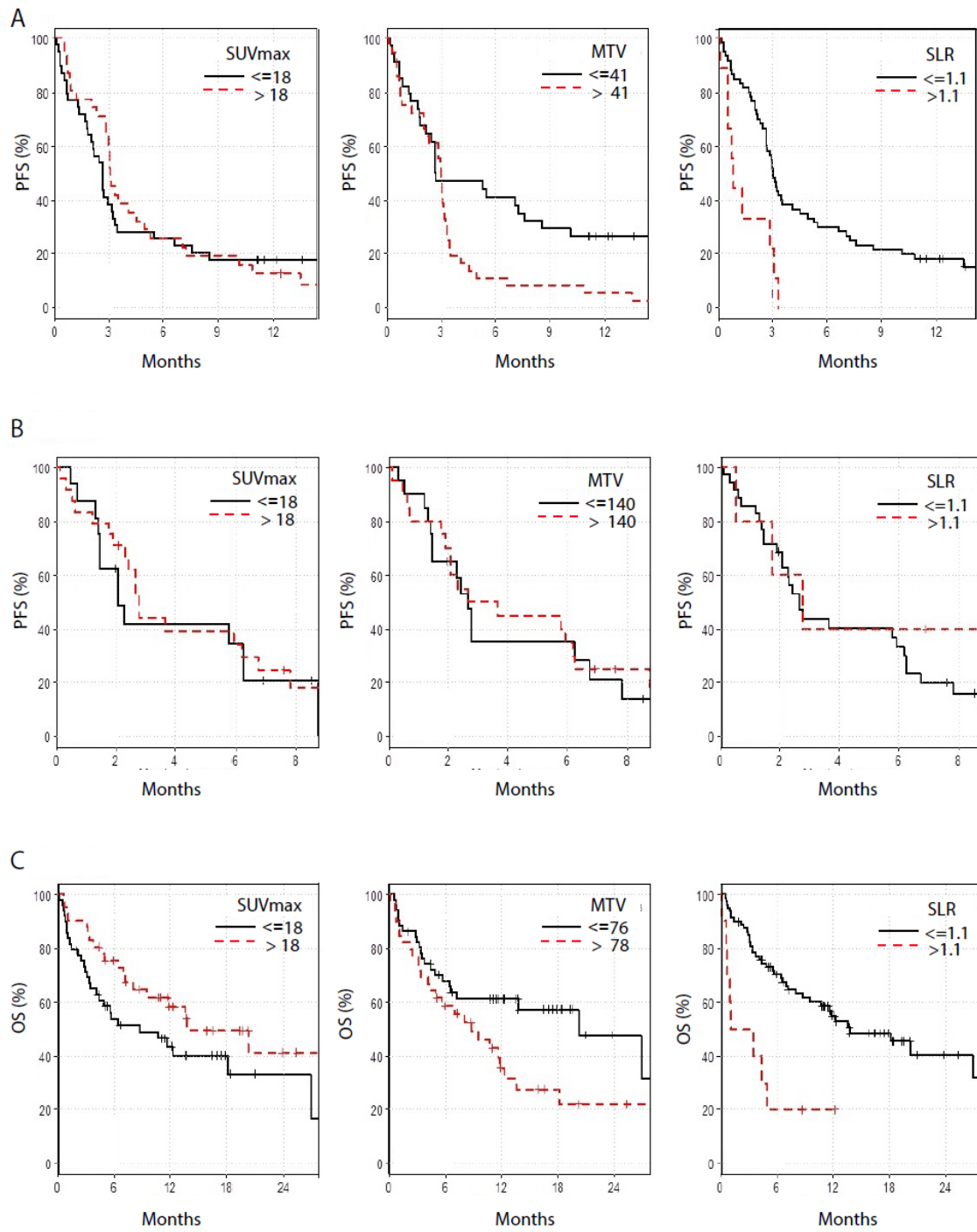
(B) Association between PET imaging parameters and overall survival, analysed as continuous variable and dichotomized at the median for the cohort

	Ipilimumab (n=70)				
PET parameter	Level	n	HR	HR 95% CI	P-value
Tumoural SUVmax			0.983	[0.95, 1.01]	0.24
MTV			1.000	[1.00, 1.001]	0.03
SLR			18.71	[1.34, 261.25]	0.02
Tumoural SUVmax	<16.07	35	1	-	
	≥16.07	35	0.83	[0.42, 1.63]	0.59
MTV (mL)	<41.38	35	1	-	
	≥41.38	35	1.09	[0.52, 2.29]	0.83
SLR	≤1.1	59	1	-	
	>1.1	10	5.83	[2.31-14.74]	0.0002

	Anti-PD-1 (n=40)				
PET parameter	Level	n	HR	HR 95% CI	P-value
Tumoural SUVmax			0.967	[0.93, 1.007]	0.10
MTV			1.000	[0.9995, 1.001]	0.49
SLR			2.419	[0.08, 76.00]	0.62
Tumoural SUVmax	<19.33	20	1	-	
	≥19.33	20	0.57	[0.21, 1.53]	0.27
MTV (mL)	<137	20	1	-	
	≥137	20	1.67	[0.64, 4.34]	0.29
SLR	≤1.1	35	1	-	
	>1.1	5	0.92	[0.26, 3.29]	0.89

	First line immunotherapy (n=90)				
PET parameter	Level	n	HR	HR 95% CI	P-value
Tumoural SUVmax			0.986	[0.964, 1.01]	0.21
MTV			1.09	[1.02, 1.15]	0.02
SLR			17.3	[1.90, 157]	0.02
Tumoural SUVmax	≤18	49	1	-	0.05
	>18	41	0.57	[0.32, 1.01]	
MTV (mL)	≤76	51	1	-	0.61
	>76	39	1.18	[0.63, 2.23]	
SLR	≤1.1	79	1	-	0.003
	>1.1	10	3.92	[1.76, 8.72]	

PET, positron emission tomography; SUVmax, maximum Standardised Uptake Value; MTV, Metabolic Tumour Volume; SLR, Spleen to Liver Ratio.



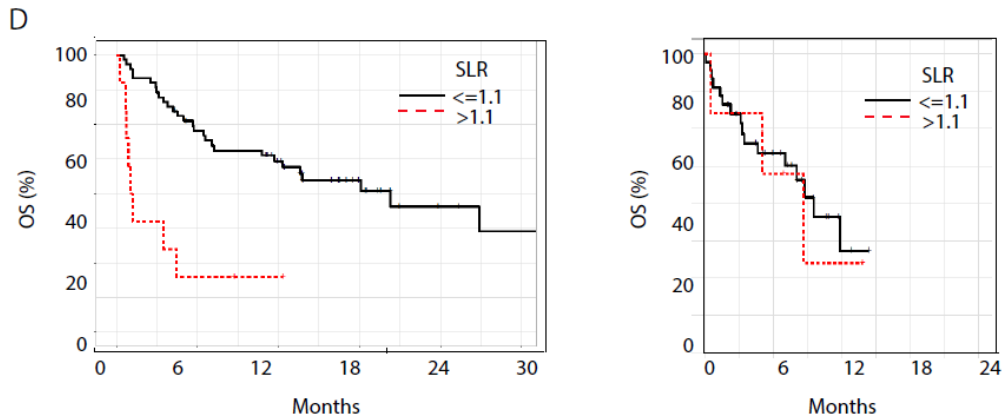


Figure 5.2 (A). Kaplan Meier curves of FDG PET parameters and Progression free survival after ipilimumab; (B). Kaplan Meier curves of FDG-PET parameters and Progression free survival after anti-PD-1; (C). Kaplan Meier curves of FDG-PET parameters and overall survival from the start of first line immunotherapy; (D). Kaplan Meier curves of Spleen to Liver Ratio (SLR) and overall survival after ipilimumab and anti-PD-1 respectively

Kaplan Meier survival curves for progression free survival after ipilimumab or anti-PD-1 and its correlation for SUVmax, Metabolic Tumour Volume (MTV) and spleen to liver ratio (SLR) respectively. High SLR was significantly correlated with PFS after ipilimumab (median PFS 1.0 vs 3.0 months, HR 3.14, $p = 0.008$), but was not associated with PFS after anti-PD-1 (median 3.0 vs 3.0 months, HR 0.56, $p = 0.324$). High SLR was associated with poor OS after first line immunotherapy (ipilimumab or anti-PD-1), median 1 vs 14 months; HR 3.92, $p=0.003$. Neither SUVmax nor MTV was significantly associated with PFS or OS after either treatment. High SLR was associated with poor OS after ipilimumab (median 1 v 21 months, HR 5.83, $p=0.0001$). High SLR was not associated with OS after anti-PD-1 treatment (median 8.8 v 9.7 months; HR 0.92, $p=0.89$).

Overall survival was calculated from date of first treatment separately for each agent, as well as for date of first line immunotherapy. High SLR was also associated with short OS as calculated from start of first line immunotherapy (median 1.0 month vs 14.0 months respectively, HR 3.92, $p = 0.003$) . See Table 5.3B and Figure 5.2C. Patients with high SLR >1.1 also had significantly worse OS compared to patients with normal SLR after ipilimumab (median 1 vs 21 months; HR 5.83, $p= 0.0001$). See Figure 5.2D. High SLR was not associated with OS after anti-PD-1 treatment (median 8.8 v 9.7 months; HR 0.92, $p=0.89$).

Again, high tumoural SUVmax was not associated with OS after first line immunotherapy compared to low tumoural SUVmax, (median OS 14.0 vs 9.0 months, HR 0.57, $p=0.053$). High tumoural SUVmax was not associated with OS after ipilimumab (HR 0.83, $p=0.59$) or after anti-PD-1 (HR 0.57, $p=0.267$). SUVmax also not significantly associated with OS after first line immunotherapy when analysed as a continuous variable, HR 0.99 with $p=0.21$.

Interestingly, high MTV were not associated with OS after first line immunotherapy compared to patients with low MTV when dichotomised at the median (median OS 9.0 vs 20.0 months, HR 1.18, $p=0.606$) but MTV was significantly associated with OS when analysed as a continuous variable (HR 1.09, $p=0.020$). Similarly, high MTV was not associated with OS after ipilimumab (HR 1.08, $p=0.827$) or after anti-PD-1 (HR 1.67, $p=0.294$). We explored this further by splitting the cohort into MTV quintiles and found that the quintile with the lowest level of disease had excellent OS whereas the remaining 4 quintiles all appeared to have similar OS. This suggests a degree of non-linearity between the MTV and OS with excellent survival in patients with very low volume of disease as shown in Figure 5.3.

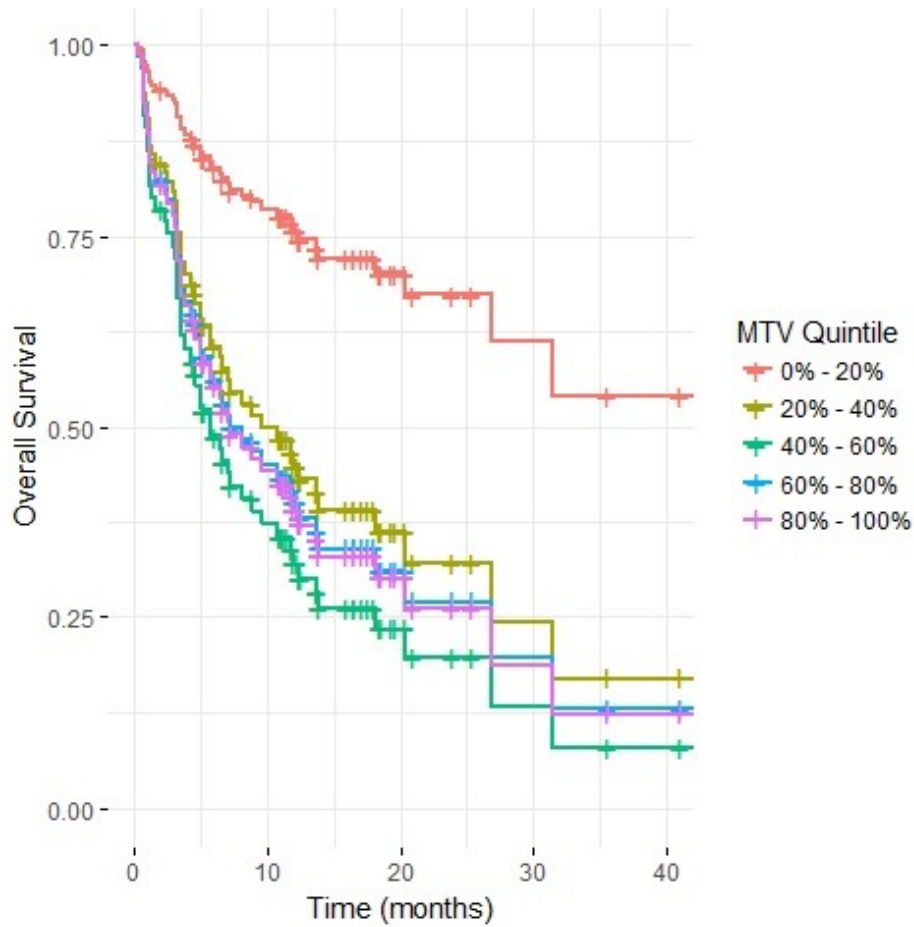


Figure 5.3 Overall survival curves stratified by metabolic tumour volume quintile

High metabolic tumour volume (MTV) was not associated with OS compared to patients with low MTV when dichotomised at the median (HR 1.18, $p=0.606$) but MTV was significantly associated with OS when analysed as a continuous variable (HR 1.09, $p=0.020$). To explore this further, the discovery cohort was split into MTV quintiles and found that the quintile with the lowest level of disease had excellent OS whereas the remaining 4 quintiles all appeared to have similar OS. This suggests a degree of non-linearity between the MTV and OS.

Multivariable analysis for progression free survival and overall survival in discovery cohort

On multivariable analysis, SLR was independently associated with survival, with HR 3.58 (95% CI 1.34-9.61), p 0.0026. The other variables including LDH, metastasis substage, MTV, absolute lymphocyte count (ALC) were all non-significant. See Table 5.4 for details. For PFS analysis, multivariable analysis was performed only for the ipilimumab-treated cohort of patients, as the univariable regressions for anti-PD-1 all resulted in non-statistically significant p -values (>0.05). For PFS after ipilimumab, SLR was associated with HR 2.67 (95% CI 1.13-6.33), $p=0.042$.

Table 5.4 (A) Multivariate analysis for progression free survival after ipilimumab

Predictor	Level	HR	HR 95% CI	P-value
LDH	≤ULN	1	-	0.738
	>ULN	1.12	[0.58, 2.14]	
Stage	not M1c	1	-	0.431
	M1c	1.32	[0.66, 2.67]	
MTV	≤41	1	-	0.334
	>41	1.39	[0.71, 2.70]	
ALC	≤1	1	-	0.083
	>1	0.55	[0.28, 1.06]	
SLR	≤1.1	1	-	0.042
	>1.1	2.67	[1.13, 6.33]	

LDH, lactate dehydrogenase; ULN, upper limit of normal; MTV, Metabolic Tumour Volume; ALC, Absolute Lymphocyte Count; SLR, Spleen to Liver Ratio

(B) Multivariate analysis for overall survival after first line immunotherapy

Predictor	Level	HR	HR 95% CI	P-value
LDH	≤ULN	1	-	0.076
	>ULN	2.13	[0.89, 5.08]	
Stage	not M1c	1	-	0.163
	M1c	1.88	[0.75, 4.75]	
MTV	≤76	1	-	0.973
	>76	1.01	[0.51, 2.02]	
ALC	≤1	1	-	0.164
	>1	0.61	[0.30, 1.21]	
SLR	≤1.1	1	-	0.026
	>1.1	3.58	[1.34, 9.61]	

LDH, lactate dehydrogenase; ULN, upper limit of normal; MTV, Metabolic Tumour Volume; ALC, Absolute Lymphocyte Count; SLR, Spleen to Liver Ratio

Validation cohort

110 patients treated with ipilimumab between May 2007 and April 2015 were included. Fifty-eight patients were from UZ Brussel, Brussels and 52 patients were from University Hospital Zurich, Zurich. Patient and disease characteristics are described in *Supplementary materials S4*. There were higher proportions of patients with M1c in the Brussels cohort but this difference was negligible when the patients were combined into a single validation cohort (68% of the discovery cohort had M1c disease compared to 73% of the validation cohort). The proportion of patients with the high SLR was also lower in the European cohort compared to Australian patients (4% compared to 13%). Despite these differences, SLR>1.1 was still associated with poor OS after ipilimumab (median 2.3 months versus 11.9 months, HR 3.74, CI 1.34-10.4), as shown in *Figure 4*.

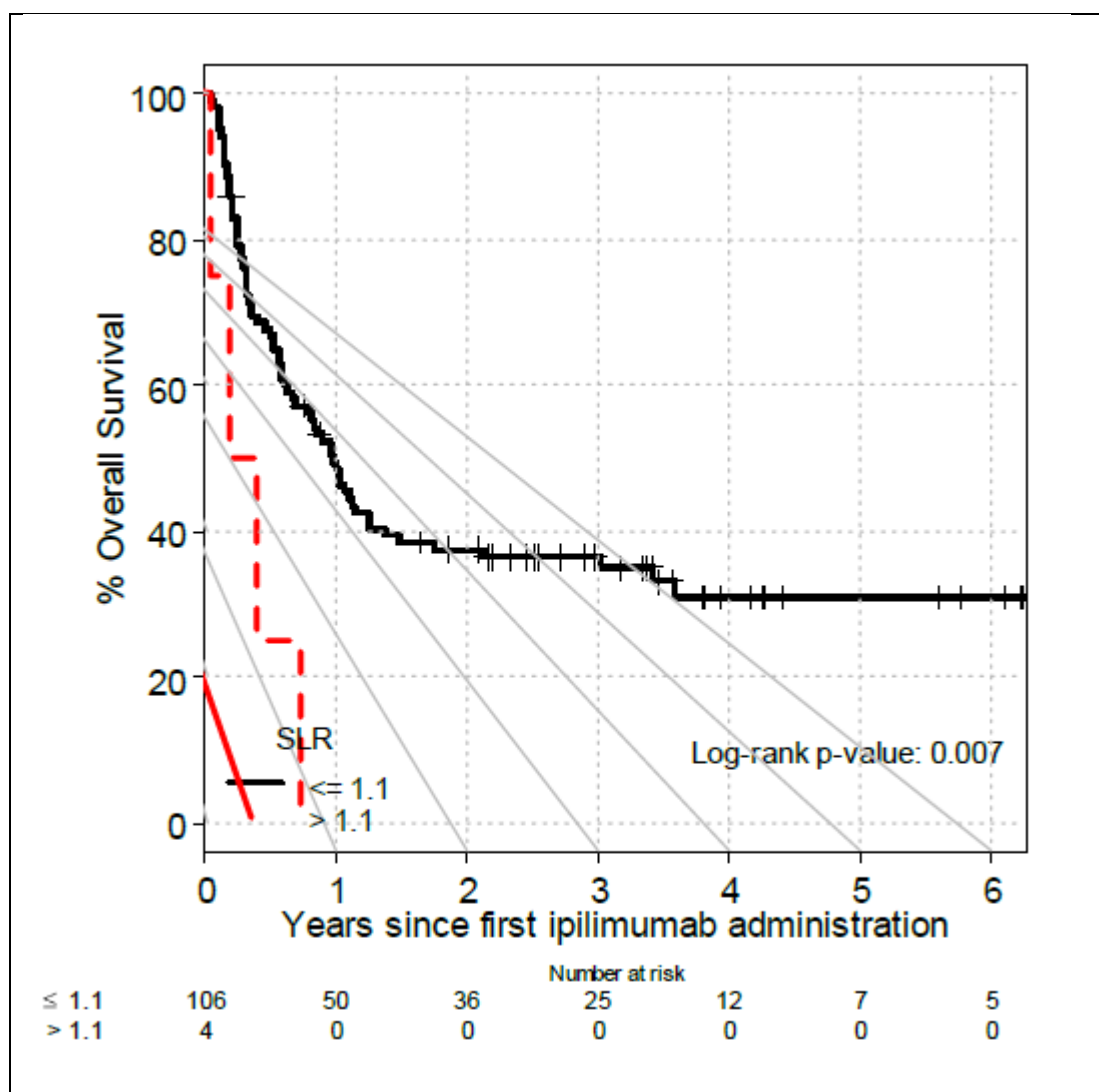


Figure 5.4 SLR and overall survival after ipilimumab in the validation cohort

High SLR was associated with poor overall survival after ipilimumab treatment. This was validated in an external cohort (n=110 patients) from Brussels and Zurich (median 2.3 months versus 11.9 months, HR 3.74, CI 1.34-10.4).

Biological analyses for discovery cohort

We sought to explore the cause of the raised SLR. We compared the PET parameters of liver and splenic SUV in patients with raised SLR and normal SLR. In patients with raised SLR, the mean and median liver SUV was 2.0 with an interquartile range (IQR) of 0.5, which is slightly lower than the normal reference range (lower limit of normal for liver SUV was 2.3)(469). In contrast, the normal SLR group had a mean and median liver SUV of 2.4, IQR 0.6; which is within the normal reference range. Within the high SLR group, the mean and median splenic SUV was 2.4, with IQR of 0.4; whereas in normal SLR group, where the mean and median splenic SUV was 2.1, IQR 0.5.

To explore the relationship of SLR and inflammatory or haematological parameters, a multivariable linear regression model for SLR was considered, considering: Neutrophil: Lymphocyte Ratio, Absolute Neutrophil Count (<7.5 vs. ≥ 7.5), Haemoglobin (<120 vs. ≥ 120), LDH ($\leq$ ULN vs. $>$ ULN), ALC $\times 10^9$ (<1 vs. ≥ 1), albumin (<35 vs. ≥ 35), Lymphocyte: Monocyte ratio and patient performance status. Only albumin was found to be significantly associated with SLR, beta -0.15 (CI -0.209, -0.091, $p < 0.001$). See *supplementary materials S2*. Given that albumin is a negative acute phase marker (i.e. decrease during inflammation), the inflammatory cytokine profile was examined in patients with stored sera.

14 patients in the discovery cohort had evaluable stored sera from baseline, week 3 and week 6 post commencement of ipilimumab. Of these patients, 13 had normal SLR, whereas one patient had high $SLR > 1.1$. There were no major differences in the baseline levels nor the dynamic changes in IL2, IL1 beta, IL4, IL6, IL10, IL17, IL12p70, IFN gamma and TNF alpha post ipilimumab, when comparing the patient

with high SLR to others with normal SLR. Patients with progressive disease and high SLR were both observed to have a logarithmic increase in IL6 and IL10 at week 3 and week 6. *See supplementary materials S3.*

Discussion

High SLR on pre-treatment FDG-PET was associated with poor OS after ipilimumab for advanced melanoma in our study. This finding was then validated in an additional combined cohort of patients from two separate institutions. Although only a small proportion of patients exhibited high SLR (i.e. only 10% of the discovery cohort and 4% of the validation cohort), it is noteworthy that these patients had a very poor prognosis after ipilimumab (OS 1-3 months compared to 10-15 months in phase III studies)(166, 167, 186). In the discovery cohort, high SLR was associated with poor PFS after ipilimumab but not after anti-PD-1. This may be due to the small sample size of the anti-PD-1 cohort or it may be due to the different mechanisms of action between the two treatments.

Caution is advised when interpreting the overall survival analyses for each immunotherapy separately. Patients in this study was treated during a transitional period where ipilimumab was the standard first line immunotherapy at the start of the study period (n=50). Patients who received first line ipilimumab were able to receive anti-PD-1 upon disease progression (n=20). Towards the end of the study period, patients were able to receive anti-PD-1 as their first line therapy (n=20). The overall survival analysis of a cancer treatment could be influenced by subsequent treatments. In the case of immunotherapy, there is also the potential that overall survival is influenced by *prior* treatments given the long half-life of these agents and possible changes to the patients' immune system following first line treatment. We attempted to address this in

the study by analysing the overall survival from the start of the first line immunotherapy treatment so that patients contributed only once to survival censoring (n=70). Using this analysis method, high SLR remained associated with poor OS after first line immunotherapy.

The mechanism for high splenic avidity is not well understood. The spleen, being the largest lymphoid organ in the human body, is the site of immune cell activation and maturation. Increased splenic avidity on FDG-PET has been previously reported in lymphoma, granulomatous diseases, GCSF injections and interferon-alpha administration in melanoma(464). Pak and colleagues have also studied this phenomenon more extensively in the context of cholangiocarcinoma(287). They showed that high splenic avidity is associated with poor survival in patients with metastatic cholangiocarcinoma(288). However, this signature has not been studied prior to immune modulation with ipilimumab or anti-PD-1 in the setting of advanced melanoma.

Pak et al demonstrated that high SLR in patients with cholangiocarcinoma is associated with markers of inflammation including leucocytosis, raised CRP, raised levels of IL-1b, IL-1RA, IL-4, IL-6, IL-7 and IL-13(287). These inflammatory cytokines are suggestive of an acute phase inflammatory response or activation of humoral immunity. In our cohort, we found that patients with high SLR post ipilimumab were more likely to have low albumin, but importantly independent of leucocytosis or high neutrophil:lymphocyte ratio. Our cohort of 14 patients with stored sera was too small to perform comparative statistics but we did not find any striking differences in the cytokine profile of the patient with high SLR. It would be of interest to explore in future studies whether this splenic avidity is a result of systemic cytokines or preferential glucose metabolism in specific immune cells within the spleen. We

postulate that the failure to control disease despite already invoking immune activation may underlie the lack of benefit from further stimulation by immunotherapy.

Additionally, the abnormal SLR group was associated with decreased hepatic uptake as well as increased splenic uptake. This raises the possibility that SLR could be related to both reduced liver SUV as well as high splenic SUV rather than either factor alone. However, our study only included a small number of patients with raised SLR signature so it would be important to explore this in larger cohorts. No evidence of association between high tumoural SUVmax and outcome after immunotherapy was found. This may be because tumoural glucose uptake is not only due to malignant cells, but also is caused by immune cells as part of immune activation. Therefore, examination of tumoural SUVmax alone may be problematic in the context of immunotherapy and diseases in which immune modulation is important for disease outcomes. MTV was not associated with PFS in either ipilimumab or anti-PD-1 in our cohort but was significantly associated with OS when analysed as a continuous variable. The patient group with very low levels of tumour volume appeared to have very good survival whereas the relationship did not appear linear at larger tumour volumes, this finding needs to be taken with caution given it has not been externally validated. However, it does appear to be in keeping with the findings of other investigators who have also correlated anti-PD-1 response with tumoural burden as measured by CT(159, 478). Our cohort predominantly evaluated patients treated with ipilimumab, and only included 40 patients treated with anti-PD-1. It may be that MTV is of higher predictive value for anti-PD-1 alone and should be evaluated in a larger cohort to better understand an optimal threshold for analysis, and its utility for that treatment.

From a technical standpoint, SLR may be an easier parameter to apply clinically than absolute thresholds of significance for parameters such as tumoural SUV or MTV. Despite effort to harmonise its measurement (475), tumoural SUV_{max} can be a more challenging parameter to reproduce in multi-centre studies, owing to the need for the same imaging systems, acquisition parameters, reconstruction parameters, and tracer uptake times in order to obtain an accurate and reproducible measurement of SUV_{max}. In the case of the SLR, the precise measure of SUV is less important. Examination of the same patient on multiple scanners, may produce different SUV but the ratio will remain the same. Consequently, this makes SLR a more robust measure for comparison between different study sites.

Our study does shift the focus of functional imaging of the tumour alone to the role of functional imaging of the host immune system. This additional use of PET scans gives a snapshot of the host immune status and may complement other biomarkers as suggested by Blank et al (376). Further understanding of the biological significance of avidity and activation of splenic avidity or draining lymphatics of the tumour may improve the understanding of not only the mechanism of action of immunotherapies, but also possible targets for future research into specific body sites contributing to treatment resistance.

Our study was limited by its retrospective nature, its relatively small sample size and lack of homogenous assessment modalities for PFS. Our findings were validated in an independent cohort albeit only in a small number of patients exhibited high SLR. The combined validation cohort had a similar proportion of patients with M1c disease but there was variation in the prior treatments received. Despite the low occurrence of high SLR, this parameter was associated with very poor clinical outcome and may be useful in identifying patients who should not receive ipilimumab treatment. This parameter

also needs to be prospectively assessed in larger cohorts of patients treated with second line ipilimumab or ipilimumab combinations (ipilimumab/nivolumab).

Conclusion

Pre-treatment high SLR on FDG PET/CT was associated with poor outcome after ipilimumab in advanced melanoma. Further prospective validation of this FDG PET/CT signature is required, particularly in larger cohorts of patients treated with anti-PD-1 or ipilimumab combination therapies.

List of abbreviations

ALC	Absolute Lymphocyte Count
Anti-PD-1	Anti-programmed death 1 monoclonal antibodies
BLR	Bone to Liver Ratio
FDG PET	Fluorodeoxyglucose Positron Emission Tomography
HR	Hazard Ratio
IQR	Interquartile Range
LDH	Lactate Dehydrogenase
MTV	Metabolic Tumour Volume
OS	Overall survival
PET	Positron Emission Tomography
PFS	Progression free survival
PMCC	Peter MacCallum Cancer Centre
SLR	Spleen to Liver Ratio
SUVmax	Maximum Standardised Uptake Value
ULN	Upper limit of normal

Declaration:

- Ethics approval and consent to participate

This study was approved by human research ethics committees and requirement for informed consent was waived at the Peter MacCallum Cancer Centre, the UZ Brussel, and the University Hospital Zurich.

- Consent for publication

Not applicable.

- Availability of data and material

The datasets generated and/or analysed during the current study are not publicly available due to restrictions on patients' consent pertaining to existing ethics-committee approved research at our respective institutions only. They may be obtained from the corresponding author on reasonable request but may require further approval from respective ethics committee approval.

- Competing interests

The authors do not have any direct competing interest relevant to this study.

- Funding

AW received research support from the Royal Australasian College of Physicians.

- Authors' contributions

AW, RH and GM contributed to the study design. AW, JC, SF and DM contributed to data collection at PMCC. AH performed the statistical analyses. MK, BN, JM and SA contributed to the collection of patient data for the validation cohort. AW wrote the manuscript, all authors reviewed and approved the manuscript.

- Acknowledgements

Nil

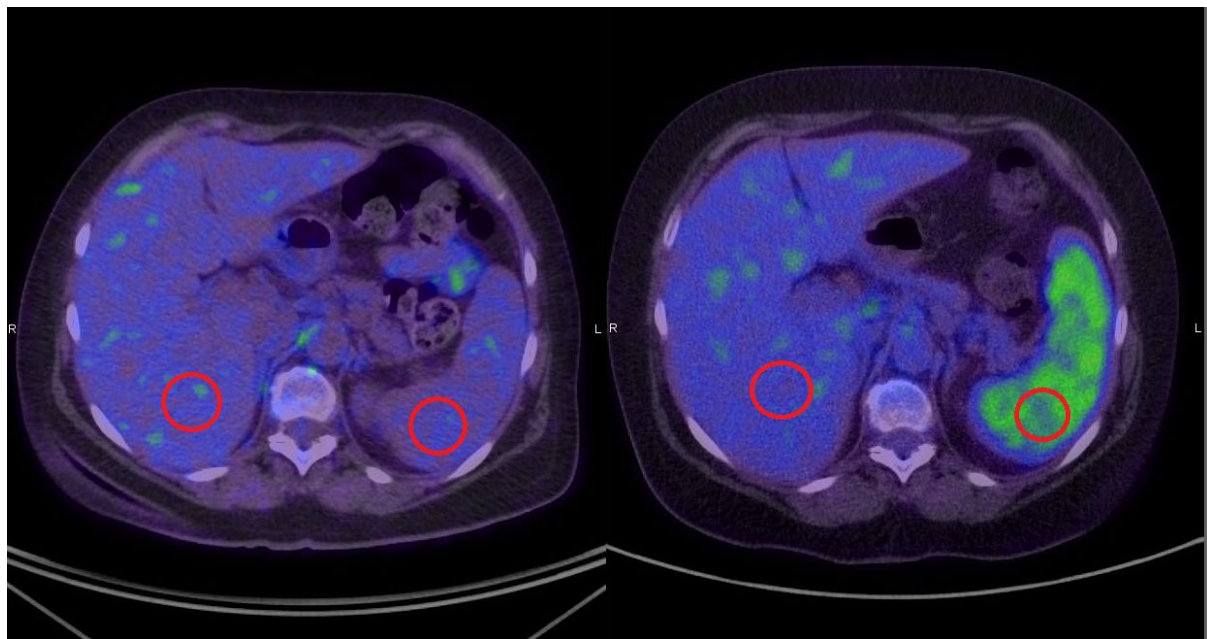
- Authors' information (optional)

Supplementary materials

S1. Detailed PET acquisition methodology

FDG-PET scans were performed after patients fasted for at least 6 hours, and plasma glucose levels were determined to document euglycemia. ^{18}F FDG was administered intravenously according to weight (0.15 mCi/kg, ranging from 3 to 16 mCi) and images were acquired after a distribution time of approximately 60 minutes. Images were obtained from the base of the skull to extremities. FDG-PET images were corrected for attenuation. Metabolic tumor volume was calculated by multiplying the number of abnormal voxels identified within the volume of interest by the known voxel volume.

The Spleen to liver ratio is calculated by placing spherical volumes of interest that are 2cm in diameter in the Liver and spleen. The ratio of the SUVmean of the spleen to the SUVmean of the liver is then calculated. A spleen to liver ratio of greater than 1.1 is considered abnormal. Below is an example of normal (left) and abnormal spleen to liver ratios (right).



The whole body metabolic tumour volume (MTV), measured in mL, is quantified on the FDG-PET/CT scans by adapting the PERCIST recommendations for contouring FDG avid disease (R. L. Wahl, H. Jacene, Y. Kasamon and M. A. Lodge, J Nucl Med 50 Suppl 1, 122S-150S (2009)). All FDG-avid disease was contoured using an SUV threshold that was applied to the whole body. The SUV threshold used was 1.5 times the mean SUV of the liver plus 2 standard deviations. All normal physiologic uptake (brain, kidneys, bladder etc) was manually excluded from the whole body volume of

interest as shown in the example below. The following parameters for the Whole Body disease volume of interest are recorded:

- SUV_{max}
- SUV_{mean}
- Metabolic Tumour Volume (ml)

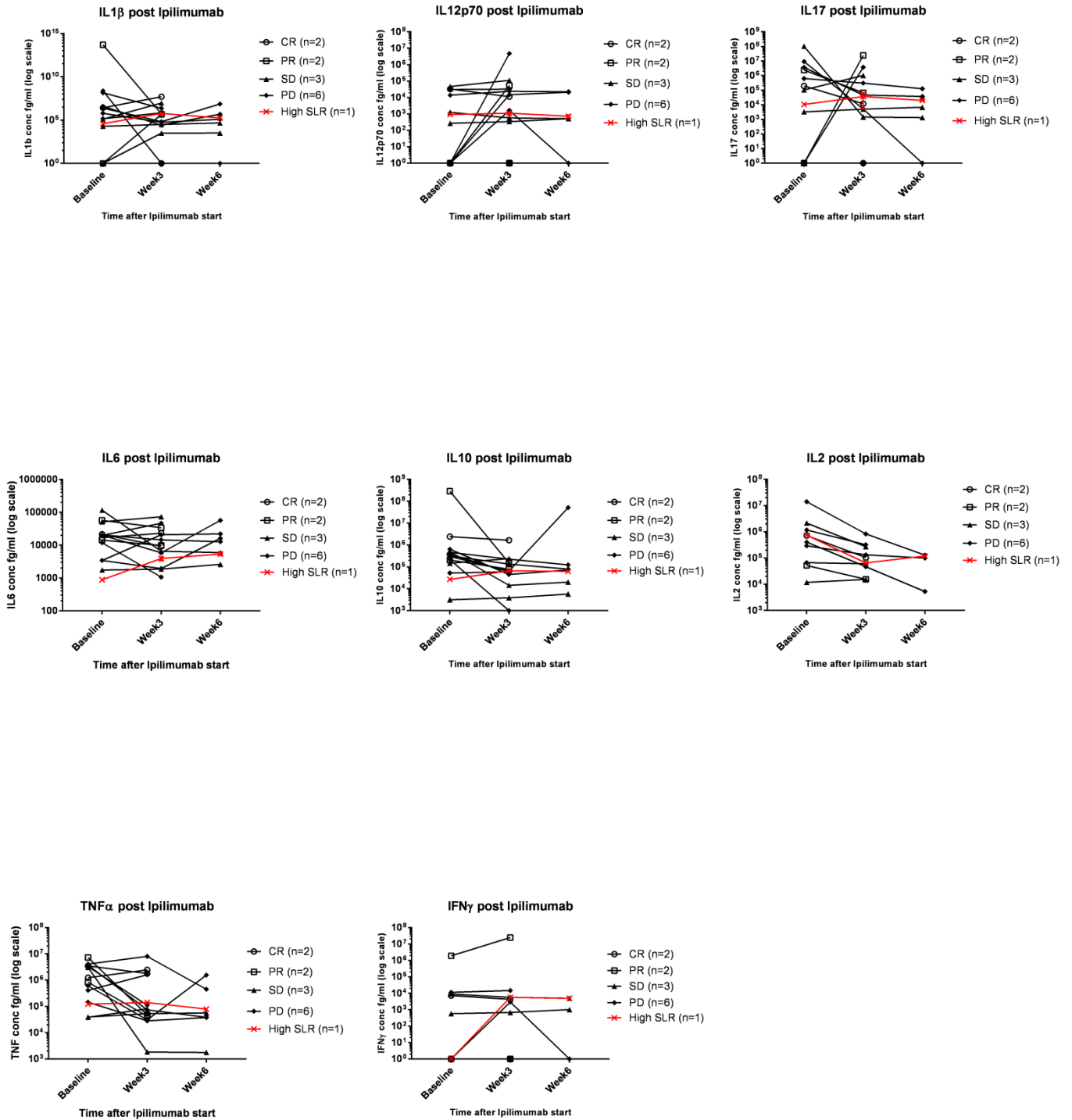
S2. Multi-variable linear regression model for spleen to liver ratio

A multivariate linear regression model for SLR was considered, with all of the following considered as candidate covariates: NLR, Neutrophils (<7.5 vs. ≥7.5), Hb (<120 vs. ≥120), LDH (≤ULN vs. >ULN), ALC x10⁹ (<1 vs. ≥1), Albumin (<35 vs. ≥35), LMR and ECOG-PS. (NLR = Neutrophil to Lymphocyte ratio, LMR = Lymphocyte to Monocyte ratio). A forward selection approach to model construction was used, whereby, commencing with the null model, the most significant covariate not in the model was added successively until no further covariates significant at the 0.05 level could be found. Using this approach only 1 covariate from the list above was found to be significant. Beta is the modelled slope of the regression line. In this case, since the predictor (Albumin) is dichotomous, it represents the modelled difference in SLR between patients with albumin <35 and patients with albumin ≥35.

Multivariate linear regression results for SLR

Predictor	Level	beta	beta 95% CI	N	P-value
Albumin	<35	0	-	21	<0.001
	≥35	-0.15	[-0.209, -0.091]	67	

S3. Cytokine profile of patients treated with ipilimumab (n=14)



S4. Baseline demographic and disease characteristics of validation cohort

Demographic or Characteristic		Site	
		Brussels (n = 58)	Zurich (n = 52)
Sex	Female	28 (48%)	26 (50%)
	Male	30 (52%)	26 (50%)
Stage	IIIC	2 (3%)	5 (10%)
	M1a	4 (7%)	4 (8%)
	M1b	3 (5%)	11 (21%)
	M1c	49 (84%)	32 (62%)
Mutation	BRAF V600E/K	16 (28%)	7 (13%)
	RAS/NRAS	0 (0%)	16 (31%)
	BRAF Wildtype	22 (38%)	25 (48%)
	Other	2 (3%)	2 (4%)
	Unknown	16 (28%)	2 (4%)
Prior lines of therapy	0	10 (17%)	25 (48%)
	1	44 (76%)	23 (44%)
	2	4 (7%)	4 (8%)
SLR	Mean (SD)	0.82 (0.1)	0.90 (0.1)
	Median [range]	0.81 [0.44 - 1.28]	0.9 [0.7 - 1.3]
	IQR	0.73 - 0.89	0.8 - 1
SLR	≤1.1	57 (98%)	49 (94%)
	>1.1	1 (2%)	3 (6%)

5.2 Early treatment PET/CT response as a biomarker

The previous subchapter explored baseline PET as a biomarker for ICI in patients with melanoma. This subchapter will focus on the value of ‘early on-treatment’(EOT) PET scans as a potential predictor of clinical outcome after ICI. As per clinical trials, disease response to a particular treatment is normally reassessed after 3 months of therapy. Some investigators have evaluated the prognostic value of an early PET scan in predicting treatment response.

Baseline and day-15 FDG PET was evaluated in 31 patients treated with vemurafenib as part of a phase 1b dose-finding clinical trial (258). In the 27 patients treated with therapeutic levels, $80\% \pm 3\%$ of patients exhibited a metabolic response on FDG PET, with reduction in SUVmax and $87\% \pm 3\%$ decrease in the percentage of injected dose (%ID). This small study did not show a relationship between reduction in SUVmax and response by RECIST criteria. It did, however, show a trend towards improved PFS in patients who experienced more than 82% reduction in the SUVmax (median SUVmax reduction of the cohort), HR 0.34; 95% CI 0-0.7.

Another study reported on baseline and day-15 FDG PET in patients treated with dabrafenib as part of a phase 1 clinical trial (479). Twenty-six percent of the 23 patients exhibited a heterogeneous response to therapy (defined as complete metabolic response/partial metabolic response with progressing or new lesions within a patient, or >10% of the lesions with a stable metabolic response). A heterogeneous response on day 15 scan was associated with a short time to progression: median 3.0 months compared to 7.4 months in patients with homogenous response ($p= 0.032$); however, it did not influence OS. Overall, these two studies indicate that the majority of patients exhibited a homogenous response to BRAF inhibitors, even as early as two weeks after the initiation of treatment.

There are also data pertaining to metabolic responses in early FDG PET scans 6 weeks after the start of treatment, being correlated with tumour response in 29 patients with non-small cell lung cancer who were treated in a phase II clinical trial of atezolizumab, an anti-PD-L1 monoclonal antibody (480). However, it is not known whether early response on FDG PET can predict long term clinical benefit in advanced melanoma.

