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Assessment of the clinical validity of ctDNA Analysis for melanoma management

Anda-Gabriela Marsavela
Edith Cowan University

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**Assessment of the Clinical Validity of ctDNA Analysis for
Melanoma Management**

This thesis is presented for the degree of
Doctor of Philosophy

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Dr Leslie Calapre

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School of Medical & Health Sciences

EDITH COWAN UNIVERSITY

2021

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DECLARATION

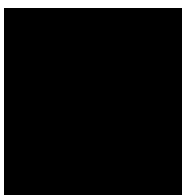
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29th October 2020

ABSTRACT

Metastatic melanoma is responsible for almost 80% of all skin cancer-related deaths and the incidence of people affected continues to rise worldwide. The emergence of targeted therapy and immune-checkpoint inhibitors has improved the clinical management of melanoma, but durable survival benefit is only seen in a minority of patients. The use of these very expensive systemic therapies on all appropriate patients also poses a high economic burden on health systems across numerous countries. Currently, surveillance for treatment failure is not optimal. Thus, reliable and accurate biomarkers of patient disease status are urgently required.

Circulating tumour DNA (ctDNA) analysis has emerged as a potential “liquid biopsy” for melanoma. Plasma-derived ctDNA are short DNA fragments released into the bloodstream by apoptotic tumour cells. Studies have shown that ctDNA levels in blood correlate with tumour burden and can comprehensively capture the molecular heterogeneity of melanoma metastases. Thus, ctDNA appears to be a viable biomarker for monitoring treatment response and disease progression in melanoma patients. However, further studies aimed at comparing ctDNA and current standard clinical assessments are needed to fully define its suitability as a complementary test to guide treatment decisions.

This thesis aims to provide important information that will assist with the implementation of ctDNA as a biomarker for melanoma in the clinical management of the disease. This thesis is comprised of 7 chapters: a comprehensive literature review (**Chapter 1. Introduction**); a materials and methods chapter (**Chapter 2**); 4 results chapters (**Chapter 3 – 6**); and a final chapter with a general discussion of main findings and future directions (**Chapter 7. General Discussion and Future Directions**).

The first chapter of the thesis includes a thorough review of the literature on ctDNA as a potential biomarker for melanoma disease (Chapter 1). This is then followed by a detailed description of our protocol for plasma ctDNA extraction and quantification using droplet digital PCR (Chapter 2). Using this methodology, we evaluated the ctDNA detection rate in untreated *BRAF* mutant melanoma patients, as a potential alternative to tumour genotyping (Chapter 3), where the potential economic benefit of implementing plasma ctDNA testing by ddPCR relative to tissue *BRAF* testing was also investigated.

The study in Chapter 4 demonstrated that pre-treatment plasma ctDNA is predictive of patient outcomes in the first-line treatment setting. However, baseline ctDNA level was not predictive of outcomes in the second-line immunotherapy setting, especially in patients that were pre-treated with BRAF/MEK inhibitors. Moreover, we found preliminary evidence that patients with high pre-treatment ctDNA may benefit from combined anti-CTLA-4/anti-PD-1 therapy.

Chapter 5 discusses the validity of ctDNA as a surveillance biomarker for melanoma. The kinetics of ctDNA decline were found delayed in patients treated with immunotherapy compared to those receiving MAPK inhibitors. Nonetheless, decreasing ctDNA levels within 12 weeks of immunotherapy or BRAF/MEK inhibitors was strongly concordant with treatment response and significantly associated with longer progression-free survival (PFS). Furthermore, exploratory analysis of nine patients commencing anti-PD-1 therapy showed a trend of high tumour mutational burden (TMB) and neoepitope load in responders compared to non-responders.

Chapter 6 evaluates the validity of ctDNA to accurately detect disease progression using both a retrospective and a prospective cohort of melanoma patients. The results indicated a moderate detection rate, suggesting that more sensitive methodologies are required to achieve a limit of detection comparable to current medical imaging.

Finally, Chapter 7 provides a general discussion of the studies covered in this thesis. It underscores the clinical validity of ctDNA as a biomarker of prognosis and therapeutic response in melanoma patients, while highlighting important limitations inherent to ctDNA analysis that need to be thoroughly addressed before it can be successfully implemented in the clinic.

USE OF THESIS

The Use of Thesis statement is not included in this version of the thesis.

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First of all, I would like to acknowledge A/Prof. Elin Gray, Dr. Leslie Calapre and Prof. Mel Ziman for giving me the opportunity to be part of this project. I can truly say that studying and working together with you has deeply enriched me as a scientist and as a person.

I really appreciate Elin's guidance throughout all these years. Your knowledge is truly astounding, and I appreciate all the time used in sharing it with me. Leslie, I would like to thank you for all your constant help in the lab and sharing your great ideas and advice with me. Also, thank you for seeing the best that I can give when I could not see it anymore. Mel, I would like to thank you for your continuous care towards your students. Your disposition to always be ready to dedicate time and energy to me and to my research it has meant a lot to me. I greatly thank the constant help from all of you. Your opinions and advice have been very valuable for me and have made me a better researcher.

I would like to thank all those that have investigated and those that will continue to investigate melanoma. It is my deepest wish that the work presented in this thesis will help even a little to fight against this terrible worldwide cancer.

I also appreciate the care and love showed to me by the ECU melanoma research group. We have had great moments and I will always carry each and every one of you in my heart. Your friendship, support and affection have meant a lot to me and I have been blessed by your friendship.

I want to thank my family, who even being away, have lived each of my ups and downs with me. I would also like to thank my husband's family, their continuous support that has been of great help to me. Special mention to my dearest husband David, who has been putting up with me through all my downs. Your unconditional understanding and support during all these years has meant a lot to me. Thanks to all my friends outside of university for being supportive in difficult times and cheerful when celebrating achievements. I also thank everyone who have helped to make this project a reality. It has not always been easy, but with all your support, it has definitely been easier and more bearable. Thank you!

LIST OF PUBLICATIONS

This thesis contains work that has been published (bolded), as well as work that is being prepared for publication, as indicated in this list and in association with their respective chapter order.

- 1. Marsavela, G. et al., *Isolation and Quantification of Plasma Circulating Tumour DNA from Melanoma Patients*. Hargadon, Kristian M. (Ed.). **Melanoma: Methods and Protocols. Methods in Molecular Biology. vol. 2265. © Springer Science+Business Media New York 2021. (Chapter 2)****
2. Marsavela, G. et al., *BRAF V600 Testing in Circulating Tumour DNA for Treatment Decisions in Metastatic Melanoma: Comparative Outcomes and Cost Analysis for Liquid versus Tissue Biopsies*. Manuscript in preparation. (Chapter 3)
- 3. Marsavela, G. et al., *Circulating Tumor DNA Predicts Outcome from First-, but not Second-line Treatment and Identifies Melanoma Patients Who May Benefit from Combination Immunotherapy*. Clinical Cancer Research, 2020. DOI: 10.1158/1078-0432.ccr-20-2251. (Chapter 4)**
- 4. Marsavela, G. et al., *The prognostic impact of circulating tumour DNA in melanoma patients treated with systemic therapies: Beyond BRAF mutant detection*. Cancers, 2020. DOI: 10.3390/cancers12123793. (Chapter 5)**
5. Marsavela, G. et al., *Plasma ctDNA fails to reliably detect clinical progression in metastatic melanoma patients*. Submitted. (Chapter 6)

OTHER RESEARCH-RELATED OUTPUT DURING CANDIDATURE

Oral presentations

1. ASMR National Conference (2019, Fremantle).
2. Melanoma Research Group Symposium (2018 and 2019, Perth).
3. ASMR Medical Research Week Symposium (2017 and 2018, Perth).

Poster presentations

1. Thomas Ashworth Symposium (31 Oct-1 Nov 2019, Sydney).
2. World Melanoma Conference/Society Melanoma Research (18-21 October 2017, Brisbane).

Other

1. CSIRO On Prime Workshop (September – December 2019).
2. Health Economics in Clinical Trials (26-28 November 2018, Perth).
3. Health Economics Symposium (27 February 2019, Perth).

Professional manuscript revision

Peer-reviewed 3 manuscripts for journals: International Journal of Cancer and Scientific Reports.

Collaboration in other publications

1. Michael Clark, Helen Rizos, Michelle R Pereira, Ashleigh McEvoy, **Gabriela Marsavela**, Leslie Calapre, Katie Meehan, Olivia Ruhlen, Muhammad A Khattak, Tarek M Meniawy, Georgina V Long, Matteo S Carlino, Alexander M Menzies, Michael Millward, Melanie Ziman, Elin S Gray. *Detection of BRAF splicing variants in plasma-derived cell-free nucleic acids and extracellular vesicles of melanoma patients failing BRAF directed therapies*. Oncotarget (accepted for publication October 2020).
2. Pauline Zaenker, Leslie Calapre, Michael Clark, **Gabriela Marsavela**, Carlos Aya-Bonilla, Dr Elin Gray, and Prof Mel Ziman. *Blood-based melanoma detection*. Dermnet NZ, 2018. <https://www.dermnetnz.org/topics/blood-based-melanoma-detection>.

STATEMENT OF CONTRIBUTION

I have completed this thesis as a full-time student at the Melanoma Research Group at Edith Cowan University under the supervision of A/Prof Elin Gray, Dr Leslie Calapre and Professor Mel Ziman.


This thesis is presented as a series of manuscripts in preparation (Chapter 3), submitted papers (Chapter 6) and published (Chapters 2, 4 and 5).

I, Gabriela Marsavela, was the primary responsible for the development of the research proposal, submission of Edith Cowan University - Human Research Ethics Committee applications, selection of research methods, optimisation of assays and laboratory protocols, processing patient samples, analysis of clinical data, interpretation of findings, drafting the manuscripts and the thesis.

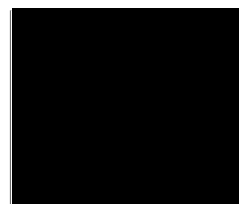
For Chapter 1 and 2, I was responsible for the review of literature, drafting and revision of the chapter.

For Chapter 3 to 6, I was responsible of the content conceptualisation, performing experiments, drafting and editing the manuscript, data curation, visualisation and interpretation.

Finally, for Chapter 7, I was responsible of the drafting, content development and final revision of the chapter.