Tan and colleagues described the FDG PET responses observed in 104 patients with metastatic melanoma treated with anti-PD-1 monotherapy (67%) or combined with ipilimumab (31%) in a retrospective study (481). In this study, baseline imaging was compared with 1-year landmark FDG PET or CT. Response rates were assessed using EORTC criteria for PET and RECIST criteria for CT. At 1 year, 75% of patients had CMR, 16% PMR and 9% SMD/PMD on PET, whereas 28% had CR, 66% had PR and 6% had SD on CT. RECIST PFS was similar in patients with CR compared to PR/SD on CT, whereas patients with CMR had a higher RECIST PFS compared to non-CMR (median not reached NR vs 12.8 months, HR 0.06, $p < 0.01$). PET may be able to further delineate the patients with PR on CT into prognostic groups, where patients with CMR within this group have durable response compared to those without CMR (median NR vs 12.8 months; HR 0.07, $p < 0.01$). This study suggests that PET is superior to CT, at one year, in predicting long term clinical benefit.

There are two studies that have evaluated early PET responses in patients with metastatic melanoma. These are both retrospective studies with only a small number of patients. The first is a published prospective study of twenty patients who were treated with immune checkpoint inhibitors (16 ipilimumab, 3 anti-PD-L1, 1 anti-PD-1) (482). In this study, FDG PET scans were performed at baseline (scan 1), 21-28 days post treatment commencement (scan2), and at 4 months post treatment commencement (scan 3). Scan 2 was able to predict the best observed response in Scan 3 (i.e. at 4 months) with 75%, 70%, 70%, and 65% accuracy when using RECIST 1.1, IrRC, PERCIST and EORTC PET criteria respectively. Authors also reported an inter-criteria discrepancy on scan 2 owing to the early FDG uptake of tumours which eventually regressed. While this study is prospective and evaluated 4 different criteria used in clinical trials, the study included different ICI which may have varying tumour responses observed on PET due to their different mechanisms of action (117).

5.2.1 Aim

The aim of this subchapter was:

To evaluate early PET scan response (after 2-3 weeks of treatment) as a predictor of clinical outcome of response at 6 months after ipilimumab or anti-PD-1 for advanced melanoma.

5.2.2 Summary of Results

There were 16 patients in each treatment group (of ipilimumab or antiPD-1) where patients had matched baseline and early on-treatment PET scans.

Table 5.5 Early on-treatment PET parameters compared to baseline PET prior to ipilimumab or anti-PD-1

EOT changes after ipilimumab	PET after	Mean	Median	Range	IQR
SUVmax		6.125	8	[-90,99]	-22 - 32
MTV		242.8	24.5	[-89,1450]	-31 - 335
SLR		-0.4	4	[-64,45]	-15 - 10

EOT changes after anti-PD-1	PET after	Mean	Median	Range	IQR
SUVmax		22.31	14	[-96,170]	-47 - 77
MTV		232.3	74.5	[-97,1557]	-23 - 196
SLR		8.188	3.5	[-27,75]	-6 - 12

Table 5.5 shows the mean, median, range and interquartile range for the changes observed between the baseline pre-treatment PET scan and the early on-treatment PET scan performed 2 to 3 weeks after the commencement of therapy. The average change in SUVmax and MTV shows an increase for both therapies on the EOT PET. The mean SLR is reduced on the EOT PET, whereas the mean SLR has increased after anti-PD-1. EOT PET, Early on-treatment positron emission tomography; IQR, interquartile range; SUVmax, maximal tumoural SUV; MTV, metabolic tumour volume; SLR, Spleen to Liver Ratio.

1. On the EOT PET after ipilimumab, 38% of patients had a decrease in SUVmax, 31% of patients had a decrease in MTV, and 38% of patients had a decrease in SLR. Changes in these EOT PET parameters of SUVmax, MTV or SLR, did not correlate with response at 6 months to ipilimumab. None of the 6 patients with a decrease in SLR had progressive disease (*see figure of waterfall plots of early on-treatment PET changes after treatment with ipilimumab monotherapy and anti-PD-1 monotherapy*).

2. On the EOT PET after anti-PD-1, 50% of patients had a decrease in SUVmax, 38% of patients had a decrease in MTV, and 31% of patients had a decrease in SLR. Changes in these EOT PET parameters did not correlate with response at 6 months to anti-PD-1.
3. Overall survival did not correlate with EOT PET changes in SUVmax, MTV using a 30% reduction as a threshold. OS did not correlate with an absolute decrease in SLR. See Figure 5.5.

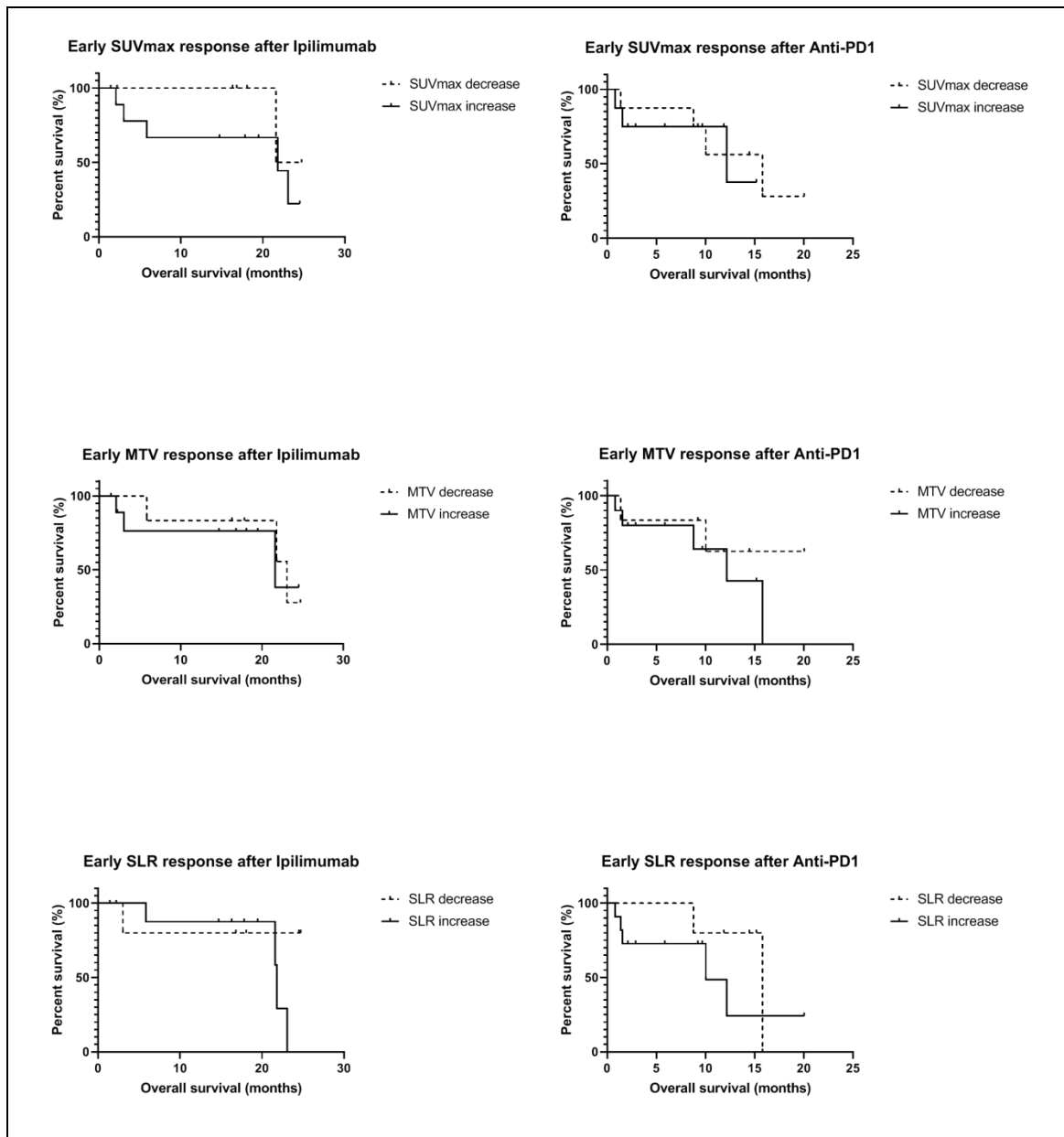


Figure 5.5 Kaplan-Meier survival curves for patients treated with either ipilimumab (n=16) or anti-PD-1 (n=16) and its association with EOT PET changes.

Survival was stratified by changes on early on-treatment PET scan, using 30% change for SUVmax or metabolic tumour volume. Given the tightly controlled threshold for SLR, an absolute increase or decrease was used to stratify the cohort. Overall survival was not significantly associated with changes in any of the parameters on EOT PET.

5.2.3 Discussion

Early on-treatment PET scans correlated poorly with subsequent treatment responses in this study. There were 16 ipilimumab treated patients who had matched baseline and EOT PET scans and 16 anti-PD-1 treated patients who had matched PET scans. Early changes in MTV, SUVmax or SLR did not correlate with subsequent treatment responses at 6 months. A limitation of the study is that the method of treatment response assessment at 6 months was heterogeneous (with a mixture of PET, CT, MRI or clinical). Despite this, changes in SUVmax, MTV and SLR did not correlate with overall survival in either ipilimumab or anti-PD-1.

Firstly, for ipilimumab treated patients, EOT scans of SUVmax showed both false positives as well as false negatives (i.e. reduction in SUVmax was seen in patients with progressive disease, and increase in SUVmax was seen in patients whose disease subsequently responded). This was similarly seen for patients treated with anti-PD-1. The variation in FDG uptake after treatment with ICI is in contrast to early responses seen in tumoural lesions after BRAF molecularly targeted treatments, where tumours showed a marked and homogenous reduction in SUVmax (258). This may be owing to the differential mechanisms of action: where molecularly targeted treatments downregulate the abrogated tumoural pathways of cellular metabolism. This is in contrast to immune checkpoint blockade where successful tumour responses involve metabolically active immune cells (158, 299) and therefore would be indistinguishable from a tumour that is highly FDG avid due to tumoural uptake.

Regarding tumour burden, 5 out of 10 patients who had an increase in MTV after ipilimumab experienced a subsequent tumour response (including 3 patients who had complete response). While this phenomenon of ‘pseudo-progression’ has been observed on CT imaging (483), it has not yet been well documented on FDG PET. In contrast, there was only 1 patient out of 10 with increase in MTV who experienced a subsequent partial response following anti-PD-1 in our small study. This may reflect the different tumoural response after anti-PD-1 compared to ipilimumab; this needs to be explored in larger studies. Given the false positives (i.e. patients who have a subsequent treatment response despite initial increase in MTV), it may be that 2-3 weeks after treatment initiation is too early for treatment response to be assessed after anti-PD-1 or ipilimumab.

Interestingly, 6 patients were observed to have a reduction in SLR on their EOT PET. None of these patients had progressive disease at 6 months. This is in contrast to SLR response after anti-PD-1, where 4 of the 6 patients with a reduction of SLR had progressive disease at 6 months. There was no statistical difference in overall survival in those with a reduction of SLR compared to those with no change or increase in SLR (median OS not reached v 22 months, $p=0.82$). Given our other findings of poor survival after ipilimumab in patients with high SLR, it was of interest to find that a reduction of this parameter was associated with response at 6 months. Future studies evaluating dynamic changes observed with normalisation of SLR may be worthwhile.

Changes on EOT PET were not correlated with survival by log-rank test in this small study. Larger studies are needed to examine PET parameters and their correlation to survival. Optimal thresholds of parameter change will also need to be identified using larger cohorts. Other larger studies examined the utility of FDG PET at one year, demonstrating that complete metabolic response was better able to predict survival compared to CT treatment response assessment alone at this later point in time (481).

This small study has raised some of the challenges of using FDG PET as an early predictor of outcome to ICI. Our study was limited by the heterogeneous methodology of response assessment at 6 months to define clinical benefit, but nonetheless there did not appear to be any correlation between EOT PET changes and survival. Future studies involving larger groups of patients in comparison with a unified treatment response assessment are needed to fully explore the value of EOT PET after ICI. PET treatment response assessment at a later time appears to be a more promising way of using FDG PET(481). Radiotracers directly targeting the PD-1/PD-L1 pathway or T cell activation may overcome some of the challenges that are encountered with FDG-PET.

5.2.4 Conclusion

Baseline FDG PET parameter of high spleen to liver ratio was associated with poor survival after ipilimumab at an Australian academic cancer centre, and validated in a combined patient cohort from two European cancer centres. The underlying mechanism of high spleen to liver ratio is not known but it was associated with low albumin, likely as part of an acute phase inflammatory response. SUVmax and MTV were not

associated with survival in our cohort but larger studies need to be used to assess for optimal thresholds for these PET parameters as biomarkers.

In a small subset of 32 patients treated with either ipilimumab or anti-PD-1, early on-treatment FDG PET scans did not correlate well with subsequent response. Several challenges are evident in the use of FDG PET as response assessment at an early on-treatment time point, particularly as an increase in MTV or SUVmax can be associated with subsequent response at 6 months. Furthermore, early decreases in MTV or SUVmax can be associated with treatment progression. Reduction in SLR was observed in patients with clinical benefit from ipilimumab but this needs to be evaluated in larger cohorts.

6 Comparison of immune profile of UV related cutaneous malignancies

6.1.1 Introduction

Immune checkpoint inhibitors have been successful in not only melanoma, but other chemotherapy-refractory cutaneous malignancies such as Merkel cell carcinoma (MCC) and cutaneous squamous cell carcinoma (cSCC). This may potentially be related to the UV-related aetiology of these cancers and the resultant high tumour mutation burden. Different mechanisms of immune activation may be highlighted by in-depth comparison of immune infiltrates, PD-L1 expression and genomic characterisation of immune processes present across these three cutaneous cancers. More in-depth study of the tumour immune profile of early stage melanoma and how it compares to other UV-related skin cancers will not only improve the understanding of tumour immuno-biology but may have implications for biomarker development in skin cancers.

This chapter characterised and compared the immune landscape of these three skin cancers by using orthogonal methods of multi-spectral immunohistochemistry and bulk RNA-sequencing. Comparison of the genomic characteristics associated with PD-L1 expression may highlight the different immune processes at play in these cancers.

6.1.1.1 Multi-spectral immunohistochemistry

Until recently, the characterisation of tumour infiltrating immune cells in pre-treatment biopsies has been performed using traditional immunohistochemistry (IHC) methodologies, where single antibody stains are applied to formalin fixed paraffin embedded tissue sections, to identify the presence of immune cells within the histological samples. While traditional IHC is a standard clinical laboratory technique that is widely available, the identification of immune cells is limited by the few antibody stains possible on a single histological section. Novel multiplexed IHC techniques were needed to improve the characterisation of the immune microenvironment within the tumour. Opal™ 7 colour Kit (PerkinElmer) is a novel IHC technique using immunofluorescence which allows 7 antibodies to be visualised

simultaneously, providing an in-depth characterisation of immune subsets within various tissue compartments e.g. tumour, peri-tumour or stroma (484, 485). This technology not only enables the enumeration of cellular subsets but identifies the exact location of cells within the section which enables an assessment of the spatial relationships between cells.

Melanoma

As previously described in the literature review [Chapter 1.3.3], tumour infiltrating lymphocytes and CD8 lymphocytes have been associated with good prognosis in melanoma. PD-L1 expression has also been extensively studied in melanoma, with studies reporting positive staining in approximately a third of samples in early stage disease and inflamed benign naevi (138, 486). These studies have also shown a strong correlation between PD-L1 staining and tumour infiltrating lymphocytes (approximately 90%) which mediated by interferon- γ signalling (138). PD-L1 expression was associated with good prognosis in metastatic disease but was not associated with prognosis in early stage disease (138). However, little is known about the spatial relationship between PD-L1 staining cells and the different subsets of T cells.

MCC

A few recent studies have characterised the immune infiltrates within MCC. Similar to melanoma, CD8 lymphocytes have been associated with good prognosis: one study of 151 cases of MCC (38-55% with localised disease) validated intratumoural CD8 lymphocytes and their correlation with improved MCC-specific survival (487). Regarding PD-L1, positive staining was observed in 29 of 67 specimens (half of which were localised disease) (488). All specimens with PD-L1 positivity were correlated with immune infiltrates and all but one sample showed PD-L1+ cells were associated with clusters of T cells nearby. PD-L1 negativity was associated with poor survival. Within this cohort, 25/32 (78%) patients had MCPyV positive tumours, and all MCPyV negative tumour were all PD-L1 negative. Given that MCPyV comprise the majority of MCC in Australia, further study of T cell subsets and PD-L1 staining in MCPyV- MCC is needed.

cSCC

Several studies have evaluated the presence of immune cells in cSCC. Interestingly, the presence of CD4 and CD8 T cells and FOXP3 cells was associated with tumour

aggressiveness and higher grade disease in one study (489). Similarly, another study of 97 cSCC samples showed that Tregs and OX40+ lymphocytes were also associated with metastatic potential and poor prognosis (490). PD-L1 expression as evaluated by tumour proportional score (percentage of viable tumour cells expressing PD-L1) was also evaluated in 40 primary cSCC and 5 metastatic cSCC in one study (491). In this study, PD-L1 expression was correlated with higher risk of metastasis, higher histological grade and increased depth of disease. This is echoed in a study of 100 patients with cSCC from the head and neck region, where PD-L1 staining was associated with increased risk for nodal metastasis (492). Macrophages and myeloid cells also appear to play a significant role in the development in cutaneous squamous cell carcinomas, with higher numbers of both M1 and M2 macrophages being observed in cSCC compared to normal skin (493, 494). In a comparative study of 21 cSCC and 52 basal cell carcinomas, a high immune infiltrate was seen in cSCC compared to BCC with higher densities of macrophages, T cells and mast cells; poorly differentiated tumours were associated with higher immune infiltrate density than well differentiated tumours (495). Given this interesting observation between aggressive cSCC and immune cells or PD-L1 staining, it would be important to study the correlation between T cell subsets and PD-L1 staining cells in cSCC.

6.1.1.2 Transcriptomic immune profile with bulk RNA sequencing

The study of immunogenomics has highlighted the prognostic significance of immune molecular subclass in melanoma, as well its potential to provide transcriptomic biomarkers for ICI response in the metastatic setting. As previously reviewed [Chapter 1.3.3], both the melanoma TCGA dataset and the Lund study identified a molecular class of melanoma associated with high immune response and improved survival, compared to other molecular subclasses (19, 44, 45). The transcriptomic biomarker studies for melanoma have focused principally on metastatic disease, and compared pre-treatment biopsies between responders and non-responders to ICI in melanoma (299, 331, 341-344). Not surprisingly these studies have identified genes related to the interferon pathway, T cell activation and T cell memory formation as important for ICI response. Given the shift of ICI into earlier stage disease, it is prudent to direct future studies to the transcriptomic immune profile of early stage cutaneous malignancies.

MCC

Similarly, an immune signature has been suggested to be associated with good prognosis in MCC (487). In this study, gene expression profiles of 35 MCC tumours were clustered based on prognosis and validated in 146 patients. Thirty genes were identified in patients with good prognosis and were associated with immune response on Gene Ontology and Panther. Genes associated with cytotoxic CD8⁺ lymphocytes (*CD8a*, *CXCL10*, *GZMB*, and *IFNG*) were overexpressed in patients with good prognosis. In the validation set, CD8 infiltration but not overall tumour infiltrating lymphocytes were associated with improved outcome. Authors concluded that overexpression of *CD8β* and *CD3E* in tumours from patients with good prognoses was consistent with CD8⁺ T cells (rather than natural killer cells) as the major infiltrating cell type. Direct assessment of NK infiltration was not feasible because greater than 90% of MCC tumours express CD56. DNA microarray analysis of 30 primary MCC also showed over-expression of immune related genes in MCPyV⁺ specimens (205).

cSCC

The transcriptomic profile of cSCC is not well characterised, and is limited by small sample numbers. Transcriptomic comparisons between cSCC with actinic keratoses (pre-cancerous lesion) or healthy skin have also revealed an over-expression of immune related genes in cSCC(496). In this study, pro-angiogenic chemokines (*CXCL1*, *CXCL10*, *CXCL11*) and CC chemokines (*CCL2*, *CCL5*) were over-expressed with enrichment of inflammatory and cytokine mediated signalling pathways. A study of 29 metastatic cSCC samples identified recurrent alterations in RAS pathway activation, cell cycle pathway inactivation, squamous cell differentiation (497). The RAS pathway activation was associated with poor prognosis. There were no immune related genes or immune process signatures highlighted in this study. Whole exome sequencing of 40 primary cSCC showed an enrichment of immune related pathways in moderately/poorly differentiated (MD/PD) cSCC compared to well differentiated cSCC (498). Pathways involved in T-cell receptor, Fc epsilon RI, RIG-I-like receptor and chemokine signalling pathways were more mutated in MD/PD cSCC.

Summary

Given the importance of ICI in skin cancer treatment, there is a need to better understand the immune landscape of primary skin cancers, particular with respect to the density of T cell subsets and the PD-L1 staining cells, as well as the cellular spatial

relationships. This not only improves the understanding of skin cancer immunobiology but may also have implications for biomarker development in the future.

6.1.2 Aims

The aim of this chapter is:

1. To characterise and contrast the immune profile of UV related skin cancers (melanoma, Merkel cell carcinoma, cutaneous squamous cell carcinoma)
2. To compare the T cell subsets (CD3, CD4, CD8, FOXP3) and PD-L1+ cellular density and its spatial distribution within 3 skin cancers
3. To compare the differential gene expression of these three skin cancers using bulk RNA sequencing, with focus on genes involved in immune related processes and genes associated with PD-L1 density by mIHC.

6.1.3 Results

To compare the immune profile of 3 skin cancers, a cohort of seventy-four primary skin cancer samples was assembled: 26 melanoma, 32 MCC, 16 cSCC. Of the 26 T4 primary melanoma samples included in the study: 14 were non-ulcerated (T4a) and 12 were ulcerated (T4b). Of the 32 MCC, 20 were MCPyV negative and 12 were MCPyV positive. The study schema and method for mIHC is shown in Figure 6.1A and 6.1B respectively.

The density of intratumoural T cells and PD-L1+ staining cells was investigated using a 7 marker mIHC: CD3 (CD3⁺CD4⁻CD8⁻ as 'double negative' T cells, DNT), CD4 (CD4⁺ helper T cells), CD8 (CD8⁺ cytotoxic T cells), FOXP3 (FOXP3⁺ regulatory T cells), tumour cells (SOX10 for melanoma, synaptophysin for MCC and p40 for cSCC), and PD-L1 staining (PD-L1) cells. Using this mIHC T-cell characterisation panel, melanoma was associated with higher density of all T cell subsets compared to MCC, including CD3 DNT cells (p=0.001), CD4 helper T cells (p=0.002), CD8 cytotoxic T cells (p<0.0001) and FOXP3 regulatory T cells (p=0.0003) using the Kruskal-Wallis test. Melanoma had higher CD8 cytotoxic T cells compared to cSCC (p=0.01) but the density of other T cell subsets was similar between the two cancers. Cutaneous SCC had higher FOXP3⁺ regulatory T cells density compared to MCC, but no further

differences were observed in the density of other T cell subsets. Figure 6.2A showed the cellular densities of T cell subsets and PD-L1⁺ cells across the three skin cancers. There was a wide range of PD-L1 density across each cancer type, but the overall the PD-L1⁺ density was higher in melanoma compared to MCC (p=0.03).

The samples were then grouped by their predominant T cell subset. Interestingly, 3 MCC samples were noted to have CD3 (CD3⁺CD4⁻CD8⁻) DNT T cells as their predominant T cell subset (i.e. comprised more than 50% of the intratumoural T cells), whereas this was not the case in any melanoma or cSCC samples, as shown in Figure 6.2B. CD4 or CD8 represented the predominant T cell subset in the majority of samples in melanoma and cSCC. Of note, PD-L1 staining, as defined by the percentage of intratumoural cells that were PD-L1⁺, was not associated with the predominant T cell subset, as shown in Figure 6.2C.

The impact of ulceration in melanoma as well as MCPyV viral status in MCC on the immune landscape was then examined. PD-L1 cellular density (absolute number of PD-L1⁺ cells/mm²) was similar in ulcerated and non-ulcerated melanoma; similarly, MCPyV status did not alter PD-L1 density, as shown in Figure 6.2D. CD8 density was higher in MCPyV⁺ MCC compared to MCPyV⁻ MCC (Mann Whitney test, p=0.003) but was not altered by ulceration status in melanoma, as shown in Figure 6.2E. CD3, CD4 and FOXP3 density was not altered by MCPyV status in MCC or ulceration in melanoma, as shown in Figure 6.2F, 6.2G and 6.2H respectively.

Figure 6.3A shows the Spearman correlation between density of T cell subsets and PD-L1⁺ cells across the 3 skin cancers. Although the proportion of intratumoural cells which were PD-L1⁺ did not appear to correlate with the *predominant* T cell subset within the tumours (Fig 6.2C), PD-L1 density was correlated to CD8 density (rho=0.41, p=0.04) in melanoma. To illustrate this, two melanoma cases of specimen 26 and specimen 8 can be compared: in the case of specimen 26, the PD-L1 density was high at 1480 cells/mm² and CD4 was the predominant T cell subset (comprised 71% of TILs). Furthermore, both the density of CD4 and CD8 was also high (508 cells/mm² and 113 cells/mm² respectively). Contrast this with specimen 8, where PD-L1 density was low at 33 cells/mm² despite CD4 still being the predominant TIL (66% of TILs): the absolute density of CD4 and CD8 was both low (250 cells/mm² and 42 cells/mm²).

These two cases illustrate how PD-L1 density was not correlated with the predominant T cell subtype, but was more correlated with the CD8 density.

Using the Spearman correlation to correlate between the density of T cells subsets and PD-L1 density in other skin cancers, multiple T cell subsets correlated with PD-L1 density in cSCC, where PD-L1 density was correlated with CD4 ($\rho = 0.73$, $p < 0.01$), CD8 ($\rho = 0.62$, $p = 0.01$) and FOXP3 ($\rho = 0.64$, $p < 0.01$). However, PD-L1 density was not correlated to other T cell subsets in MCC.

In terms of correlation between other T cell subsets, CD3 DNT cells were correlated with all other T cell subsets in melanoma (CD4 $\rho = 0.54$, $p < 0.01$; CD8 $\rho = 0.57$, $p < 0.01$; FOXP3 $\rho = 0.50$, $p = 0.01$). In MCC, CD3 DNT was correlated with CD8 ($\rho = 0.37$, $p = 0.04$) and FOXP3 ($\rho = 0.43$, $p = 0.01$). In cSCC, the T cell subsets were highly correlated with each other, where CD3 DNT was correlated with all other T cell subsets (CD4 $\rho = 0.073$, $p < 0.01$; CD8 $\rho = 0.70$, $p < 0.01$; FOXP3 $\rho = 0.66$, $p < 0.01$); CD4 was correlated with CD8 ($\rho = 0.66$, $p < 0.01$) and FOXP3 ($\rho = 0.64$, $p < 0.01$); CD8 was correlated with FOXP3 ($\rho = 0.73$, $p < 0.01$). When Fig.6.2A and Fig 6.3A are taken together, CD3 DNT cells are present in small numbers across all three cancer types, and appear to correlate with the presence of other T cell subsets.

The spatial relationships between T cell subsets and PD-L1 or tumour cells were examined in Figure 6.3B. The average distance between CD3 cells and PD-L1 cells was shortest in MCC compared to both melanoma (Kruskal Wallis test $p = 0.0003$) and cSCC (Kruskal Wallis test $p = 0.003$). The average distances between PD-L1 and CD4, PD-L1 and CD8 were similar in the different cancers (Kruskal Wallis test, $p < 0.05$). The spatial relationships between T cell subsets and tumour cells were also explored, where a shorter average distance between CD8 and tumour cells was seen in MCC compared to melanoma (Kruskal Wallis test, $p = 0.02$), but was similar between MCC and SCC, as well as SCC and melanoma. The average distances between CD4 and tumour cells, tumour cells and CD3 cells were similar in the different cancers (Kruskal Wallis test, $p < 0.05$). The HALO software program was not able to immunophenotype FOXP3 cells and therefore the average distance between FOXP3 cells and other cells was not able to be examined.

A subset of the samples studied by m-IHC was also profiled by bulk RNA-sequencing: melanoma ($n = 17$), MCC ($n = 19$) and SCC ($n = 5$). Principal component analysis

confirmed that the tumour type was the main determinant of differentially expressed genes observed (Figure 6.4A). Unsupervised hierarchical clustering grouped melanoma with cSCC, and separately from MCC. Melanoma again clustered with cSCC and separately from MCC when unsupervised hierarchical clustering of a curated list of 1270 genes related to immune cell types or immune related processes was performed (383, 384), as shown in Figure 6.4B.

In an attempt to understand the functional correlates of PD-L1 staining by mIHC, gene expression of a number of immune related genes was examined. Surprisingly, PD-L1 mRNA expression was not correlated with PD-L1 staining in any of the cancer types (Figure 6.5A). There was also no relationship observed between tumour mutational burden (TMB) and PD-L1 staining by mIHC in any of the cancer types (See Figure 6.5B).

As the number of cSCC samples was very small, the subsequent analyses focused on the comparison between melanoma and MCC. Samples were ranked by the PD-L1 density, and dichotomized by the median of the cohort into groups of 'high PD-L1' and 'low PD-L1' samples. When the high PD-L1 melanoma were compared with low PD-L1 melanoma, with upregulation of 6 genes in high PD-L1 melanoma: *IRF4*, *STAT5B*, *IFI27*, *MAP3K7*, *IL12RB2* and *CHIT1*, and downregulation of *CXCL6*, *TNFRSF10C*, *NT5E* and *MCAM* (Figure 6.6A). When high PD-L1 MCC was compared to low PD-L1 MCC: *VEGFA* was upregulated, whereas *TNFRSF11a* and *IL1RL2* were downregulated (Figure 6.6B).

Figure 6.6C showed the comparison was then made of differentially expressed genes between high PD-L1 melanoma and high PD-L1 MCC. Using a log-fold change of 2, 134 genes were significantly upregulated in melanoma and 15 genes upregulated in MCC. Gene set enrichment pathway analyses were performed on these genes (Figure 6.7A, Figure 6.7B, Figure 6.7C, Figure 6.7D). Pathways associated with leucocyte proliferation, migration and cytokine signalling were highly enriched in melanoma, whereas a number of less significantly enriched pathways involved in interleukin and MAPK1/MAPK3 signalling were enriched in MCC.

6.1.4 Discussion

The skin is an immunologically active organ and presents as a unique tumour microenvironment for cancer development (499, 500). Despite the shared aetiology of UV radiation, high mutational burden and the responsiveness to ICI treatments (500), the comparison of three cutaneous malignancies using mIHC and RNA sequencing has revealed distinct immune profiles. These contrasts provide a foundation to further the understanding of the T cell and tumour environment, as well as their relation to PD-L1+ staining cells. Overall, melanoma had a significantly higher density of T cells of all subsets compared to MCC and higher CD8 density than cSCC. There was also a subset of MCC where CD3⁺ DNT cells were the predominant T cell subset. This was not observed in melanoma and cSCC. The PD-L1 density was highly variable across the three cancers, with no correlation observed between PD-L1 density and T cell subsets in MCC. Lastly, the transcriptomic immune profile also shows similarities between melanoma and cSCC, and again they are both distinct from MCC.

Distinct T cell profiles in 3 skin cancers

Overall, melanoma appeared to be a highly ‘inflamed’ tumour compared to both MCC and cSCC, as shown by the higher density of all T cell subsets compared to MCC, and the higher CD8 density in melanoma compared to cSCC. When the samples were categorised by the predominant T cell subset, CD3⁺ (CD3⁺CD4⁺CD8⁻) DNT cells was the predominant T cell subset in a subset of MCC samples, whereas DNT was not the predominant T cell subset in any melanoma or SCC samples. Furthermore, MCPyV positive MCC were associated with a higher abundance of CD8⁺ T cells compared to MCPyV negative MCC.

This observation of low tumour infiltrating lymphocytes (TILs) in MCC is somewhat surprising, given the immunogenic aetiology (i.e. either viral or UV-mediated) and its high response to ICI in the advanced setting. Previous studies have also found lower TIL in MCPyV negative MCC compared to MCPyV positive samples (501), so the high proportion of virally negative MCCs in our cohort (2/3 samples are MCPV-) may also have contributed to the lower T cell infiltration. Given that the present study only compared the intratumoural T cell subsets, it is possible that the immunogenicity seen in MCC is attributed to other immune cell types or T cells subsets present within the stromal compartment. Furthermore, comparison of paired primary and metastatic

disease will be needed to assess whether T cell infiltration occurs at a later stage of MCC development compared to melanoma and cSCC.

Cellular spatial relationships

Spatial distance relationships between cells were explored using the HALO analytical software program. Firstly, CD8 and tumour cells were in closer proximity in MCC than in melanoma. This may be owing to the morphological differences between MCC and melanoma, where the densely packed small round blue cells of MCC would result in closer proximity to CD8 T cells. Secondly, PD-L1⁺ cells were in closer proximity to CD3 DNT cells in MCC compared to melanoma and cSCC. The prognostic value of proximity between PD-L1 staining cells and CD3 DNT cells observed in MCC is not currently known but will be important to evaluate in future studies, particularly as this CD3 DNT population becomes more fully characterised.

There are a number of recent studies in pancreatic cancer (502) and colon cancer (503) that have associated the proximity of immune cells to tumour cells to positive clinical outcome. Proximity between Tregs and tumour cells was also associated with poor prognosis in non-small cell lung cancer (504). More recently, cellular spatial relationships have been evaluated as in 27 melanoma samples prior to ICI treatment (505). Response and PFS were associated with proximity of CD8⁺ cells to tumour cells for both anti-PD-1 monotherapy and combination anti-PD-1/anti-CTLA4 therapy. Furthermore, proximity between PD-L1 cells and tumour cells was associated with 12-month PFS for anti-PD-1 monotherapy, while proximity between tumour cells and CD8⁺ cells was significant for anti-PD-1/anti-CTLA4 combination therapy. Therefore, functional studies of CD3 DNT population in MCC are needed to explore whether the cellular proximity of CD3 DNT to PD-L1⁺ cells may also have implications for ICI treatment.

PD-L1 density

Interestingly, PD-L1⁺ density was highly variable across the three cancers, despite density of T cell subsets in MCC compared to melanoma. Furthermore, there was no significant difference in PD-L1 density between MCPyV positive and MCPyV negative tumours in our study. Previous studies have reported that MCPyV negative MCC all stained negatively for PD-L1 but this study used a different PD-L1 antibody assay and

different scoring threshold for PD-L1 positivity (501). In an attempt to explore this further, the correlation of PD-L1 density and T cell subset density was examined. In contrast to melanoma and cSCC, PD-L1 density was not correlated to any of the T cell subsets in MCC.

The correlation between T cells and PD-L1 in melanoma is not surprising, given the inducible expression of PD-L1 upon T cell activation (138). As such, PD-L1 density was also highly correlated with all T cell subsets in cSCC. Correlation between PD-L1 density and other T cell subsets does not appear to have been evaluated in previous studies of cSCC. Interestingly, FOXP3, a known poor prognostic factor (490), was correlated with PD-L1+ density in cSCC in our cohort. This may provide a possible rationale for the poor prognosis previously observed in association with PD-L1 in cSCC (492), which is in contrast to positive prognoses seen in high PD-L1 staining in melanoma and MCC.

Comparison of bulk RNAseq in high PD-L1 density melanoma and MCC samples

Important questions remain as to the correlates of PD-L1 in MCC. While PD-L1 density correlated with the density of other T cell subsets in melanoma and cSCC, this was not the case with MCC. The differential gene expression analyses in MCC highlighted an up-regulation of *VEGFA* in high PD-L1 samples, whereas *TNFRSF11a* and *IL1RL2* were upregulated in low PD-L1 MCC. Vascular endothelial growth factor (VEGF) is a known immunosuppressive cytokine induced by hypoxic factors in the tumour microenvironment. VEGF has been correlated with PD-L1 expression in other cancers such as clear cell renal cancer and Hodgkins lymphoma (506, 507). More in-depth analysis of the immune cell types, genes and pathways associated with angiogenesis may enable this pathological process to be targeted in combination with ICI (508). *TNFRSF11a* encodes for RANK protein, which is integral to bone homeostasis. There is an increasing recognition of RANK and RANK-ligand in immune processes, where RANK is expressed on tumour infiltrating myeloid (TAM) cells, and RANKL is expressed on CD4 and CD8 cells(509). Active investigation is underway both pre-clinically and clinically targeting the RANK/RANKL pathway in conjunction with ICI, including the CHARLI study evaluating Denosumab in combination with anti-CTLA4 or anti-PD-1 in advanced melanoma (*NCT03161756*) (510, 511). *IL1RL2* encodes for a member of the interleukin 1 receptor family and acts

as a decoy for IL1 binding. An association of IL1RL2 and PD-L1 has not been reported previously, though efforts are being made to combine cytokine signaling modulators and ICI (512, 513). These differentially expressed genes pose some possible correlates with PD-L1 in MCC, which highlights firstly the need to investigate the tumour immune environment beyond the T cell compartment and, secondly, to learn how to integrate additional immune pathways such as RANK or cytokine signaling pathways to maximise anti-tumour killing.

A comparison of transcriptomic profiles of high PD-L1 staining melanoma and MCC found that genes involved in adaptive immune processes, leucocyte proliferation and migration are highly upregulated in melanoma and not in MCC. However, it is not known whether the transcriptomic profiles purely relative to the high infiltrations of T cells in melanoma compared to MCC. One could consider a bioinformatics method of normalisation for T cell density in the statistical analysis of the differentially expressed genes, by normalising for the T cell density within the *edgeR* linear model. However such normalization methods within the linear model are designed to correct for technical effects, and not to adjust for genuine biological differences. Hence it remains that the adaptive immune response and leucocyte pathways enhanced in the melanoma specimens may be representative of the higher T cell infiltration, rather than unique immune processes being present in MCC. One possible way to overcome this in future studies would be to employ techniques such as single cell RNA (scRNA) sequencing. scRNA sequencing is a method by which the functional gene expression can be directly exemplified for each immune cell type, as has been performed in other biomarker studies. While this exciting technology is fundamental in dissecting the underlying genomic pathways differentially expressed by each cell type, the cellular architecture and spatial relationships are lost during tissue processing, therefore it is anticipated these two methods of immune profiling would be complementary.

Limitations with PD-L1 mIHC

It must be noted that the previous studies have utilised different methods for PD-L1 assessment, where only a subset of cells is evaluated and the proportion of cells with membranous or cytoplasmic expression of PD-L1 stain at particular thresholds were deemed positive [as discussed in detail in Chapter 1.3.3] (138, 312, 488). In contrast, our study enumerated the absolute number of cells with membranous PD-L1 staining

and was able to provide a quantitative measure of the density of PD-L1 staining cells. While the assessment of PD-L1 cellular density is more quantitative using the novel OPAL method, the landmark clinical trials have not used PD-L1 density as a biomarker for immunotherapy. This automated technique may overcome the relative subjectivity of PD-L1 assessment using contemporary methods (312), but again would require validation using tissue from the pre-existing clinical studies to establish its clinical significance.

The PD-L1 assay used in this study was the SP142 antibody, which is the companion PD-L1 antibody for atezolizumab, an anti-PD-L1 monoclonal antibody. As discussed in the literature review [Chapter 1.3.3], there has been one study comparing PD-L1 antibody assays in lung cancer specimens, where SP142 was an outlier and did not show the same sensitivity as the other three commercially available antibody assays, SP142, 28-8, 22C3, and SP263 clones (310). However, a subsequent study in melanoma showed that SP142 showed comparable results to the other antibodies of 5H1, SP142, 28-8, 22C3, and SP263 (312).

A limitation of our study was that PD-L1 co-staining (i.e. whether PD-L1 staining was expressed on tumour cells or the immune cells) was not examined. In future studies, it would be important to incorporate PD-L1 co-staining, examining not only the tumoural and T cell compartment but also PD-L1 expression on other immune cells. There is an increasing recognition of the importance of non-T immune subsets in the mechanism of action for ICI (163, 165). It is highly possible that PD-L1 expression within MCC is more dependent on these other immune cell compartments, given the lack of correlation between any of the other T cell subsets and PD-L1 in our MCC cohort.

There were several technical challenges with mIHC staining and image analysis. The staining process itself was time consuming, with the staining of 7 antibodies took a minimum of three days. Furthermore, such an intensive antigen retrieval also meant that more friable tissue was less likely to remain on the slide after processing. Small tumour samples were particularly susceptible to damage through sequential heating and washing and therefore may be troublesome for utilisation in cancer types where only small core biopsies are available. In terms of image analysis, selection of a representative high powered field for analysis can also generate bias, as there can be significant variability of immune cells between different sections within the tumour,

tumoural margin or stroma. This is particularly relevant for PD-L1 expression, where substantial heterogeneity both spatially and longitudinally is well documented (314). Lastly, enormous amounts of data are generated with these high resolution image analyses. Consequently, an efficient bioinformatics pipeline is needed to accompany such technology. While this m-IHC technique gives extremely high resolution into the presence of specific cellular subtypes present and the spatial interactions between these cells, functional studies are also needed to supplement this in order to understand biological significance.

PD-L1 mRNA expression

PD-L1 mRNA expression has been proposed as an alternative to PD-L1 staining as a potential biomarker, in order to overcome the inter-observer variability, the technical variabilities in antibody acquisition, and the variable thresholds for positivity across clinical trials. In our study, PD-L1+ density correlated poorly with mRNA expression of CD274 and PDCD1 in all cancer types using Spearman correlation: melanoma $\rho = 0.023$, MCC $\rho = -0.07$ and cSCC $\rho = 0.04$. This lack of correlation may possibly be driven by PD+ cells outside the tumoural tissue compartment, as our PD-L1+ density is only reported for the intratumoural tissue compartment. Since the cSCC sample size is small, only limited conclusions can be drawn from these observations.

The small studies that have correlated PD-L1 mRNA levels with PD-L1 staining have produced conflicting results (514-516). One study compared PD-L1 staining by ab58810 polyclonal (Abcam) and 5H1 antibodies and PD-L1 mRNA detection by Reverse Transcriptase quantitative Polymerase Chain Reaction (RT-qPCR) in 34 metastatic melanoma specimens, where PD-L1 staining was not correlated with PD-L1 mRNA (Cohen's $\kappa < 3\%$, $P = 0.4273$). These findings are in contrast to a study of 21 metastatic melanoma samples evaluated by the SP142 and E1L3N antibodies where a significant correlation was found between the two antibodies and mRNA as quantified by Nanostring's nCounter PanCancer Immune panel, with Spearman's coefficients of 0.80 (SP142) and 0.90 (E1LSN) respectively(515). Finally, positive correlation was found between PD-L1 staining on tumour cells (but not immune cells) by 28-8 antibody (but not E1L3N antibody) with mRNA detection by RT-qPCR in a study of 22 NSCLC specimens (516). Therefore, there may be some variability in the different staining antibodies and their correlation to PD-L1 mRNA expression. Our study did not

examine the relative PD-L1 co-staining on tumour cells compared to immune cells so it is not known whether this may account for part of the variability of mRNA to PD-L1 expression by mIHC.