Anda-Gabriela Marsavela
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A/Prof Elin Gray
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Date: 29th October 2020

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LIST OF COMMON ABBREVIATIONS

AJCC: American Joint Commission on Cancer
BRAF: B-raf Proto-Oncogene, Serine/Threonine Kinase
CDK4: Cyclin-Dependent Kinase 4
CDKN2A: Cyclin-Dependent Kinase Inhibitor 2A
cfDNA: Cell-free DNA
CNS: Central Nervous System
CSF: Cerebrospinal Fluid
CT: Computerised Tomography
CTCs: Circulating Tumour Cells
ctDNA: Circulating Tumour DNA
CTLA-4: Cytotoxic T-Lymphocyte Antigen 4
ddPCR: Droplet Digital PCR
EMA: European Medicines Agency
FDA: Food and Drug Administration
ICI: Immune Checkpoint Inhibitors
LDH: Lactate Dehydrogenase
LOD: Limit of Detection
MAPK: Mitogen Activated Protein Kinase
miRNAs: MicroRNAs
MITF: Microphthalmia-Associated Transcription Factor
MRD: Minimal Residual Disease
MRI: Magnetic Resonance Imaging
NF1: Neurofibromin Type 1
NRAS: Neuroblastoma RAS Viral (V-Ras) Oncogene Homolog
NSCLC: Non-Small Cell Lung Cancer
ORR: Overall Response Rate
OS: Overall Survival
PD: Progressive Disease
PD-1: Programmed Death-1
PET: Positron Emission Tomography
PFS: Progression-Free Survival
SNP: Single Nucleotide Polymorphisms
TERT: Telomerase Reverse Transcriptase
TMB: Tumour Mutational Burden
UMI: Unique Molecular Identifiers
UV: Ultraviolet Radiation
WES: Whole Exome Sequencing
WGS: Whole Genome Sequencing
WLE: Wide Local Excision
WT: Wild-type

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Melanoma is an aggressive cutaneous cancer responsible for the majority of skin cancer-related deaths [1, 2]. In addition, the incidence of melanoma is increasing worldwide, which results in significant costs to the healthcare systems [3]. Generally, patients are considered cured after complete surgical resection of the primary tumour [4]. Nevertheless, around 20% of patients will develop metastatic disease, which is extremely difficult to treat [5].

In the last ten years, the use of targeted therapies and immunotherapies has improved the survival of metastatic melanoma patients. However, the low response rate to immunotherapies and the risk of developing resistance to both targeted and immunotherapies contribute to the sub-optimal prognosis for most metastatic patients. Moreover, adverse side-effects associated with current immunotherapies restrict treatment efficacy in some cases [6]. Therefore, new biomarkers are urgently needed for personalised monitoring of the disease, with the aim to determine treatment response and recurrence at earlier stages. This will allow optimal selection and/or timely treatment modification to improve survival rates in patients.

Plasma ctDNA are short DNA fragments released into the bloodstream by the tumours within a patient. Since ctDNA can be detected in the blood of melanoma patients, these DNA fragments can be used as a “liquid biopsy”, providing critical insight into each person’s melanoma status. Studies have previously shown that ctDNA levels correlate with tumour burden and disease stage, characteristics that have been exploited in different cancer types to determine the clinical value of ctDNA in monitoring clonal evolution and identifying mechanisms of resistance [7-10]. Outcomes of these studies have driven the Food and Drug Administration (FDA) and European Medicines Agency (EMA) to approve the analysis of ctDNA in patients with non-small cell lung cancer (NSCLC) to identify patients carrying a specific mutation that makes them resistant to first-line treatment [11].

Plasma ctDNA in melanoma has been defined as a biomarker of disease status used to monitor clonal evolution, detect the emergence of resistance to treatment and predict

response to therapy [12-16]. Detection of *BRAF* mutations in plasma cfDNA has been associated with higher disease burden and worse prognosis. Similarly, the presence of *BRAF* or *NRAS* mutation prior to or during systemic treatment has been associated with larger tumours, increased lactate dehydrogenase (LDH) levels, and development of brain metastases [17]. Studies investigating circulating tumour DNA (ctDNA) have been conducted mainly in small cohorts of patients treated with targeted therapies and only a few studies have focused on *BRAF* wild-type patients treated with immunotherapy. Constant changes in the pharmacotherapies used in melanoma have shaped an urgent need to effectively evaluate the detection rate of ctDNA in large cohorts and assess its proficiency to inform response to therapy and progressive disease. Further studies are needed to compare this biomarker with current standard clinical assessments and to assess its suitability as a complementary test that guides treatment decisions.

1.2 Cutaneous Melanoma

The skin is the largest organ in the body and serves to protect us from ultraviolet (UV) light, injuries and infections, among other important functions [18]. The main layers of the skin include the subcutaneous layer, the dermis, and the epidermis. The epidermis is the outermost layer and contains three different types of skin cells: squamous cells, basal cells, and melanocytes. Melanocytes are in the basal layer of the epidermis and are responsible for melanin pigment production (Figure 1.1).



Figure 1.1. Representation of normal skin anatomy. Melanocytes are present in the basal layer of the epidermis. Retrieved from Winslow *et al.* [19].

Cutaneous Melanoma is a highly aggressive skin tumour originating from the neoplastic transformation of melanocytes. Melanoma occurs principally in the skin but may also develop in the conjunctiva and uvea of the eye (uveal melanoma), on various mucosal membranes with pigmented tissues, such as the gastrointestinal tract, oral or genital membranes, meninges, and in internal organs, such as in the central nervous system [20].

1.2.1 Epidemiology

Cutaneous melanoma is the most lethal form of skin cancer responsible for approximately 80% of skin cancer deaths despite representing only <5% of all dermatological neoplasms [21]. The incidence of melanoma is increasing worldwide, but New Zealand and Australia continue to have the highest incidence and mortality rates [22, 23]. In Australia, melanoma was the 3rd most commonly diagnosed cancer in 2019 and new cases of melanoma skin cancer are estimated to increase to 16,221 in 2020 with approximately 1,375 estimated deaths [1] (Figure 1.2). In fact, the Australian melanoma health expenditure each year is the highest in the world relative to the population [24]. Moreover, melanoma accounts for 11% of all newly diagnosed cancers [1]. The anatomical location of the primary melanoma is commonly found in the trunk in men and the extremities in women [25]. The location of the tumour also varies with age, being on the trunk or extremities more commonly in younger ages, but on head and neck locations in advanced ages [26, 27].

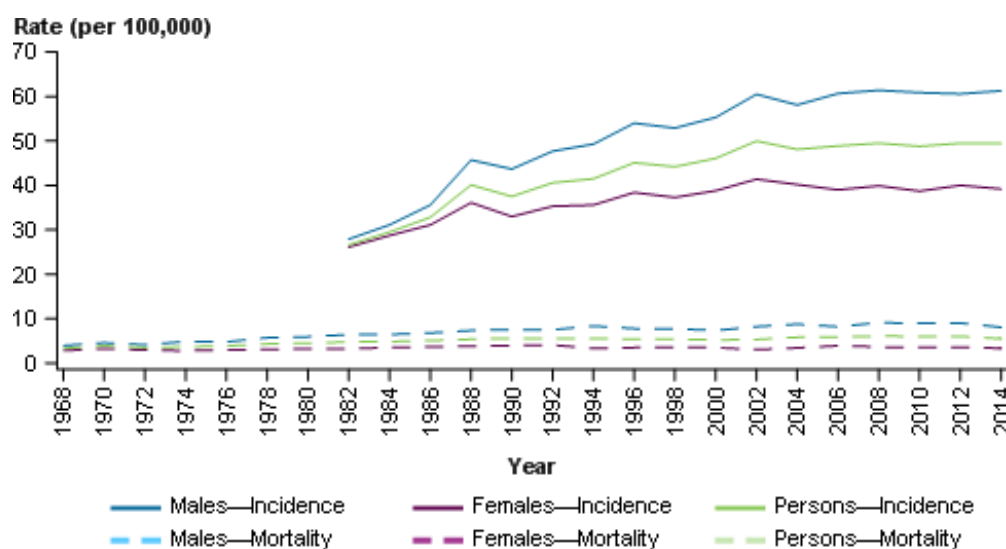


Figure 1.2. Age-standardised incidence rates for melanoma skin cancer 1982–2013 and age-standardised mortality rates for melanoma skin cancer 1968–2014, by sex, in Australia. Retrieved from the AIHW 2017 [2].

1.2.2 Aetiology

Sun exposure is a major risk factor associated with cutaneous melanoma formation. UV radiation induces the formation of mutagenic cyclobutane pyrimidine dimers. A significant number of melanoma-associated genes bear UV-induced signature C>T transition mutations in cellular DNA [28-32]. Melanoma also has the highest median number of somatic mutations across all human cancer types [33].

Trends in melanoma incidence show that Caucasian race, male gender, and older age are characteristics associated with an increased risk of developing melanoma [34]. Also, fair-skinned individuals with red or blond hair and many freckles are more likely to suffer from the disease [35, 36]. Having a higher number of benign melanocytic naevi, atypical naevi or giant congenital naevi also increases the risk of developing melanoma [37, 38]. Other host factors such as a personal history of melanoma or non-melanoma tumours, including other skin cancers, can increase the risk of developing melanoma [39-41]. Moreover, a weak immune system or having a disease that weakens the immune system, increases the cancer risk, with a higher incidence of cutaneous melanoma in patients after organ transplantation proposed to be due to medical immunosuppression [42].

Genetic predisposition is also a factor involved in melanoma development. About 10% of all melanomas are estimated to be due to hereditary susceptibility [43]. Since William Norris [44] first suggested that melanomas have a hereditary component, the knowledge of melanoma genetics has advanced significantly. It is now known that having a family history of melanoma increases the risk 1.74 times compared with a negative family history of the disease [45]. Inherited genetic risk factors can be classified by their penetrance and prevalence [46].

Rare but highly penetrant genetic mutations in *CDKN2A* and *CDK4* genes are commonly found in high-risk melanoma families [47]. These genes are involved in cell-cycle regulation and melanocyte senescence. As an example, the *CDKN2A* gene located on chromosome 9p21.3, encodes two proteins. p14ARF and p16INK4A, which control cell cycle entry at the G1 checkpoint and stabilise p53 expression [48]. Low melanoma penetrance mutations present in the general population, often referred to as single nucleotide polymorphisms (SNPs), have also been detected in genes involved in hair and

skin pigmentation, such as *MC1R*, *ASIP*, *TYR* and *TYRP1* [49-52]. In addition, SNPs in *BAP1*, *POT1*, *ACD*, *TERF2IP*, and *TERT* are also considered risk genes for inherited melanoma [53].