Summary

In summary, the three primary cutaneous malignancies in our cohort showed distinct tumour immune profiles. Melanoma was associated with a high density of T cells of all subsets compared to MCC, and higher CD8 compared to cSCC. PD-L1 density varied across samples in all three cancer types, though PD-L1 was higher in melanoma compared to MCC. Contrasting the three skin cancers highlighted the predominance of CD3 DNT in a subset of MCC, as well as different spatial cellular relationships and correlates to PD-L1 density across the three cancers. Further analysis of immune cells outside the T cell compartment as well as exploration of the stromal compartment may yield further insights into the immune landscape of these cutaneous malignancies.

6.1.5 Figures

Fig 6.1(A)

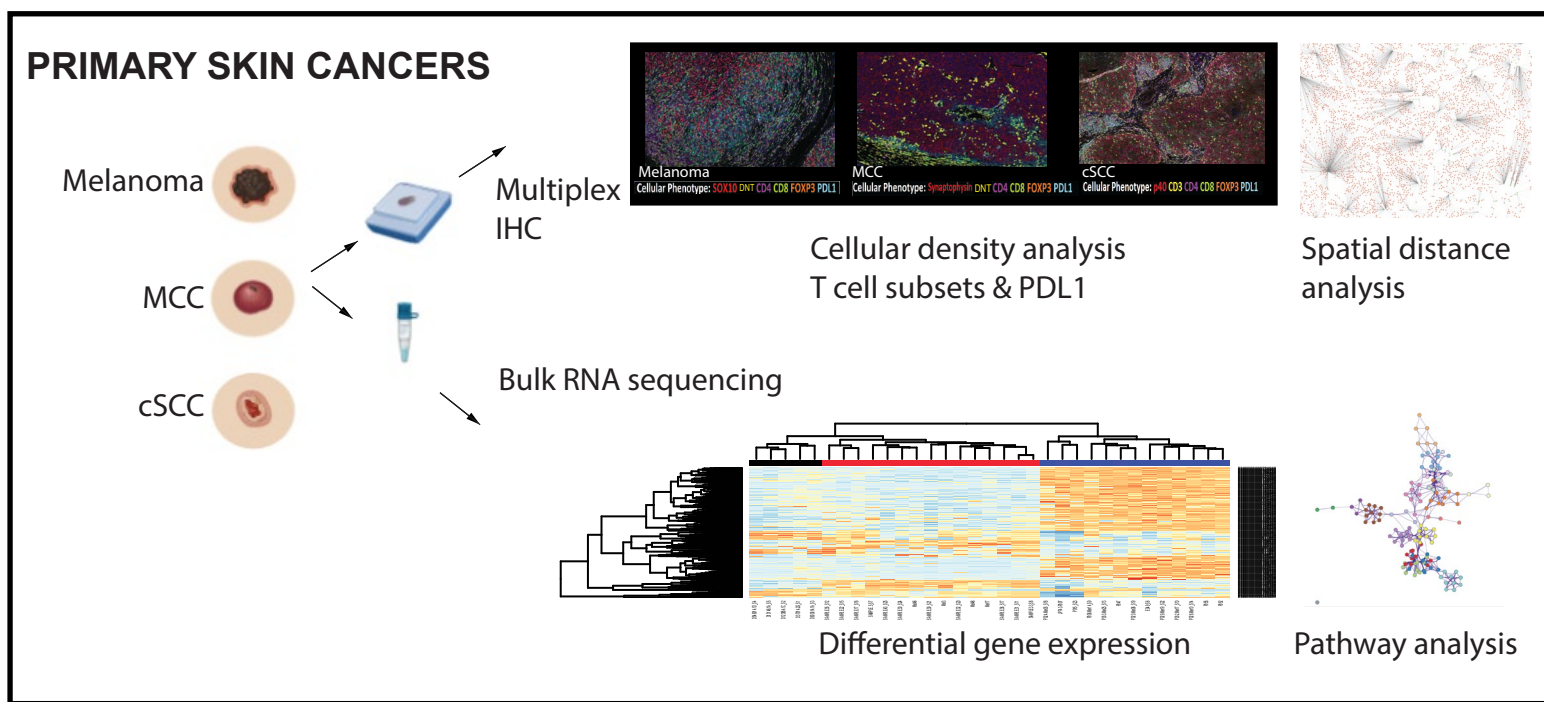


Fig 6.1(B)

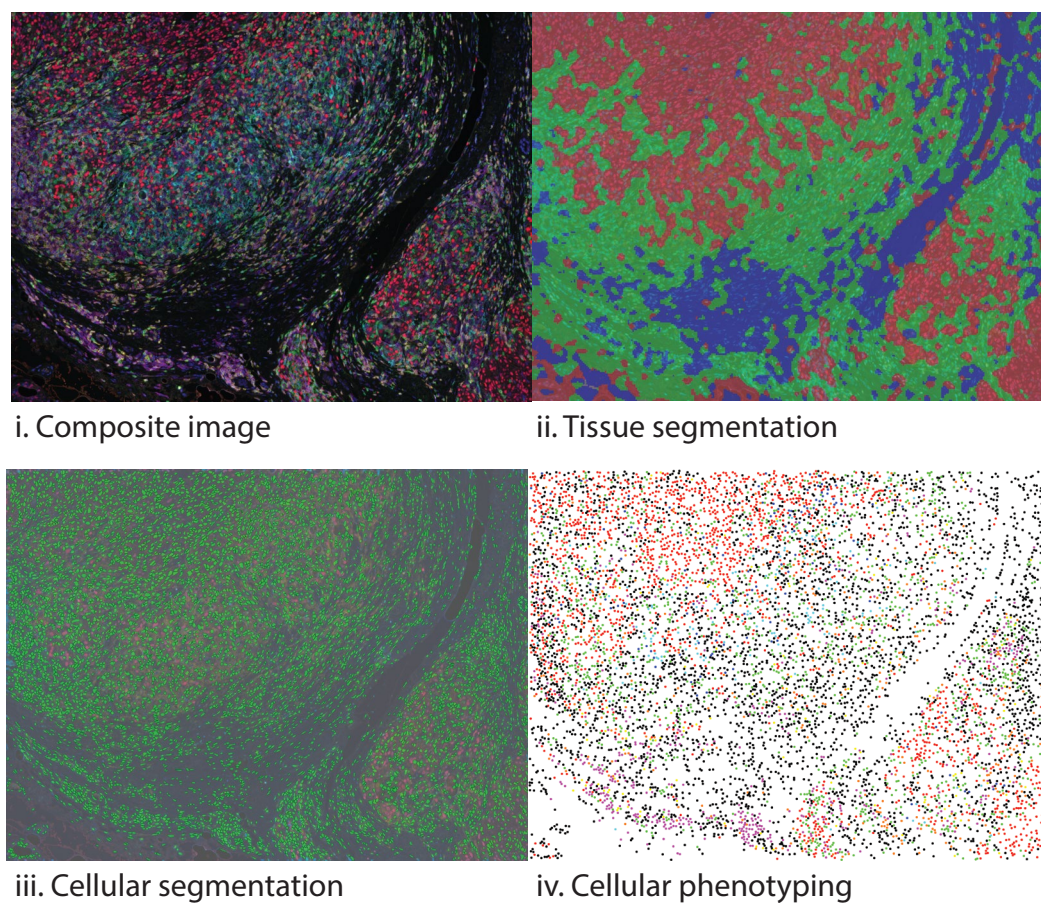


Figure 6.1 (A) Study schema and (B) methodology for multi-spectral immunohistochemistry (mIHC)

(A) Chapter 6 is a comparative study of the immune landscape of primary skin cancers using orthogonal methods of mIHC and RNA-sequencing on matched samples. Thirty-four melanomas, 32 Merkel cell carcinoma (MCC) and 16 cutaneous squamous cell carcinoma (cSCC) were included in the study. Multiplex IHC allows for analysis of density of T cell subsets, tumour cells and PD-L1 staining cells within the tumour microenvironment. The spatial relationships between the different cell types were also assessed. Bulk RNA sequencing allows comparison of differentially expressed genes in 3 skin cancers as well as analysis of enriched pathways.

(B) 4µm formalin fixed paraffin embedded tissue sections were stained using the novel 7 antibody multi-spectral immunohistochemistry panel to characterise the T cell subsets in three skin cancers. The InForm software is used to perform on i) composite image is used for ii) tissue segmentation into tumour compartment which is a composite of the intratumoural (red) and peri-tumoural (green) compartments, and separate from the stromal compartment (blue). iii) Cells are segmented and enumerated for each tissue compartment. iv) Cellular phenotyping is performed, with seven cellular phenotypes being identified: CD3+CD4-CD8- 'Double Negative' T cells (DNT), CD4+ T helper cells, CD8+ cytotoxic T cells, FOXP3+ regulatory T cells as well as PD-L1+ cells. Tumour cells are stained (SOX10 for melanoma, synaptophysin for Merkel, p40 for cSCC) and DAPI as nuclear counterstain. See Methods for full details.

Fig 6.2(A)

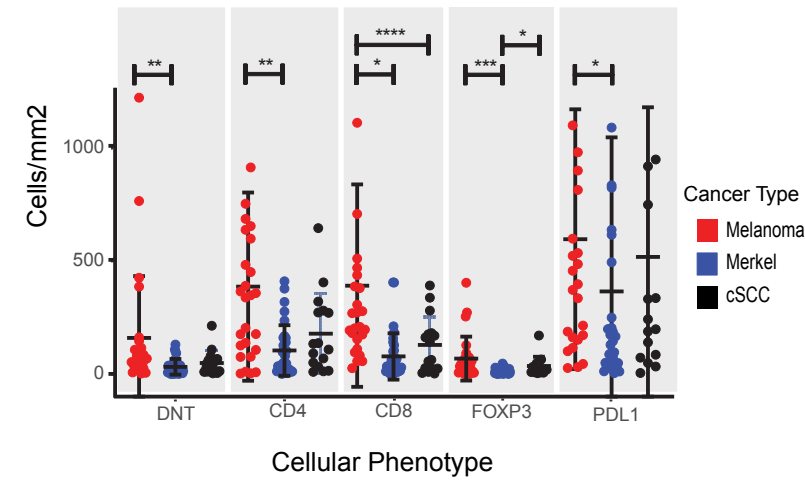


Fig 6.2(B)

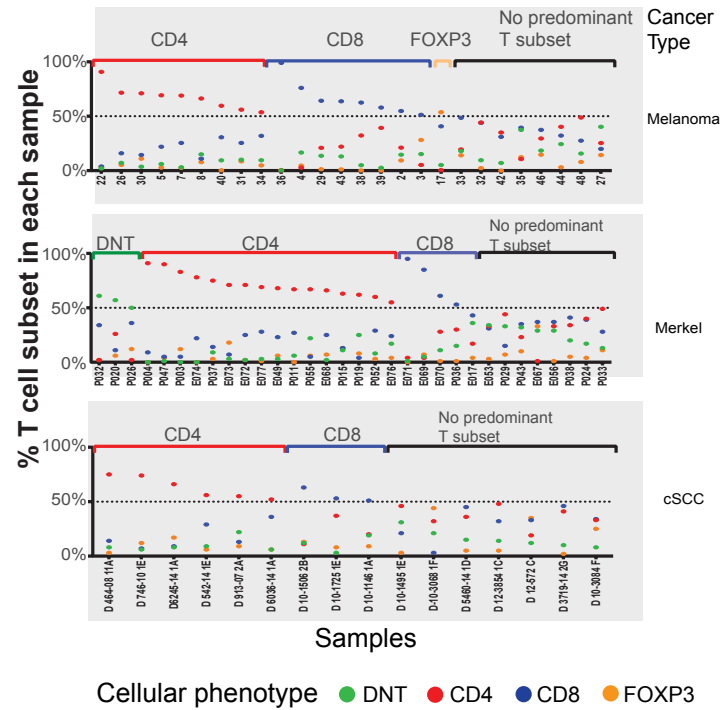


Fig 6.2(C)

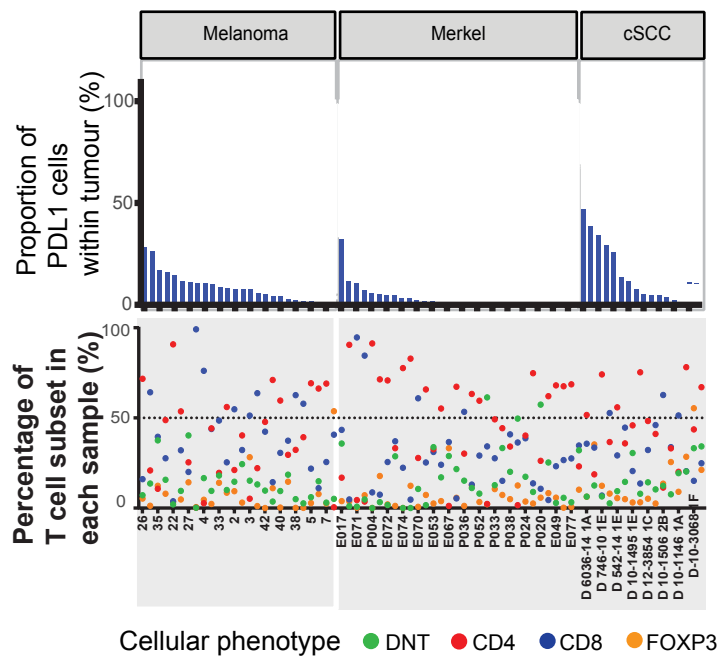


Fig 6.2(D)

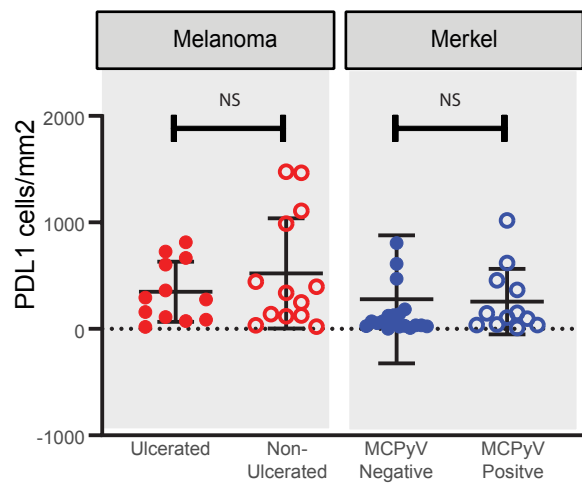


Fig 6.2(E)

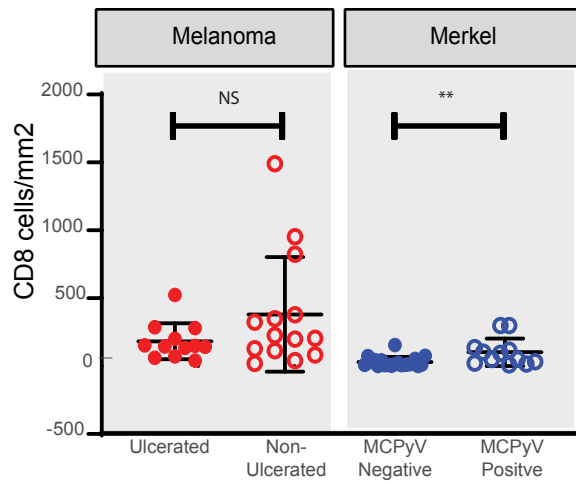


Fig 6.2(F)

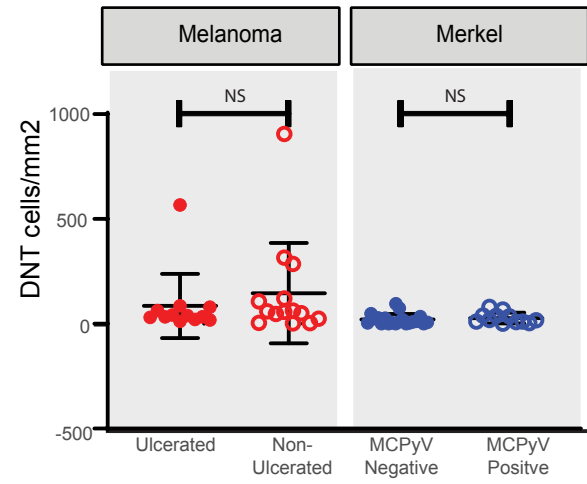


Fig 6.2(G)

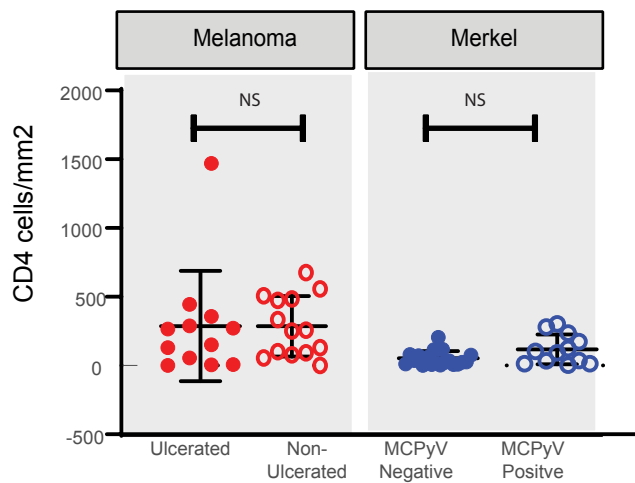


Fig 6.2(H)

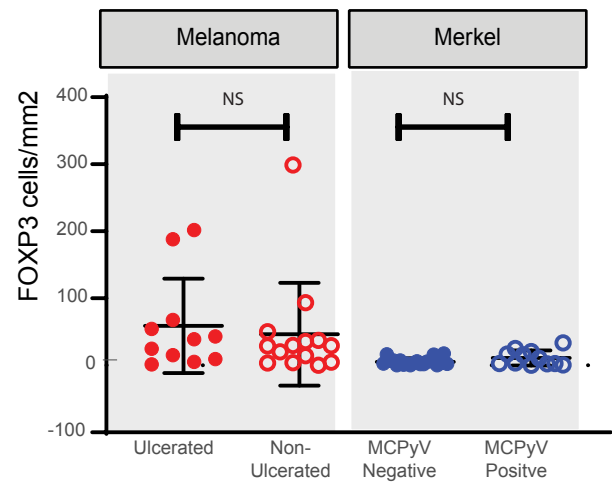


Figure 6.2 (A) Comparison of intratumoural T cell subsets and PD-L1⁺ cells in 3 skin cancers by mIHC; (B) DNT is the predominant T cell subset in a subset of MCC samples; (C) PD-L1⁺ density is not associated with predominant T cell subset; (D) PD-L1 is not associated with Merkel polyomavirus status in MCC or ulceration status in melanoma; (E) CD8 was higher in MCPyV⁺ MCC than MCPyV⁻ MCC but did not differ with ulceration in melanoma; (F) CD3 was not associated with MCPyV in MCC or ulceration in melanoma; (G) CD4 was not associated with MCPyV in MCC or ulceration in melanoma; (H) FOXP3 was not associated with MCPyV in MCC or ulceration in melanoma

(A) Melanoma has higher T cell infiltrates (CD3, CD4, CD8, FOXP3) than Merkel; Kruskal-Wallis test, all $p \leq 0.01$. Melanoma has higher CD8 than SCC, $p=0.01$. SCC has higher FOXP3 than Merkel, $p=0.01$. Melanoma has higher PD-L1 compared to Merkel ($p=0.03$) but there is variation of PDL1 staining within each tumour type. Double negative T cells (DNT) are CD3⁺CD4⁻CD8⁻ throughout the figures unless otherwise stated. Melanoma shown in red, Merkel in blue, SCC in black. Mean density of each cellular phenotype (cells/mm²) and error bars of standard deviation are shown. Kruskal-Wallis test was performed for each cellular phenotype across three cancers, with significantly different data presented as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

(B) Predominant T cell subset within each cancer type. The density of T cell subsets was assessed using mIHC. The relative proportions of each T cell subset (i.e. DNT, CD4⁺, CD8⁺ and FOXP3) are presented as percentages for each sample. The T cell subsets are shown as DNT (green), CD4 (red), CD8 (blue), FOXP3 (orange). The samples were grouped by the predominant T cell subset (as shown by the coloured bars above each sample); with the 'predominant' T cell subset defined as the T cell subset that comprise more than 50% of tumour infiltrating T cells. Three MCC samples had DNT as the predominant T cell subset, whereas DNT was not the predominant T cell subset in melanoma or SCC samples.

(C) PD-L1 expression is not correlated with predominant T cell subset. The proportion of cells expressing PD-L1⁺ and its correlating T cell subset proportions are shown. Each column represents one sample.

(D) PD-L1 expression did not differ with ulceration status in melanoma (Mann-Whitney test, $p=0.49$). PD-L1 expression did not differ with MCPyV (MCPyV) status in MCC (Mann-Whitney test, $p=0.27$).

(E) Interestingly, CD8 density was higher in MCPyV⁺ MCC compared to virally negative MCC (Mann Whitney test, $p=0.003$), but CD8 density did not differ with ulceration in melanoma (Mann Whitney test, $p=0.30$).

(F) DNT density did not differ with ulceration in melanoma or MCPyV status in MCC (Mann-Whitney test, $p=0.43$ and $p=0.25$ respectively).

(G) CD4 density did not differ with ulceration in melanoma or MCPyV

status in MCC (Mann Whitney test, $p=0.60$ and $p=0.09$ respectively).

(H) FOXP3 density also did not differ with ulceration in melanoma or MCPyV status in MCC (Mann Whitney test, $p=0.43$ and $p=0.28$).

Fig 6.3(A)

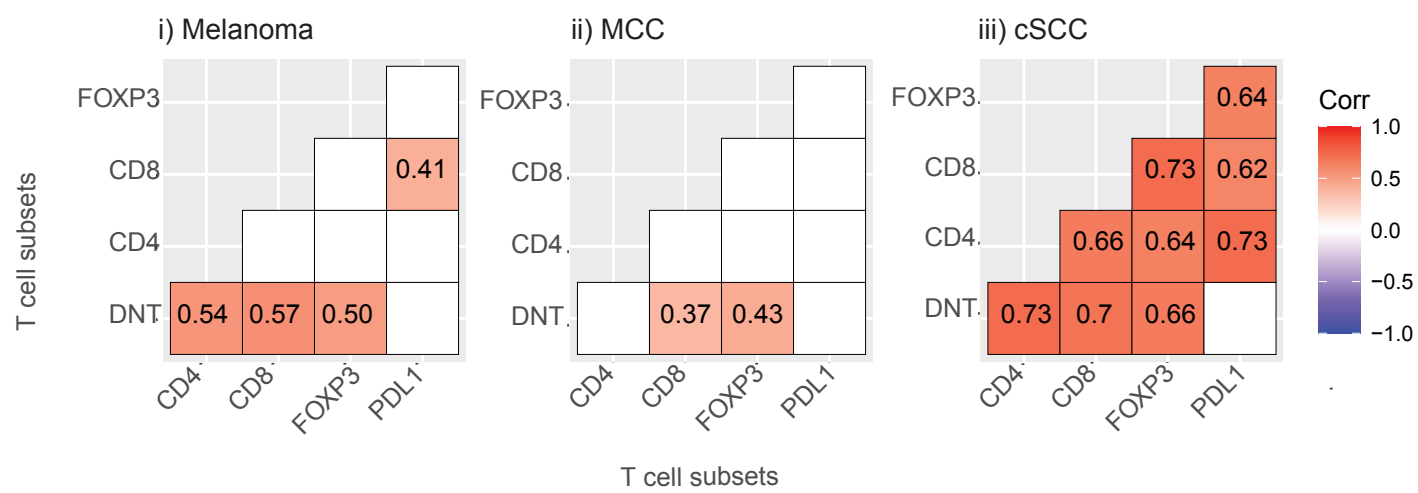


Fig 6.3(B)

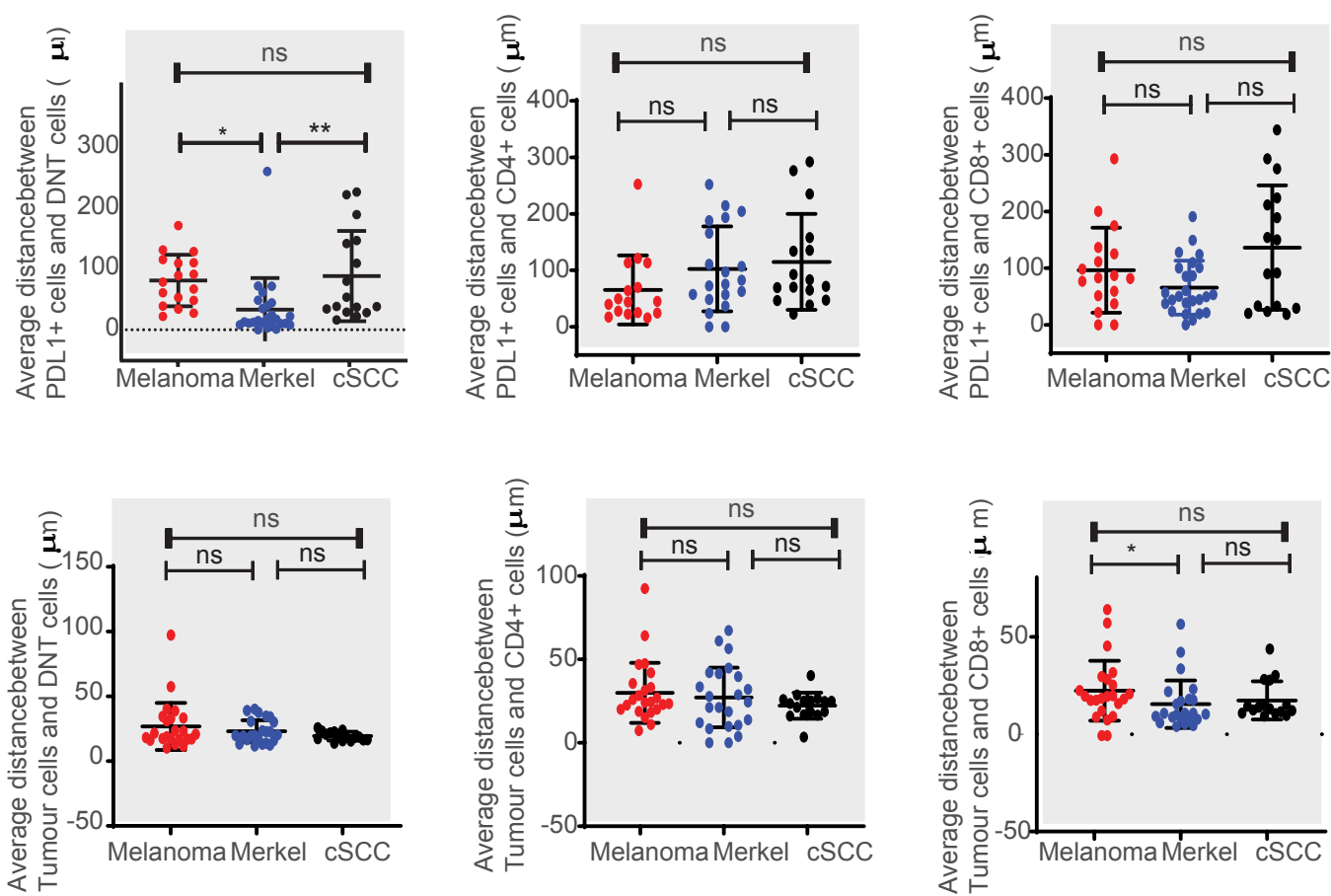


**Figure 6.3 (A) Spearman correlation of T cell subsets and PD-L1 cells;
(B) Spatial distance between cell types**

(A) Spearman correlation of T cell subsets and PD-L1 cells. Only significant results are displayed in the figure (all $p < 0.05$).

- i. DNT are correlated with all other T cell subsets (CD4 $\rho = 0.54$, $p < 0.01$; CD8 $\rho = 0.57$, $p < 0.01$; FOXP3 $\rho = 0.50$, $p = 0.01$). PD-L1 was correlated with CD8 ($\rho = 0.41$, $p = 0.04$).
- ii. For MCC, Spearman correlation of T cell subsets density and PDL1+ cellular density for MCC. DNT are correlated with CD8 ($\rho = 0.37$, $p = 0.04$) and FOXP3 ($\rho = 0.43$, $p = 0.01$). PD-L1 was not correlated with any other cellular densities.
- iii. Spearman correlation of T cell subsets density and PD-L1 cellular density for cSCC. DNT was correlated with all other T cell subsets (CD4 $\rho = 0.073$, $p < 0.01$; CD8 $\rho = 0.70$, $p < 0.01$; FOXP3 $\rho = 0.66$, $p < 0.01$). CD4 was correlated with CD8 ($\rho = 0.66$, $p < 0.01$) and FOXP3 ($\rho = 0.64$, $p < 0.01$). CD8 was correlated with FOXP3 ($\rho = 0.73$, $p < 0.01$). PD-L1 was correlated with CD4 ($\rho = 0.73$, $p < 0.01$), CD8 ($\rho = 0.62$, $p = 0.01$) and FOXP3 ($\rho = 0.64$, $p < 0.01$).

(B) Cellular distance between PD-L1 cells and DNT is smaller in Merkel compared to melanoma (Kruskal-Wallis test, $p = 0.0003$) or SCC (KW test, $p = 0.0025$). Cellular distance between tumour cells and CD8 cells is smaller in Merkel compared to melanoma (Kruskal-Wallis test, $p = 0.0215$), but is not significantly different between MCC and SCC (KW test, $p = 0.7998$). Cellular distance between tumour cells is not different in melanoma compared to SCC (KW test, $p = 0.6493$). *Significant values are indicated where, $**p \leq 0.01$.* Error bars of standard deviation are shown. Other comparisons between cellular populations non-significant e.g. PD-L1 to CD4, CD8, FOXP3 or tumour cells (data not shown).

Fig 6.4(A)

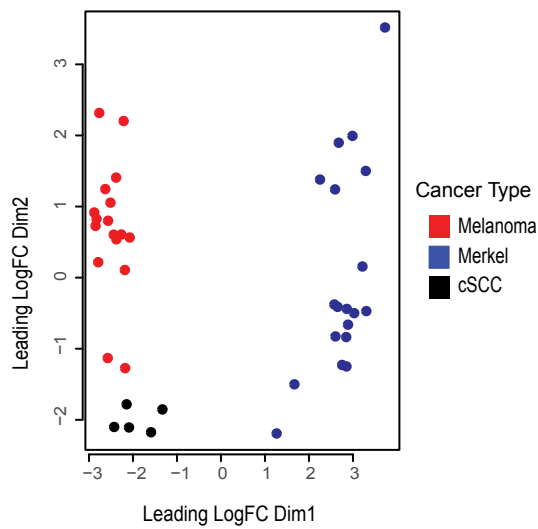


Fig 6.4(B)

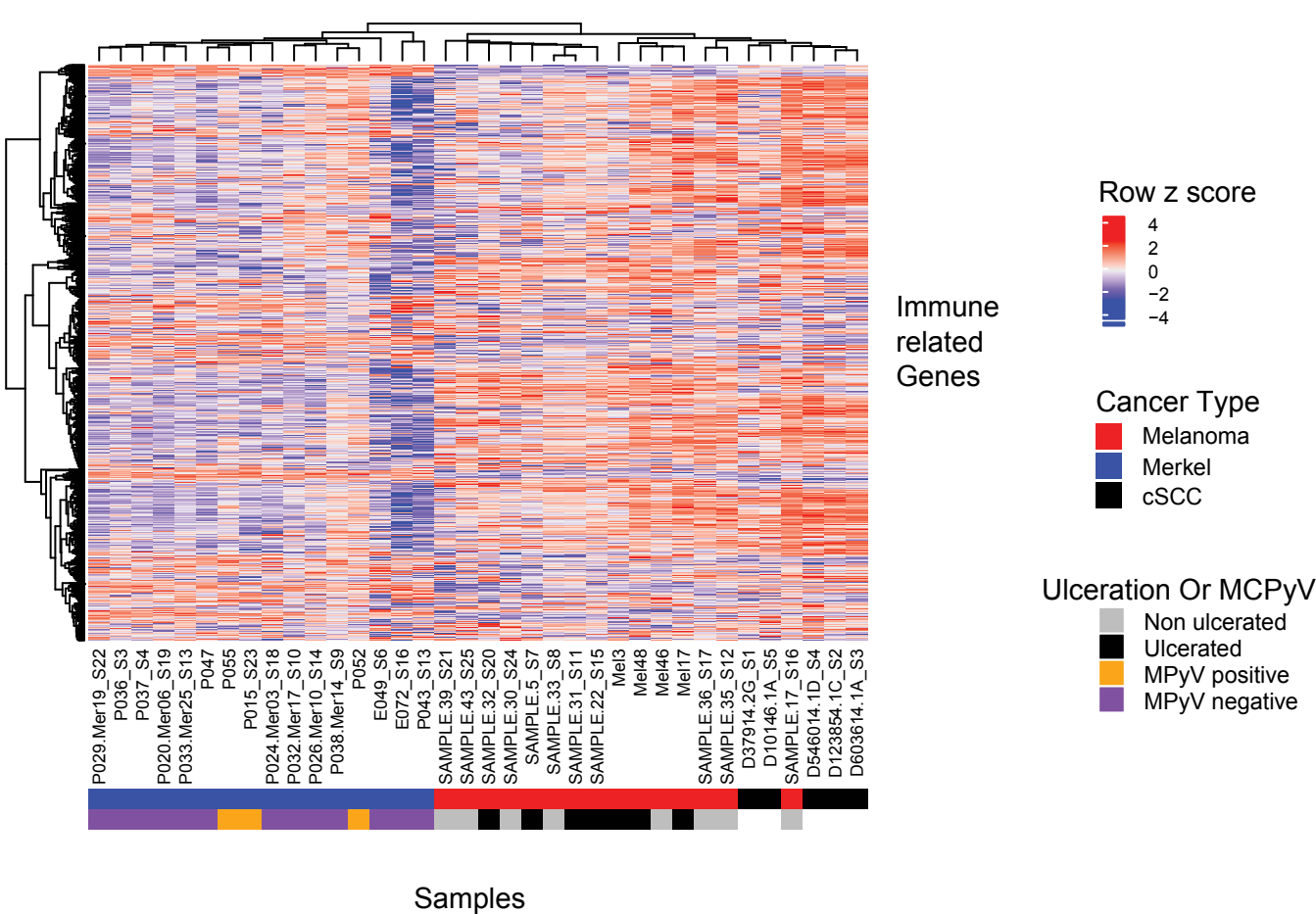


Figure 6.4 (A) Principal components analysis of differentially expressed genes across 3 skin cancers; (B) Unsupervised clustering of immune related genes

(A) Principal components analysis using Multi-Dimensional Scaling plot of differentially expressed genes showing that cancer type is the main determinant of variation.

(B) Unsupervised clustering of differentially expressed genes associated with immune cell types or immune processes (curated list of Galon immune gene set and Nanostring immune gene set) (383, 384). Melanoma and SCC cluster together, whereas Merkel cluster separately from the other two cancers. Each column represents one sample. Ulceration status and viral status are shown for melanoma and Merkel samples respectively.

Fig 6.5(A)

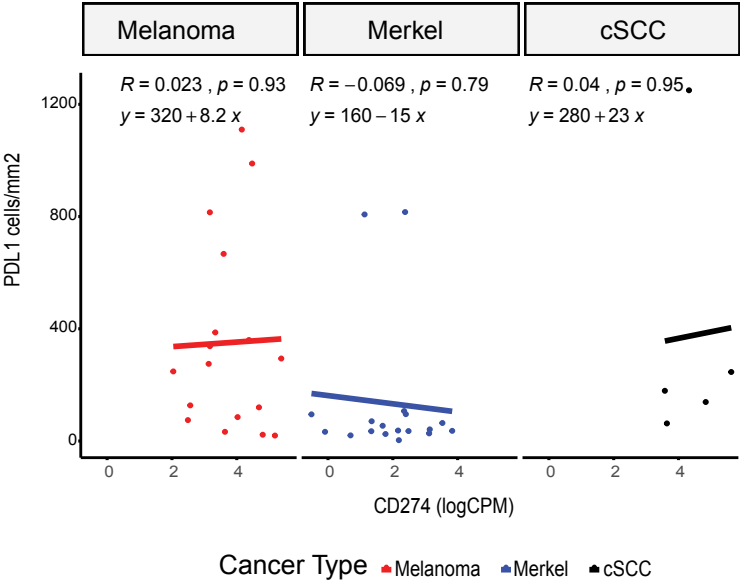


Fig 6.5(B)

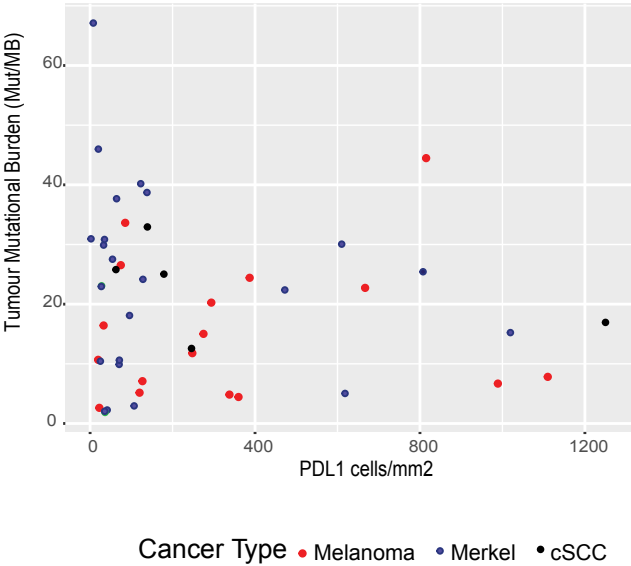


Figure 6.5 (A) Correlation between PD-L1 staining by mIHC and PD-L1 expression by mRNA; (B) Correlation between PD-L1 density by tumour mutational burden

(A) PD-L1 density by mIHC is not significantly correlated to PDL1 mRNA(CD274) gene expression; Spearman correlation, all non-significant.

(B) PD-L1 density is not associated with tumour mutational load derived from bulk RNA-sequencing using FFPE samples. See methods for full details.

Fig 6.6(A)

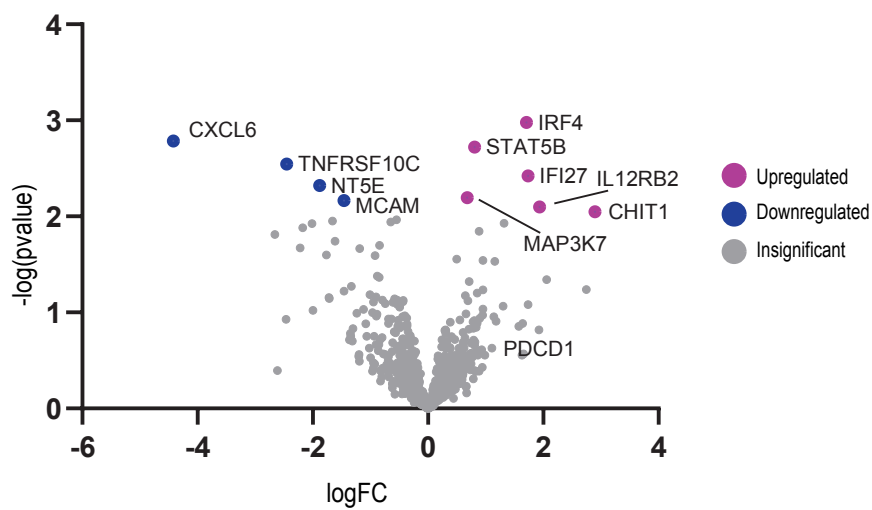


Fig 6.6(B)

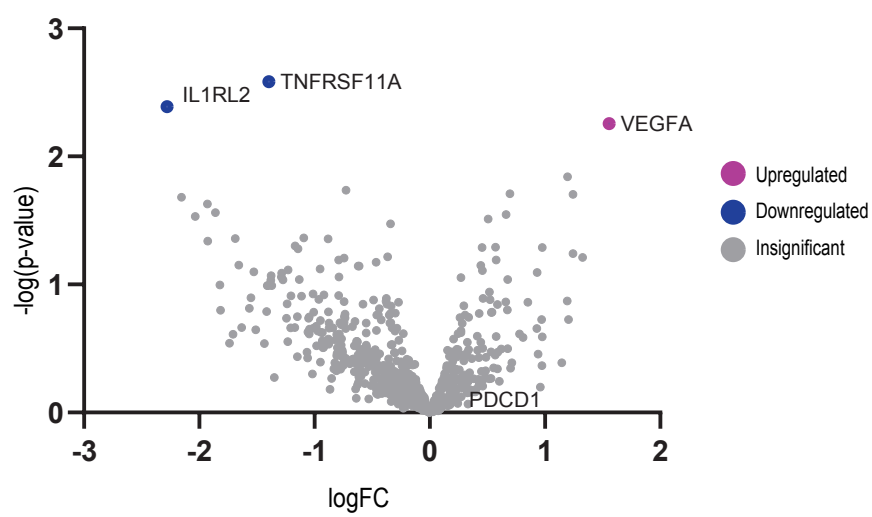


Fig 6.6(C)

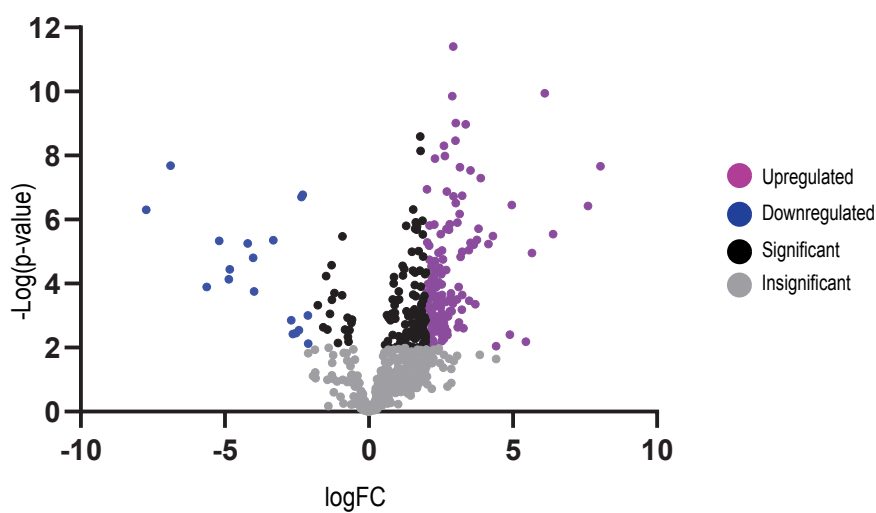


Figure 6.6 Differential gene expression analyses of melanoma (n=17) and Merkel cell carcinoma (MCC, n=19) samples

Differential expression analysis was performed on the melanoma and MCC samples with the *limma-voom* package (390) with the design 0 + condition. Melanoma and MCC samples were dichotomized at the median of the group for PDL1 density to categorise the group into high PD-L1 staining and low PD-L1 staining. Volcano plots were used to visualise genes that were expressed differentially between 1) high and low PD-L1 expressing melanoma, 2) high and low PD-L1 expressing MCC and 3) high PD-L1 expressing melanoma compared to high PD-L1 expressing MCC. Genes with an adjusted *P*-value of <0.01 (based on the moderated t-statistic using the Benjamini-Hochberg (BH) method to control the false discovery rate and a $|\log_2FC| > 2$ were considered significantly differentially expressed.

(A) Volcano plot of genes differentially expressed in high PD-L1 staining melanoma compared to low PD-L1 staining melanoma. Six genes were upregulated (shown in purple) and 4 genes were downregulated (shown in blue). These changes were non-significant after correction for multiple testing using the Benjamini-Hochberg method (false discovery rate, $FDR > 0.01$). PD-L1 is highlighted for completeness but its expression is not significantly different between high and low PD-L1 staining melanoma.

(B) Volcano plot of genes differentially expressed in high PD-L1 staining Merkel compared to low PD-L1 staining Merkel. One gene was upregulated and 2 genes were downregulated, but again these genes were non-significant after correction for multiple testing using the Benjamini-Hochberg method ($FDR > 0.01$). PD-L1 is highlighted for completeness but its expression is not significantly different between high and low PD-L1 staining Merkel.

(C) Volcano plot of genes differentially expressed in high PD-L1 staining melanoma compared to high PD-L1 staining Merkel. 296 genes were significantly expressed ($p < 0.01$), with 134 genes upregulated in melanoma ($\log_2FC > 2$, *purple*) and 15 genes upregulated in Merkel ($\log_2FC < -2$, *blue*).

Fig 6.7(A)

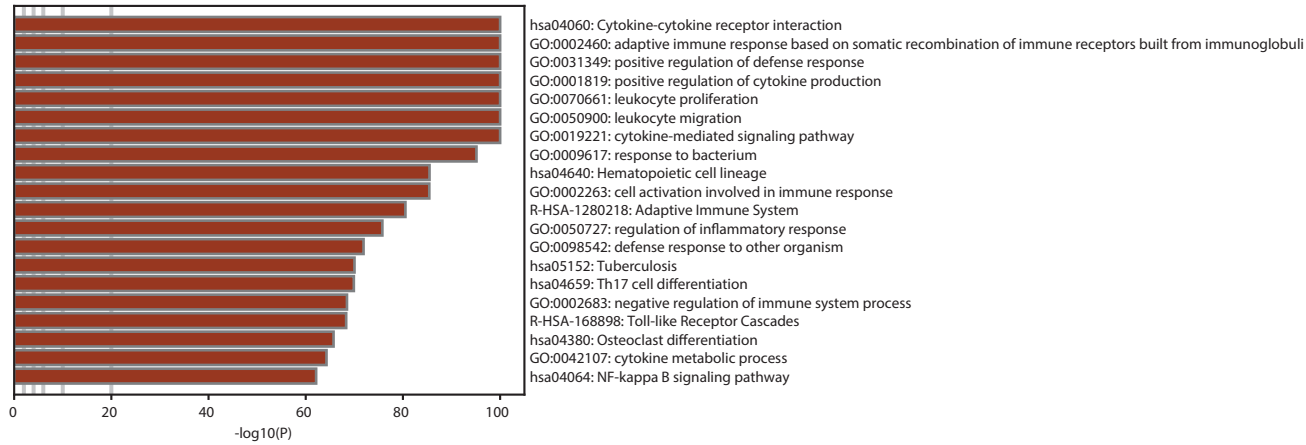


Fig 6.7(B)

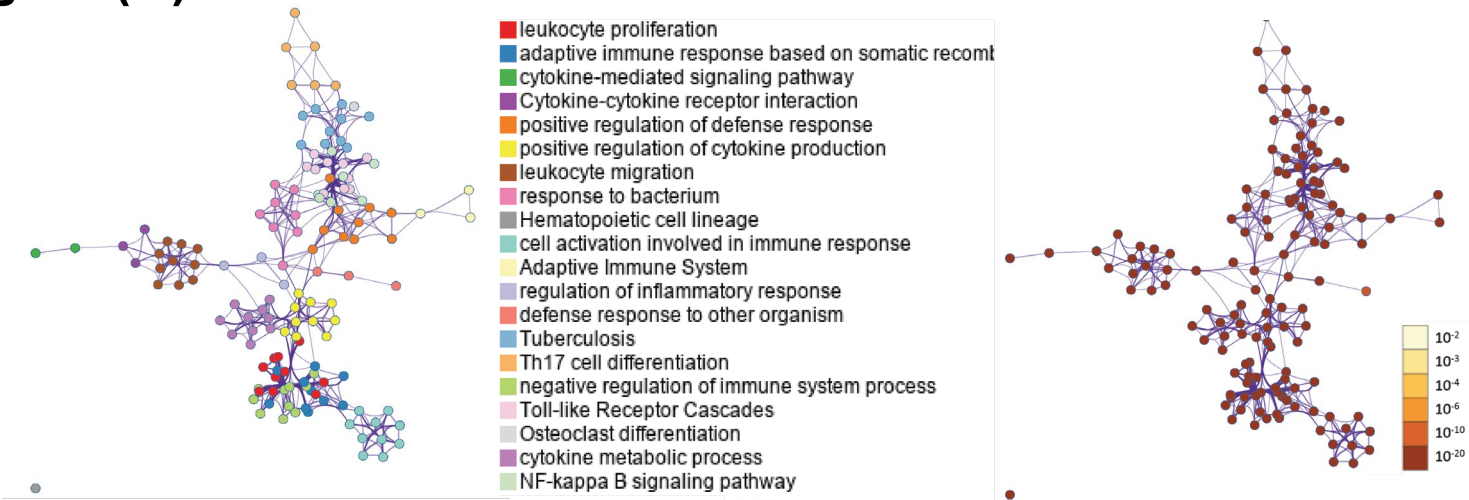


Fig 6.7(C)

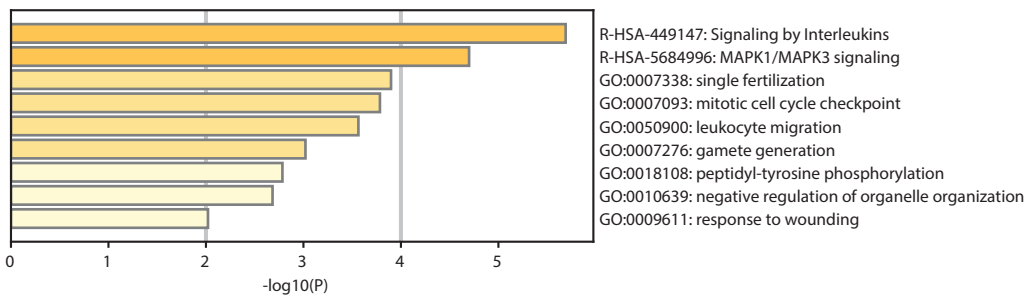


Fig 6.7(D)



Figure 6.7 (A) Enriched GO Biological Processes associated with high-PDL1 melanoma; (B) Network analysis for genes associated with high PD-L1 melanoma; (C) Enriched GO Biological Processes associated with high-PDL1 melanoma; (D) Network analysis for genes associated with high PD-L1 melanoma

Gene set enrichment and network analyses were performed on the genes upregulated in melanoma compared to MCC using the Metascape gene set enrichment analyses program.

(A)The 296 upregulated genes in high PD-L1 staining melanoma were analysed and a bar graph of enriched GO Biological Processes Terms is shown. Genes associated with adaptive immune processes, cytokine signalling and leucocyte proliferation and migration are over-expressed in high PD-L1 staining melanoma samples.

(B)Network of enriched terms with high PD-L1 staining melanoma are coloured by cluster ID (left) and are coloured by p-value (right).

(C)Gene set enrichment and network analyses were performed on the genes downregulated in melanoma compared to MCC using the Metascape gene set enrichment analyses program. The 15 genes in high PD-L1 Merkel were studied using the Metascape Gene Set Enrichment analyses program. Bar graph of enriched Reactome Gene Sets and GO Biological Processes Terms are shown. Genes associated with interleukin signalling, leucocyte migration along with non-immune terms are highlighted.

(D)Network of enriched terms with high PD-L1 staining Merkel are coloured by cluster ID (left) and are coloured by p-value (right).

7 Application of biomarkers: case studies

7.1.1 Introduction

One of the challenges of biomarker discovery and application is how to implement a combination of biomarkers for an individual patient. As highlighted in the literature review [Chapter 1.3], a number of potential biomarkers have been evaluated to predict outcome after ICI for melanoma. However, these biomarkers have mainly been examined individually, and have yet to be examined in combination with each other. Hence, the different determinants of response have not been prioritised in any way or assigned any relative weighting, and it is not known whether a single positive factor can override a number of other negative factors.

Some predictive models have attempted to incorporate a number of different patient characteristics in order to stratify outcome after ICI treatment. A predictive model was proposed for anti-PD-1 using the clinical characteristics of 228 patients treated with pembrolizumab (517). This study found female sex (OR=0.51 (0.27, 0.94), $P=0.03$); age <65 years (OR=0.55 (0.30, 0.98), $P=0.04$); previous ipilimumab treatment (OR=0.38 (0.20, 0.69), $P<0.001$); elevated LDH (OR=0.48 (0.25, 0.90), $P=0.02$) and presence of liver metastasis (OR=0.34 (0.17, 0.66), $P<0.001$) to be significantly associated with worse survival. Five factors were used to develop a 7-point predictive scale: female sex (1 point), age <65 years (1 point), previous ipilimumab treatment (2 points), elevated LDH (1 point), and the presence of liver metastasis (2 points). The proposed scale showed a reasonable performance in predicting response to anti-PD-1: AUC 0.73 (95% CI 0.67, 0.80), which was validated in a cohort of 87 patients (AUC of 0.7). However, this model was not able to accurately predict non-responders (patients predicted to be unlikely to respond still had a response rate of 29%) and the model has yet to be validated in patients outside of the clinical trial setting. Furthermore, this model does not take into account the patient's immune status or the tumoural immune-profile. Lastly, anti-PD-1 as monotherapy or in combination with anti-CTLA4 is now the standard of care treatment in the first line setting for metastatic melanoma, so few contemporary patients would have a prior use of ipilimumab monotherapy.

On the other hand, other predictive models consider *only* the tumoural immune profile without considering patient demographics or disease characteristics. For example, the Omniseq Immune Report Card is a response score based on a combination of tumour mutational burden, CD8+ T cell infiltration, gene expression profiles for PD-L1 and CD8, as well as an immune transcriptomic signature(518). In this study, the composite Response Score (RS) was evaluated in 48 patients with metastatic melanoma treated with ICI, and validated on a cohort of 29 patients from different institutions. The Response Score was associated with higher sensitivity but similar specificity in predicting tumour response than either PD-L1 IHC (28-8 assay) or tumour mutational burden alone. Using the proposed threshold of $RS \geq 50$, patients had significantly improved OS and ORR of 82.9% as compared to 23.8% for patients with $RS < 50$ ($p=0.0012$). While this study highlights the potential to study the different tumoural immune factors in concert, the reasonably high tumoural response rate in patients with a low RS does not help to exclude patients for ICI treatment. These predictive scores also need to be prospectively validated in clinical studies to understand how they can be used in conjunction with other patient and disease characteristics.

Blank and colleagues proposed the ‘cancer immunogram’ (376), a framework by which different biomarkers characterising the patient’s immune status and tumoural immune profile can be considered. Regarding the patient’s immune status, there are 3 different domains of general immune status (as measured by lymphocyte count), soluble inhibitors (IL6 and CRP), and inhibitory metabolism (LDH, glucose utilisation). Four other domains are included when considering the tumoural determinants of ICI effectiveness: immune cell infiltration (intratumoural T cells), checkpoint expression (PD-L1), tumour foreignness (mutational load) and tumoural sensitivity to immune effectors (MHC expression and IFN- γ sensitivity). Together, this framework highlights the importance of considering the different components of immune activation, in order to understand possible mechanisms of response as well as potential mechanisms of resistance.

The ‘cancer immunogram’ is expanded to assess the biomarkers explored in this PhD thesis, as in Figure 7.1. Clinical factors to be considered include pre-treatment performance status and presence of brain metastases, as highlighted in chapters 3 and 4 in this PhD thesis. Functional FDG PET biomarkers can also be considered, such as raised spleen to liver ratio (SLR) or metabolic tumour volume (MTV) [Chapter 5].

Although a significant threshold to stratify overall survival using metabolic tumour volume on pre-treatment FDG PET scans were not found in chapter 5, a number of other studies support a threshold of approximately 25ml to stratify patients into low and high tumour burden(458, 459). In terms of routine laboratory tests, LDH is a readily available biomarker and elevated LDH has remained an important indicator of poor survival after ICI treatment. Markers of generalised inflammation could also be considered, given the potential association of inflammation and poor prognosis as described in this thesis, with high CRP in brain metastases, as well as low albumin in patients with high SLR. A number of studies have also evaluated high neutrophil: lymphocyte ratio (NLR)>5 to be associated with inflammation and poor outcome after ICI. Lastly, the study of pre-treatment tumoural biopsies using the novel multiplexed immunohistochemistry platform can provide a more in-depth assessment of the tumoural immune landscape, assessing not only the intratumoural T cell subsets (CD3+CD4-CD8- unconventional T cells, CD4+ helper T cells, CD8+ cytotoxic T cells, FOXP3+ regulatory T cells) but also other immune cells within the tumour microenvironment such as CD68 macrophages, CD11c dendritic cells or CD20 B cells. The mIHC platform can also evaluate PD-L1 expression in more detail than tumoural PD-L1 expression alone: it can provide the proportion of tumour cells that stain positively for PD-L1, as well as the relative proportions of tumour cells compared to immune cells which stain positively for PD-L1.

In this chapter, case studies of six patients with advanced melanoma treated with ICI (ipilimumab or anti-PD-1) treatments are presented to demonstrate the application of comprehensive pre-treatment biomarker sampling.

7.1.2 Aims

To assess the application of a combination of clinical factors, peripheral blood tests, FDG PET imaging, peripheral cytokine profiling and tumoural mIHC on a cohort of patients with advanced melanoma treated with ICI

7.1.3 Results

Patients with advanced melanoma who were treated at Peter MacCallum Cancer Centre were offered the option of participating in a voluntary biomarker research study, whereby their clinical, imaging, blood and tissue samples were stored for research.

Patient demographics, disease characteristics, treatment history, outcomes and pre-treatment biomarkers are presented in Table 7.1. The median age of the cohort was 61 (range 45-70 years). All patients had stage IV cutaneous melanoma and only one patient (Patient A) had known brain metastases at the time of commencing ICI treatment.

Four patients (Patients A, D, E, F) were treated with ipilimumab monotherapy, followed by anti-PD-1 monotherapy upon progression. Two patients only received one line of ICI treatment: Patient B only received ipilimumab monotherapy, while Patient C received only anti-PD-1 monotherapy. The patient treatment histories are presented in full in the Appendix A.1. One patient had an objective response to ipilimumab, but this response was not durable (5 months). Complete response was observed in 2 of the 5 patients treated with anti-PD-1. There was one patient (patient B) who experienced a significant treatment related toxicity of colitis and hypophysitis after the cessation of his treatment for progressive disease.

Regarding baseline routine laboratory biomarkers, pre-treatment LDH was elevated in majority of cases (6 of 7), while two patients did not have a pre-treatment LDH documented. While LDH is well known to be a poor prognostic marker, the two complete responses occurred in patients with elevated LDH. Progressive disease was associated with 2 of the 3 cases with neutrophil: lymphocyte ratio > 5. NLR>5 did not always co-occur with lymphopenia (absolute lymphocyte count, ALC, less than 1), but was associated with low albumin (<30g/L) in 2 of 3 cases. Two patients had lymphopenia (ALC<1) prior to their treatment with ipilimumab; one of these patients had progressive disease while the other experienced partial response.

Seven of the nine ICI treatments had an evaluable FDG PET scan prior to commencing therapy. The majority (7 of 9 cases) had metabolic tumour volume over the threshold of 25mL. In the two patients with very low volume disease, one patient experienced stable disease following ipilimumab, while the other had complete response to the anti-PD-1 treatment. Interestingly, the patient with a large volume of disease (1560mL) experienced a complete response following his anti-PD-1 treatment, suggesting that large volume of tumour burden may not negate the possibility of tumour response.

Seven of the nine ICI treatments had a pre-treatment biopsy for mIHC examination with both the T-cell panel and the pan-immune panel to study T cell subsets and immune

cells respectively. Interestingly, all 3 patients with CD68 macrophage as their predominant immune cell type (i.e. >50% immune cells were CD68 cells) experienced progressive disease to their ICI treatment, as did the patient with predominantly CD11c dendritic cells. On the other hand, the two patients who experienced complete response to anti-PD-1 treatment had T cells as their predominant immune cell type. The majority of patients had CD4 helper cells as their predominant (>50%) T cell subset and this was not particularly discriminating between responders and non-responders to either ICI. PD-L1 density was evaluated in our study using the SP142 assay, and the proportion of tumour cells which stained PD-L1 are presented: the majority of cases had a very low proportion of tumour cells expressing PD-L1+ staining. The ratio of PD-L1 staining on tumour cells compared to immune cells was explored and four of the five patients had PD-L1 expression predominantly on tumour cells, rather than immune cells.

The prognostic factors could be visualised in a modified ‘cancer immunogram’ using a traffic-light coloured schema, such as in Figure 7.2. Such a diagram could incorporate prognostic factors at particular thresholds to indicate good, intermediate or poor prognosis (presented as green, orange and red respectively) to allow for an overview of the different factors together. However, systematic review of these prognostic factors in prospective ICI studies involving large number of patients would be needed to establish the optimal thresholds for survival and toxicity.

Table 7.1 Patient demographics, disease characteristics, treatment outcome and biomarkers

Pt	Age	BRAF Mut	Treatment					Clinical biomarkers		Blood tests				FDG PET Imaging		Pre-treatment mIHC			
			Line of systemic treatment	ICI Treatment	Best tumoural response	Duration of response (months)	G3/4 Toxicity	PS	Presence of brain metastases	LDH >ULN [#]	NLR	ALC	Alb	MTV	SLR	Pre-dominant Immune cell type	Pre-dominant T cell subset	PD-L1+ TC (%)	PD-L1+ (TC: IC %)
A	45	V600E	2	Ipilimumab	PR	5	No	1	Yes	NR	9.4	0.5	40	NR	NR	NR	NR	NR	NR
			3	Anti-PD-1	PD	1	No	2	Yes	Yes	14.0	1.14	28	223	0.78	CD68 Macrophage	CD4	2%	90%
B	59	Wt	1	Ipilimumab	SD	8	Yes Colitis, hypophysitis	0	No	Yes	1.8	1.53	44	1.73	0.82	CD3 T cells	CD8	4%	65%
C	55	V600E	2	Anti-PD-1	PD	1	No	1	No	NR	5	1.66	36	NR	NR	CD68 Macrophage	CD4	1%	2%
D	70	Wt	2	Anti-PD-1	PD	2	No	NR	No	Yes	3.71	1.82	32	153	0.90	CD11c Dendritic cells	CD4	2%	30%
E	66	Wt	1	Ipilimumab	PD	3	No	0	No	Yes	2.5	1.61	42	541	0.76	NR	NR	NR	NR
			2	Anti-PD-1*	CR	60+	No	1	No	Yes	3.5	1.06	42	1560	0.82	CD3 T cells	CD4	1%	79%

F	62	V600E	2	Ipilimumab	PD	3	No	1	No	No	9.6	0.85	31	64	1.22	CD68 Macroph -ages	CD4	NR	NR
			3	Anti-PD-1	CR	60+	No	1	No	Yes	4.8	1.88	35	1	0.95	CD3 T cells	CD4	NR	NR

Pt, patient; BRAF Mut, BRAF mutation; FDG PET, Fluoro-deoxyglucose positron emission tomography; ICI, immune checkpoint inhibitor; G3/4, CTCAE Grade 3 or 4 toxicity; PS, Performance Status; LDH>ULN, Lactate dehydrogenase greater than upper limit of normal; NLR, Neutrophil: Lymphocyte Ratio; ALC, absolute lymphocyte ratio; Alb, Albumin; MTV, metabolic tumour volume; SLR, spleen to liver ratio; PR, partial response; PD, progressive disease; SD, stable disease; NR, not recorded; Wt, Wildtype. PD-L1+ TC, PD-L1+ Tumour cells (%) represents the proportion of tumour cells which stain positively for PD-L1. PD-L1+ (TC: IC %) represents the proportion of PD-L1+ cells that are tumour cells, compared to immune cells.

#All patients with elevated LDH were less than 2 times upper limit of normal.

*Patient E also received denosumab, an anti-RANKL monoclonal antibody treatment for bone metastases.

For pre-treatment mIHC, the predominant immune cell type (out of CD3 T cells, CD68 macrophages, CD11c dendritic cells or CD20 B cells) and the predominant T cell subset (out of CD3+CD4-CD8- unconventional T cells, CD4+ helper T cells, CD8+ cytotoxic T cells, or FOXP3+ regulatory T cells) are presented. Tumoural PD-L1 mIHC expression is denoted as the proportion of intra-tumoural tumour cells that stain positively for PD-L1 (presented as a %).

7.1.4 Discussion

While it is important to have a framework such as the ‘cancer immunogram’ to consider the different determinants of response to ICI, there are several challenges when using such a framework to consider biomarkers for an individual patient. Although these case studies only described a very small number of patients, there are some interesting observations worthy of further investigation.

Clinical factors

Patient A highlights the poor clinical prognostic impact of poor baseline performance status and presence of brain metastases. Prior to her anti-PD-1 treatment, she also had systemic markers of inflammation with high NLR and low albumin. Not surprisingly, she quickly deteriorated after the initiation of the anti-PD-1 treatment. As Chapter 3 has described, patients with poor baseline performance status have a lower likelihood of response to anti-PD-1 treatment. The remaining patients in this cohort did not have brain metastases prior to commencing ICI treatment and the pre-treatment performance status was ECOG 0 or 1.

Blood parameters

The majority of patients had elevated LDH in this case series. LDH is a well-known prognostic marker in melanoma, and remains as an important prognostic marker in the setting of anti-PD-1 treated patients based on post-hoc analyses of clinical trial data . However, durable responses are still seen in patients with elevated LDH in the clinical trials and therefore cannot be used as a discriminatory marker for ICI treatment. From our small cohort, the three patients who had other markers of inflammation ($\text{NLR} \geq 5$, $\text{albumin} < 34\text{g/L}$) in addition to elevated LDH had progressive disease and short survival. Further validation of the prognostic value of these three blood markers combined as a composite factor, compared to any individual marker alone, is worthy of exploration in larger cohorts.

PET imaging

In this study there was one patient with raised spleen to liver ratio (SLR) > 1.1 and indeed this patient had a short duration of response to ipilimumab treatment. However,

it is interesting that she had a subsequent complete response to anti-PD-1 treatment. This raises the possibility of whether biomarkers are context dependent, and indeed some biomarkers such as tumour mutation burden, have only been shown to be prognostic in anti-PD-1 treated patients who are ipilimumab naïve (331). Furthermore, ipilimumab monotherapy has been replaced by either combination ipilimumab/nivolumab or anti-PD-1 monotherapy as standard of care treatment in advanced melanoma. Therefore, the more important question regarding high SLR is whether it is still prognostic in patients treated with combination ICI or anti-PD-1 monotherapy.

We were not able to determine a significant threshold for metabolic tumour volume in our study presented in Chapter 5 of this thesis, although others have shown that $MTV < 25\text{mL}$ is associated with good prognosis (458, 459). However, Patient E had an exceptionally large tumour burden of 1.5L of metabolically avid tumour on FDG PET in addition to elevated LDH prior to the commencement of anti-PD-1 treatment. Despite these poor prognostic factors, complete resolution of tumour was seen in association with durable response (the patient was last seen at 5 years after the commencement of treatment). It is noteworthy that he also received anti-RANK-L monoclonal antibody for the treatment of his bony metastases and interesting to hypothesise that this additional treatment was sufficient to overcome the poor prognostic factors.

Pre-treatment biopsy

The two mIHC panel enabled the study of not only the T cell compartment but other immune cell types. Interestingly, there were three cases with a predominantly CD68 immune infiltrate within the tumour in non-responders to ICI. In all three cases, patients had both high tumour burden as well as other markers of generalised inflammation (i.e. high NLR and low albumin). Previous biomarker series have highlighted the importance of CD8 cells and PD-L1 cells at the invasive margin in predicting response, but have not found CD68 cells to be significant (296). Given the development of mIHC technologies to evaluate multiple immune panels concurrently on the same slide, it would be of interest to incorporate CD68 density into future studies. Furthermore, it is also not known whether high NLR could be a surrogate for high tumoural myeloid infiltration; this may be important to evaluate in future studies.

Patient D's pre-antiPD-1 pre-treatment biopsy comprised mostly of CD11c dendritic cells, with relatively few T cells. The significance of high dendritic cell density is not known. A study recently highlighted the importance of dendritic cells for anti-PD-L1 function: insofar as this therapy unleashed PD-L1- and CD80- expressing DC to promote an anti-tumour effect (519). However, it is not known from that study whether anti-PD-1 would have the same effect, or whether the application of anti-PD-1 into a DC rich environment could escalate tumour progression as seen in Patient D. Another clinical question would be whether DC rich disease would benefit more from anti-PD-L1 therapy instead of anti-PD-1 therapy. Currently anti-PD-L1 agents are not used in melanoma but this may be because therapies to date have not been rationally selected depending on the immune cell types present. Improved understanding of the mechanisms of response may see a shift to a more rational selection of therapy dependent on the immune cell types present in pre-treatment biopsies.

One of the limitations of this patient case series is the lack of PD-L1 testing using a validated assay for melanoma. There are four validated companion diagnostic assays in clinical practice: PD-L1 28-8 assay for nivolumab, 22C3 for pembrolizumab, SP142 for atezolizumab and SP263 for durvalumab. The SP142 assay was used as part of the multiplexed immunohistochemistry panel for our patient cohort study, and this assay is not one approved for clinical use in melanoma (i.e. PD-L1 28-8 and 22C3). There were initial concerns regarding the SP142 assay in lung cancer (310), although this was not observed in a subsequent study in melanoma specimens, where five PD-L1 assays showed comparable staining (312). Though it must be noted that despite the approval of PD-L1 28-8 and 22C3 assays for stratification of patients in clinical trials for melanoma, these two assays have not been used for routine clinical decision making. This is largely owing to the fact that the patients with PD-L1 negative tumours can still derive clinical benefit from treatment. These two assays only assess PD-L1 staining on tumour cells, and the importance of PD-L1 expression on immune cells is increasingly recognised. While we have sub-categorised PD-L1 expression into tumoural (PD-L1+SOX10+) or immune cell expression (PD+CD3+), the implications of the relative expression on tumoural response is not yet known. Secondly, PD-L1 expression on other immune cells such as the myeloid and dendritic cell compartment has been ignored. Future studies of PD-L1 in these latter immune cell compartments will be

important to further the understanding of how ICI achieve tumoural responses, and whether PD-L1 positivity of these other immune cells can also be prognostic.

Summary

Using these illustrative case studies, we have explored the clinical, blood, FDG PET imaging and pre-treatment immune profiling biomarkers for ICI. Some of these cases echo the factors described in other chapters of this thesis such as performance status, inflammatory markers and FDG PET biomarkers. The pre-treatment mIHC uses a pan-immune and T cell panel to study the different immune infiltrates as an evaluation of the tumoural immune microenvironment. While potential biomarkers have been investigated as single components, these case studies highlight the possibility of achieving tumour response despite multiple negative prognostic factors. As these biomarkers have been investigated separately in the literature, it is not currently possible to give them weightings to incorporate into a composite model. Understanding how these multi-factorial biomarkers can be considered together will be crucial in future studies.

7.1.5 Conclusion

Comprehensive immune profiling of both patient and tumoural immune factors provides a framework to consider the various determinants of immune response. In this case study of 6 patients, multi-factorial biomarkers have been explored in relation to their ICI treatment outcomes. Future prospective studies of multi-factorial biomarker models will be required in order to establish relative weightings of each biomarker.

7.1.6 Figures

Modified Immunogram for melanoma immune checkpoint inhibitors

Clinical factors

Performance status

Brain metastases

General immune status

Neutrophil to Lymphocyte Ratio

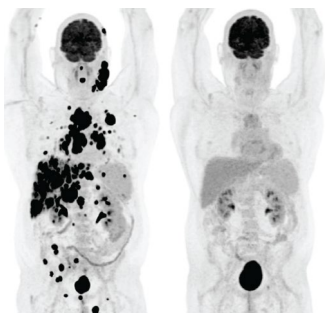
Absolute Lymphocyte Count

Albumin

Functional Imaging

Spleen to Liver Ratio

Metabolic Tumour Volume



Absence of inhibitory tumour metabolism

LDH

Absence of soluble inhibitors

IL-6, CRP

Immune cell infiltration

Predominant T cells subset

Predominant immune subset:

*T cells, myeloid cells, B cells,
dendritic cells*



CD11C
CD20
CD3
CD68



CD3
CD4
CD8
FOXP3



PDL1+CD3+
PDL1+SOX10+

Absence of checkpoints

PD-L1

*Proportion of PD-L1 on tumour cells
vs immune cells*

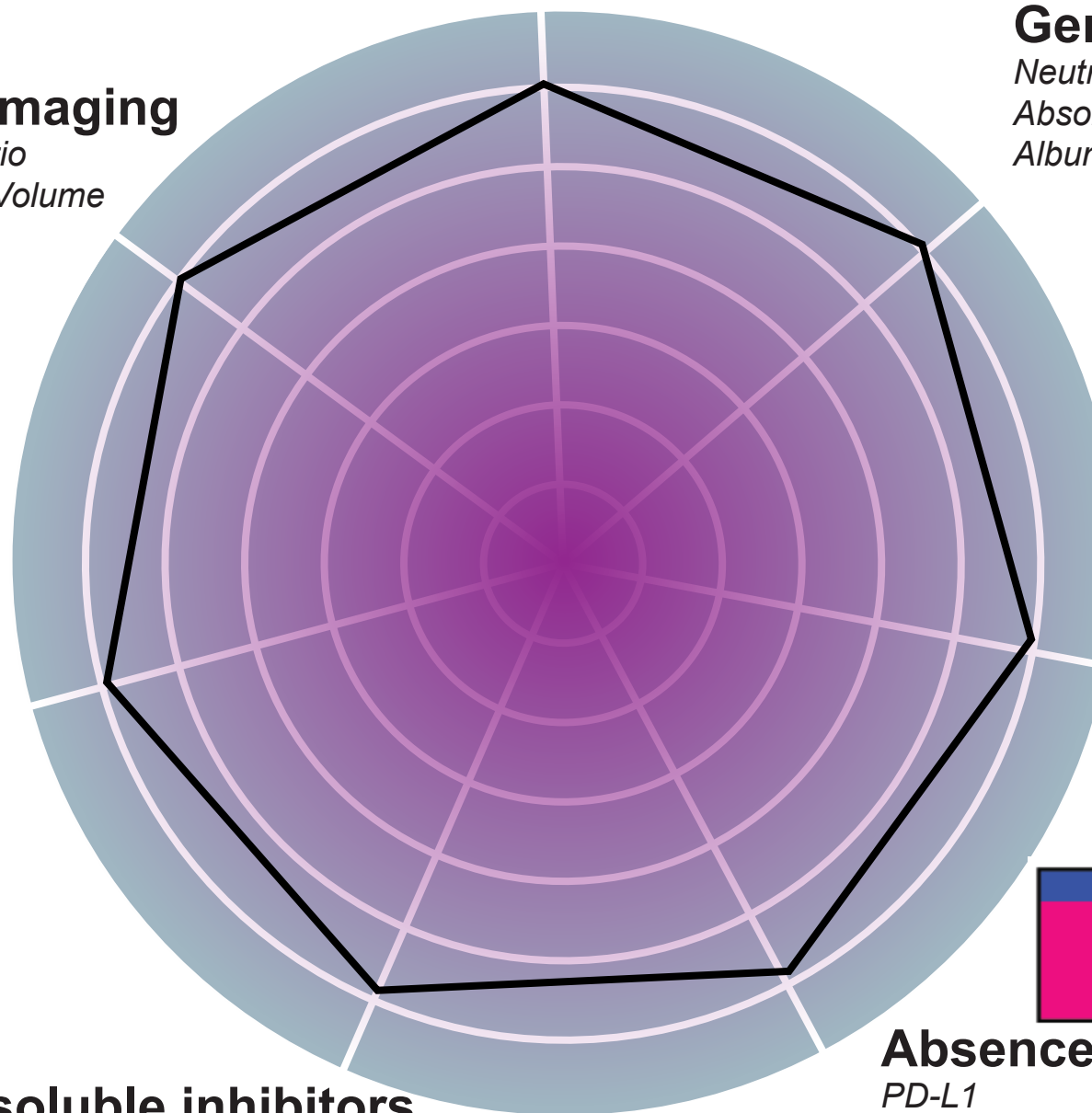


Figure 7.1 Modified Immunogram for immune checkpoint inhibitors for advanced melanoma

The immunogram proposed by Blank and colleagues (376) can be modified to incorporate the prognostic factors explored in this thesis as well as the more established biomarkers for immune checkpoint inhibitors in advanced melanoma. Clinical biomarkers of performance status and presence of brain metastases can be incorporated as well as blood based biomarkers such as neutrophil to lymphocyte, absolute lymphocyte count, albumin, serum IL-6, CRP and LDH. Functional FDG PET CT biomarkers such as spleen to liver ratio and metabolic tumour volume can also be considered. Pre-treatment tumour biopsy can also be examined for predominant intratumoural T cell subset, as well as ratio of PD-L1 expression on tumour cells or immune cells. These factors can be explored visually in concert in a multi-factorial schema, rather than as single components.

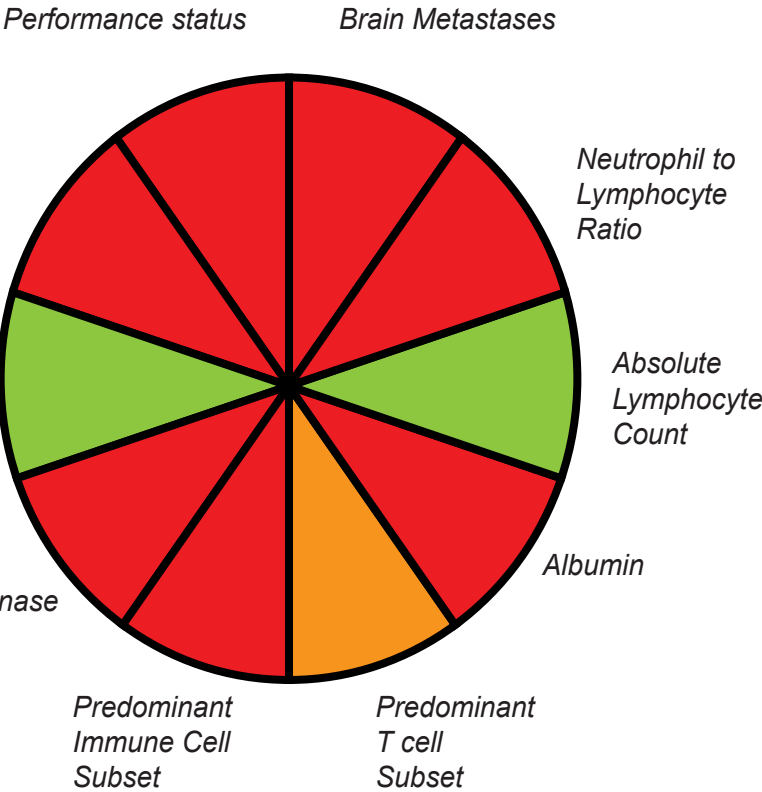
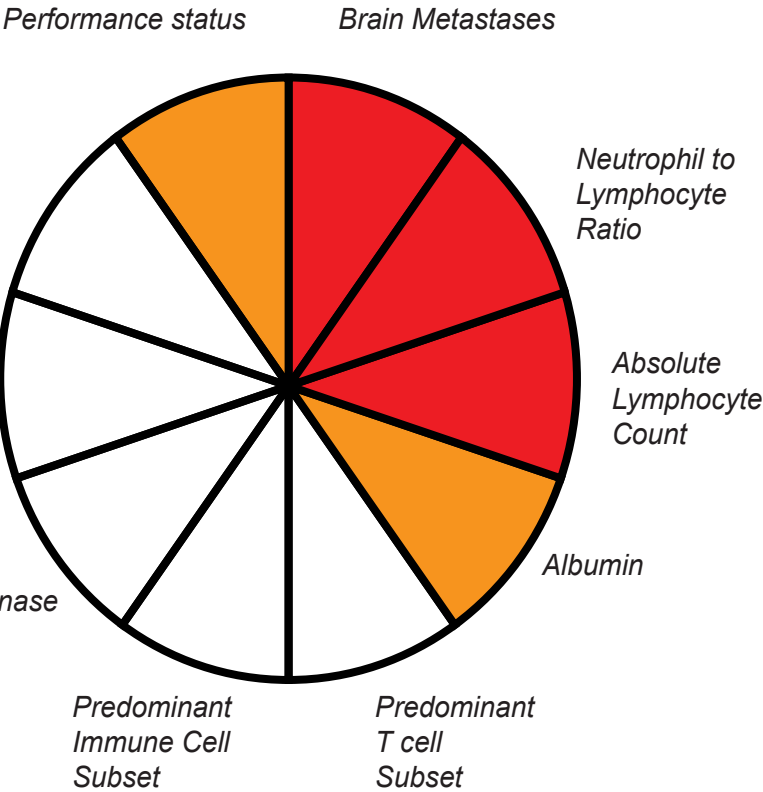
Figure 7.2 Modified immunograms for patients in Chapter 7

The modified immunograms are presented for the patients described in Chapter 7. The prognostic factors can also be presented visually using a 'traffic light' system of red, orange and green to indicate whether the factors are associated with poor, intermediate or good prognosis respectively. PD-L1 density was not included in this schema as the optimal threshold for this factor has not been explored.