1.2.3 Diagnosis

The first step in melanoma diagnosis is the visual characterisation of suspicious lesions. Nonetheless, diagnosis of melanoma is often complicated by the resemblance of early stage melanoma to naevi, which are chronic lesions found in the skin or mucosa [54]. While naevi are often considered benign, more than 20% of malignant melanomas arise from pre-existing naevi [55]. Visual characteristics of a transformed mole can be identified via the "ABCDE criteria": *A*symmetry, a *B*order that is irregular, *C*olour that is uneven, a *D*iameter longer than 6 millimetres and, a shape, size or colour that is *E*volving (Figure 1.3). Other early signs of a malignant change are itching, ulceration or bleeding. By contrast, nodular melanomas do not follow these criteria. They have their own "EFG criteria"; the lesion is *E*levated above the surrounding the skin, the nodule is *F*irm to the touch and *G*rowing in size.

Image is available online (Figure 5) at <https://www.cancer.gov/types/skin/hp/skin-genetics-pdq>

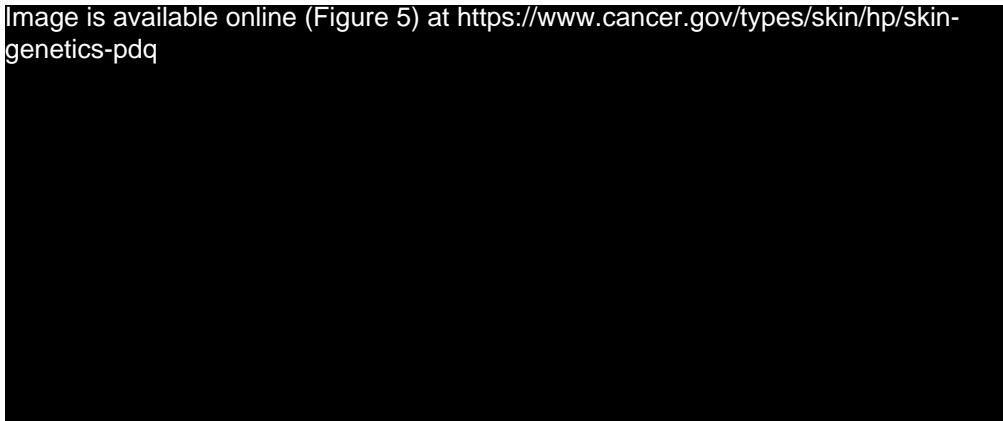


Figure 1.3. Melanomas with characteristic asymmetry, border irregularity, colour variation and large diameter. Retrieved from the National Cancer Institute [56].

The majority of abnormal lesions/naevi/moles are primarily detected through skin examination and assessed using dermoscopic tools that can differentiate melanoma from other types of carcinoma or benign lesions [57]. Some of the diagnostic techniques available for better identification of skin cancer include; total body photography [58], multispectral imaging, confocal scanning laser microscopy, ultrasound imaging [59], magnetic resonance imaging [60], and optical coherence tomography. A histological

examination of a tissue biopsy from a suspected lesion is necessary to confirm melanoma diagnosis and tumour staging. The histopathological criteria of melanoma are based on abnormal characteristics of the lesion such as ulceration, the extent and penetrance of the tumour, and the mitotic rate of the cells in the lesion.

The discovery of histologic markers that uniquely identify melanocytes in melanoma has aided melanoma diagnosis. The most commonly used markers are S100 [61], MART-1/Melan-A [62], HMB-45 [63, 64], SOX-10 [65] and MC1R [66]. Currently, a combination of multiple positive histologic markers and histopathologic criteria provides the most reliable method of diagnosis. If melanoma cells are detected in a lesion, the spread of the disease will be further assessed, to stage the melanoma.

1.2.4 Staging of melanoma

To determine the optimal treatment strategy and the prognosis of a patient with melanoma, staging of the biopsied material is needed. The American Joint Commission on Cancer (AJCC) updated the melanoma classifications in 2017 [67] using the most significant prognostic values as shown in Table 1.1. The clinical staging of melanoma is based primarily on the micro-staging of the excised tumour and the clinical and radiological examination of the regional lymph nodes and distant organ involvement.

Patients are then classified into five main groups. In patients with stage 0 (*in situ* melanoma), melanoma is confined to the epidermis and has not invaded deeper skin layers. Patients with clinical stages I and II, are those with primary melanoma and no evidence of metastases. Stage III is indicated for patients with evidence of regional lymph node metastases. Lastly, stage IV melanoma patients are those that have been diagnosed with one or more distant metastases [68]. Pathological staging includes micro-staging of the primary melanoma and pathological information about the regional lymph nodes after lymph node biopsy. Both clinical and pathological staging are important in melanoma diagnosis and treatment decisions.

The TNM classification is used to stage patients based on the extent of the primary tumour (T), the presence or not and the extent of regional lymph node metastases and non-nodal locoregional sites, such as in-transit, satellite and/or microsatellite metastases (N), and the presence or absence of distant metastases (M) [69].

Table 1.1. Classification of the pathological stage and the main features related to melanoma staging [67, 70]. MN=matted nodes; ISM=In-transit, satellite or microsattelites.

Pathologic Stage	TNM	Breslow's Thickness (mm)	Ulceration	No. Positive Nodes	Nodal Type	Metastasis	Survival (%)	
							5 years	10 years
0	Tis	-	-	0	-	-	≥99	≥99
IA	T1a	<0.8	No	0	-	-	99	98
	T1b	<0.8 0.8 - 1.0	Yes Yes/No	0	-	-	99	96
IB	T2a	>1.0 - 2.0	No	0	-	-	96	92
IIA	T2b	>1.0 - 2.0	Yes	0	-	-	93	88
	T3a	>2.0 - 4.0	No	0	-	-	94	88
IIB	T3b	>2.0 - 4.0	Yes	0	-	-	86	81
	T4a	>4.0	No	0	-	-	90	83
IIC	T4b	>4.0	Yes	0	-	-	82	75
IIIA	N1a-	<0.8	Yes/No	1	Occult	-	93	88
	N2a	0.8 - 1.0 >1.0 - 2.0		2-3	Occult	-		
IIIB	N1a	>1.0 - 2.0	Yes	1-3	Any	-	83	77
	N2b	>2.0 - 4.0	No			ISM		
	N1b	-	-	1	Apparent	-		
	N1c	-	-	0	-	ISM		
IIIC	N1b	-	-	1	Apparent	-	69	60
	N1c	<0.8 - >1.0 - 2.0	Yes/No	0	-	ISM		
	N2b	-	-	2-3	≥1 App.	-		
	N1a - N2c	>4.0	Yes	1-3	Any	-		
	N2b	-	-	2-3	≥1 App.	ISM		
	N2c	-	-	1	Any	ISM		
	N3b	-	-	≥4	≥1 App. MN	-		
	N3c	-	-	≥2	≥2 App. MN	ISM		
	N2c	-	-	1	Any	ISM		
	N3a	<0.8 - >2.0 - 4.0	Any	≥4	Occult	-		
N3b	-	-	≥4	≥1 App. MN	-			
N3c	-	-	≥2	≥2 App. MN	ISM			
IIID	N≥1	>2.0 - 4.0 >4.0	Yes No	1- ≥4	Any	Any	32	24
	N3a	>4.0	Yes	≥4	Occult	-		
	N3b	>4.0	Yes	≥4	≥1 App. MN	-		
	N3c	>4.0	Yes	≥2	Occult; Apparent; Any MN	ISM		
IV	M1a	Any	Any	Any	Any	Skin	n/a	n/a
	M1b	Any	Any	Any	Any	Skin; Lung	n/a	n/a
	M1c	Any	Any	Any	Any	Other; Non-CNS Visceral	n/a	n/a
	M1d	Any	Any	Any	Any	Other; CNS Visceral	n/a	n/a

The N category defines the number of clinically occult or clinically apparent regional nodes or macro-metastatic nodes determined by standard immunohistochemical staining using melanocytic markers. The presence of intra-lymphatic metastases including the presence or absence of satellites or in-transit metastases are also taken into consideration in this category. Regional nodal metastasis is defined as a disease confined

to one nodal basin, while patients with distant nodal metastases will be classified as having M stage disease [69].

The M category defines the presence of distant metastases in the skin, subcutaneous tissue, or distant lymph nodes or organs. Importantly, based on the anatomic sites of metastasis, patients are assigned into 4 M subcategories: M1a, M1b, M1c, and M1d. Patients with distant metastasis to the skin, subcutaneous tissue, muscle, or distant lymph nodes are categorized as M1a. Patients with metastasis to lung (with or without M1a disease) are categorized as M1b. Patients with metastases to any other visceral site(s) (excluding central nervous system (CNS) disease) are designated as M1c, while patients with metastases to the CNS are designated as M1d.

Cutaneous melanomas are also subdivided into several subtypes, based on common anatomical locations and different patterns of growth. Superficial spreading melanoma is the most frequent, followed by nodular melanoma, acral lentiginous melanoma and lentigo maligna melanoma [71].

1.2.5 Genetic and histopathological changes in melanoma development

As explained above, both genetic predisposition and exposure to environmental agents are risk factors for melanoma development. It is important to highlight that melanocyte malignant transformation involves the progressive accumulation of mutations in genes that are involved in cell-cycle control, proliferation, differentiation, and cell death [72], until finally, melanoma cells acquire the ability to initiate and sustain angiogenesis. This process, by which melanocytes progress to a malignant phenotype through several steps, is known as melanomagenesis [73] (Figure 1.4).

Based on the pattern of the most prevalent and significant mutated genes, melanomas can be classified into four genomic sub-types: mutant *BRAF*, mutant *NRAS*, mutant neurofibromin type 1 (*NF1*), and triple wild-type (WT; Figure 1.5) [74]. Molecular classification of melanomas is important as it can guide treatment strategies.

Image is available (Figure 2) at <https://www.nejm.org/doi/full/10.1056/NEJMra052166>

Figure 1.4. Biologic events that take place in melanoma progression. The benign nevus commences a dysplastic transformation, through radial and vertical growth until the metastatic phenotype arises. Adapted from Miller *et al.* [5].