Patient A

Pre-ipilimumab:
partial response

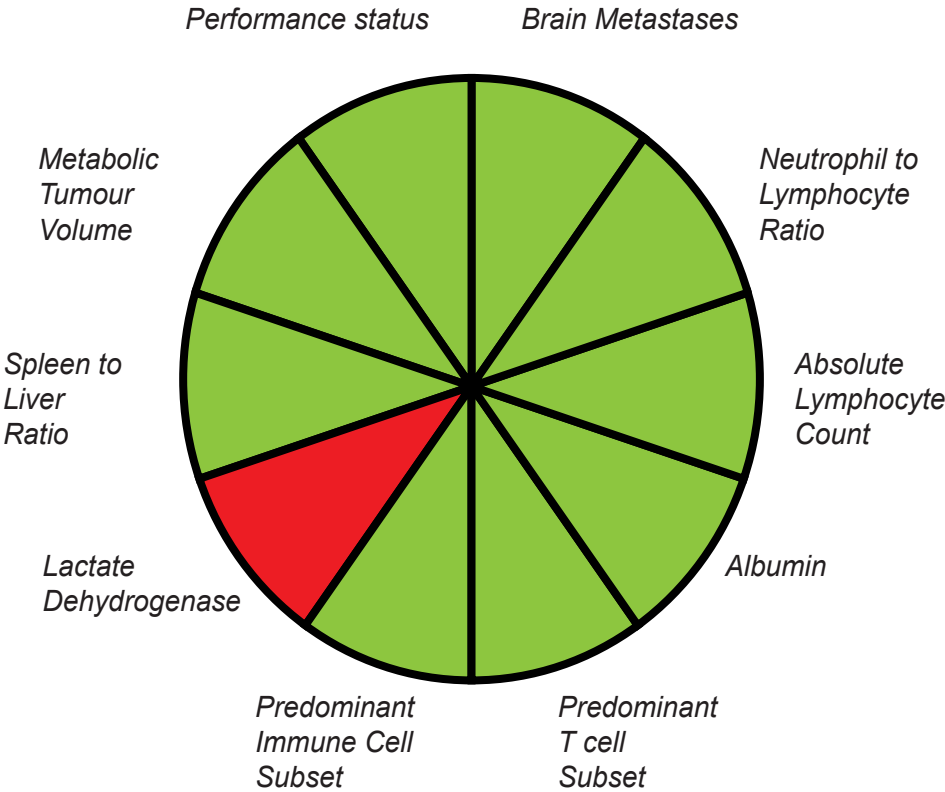
Pre-anti-PD-1:
progressive disease



Prognostic factors	Poor	Intermediate	Good
Performance status	≥2	1	0
Brain Metastases	Yes		No
Neutrophil to Lymphocyte Ratio	>5		≤ 5
Metabolic Tumour Volume	>25mL		≤ 5mL
Absolute Lymphocyte Count	<1.0		≥ 1.0
Spleen to Liver Ratio	>1.1		≤ 1.1
Lactate Dehydrogenase	>ULN		≤ ULN
Albumin	<30g/L	<LLN - 30g/L	>LLN
Predominant T cell	FOXP3	CD4	CD8
Predominant Immune Cell	Macrophages	Dendritic cells	T cells

Patient B

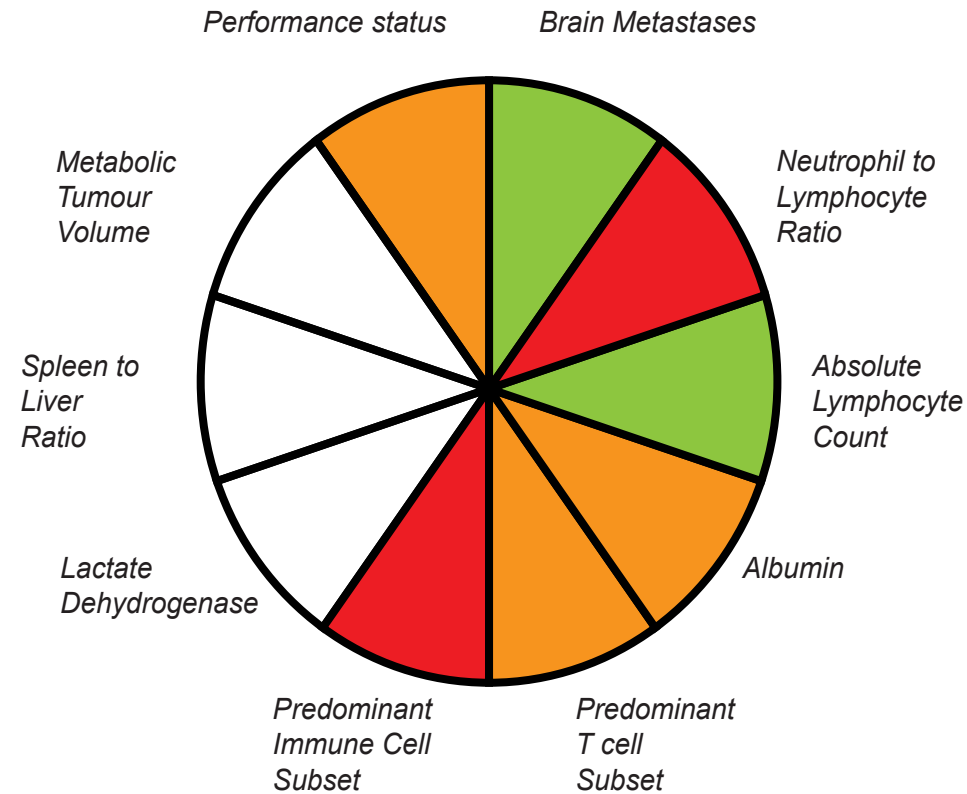
Pre-ipilimumab:
stable disease



Prognostic factors	Poor	Intermediate	Good
Performance status	≥2	1	0
Brain Metastases	Yes		No
Neutrophil to Lymphocyte Ratio	>5		≤ 5
Metabolic Tumour Volume	>25mL		≤ 5mL
Absolute Lymphocyte Count	<1.0		≥ 1.0
Spleen to Liver Ratio	>1.1		≤ 1.1
Lactate Dehydrogenase	>ULN		≤ ULN
Albumin	<30g/L	<LLN - 30g/L	>LLN
Predominant T cell	FOXP3	CD4	CD8
Predominant Immune Cell	Macrophages	Dendritic cells	T cells
	Not recorded		

Patient C

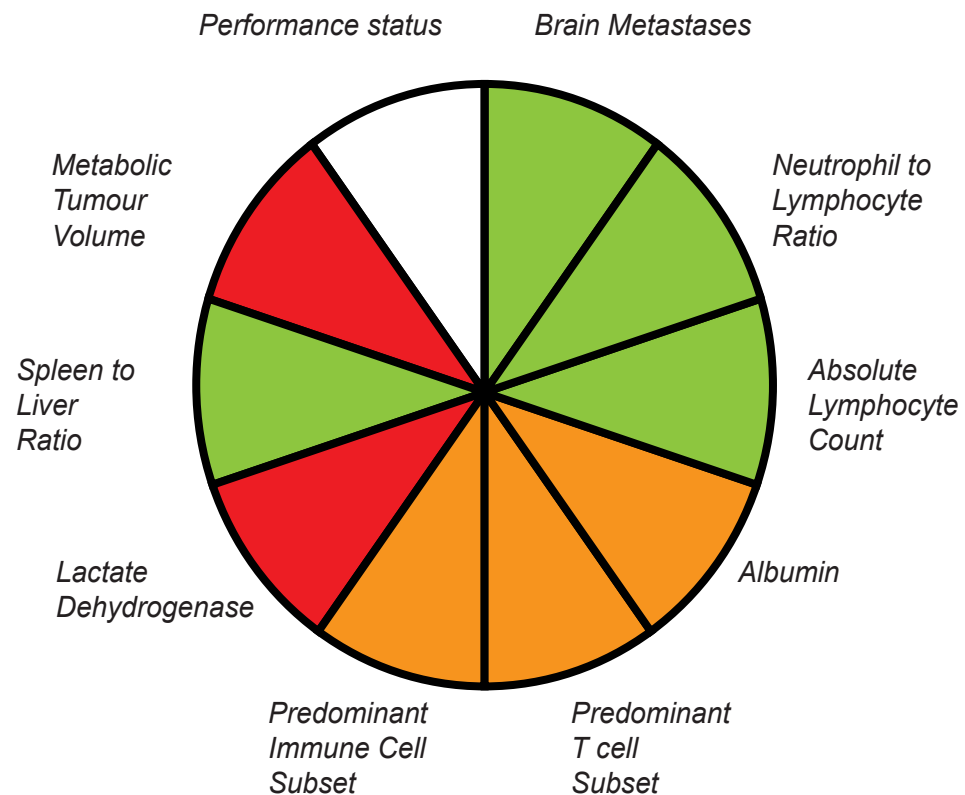
Pre-anti-PD-1:
progressive disease



Prognostic factors	Poor	Intermediate	Good
Performance status	≥2	1	0
Brain Metastases	Yes		No
Neutrophil to Lymphocyte Ratio	>5		≤ 5
Metabolic Tumour Volume	>25mL		≤ 5mL
Absolute Lymphocyte Count	<1.0		≥ 1.0
Spleen to Liver Ratio	>1.1		≤ 1.1
Lactate Dehydrogenase	>ULN		≤ ULN
Albumin	<30g/L	<LLN - 30g/L	>LLN
Predominant T cell	FOXP3	CD4	CD8
Predominant Immune Cell	Macrophages	Dendritic cells	T cells

Patient D

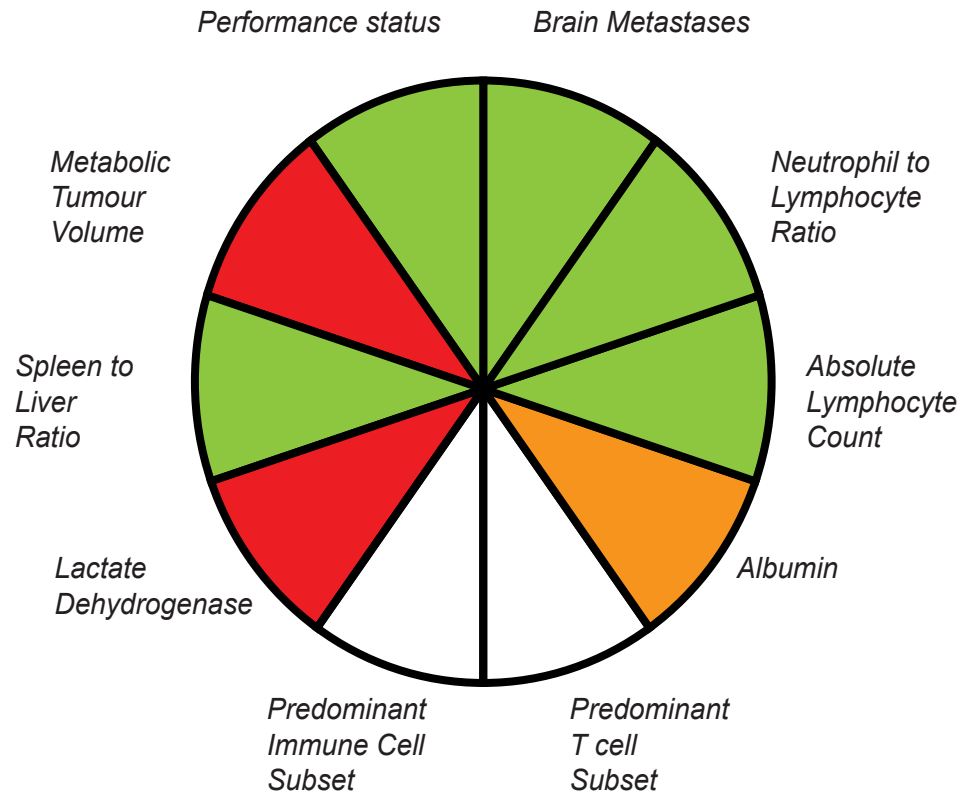
Pre-anti-PD-1:
progressive disease



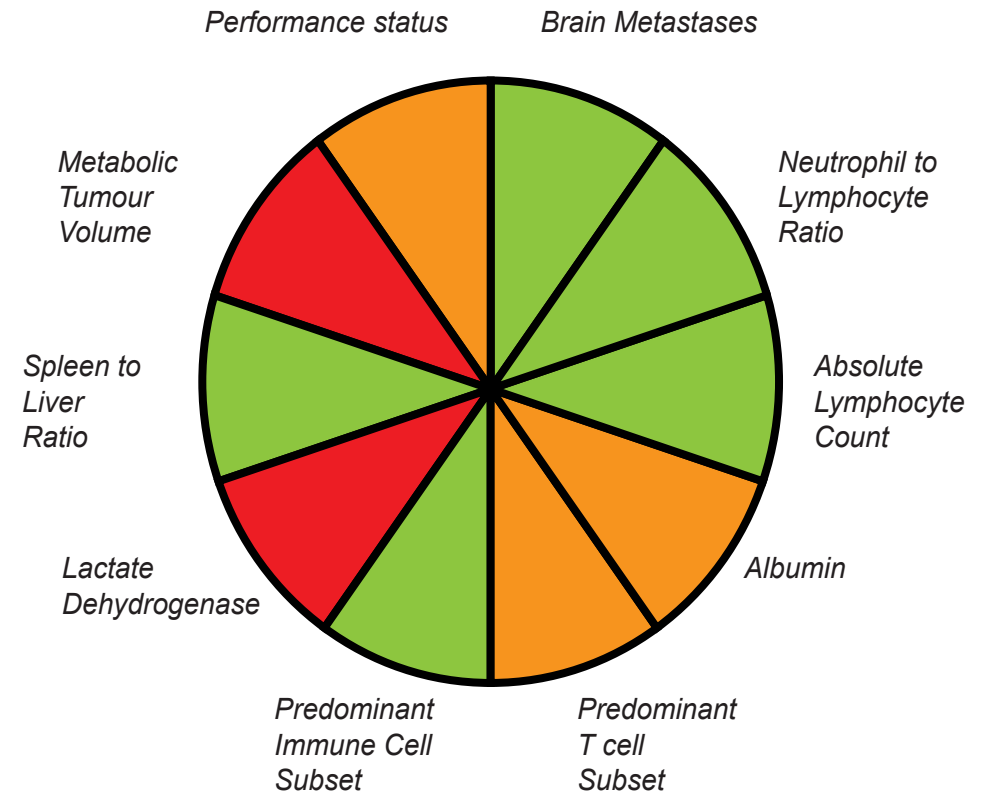
Prognostic factors	Poor	Intermediate	Good
Performance status	≥2	1	0
Brain Metastases	Yes		No
Neutrophil to Lymphocyte Ratio	>5		≤ 5
Metabolic Tumour Volume	>25mL		≤ 5mL
Absolute Lymphocyte Count	<1.0		≥ 1.0
Spleen to Liver Ratio	>1.1		≤ 1.1
Lactate Dehydrogenase	>ULN		≤ ULN
Albumin	<30g/L	<LLN - 30g/L	>LLN
Predominant T cell	FOXP3	CD4	CD8
Predominant Immune Cell	Macrophages	Dendritic cells	T cells

Patient E

Pre-ipilimumab:
progressive disease



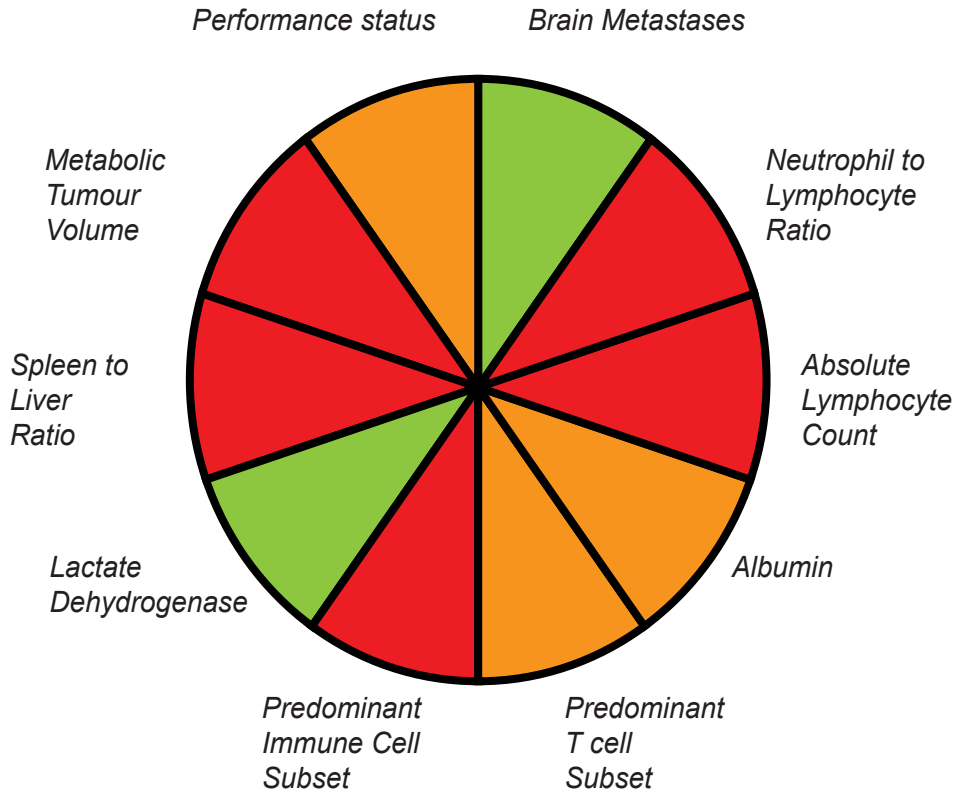
Pre-anti-PD-1:
complete response



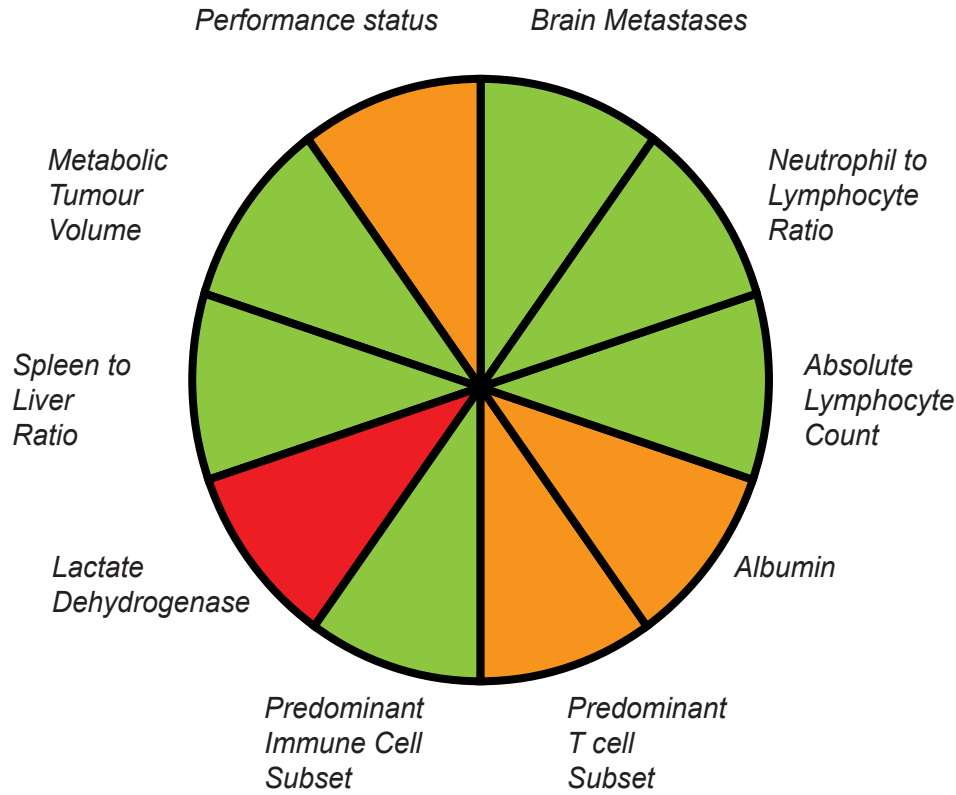
Prognostic factors	Poor	Intermediate	Good
Performance status	≥2	1	0
Brain Metastases	Yes		No
Neutrophil to Lymphocyte Ratio	>5		≤ 5
Metabolic Tumour Volume	>25mL		≤ 5mL
Absolute Lymphocyte Count	<1.0		≥ 1.0
Spleen to Liver Ratio	>1.1		≤ 1.1
Lactate Dehydrogenase	>ULN		≤ ULN
Albumin	<30g/L	<LLN - 30g/L	>LLN
Predominant T cell	FOXP3	CD4	CD8
Predominant Immune Cell	Macrophages	Dendritic cells	T cells

Patient F

Pre-ipilimumab:
progressive disease



Pre-anti-PD-1:
complete response



Prognostic factors	Poor	Intermediate	Good
Performance status	≥2	1	0
Brain Metastases	Yes		No
Neutrophil to Lymphocyte Ratio	>5		≤ 5
Metabolic Tumour Volume	>25mL		≤ 5mL
Absolute Lymphocyte Count	<1.0		≥ 1.0
Spleen to Liver Ratio	>1.1		≤ 1.1
Lactate Dehydrogenase	>ULN		≤ ULN
Albumin	<30g/L	<LLN - 30g/L	>LLN
Predominant T cell	FOXP3	CD4	CD8
Predominant Immune Cell	Macrophages	Dendritic cells	T cells

8 Conclusions

8.1 Discussion

Over the past decade, immune checkpoint inhibitors have dramatically transformed the treatment landscape for metastatic melanoma. However, only a subset of patients enjoy a durable response, coupled with potential serious treatment related toxicities and substantial financial cost. Therefore, biomarkers are urgently needed to aid treatment selection. This thesis explored a range of potential biomarkers including performance status (PS), biomarkers in melanoma brain metastases, functional PET imaging biomarkers and an in-depth tumoural immune-profiling using novel technologies.

Clinical biomarkers

Clinical biomarkers such as performance status are fundamental in the prognostication of patients with melanoma. Our retrospective study showed that poor PS remains significantly associated with poor survival and low treatment response rate in patients treated with anti-PD-1(259). A recent prospective study of anti-PD-1 in those with ECOG 2 has echoed our findings (397). These observations have a number of clinical implications, including the need for improved surveillance of patients with high risk of metastatic disease and early treatment of disease. The shift towards treatment of early stage melanoma such as in the adjuvant and neo-adjuvant setting are also potential strategies to treat those with potential micro-metastatic disease (that is, prior to the development of symptomatic metastatic disease). However, the long-term survival data from the landmark adjuvant ICI studies are still eagerly awaited.

In our study we also found that patients with poor baseline performance status were more likely to receive treatment within the last month of life and more likely to die in an acute hospital. The promise of durable survival coupled with the lack of predictive biomarkers have posed a number of challenges for health care providers trying to balance hope while keeping patient expectations realistic (394, 398). Future prospective studies are needed for patients with poor performance status using validated quality of life measurements or patient reported outcomes. It is not known whether anti-PD-1 in this population is able to improve cancer related symptoms or whether the treatment toxicities and lack of steroid use is actually detrimental to patient care.

Routine laboratory biomarkers

In addition to LDH, routine laboratory tests such as raised white cell count or neutrophil: lymphocyte ratio as markers of inflammation have been studied as prognostic biomarkers after ICI treatment (248, 268, 520, 521). More recently, CRP and serum IL6 have also been identified as potential prognostic markers in patients treated with combination anti-CTLA4 and anti-PD-1 in randomised clinical trials (273). However, these studies have routinely excluded patients with brain metastases. In Chapter 4 of this thesis, the post-hoc analysis of clinical biomarkers in patients treated in the Anti-PD-1 Brain Collaboration study showed a statistically significant association between CRP and poor outcome in patients treated with either nivolumab or its combination with anti-CTLA4. The two candidate variables of CRP and raised white cell count were able to stratify patients into three prognostic groups, with excellent survival in patients with normal CRP and normal white cell count. These results need to be validated in a larger, independent cohort such as the phase II study of combination ipilimumab and nivolumab in 94 patients (4). If CRP and white cell count were validated as prognostic biomarkers in melanoma brain metastases, these readily available tests could potentially help stratify patients in future clinical trials for melanoma brain metastases. Furthermore, there may be an opportunity to therapeutically target these pathways of inflammation with agents such as Tocilizumab, an IL6 inhibitor which may present as a novel combination with ICI for patients with brain metastases. Our post-hoc analysis was limited to a specific subpopulation of patients with brain metastases who were asymptomatic and had not received prior local therapy. Future studies also need to assess biomarkers in patients treated for symptomatic disease and patients who have received local radiotherapy, as these patients represent a significant proportion of patients in clinical practice.

FDG PET imaging biomarkers

Baseline tumour size on CT was found to be an important prognostic factor after anti-PD1 treatment (159, 274). Functional FDG PET imaging presents as a way of surveying not only the metabolic activity of the tumour but also the patient's immune organs prior to ICI treatment. High spleen to liver ratio (SLR) on pre-treatment FDG PET was associated with poor survival after ipilimumab for advanced melanoma [Chapter 5]. The underlying mechanism of this PET signature is not known, but

patients with high SLR had low albumin in a multivariate analysis, and echoes the findings of inflammation being an important prognostic factor in Chapter 4.

In contrast to published literature, MTV was not associated with survival after ICI when MTV was dichotomized at the median of the cohort for our study (458, 459). However, MTV was associated with OS when analysed as a continuous variable, suggesting a non-linear relationship between MTV and survival. This was further explored by splitting the cohort into 5 MTV quantiles, where the subgroup with the lowest quantile had excellent survival after ICI. This highlights the risk of type II errors in dichotomising continuous variables in discovery cohorts and also the need to validate biomarkers in independent cohorts.

Our PET study also captured a transitional time during ICI development, when majority of patients received ipilimumab monotherapy as their first line of ICI treatment, and only small numbers of anti-PD-1 treated patients were included. The treatment paradigm has moved on, to where anti-PD-1 monotherapy or anti-PD-1 in combination with anti-CTLA4 would be standard of care first line ICI for melanoma. High SLR was associated with poor PFS with ipilimumab and not anti-PD-1 in our small study. It is not known whether the lack of association with anti-PD-1 outcome is genuine or owing to the small numbers of anti-PD-1 treated patients in the study. Nonetheless, it will be important to evaluate the utility of this high SLR in anti-CTLA4 based combinations, particularly as this is now the standard of care treatment.

Functional PET biomarkers have great clinical potential, given the non-invasive nature of the testing and that the technology is already used in clinical practice. Our study highlights the importance of PET imaging not only in the functional assessment of the tumour, but also as a semi-quantitative assessment of the patient's immune system. Improved characterisation of immune organs such as spleen, draining lymph nodes and bone marrow will be complementary to other methods of immune profiling. The major limitation of our PET biomarker study is that the biological determinant of high SLR is not known. While it would not be feasible to have splenic biopsies to correlate FDG PET imaging, a more comprehensive serological assessment of other inflammatory markers such as CRP, white cell count, and profiling of immune cell subsets in the peripheral blood matched with functional imaging would be of interest.

Biological biomarkers

In-depth immune profiling of the tumour can be performed by orthogonal methods of multi-spectral immunohistochemistry (mIHC) and RNA-sequencing to further the understanding of the immune landscape of tumour microenvironments. Comparison of the density and spatial relationships of T cell subsets and PD-L1 staining cells has highlighted the distinct immune profile of 3 UV-related skin cancers (melanoma, compared to MCC and cSCC). The novel mIHC panel allowed characterisation of T cell subsets, with melanoma displaying an abundance of all T cell subsets compared to MCC and a higher infiltration of CD8 compared to cSCC. There was also a subset of MCC samples with a high proportion of CD3+CD4-CD8- T cells which require further characterisation. It would be intriguing to study the double negative T cells in MCC further and assess how this cellular population is responsible for PD-L1 expression in the tumour. It may be possible to study these cells more specifically using single cell RNA sequencing but the feasibility of this may be limited by the overall low frequency of these T cell subsets. The comparison of the 3 skin cancer types has also highlighted the correlates to PD-L1 density in each cancer type: where PD-L1 density appears to be highly correlated to other T cell subsets in both melanoma and cSCC but not in MCC. This study could be improved by further classification of the PD-L1 expression, to assess whether it is co-expressed on tumour cells, T cell subsets or other immune cell types. Furthermore, it may be of interest to assess the PD-L1 status using some of the other commercially available assays so to compare whether the different assays are taken up differently in different cutaneous cancers.

Furthermore, differential gene expression and gene set enrichment analyses suggest that adaptive immune processes and leucocyte proliferation and migration processes are highly upregulated in melanoma and not in MCC. This highlights the importance of examining the non-T cell compartment in skin cancers such as MCC, not only to further our understanding of the immune processes at play but also to identify novel immune therapeutic targets. Indeed, recent evaluation of both the myeloid and B cell compartments of the tumoural immune landscape has identified possible future biomarkers and therapeutic targets(163, 165). Ideally future tumoural immune-profiling would study not only the T cell compartment, PD-L1 status but also the non-T cell immune compartments.

There are a several challenges limiting comprehensive tumoural immune-profiling in routine practice. The technique used to acquire tissue is invasive, the technologies are

available only in research setting in academic centres, requiring sophisticated tissue processing and bioinformatics pipelines for analyses. However, these research tools are immensely valuable to further the understanding of underlying mechanism of treatment response and resistance. Importantly, these detailed descriptions of the immune cellular subtypes or immune processes will be paramount in drug development and rational clinical trial design where potentially basket clinical trial design can rationally select appropriate ICI combinations dependent on the immune cell type present or the resistant mechanisms at play.

Future of biomarkers in the use of ICI

A major limitation of biomarker discovery is that candidate biomarkers have largely been evaluated as single elements, rather than as part of multi-factorial biomarker panels. Attempts to encompass multi-factorial clinical and tumoural biomarkers into clinical utility indices would require well annotated clinical data and tumoural material to test and validate multiple biomarkers and determine the relative weighting of each biomarker. This, in turn, would allow the generation of patient decision aids that can influence therapeutic decision making. However, this type of comprehensive clinical data collection and biobank is seldom available in clinical practice due to time and financial constraints. Some international consortia have been established by investigators from major academic centres; these alliances have the potential to use this powerful resource as the framework for research collaborations using real-world data. Currently, most well annotated clinical data and ICI outcomes are derived from pharmaceutical company sponsored clinical trials. These clinical trials also have substantial biobanks with tumoural tissue and blood stored for biomarker discovery. Strong collaboration between clinicians, scientists and pharmaceutical companies is needed in order to assess these biomarkers in multi-factorial panels.

Candidate biomarkers identified to date have not been discriminatory and have failed to separate responders from non-responders. This is not surprising, given the complexity of factors needed to initiate and maintain an anti-tumoural response. In the ideal setting, biomarkers would not only discriminate responders from non-responders but have the ability to identify resistance mechanisms in the non-responders. Detailed tumoural and patient profiling would be needed in order to have a truly ‘personalised’ form of immunotherapy, where resistance mechanism are identified and matched with

treatments that can neutralise inhibitors or activate the immune system as needed. Clinical trial design will also need to be modified in order to test adaptive strategies where resistant tumours can then be tested with different immunotherapy combinations.

Given the need for improved profiling of both the tumoural microenvironment as well as the patient's immune system, it is important to direct research efforts into non-invasive means of immune-profiling. Our work assessing FDG PET is one form of non-invasive immune monitoring but future functional imaging could potentially involve specific immune checkpoints to assess the nature of tumour micro-environment. There is also an emerging field of radio-therapeutics where radio-isotopes can be used not only in imaging or monitoring response but potentially used therapeutically. These functioning imaging techniques also need to be developed in conjunction with other non-invasive techniques such as monitoring of circulating tumour DNA.

Lastly, while it is important to have biomarkers to enrich for likely responders for the purpose of clinical trial stratification, these types of biomarkers are less helpful for individual patients trying to balance possible toxicities with the potential for durable response. This therapeutic decision-making may then be left to individual judgements; however, it is known from breast cancer literature that patients are prepared to undergo substantial toxic chemotherapies for a 1% absolute gain in survival (522). Research into factors that are helpful for treatment decision making for patients due to undergo ICI for metastatic melanoma may also focus biomarker development. Previous studies have highlighted treatment toxicities and quality of life as important factors that influence treatment decision making. Consequently, more dedicated research into biomarkers for treatment toxicities, as well as patient reported quality of life outcomes in ICI are also needed.

8.2 Conclusion

In conclusion, this thesis identified a number of candidate biomarkers for ICI in patients with melanoma, using clinical factors, functional PET imaging and tumoural immune-profiling. This body of work presents some clinical scenarios associated with poor outcome after ICI, namely patients with poor baseline performance status, high CRP and high SLR on FDG PET imaging. The comprehensive characterisation of intratumoural T cell subsets and PD-L1 density across three UV-related skin cancers

highlighted distant immune profiles using orthogonal methods of mIHC and bulk RNA sequencing, with possible implications for biomarker development. Improved understanding of both the patient's immune status as well as characterisation of the tumoural immune landscape will be paramount in aiding rational treatment ICI selection in the future.

Appendix

A.1 Patient cohort treatment and biomarker summaries

Patient A

Patient A is a 45-year-old woman with BRAF V600E mutated metastatic melanoma. She presented with de-novo metastatic disease with an intracranial haemorrhage into a left fronto-parietal mass (14x22x16mm) at diagnosis. This was managed with neurosurgical debulking. Her staging CT scans showed metastatic disease in the lung, liver, mediastinal lymphadenopathy and subcutaneous metastases. She was treated systemically with vemurafenib with partial response. Subsequently her disease had isolated progression in her liver after 8 months of vemurafenib. Stereotactic radiation was directed at the oligo-metastasis in the liver, while the vemurafenib was continued systemically. Three months later, the disease progressed both extra-cranially in her lungs and subcutaneous sites, as well as intra-cranially, with an asymptomatic 9mm right precentral gyrus metastasis. Prior to the commencement of whole brain radiation, she developed left arm weakness and seizures. She was treated with corticosteroids with good symptomatic improvement. MRI brain showed haemorrhage into the right pre-central gyrus lesion. FDG PET confirmed progressive disease in lung but otherwise controlled disease. Neurosurgery was performed and the haemorrhagic metastasis was resected.

She was commenced on ipilimumab, with a baseline performance ECOG of 1. Pre-treatment biomarkers showed a raised Neutrophil to Lymphocyte Ratio (NLR) ratio of 9.4, where $NLR > 5$ has been shown to be a poor prognostic feature. Her intracranial involvement was also a poor prognostic factor. FDG PET scan prior to treatment showed normal SLR. The post treatment FDG PET showed partial response after 4 treatments. This was only maintained for 2 months until she developed further progressive disease.

Anti-PD-1 was commenced, by which time her baseline performance status fell to ECOG 2. Her blood tests also showed a persistently raised NLR of 14, with low albumin. Her pre-anti-PD-1 scan showed a normal SLR. Her pre-antiPD-1 biopsy showed a high CD68+ myeloid infiltrate, which may also be a predictor of poor outcome(295). The T cell subset also showed a high CD4 phenotype, rather than the

effector CD8 phenotype, the latter of which is associated with a good response to anti-PD-1(296). The PD-L1 co-staining was predominantly on tumour cells rather than CD3 T cells. Her baseline cytokine profile was unremarkable, except that her IL1 β levels were low, though this pro-inflammatory cytokine has not been correlated with response to ICI previously.

She was admitted 3 weeks after anti-PD-1 commencement with the development of an intra-abdominal necrotic tumoural collection. She died shortly of systemic progressive disease.

Patient B

Patient B is a 59-year-old man who initially presented in 2004 with a 2.1mm BRAF wildtype melanoma of the right cheek (other details of the primary melanoma were not available). Interestingly, he developed a late metastasis to his right testis in 2008, which was managed with a right orchidectomy. Over the next two years his disease relapsed with more local regional spread from his primary tumour, with right cervical nodal involvement and subsequently right arm subcutaneous and axillary nodal disease; all managed with surgery. Further surgery was performed in 2011 and 2014 when he presented with non-locoregional left gluteal and left thigh adductor metastases.

Finally he presented in 2014 with left external iliac nodal disease and was commenced on ipilimumab. He had a very low burden of disease on PET with no involvement of high risk organs such as brain or liver, with a normal SLR. His baseline performance status was not available, but his blood tests showed normal levels of LDH and NLR. His pre-treatment biopsies indicate a predominantly T cell rich immune profile with a high proportion of CD8 T cells. His best response to ipilimumab was stable disease but the treatment was complicated by Bell's palsy and ACTH-cortisol axis endocrinopathy. Of note, he did not have any history of auto-immunity but as mentioned in the thesis introduction, clinically validated biomarkers of ICI induced immune related toxicities are currently lacking. He subsequently developed oligo-progressive disease in a left iliac lymph node and left leg which was managed with local resection.

Patient C

Patient C is a 55-year-old woman with BRAFV600E mutated melanoma. She presented with a 1.25mm left scalp superficial spreading melanoma in 2010. Her disease relapsed in 2012 with left neck, left scalp recurrence which was treated with surgery and post-operative radiotherapy (48Gy in 20 fractions to the left posterior scalp and neck completed 11 September 2012). She was subsequently diagnosed with metastatic disease on surveillance imaging October 2013, with bone and small soft tissue metastases. She was commenced on dabrafenib and trametinib January 2014. This resulted in good response confirmed on imaging for ten months. She was noted to have uptake in her left occiput and T9 bony involvement with possible inflammatory changes on her lungs on FDG PET November 2014. She received radiation to the T9 vertebra (25Gy/5#) March 2015 and underwent wide local excision and split skin graft May 2015 for the scalp metastasis.

Her disease progressed despite pembrolizumab monotherapy which was commenced in June 2015. Her pre-treatment ECOG was 1, and her LDH was normal but she had a notably high NLR ratio, a marker of poor prognosis. On her pre-treatment FDG PET scan, her SLR was normal but she did have bony involvement, which is a metastatic site associated with poor response to anti-PD-1(237). Pre-treatment immune profile with mIHC showed almost no T cell infiltrates, but a large proportion of immune cells were CD68⁺ myeloid subsets. While CD68⁺ are known to be immunosuppressive and associated in early stage disease (43, 523), it has yet to be shown to be prognostic in pre-ICI biopsies in small cohorts (299). Of the small amounts of T cell infiltrates, CD4 was the predominant phenotype. The majority of PD-L1 co-staining was on CD3 cells (i.e. PD-L1+CD3⁺ cells). Palliative radiation was administered in July 2015 for symptomatic progression.

Patient D

Patient D is a 70-year-old man with BRAF wildtype, NRAS mutated melanoma. He presented in 2004 with right forearm melanoma which was excised (no other details available on the primary tumour). Over the next 4 years he had annual recurrences of in-transit disease which required surgical resection. In 2012, his disease relapsed in the axilla, and this was excised with an axillary clearance with 1 of 15 lymph nodes involved. He received post-operative radiotherapy to the axilla. In April 2014, he had further local recurrences to the chest wall which were treated with radiation.

He was commenced on ipilimumab for unresectable localised disease in April 2014, with progressive disease as the best treatment response. No pre-treatment biomarkers were available prior to his anti-CTLA4 treatment.

His FDG PET imaging in March 2015 confirmed progressive loco-regional disease and was commenced on pembrolizumab monotherapy. His pretreatment FDG PET scan showed large volume disease but limited to his axilla and chest wall, with normal SLR. His peripheral blood tests and cytokine profile were unremarkable. His lack of anti-tumour response may also be the result of the lack of T cells and B cells, and strategies to stimulate the activation of these cell types are crucial in overcoming innate resistance. Of the small proportion of T cells present, this comprised mostly CD4 and CD8. His PD-L1 co-staining was mainly on T cells (PD-L1+CD3+ cells). His disease also progressed on anti-PD-1 and he was treated with further palliative radiation in July 2015. On imaging in October 2015, he had progressive disease in the chest wall, with low volume lung metastases and a new 14mm left basal ganglia metastasis on MRI brain. He was commenced on temozolamide but his disease progressed shortly despite this therapy.

Patient E

Patient E is a 66-year-old man with BRAF wild type melanoma. He presented in 2013 with a thick left cheek primary melanoma (details unavailable). His disease relapsed locally shortly after his original surgery for primary disease. Within one year (2014), systemic relapse occurred with extensive metastases in his left neck, local and distant lymph nodes, liver, lung, and bone involvement. His pre-treatment PET scan confirmed this high volume of disease but the SLR was normal. Surprisingly, his performance status was maximal with ECOG of 0. His FDG PET imaging showed a very large volume of disease. His pre-ipilimumab LDH was above normal limit of normal and his cytokine level did show a high level of serum IL2 but the other cytokines associated with inflammation such as TNF α were comparable to those in other patients. He completed four doses of ipilimumab in June with progressive disease. He was treated with radiation to his left neck nodal disease, mediastinum and C1-C3 vertebrae, completed 12 June 2014.

He commenced nivolumab in July 2014, with further radiation to his thoracic spine. Denosumab was added in October 2014 and remarkably, he had complete metabolic response by March 2015. His pre-antiPD-1 tumoural biopsy confirmed a predominantly T cell infiltrated immune profile with a mixture of CD4 and CD8 T cell phenotypes. His PD-L1 co-staining was mostly on tumour cells (PD-L1+SOX10+ cells). Given his durable response, he was able to discontinue treatment in June 2018. His most recent imaging in 2020 still confirmed ongoing complete metabolic response.

This case highlights the exceptional durable tumoural responses that are observed, despite multiple bad prognostic indicators such as high disease burden, bone involvement, raised LDH and high PD-L1 expression on tumour cells as opposed to PD-L1 on T cells. It is not known whether the short interval between anti-CTLA4 and anti-PD-1 therapy meant that he effectively received combination ICI, owing to the long half-life of anti-CTLA4. Furthermore, the addition of both radiation and denosumab may have had a synergistic effect on anti-PD-1. Multiple reports have highlighted the potential of abscopal effects of radiation, which may lead to an enhanced immune response. Lastly, there is mounting pre-clinical and clinical evidence to suggest the combination of denosumab, a RANKL inhibitor, may be synergistic to anti-PD-1(510,

524). More in-depth analyses of exceptional responders such as Patient E may identify some mechanism to overcome ICI resistance.

Patient F

62-year-old woman presented with 2.6mm nodular melanoma in 1990 on her left lateral thigh. She was treated with wide local excision and inguinal lymph node dissection which showed 0 of the 5 nodes involved. Her disease recurred loco-regionally in February 2013 with a 7.2 x 6.5 x 5.9cm mass in the left upper thigh subcutaneously. This was treated with wide local excision and post-operative radiation(48Gy/20 fractions). In November 2013, her disease recurred again in the left inguinal region with a new right lower lobe metastases identified on FDG PET. She was commenced on dabrafenib in December 2013, and trametinib was added in Jan 2014. There was good partial response initially to BRAF targeted treatment but the disease subsequently progressed in July 2014. Further left inguinal dissection and reconstruction was attempted but was complicated by post-operative wound break down. At this stage her ECOG was 1 and she was commenced on ipilimumab. Her pre-anti-CTLA4 FDG PET also showed a raised SLR. Her pre-ipilimumab blood tests show a raised NLR of 9.6 with unremarkable peripheral cytokines. Her tumoural immune profile showed a high CD68 myeloid infiltrate with predominantly CD4 T cell infiltrate. FDG PET scan after 4 doses of ipilimumab showed an interval progression of avid disease in the left groin.

She was commenced on nivolumab in January 2015 and received antibiotics as well as radiation to the groin. It is interesting to speculate whether her interval antibiotics may have contributed to her treatment response; furthermore, immunoprofiling of stool microbes through this period may have provided additional biomarkers. Prior to starting anti-PD-1, her ECOG was 1, her NLR had normalised compared to pre-ipilimumab. The SLR on her pre-antiPD-1 FDG PET scan had also normalised. LDH was normal prior to both ICI treatments. Pre-antiPD-1 tumoural immune profile showed an expansion of the T cell compartment and reduction of the myeloid compartment compared to pre-ipilimumab biopsy. CD4 was again the predominant T cell phenotype within her tumour. Subsequently she gained complete metabolic response to treatment. She received 2 years of nivolumab treatment, then ceased therapy after ongoing response was demonstrated. This case is an example of the short progression-free survival seen after ipilimumab in patients with high SLR. However, a

subsequent switch to anti-PD-1 still resulted in a durable response. It is not known whether this is due to the non-overlapping mechanisms of action between the two agents or whether the antibiotics administered between the two therapies have had a synergistic response. To date, preclinical and retrospective data have associated antibiotics with poor response to anti-PD-1 therapy but investigations are underway to understand how faecal microbiota can be manipulated to induce a tumoural response.

9 References

1. Davies MA, Liu P, McIntyre S, Kim KB, Papadopoulos N, Hwu W-J, et al. Prognostic factors for survival in melanoma patients with brain metastases. *Cancer*. 2011;117(8):1687-96.
2. Raizer JJ, Hwu W-J, Panageas KS, Wilton A, Baldwin DE, Bailey E, et al. Brain and leptomeningeal metastases from cutaneous melanoma: survival outcomes based on clinical features. *Neuro-oncology*. 2008;10(2):199-207.
3. Long GV, Atkinson V, Lo S, Sandhu S, Guminski AD, Brown MP, et al. Combination nivolumab and ipilimumab or nivolumab alone in melanoma brain metastases: a multicentre randomised phase 2 study. *The Lancet Oncology*. 2018;19(5):672-81.
4. Tawbi HA, Forsyth PA, Algazi A, Hamid O, Hodi FS, Moschos SJ, et al. Combined Nivolumab and Ipilimumab in Melanoma Metastatic to the Brain. *New England Journal of Medicine*. 2018;379(8):722-30.
5. Gaspar L, Scott C, Rotman M, Asbell S, Phillips T, Wasserman T, et al. Recursive partitioning analysis (RPA) of prognostic factors in three Radiation Therapy Oncology Group (RTOG) brain metastases trials. *International journal of radiation oncology, biology, physics*. 1997;37(4):745-51.
6. Gaspar LE, Scott C, Murray K, Curran W. Validation of the RTOG recursive partitioning analysis (RPA) classification for brain metastases. *International Journal of Radiation Oncology Biology Physics*. 2000;47(4):1001-6.
7. Fife KM, Colman MH, Stevens GN, Firth IC, Moon D, Shannon KF, et al. Determinants of outcome in melanoma patients with cerebral metastases. *Journal of Clinical Oncology*. 2004;22(7):1293-300.
8. Staudt M, Lasithiotakis K, Leiter U, Meier F, Eigentler T, Bamberg M, et al. Determinants of survival in patients with brain metastases from cutaneous melanoma. *British Journal of Cancer*. 2010;102(8):1213-8.
9. Health AIo, Welfare. *Cancer in Australia 2019*. Canberra: AIHW, 2019.
10. Health AIo, Welfare. *Skin cancer in Australia*. Canberra: AIHW, 2016.
11. Balch CM, Gershenwald JE, Soong S-j, Thompson JF, Atkins MB, Byrd DR, et al. Final version of 2009 AJCC melanoma staging and classification. *Journal of Clinical Oncology*. 2009;27(36):6199-206.
12. Armstrong BK, Kricker A. The epidemiology of UV induced skin cancer. *Journal of photochemistry and photobiology B: Biology*. 2001;63(1-3):8-18.
13. International Agency for Research on Cancer Working Group on Artificial Ultraviolet L, Skin C. The association of use of sunbeds with cutaneous malignant melanoma and other skin cancers: a systematic review. *International journal of cancer*. 2007;120(5):1116-22.
14. Lew RA, Sober AJ, Cook N, Marvell R, Fitzpatrick TB. Sun exposure habits in patients with cutaneous melanoma: a case control study. *The Journal of dermatologic surgery and oncology*. 1983;9(12):981-6.
15. Gilchrest BA, Eller MS, Geller AC, Yaar M. The pathogenesis of melanoma induced by ultraviolet radiation. *New England Journal of Medicine*. 1999;340(17):1341-8.

16. Noonan FP, Dudek J, Merlino G, De Fabo EC. Animal models of melanoma: an HGF/SF transgenic mouse model may facilitate experimental access to UV initiating events. *Pigment cell research*. 2003;16(1):16-25.
17. Husain Z, Pathak MA, Flotte T, Wick MM. Role of ultraviolet radiation in the induction of melanocytic tumors in hairless mice following 7, 12-dimethylbenz (a) anthracene application and ultraviolet irradiation. *Cancer Research*. 1991;51(18):4964-70.
18. Hayward NK, Wilmott JS, Waddell N, Johansson PA, Field MA, Nones K, et al. Whole-genome landscapes of major melanoma subtypes. *Nature*. 2017;545(7653):175.
19. Akbani R, Akdemir KC, Aksoy BA, Albert M, Ally A, Amin SB, et al. Genomic classification of cutaneous melanoma. *Cell*. 2015;161(7):1681-96.
20. Goldstein AM, Tucker MA. Genetic epidemiology of cutaneous melanoma: a global perspective. *Archives of dermatology*. 2001;137(11):1493-6.
21. Goldstein AM, Tucker MA. Screening for CDKN2A mutations in hereditary melanoma. Oxford University Press; 1997.
22. Liu L, Lassam NJ, Slingerland JM, Bailey D, Cole D, Jenkins R, et al. Germline p16INK4A mutation and protein dysfunction in a family with inherited melanoma. *Oncogene*. 1995;11(2):405-12.
23. Hansson J. Familial cutaneous melanoma. *Diseases of DNA Repair: Springer*; 2010. p. 134-45.
24. Koh HK. Cutaneous melanoma. *New England Journal of Medicine*. 1991;325(3):171-82.
25. Gershenwald JE, Scolyer RA, Hess KR, Sondak VK, Long GV, Ross MI, et al. Melanoma staging: Evidence-based changes in the American Joint Committee on Cancer eighth edition cancer staging manual. *CA Cancer J Clin*. 2017;67(6):472-92.
26. Dickson PV, Gershenwald JE. Staging and prognosis of cutaneous melanoma. *Surgical oncology clinics of North America*. 2011;20(1):1-17.
27. Morton DL, Wen D-R, Wong JH, Economou JS, Cagle LA, Storm FK, et al. Technical Details of Intraoperative Lymphatic Mapping for Early Stage Melanoma. *Archives of Surgery*. 1992;127(4):392-9.
28. Gershenwald JE, Thompson W, Mansfield PF, Lee JE, Colome MI, Tseng C-h, et al. Multi-Institutional Melanoma Lymphatic Mapping Experience: The Prognostic Value of Sentinel Lymph Node Status in 612 Stage I or II Melanoma Patients. *Journal of Clinical Oncology*. 1999;17(3):976.
29. Morton DL, Thompson JF, Cochran AJ, Mozzillo N, Nieweg OE, Roses DF, et al. Final Trial Report of Sentinel-Node Biopsy versus Nodal Observation in Melanoma. *New England Journal of Medicine*. 2014;370(7):599-609.
30. Edge SB, Byrd DR, Carducci MA, Compton CC, Fritz AG, Greene FL. *AJCC cancer staging manual*: Springer New York; 2010.
31. Bedikian AY, Johnson MM, Warneke CL, Papadopoulos NE, Kim K, Hwu W-J, et al. Prognostic Factors That Determine the Long-Term Survival of Patients with Unresectable Metastatic Melanoma. *Cancer Investigation*. 2008;26(6):624-33.
32. Sirott MN, Bajorin DF, Wong GY, Tao Y, Chapman PB, Templeton MA, et al. Prognostic factors in patients with metastatic malignant melanoma. A multivariate analysis. *Cancer*. 1993;72(10):3091-8.
33. Fridman WH, Galon J, Pagès F, Tartour E, Sautès-Fridman C, Kroemer G. Prognostic and predictive impact of intra-and peritumoral immune infiltrates. *Cancer research*. 2011;71(17):5601-5.

34. Fridman WH, Zitvogel L, Sautès-Fridman C, Kroemer G. The immune contexture in cancer prognosis and treatment. *Nature Reviews Clinical oncology*. 2017;14(12):717.
35. Clark Jr WH, Elder DE, Guerry Iv D, Braitman LE, Trock BJ, Schultz D, et al. Model predicting survival in stage I melanoma based on tumor progression. *JNCI: Journal of the National Cancer Institute*. 1989;81(24):1893-904.
36. Clemente CG, Mihm Jr MC, Bufalino R, Zurrida S, Collini P, Cascinelli N. Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. *Cancer: Interdisciplinary International Journal of the American Cancer Society*. 1996;77(7):1303-10.
37. Azimi F, Scolyer RA, Rumcheva P, Moncrieff M, Murali R, McCarthy SW, et al. Tumor-Infiltrating Lymphocyte Grade Is an Independent Predictor of Sentinel Lymph Node Status and Survival in Patients With Cutaneous Melanoma. *Journal of Clinical Oncology*. 2012;30(21):2678-83.
38. Piras F, Colombari R, Minerba L, Murtas D, Floris C, Maxia C, et al. The predictive value of CD8, CD4, CD68, and human leukocyte antigen-D-related cells in the prognosis of cutaneous malignant melanoma with vertical growth phase. *Cancer: Interdisciplinary International Journal of the American Cancer Society*. 2005;104(6):1246-54.
39. Ladányi A, Kiss J, Somlai B, Gilde K, Fejős Z, Mohos A, et al. Density of DC-LAMP+ mature dendritic cells in combination with activated T lymphocytes infiltrating primary cutaneous melanoma is a strong independent prognostic factor. *Cancer immunology, immunotherapy*. 2007;56(9):1459-69.
40. Hillen F, Baeten CIM, van de Winkel A, Creytens D, van der Schaft DWJ, Winnepeninckx V, et al. Leukocyte infiltration and tumor cell plasticity are parameters of aggressiveness in primary cutaneous melanoma. *Cancer Immunology, Immunotherapy*. 2008;57(1):97-106.
41. Miracco C, Mourmouras V, Biagioli M, Rubegni P, Mannucci S, Monciatti I, et al. Utility of tumour-infiltrating CD25+ FOXP3+ regulatory T cell evaluation in predicting local recurrence in vertical growth phase cutaneous melanoma. *Oncology reports*. 2007;18(5):1115-22.
42. Mougiakakos D, Johansson CC, Trocme E, All-Ericsson C, Economou MA, Larsson O, et al. Intratumoral forkhead box P3-positive regulatory T cells predict poor survival in cyclooxygenase-2-positive uveal melanoma. *Cancer*. 2010;116(9):2224-33.
43. Jensen TO, Schmidt H, Moller HJ, Hoyer M, Maniecki MB, Sjoegren P, et al. Macrophage markers in serum and tumor have prognostic impact in American Joint Committee on Cancer stage I/II melanoma. *Journal of Clinical Oncology*. 2009;27(20):3330-7.
44. Jönsson G, Busch C, Knappskog S, Geisler J, Miletic H, Ringnér M, et al. Gene expression profiling-based identification of molecular subtypes in stage IV melanomas with different clinical outcome. *Clinical cancer research*. 2010;16(13):3356-67.
45. Harbst K, Staaf J, Lauss M, Karlsson A, Måsbäck A, Johansson I, et al. Molecular profiling reveals low-and high-grade forms of primary melanoma. *Clinical Cancer Research*. 2012;18(15):4026-36.
46. Nsengimana J, Laye J, Filia A, Walker C, Jewell R, Van den Oord JJ, et al. Independent replication of a melanoma subtype gene signature and evaluation of

- its prognostic value and biological correlates in a population cohort. *Oncotarget*. 2015;6(13):11683.
47. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob J-J, Rutkowski P, Lao CD, et al. Five-year survival with combined Nivolumab and Ipilimumab in advanced melanoma. *New England Journal of Medicine*. 2019;381(16):1535-46.
 48. Essner R, Lee JH, Wanek LA, Itakura H, Morton DL. Contemporary surgical treatment of advanced-stage melanoma. *Archives of Surgery*. 2004;139(9):961-7.
 49. Hena MA, Emrich LJ, Nambisan RN, Karakousis CP. Effect of surgical treatment on stage IV melanoma. *The American Journal of Surgery*. 1987;153(3):270-5.
 50. Meyer T, Merkel S, Goehl J, Hohenberger W. Surgical therapy for distant metastases of malignant melanoma. *Cancer: Interdisciplinary International Journal of the American Cancer Society*. 2000;89(9):1983-91.
 51. Lee ML, Tomsu K, Von KBE. Duration of survival for disseminated malignant melanoma: results of a meta-analysis. 2000.
 52. Twyman-Saint Victor C, Rech AJ, Maity A, Rengan R, Pauken KE, Stelekati E, et al. Radiation and dual checkpoint blockade activate non-redundant immune mechanisms in cancer. *Nature*. 2015;520(7547):373.
 53. Hiniker SM, Reddy SA, Maecker HT, Subrahmanyam PB, Rosenberg-Hasson Y, Swetter SM, et al. A prospective clinical trial combining radiation therapy with systemic immunotherapy in metastatic melanoma. *International Journal of Radiation Oncology Biology Physics*. 2016;96(3):578-88.
 54. Shabason JE, Minn AJ, editors. *Radiation and immune checkpoint blockade: from bench to clinic* 2017: Elsevier.
 55. Elias ML, Behbahani S, Maddukuri S, John AM, Schwartz RA, Lambert WC. Prolonged overall survival following metastasectomy in stage IV melanoma. *Journal of the European Academy of Dermatology and Venereology*. 2019.
 56. Eggermont AMM, Kirkwood JM. Re-evaluating the role of dacarbazine in metastatic melanoma: what have we learned in 30 years? *European Journal of Cancer*. 2004;40(12):1825-36.
 57. Chapman PB, Einhorn LH, Meyers ML, Saxman S, Destro AN, Panageas KS, et al. Phase III multicenter randomized trial of the Dartmouth regimen versus dacarbazine in patients with metastatic melanoma. *Journal of Clinical Oncology*. 1999;17(9):2745.
 58. Luikart SD, Kennealey GT, Kirkwood JM. Randomized phase III trial of vinblastine, bleomycin, and cis-dichlorodiammine-platinum versus dacarbazine in malignant melanoma. *Journal of Clinical Oncology*. 1984;2(3):164-8.
 59. Brown CK, Kirkwood JM. Medical management of melanoma. *The Surgical clinics of North America*. 2003;83(2):283-322.
 60. Creagan ET, Suman VJ, Dalton RJ, Pitot HC, Long HJ, Veeder MH, et al. Phase III clinical trial of the combination of cisplatin, dacarbazine, and carmustine with or without tamoxifen in patients with advanced malignant melanoma. *Journal of Clinical Oncology*. 1999;17(6):1884.
 61. Jungnelius U, Ringborg U, Aamdal S, Mattsson J, Stierner U, Ingvar C, et al. Dacarbazine–vindesine versus dacarbazine–vindesine–cisplatin in disseminated malignant melanoma. A randomised phase III trial. *European Journal of Cancer*. 1998;34(9):1368-74.
 62. Jelic S, Babovic N, Kovcin V, Milicevic N, Milanovic N, Popov I, et al. Comparison of the efficacy of two different dosage dacarbazine-based regimens

- and two regimens without dacarbazine in metastatic melanoma: a single-centre randomized four-arm study. *Melanoma Research*. 2002;12(1):91-8.
63. Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, et al. Prospective randomized trial of the treatment of patients with metastatic melanoma using chemotherapy with cisplatin, dacarbazine, and tamoxifen alone or in combination with interleukin-2 and interferon alfa-2b. *Journal of Clinical Oncology*. 1999;17(3):968-75.
 64. Falkson CI, Ibrahim J, Kirkwood JM, Coates AS, Atkins MB, Blum RH. Phase III trial of dacarbazine versus dacarbazine with interferon alpha-2b versus dacarbazine with tamoxifen versus dacarbazine with interferon alpha-2b and tamoxifen in patients with metastatic malignant melanoma: an Eastern Cooperative Oncology Group study. *Journal of Clinical Oncology*. 1998;16(5):1743-51.
 65. Patel M, McCully C, Godwin K, Balis FM. Plasma and cerebrospinal fluid pharmacokinetics of intravenous temozolomide in non-human primates. *Journal of Neuro-oncology*. 2003;61(3):203-7.
 66. Middleton MR, Grob JJ, Aaronson N, Fierlbeck G, Tilgen W, Seiter S, et al. Randomized phase III study of temozolomide versus dacarbazine in the treatment of patients with advanced metastatic malignant melanoma. *Journal of Clinical Oncology*. 2000;18(1):158-66.
 67. Avril MF, Aamdal S, Grob JJ, Hauschild A, Mohr P, Bonerandi JJ, et al. Fotemustine compared with dacarbazine in patients with disseminated malignant melanoma: a phase III study. *Journal of Clinical Oncology*. 2004;22(6):1118-25.
 68. Flaherty KT, Lee SJ, Zhao F, Schuchter LM, Flaherty L, Kefford R, et al. Phase III trial of carboplatin and paclitaxel with or without sorafenib in metastatic melanoma. *Journal of Clinical Oncology*. 2013;31(3):373.
 69. Agarwala SS. Current systemic therapy for metastatic melanoma. *Expert review of anticancer therapy*. 2009;9(5):587-95.
 70. Trinh VA. Current management of metastatic melanoma. *American Journal of Health-System Pharmacy*. 2008;65(24_Supplement_9):S3-S9.
 71. Albino AP, Nanus DM, Mentle IR, Cordon-Cardo C, McNutt NS, Bressler J, et al. Analysis of ras oncogenes in malignant melanoma and precursor lesions: correlation of point mutations with differentiation phenotype. *Oncogene*. 1989;4(11):1363-74.
 72. Brose MS, Volpe P, Feldman M, Kumar M, Rishi I, Gerrero R, et al. BRAF and RAS mutations in human lung cancer and melanoma. *Cancer Research*. 2002;62(23):6997-7000.
 73. Omholt K, Platz A, Kanter L, Ringborg U, Hansson J. NRAS and BRAF mutations arise early during melanoma pathogenesis and are preserved throughout tumor progression. *Clinical Cancer Research*. 2003;9(17):6483-8.
 74. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. Mutations of the BRAF gene in human cancer. *Nature*. 2002;417(6892):949.
 75. Bowyer SE, Rao AD, Lyle M, Sandhu S, Long GV, McArthur GA, et al. Activity of trametinib in K601E and L597Q BRAF mutation-positive metastatic melanoma. *Melanoma Research*. 2014;24(5):504-8.
 76. Porcelli L, Guida G, Tommasi S, Guida M, Azzariti A. Metastatic melanoma cells with BRAF G469A mutation: nab-paclitaxel better than vemurafenib? *Cancer chemotherapy and pharmacology*. 2015;76(2):433-8.

77. Heidorn SJ, Milagre C, Whittaker S, Nourry A, Niculescu-Duvas I, Dhomen N, et al. Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. *Cell*. 2010;140(2):209-21.
78. McArthur GA, Chapman PB, Robert C, Larkin J, Haanen JB, Dummer R, et al. Safety and efficacy of vemurafenib in BRAF V600E and BRAF V600K mutation-positive melanoma (BRIM-3): extended follow-up of a phase 3, randomised, open-label study. *The Lancet Oncology*. 2014;15(3):323-32.
79. Hauschild A, Grob J-J, Demidov LV, Jouary T, Gutzmer R, Millward M, et al. Dabrafenib in BRAF-mutated metastatic melanoma: a multicentre, open-label, phase 3 randomised controlled trial. *The Lancet*. 2012;380(9839):358-65.
80. Delord J-P, Robert C, Nyakas M, McArthur GA, Kudchakar R, Mahipal A, et al. Phase I dose-escalation and-expansion study of the BRAF inhibitor encorafenib (LGX818) in metastatic BRAF-mutant melanoma. *Clinical Cancer Research*. 2017;23(18):5339-48.
81. Dummer R, Ascierto PA, Gogas HJ, Arance A, Mandala M, Liskay G, et al. Encorafenib plus binimetinib versus vemurafenib or encorafenib in patients with BRAF-mutant melanoma (COLUMBUS): a multicentre, open-label, randomised phase 3 trial. *The Lancet Oncology*. 2018;19(5):603-15.
82. Van Allen EM, Wagle N, Sucker A, Treacy DJ, Johannessen CM, Goetz EM, et al. The genetic landscape of clinical resistance to RAF inhibition in metastatic melanoma. *Cancer discovery*. 2014;4(1):94-109.
83. Trunzer K, Pavlick AC, Schuchter L, Gonzalez R, McArthur GA, Hutson TE, et al. Pharmacodynamic effects and mechanisms of resistance to vemurafenib in patients with metastatic melanoma. *Journal of Clinical Oncology*. 2013;JCO. 2012.44. 7888.
84. Rizos H, Menzies AM, Pupo GM, Carlino MS, Fung C, Hyman J, et al. BRAF inhibitor resistance mechanisms in metastatic melanoma: spectrum and clinical impact. *Clinical Cancer Research*. 2014;20(7):1965-77.
85. Shi H, Hugo W, Kong X, Hong A, Koya RC, Moriceau G, et al. Acquired resistance and clonal evolution in melanoma during BRAF inhibitor therapy. *Cancer Discovery*. 2014;4(1):80-93.
86. Long GV, Stroyakovskiy D, Gogas H, Levchenko E, De Braud F, Larkin J, et al. Dabrafenib and trametinib versus dabrafenib and placebo for Val600 BRAF-mutant melanoma: a multicentre, double-blind, phase 3 randomised controlled trial. *The Lancet*. 2015;386(9992):444-51.
87. Robert C, Karaszewska B, Schachter J, Rutkowski P, Mackiewicz A, Stroiakovski D, et al. Improved overall survival in melanoma with combined dabrafenib and trametinib. *New England Journal of Medicine*. 2015;372(1):30-9.
88. Ascierto PA, McArthur GA, Dréno B, Atkinson V, Liskay G, Di Giacomo AM, et al. Cobimetinib combined with vemurafenib in advanced BRAF V600-mutant melanoma (coBRIM): updated efficacy results from a randomised, double-blind, phase 3 trial. *The Lancet Oncology*. 2016;17(9):1248-60.
89. Oberholzer PA, Kee D, Dziunycz P, Sucker A, Kamsukom N, Jones R, et al. RAS mutations are associated with the development of cutaneous squamous cell tumors in patients treated with RAF inhibitors. *Journal of Clinical Oncology*. 2012;30(3):316-21.
90. Su F, Viros A, Milagre C, Trunzer K, Bollag G, Spleiss O, et al. RAS mutations in cutaneous squamous-cell carcinomas in patients treated with BRAF inhibitors. *New England Journal of Medicine*. 2012;366(3):207-15.

91. Ribas A, Hodi FS, Callahan M, Konto C, Wolchok J. Hepatotoxicity with combination of vemurafenib and ipilimumab. *New England Journal of Medicine*. 2013;368(14):1365-6.
92. Puzanov I, Callahan MK, Linette GP, Patel SP, Luke JJ, Sosman JA, et al. Phase 1 study of the BRAF inhibitor dabrafenib (D) with or without the MEK inhibitor trametinib (T) in combination with ipilimumab (Ipi) for V600E/K mutation-positive unresectable or metastatic melanoma (MM). *American Society of Clinical Oncology*; 2014.
93. Kalialis LV, Drzewiecki KT, Klyver H. Spontaneous regression of metastases from melanoma: review of the literature. *Melanoma research*. 2009;19(5):275-82.
94. Jensen P, Hansen S, Møller B, Leivestad T, Pfeffer P, Geiran O, et al. Skin cancer in kidney and heart transplant recipients and different long-term immunosuppressive therapy regimens. *Journal of the American Academy of Dermatology*. 1999;40(2):177-86.
95. Birkeland SA, Storm HH. Cancer risk in patients on dialysis and after renal transplantation. *The Lancet*. 2000;355(9218):1886-7.
96. Tsimberidou A-M, Wen S, McLaughlin P, O'Brien S, Wierda WG, Lerner S, et al. Other malignancies in chronic lymphocytic leukemia/small lymphocytic lymphoma. *Journal of Clinical Oncology*. 2009;27(6):904.
97. Burgi A, Brodine S, Wegner S, Milazzo M, Wallace MR, Spooner K, et al. Incidence and risk factors for the occurrence of non-AIDS-defining cancers among human immunodeficiency virus-infected individuals. *Cancer*. 2005;104(7):1505-11.
98. Kubica AW, Brewer JD, editors. *Melanoma in immunosuppressed patients 2012*: Elsevier.
99. Coley WB. The Treatment of inoperable sarcoma with the mixed toxins of erysipelas and basillus prodigiosus: immediate and final results in one hundred and forty cases. *Journal of the American Medical Association*. 1898;31(9):456-65.
100. Sparano JA, Fisher RI, Sunderland M, Margolin K, Ernest ML, Sznol M, et al. Randomized phase III trial of treatment with high-dose interleukin-2 either alone or in combination with interferon alfa-2a in patients with advanced melanoma. *Journal of Clinical Oncology*. 1993;11(10):1969-77.
101. Rosenberg SA, Lotze MT, Yang JC, Topalian SL, Chang AE, Schwartzentruber DJ, et al. Prospective randomized trial of high-dose interleukin-2 alone or in conjunction with lymphokine-activated killer cells for the treatment of patients with advanced cancer. *Journal of the National Cancer Institute*. 1993;85(8):622-32.
102. McCabe MS, Stablein D, Hawkins MJ, editors. *The modified group C experience—phase III randomized trials of IL-2 vs IL-2LAK in advanced renal cell carcinoma and advanced melanoma 1991*.
103. Petrella T, Quirt I, Verma S, Haynes AE, Charette M, Bak K, et al. Single-agent interleukin-2 in the treatment of metastatic melanoma: a systematic review. *Cancer treatment reviews*. 2007;33(5):484-96.
104. Mocellin S, Pasquali S, Rossi CR, Nitti D. Interferon Alpha Adjuvant Therapy in Patients With High-Risk Melanoma: A Systematic Review and Meta-analysis. *JNCI: Journal of the National Cancer Institute*. 2010;102(7):493-501.
105. Creagan ET, Ahmann DL, Frytak S, Long HJ, Chang MN, Itri LM. Phase II trials of recombinant leukocyte A interferon in disseminated malignant melanoma: results in 96 patients. *Cancer Treatment Reports*. 1986;70(5):619-24.

106. Dummer R, Garbe C, Thompson JA, Eggermont AM, Yoo K, Maier T, et al. Randomized dose-escalation study evaluating peginterferon alfa-2a in patients with metastatic malignant melanoma. *Journal of Clinical Oncology*. 2006;24(7):1188-94.
107. Lurquin C, Lethé B, De Plaen E, Corbière V, Théate I, Van Baren N, et al. Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen. *Journal of Experimental Medicine*. 2005;201(2):249-57.
108. Germeau C, Ma W, Schiavetti F, Lurquin C, Henry E, Vigneron N, et al. High frequency of antitumor T cells in the blood of melanoma patients before and after vaccination with tumor antigens. *Journal of Experimental Medicine*. 2005;201(2):241-8.
109. Palucka K, Banchereau J. Dendritic-cell-based therapeutic cancer vaccines. *Immunity*. 2013;39(1):38-48.
110. Rosenberg SA, Spiess P, Lafreniere R. A new approach to the adoptive immunotherapy of cancer with tumor-infiltrating lymphocytes. *Science*. 1986;233(4770):1318-21.
111. Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science*. 2002;298(5594):850-4.
112. Dudley ME, Wunderlich JR, Yang JC, Sherry RM, Topalian SL, Restifo NP, et al. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. *Journal of Clinical Oncology*. 2005;23(10):2346.
113. Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM, et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science*. 2006;314(5796):126-9.
114. Morton DL, Eilber FR, Holmes EC, Hunt JS, Ketcham AS, Silverstein MJ, et al. BCG immunotherapy of malignant melanoma: summary of a seven-year experience. *Annals of Surgery*. 1974;180(4):635-43.
115. Andtbacka RH, Kaufman HL, Collichio F, Amatruda T, Senzer N, Chesney J, et al. Talimogene laherparepvec improves durable response rate in patients with advanced melanoma. *Journal of Clinical Oncology*. 2015;33(25):2780-8.
116. Food US, Drug A. The Department of Health and Human Services (2018a): List of Cleared or Approved Companion Diagnostic Devices (In Vitro and Imaging Tools). 2018.
117. Wei SC, Duffy CR, Allison JP. Fundamental Mechanisms of Immune Checkpoint Blockade Therapy. *Cancer Discovery*. 2018.
118. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. *Annual Review Immunology*. 2004;22:329-60.
119. Sakaguchi S, Sakaguchi N, Shimizu J, Yamazaki S, Sakihama T, Itoh M, et al. Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunological Reviews*. 2001;182(1):18-32.
120. Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. *Nature Immunology*. 2002;3(11):999.
121. Marincola FM, Jaffee EM, Hicklin DJ, Ferrone S. Escape of human solid tumors from T-cell recognition: Molecular mechanisms and functional significance. *Advances in Immunology*. 74: Elsevier; 1999. p. 181-273.

122. Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. *Immunity*. 2013;39(1):1-10.
123. Brunet J-F, Denizot F, Luciani M-F, Roux-Dosseto M, Suzan M, Mattei M-G, et al. A new member of the immunoglobulin superfamily—CTLA-4. *Nature*. 1987;328(6127):267.
124. Krummel MF, Allison JP. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *Journal of Experimental Medicine*. 1995;182(2):459-65.
125. Friedline RH, Brown DS, Nguyen H, Kornfeld H, Lee J, Zhang Y, et al. CD4+ regulatory T cells require CTLA-4 for the maintenance of systemic tolerance. *Journal of Experimental Medicine*. 2009;206(2):421-34.
126. Read S, Greenwald R, Izcue A, Robinson N, Mandelbrot D, Francisco L, et al. Blockade of CTLA-4 on CD4+ CD25+ regulatory T cells abrogates their function in vivo. *The Journal of Immunology*. 2006;177(7):4376-83.
127. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, et al. CTLA-4 control over Foxp3+ regulatory T cell function. *Science*. 2008;322(5899):271-5.
128. Qureshi OS, Zheng Y, Nakamura K, Attridge K, Manzotti C, Schmidt EM, et al. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science*. 2011;332(6029):600-3.
129. Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *The EMBO journal*. 1992;11(11):3887-95.
130. Keir ME, Liang SC, Guleria I, Latchman YE, Qipo A, Albacker LA, et al. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *Journal of Experimental Medicine*. 2006;203(4):883-95.
131. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nature immunology*. 2001;2(3):261.
132. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *Journal of Experimental Medicine*. 2000;192(7):1027-34.
133. Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity*. 1999;11(2):141-51.
134. Nishimura H, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matsumori A, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science*. 2001;291(5502):319-22.
135. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nature medicine*. 2002;8(8):793-800.
136. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nature Reviews Cancer*. 2012;12(4):252-64.
137. Gajewski TF, Louahed J, Brichard VG. Gene signature in melanoma associated with clinical activity: a potential clue to unlock cancer immunotherapy. *The Cancer Journal*. 2010;16(4):399-403.
138. Taube JM, Anders RA, Young GD, Xu H, Sharma R, McMiller TL, et al. Colocalization of Inflammatory Response with B7-H1 Expression in Human

- Melanocytic Lesions Supports an Adaptive Resistance Mechanism of Immune Escape. *Science Translational Medicine*. 2012;4(127):127ra37.
139. Marzec M, Zhang Q, Goradia A, Raghunath PN, Liu X, Paessler M, et al. Oncogenic kinase NPM/ALK induces through STAT3 expression of immunosuppressive protein CD274 (PD-L1, B7-H1). *Proceedings of the National Academy of Sciences*. 2008;105(52):20852-7.
 140. Green MR, Rodig S, Juszczynski P, Ouyang J, Sinha P, O'Donnell E, et al. Constitutive AP-1 activity and EBV infection induce PD-L1 in Hodgkin lymphomas and posttransplant lymphoproliferative disorders: implications for targeted therapy. *Clinical Cancer Research*. 2012;18(6):1611-8.
 141. Jiang X, Zhou J, Giobbie-Hurder A, Wargo J, Hodi FS. The activation of MAPK in melanoma cells resistant to BRAF inhibition promotes PD-L1 expression that is reversible by MEK and PI3K inhibition. *Clinical Cancer Research*. 2013;19(3):598-609.
 142. Crane C, Panner A, Pieper RO, Arbiser J, Parsa AT. Honokiol mediated inhibition of PI3K/mTOR pathway: A potential strategy to overcome immunoresistance in glioma, breast and prostate carcinoma without impacting T cell function. *Journal of Immunotherapy*. 2009;32(6):585.
 143. Ramagopal UA, Liu W, Garrett-Thomson SC, Bonanno JB, Yan Q, Srinivasan M, et al. Structural basis for cancer immunotherapy by the first-in-class checkpoint inhibitor ipilimumab. *Proceedings of the National Academy of Sciences*. 2017;114(21):E4223-E32.
 144. Fehlings M, Simoni Y, Penny HL, Becht E, Loh CY, Gubin MM, et al. Checkpoint blockade immunotherapy reshapes the high-dimensional phenotypic heterogeneity of murine intratumoural neoantigen-specific CD8⁺ T cells. *Nature Communications*. 2017;8(1):562.
 145. Liakou CI, Kamat A, Tang DN, Chen H, Sun J, Troncso P, et al. CTLA-4 blockade increases IFN γ -producing CD4⁺ & ICOS⁺ cells to shift the ratio of effector to regulatory T cells in cancer patients. *Proceedings of the National Academy of Sciences*. 2008;105(39):14987.
 146. Carthon BC, Wolchok JD, Yuan J, Kamat A, Ng Tang DS, Sun J, et al. Preoperative CTLA-4 Blockade: Tolerability and Immune Monitoring in the Setting of a Presurgical Clinical Trial. *Clinical Cancer Research*. 2010;16(10):2861.
 147. Chaput N, Lepage P, Coutzac C, Soularue E, Le Roux K, Monot C, et al. Baseline gut microbiota predicts clinical response and colitis in metastatic melanoma patients treated with ipilimumab. *Annals of Oncology*. 2017;28(6):1368-79.
 148. Ng Tang D, Shen Y, Sun J, Wen S, Wolchok JD, Yuan J, et al. Increased Frequency of ICOS⁺ CD4⁺ T Cells as a Pharmacodynamic Biomarker for Anti-CTLA-4 Therapy. *Cancer Immunology Research*. 2013;1(4):229.
 149. Simpson TR, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F, et al. Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. *The Journal of Experimental Medicine*. 2013;210(9):1695-710.
 150. Selby MJ, Engelhardt JJ, Quigley M, Henning KA, Chen T, Srinivasan M, et al. Anti-CTLA-4 Antibodies of IgG2a Isotype Enhance Antitumor Activity through Reduction of Intratumoral Regulatory T Cells. *Cancer Immunology Research*. 2013.

151. Bulliard Y, Jolicoeur R, Windman M, Rue SM, Ettenberg S, Knee DA, et al. Activating Fc γ receptors contribute to the antitumor activities of immunoregulatory receptor-targeting antibodies. *The Journal of Experimental Medicine*. 2013;210(9):1685-93.
152. Peggs KS, Quezada SA, Chambers CA, Korman AJ, Allison JP. Blockade of CTLA-4 on both effector and regulatory T cell compartments contributes to the antitumor activity of anti-CTLA-4 antibodies. *The Journal of Experimental Medicine*. 2009;206(8):1717-25.
153. Romano E, Kusio-Kobialka M, Foukas PG, Baumgaertner P, Meyer C, Ballabeni P, et al. Ipilimumab-dependent cell-mediated cytotoxicity of regulatory T cells ex vivo by nonclassical monocytes in melanoma patients. *Proceedings of the National Academy of Sciences*. 2015;112(19):6140.
154. Vargas FA, Furness AJS, Litchfield K, Joshi K, Rosenthal R, Ghorani E, et al. Fc effector function contributes to the activity of human anti-CTLA-4 antibodies. *Cancer Cell*. 2018;33(4):649-63.
155. Robert L, Tsoi J, Wang X, Emerson R, Homet B, Chodon T, et al. CTLA4 blockade broadens the peripheral T-cell receptor repertoire. *Clinical Cancer Research*. 2014;20(9):2424-32.
156. Cha E, Klinger M, Hou Y, Cummings C, Ribas A, Faham M, et al. Improved survival with T cell clonotype stability after anti-CTLA-4 treatment in cancer patients. *Science translational medicine*. 2014;6(238):238ra70-ra70.
157. Herbst RS, Soria JC, Kowanetz M, Fine GD, Hamid O, Gordon MS, et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature*. 2014;515(7528):563-7.
158. Tumeh PC, Harview CL, Yearley JH, Shintaku IP, Taylor EJ, Robert L, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature*. 2014;515(7528):568-71.
159. Huang AC, Postow MA, Orlowski RJ, Mick R, Bengsch B, Manne S, et al. T-cell invigoration to tumour burden ratio associated with anti-PD-1 response. *Nature*. 2017;545(7652):60-5.
160. Im SJ, Hashimoto M, Gerner MY, Lee J, Kissick HT, Burger MC, et al. Defining CD8⁺ T cells that provide the proliferative burst after PD-1 therapy. *Nature*. 2016;537(7620):417.
161. Spitzer MH, Carmi Y, Reticker-Flynn NE, Kwek SS, Madhiredy D, Martins MM, et al. Systemic immunity is required for effective cancer immunotherapy. *Cell*. 2017;168(3):487-502.
162. Griss J, Bauer W, Wagner C, Simon M, Chen M, Grabmeier-Pfistershammer K, et al. B cells sustain inflammation and predict response to immune checkpoint blockade in human melanoma. *Nature Communications*. 2019;10(1):1-14.
163. Strauss L, Mahmoud MAA, Weaver JD, Tijaro-Ovalle NM, Christofides A, Wang Q, et al. Targeted deletion of PD-1 in myeloid cells induces antitumor immunity. *Science Immunology*. 2020;5(43).
164. House IG, Savas P, Lai J, Chen AXY, Oliver AJ, Teo ZL, et al. Macrophage derived CXCL9 and CXCL10 are required for anti-tumor immune responses following immune checkpoint blockade. *Clinical Cancer Research*. 2020;26(2):487-504.
165. Helmink BA, Reddy SM, Gao J, Zhang S, Basar R, Thakur R, et al. B cells and tertiary lymphoid structures promote immunotherapy response. *Nature*. 2020;577(7791):549-55.

166. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *New England Journal of Medicine*. 2010;363(8):711-23.
167. Ascierto PA, Del Vecchio M, Robert C, Mackiewicz A, Chiarion-Sileni V, Arance A, et al. Ipilimumab 10 mg/kg versus ipilimumab 3 mg/kg in patients with unresectable or metastatic melanoma: a randomised, double-blind, multicentre, phase 3 trial. *The Lancet Oncology*. 2017;18(5):611-22.
168. Menzies AM, Long GV. Optimum dosing of ipilimumab in melanoma: too little, too late? *The Lancet Oncology*. 2017;18(5):558-9.
169. Schadendorf D, Hodi FS, Robert C, Weber JS, Margolin K, Hamid O, et al. Pooled analysis of long-term survival data from phase II and phase III trials of ipilimumab in unresectable or metastatic melanoma. *Journal of Clinical Oncology*. 2015;JCO. 2014.56. 736.
170. Schachter J, Ribas A, Long GV, Arance A, Grob J-J, Mortier L, et al. Pembrolizumab versus ipilimumab for advanced melanoma: final overall survival results of a multicentre, randomised, open-label phase 3 study (KEYNOTE-006). *The Lancet*. 2017;390(10105):1853-62.
171. Hamid O, Robert C, Daud A, Hodi FS, Hwu W-J, Kefford R, et al. Five-year survival outcomes for patients with advanced melanoma treated with pembrolizumab in KEYNOTE-001. *Annals of Oncology*. 2019;30(4):582-8.
172. Robert C, Long GV, Brady B, Dutriaux C, Maio M, Mortier L, et al. Nivolumab in previously untreated melanoma without BRAF mutation. *New England Journal of Medicine*. 2015;372(4):320-30.
173. Ascierto PA, Long GV, Robert C, Brady B, Dutriaux C, Di Giacomo AM, et al. Survival outcomes in patients with previously untreated BRAF wild-type advanced melanoma treated with Nivolumab therapy: three-year follow-up of a randomized phase 3 trial. *JAMA Oncology*. 2019;5(2):187-94.
174. Robert C, Schachter J, Long GV, Arance A, Grob JJ, Mortier L, et al. Pembrolizumab versus ipilimumab in advanced melanoma. *New England Journal of Medicine*. 2015;372(26):2521-32.
175. Robert C, Ribas A, Schachter J, Arance A, Grob J-J, Mortier L, et al. Pembrolizumab versus ipilimumab in advanced melanoma (KEYNOTE-006): post-hoc 5-year results from an open-label, multicentre, randomised, controlled, phase 3 study. *The Lancet Oncology*. 2019;20(9):1239-51.
176. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, et al. Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. *New England Journal of Medicine*. 2015;373(1):23-34.
177. Larkin J, Chiarion-Sileni V, Gonzalez R, Grob J-J, Rutkowski P, Lao CD, et al. Five-Year Survival with Combined Nivolumab and Ipilimumab in Advanced Melanoma. *New England Journal of Medicine*. 2019;381(16):1535-46.
178. Alexander M. Ipilimumab in pretreated patients with unresectable or metastatic cutaneous, uveal and mucosal melanoma. *survival*. 2014;7(9):10.
179. Fecher LA, Agarwala SS, Hodi FS, Weber JS. Ipilimumab and its toxicities: a multidisciplinary approach. *Oncologist*. 2013;18(6):733-43.
180. Friedman CF, Navid-Azarbaijani P, Shoushtari AN, Campbell SC, Callahan MK, Momtaz P, et al. Toxicity associated with ipilimumab and nivolumab (Ipi+Nivo) combination therapy in melanoma patients (pts) treated at a single-institution under an expanded-access program (EAP). *American Society of Clinical Oncology*; 2016.

181. Mariotto AB, Robin Yabroff K, Shao Y, Feuer EJ, Brown ML. Projections of the cost of cancer care in the United States: 2010–2020. *Journal of the National Cancer Institute*. 2011;103(2):117-28.
182. Karikios DJ, Schofield D, Salkeld G, Mann KP, Trotman J, Stockler MR. Rising cost of anticancer drugs in Australia. *Internal Medicine Journal*. 2014;44(5):458-63.
183. Health AGDo. Pharmaceutical Benefits Scheme and Repatriation Pharmaceutical Benefits Scheme Section 85 Supply Data 2020 [
184. Long GV, Tykodi SS, Schneider JG, Garbe C, Gravis G, Rashford M, et al. Assessment of nivolumab exposure and clinical safety of 480 mg every 4 weeks flat-dosing schedule in patients with cancer. *Annals of Oncology*. 2018;29(11):2208-13.
185. Centanni M, Moes DJAR, Trocóniz IF, Ciccolini J, van Hasselt JGC. Clinical pharmacokinetics and pharmacodynamics of immune checkpoint inhibitors. *Clinical Pharmacokinetics*. 2019:1-23.
186. Robert C, Thomas L, Bondarenko I, O'day S, Weber J, Garbe C, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *New England Journal of Medicine*. 2011;364(26):2517-26.
187. Weber JS, D'Angelo SP, Minor D, Hodi FS, Gutzmer R, Neyns B, et al. Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial. *The Lancet Oncology*. 2015;16(4):375-84.
188. Larkin J, Minor D, D'Angelo S, Neyns B, Smylie M, Miller WH, et al. Overall survival in patients with advanced melanoma who received nivolumab versus investigator's choice chemotherapy in CheckMate 037: a randomized, controlled, open-label phase III trial. *Journal of Clinical Oncology*. 2018;36(4):383-90.
189. Wolchok JD, Chiarion-Sileni V, Gonzalez R, Rutkowski P, Grob J-J, Cowey CL, et al. Overall survival with combined nivolumab and ipilimumab in advanced melanoma. *New England Journal of Medicine*. 2017;377(14):1345-56.
190. Hodi FS, Chiarion-Sileni V, Gonzalez R, Grob J-J, Rutkowski P, Cowey CL, et al. Nivolumab plus ipilimumab or nivolumab alone versus ipilimumab alone in advanced melanoma (CheckMate 067): 4-year outcomes of a multicentre, randomised, phase 3 trial. *The Lancet Oncology*. 2018;19(11):1480-92.
191. Luke JJ. Comprehensive Clinical Trial Data Summation for BRAF-MEK Inhibition and Checkpoint Immunotherapy in Metastatic Melanoma. *The oncologist*. 2019:theoncologist-2018.
192. Nghiem PT, Bhatia S, Lipson EJ, Kudchadkar RR, Miller NJ, Annamalai L, et al. PD-1 Blockade with pembrolizumab in advanced Merkel-cell carcinoma. *New England Journal of Medicine*. 2016.
193. D'Angelo SP, Russell J, Lebbé C, Chmielowski B, Gambichler T, Grob J-J, et al. Efficacy and safety of first-line avelumab treatment in patients with stage IV metastatic Merkel cell carcinoma: a preplanned interim analysis of a clinical trial. *JAMA oncology*. 2018;4(9):e180077-e.
194. Kaufman HL, Russell J, Hamid O, Bhatia S, Terheyden P, D'Angelo SP, et al. Avelumab in patients with chemotherapy-refractory metastatic Merkel cell carcinoma: a multicentre, single-group, open-label, phase 2 trial. *The Lancet Oncology*. 2016;17(10):1374-85.
195. Topalian SL, Bhatia S, Hollebecque A, Awada A, De Boer JP, Kudchadkar RR, et al. Abstract CT074: non-comparative, open-label, multiple cohort, phase 1/2

study to evaluate nivolumab (NIVO) in patients with virus-associated tumors (CheckMate 358): efficacy and safety in Merkel cell carcinoma (MCC). *AACR*; 2017.