The RAS/RAF/MEK/ERK mitogen-activated protein kinase (MAPK) signal transduction pathway is one of the most important pathways in the origin and progression of melanoma. BRAF is a member of the RAF family of serine-threonine kinases, along with ARAF and CRAF (also called RAF1). Mutations in the *BRAF* gene have been described in 40-60% [75, 76] of all melanoma cases. Among mutations in the *BRAF* gene, approximately 80% of the mutations result in the substitution of glutamic acid (E) for valine (V) in codon 600, known as the *BRAF V600E* mutation. About 16% of mutations in this gene result in a lysine (K) substitution at the same *BRAF V600K* codon, and 3% result in an aspartic acid (D) or arginine (R) substitution that produces a *V600D/R* codon [77]. These last two mutant forms of *BRAF* tend to be present in melanomas arising in older patients [78]. All of these mutations occur as early events in melanomagenesis and result in a mutant protein that is constitutively active in the cell without the need for activation signals. The consequence is uncontrolled proliferation and resistance to apoptosis. Nevertheless, the presence of *BRAF* mutations in 80% of benign naevi suggests that mutational activation of the MAPK pathway is a critical step in melanocytic transformation but alone is insufficient for melanoma tumourigenesis [79].

Mutations in the *NRAS* gene are present in approximately 15 to 20% of all cutaneous melanomas [80]. Commonly, *NRAS* mutations occur in codon 61, resulting in replacement of a glutamine residue by an arginine (Q61R) or lysine (Q61K) in the protein. However, other forms of *NRAS* mutations result in a substitution of glutamine at position 61 by leucine (Q61L) or histidine (Q61H) or substitution of glycine at position 12 or 13 by aspartic acid (G12D/G13D). Mutated *NRAS* results in constitutive activation of the MAPK signalling pathway resulting in increased cell proliferation and advancement of tumour growth. Tumours that carry *NRAS* mutations represent a distinct subpopulation from mutant *BRAF* melanomas, since *BRAF* and *NRAS* mutations are generally mutually exclusive [81, 82]. Together, *NRAS* and *BRAF* mutations are present in about 70% of the most common types of melanoma [74].

NF1 is the third most commonly mutated gene in melanoma and is present in up to 46% of *BRAF* and *NRAS* wild-type melanomas or ~12-18% of all melanomas. *NF1* shows a high frequency of non-silent exonic mutations and a low frequency of synonymous or intronic mutations but there are no hot spot mutations in *NF1* [83]. This tumour suppressor gene encodes for a direct negative regulator of RAS, which cooperates with mutated *BRAF* in melanomagenesis [84].

Image is available (Figure 1 - middle section of A) at <https://doi.org/10.1016/j.cell.2015.05.044>




Figure 1.5. Frequency and mutation subtypes associated with melanoma. Retrieved from the Cancer Genome Atlas [74].

Another important genetic alteration present in melanoma is found on a well-characterised tumour-suppressor, the phosphatase and tensin homolog (*PTEN*) gene. The encoded protein is a key initiator of the PI3K signalling pathway. Somatic *PTEN* mutations or deletions have been identified in 10-30% of melanomas [74, 85]. Loss of *PTEN* frequently coexists with *BRAF* mutations, but not with mutant *NRAS*, which can activate the PI3K pathway independently.

The Ras-related C3 botulinum toxin substrate 1 (*RAC1*) gene encodes for an RHO GTPase protein which plays a key role in cellular cytoskeleton organization. Dysregulation of *RAC1* leads to cell proliferation and suppression of antitumor immune responses [86]. A recurrent mutation at codon 29 (P29S) of *RAC1* that leads to a proline-to-serine conversion has been identified in 5% to 9% of melanomas.

The telomerase reverse transcriptase (*TERT*) gene encodes for a catalytic subunit of the holoenzyme telomerase, which sustains telomere length and chromosomal stability. Reactivation of the *TERT* gene enables cells to overcome replication-induced senescence, a critical step in tumour initiation. There are two recurrent mutations, located within 100 base pairs of the *TERT* transcriptional start site on chromosome 5; cysteine-to-threonine mutations at codon 228 (C228T) and codon 250 (C250T). These mutations are consistent with an ultraviolet signature (C>T or CC>TT), thus implicating a role for ultraviolet radiation in their induction. These mutations have been identified in sporadic primary melanomas (33%), metastatic melanomas (85%), and melanoma cell lines (76%) [87]. In addition, 55% of melanomas have *TERT* mutations co-existing with *BRAF* or *NRAS* mutations [88].

Melanomas may also carry mutations that activate the KIT receptor protein tyrosine kinase, tumour protein 53 (*TP53*) or cyclin-dependent kinase inhibitor 2A (*CDKN2A*). The *KIT* gene is located on the long (q) arm of chromosome 4. Most *KIT* mutations are located in exon 11, which encodes for the juxtamembrane domain, and in exon 13, which encodes for a kinase domain [89]. Mutations in *TP53* are prevalent in approximately 20% of melanomas [90] and *CDKN2A*, which encodes for the p16INK4A protein, is found mutated in approximately 30% to 40% of familial melanomas [91, 92]. Altogether, studies have shown that *BRAF*, *CDKN2A*, *NRAS* and *TP53* are significantly mutated in cutaneous melanoma; *BRAF*, *NRAS* and *NF1* in acral melanoma, and *SF3B1* in mucosal melanoma [93].

1.2.6 Progression

Metastatic melanoma can be distinguished as either a local recurrence, in-transit metastasis, nodal metastasis and/or distal metastasis, based on the morphological characteristics and location. Local recurrence is defined as a recurrence of melanoma

within 2 cm of the surgical scar of a primary melanoma [94]. This recurrence can result from either the extension of the primary melanoma or from the spread via lymphatic or haematogenous vessels [95]. In-transit metastasis is defined as melanoma deposits within the lymphatic vessels located more than 2 cm from the site of the primary melanoma. Nodal metastasis involves the spread of tumour cells into the lymph nodes. Haematogenous spread of metastatic melanoma results in the development of distal metastasis [96].

A variety of sophisticated imaging techniques are now used to follow and evaluate patients with melanoma, including chest radiographs, regional nodal ultrasound imaging, computerised tomography (CT) scans, positron emission tomography (PET), PET/CT scans, and brain/spine and hepatic magnetic resonance imaging (MRI) to detect nodal disease and distant metastases [68, 97]. Among all these modalities, PET scans have shown an improved performance in depicting metastatic lesions over conventional imaging modalities, such as CT has been commonly described in the literature [98, 99].

In addition, LDH blood levels have been considered an important prognostic marker for patients with stage IV disease [100]. However, LDH levels are highly affected by other normal biological processes including inflammation and tissue damage. Therefore, new biomarkers are required to identify patients likely to develop distant metastases.

1.2.7 Treatment and prognosis

The standard treatment for *in situ* and primary melanoma is wide local excision (WLE) of the skin and subcutaneous tissues around the melanoma. Surgical removal of melanoma offers the best chance for a complete cure and is usually successful in patients categorised as stage 0, I and stage II. Most patients do not need either radiotherapy or chemotherapy since the tumour is still localised and there are no other existing metastases. However, greater tumour thickness is associated with an increased risk of local recurrence [101]. WLE is the treatment of choice in local recurrences, with consideration given to adjuvant therapy in some situations [68], since there is an increased risk of systematic or regional metastasis.

Currently, sentinel lymph node biopsies are carried out in patients with melanomas thicker than 1mm, or >0.8mm if the tumour is ulcerated [67, 102]. If the result is positive,

the patient will be diagnosed with melanoma stage III. Since patients with positive lymph nodes are at high risk of systemic dissemination, a therapeutic lymph node dissection may be considered. However, the MSLT-I study together with the Cochrane Review found no improved melanoma-specific survival for patients with intermediate or thick melanomas treated with a sentinel lymph node biopsy [103, 104]. In addition, adjuvant treatment with targeted therapies and immunotherapies is the standard of care due to their proven increase in survival and delayed disease recurrence [105, 106].

In regards to prognosis, the main clinical and histopathologic predictors of outcome are Breslow's thickness, the presence of ulceration, the sentinel lymph node status [107] and the presence of distant metastases.

The choice of therapy for the management of unresectable or metastatic melanoma depends on the number of lesions present within a patient, as well as the anatomical location, and size of the lesions. Treatment for metastatic melanoma aims to control the melanoma and to relieve any symptoms, with curable intentions. When surgery is not possible, treatments such as targeted therapies, immunotherapy, chemotherapy, radiotherapy and diverse clinical trials of these and other new drugs individually or in combination are available. Since 2011, treatment combinations using targeted therapy and/or immunotherapy have revolutionised the clinical management of melanoma patients, improving the length of survival of patients with stage III or stage IV melanoma [108-110].

Targeted Therapy

Since the discovery of the presence of activating mutations in the *BRAF* oncogene in approximately 50% of melanomas [75], there has been notable progress in the development of targeted therapies for unresectable and metastatic melanoma of patients carrying this mutation [111]. The FDA approved the use of BRAF inhibitors vemurafenib, dabrafenib and encorafenib as first line therapy for *BRAF* positive metastatic or unresectable melanoma [112, 113].

The great success of BRAF inhibitors in some patients and at the same time, the disease relapse in other patients treated with BRAF inhibitors, prompted the development of MEK inhibitors to further inhibit the MAPK signalling pathway at the next downstream

MEK1/2 activation point. Nowadays, the FDA approved MEK inhibitor trametinib, is less commonly indicated as a monotherapy, being used more commonly in combination with dabrafenib. The other FDA-approved MEK inhibitor, cobimetinib, is indicated for use in combination with vemurafenib [114]. Additional MAPK targeted drugs have been developed, such as BRAF inhibitor, encorafenib and MEK inhibitor, binimetinib. Results from an open-label phase III COLUMBUS trial showed that encorafenib and binimetinib combination offer longer median PFS and OS than vemurafenib monotherapy [115].

As a result of the positive outcomes from the clinical trials involving these combination therapies (Figure 1.6), BRAF/MEK-inhibitor combination therapy is the standard-of-care for *BRAF*-mutant metastatic melanoma.

While targeted therapies are effective in most patients (~65%) [116], unfortunately, the impressive initial tumour shrinkage is relatively short-lived and acquired resistance occurs in a high proportion of cases (58%) [117-119]. Another disadvantage of this type of therapy is that significant toxicities are associated with BRAF inhibitors [120] and combination therapies [121, 122]. Combination dabrafenib/trametinib regimens are associated with high fever whereas vemurafenib/cobimetinib combination therapy causes cutaneous and gastrointestinal adverse events [123]. Similarly, common adverse events with encorafenib/binimetinib included nausea, vomits, muscle spasms and altered liver function [124].

Immunotherapy

Immunotherapy aims to strengthen the anti-tumour immune response in patients with stages III or IV malignant melanoma. In 2011, the FDA approved the use of immune checkpoint inhibitor, ipilimumab for unresectable metastatic melanoma. This antibody binds to the cytotoxic T-lymphocyte antigen 4 (CTLA-4) on tumour cells and blocks its activity, allowing immune cells to target the melanoma cells. Unfortunately, the patient response rate is low (11.9%) and immune-related adverse effects, such as, dermatitis, diarrhoea and colitis, are severe and common [125].