196. Lemos BD, Storer BE, Iyer JG, Phillips JL, Bichakjian CK, Fang LC, et al. Pathologic nodal evaluation improves prognostic accuracy in Merkel cell carcinoma: analysis of 5823 cases as the basis of the first consensus staging system. *Journal of the American Academy of Dermatology*. 2010;63(5):751-61.

197. Stang A, Becker JC, Nghiem P, Ferlay J. The association between geographic location and incidence of Merkel cell carcinoma in comparison to melanoma: An international assessment. *European journal of cancer*. 2018;94:47-60.

198. Youlden DR, Soyer HP, Youl PH, Fritschi L, Baade PD. Incidence and survival for Merkel cell carcinoma in Queensland, Australia, 1993-2010. *JAMA Dermatology*. 2014;150(8):864-72.

199. Garbutcheon-Singh KB, Curchin DJ, McCormack CJ, Smith SD. Trends in the incidence of Merkel cell carcinoma in Victoria, Australia, between 1986 and 2016. *Australasian Journal of Dermatology*. 2019.

200. Lanoy E, Dores GM, Madeleine MM, Toro JR, Fraumeni JF, Engels EA. Epidemiology of non-keratinocytic skin cancers among persons with AIDS in the United States. *Infectious Agents and Cancer*. 2009;4(S2):O10.

201. Clarke CA, Robbins HA, Tatalovich Z, Lynch CF, Pawlish KS, Finch JL, et al. Risk of merkel cell carcinoma after solid organ transplantation. *Journal of the National Cancer Institute*. 2015;107(2):dju382.

202. Brewer JD, Shanafelt TD, Otley CC, Roenigk RK, Cerhan JR, Kay NE, et al. Chronic lymphocytic leukemia is associated with decreased survival of patients with malignant melanoma and Merkel cell carcinoma in a SEER population-based study. *Journal of Clinical Oncology*. 2012;30(8):843-9.

203. Feng H, Shuda M, Chang Y, Moore PS. Clonal integration of a polyomavirus in human Merkel cell carcinoma. *Science*. 2008;319(5866):1096-100.

204. Wong SQ, Waldeck K, Vergara IA, Schröder J, Madore J, Wilmott JS, et al. UV-Associated Mutations Underlie the Etiology of MCV-Negative Merkel Cell Carcinomas. *Cancer Research*. 2015;75(24):5228.

205. Harms PW, Vats P, Verhaegen ME, Robinson DR, Wu Y-M, Dhanasekaran SM, et al. The distinctive mutational spectra of polyomavirus-negative Merkel cell carcinoma. *Cancer research*. 2015;75(18):3720-7.

206. Knepper TC, Montesion M, Russell JS, Sokol ES, Frampton GM, Miller VA, et al. The genomic landscape of Merkel cell carcinoma and clinicogenomic biomarkers of response to immune checkpoint Inhibitor therapy. *Clinical Cancer Research*. 2019;25(19):5961-71.

207. Iyer JG, Blom A, Doumani R, Lewis C, Tarabadkar ES, Anderson A, et al. Response rates and durability of chemotherapy among 62 patients with metastatic Merkel cell carcinoma. *Cancer medicine*. 2016;5(9):2294-301.

208. Cowey CL, Mahnke L, Espirito J, Helwig C, Oksen D, Bharmal M. Real-world treatment outcomes in patients with metastatic Merkel cell carcinoma treated with chemotherapy in the USA. *Future Oncology*. 2017;13(19):1699-710.

209. Becker JC, Lorenz E, Ugurel S, Eigentler TK, Kiecker F, Pföhler C, et al. Evaluation of real-world treatment outcomes in patients with distant metastatic Merkel cell carcinoma following second-line chemotherapy in Europe. *Oncotarget*. 2017;8(45):79731.

210. Nghiem P, Bhatia S, Lipson EJ, Sharfman WH, Kudchadkar RR, Brohl AS, et al. Durable tumor regression and overall survival in patients with advanced merkel

- cell carcinoma receiving pembrolizumab as first-line therapy. *Journal of Clinical Oncology*. 2019;37(9):693-702.
211. Topalian SL, Bhatia S, Kudchadkar RR, Amin A, Sharfman WH, Lebbe C, et al. Nivolumab (Nivo) as neoadjuvant therapy in patients with resectable Merkel cell carcinoma (MCC) in CheckMate 358. American Society of Clinical Oncology; 2018.
 212. Pondicherry A, Martin R, Meredith I, Rolfe J, Emanuel P, Elwood M. The burden of non-melanoma skin cancers in Auckland, New Zealand. *Australasian Journal of Dermatology*. 2018;59(3):210-3.
 213. Stratigos A, Garbe C, Lebbe C, Malvehy J, Del Marmol V, Pehamberger H, et al. Diagnosis and treatment of invasive squamous cell carcinoma of the skin: European consensus-based interdisciplinary guideline. *European Journal of Cancer*. 2015;51(14):1989-2007.
 214. Jarkowski Iii A, Hare R, Loud P, Skitzki JJ, Kane Iii JM, May KS, et al. Systemic therapy in advanced cutaneous squamous cell carcinoma (CSCC): The Roswell Park experience and a review of the literature. *American journal of Clinical Oncology*. 2016;39(6):545-8.
 215. Cranmer LD, Engelhardt C, Morgan SS. Treatment of unresectable and metastatic cutaneous squamous cell carcinoma. *The oncologist*. 2010;15(12):1320.
 216. Shin DM, Glisson BS, Khuri FR, Clifford JL, Clayman G, Benner SE, et al. Phase II and biologic study of interferon alfa, retinoic acid, and cisplatin in advanced squamous skin cancer. *Journal of Clinical Oncology*. 2002;20(2):364-70.
 217. Lippman SM, Parkinson DR, Itri LM, Weber RS, Schantz SP, Ota DM, et al. 13-cis-retinoic acid and interferon α -2a: effective combination. Therapy for advanced squamous cell carcinoma of the skin. *JNCI: Journal of the National Cancer Institute*. 1992;84(4):235-41.
 218. Migden MR, Rischin D, Schmults CD, Guminski A, Hauschild A, Lewis KD, et al. PD-1 blockade with cemiplimab in advanced cutaneous squamous-cell carcinoma. *New England Journal of Medicine*. 2018;379(4):341-51.
 219. Hillen U, Leiter U, Haase S, Kaufmann R, Becker J, Gutzmer R, et al. Advanced cutaneous squamous cell carcinoma: A retrospective analysis of patient profiles and treatment patterns—Results of a non-interventional study of the DeCOG. *European Journal of Cancer*. 2018;96:34-43.
 220. Maubec E, Petrow P, Scheer-Senyarich I, Duvillard P, Lacroix L, Gelly J, et al. Phase II study of cetuximab as first-line single-drug therapy in patients with unresectable squamous cell carcinoma of the skin. *Journal of Clinical Oncology*. 2011;29(25):3419-26.
 221. William Jr WN, Feng L, Ferrarotto R, Ginsberg L, Kies M, Lippman S, et al. Gefitinib for patients with incurable cutaneous squamous cell carcinoma: A single-arm phase II clinical trial. *Journal of the American Academy of Dermatology*. 2017;77(6):1110-3.
 222. Cavalieri S, Perrone F, Miceli R, Ascierto PA, Locati LD, Bergamini C, et al. Efficacy and safety of single-agent pan-human epidermal growth factor receptor (HER) inhibitor dacomitinib in locally advanced unresectable or metastatic skin squamous cell cancer. *European Journal of Cancer*. 2018;97:7-15.
 223. Foote MC, McGrath M, Guminski A, Hughes BGM, Meakin J, Thomson D, et al. Phase II study of single-agent panitumumab in patients with incurable cutaneous squamous cell carcinoma. *Annals of Oncology*. 2014;25(10):2047-52.

224. Gold KA, Kies MS, William Jr WN, Johnson FM, Lee JJ, Glisson BS. Erlotinib in the treatment of recurrent or metastatic cutaneous squamous cell carcinoma: A single-arm phase 2 clinical trial. *Cancer*. 2018;124(10):2169-73.
225. Migden MR, Khushalani NI, Chang ALS, Lewis KD, Schmults CD, Hernandez-Aya L, et al. Cemiplimab in locally advanced cutaneous squamous cell carcinoma: results from an open-label, phase 2, single-arm trial. *The Lancet Oncology*. 2020;21(2):294-305.
226. Tang J, Yu JX, Hubbard-Lucey VM, Neftelinov ST, Hodge JP, Lin Y. The clinical trial landscape for PD1/PDL1 immune checkpoint inhibitors. *Nature Publishing Group*; 2018.
227. Kirkwood JM, Strawderman MH, Ernstoff MS, Smith TJ, Borden EC, Blum RH. Interferon alfa-2b adjuvant therapy of high-risk resected cutaneous melanoma: the Eastern Cooperative Oncology Group Trial EST 1684. *Journal of Clinical Oncology*. 1996;14(1):7-17.
228. Eggermont AMM, Suci S, Santinami M, Testori A, Kruit WHJ, Marsden J, et al. Adjuvant therapy with pegylated interferon alfa-2b versus observation alone in resected stage III melanoma: final results of EORTC 18991, a randomised phase III trial. *The Lancet*. 2008;372(9633):117-26.
229. Eggermont AMM, Chiarion-Sileni V, Grob J-J, Dummer R, Wolchok JD, Schmidt H, et al. Prolonged Survival in Stage III Melanoma with Ipilimumab Adjuvant Therapy. *New England Journal of Medicine*. 2016;375(19):1845-55.
230. Weber J, Mandala M, Del Vecchio M, Gogas HJ, Arance AM, Cowey CL, et al. Adjuvant Nivolumab versus Ipilimumab in Resected Stage III or IV Melanoma. *New England Journal of Medicine*. 2017;377(19):1824-35.
231. Eggermont AMM, Blank CU, Mandala M, Long GV, Atkinson V, Dalle S, et al. Adjuvant Pembrolizumab versus Placebo in Resected Stage III Melanoma. *New England Journal of Medicine*. 2018;378(19):1789-801.
232. Colburn W, DeGruttola V, DeMets D, Downing G, Hoth D, Oates J, et al. Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework. Biomarkers Definitions Working Group. *Clinical Pharmacol & Therapeutics*. 2001;69:89-95.
233. Goossens N, Nakagawa S, Sun X, Hoshida Y. Cancer biomarker discovery and validation. *Translational cancer research*. 2015;4(3):256.
234. Poste G. Bring on the biomarkers. *Nature*. 2011;469(7329):156.
235. Manson G, Norwood J, Marabelle A, Kohrt H, Houot R. Biomarkers associated with checkpoint inhibitors. *Annals of Oncology*. 2016:mdw181.
236. Havel JJ, Chowell D, Chan TA. The evolving landscape of biomarkers for checkpoint inhibitor immunotherapy. *Nature Reviews Cancer*. 2019;19(3):133-50.
237. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Five-Year Survival and Correlates Among Patients With Advanced Melanoma, Renal Cell Carcinoma, or Non-Small Cell Lung Cancer Treated With Nivolumab. *JAMA Oncology*. 2019.
238. Tumei PC, Hellmann MD, Hamid O, Tsai KK, Loo KL, Gubens MA, et al. Liver metastasis and treatment outcome with anti-PD-1 monoclonal antibody in patients with melanoma and NSCLC. *Cancer immunology research*. 2017:canimm-0325.
239. Hauschild A, Larkin J, Ribas A, Dréno B, Flaherty KT, Ascierto PA, et al. Modeled prognostic subgroups for survival and treatment outcomes in BRAF V600-mutated metastatic melanoma: pooled analysis of 4 randomized clinical Trials. *JAMA oncology*. 2018;4(10):1382-8.

240. Ribas A, Puzanov I, Dummer R, Schadendorf D, Hamid O, Robert C, et al. Pembrolizumab versus investigator-choice chemotherapy for ipilimumab-refractory melanoma (KEYNOTE-002): a randomised, controlled, phase 2 trial. *The Lancet Oncology*. 2015;16(8):908-18.
241. Margolin K, Ernstoff MS, Hamid O, Lawrence D, McDermott D, Puzanov I, et al. Ipilimumab in patients with melanoma and brain metastases: an open-label, phase 2 trial. *The Lancet Oncology*. 2012;13(5):459-65.
242. Parakh S, Park JJ, Mendis S, Rai R, Xu W, Lo S, et al. Efficacy of anti-PD-1 therapy in patients with melanoma brain metastases. *British Journal of Cancer*. 2017;116(12):1558-63.
243. Long GV, Atkinson VG, Lo S, Sandhu SK, Brown M, Gonzalez M, et al. 13110Long-term outcomes from the randomized phase II study of nivolumab (nivo) or nivo+ipilimumab (ipi) in patients (pts) with melanoma brain metastases (mets): Anti-PD1 brain collaboration (ABC). *Annals of Oncology*. 2019;30(Supplement_5).
244. Long GV, Trefzer U, Davies MA, Kefford RF, Ascierto PA, Chapman PB, et al. Dabrafenib in patients with Val600Glu or Val600Lys BRAF-mutant melanoma metastatic to the brain (BREAK-MB): a multicentre, open-label, phase 2 trial. *The lancet oncology*. 2012;13(11):1087-95.
245. Eton O, Legha SS, Moon TE, Buzaid AC, Papadopoulos NE, Plager C, et al. Prognostic factors for survival of patients treated systemically for disseminated melanoma. *Journal of Clinical Oncology*. 1998;16(3):1103-11.
246. Weide B, Richter S, Büttner P, Leiter U, Forschner A, Bauer J, et al. Serum S100B, lactate dehydrogenase and brain metastasis are prognostic factors in patients with distant melanoma metastasis and systemic therapy. *PloS one*. 2013;8(11):e81624.
247. Diem S, Kasenda B, Martin-Liberal J, Lee A, Chauhan D, Gore M, et al. Prognostic score for patients with advanced melanoma treated with ipilimumab. *European Journal of Cancer*. 2015;51(18):2785-91.
248. Weide B, Martens A, Hassel JC, Berking C, Postow MA, Bisschop K, et al. Baseline biomarkers for outcome of melanoma patients treated with pembrolizumab. *Clinical Cancer Research*. 2016.
249. Long GV, Grob J-J, Nathan P, Ribas A, Robert C, Schadendorf D, et al. Factors predictive of response, disease progression, and overall survival after dabrafenib and trametinib combination treatment: a pooled analysis of individual patient data from randomised trials. *The Lancet Oncology*. 2016;17(12):1743-54.
250. Hauschild A, Larkin J, Ribas A, Dréno B, Flaherty KT, Ascierto PA, et al. Modeled Prognostic Subgroups for Survival and Treatment Outcomes in BRAF V600-Mutated Metastatic Melanoma: Pooled Analysis of 4 Randomized Clinical Trials. *JAMA Oncology*. 2018;4(10):1382-8.
251. Stanley KE. Prognostic factors for survival in patients with inoperable lung cancer. *Journal of the National Cancer Institute*. 1980;65(1):25-32.
252. Manola J, Atkins M, Ibrahim J, Kirkwood J. Prognostic factors in metastatic melanoma: a pooled analysis of Eastern Cooperative Oncology Group trials. *Journal of Clinical Oncology*. 2000;18(22):3782-93.
253. Pater JL, Loeb M. Nonanatomic prognostic factors in carcinoma of the lung. A multivariate analysis. *Cancer*. 1982;50(2):326-31.

254. Oken MM, Creech RH, Tormey DC, Horton J, Davis TE, McFadden ET, et al. Toxicity and response criteria of the Eastern Cooperative Oncology Group. *American journal of Clinical Oncology*. 1982;5(6):649-56.
255. Schnipper LE, Smith TJ, Raghavan D, Blayney DW, Ganz PA, Mulvey TM, et al. American Society of Clinical Oncology Identifies Five Key Opportunities to Improve Care and Reduce Costs: The Top Five List for Oncology. *Journal of Clinical Oncology*. 2012;30(14):1715-24.
256. Shaw AT, Yeap BY, Solomon BJ, Riely GJ, Gainor J, Engelman JA, et al. Effect of crizotinib on overall survival in patients with advanced non-small-cell lung cancer harbouring ALK gene rearrangement: a retrospective analysis. *The Lancet Oncology*. 2011;12(11):1004-12.
257. Robert C, Grob JJ, Stroyakovskiy D, Karaszewska B, Hauschild A, Levchenko E, et al. Five-Year Outcomes with Dabrafenib plus Trametinib in Metastatic Melanoma. *New England Journal of Medicine*. 2019;381(7):626-36.
258. McArthur GA, Puzanov I, Amaravadi R, Ribas A, Chapman P, Kim KB, et al. Marked, homogeneous, and early [18F] fluorodeoxyglucose-positron emission tomography responses to vemurafenib in BRAF-mutant advanced melanoma. *Journal of Clinical Oncology*. 2012;30(14):1628-34.
259. Wong A, Williams M, Milne D, Morris K, Lau P, Spruyt O, et al. Clinical and palliative care outcomes for patients of poor performance status treated with antiprogrammed death-1 monoclonal antibodies for advanced melanoma. *Asia-Pacific Journal of Clinical Oncology*. 2017;13(6):385-90.
260. Kartolo A, Sattar J, Sahai V, Baetz T, Lakoff JM. Predictors of immunotherapy-induced immune-related adverse events. *Current Oncology (Toronto, Ont)*. 2018;25(5):e403-e10.
261. Schadendorf D, Wolchok JD, Hodi FS, Chiarion-Sileni V, Gonzalez R, Rutkowski P, et al. Efficacy and safety outcomes in patients with advanced melanoma who discontinued treatment with nivolumab and ipilimumab because of adverse events: a pooled analysis of randomized phase II and III trials. *Journal of Clinical Oncology*. 2017;35(34):3807.
262. Freeman-Keller M, Kim Y, Cronin H, Richards A, Gibney G, Weber JS. Nivolumab in resected and unresectable metastatic melanoma: characteristics of immune-related adverse events and association with outcomes. *Clinical Cancer Research*. 2016;22(4):886-94.
263. Indini A, Di Guardo L, Cimminiello C, Prisciandaro M, Randon G, De Braud F, et al. Immune-related adverse events correlate with improved survival in patients undergoing anti-PD1 immunotherapy for metastatic melanoma. *Journal of Cancer Research and Clinical Oncology*. 2019;145(2):511-21.
264. Attia P, Phan GQ, Maker AV, Robinson MR, Quezado MM, Yang JC, et al. Autoimmunity correlates with tumor regression in patients with metastatic melanoma treated with anti-cytotoxic T-lymphocyte antigen-4. *Journal of Clinical Oncology: official journal of the American Society of Clinical Oncology*. 2005;23(25):6043.
265. Ku GY, Yuan J, Page DB, Schroeder SEA, Panageas KS, Carvajal RD, et al. Single-institution experience with ipilimumab in advanced melanoma patients in the compassionate use setting: lymphocyte count after 2 doses correlates with survival. *Cancer: Interdisciplinary International Journal of the American Cancer Society*. 2010;116(7):1767-75.

266. Martens A, Wistuba-Hamprecht K, Foppen MG, Yuan J, Postow MA, Wong P, et al. Baseline peripheral blood biomarkers associated with clinical outcome of advanced melanoma patients treated with ipilimumab. *Clinical Cancer Research*. 2016;22(12):2908-18.
267. Schindler K, Harmankaya K, Postow MA, Frantal S, Bello D, Ariyan CE, et al. Pretreatment levels of absolute and relative eosinophil count to improve overall survival (OS) in patients with metastatic melanoma under treatment with ipilimumab, an anti CTLA-4 antibody. *Journal of Clinical Oncology*. 2013;31(15_suppl):9024.
268. Ferrucci P, Gandini S, Battaglia A, Alfieri S, Di Giacomo A, Giannarelli D, et al. Baseline neutrophil-to-lymphocyte ratio is associated with outcome of ipilimumab-treated metastatic melanoma patients. *British journal of cancer*. 2015;112(12):1904-10.
269. Ferrucci PF, Gandini S, Battaglia A, Alfieri S, Di Giacomo AM, Giannarelli D, et al. Baseline neutrophil-to-lymphocyte ratio is associated with outcome of ipilimumab-treated metastatic melanoma patients. *British Journal of Cancer*. 2015;112(12):1904-10.
270. Garnier M, Zaragoza J, Bénétou N, Bens G, Meurisse V, Samimi M, et al. High neutrophil-to-lymphocyte ratio before starting anti-programmed cell death 1 immunotherapy predicts poor outcome in patients with metastatic melanoma. *Journal of the American Academy of Dermatology*. 2018;79(1):165-7.e2.
271. Di Giacomo AM, Calabrò L, Danielli R, Fonsatti E, Bertocci E, Pesce I, et al. Long-term survival and immunological parameters in metastatic melanoma patients who responded to ipilimumab 10 mg/kg within an expanded access programme. *Cancer Immunology, Immunotherapy*. 2013;62(6):1021-8.
272. Weide B, Martens A, Hassel JC, Berking C, Postow MA, Bisschop K, et al. Baseline Biomarkers for Outcome of Melanoma Patients Treated with Pembrolizumab. *Clinical Cancer Research*. 2016.
273. Weber JS, Tang H, Hippeli L, Qian M, Wind-Rotolo M, Larkin JMG, et al. Serum IL-6 and CRP as prognostic factors in melanoma patients receiving single agent and combination checkpoint inhibition. *Journal of Clinical Oncology*. 2019;37(15_suppl):100.
274. Joseph RW, Elassaiss-Schaap J, Kefford R, Hwu W-J, Wolchok JD, Joshua AM, et al. Baseline Tumor Size Is an Independent Prognostic Factor for Overall Survival in Patients with Melanoma Treated with Pembrolizumab. *Clinical Cancer Research*. 2018;24(20):4960.
275. Floberg JM, Zhang J, DeWees TA, Markovina S, Grigsby PW, Schwarz JK. Pre-Treatment [F-18]FDG-PET SUV_{max} as a Prognostic and Radiogenomic Marker in Cervical Cancer. *International Journal of Radiation Oncology • Biology • Physics*. 2018;102(3):S82-S3.
276. Nakaigawa N, Kondo K, Tateishi U, Minamimoto R, Kaneta T, Namura K, et al. FDG PET/CT as a prognostic biomarker in the era of molecular-targeting therapies: max SUV_{max} predicts survival of patients with advanced renal cell carcinoma. *BMC Cancer*. 2016;16(1):67.
277. Son SH, Kang SM, Jeong SY, Lee S-W, Lee S-J, Lee J, et al. Prognostic value of volumetric parameters measured by pretreatment 18F FDG PET/CT in patients with cutaneous malignant melanoma. *Clinical Nuclear Medicine*. 2016;41(6):e266-e73.

278. Bastiaannet E, Hoekstra OS, de Jong JR, Brouwers AH, Suurmeijer AJH, Hoekstra HJ. Prognostic value of the standardized uptake value for (18)F-fluorodeoxyglucose in patients with stage IIIB melanoma. *European Journal of Nuclear Medicine and Molecular Imaging*. 2012;39(10):1592-8.
279. Pampaloni MH, Ashworth MT, Algazi AP, Daud A. 18F-fluorodeoxyglucose PET/CT imaging for predicting treatment response in patients with advanced melanoma treated with anti-PD1 immunotherapy. *Journal of Clinical Oncology*; 2014; 32, no. 15_suppl: 3084-3084
280. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer*. 2009;45(2):228-47.
281. Wahl RL, Jacene H, Kasamon Y, Lodge MA. From RECIST to PERCIST: Evolving Considerations for PET response criteria in solid tumors. *Journal of Nuclear Medicine*. 2009;50 Suppl 1:122S-50S.
282. Pinker K, Riedl C, Weber WA. Evaluating tumor response with FDG PET: updates on PERCIST, comparison with EORTC criteria and clues to future developments. *European Journal of Nuclear Medicine and Molecular Imaging*. 2017;44(Suppl 1):55-66.
283. Bastiaannet E, Hoekstra O, De Jong J, Brouwers A, Suurmeijer A, Hoekstra H. Prognostic value of the standardized uptake value for 18 F-fluorodeoxyglucose in patients with stage IIIB melanoma. *European Journal of Nuclear Medicine and Molecular Imaging*. 2012;39(10):1592-8.
284. Im H-J, Pak K, Cheon GJ, Kang KW, Kim S-J, Kim I-J, et al. Prognostic value of volumetric parameters of 18 F-FDG PET in non-small-cell lung cancer: a meta-analysis. *European Journal of Nuclear Medicine and Molecular Imaging*. 2015;42(2):241-51.
285. McArthur GA, Callahan J, Ribas A, Gonzalez R, Pavlick AC, Hamid O, et al., editors. Metabolic tumor burden for prediction of overall survival following combined BRAF/MEK inhibition in patients with advanced BRAF mutant melanoma. *ASCO Annual Meeting Proceedings*; 2014.
286. Pak K, Kim S-J, Kim IJ, Kim DU, Kim K, Kim H. Impact of cytokines on diffuse splenic 18F-fluorodeoxyglucose uptake during positron emission tomography/computed tomography. *Nuclear Medicine Communications*. 2013;34(1):64-70.
287. Kim K, Kim S-J, Kim I-J, Kim DU, Kim H, Kim S, et al. Factors Associated with Diffusely Increased Splenic F-18 FDG Uptake in Patients with Cholangiocarcinoma. *Nuclear Medicine and Molecular Imaging*. 2014;48(2):137-43.
288. Pak K, Kim S, Kim I, Kim D, Kim K, Kim H, et al. Splenic FDG uptake predicts poor prognosis in patients with unresectable cholangiocarcinoma. *Nuklearmedizin*. 2014;53(2):26-31.
289. Sugawara Y, Zasadny KR, Kison PV, Baker LH, Wahl RL. Splenic fluorodeoxyglucose uptake increased by granulocyte colony-stimulating factor therapy: PET imaging results. *Journal of Nuclear Medicine*. 1999;40:1456-62.
290. Ribas A, Benz MR, Allen-Auerbach MS, Radu C, Chmielowski B, Seja E, et al. Imaging of CTLA4 blockade-induced cell replication with (18)F-FLT PET in patients with advanced melanoma treated with tremelimumab. *Journal of Nuclear Medicine*. 2010;51(3):340-6.
291. Ribas A, Kefford R, Marshall MA, Punt CJ, Haanen JB, Marmol M, et al. Phase III randomized clinical trial comparing tremelimumab with standard-of-care

chemotherapy in patients with advanced melanoma. *Journal of Clinical Oncology*. 2013;31(5):616-22.

292. Galon J, Pagès F, Marincola FM, Thurin M, Trinchieri G, Fox BA, et al. The immune score as a new possible approach for the classification of cancer. *Journal of Translational Medicine*. 2012;10(1):1.

293. Hamid O, Schmidt H, Nissan A, Ridolfi L, Aamdal S, Hansson J, et al. A prospective phase II trial exploring the association between tumor microenvironment biomarkers and clinical activity of ipilimumab in advanced melanoma. *Journal of Translational Medicine*. 2011;9(1):204.

294. Hodi FS, Butler M, Oble DA, Seiden MV, Haluska FG, Kruse A, et al. Immunologic and clinical effects of antibody blockade of cytotoxic T lymphocyte-associated antigen 4 in previously vaccinated cancer patients. *Proceedings of the National Academy of Sciences*. 2008;105(8):3005-10.

295. Neubert NJ, Schmittnaegel M, Bordry N, Nassiri S, Wald N, Martignier C, et al. T cell-induced CSF1 promotes melanoma resistance to PD1 blockade. *Science translational medicine*. 2018;10(436):eaan3311.

296. Tumeh PC, Harview CL, Yearley JH, Shintaku IP, Taylor EJ, Robert L, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature*. 2014;515(7528):568-71.

297. Teng MW, Ngiow SF, Ribas A, Smyth MJ. Classifying cancers based on T-cell infiltration and PD-L1. *Cancer Research*. 2015;75(11):2139-45.

298. Galon J, Bruni D. Approaches to treat immune hot, altered and cold tumours with combination immunotherapies. *Nature Reviews Drug Discovery*. 2019;18(3):197-218.

299. Chen P-L, Roh W, Reuben A, Cooper ZA, Spencer CN, Prieto PA, et al. Analysis of immune signatures in longitudinal tumor samples yields insight into biomarkers of response and mechanisms of resistance to immune checkpoint blockade. *Cancer Discovery*. 2016.

300. Postow MA, Cardona DM, Taube JM, Anders RA, Taylor CR, Wolchok JD, et al. Peripheral and tumor immune correlates in patients with advanced melanoma treated with nivolumab (anti-PD-1, BMS-936558, ONO-4538) monotherapy or in combination with ipilimumab. *Journal of Translational Medicine*. 2014;12(1):O8.

301. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *New England Journal of Medicine*. 2012;366(26):2443-54.

302. Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, et al. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *Journal of Clinical Oncology*. 2010;28(19):3167.

303. Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, et al. Nivolumab versus docetaxel in advanced nonsquamous non-small-cell lung cancer. *New England Journal of Medicine*. 2015;373(17):1627-39.

304. Rosenberg JE, Hoffman-Censits J, Powles T, Van Der Heijden MS, Balar AV, Necchi A, et al. Atezolizumab in patients with locally advanced and metastatic urothelial carcinoma who have progressed following treatment with platinum-based chemotherapy: a single-arm, multicentre, phase 2 trial. *The Lancet*. 2016;387(10031):1909-20.

305. Garon EB, Rizvi NA, Hui R, Leighl N, Balmanoukian AS, Eder JP, et al. Pembrolizumab for the treatment of non–small-cell lung cancer. *New England Journal of Medicine*. 2015;372(21):2018-28.
306. Reck M, Rodríguez-Abreu D, Robinson AG, Hui R, Csőszi T, Fülöp A, et al. Pembrolizumab versus Chemotherapy for PD-L1–Positive Non–Small-Cell Lung Cancer. *New England Journal of Medicine*. 2016;375(19):1823-33.
307. Motzer RJ, Escudier B, McDermott DF, George S, Hammers HJ, Srinivas S, et al. Nivolumab versus everolimus in advanced renal-cell carcinoma. *New England Journal of Medicine*. 2015;373(19):1803-13.
308. Motzer RJ, Tannir NM, McDermott DF, Arén Frontera O, Melichar B, Choueiri TK, et al. Nivolumab plus ipilimumab versus sunitinib in advanced renal-cell carcinoma. *New England Journal of Medicine*. 2018;378(14):1277-90.
309. Brahmer J, Reckamp KL, Baas P, Crinò L, Eberhardt WEE, Poddubskaya E, et al. Nivolumab versus docetaxel in advanced squamous-cell non–small-cell lung cancer. *New England Journal of Medicine*. 2015;373(2):123-35.
310. Hirsch FR, McElhinny A, Stanforth D, Ranger-Moore J, Jansson M, Kulangara K, et al. PD-L1 Immunohistochemistry Assays for Lung Cancer: Results from Phase 1 of the Blueprint PD-L1 IHC Assay Comparison Project. *Journal of Thoracic Oncology*. 2017;12(2):208-22.
311. Rimm DL, Han G, Taube JM, et al. A prospective, multi-institutional, pathologist-based assessment of 4 immunohistochemistry assays for pd-l1 expression in non–small cell lung cancer. *JAMA Oncology*. 2017;3(8):1051-8.
312. Sunshine JC, Nguyen PL, Kaunitz GJ, Cottrell TR, Berry S, Esandrio J, et al. PD-L1 Expression in Melanoma: A Quantitative Immunohistochemical Antibody Comparison. *Clinical Cancer Research*. 2017;23(16):4938.
313. Kluger HM, Zito CR, Barr ML, Baine MK, Chiang VLS, Sznol M, et al. Characterization of PD-L1 expression and associated T-cell infiltrates in metastatic melanoma samples from variable anatomic sites. *Clinical Cancer Research*. 2015;21(13):3052-60.
314. Mansfield AS, Aubry MC, Moser JC, Harrington SM, Dronca RS, Park SS, et al. Temporal and spatial discordance of programmed cell death-ligand 1 expression and lymphocyte tumor infiltration between paired primary lesions and brain metastases in lung cancer. *Annals of Oncology*. 2016;27(10):1953-8.
315. Madore J, Vilain RE, Menzies AM, Kakavand H, Wilmott JS, Hyman J, et al. PD-L1 expression in melanoma shows marked heterogeneity within and between patients: implications for anti-PD-1/PD-L1 clinical trials. *Pigment Cell & Melanoma Research*. 2014;28(3):245-53.
316. Kluger HM, Zito CR, Turcu G, Baine MK, Zhang H, Adeniran A, et al. PD-L1 studies across tumor types, its differential expression and predictive value in patients treated with immune checkpoint inhibitors. *Clinical Cancer Research*. 2017;23(15):4270-9.
317. Tang H, Liang Y, Anders RA, Taube JM, Qiu X, Mulgaonkar A, et al. PD-L1 on host cells is essential for PD-L1 blockade–mediated tumor regression. *The Journal of Clinical Investigation*. 2018;128(2):580-8.
318. Mariathasan S, Turley SJ, Nickles D, Castiglioni A, Yuen K, Wang Y, et al. TGFβ attenuates tumour response to PD-L1 blockade by contributing to exclusion of T cells. *Nature*. 2018;554(7693):544.
319. Kowanetz M, Zou W, Gettinger SN, Koeppen H, Kockx M, Schmid P, et al. Differential regulation of PD-L1 expression by immune and tumor cells in NSCLC

and the response to treatment with atezolizumab (anti-PD-L1). *Proceedings of the National Academy of Sciences*. 2018;115(43):E10119.

320. Bendall SC, Simonds EF, Qiu P, El-ad DA, Krutzik PO, Finck R, et al. Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. *Science*. 2011;332(6030):687-96.

321. Gide TN, Quek C, Menzies AM, Tasker AT, Shang P, Holst J, et al. Distinct immune cell populations define response to anti-PD-1 monotherapy and anti-PD-1/anti-CTLA-4 combined therapy. *Cancer Cell*. 2019;35(2):238-55.

322. Coulie PG, Van den Eynde BJ, van der Bruggen P, Boon T. Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy. *Nature Reviews Cancer*. 2014;14(2):135-46.

323. Heemskerk B, Kvistborg P, Schumacher TNM. The cancer antigenome. *The EMBO journal*. 2013;32(2):194-203.

324. Shukla SA, Bachireddy P, Schilling B, Galonska C, Zhan Q, Bango C, et al. Cancer-germline antigen expression discriminates clinical outcome to CTLA-4 blockade. *Cell*. 2018;173(3):624-33.

325. Tran E, Turcotte S, Gros A, Robbins PF, Lu Y-C, Dudley ME, et al. Cancer immunotherapy based on mutation-specific CD4⁺ T cells in a patient with epithelial cancer. *Science*. 2014;344(6184):641-5.

326. Tran E, Ahmadzadeh M, Lu Y-C, Gros A, Turcotte S, Robbins PF, et al. Immunogenicity of somatic mutations in human gastrointestinal cancers. *Science*. 2015;350(6266):1387-90.

327. Brahmer JR, Tykodi SS, Chow LQM, Hwu W-J, Topalian SL, Hwu P, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *New England Journal of Medicine*. 2012;366(26):2455-65.

328. Yarchoan M, Hopkins A, Jaffee EM. Tumor mutational burden and response rate to PD-1 inhibition. *The New England Journal of Medicine*. 2017;377(25):2500.

329. Snyder A, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, et al. Genetic basis for clinical response to CTLA-4 blockade in melanoma. *New England Journal of Medicine*. 2014;371(23):2189-99.

330. Van Allen EM, Miao D, Schilling B, Shukla SA, Blank C, Zimmer L, et al. Genomic correlates of response to CTLA-4 blockade in metastatic melanoma. *Science*. 2015;350(6257):207.

331. Riaz N, Havel JJ, Makarov V, Desrichard A, Urba WJ, Sims JS, et al. Tumor and Microenvironment Evolution during Immunotherapy with Nivolumab. *Cell*. 2017;171(4):934-49.e16.

332. Peng W, Chen JQ, Liu C, Malu S, Creasy C, Tetzlaff MT, et al. Loss of PTEN promotes resistance to T cell-mediated immunotherapy. *Cancer Discovery*. 2016;6(2):202-16.

333. Riaz N, Havel JJ, Kendall SM, Makarov V, Walsh LA, Desrichard A, et al. Recurrent SERPINB3 and SERPINB4 mutations in patients who respond to anti-CTLA4 immunotherapy. *Nature Genetics*. 2016;48(11):1327.

334. Gao J, Shi LZ, Zhao H, Chen J, Xiong L, He Q, et al. Loss of IFN- γ pathway genes in tumor cells as a mechanism of resistance to anti-CTLA-4 therapy. *Cell*. 2016;167(2):397-404.

335. Zaretsky JM, Garcia-Diaz A, Shin DS, Escuin-Ordinas H, Hugo W, Hu-Lieskovan S, et al. Mutations associated with acquired resistance to PD-1 blockade in melanoma. *New England Journal of Medicine*. 2016;375(9):819-29.