Alternate immune attenuating checkpoints have been identified including the interaction between programmed death-1 factor (PD-1) with its ligand (PD-L1) on diverse cells, such as, antigen-presenting cells and tumour cells. This interaction is now routinely targeted

for the treatment of metastatic melanoma [110, 126]. Pembrolizumab and nivolumab are anti-PD-1 antibodies approved by the FDA for the treatment of advanced melanoma in *BRAF* negative melanomas [127, 128] or as a second-line therapy in patients that acquire resistance to BRAF inhibitors. Although the toxicities associated with these agents are severe, the frequency is lower than for ipilimumab. The objective response rates of melanoma patients to nivolumab, and pembrolizumab are 43.7% and 33.7% respectively [109, 129].

Although immunotherapies have shown long-lasting effects in a moderate proportion of metastatic melanoma patients, approximately one-fourth/one-third of the patients will eventually relapse after showing initially an objective response to treatment [130]. Currently, much effort is placed on developing different therapeutical strategies to transform immunologically “cold” tumours into “hot” tumours. These approaches involve combination therapies [109, 131, 132] and the use of targeted therapy plus immunotherapy. Clinical trials of combination immune therapies [133-136] which compare the efficacy of single-agent nivolumab or ipilimumab versus ipilimumab plus nivolumab demonstrate increased response rates and overall survival in the combination therapy (Figure 1.6).

The increase in overall survival offered by immunotherapy and targeted therapy generated considerable interest to investigate these therapeutic agents in the neoadjuvant and adjuvant settings. Neoadjuvant therapy would allow curative surgical resection in melanoma patients with locally advanced disease. The use of neoadjuvant therapy is currently an active field of research, especially for patients with stage III that are at high risk of relapse. Numerous completed and ongoing trials are investigating the use of immunotherapies in the neoadjuvant setting.

Systemic immunotherapies have also shown a positive effect in stage III and IV post-surgery patients as an adjuvant treatment. Recent studies have demonstrated that adjuvant ipilimumab following resection of stage III disease improves PFS and OS rates at 5 years versus placebo (65% and 54% respectively).

However, in the ipilimumab cohort, grade 3 or 4 adverse events occurred in 41.6% of patients, together with 5 deaths (1.1%) [137]. Studies comparing ipilimumab with

nivolumab for resected stage III and IV disease, identified nivolumab as more effective and less toxic than ipilimumab [106]. However, further studies are needed to identify the best adjuvant therapy, as well as the dose and the duration of treatment that will benefit most patients.

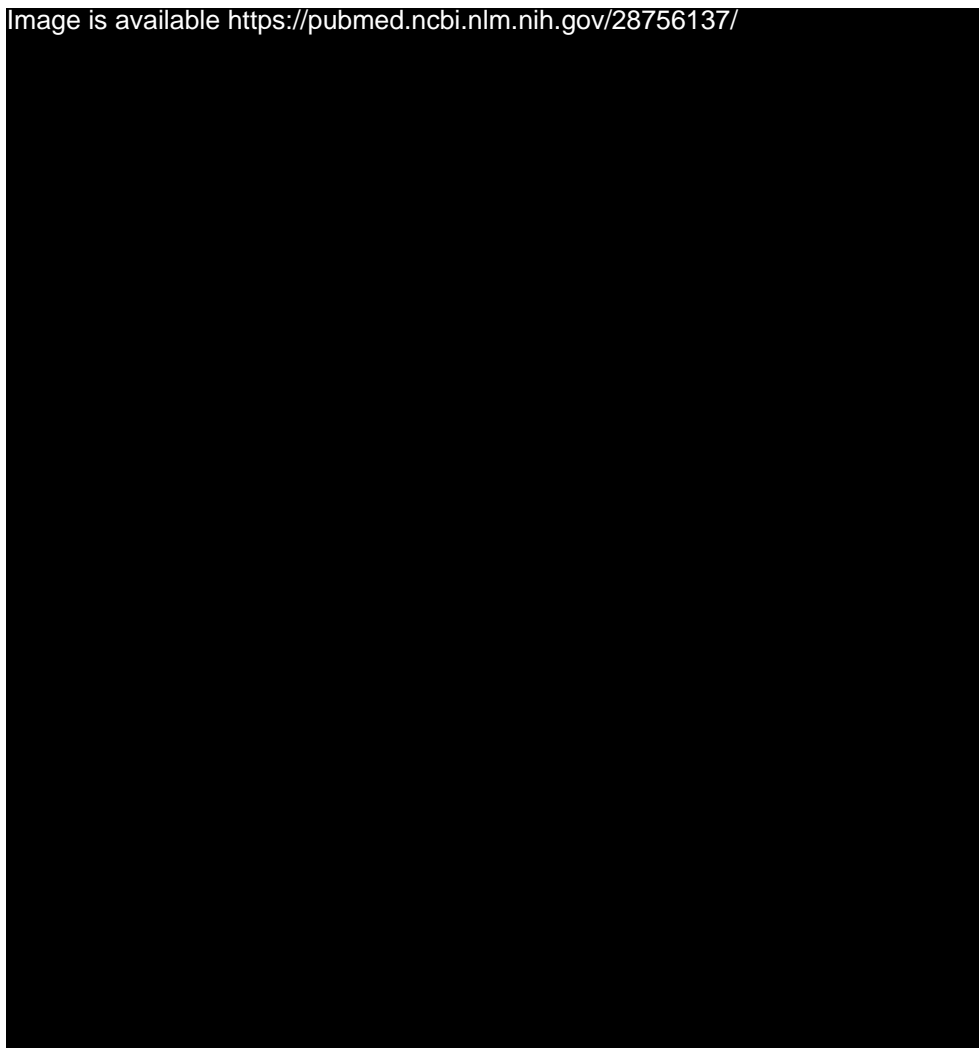


Figure 1.6. Summary of **A)** progression-free and **B)** overall survival by Kaplan–Meier analysis across clinical trials in patients with advanced-stage melanoma. Retrieved from Ugurel *et al.* [138].

Combinations of BRAFi and immunotherapy

Since BRAF-targeted therapy is associated with favourable effects in the tumour microenvironment [139-141], it was hypothesised that multi-modality treatment approaches that combine targeted therapies and immunotherapies might be of clinical value. Clinical trials comprising targeted therapies together with immunotherapies are also now underway, to explore the safety of the triple-combination of BRAF, MEK, and

PD-1 or PD-L1 inhibitors (CD-ON-MEDI4736-1161; KEYNOTE-022; COMBI-I; GP28384/TRILOGY). Initial data suggests that this combination is well tolerated, and response rates are similar to those observed with BRAF-MEK inhibitor combinations [142-146]. Further investigations will provide a more extensive understanding of the clinical utility of these therapies and their long-term efficacies.

Intratumoral Immunotherapy

More recently, melanoma treatments may include T-VEC, a genetically modified herpes virus that replicates in carcinogenic cells when directly administered into tumours. This oncolytic virus induces the production of granulocyte-macrophage colony-stimulating factor, improving the antigen presentation by macrophages, and enhancing a broader immune response to tumour antigens [147].

T-VEC is the most recent agent approved by the FDA for local treatment of advanced-stage, unresectable cutaneous, subcutaneous, and nodal lesions in patients with recurrent melanoma after surgery. Preliminary data from clinical trials indicate that lower toxicities and higher response rates are observed in patients treated with T-VEC in combination with ipilimumab (~50%) [148] or pembrolizumab (~46%) [149]. Ongoing trials are now comparing T-VEC alone or in combination with checkpoint inhibitors in advanced melanoma [150].

1.2.8 Melanoma blood-based biomarkers

Current therapeutic options for late stage melanoma disease are more effective in patients with a lower disease burden [151, 152]. The ability to identify melanoma at earlier stages or to recognise signs of disseminating disease prior to overt metastatic disease would be of significant clinical benefit. In addition, the rapidly evolving clinical landscape of melanoma therapy would be dramatically improved by the development of reliable and accurate biomarkers to provide accurate molecular classification and prognostication, and to monitor patient surveillance, during and/or post-treatment [153].

Currently, there are limited blood-based or tissue-specific biomarkers that are used for the clinical assessment of melanoma. One such marker routinely used to determine

prognosis in later disease stages is LDH, now incorporated into the TNM staging of melanoma [154]. However, the sensitivity of this marker is reduced during progression because it has a narrow dynamic range and its increase can be associated with non-specific inflammatory conditions [155].

MicroRNAs (miRNAs) present in the blood of melanoma patients can discriminate between healthy individuals and cancer patients [156]. Moreover, miRNA 150-5P [156] and miRNA 206 [157] have been found to predict melanoma patients with shortened PFS.

Melanoma exosomal proteins, such as tyrosinase-related protein 1 (TYRP1), tetraspanin CD63 and caveolin-1 have been found elevated in patients with advanced stage melanoma [158] and related to the development of resistance to treatment [159, 160].

Circulating tumour cells (CTCs) have also been indicated as a prognostic marker for melanoma [161-164], with the presence of these cells in the blood correlates with poor prognosis and short survival despite systemic treatment [165-167]. Additionally, heterogeneous CTCs have been identified with distinct populations shown to respond differently to targeted and immune therapies [168-170].

Although the prognostic utility of novel melanoma biomarkers, such as miRNA, exosomes, and CTCs have been evaluated with promising results, none have been utilised in the clinic to date for melanoma. Therefore, the ability to measure disease burden and treatment responses is limited and there is a need for biomarkers that are sensitive or specific enough to be beneficial for early detection of melanoma and prediction of treatment response and, to monitor melanoma and assess therapeutic responses are required.

1.3 Circulating tumour DNA (ctDNA)

The presence of fragments of cell-free nucleic acids (cfDNA) in human blood was first described by Mandel and Métais in 1948 [171]. These cfDNA are short nucleic fragments (~166 bp) found in plasma, serum, urine, cerebrospinal fluid, pleural fluid, and saliva [172-174]. It is thought that cfDNA is released as a result of cell apoptosis and/or necrosis and may also be released by active secretion (Figure 1.7).

In healthy individuals, cfDNA is released from apoptotic and necrotic cells into the bloodstream to reach concentrations that range from 1 to 10 ng ml⁻¹ in plasma [175-177]. However, these concentrations appear to be raised in patients with cancer, acute trauma, cerebral infarction or, as a result of exercise, transplantation and, infection, among other factors. In addition, cfDNA analysis provides “real-time” information about the mutations present in the tumour(s) since its half-life is between 15 minutes to 2.5 hours in circulation before it is cleared via degradation by the liver and kidney [178].

Image is available <https://doi.org/10.1038/nrclinonc.2013.110>



Figure 1.7. Analysis of cfDNA from the blood. cfDNA is released from cells undergoing apoptosis or necrosis. Retrieved from Crowley *et al.* [179].

Stroun and colleagues reported in 1989, that a proportion of cfDNA in the plasma of cancer patients originates from cancer cells [180]. However, only five years later, mutant *KRAS* sequences were first reported to be detected in the cfDNA of patients with pancreatic cancer, with the *KRAS* mutation matching that found in the patient’s tumour [181, 182], thus confirming that the mutations found in cfDNA are of tumour origin. Those tumour-specific DNA fragments were termed “circulating cell-free tumour DNA” (ctDNA) and are found to be shorter (134 - 144bp) than most cfDNA fragments [183].

Circulating tumour DNA (ctDNA) is one marker with great potential to provide information pertaining to treatment response early during treatment. Plasma ctDNA is derived from tumour cells that undergo apoptosis and so molecular characterisation provides information that can guide therapeutic decisions. In addition, ctDNA levels are indicative of tumour burden in patients with advanced melanoma and may similarly indicate residual localised disease.

Notably, at any one-time point, the blood of cancer patients contains ctDNA from multiple tumour sites, allowing faster, more accurate/complete analysis than that of individual tumour biopsies, which are from selected tumour sites. The short half-life of ctDNA [184] and the associated low risk of repeated collections enables the use of this liquid biopsy for detection of residual disease and to monitor cancer burden in response to a given therapy. In addition, genomic analysis of the ctDNA allows for early cancer detection and diagnosis (Figure 1.8). During the last decade, the development of more sensitive detection methods has allowed the study of this minimally invasive “liquid biopsy” for improved cancer management and monitoring.

Image is available <https://pubmed.ncbi.nlm.nih.gov/28233803/>



Figure 1.8. Applications of circulating tumour DNA analysis during the course of disease management. Retrieved from Wan *et al.* [185].

1.3.1 ctDNA detection methods

The detection in ctDNA from the blood of melanoma patients poses a significant challenge. Sensitive methods are required to detect low amounts of ctDNA, quantify the number of mutant fragments of ctDNA, and discriminate between normal cfDNA and tumour ctDNA. The analysis of ctDNA can be separated into two approaches based on whether the objective is to monitor specific genes or mutations in a targeted approach or to examine all genes in an untargeted approach (Table 1.2).

Table 1.2. Technologies for detecting circulating tumour DNA (ctDNA). Adapted from Elazezy *et al.* [186].

Method	Platform	Sensitivity	Specificity	Input	Targets	Type of alteration	Limitations
NGS	TAm-Seq	0.02%	99.9997%	0.9-20 ng	Panel	Known mutations	Detects only known mutations
	Safe-SeqS	0.1%	98.9%	3 ng	Panel	Known mutations and CNV	Less comprehensive than WES
	FASTSeqS	>10%	80%	5-10 ng	Panel	Genome-wide copy number changes	Low sensitivity and specificity
	CAPP-Seq	0.004%	>99.99%	32 ng	Panel	Known mutations, CNV, and rearrangements	High cfDNA input; detects only known mutations
	MCTA-Seq	0.25%	89%	7.5 pg	Panel	Known methylation sites	Low sensitivity and high cfDNA input
	Bias-Corrected Targeted NGS	>0.4%	100%		Panel	Known mutations, CNV, and rearrangements	Known mutations.
	Multiplex-PCR NGS	>0.1%	99.6%	2-50 ng	Panel	Known mutations	Detects only known mutations
Digital-PCR	ddPCR	0.1%	100%	25 ng	1 to 3	Known mutations	Detects specific genomic loci; limited in multiplexing
	BEAMing	0.01%	100%	1 ng	1 to 20	Known mutations	Detects only known mutations; no multiplexing
Real-Time PCR	AS-PCR	1%	98%	3-50 ng	1	Known mutations	Low sensitivity; detects known mutations
	AS-NEPB-PCR	0.1%	100%	20 ng	1	Known mutations	Detects only known point mutations
	(PNA-LNA) PCR clamp	0.1-1%	79%	30 ng	1	Known mutations	Low specificity; detects only known point mutations
	(COLD-PCR)	0.1%	94.9%	1-10 ng	1-3	Known mutations	Detect limited genomic loci; limited in multiplexing
	MS-PCR	0.62%	100%	20-100 ng	1	Known methylation sites	Detects only specific CpG islands
Mass-spectrometry technology	SERS	0.1%	100%	5 ng	3 to 10	Known mutations	Limited loci
	UltraSEEK	0.1%	100%	9 pg-4.2 ng	Up to 40	Known mutations	Limited loci

Assays that target individual mutations can achieve high sensitivity using a relatively simple workflow. Allele-specific PCR methods have been applied for the detection of hot-spot mutations in melanoma [187]. Similarly, a melanoma-specific panel was developed based on UltraSEEK technology [188]. Nevertheless, the most common methods to quantify plasma ctDNA are based on detection of single melanoma mutations by droplet digital PCR (ddPCR) [189] and BEAMing (beads, emulsion, amplification and magnetics) [190].

Due to its high sensitivity and specificity, ddPCR has been recognised as one of the most accurate and reliable tools to examine genetic aberrations in a wide variety of cancers [191]. Studies assessing the validity of ctDNA as a biomarker of disease status in stage IV melanoma, show that the sensitivity of ddPCR appears to be similar to the BEAMing method, with ctDNA detection values ranging from 73% to 89% [12, 14, 17, 192, 193].

Since around 70% of melanomas harbour *BRAF* V600 or *NRAS* Q61 mutations, targeted approaches for ctDNA analysis are highly efficient in most melanoma cases. However, patients that are wild-type for *BRAF* and *NRAS* (approximately 30% overall) are more challenging to monitor since new, personalised targets have to be identified. While the sensitivity of ddPCR and BEAMing allow the detection of low frequency, specific mutations, they are most suited for investigating a small number of pre-identified targets.

The development of technologies based on next-generation sequencing allows a broader study of the genome, enabling the monitoring of multiple tumour-specific mutations in a single assay. Targeted sequencing can be used to study small regions, such as individual exons, or a larger number of loci, expanding our ability to detect multiple genes of interest [194-197] (Table 1.2).

1.3.2 ctDNA as a biomarker of disease status in metastatic cancers

Various studies have shown that ctDNA levels correlate with tumour burden and disease stage in different cancer types [195, 198-201]. A study focused on comparing tumour volume with ctDNA levels in ovarian cancer, reported that ctDNA levels and disease volume were significantly correlated. In addition, the extent of the decrease in ctDNA levels after chemotherapy initiation was significantly associated with time to progression [202]. In patients with colorectal and breast cancer, detectable or increased ctDNA detection is a biomarker of worse survival compared with undetectable or decreasing ctDNA levels [195, 203].

Studies have also demonstrated that ctDNA can be used to monitor clonal evolution and identify mechanisms of resistance to treatment [7-10]. Serial ctDNA analysis in patients with colorectal cancer demonstrated the positive selection of *KRAS* mutant clones during *EGFR* blockade, and a later decline in the ctDNA levels from *KRAS* mutant clones upon the withdrawal of anti-EGFR therapy [204, 205]. In patients with NSCLC undergoing

treatment with EGFR inhibitors, resistance-conferring mutations emerged in plasma ctDNA prior to clinical progression determination [206, 207]. Studies in metastatic breast cancer comparing the sensitivity of ctDNA detection versus the widely used cancer antigen 15-3 biomarker showed ctDNA detection in 97%, in comparison with CA 15-3 detection in only 78% of 30 women with metastatic breast cancer [195]. Similar results were found in colon cancer, where ctDNA anticipated tumour recurrence earlier than the carcinoembryonic antigen biomarker [208].

Taken together, this evidence led the FDA and the EMA to approve the use of ctDNA analysis in non-small cell lung cancer (NSCLC), to detect the tyrosine kinase resistant clone EGFR p.T790M mutation allowing the early identification of patients that will benefit from second-line drugs [11]. Current FDA and EMA recommendations for the use of ctDNA in NSCLC, state that, if liquid biopsies are performed in advance of a tumour biopsy, ctDNA detection may abrogate the need for tissue biopsy, but if ctDNA analysis is negative, a tissue biopsy may still provide valuable genomic information. In addition, ctDNA analysis has now been included in numerous clinical trials, shortening the gap between research and clinical practice.

1.3.3 ctDNA as a biomarker for melanoma

ctDNA as a prognostic biomarker

The potential clinical validity of ctDNA for melanoma management has been addressed in previous studies. In particular, baseline ctDNA levels have been shown to significantly correlate with response to therapy [13, 209, 210], and were significantly associated with overall response rate (ORR) and PFS in melanoma patients undergoing targeted therapy [12, 15, 187, 193]. Ongoing clinical trials show that high basal levels of circulating *BRAF* V600E correlated with lower ORR and lower PFS to targeted therapy [12, 15]. These studies indicate that elevated ctDNA levels are a valid predictor of poor clinical outcome.

Various studies have evaluated the prognostic value of ctDNA in melanoma management. Baseline ctDNA levels have been shown to significantly correlate with response to therapy and survival outcomes in melanoma patients receiving targeted therapy [12, 15, 187, 193, 211]. A low baseline ctDNA level has been previously associated with long PFS in melanoma patients treated with immunotherapies [14, 212] and was found to

correlate with response to treatment [213-216]. In contrast, Lee et al. showed that a decline in ctDNA during treatment, but not low baseline levels, predicted longer PFS and OS [217]. Moreover, decreasing or undetectable ctDNA levels can distinguish between pseudoprogession or definite disease progression [218].

ctDNA as an indicator of treatment response

Similarly, in melanoma, multiple studies have shown evidence of a significant correlation between ctDNA levels and tumour burden [14, 16, 193]. Since ctDNA is tumour specific and can expand over a dynamic range, it appears to be a more accurate blood-based biomarker than the currently used LDH marker for defining patient tumour status [13]. Tsao and colleagues demonstrated that ctDNA levels correlate with tumour burden as assessed by radiological scans in patients treated with immunotherapies [219]. Furthermore, a close correlation ($p < 0.001$), between ctDNA levels per mL of plasma and metabolic tumour burden (MTB) (FDG uptake by the tumour assessed by PET/CT [16, 220]) has been identified. Interestingly, PET/CT remains more sensitive than ctDNA analysis at this stage, since ctDNA was not detectable in patients with an MTB value of less than 10, defining the limit of detection of ctDNA to date [221]. On the other hand, an increase in ctDNA levels during or after treatment can indicate disease progression. Frequent ctDNA analysis may lead to early detection of disease progression [14].