336. Shin DS, Zaretsky JM, Escuin-Ordinas H, Garcia-Diaz A, Hu-Lieskovan S, Kalbasi A, et al. Primary resistance to PD-1 blockade mediated by JAK1/2 mutations. *Cancer Discovery*. 2017;7(2):188-201.
337. Chowell D, Morris LGT, Grigg CM, Weber JK, Samstein RM, Makarov V, et al. Patient HLA class I genotype influences cancer response to checkpoint blockade immunotherapy. *Science*. 2018;359(6375):582-7.
338. Turajlic S, Litchfield K, Xu H, Rosenthal R, McGranahan N, Reading JL, et al. Insertion-and-deletion-derived tumour-specific neoantigens and the immunogenic phenotype: a pan-cancer analysis. *The Lancet Oncology*. 2017;18(8):1009-21.
339. Davoli T, Uno H, Wooten EC, Elledge SJ. Tumor aneuploidy correlates with markers of immune evasion and with reduced response to immunotherapy. *Science*. 2017;355(6322):eaaf8399.
340. Roh W, Chen P-L, Reuben A, Spencer CN, Prieto PA, Miller JP, et al. Integrated molecular analysis of tumor biopsies on sequential CTLA-4 and PD-1 blockade reveals markers of response and resistance. *Science Translational Medicine*. 2017;9(379):eaah3560.
341. Ayers M, Lunceford J, Nebozhyn M, Murphy E, Loboda A, Kaufman DR, et al. IFN- γ -related mRNA profile predicts clinical response to PD-1 blockade. *The Journal of Clinical Investigation*. 2017;127(8):2930-40.
342. Das R, Verma R, Sznol M, Boddupalli CS, Gettinger SN, Kluger H, et al. Combination Therapy with Anti-CTLA-4 and Anti-PD-1 Leads to Distinct Immunologic Changes In Vivo. *The Journal of Immunology*. 2015;194(3):950.
343. Ji R-R, Chasalow SD, Wang L, Hamid O, Schmidt H, Cogswell J, et al. An immune-active tumor microenvironment favors clinical response to ipilimumab. *Cancer Immunology, Immunotherapy*. 2012;61(7):1019-31.
344. Hugo W, Zaretsky JM, Sun L, Song C, Moreno BH, Hu-Lieskovan S, et al. Genomic and transcriptomic features of response to anti-PD-1 therapy in metastatic melanoma. *Cell*. 2016;165(1):35-44.
345. Jerby-Arnon L, Shah P, Cuoco MS, Rodman C, Su M-J, Melms JC, et al. A cancer cell program promotes T cell exclusion and resistance to checkpoint blockade. *Cell*. 2018;175(4):984-97.
346. Sade-Feldman M, Yizhak K, Bjorgaard SL, Ray JP, de Boer CG, Jenkins RW, et al. Defining T Cell States Associated with Response to Checkpoint Immunotherapy in Melanoma. *Cell*. 2018;175(4):998-1013. e20.
347. Tarhini AA, Edington H, Butterfield LH, Lin Y, Shuai Y, Tawbi H, et al. Immune monitoring of the circulation and the tumor microenvironment in patients with regionally advanced melanoma receiving neoadjuvant ipilimumab. *PloS one*. 2014;9(2):e87705.
348. de Coaña YP, Poschke I, Gentilcore G, Mao Y, Nyström M, Hansson J, et al. Ipilimumab treatment results in an early decrease in the frequency of circulating granulocytic myeloid-derived suppressor cells as well as their Arginase1 production. *Cancer Immunology Research*. 2013;1(3):158-62.
349. Kitano S, Postow MA, Ziegler CGK, Kuk D, Panageas KS, Cortez C, et al. Computational algorithm-driven evaluation of monocytic myeloid-derived suppressor cell frequency for prediction of clinical outcomes. *Cancer Immunology Research*. 2014;2(8):812-21.
350. Gandara DR, Paul SM, Kowanetz M, Schleifman E, Zou W, Li Y, et al. Blood-based tumor mutational burden as a predictor of clinical benefit in non-small-cell

- lung cancer patients treated with atezolizumab. *Nature Medicine*. 2018;24(9):1441.
351. Lee JH, Long GV, Boyd S, Lo S, Menzies AM, Tembe V, et al. Circulating tumour DNA predicts response to anti-PD1 antibodies in metastatic melanoma. *Annals of Oncology*. 2017;28(5):1130-6.
 352. Sanmamed MF, Fernández-Landázuri S, Rodríguez C, Zárata R, Lozano MD, Zubiri L, et al. Quantitative cell-free circulating BRAFV600E mutation analysis by use of droplet digital PCR in the follow-up of patients with melanoma being treated with BRAF inhibitors. *Clinical Chemistry*. 2015;61(1):297-304.
 353. Gray ES, Rizos H, Reid AL, Boyd SC, Pereira MR, Lo J, et al. Circulating tumor DNA to monitor treatment response and detect acquired resistance in patients with metastatic melanoma. *Oncotarget*. 2015;6(39):42008.
 354. Tsao SC-H, Weiss J, Hudson C, Christophi C, Cebon J, Behren A, et al. Monitoring response to therapy in melanoma by quantifying circulating tumour DNA with droplet digital PCR for BRAF and NRAS mutations. *Scientific Reports*. 2015;5:11198.
 355. Lee JH, Long GV, Boyd S, Lo S, Menzies AM, Tembe V, et al. Circulating tumour DNA predicts response to anti-PD1 antibodies in metastatic melanoma. *Annals of Oncology*. 2017;28(5):1130-6.
 356. Lee JH, Long GV, Menzies AM, Lo S, Guminski A, Whitbourne K, et al. Association between circulating tumor DNA and pseudoprogression in patients with metastatic melanoma treated with anti-programmed cell death 1 antibodies. *JAMA Oncology*. 2018;4(5):717-21.
 357. Ashida A, Sakaizawa K, Uhara H, Okuyama R. Circulating tumour DNA for monitoring treatment response to anti-PD-1 immunotherapy in melanoma patients. *Acta dermato-venereologica*. 2017;97(8-9):1212-8.
 358. Lim SY, Lee JH, Gide TN, Menzies AM, Guminski A, Carlino MS, et al. Circulating Cytokines Predict Immune-Related Toxicity in Melanoma Patients Receiving Anti-PD-1–Based Immunotherapy. *Clinical Cancer Research*. 2018.
 359. Sivan A, Corrales L, Hubert N, Williams JB, Aquino-Michaels K, Earley ZM, et al. Commensal *Bifidobacterium*; promotes antitumor immunity and facilitates anti-PD-L1 efficacy. *Science*. 2015;350(6264):1084.
 360. Vétizou M, Pitt JM, Daillère R, Lepage P, Waldschmitt N, Flament C, et al. Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. *Science*. 2015;350(6264):1079.
 361. Matson V, Fessler J, Bao R, Chongsuwat T, Zha Y, Alegre M-L, et al. The commensal microbiome is associated with anti-PD-1 efficacy in metastatic melanoma patients. *Science*. 2018;359(6371):104.
 362. Gopalakrishnan V, Spencer CN, Nezi L, Reuben A, Andrews MC, Karpinets TV, et al. Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. *Science*. 2018;359(6371):97-103.
 363. Pinato DJ, Howlett S, Ottaviani D, Urus H, Patel A, Mineo T, et al. Association of prior antibiotic treatment with survival and response to immune checkpoint inhibitor therapy in patients with cancer. *JAMA oncology*. 2019;5(12):1774-8.
 364. Tinsley N, Zhou C, Villa S, Tan G, Lorigan P, Blackhall FH, et al. Cumulative antibiotic use and efficacy of immune checkpoint inhibitors in patients with advanced cancer. *American Society of Clinical Oncology*; 2018.
 365. Elkrif A, El Raichani L, Richard C, Messaoudene M, Belkaid W, Malo J, et al. Antibiotics are associated with decreased progression-free survival of advanced

- melanoma patients treated with immune checkpoint inhibitors. *Oncoimmunology*. 2019;8(4):e1568812.
366. Kelderman S, Heemskerk B, van Tinteren H, van den Brom RR, Hospers GA, van den Eertwegh AJ, et al. Lactate dehydrogenase as a selection criterion for ipilimumab treatment in metastatic melanoma. *Cancer Immunology, Immunotherapy*. 2014;63(5):449-58.
367. Delyon J, Mateus C, Lefeuvre D, Lanoy E, Zitvogel L, Chaput N, et al. Experience in daily practice with ipilimumab for the treatment of patients with metastatic melanoma: an early increase in lymphocyte and eosinophil counts is associated with improved survival. *Annals of Oncology*. 2013;24(6):1697-703.
368. Schindler K, Harmankaya K, Kuk D, Mangana J, Michielin O, Hoeller C, et al. Correlation of absolute and relative eosinophil counts with immune-related adverse events in melanoma patients treated with ipilimumab. *Journal of Clinical Oncology*. 2014;32(15_suppl):9096.
369. Yuan J, Adamow M, Ginsberg BA, Rasalan TS, Ritter E, Gallardo HF, et al. Integrated NY-ESO-1 antibody and CD8+ T-cell responses correlate with clinical benefit in advanced melanoma patients treated with ipilimumab. *Proceedings of the National Academy of Sciences*. 2011;108(40):16723-8.
370. Postow MA, Manuel M, Wong P, Yuan J, Dong Z, Liu C, et al. Peripheral T cell receptor diversity is associated with clinical outcomes following ipilimumab treatment in metastatic melanoma. *Journal for Immunotherapy of Cancer*. 2015;3(1):23.
371. Ferrucci PF, Ascierto PA, Pigozzo J, Del Vecchio M, Maio M, Antonini Cappellini GC, et al. Baseline neutrophils and derived neutrophil-to-lymphocyte ratio: prognostic relevance in metastatic melanoma patients receiving ipilimumab. *Annals of Oncology*. 2016;27(4):732-8.
372. Hannani D, Vetizou M, Enot D, Rusakiewicz S, Chaput N, Klatzmann D, et al. Anticancer immunotherapy by CTLA-4 blockade: obligatory contribution of IL-2 receptors and negative prognostic impact of soluble CD25. *Cell Research*. 2015;25(2):208.
373. Ji R-R, Chasalow SD, Wang L, Hamid O, Schmidt H, Cogswell J, et al. An immune-active tumor microenvironment favors clinical response to ipilimumab. *Cancer Immunology, Immunotherapy*. 2012;61(7):1019-31.
374. Sade-Feldman M, Yizhak K, Bjorgaard SL, Ray JP, de Boer CG, Jenkins RW, et al. Defining T Cell States Associated with Response to Checkpoint Immunotherapy in Melanoma. *Cell*. 2018;175(4):998-1013.e20.
375. Prat A, Navarro A, Paré L, Reguart N, Galván P, Pascual T, et al. Immune-related gene expression profiling after PD-1 blockade in non-small cell lung carcinoma, head and neck squamous cell carcinoma, and melanoma. *Cancer Research*. 2017;77(13):3540-50.
376. Blank CU, Haanen JB, Ribas A, Schumacher TN. The “cancer immunogram”. *Science*. 2016;352(6286):658-60.
377. Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, et al. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science*. 2015;348(6230):124.
378. Wong A, Callahan J, Keyaerts M, Neyns B, Mangana J, Aberle S, et al. 18 F-FDG PET/CT based spleen to liver ratio associates with clinical outcome to ipilimumab in patients with metastatic melanoma. *Cancer Imaging*. 2020;20:1-12.

379. Weber JS, Gibney G, Sullivan RJ, Sosman JA, Slingluff CL, Jr., Lawrence DP, et al. Sequential administration of nivolumab and ipilimumab with a planned switch in patients with advanced melanoma (CheckMate 064): an open-label, randomised, phase 2 trial. *The Lancet Oncology*. 2016;17(7):943-55.
380. Halse H, Colebatch AJ, Petrone P, Henderson MA, Mills JK, Snow H, et al. Multiplex immunohistochemistry accurately defines the immune context of metastatic melanoma. *Scientific Reports*. 2018;8(1):11158.
381. Koelzer VH, Sirinukunwattana K, Rittscher J, Mertz KD. Precision immunoprofiling by image analysis and artificial intelligence. *Virchows Archiv*. 2019;474(4):511-22.
382. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 2010;26(1):139-40.
383. Bindea G, Mlecnik B, Tosolini M, Kirilovsky A, Waldner M, Obenauf Anna C, et al. Spatiotemporal Dynamics of Intratumoral Immune Cells Reveal the Immune Landscape in Human Cancer. *Immunity*. 2013;39(4):782-95.
384. Cesano A. nCounter® PanCancer immune profiling panel (NanoString technologies, Inc., Seattle, WA). *Journal for immunotherapy of cancer*. 2015;3(1):42.
385. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome research*. 2010;20(9):1297-303.
386. DePristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, Hartl C, et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nature Genetics*. 2011;43(5):491.
387. Koboldt DC, Zhang Q, Larson DE, Shen D, McLellan MD, Lin L, et al. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome research*. 2012;22(3):568-76.
388. Ramaswami G, Li JB. RADAR: a rigorously annotated database of A-to-I RNA editing. *Nucleic acids research*. 2013;42(D1):D109-D13.
389. Pedersen BS, Quinlan AR. Mosdepth: quick coverage calculation for genomes and exomes. *Bioinformatics*. 2017;34(5):867-8.
390. Law CW, Chen Y, Shi W, Smyth GK. voom: Precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome biology*. 2014;15(2):R29.
391. Zhou Y, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, et al. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. *Nature communications*. 2019;10(1):1-10.
392. Prigerson HG, Bao Y, Shah MA, Paulk ME, LeBlanc TW, Schneider BJ, et al. Chemotherapy Use, Performance Status, and Quality of Life at the End of Life. *JAMA oncology*. 2015.
393. Kolata G. 'Desperation Oncology': When Patients Are Dying, Some Cancer Doctors Turn to Immunotherapy, in *New York Times*. The New York Times Company New York, NY; 2018.
394. Temel JS, Gainor JF, Sullivan RJ, Greer JA. Keeping Expectations in Check With Immune Checkpoint Inhibitors. *Journal of Clinical Oncology*. 2018;JCO. 2017.76. 146.
395. LeBlanc TW, Temel JS, Helft PR. "How much time do I have?": communicating prognosis in the era of exceptional responders. *American Society of Clinical Oncology Educational Book*. 2018;38:787-94.

396. Chapman PB, D'Angelo SP, Wolchok JD. Rapid Eradication of a Bulky Melanoma Mass with One Dose of Immunotherapy. *New England Journal of Medicine*. 2015.
397. Schadendorf D, Ascierto PA, Haanen J, Espinosa E, Demidov L, Garbe C, et al. Safety and efficacy of nivolumab in challenging subgroups with advanced melanoma who progressed on or after ipilimumab treatment: A single-arm, open-label, phase II study (CheckMate 172). *European Journal of Cancer*. 2019;121:144-53.
398. Wong A, Fullerton S, Spruyt O, Brady B, McArthur G, Sandhu S. Integration of immuno-oncology and palliative care. *Journal of Clinical Oncology*. 2016;34(13):1561-2.
399. Johnson DB, Sullivan RJ, Menzies AM. Immune checkpoint inhibitors in challenging populations. *Cancer*. 2017;123(11):1904-11.
400. Yennurajalingam S, Frisbee-Hume S, Palmer JL, Delgado-Guay MO, Bull J, Phan AT, et al. Reduction of cancer-related fatigue with dexamethasone: a double-blind, randomized, placebo-controlled trial in patients with advanced cancer. *Journal of Clinical Oncology*. 2013;31(25):3076-82.
401. Sloan AE, Nock CJ, Einstein DB. Diagnosis and Treatment of melanoma Brain Metastasis: A Literature Review. *Cancer Control*. 2009;16(3):248-55.
402. Agarwala SS, Kirkwood JM, Gore M, Dreno B, Thatcher N, Czarnetski B, et al. Temozolomide for the treatment of brain metastases associated with metastatic melanoma: a phase II study. *Journal of Clinical Oncology*. 2004;22(11):2101-7.
403. Margolin K, Ernstoff MS, Hamid O, Lawrence D, McDermott D, Puzanov I, et al. Ipilimumab in patients with melanoma and brain metastases: an open-label, phase 2 trial. *The Lancet Oncology*. 2012;13(5):459-65.
404. Goldberg SB, Gettinger SN, Mahajan A, Chiang AC, Herbst RS, Sznol M, et al. Pembrolizumab for patients with melanoma or non-small-cell lung cancer and untreated brain metastases: early analysis of a non-randomised, open-label, phase 2 trial. *The Lancet Oncology*. 2016;17(7):976-83.
405. Vosoughi E, Lee JM, Miller JR, Nosrati M, Minor DR, Abendroth R, et al. Survival and clinical outcomes of patients with melanoma brain metastasis in the era of checkpoint inhibitors and targeted therapies. *BMC Cancer*. 2018;18(1):490.
406. Hefler LA, Concin N, Hofstetter G, Marth C, Mustea A, Sehouli J, et al. Serum C-Reactive Protein as Independent Prognostic Variable in Patients with Ovarian Cancer. *Clinical Cancer Research*. 2008;14(3):710.
407. Erlinger TP, Platz EA, Rifai N, Helzlsouer KJ. C-reactive protein and the risk of incident colorectal cancer. *JAMA*. 2004;291(5):585-90.
408. Hashimoto K, Ikeda Y, Korenaga D, Tanoue K, Hamatake M, Kawasaki K, et al. The impact of preoperative serum C-reactive protein on the prognosis of patients with hepatocellular carcinoma. *Cancer*. 2005;103(9):1856-64.
409. Shimada H, Nabeya Y, Okazumi SI, Matsubara H, Shiratori T, Aoki T, et al. Elevation of preoperative serum C-reactive protein level is related to poor prognosis in esophageal squamous cell carcinoma. *Journal of Surgical Oncology*. 2003;83(4):248-52.
410. Fang S, Wang Y, Sui D, Liu H, Ross MI, Gershenwald JE, et al. C-reactive protein as a marker of melanoma progression. *Journal of Clinical Oncology : official journal of the American Society of Clinical Oncology*. 2015;33(12):1389-96.

411. Allin KH, Bojesen SE, Nordestgaard BG. Baseline C-Reactive Protein Is Associated With Incident Cancer and Survival in Patients With Cancer. *Journal of Clinical Oncology*. 2009;27(13):2217-24.
412. Deichmann M, Kahle B, Moser K, Wacker J, Wüst K. Diagnosing melanoma patients entering American Joint Committee on Cancer stage IV, C-reactive protein in serum is superior to lactate dehydrogenase. *British Journal of Cancer*. 2004;91(4):699-702.
413. Cassidy MR, Wolchok RE, Zheng J, Panageas KS, Wolchok JD, Coit D, et al. Neutrophil to Lymphocyte Ratio is Associated With Outcome During Ipilimumab Treatment. *EBioMedicine*. 2017;18:56-61.
414. Black S, Kushner I, Samols D. C-reactive protein. *Journal of Biological Chemistry*. 2004;279(47):48487-90.
415. Castell JV, Gómez-Lechón MJ, David M, Andus T, Geiger T, Trullenque R, et al. Interleukin-6 is the major regulator of acute phase protein synthesis in adult human hepatocytes. *FEBS letters*. 1989;242(2):237-9.
416. Moshage HJ, Roelofs HMJ, Van Pelt JF, Hazenberg BPC, Van Leeuwen MA, Limburg PC, et al. The effect of interleukin-1, interleukin-6 and its interrelationship on the synthesis of serum amyloid A and C-reactive protein in primary cultures of adult human hepatocytes. *Biochemical and Biophysical Research Communications*. 1988;155(1):112-7.
417. Kishimoto T. The biology of interleukin-6. *Blood*. 1989;74(1):1-10.
418. Hirano T. Interleukin-6 and its relation to inflammation and disease. *Clinical Immunology and Immunopathology*. 1992;62(1):S60-S5.
419. Colombo MP, Maccalli C, Mattei S, Melani C, Radrizzani M, Parmiani G. Expression of cytokine genes, including IL-6, in human malignant melanoma cell lines. *Melanoma Research*. 1992;2(3):181-9.
420. Betof AS, Nipp RD, Giobbie-Hurder A, Johnpulle RAN, Rubin K, Rubinstein SM, et al. Impact of Age on Outcomes with Immunotherapy for Patients with Melanoma. *The Oncologist*. 2017;22(8):963-71.
421. Postow MA, Chesney J, Pavlick AC, Robert C, Grossmann K, McDermott D, et al. Nivolumab and ipilimumab versus ipilimumab in untreated melanoma. *New England Journal of Medicine*. 2015;372(21):2006-17.
422. McQuade JL, Daniel CR, Hess KR, Mak C, Wang DY, Rai RR, et al. Association of body-mass index and outcomes in patients with metastatic melanoma treated with targeted therapy, immunotherapy, or chemotherapy: a retrospective, multicohort analysis. *The Lancet Oncology*. 2018;19(3):310-22.
423. Haanen J, Carbonnel F, Robert C, Kerr KM, Peters S, Larkin J, et al. Management of toxicities from immunotherapy: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of Oncology*. 2017;28(suppl_4):iv119-iv42.
424. Brahmer JR, Lacchetti C, Schneider BJ, Atkins MB, Brassil KJ, Caterino JM, et al. Management of Immune-Related Adverse Events in Patients Treated With Immune Checkpoint Inhibitor Therapy: American Society of Clinical Oncology Clinical Practice Guideline. *Journal of Clinical Oncology*. 2018;36(17):1714-68.
425. Hopkins AM, Rowland A, Kichenadasse G, Wiese MD, Gurney H, McKinnon RA, et al. Predicting response and toxicity to immune checkpoint inhibitors using routinely available blood and clinical markers. *British journal of cancer*. 2017;117(7):913-20.

426. Simeone E, Grimaldi AM, Festino L, Giannarelli D, Vanella V, Palla M, et al. Correlation between previous treatment with BRAF inhibitors and clinical response to pembrolizumab in patients with advanced melanoma. *Oncoimmunology*. 2017;6(3):e1283462-e.
427. Amini-Adle M, Khanafer N, Le-Bouar M, Duru G, Dalle S, Thomas L. Ineffective anti PD-1 therapy after BRAF inhibitor failure in advanced melanoma. *BMC Cancer*. 2018;18(1):705.
428. Kelloff GJ, Hoffman JM, Johnson B, Scher HI, Siegel BA, Cheng EY, et al. Progress and Promise of FDG-PET Imaging for Cancer Patient Management and Oncologic Drug Development. *Clinical Cancer Research*. 2005;11(8):2785-808.
429. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *science*. 2009;324(5930):1029-33.
430. Kim J-w, Dang CV. Cancer's molecular sweet tooth and the Warburg effect. *Cancer research*. 2006;66(18):8927-30.
431. Warburg O. On the Origin of Cancer Cells. *Science*. 1956;123(3191):309-14.
432. Lewin J, Sayers L, Kee D, Walpole I, Sanelli A, te Marvelde L, et al. Surveillance imaging with FDG-PET/CT in the post-operative follow-up of stage 3 melanoma. *Annals of Oncology*. 2018;29(7):1569-74.
433. Xing Y, Bronstein Y, Ross MI, Askew RL, Lee JE, Gershenwald JE, et al. Contemporary Diagnostic Imaging Modalities for the Staging and Surveillance of Melanoma Patients: a Meta-analysis. *JNCI: Journal of the National Cancer Institute*. 2011;103(2):129-42.
434. Bastiaannet E, Wobbes T, Hoekstra OS, van der Jagt EJ, Brouwers AH, Koelemij R, et al. Prospective Comparison of [18F]Fluorodeoxyglucose Positron Emission Tomography and Computed Tomography in Patients With Melanoma With Palpable Lymph Node Metastases: Diagnostic Accuracy and Impact on Treatment. *Journal of Clinical Oncology*. 2009;27(28):4774-80.
435. Oyama N, Akino H, Suzuki Y, Kanamaru H, Miwa Y, Tsuka H, et al. Prognostic Value of 2-Deoxy-2-[F-18]Fluoro-D-Glucose Positron Emission Tomography Imaging for Patients with Prostate Cancer. *Molecular Imaging & Biology*. 2002;4(1):99-104.
436. Oshida M, Uno K, Suzuki M, Nagashima T, Hashimoto H, Yagata H, et al. Predicting the prognoses of breast carcinoma patients with positron emission tomography using 2-deoxy-2-fluoro[18F]-D-glucose. *Cancer*. 1998;82(11):2227-34.
437. Forschner A, Olthof S-C, Gückel B, Martus P, Vach W, la Fougère C, et al. Impact of 18F-FDG-PET/CT on surgical management in patients with advanced melanoma: an outcome based analysis. *European Journal of Nuclear Medicine and Molecular Imaging*. 2017;44(8):1312-8.
438. Gulec SA, Faries MB, Lee CC, Kirgan D, Glass C, Morton DL, et al. The role of fluorine-18 deoxyglucose positron emission tomography in the management of patients with metastatic melanoma: impact on surgical decision making. *Clinical Nuclear Medicine*. 2003;28(12):961-5.
439. Krug B, Crott R, Lonneux M, Baurain J-F, Pirson A-S, Vander Borgh T. Role of PET in the Initial Staging of Cutaneous Malignant Melanoma: Systematic Review. *Radiology*. 2008;249(3):836-44.
440. Guo C, Lewin J, Shackleton M. Personalised surveillance after treatment for high-risk cancer. *Oncotarget*. 2019;10(7):694-5.

441. Bauml JM, Troxel A, Epperson CN, Cohen RB, Schmitz K, Stricker C, et al. Scan-associated distress in lung cancer: Quantifying the impact of "scanxiety". *Lung cancer (Amsterdam, Netherlands)*. 2016;100:110-3.
442. Mac Manus MP, Hicks RJ, Matthews JP, McKenzie A, Rischin D, Salminen EK, et al. Positron emission tomography is superior to computed tomography scanning for response-assessment after radical radiotherapy or chemoradiotherapy in patients with non-small-cell lung cancer. *Journal of Clinical Oncology*. 2003;21(7):1285-92.
443. Choi NC, Fischman AJ, Niemierko A, Ryu J-S, Lynch T, Wain J, et al. Dose-response relationship between probability of pathologic tumor control and glucose metabolic rate measured with FDG PET after preoperative chemoradiotherapy in locally advanced non-small-cell lung cancer. *International Journal of Radiation Oncology Biology Physics*. 2002;54(4):1024-35.
444. Ryu J-S, Choi NC, Fischman AJ, Lynch TJ, Mathisen DJ. FDG-PET in staging and restaging non-small cell lung cancer after neoadjuvant chemoradiotherapy: correlation with histopathology. *Lung Cancer*. 2002;35(2):179-87.
445. Hutchings M, Loft A, Hansen M, Pedersen LM, Buhl T, Jurlander J, et al. FDG-PET after two cycles of chemotherapy predicts treatment failure and progression-free survival in Hodgkin lymphoma. *Blood*. 2006;107(1):52-9.
446. Kostakoglu L, Goldsmith SJ. 18F-FDG PET evaluation of the response to therapy for lymphoma and for breast, lung, and colorectal carcinoma. *Journal of Nuclear Medicine*. 2003;44(2):224-39.
447. Spaepen K, Stroobants S, Dupont P, Van Steenweghen S, Thomas J, Vandenberghe P, et al. Prognostic value of positron emission tomography (PET) with fluorine-18 fluorodeoxyglucose ([18F] FDG) after first-line chemotherapy in non-Hodgkin's lymphoma: is [18F] FDG-PET a valid alternative to conventional diagnostic methods? *Journal of Clinical Oncology*. 2001;19(2):414-9.
448. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, et al. New Guidelines to Evaluate the Response to Treatment in Solid Tumors. *JNCI: Journal of the National Cancer Institute*. 2000;92(3):205-16.
449. Young H, Baum R, Cremerius U, Herholz K, Hoekstra O, Lammertsma AA, et al. Measurement of clinical and subclinical tumour response using [18F]-fluorodeoxyglucose and positron emission tomography: review and 1999 EORTC recommendations. *European Journal of Cancer*. 1999;35(13):1773-82.
450. Strobel K, Dummer R, Steinert HC, Conzett KB, Schad K, Lago MP, et al. Chemotherapy response assessment in stage IV melanoma patients—comparison of 18F-FDG-PET/CT, CT, brain MRI, and tumormarker S-100B. *European Journal of Nuclear Medicine and Molecular Imaging*. 2008;35(10):1786.
451. Zheng J, Menda Y, Graham M, Milhem M. F-18 FDG PET/CT evaluation of treatment response to ipilimumab therapy in advanced stage melanoma. *Journal of Nuclear Medicine*. 2015;56(supplement 3):1464.
452. Wolchok JD, Hoos A, O'Day S, Weber JS, Hamid O, Lebbé C, et al. Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. *Clinical Cancer Research*. 2009;15(23):7412-20.
453. Hodi FS, Hwu W-J, Kefford R, Weber JS, Daud A, Hamid O, et al. Evaluation of immune-related response criteria and RECIST v1. 1 in patients with advanced melanoma treated with pembrolizumab. *Journal of Clinical Oncology*. 2016;JCO640391.

454. Chiou VL, Burotto M. Pseudoprogression and Immune-Related Response in Solid Tumors. *Journal of Clinical Oncology*. 2015.
455. Nishino M, Gargano M, Suda M, Ramaiya NH, Hodi FS. Optimizing immune-related tumor response assessment: does reducing the number of lesions impact response assessment in melanoma patients treated with ipilimumab? *Journal for ImmunoTherapy of Cancer*. 2014;2(1):17.
456. Nishino M. Immune-related response evaluations during immune-checkpoint inhibitor therapy: establishing a “common language” for the new arena of cancer treatment. *Journal for ImmunoTherapy of Cancer*. 2016;4(1):30.
457. Wong ANM, McArthur GA, Hofman MS, Hicks RJ. The Advantages and Challenges of Using FDG PET/CT for Response Assessment in Melanoma in the Era of Targeted Agents and Immunotherapy. *European Journal of Nuclear Medicine and Molecular Imaging*. 2017;44(1):67-77.
458. Ito K, Schöder H, Teng R, Humm JL, Ni A, Wolchok JD, et al. Prognostic value of baseline metabolic tumor volume measured on 18F-fluorodeoxyglucose positron emission tomography/computed tomography in melanoma patients treated with ipilimumab therapy. *European Journal of Nuclear Medicine and Molecular Imaging*. 2019;46(4):930-9.
459. Seban R-D, Nemer JS, Marabelle A, Yeh R, Deutsch E, Ammari S, et al. Prognostic and theranostic 18F-FDG PET biomarkers for anti-PD1 immunotherapy in metastatic melanoma: association with outcome and transcriptomics. *European Journal of Nuclear Medicine and Molecular Imaging*. 2019;46(11):2298-310.
460. Unal I. Defining an optimal cut-point value in ROC analysis: an alternative approach. *Computational and mathematical methods in medicine*. 2017;2017.
461. Zou Kelly H, O'Malley AJ, Mauri L. Receiver-Operating Characteristic Analysis for Evaluating Diagnostic Tests and Predictive Models. *Circulation*. 2007;115(5):654-7.
462. Kim SY, Moon CM, Yoon H-J, Kim BS, Lim JY, Kim TO, et al. Diffuse splenic FDG uptake is predictive of clinical outcomes in patients with rectal cancer. *Scientific Reports*. 2019;9(1):1313.
463. Zasadny KR, Wahl RL. Standardized uptake values of normal tissues at PET with 2-[fluorine-18]-fluoro-2-deoxy-D-glucose: variations with body weight and a method for correction. *Radiology*. 1993;189(3):847-50.
464. Liu Y. Clinical significance of diffusely increased splenic uptake on FDG-PET. *Nuclear medicine communications*. 2009;30(10):763-9.
465. Ribas A, Benz MR, Allen-Auerbach MS, Radu C, Chmielowski B, Seja E, et al. Imaging of CTLA4 Blockade-Induced Cell Replication with 18F-FLT PET in Patients with Advanced Melanoma Treated with Tremelimumab. *Journal of Nuclear Medicine*. 2010;51(3):340-6.
466. Mebius RE, Kraal G. Structure and function of the spleen. *Nature Reviews Immunology*. 2005;5(8):606-16.
467. Nam H-Y, Kim S-J, Kim I-J, Kim B-S, Pak K, Kim K. The clinical implication and prediction of diffuse splenic FDG uptake during cancer surveillance. *Clinical Nuclear Medicine*. 2010;35(10):759-63.
468. Johnson DB, Balko JM. Biomarkers for Immunotherapy Toxicity: Are Cytokines the Answer? *Clinical Cancer Research*. 2019;25(5):1452.
469. Zincirkeser S, Şahin E, Halac M, Sager S. Standardized uptake values of normal organs on 18F-fluorodeoxyglucose positron emission tomography and

- computed tomography imaging. *Journal of International Medical Research*. 2007;35(2):231-6.
470. Hu C, Pang B, Lin G, Zhen Y, Yi H. Energy metabolism manipulates the fate and function of tumour myeloid-derived suppressor cells. *British Journal of Cancer*. 2019.
471. Weber J, Gibney G, Kudchadkar R, Yu B, Cheng P, Martinez AJ, et al. Phase I/II study of metastatic melanoma patients treated with nivolumab who had progressed after ipilimumab. *Cancer Immunology Research*. 2016;4(4):345-53.
472. Daud AI, Wolchok JD, Robert C, Hwu W-J, Weber JS, Ribas A, et al. Programmed Death-Ligand 1 Expression and Response to the Anti-Programmed Death 1 Antibody Pembrolizumab in Melanoma. *Journal of Clinical Oncology*. 2016;34(34):4102-9.
473. Kalff V, Hicks RJ, Ware RE, Greer B, Binns DS, Hogg A. Evaluation of high-risk melanoma: comparison of [18 F] FDG PET and high-dose 67 Ga SPET. *European Journal of Nuclear Medicine and Molecular Imaging*. 2002;29(4):506-15.
474. Hofman MS, Hicks RJ. How we read oncologic FDG PET/CT. *Cancer Imaging*. 2016;16(1):35.
475. Quak E, Le Roux P-Y, Hofman MS, Robin P, Bourhis D, Callahan J, et al. Harmonizing FDG PET quantification while maintaining optimal lesion detection: prospective multicentre validation in 517 oncology patients. *European Journal of Nuclear Medicine and Molecular Imaging*. 2015;42(13):2072-82.
476. Sugawara Y, Zasadny KR, Neuhoff AW, Wahl RL. Reevaluation of the standardized uptake value for FDG: variations with body weight and methods for correction. *Radiology*. 1999;213(2):521-5.
477. Salaun PY, Gastine T, Campion L, Cambefort P, Bodet-Milin C, Moreau A, et al. Analysis of FDG PET Diffuse Bone Marrow Uptake in Staging of Hodgkin's Disease: A Reflect of Disease Infiltration or Just Inflammation? : *American Society of Hematology*; 2008.
478. Valpione S, Gremel G, Mundra P, Middlehurst P, Galvani E, Girotti MR, et al. Plasma total cell-free DNA (cfDNA) is a surrogate biomarker for tumour burden and a prognostic biomarker for survival in metastatic melanoma patients. *European Journal of Cancer*. 2018;88:1-9.
479. Carlino MS, Saunders CA, Haydu LE, Menzies AM, Curtis CM, Lebowitz PF, et al. 18 F-labelled fluorodeoxyglucose-positron emission tomography (FDG-PET) heterogeneity of response is prognostic in dabrafenib treated BRAF mutant metastatic melanoma. *European Journal of Cancer*. 2013;49(2):395-402.
480. Fredrickson J, Callahan J, Funke R, Sanabria S, Weber W, de Crespigny A, et al. Utility of FDG-PET in immunotherapy: results from a Phase II study of NSCLC patients undergoing therapy with the PD-L1 inhibitor, atezolizumab (MPDL3280A). *Journal of Nuclear Medicine*. 2016;57(supplement 2):134.
481. Tan AC, Emmett L, Lo S, Liu V, Kapoor R, Carlino MS, et al. FDG-PET response and outcome from anti-PD-1 therapy in metastatic melanoma. *Annals of Oncology*. 2018;29(10):2115-20.
482. Cho SY, Lipson EJ, Im H-J, Rowe SP, Gonzalez EM, Blackford A, et al. Prediction of response to immune checkpoint inhibitor therapy using early-time-point 18F-FDG PET/CT imaging in patients with advanced melanoma. *Journal of Nuclear Medicine*. 2017;58(9):1421-8.

483. Wolchok JD, Hoos A, Day S, Weber JS, Hamid O, Lebbé C, et al. Guidelines for the Evaluation of Immune Therapy Activity in Solid Tumors: Immune-Related Response Criteria. *Clinical Cancer Research*. 2009;15(23):7412.
484. Parra ER, Uraoka N, Jiang M, Cook P, Gibbons D, Forget M-A, et al. Validation of multiplex immunofluorescence panels using multispectral microscopy for immune-profiling of formalin-fixed and paraffin-embedded human tumor tissues. *Scientific reports*. 2017;7(1):1-11.
485. Parra ER, Jiang M, Solis L, Mino B, Laberiano C, Hernandez S, et al. Procedural Requirements and Recommendations for Multiplex Immunofluorescence Tyramide Signal Amplification Assays to Support Translational Oncology Studies. *Cancers*. 2020;12(2):255.
486. Kaunitz GJ, Cottrell TR, Lilo M, Muthappan V, Esandrio J, Berry S, et al. Melanoma subtypes demonstrate distinct PD-L1 expression profiles. *Laboratory investigation*. 2017;97(9):1063-71.
487. Paulson KG, Iyer JG, Tegeder AR, Thibodeau R, Schelter J, Koba S, et al. Transcriptome-Wide Studies of Merkel Cell Carcinoma and Validation of Intratumoral CD8+ Lymphocyte Invasion As an Independent Predictor of Survival. *Journal of Clinical Oncology*. 2011;29(12):1539-46.
488. Lipson EJ, Vincent JG, Loyo M, Kagohara LT, Lubner BS, Wang H, et al. PD-L1 Expression in the Merkel Cell Carcinoma Microenvironment: Association with Inflammation, Merkel Cell Polyomavirus, and Overall Survival. *Cancer Immunology Research*. 2013;1(1):54.
489. Azzimonti B, Zavattaro E, Provati M, Vidali M, Conca A, Catalano E, et al. Intense Foxp3+ CD25+ regulatory T-cell infiltration is associated with high-grade cutaneous squamous cell carcinoma and counterbalanced by CD8+/Foxp3+ CD25+ ratio. *British Journal of Dermatology*. 2015;172(1):64-73.
490. Lai C, August S, Albibas A, Behar R, Cho S-Y, Polak ME, et al. OX40+ regulatory T cells in cutaneous squamous cell carcinoma suppress effector T-cell responses and associate with metastatic potential. *Clinical Cancer Research*. 2016;22(16):4236-48.
491. Slater NA, Gooe PB. PD-L1 expression in cutaneous squamous cell carcinoma correlates with risk of metastasis. *Journal of Cutaneous Pathology*. 2016;43(8):663-70.
492. García-Pedrero JM, Martínez-Camblor P, Diaz-Coto S, Munguia-Calzada P, Vallina-Alvarez A, Vazquez-Lopez F, et al. Tumor programmed cell death ligand 1 expression correlates with nodal metastasis in patients with cutaneous squamous cell carcinoma of the head and neck. *Journal of the American Academy of Dermatology*. 2017;77(3):527-33.
493. Pettersen JS, Fuentes-Duculan J, Suárez-Fariñas M, Pierson KC, Pitts-Kiefer A, Fan L, et al. Tumor-associated macrophages in the cutaneous SCC microenvironment are heterogeneously activated. *Journal of Investigative Dermatology*. 2011;131(6):1322-30.
494. Takahara M, Chen S, Kido M, Takeuchi S, Uchi H, Tu Y, et al. Stromal CD10 expression, as well as increased dermal macrophages and decreased Langerhans cells, are associated with malignant transformation of keratinocytes. *Journal of Cutaneous Pathology*. 2009;36(6):668-74.
495. Avrămoiu I, Petrescu IO, Ciurea ME, Bold A, Siloși I, TânȚu MM, et al. Peritumoral inflammatory reaction in non-melanoma skin cancers-histological and immunohistochemical study. *Rom J Morphol Embryol*. 2016;57(3):943-50.

496. Mahapatra KD, Pasquali L, Søndergaard JN, Lapins J, Nemeth IB, Baltás E, et al. A comprehensive analysis of coding and non-coding transcriptomic changes in cutaneous squamous cell carcinoma. *Scientific Reports*. 2020;10(1):1-12.
497. Li YY, Hanna GJ, Laga AC, Haddad RI, Lorch JH, Hammerman PS. Genomic Analysis of Metastatic Cutaneous Squamous Cell Carcinoma. *Clinical Cancer Research*. 2015;21(6):1447.
498. Inman GJ, Wang J, Nagano A, Alexandrov LB, Purdie KJ, Taylor RG, et al. The genomic landscape of cutaneous SCC reveals drivers and a novel azathioprine associated mutational signature. *Nature Communications*. 2018;9(1):3667.
499. Kabashima K, Honda T, Ginhoux F, Egawa G. The immunological anatomy of the skin. *Nature Reviews Immunology*. 2019;19(1):19-30.
500. Paulson KG, Lahman MC, Chapuis AG, Brownell I. Immunotherapy for skin cancer. *International immunology*. 2019;31(7):465-75.
501. Lipson EJ, Vincent JG, Loyo M, Kagohara LT, Lubner BS, Wang H, et al. PD-L1 expression in the Merkel cell carcinoma microenvironment: association with inflammation, Merkel cell polyomavirus, and overall survival. *Cancer immunology research*. 2013;1(1):54-63.
502. Carstens JL, Correa de Sampaio P, Yang D, Barua S, Wang H, Rao A, et al. Spatial computation of intratumoral T cells correlates with survival of patients with pancreatic cancer. *Nature communications*. 2017;8:15095-.
503. Berthel A, Zoernig I, Valous NA, Kahlert C, Klupp F, Ulrich A, et al. Detailed resolution analysis reveals spatial T cell heterogeneity in the invasive margin of colorectal cancer liver metastases associated with improved survival. *Oncoimmunology*. 2017;6(3):e1286436.
504. Barua S, Fang P, Sharma A, Fujimoto J, Wistuba I, Rao AUK, et al. Spatial interaction of tumor cells and regulatory T cells correlates with survival in non-small cell lung cancer. *Lung Cancer*. 2018;117:73-9.
505. Gide TN, Silva IP, Quek C, Ahmed T, Menzies AM, Carlino MS, et al. Close proximity of immune and tumor cells underlies response to anti-PD-1 based therapies in metastatic melanoma patients. *OncoImmunology*. 2020;9(1):1659093.
506. Shin S-J, Jeon YK, Kim P-J, Cho YM, Koh J, Chung DH, et al. Clinicopathologic analysis of PD-L1 and PD-L2 expression in renal cell carcinoma: association with oncogenic proteins status. *Annals of surgical oncology*. 2016;23(2):694-702.
507. Koh YW, Han J-H, Yoon DH, Suh C, Huh J. PD-L1 expression correlates with VEGF and microvessel density in patients with uniformly treated classical Hodgkin lymphoma. *Annals of Hematology*. 2017;96(11):1883-90.
508. Huang Y, Goel S, Duda DG, Fukumura D, Jain RK. Vascular normalization as an emerging strategy to enhance cancer immunotherapy. *Cancer research*. 2013;73(10):2943-8.
509. Anderson DM, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER, et al. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature*. 1997;390(6656):175-9.
510. Ahern E, Harjunpää H, Barkauskas D, Allen S, Takeda K, Yagita H, et al. Co-administration of RANKL and CTLA4 antibodies enhances lymphocyte-mediated antitumor immunity in mice. *Clinical Cancer Research*. 2017;23(19):5789-801.
511. Peters S, Clézardin P, Márquez-Rodas I, Niepel D, Gedy C. The RANK–RANKL axis: an opportunity for drug repurposing in cancer? *Clinical and Translational Oncology*. 2019:1-15.

512. Colotta F, Re F, Muzio M, Bertini R, Polentarutti N, Sironi M, et al. Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. *Science*. 1993;261(5120):472-5.
513. Rahimi Kalateh Shah Mohammad G, Ghahremanloo A, Soltani A, Fathi E, Hashemy SI. Cytokines as potential combination agents with PD-1/PD-L1 blockade for cancer treatment. *Journal of Cellular Physiology*. 2020.
514. Massi D, Brusa D, Merelli B, Ciano M, Audrito V, Serra S, et al. PD-L1 marks a subset of melanomas with a shorter overall survival and distinct genetic and morphological characteristics. *Annals of oncology*. 2014;25(12):2433-42.
515. Schats KA, Van Vré EA, De Schepper S, Boeckx C, Schrijvers DM, Waelpuut W, et al. Validated programmed cell death ligand 1 immunohistochemistry assays (E1L3N and SP 142) reveal similar immune cell staining patterns in melanoma when using the same sensitive detection system. *Histopathology*. 2017;70(2):253-63.
516. Erber R, Stoehr R, Herlein S, Giedl C, Rieker RJ, Fuchs F, et al. Comparison of PD-L1 mRNA expression measured with the CheckPoint Typer® Assay with PD-L1 protein expression assessed with immunohistochemistry in non-small cell lung cancer. *Anticancer Research*. 2017;37(12):6771-8.
517. Nosrati A, Tsai KK, Goldinger SM, Tumei P, Grimes B, Loo K, et al. Evaluation of clinicopathological factors in PD-1 response: derivation and validation of a prediction scale for response to PD-1 monotherapy. *British Journal Of Cancer*. 2017;116:1141.
518. Morrison C, Pabla S, Conroy JM, Nesline MK, Glenn ST, Dressman D, et al. Predicting response to checkpoint inhibitors in melanoma beyond PD-L1 and mutational burden. *Journal for ImmunoTherapy of Cancer*. 2018;6(1):32.
519. Mayoux M, Roller A, Pulko V, Sammiceli S, Chen S, Sum E, et al. Dendritic cells dictate responses to PD-L1 blockade cancer immunotherapy. *Science Translational Medicine*. 2020;12(534).
520. Cocorocchio E, Ferrucci PF, Martinoli C, Mandalà M, Gandini S, Giannarelli D, et al. Baseline neutrophils and derived neutrophil-to-lymphocyte ratio: prognostic relevance in metastatic melanoma patients receiving ipilimumab. *Annals of Oncology*. 2016;27(4):732-8.
521. Araujo DV, de Moraes RV, Ramos Sousa VA, Donadio MDS, Fares AF, Freitas HC, et al. Prognostic relevance of neutrophil to lymphocyte ratio (NLR) before anti-PD1 therapy in metastatic melanoma patients. *American Society of Clinical Oncology*; 2017.
522. Duric VM, Fallowfield LJ, Saunders C, Houghton J, Coates AS, Stockler MR. Patients' preferences for adjuvant endocrine therapy in early breast cancer: what makes it worthwhile? *British Journal of Cancer*. 2005;93(12):1319-23.
523. Salmi S, Siiskonen H, Sironen R, Tyynelä-Korhonen K, Hirschovits-Gerz B, Valkonen M, et al. The number and localization of CD68+ and CD163+ macrophages in different stages of cutaneous melanoma. *Melanoma Research*. 2019;29(3):237.
524. Smyth MJ, Yagita H, McArthur GA. Combination anti-CTLA-4 and anti-RANKL in metastatic melanoma. *Journal of Clinical Oncology*. 2016;34(12):e104-e6.