The surveillance of patients after receiving treatment is another potential clinical application of ctDNA. For patients who have achieved a complete response, ctDNA levels can be analysed to determine if the cessation of treatment is warranted. Conversely, for patients that have ceased treatment, monitoring of ctDNA levels, can help identify the emergence of recurrence.

In regards to early-stage patients, a study in early-stage colorectal cancer involving 230 patients indicated that recurrence-free survival at 3 years was 0% for the patients that had ctDNA-positive results at first follow-up after surgical resection, and was 90% for patients with undetectable ctDNA [222]. Similarly, in an early study of 55 breast cancer patients, ctDNA detection at first follow-up also indicated poor prognosis [223]. Thus, the detection of ctDNA following surgery or treatment with curative intent may signal the presence of minimal residual disease (MRD) even in the absence of any other clinical

evidence of disease. This has not been fully interrogated for melanoma. Further studies are required to elucidate whether ctDNA detection may indicate the presence of residual tumour mass that was not eradicated by standard treatment.

ctDNA analysis for comprehensive mutational profile

In melanoma tumours, clonal heterogeneity plays an important role in patient response to treatment and the development of resistance to treatment. While targeted therapies can efficiently target dominant clones, they can also drive an increase in the population of resistant clones. For example, recent studies have shown that ctDNA can be used to monitor the emergence of mutations responsible for driving acquired resistance to BRAF and/or MEK inhibitors in melanoma, such as, the *NRAS* (Q61K), *MAP2K1* (E203K), *PTEN*, *AKT1* [13, 14, 16]. These mutations appear in the ctDNA of patients that developed acquired resistance to BRAF inhibitor treatments, in some cases earlier than radiological evidence of disease resistance [224, 225]. In addition, low-coverage WGS and WES analyses of ctDNA identified *BRAF* amplification as a potential mechanism of resistance to MAPK inhibitors [16]. Therefore, monitoring *BRAF* V600 and *NRAS* (Q61K/R) ctDNA levels together with other mutations related to acquired resistance will aid in identifying when resistance emerges, allowing earlier transition to second-line treatment that leads to increased response rates and improved patient outcomes [226].

Overall, ctDNA appears to be a clinically valid biomarker with great potential to inform clinical decisions in melanoma. The ability to reflect tumour burden and clonal evolution makes it a reliable biomarker for disease monitoring and prediction of response to treatment. However, important limitations need to be addressed to define the real clinical utility of ctDNA as a melanoma-specific biomarker.

Firstly, most ctDNA studies thus far have only analysed *BRAF* or *NRAS* mutant cases, which represent approximately 70% of patients. These studies have remarked on the high fidelity of *BRAF* mutant ctDNA to reflect disease burden and tumour status of patients prior to and during treatment [12-14, 210, 227]. However, there is a need to ascertain the detection rate and kinetics of other common mutations found in melanoma to determine whether they can be effectively used for patient surveillance during and post-treatment of *BRAF* wild-type patients.

Secondly, the improvement in the clinical management of melanoma and proactive approach to surveillance by imaging scans means that an increasingly number of patients are provided BRAF inhibitors and immunotherapy at the early signs of metastatic disease. Thus, there is now an increase in melanoma patients with low disease burden receiving therapy. It is therefore imperative to improve the detection rate of ctDNA in melanoma and to ascertain its utility as a liquid biopsy as a suitable alternative for tissue biopsy for mutational analysis.

Thirdly, limited studies have evaluated the predictive value of baseline ctDNA in patients treated with immunotherapy. Hence, studies that clarify the ability of ctDNA to predict survival in patients treated with immunotherapies are required, particularly examining prior treatment regimens and mutation status.

Lastly, while previous studies have demonstrated the efficiency of ctDNA to indicate disease progression earlier than conventional imaging, such comparisons need to be conducted in a large cohort of patients. Cross-sectional studies aimed at comparing the efficiency of ctDNA to indicate treatment response or failure and disease progression against conventional clinical standards will inform whether this liquid biopsy is a suitable complementary, if not alternative, test for melanoma patient surveillance in the oncology clinic.

1.4 Rationale for this study

There is a clear need for novel diagnostic and molecular tools in oncology, particularly in melanoma. Australia has one of the highest melanoma incidences world-wide and the annual estimated treatment cost for all new melanoma cases in Australia has risen to AU\$201 million [228]. Numerous health resources are used in melanoma management, including doctor consultations, tissue biopsies, tumour surgeries, imaging examinations, targeted treatments, radiation therapies and chemotherapies. Currently, targeted therapies and immunotherapies are within the standard treatment regimens for metastatic melanoma in Australia and several other countries [229]. However, these novel therapies are exceptionally expensive [230, 231] and can have severe side effects. Therefore, non-invasive and cost-effective biomarkers are required for the early detection of therapeutic response and disease progression to aid with timely treatment adjustments.

In recent years, ctDNA has emerged as a clinically relevant oncological biomarker for tumour genotyping, prognostication and disease monitoring, as demonstrated by the multiple studies in breast, lung and ovarian cancers [192, 232, 233]. In melanoma, ctDNA levels before therapy initiation of BRAF inhibitor or immunotherapies, correlated with tumour burden and were associated with ORR and PFS [12, 15, 187, 193]. Changes in plasma ctDNA level during treatment was indicative of either response (decreased) or treatment failure (maintained or increased), even prior to clinically identifiable progressive disease in some cases [14, 16]. Furthermore, this blood-based biomarker is isolated from blood using minimally invasive procedures and can be analysed using relatively low-cost tests, such as ddPCR assays. Thus, ctDNA may provide a holistic, cost-effective, and highly specific analysis and tracking of each patient's disease status for improved clinical management of melanoma.

Although tumour tissue biopsy remains the gold standard in diagnosis and mutational analysis for clinical management of melanoma, the acquisition of tumour tissue is often limited by the need for highly invasive surgical procedures and/or inaccessible tumour locations. A significant concordance between the mutational profile obtained from tissue and plasma biopsies has been previously reported [15, 234]. These studies highlight the ability of ctDNA to capture the genetic heterogeneity of melanoma tumours and its

potential inclusion in the clinical setting as an alternative to tumour genotyping. It is, therefore, important to evaluate the clinical validity of ctDNA for the detection of *BRAF* V600 mutant patients that will be eligible for BRAF/MEK inhibitor therapy.

The development of new therapies for melanoma has triggered the urgent need to investigate biomarkers that allow efficient monitoring of patient disease. Baseline ctDNA levels and subsequent decline with treatment have been indicated as an early predictor of tumour response and clinical benefit [14, 193, 217]. In melanoma, *BRAF* mutant ctDNA is a robust biomarker for disease burden and tumour status of patients before and during targeted treatment [14, 16, 234, 235]. However, many patients receiving first-line immunotherapy, especially in Australia, are *BRAF* *WT* and may carry *NRAS* or other mutations. Therefore, further studies are needed to evaluate the prognostic and predictive value of early changes in ctDNA levels to inform therapeutic outcomes in *BRAF* as well as in non-*BRAF* metastatic melanoma patients receiving systemic therapies.

Studies have suggested that ctDNA can serve as an indicator of treatment failure prior to clinically identifiable progressive disease, providing a lead time that may be important for effective subsequent treatments [14, 16]. However, very few studies [224, 225] have evaluated the efficacy of ctDNA to identify disease progression in a large cohort of melanoma patients and compare it to medical imaging, the current gold standard for disease monitoring.

In conclusion, the overarching aim of this thesis is to evaluate the clinical validity of ctDNA within specific contexts of use for the management of metastatic melanoma. The result of these studies provides valuable information to aid the implementation of this biomarker in the clinic, ultimately improving clinical outcomes of melanoma patients.

1.4.1 Hypothesis and aims

Hypothesis: *Analysis of plasma ctDNA can aid treatment selection, disease monitoring and surveillance of metastatic melanoma patients.*

- Aim 1. To determine the detection rate of *BRAF* mutations in plasma ctDNA and compare the efficacy of ctDNA as a medium for tumour genotyping relative to current clinical standards, including a cost analysis.
- Aim 2. To evaluate the ability of pre-treatment ctDNA levels to provide prognostic information in different treatment types and lines of therapies.
- Aim 3. To investigate how changes in ctDNA levels early during treatment correlate with response.
- Aim 4. To examine the validity of ctDNA to inform of disease progression in comparison to conventional clinical imaging.

CHAPTER 2. ISOLATION AND QUANTIFICATION OF PLASMA CIRCULATING TUMOUR DNA FROM MELANOMA PATIENTS

Publication details

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Chapter 2 is not available in this version of the thesis

CHAPTER 3. BRAF V600 TESTING IN CIRCULATING TUMOUR DNA FOR TREATMENT DECISIONS IN METASTATIC MELANOMA: COMPARATIVE OUTCOMES AND COST ANALYSIS FOR LIQUID VERSUS TISSUE BIOPSIES

Publication details

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Chapter 3 is not available in this version of the thesis

CHAPTER 4. CIRCULATING TUMOUR DNA PREDICTS OUTCOME FROM FIRST-, BUT NOT SECOND-LINE TREATMENT AND IDENTIFIES MELANOMA PATIENTS WHO MAY BENEFIT FROM COMBINATION IMMUNOTHERAPY

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4.1 Abstract

We evaluated the predictive value of pre-treatment ctDNA to inform therapeutic outcomes in metastatic melanoma patients relative to type and line of treatment.

Plasma ctDNA was quantified in 125 samples collected from 110 patients prior to commencing treatment with immune checkpoint inhibitors (ICI), as first- ($N=32$) or second-line ($N=27$) regimens, or prior to commencing first-line BRAF/MEK inhibitors therapy ($N=66$). An external validation cohort included 128 patients commencing ICI therapies in the first- ($N=77$) or second-line ($N=51$) settings.

In the discovery cohort, low ctDNA (≤ 20 copies/mL) prior to commencing therapy predicted longer progression-free survival (PFS) in patients treated with first-line ICIs (Hazard Ratio (HR) 0.20, 95% Confidence Interval (CI) 0.07-0.53, $P < 0.0001$), but not in the second-line setting. An independent cohort validated that ctDNA is predictive of PFS in the first-line setting (HR 0.42, 95% CI 0.22-0.83, $P = 0.006$), but not in the second-line ICI setting. Moreover, ctDNA prior to commencing ICI treatment was not predictive of PFS for patients pre-treated with BRAF/MEK inhibitors in either the discovery or validation cohorts. Reduced PFS and overall survival were observed in patients with high ctDNA receiving anti-PD-1 monotherapy, relative to those treated with combination anti-CTLA-4/anti-PD-1 inhibitors.

Pre-treatment ctDNA is a reliable indicator of patient outcome in the first-line ICI treatment setting but not in the second-line ICI setting, especially in patients pre-treated with BRAF/MEK inhibitors. Preliminary evidence indicated that treatment-naïve patients with high ctDNA may preferentially benefit from combined ICI.

Keywords: circulating tumour DNA (ctDNA), melanoma, immunotherapy, immune checkpoint inhibitors (ICI), progression-free survival, treatment.

4.2 Introduction

Metastatic melanoma is an aggressive type of skin cancer and responsible for most skin cancer-related deaths [293, 294]. In the last decade, the emergence of targeted therapy and immune-checkpoint inhibitor/s (ICI/s) has significantly changed the clinical management and outcome of melanoma patients [138, 295]. Current treatment options for unresectable stage III and stage IV disease [296] include BRAF targeted therapies for patients that have *BRAF* mutant melanomas, and ICIs with anti-PD-1 alone or in combination with anti-CTLA-4. However, durable response is only seen in a minority of patients, and the optimal sequencing of therapies and the selection of the most effective first-line therapy, remain controversial [297, 298]. The CheckMate 067 trial demonstrated that the combination ipilimumab and nivolumab resulted in superior long-term survival outcomes compared to either nivolumab or ipilimumab monotherapy, with a 5-year overall survival of 52% [136]. However, in addition to being a more costly regimen [299], patients treated with combination therapy experienced more grade 3 or 4 treatment-related adverse events relative to those treated with nivolumab or ipilimumab alone [299]. Thus, there is a need to better stratify patients who will require upfront combination therapy from those who may derive a similar benefit from anti-PD-1 monotherapy.

The potential clinical utility of ctDNA for melanoma management has been demonstrated in multiple studies. Elevated baseline ctDNA levels have been shown to significantly correlate with low overall response rate (ORR) and short progression-free survival (PFS) in melanoma patients receiving targeted therapy [12, 15, 187, 193]. However, there is a paucity of studies evaluating the predictive value of baseline ctDNA in patients treated with immunotherapy. Low baseline ctDNA level has been previously associated with long PFS in melanoma patients treated with ICI [14] and was found to correlate with tumour shrinkage on radiology [213]. In contrast, Lee et al. showed that a decline in ctDNA during treatment, but not low baseline levels, predicted longer PFS and OS [217]. Thus, more studies are needed to clarify and further refine the predictive value of ctDNA in patients treated with immunotherapies, particularly taking into consideration the line of therapy, prior treatment regimens and mutation status.

In this study, we analysed a prospective cohort of melanoma patients receiving systemic therapies, including a large proportion of *BRAF* wild-type (WT) cases. We compared the predictive value of pre-treatment ctDNA levels to inform survival outcomes in metastatic melanoma patients receiving first-line or second-line systemic ICI. Our observations were validated using published datasets from two independent cohort studies [16, 217, 218, 300].

4.3 Materials and methods

4.3.1 Discovery cohort

We analysed a total of 125 baseline plasma samples collected prior to commencing systemic therapy from 110 patients with unresectable stage IV cutaneous melanoma enrolled in the study between 2013-2018 at Sir Charles Gairdner Hospital (SCGH) and Fiona Stanley Hospital (FSH) in Perth, Western Australia. Patients' characteristics are presented in Figure 4.1 and Table 4.1. A subset of 15 patients were considered as baseline for their first- and second-line therapy. This study received approval from the Human Research Ethics Committee of Edith Cowan University (No. 11543 and No. 18957) and Sir Charles Gairdner Hospital (No. 2013-246).

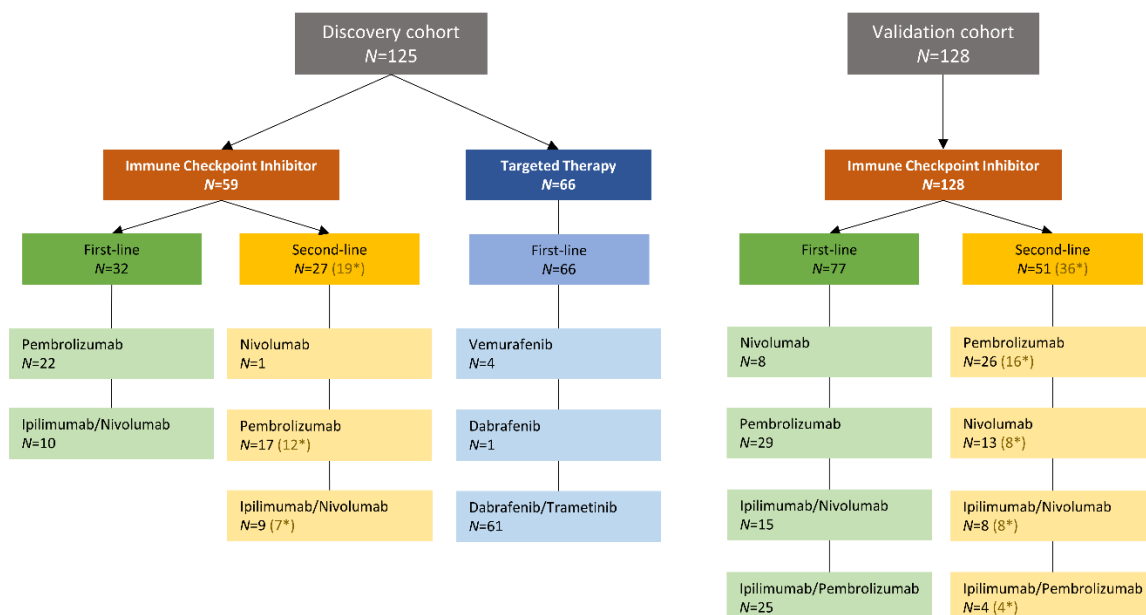


Figure 4.1. Study cohorts. Flow charts of the samples included in the analyses, which is comprised of 125 patients treated with first- or second-line ICI and first-line targeted therapy from the discovery cohort and 128 validation samples from two independent sites. *Samples obtained from patients treated with second-line ICI after failing first-line therapy with *BRAF*±*MEK* inhibitors.

Table 4.1. Clinical characteristics at baseline of the melanoma patients included in the study.

Variable	Discovery Cohort			Validation Cohort	
	1st line ICI N=32 (%)	2nd line ICI N=27 (%)	1st line TT N=66 (%)	1st line ICI N=77 (%)	2nd line ICI N=51 (%)
Age					
≤65	12 (38)	14 (52)	41 (62)	38 (49)	30 (59)
>65	20 (62)	13 (48)	25 (38)	39 (51)	21 (41)
Sex					
Female	7 (22)	7 (26)	24 (36)	25 (32)	20 (39)
Male	25 (78)	20 (74)	42 (64)	52 (68)	31 (61)
AJCC Stage/M Classification					
IIIc				1 (1)	2 (4)
M1a	4 (13)	7 (26)	14 (21)	16 (21)	1 (2)
M1b	6 (19)	1 (3)	6 (9)	18 (23)	9 (18)
M1c	14 (43)	11 (41)	30 (46)	13 (17)	17 (33)
M1d	8 (25)	8 (30)	16 (24)	29 (38)	22 (43)
Brain only metastasis					
Yes	2 (6)	5 (19)	2 (3)	1 (1)	8 (16)
No	30 (94)	22 (81)	64 (97)	76 (99)	43 (84)
LDH levels					
Normal	8 (25)	10 (37)	29 (44)	55 (71)	35 (69)
Elevated	6 (19)	9 (33)	19 (29)	19 (25)	16 (31)
Not available	18 (56)	8 (30)	18 (27)	3 (4)	
Treatment					
<u>ICI</u>					
Nivolumab		1 (3)		8 (10)	13 (25)
Pembrolizumab	22 (69)	17 (64)		29 (38)	26 (51)
Ipilimumab/Nivolumab	10 (31)	9 (33)		15 (20)	8 (16)
Ipilimumab/Pembrolizumab				25 (32)	4 (8)
<u>Targeted Therapies</u>					
Vemurafenib			4 (6)		
Dabrafenib			1 (2)		
Dabrafenib/Trametinib			61 (92)		
Prior lines of therapy					
<u>BRAF±MEK inhibitors</u>					
Dabrafenib/Trametinib		19 (70)			34 (67)
Encorafenib/Binimetinib					1 (2)
Vemurafenib					1 (2)
<u>ICI</u>					
Ipilimumab		6 (22)			14 (27)
Pembrolizumab		2 (8)			
Ipilimumab/Nivolumab					1 (2)
BRAF mutation status					
BRAF Mutant	2 (6)	21 (78)	66 (100)	35 (45)	38 (75)
NRAS Mutant	17 (53)	1 (3)		31 (41)	11 (21)
BRAF/NRAS WT	13 (41)	5 (19)		11 (14)	2 (4)

TT: targeted therapy, ICI: Immune checkpoint inhibitors.

Written informed consent was obtained from patients prior to sample collection and analysis. Patients were clinically monitored, and the median follow-up period was 95 weeks (range: 16-257 weeks).

4.3.2 Validation cohort

We pooled data from two independent cohorts of 128 unresectable stage III ($N=3$) and stage IV melanoma ($N=125$) patients recruited from the Melanoma Institute Australia (NSW) affiliated hospitals and Peter MacCallum Cancer Centre (Victoria), as previously described by Lee *et al.* [217, 218, 300] and Wong *et al.* [16]. Additional details and patient characteristics are shown in Figure 4.1 and Table 4.1. Further comparison between the discovery and validation cohorts is presented in Table S4.1.

4.3.3 Treatment response and disease progression assessment

Radiological assessment of treatment response and disease progression was performed at two to three monthly intervals by computed tomography (CT) and/or ^{18}F -labeled fluorodeoxyglucose positron emission tomography (FDG-PET) scans. Magnetic resonance imaging (MRI) of the brain was also used where indicated. PFS was defined as the time interval between the start of therapy and the date of first clinical or radiological progression.

4.3.4 Plasma sample preparation and cfDNA extractions

Pre-treatment blood samples were collected into EDTA vacutainer or Cell-Free DNA BCT® (Streck, La Vista, NE) tubes. Isolated plasma was stored at -80°C until extraction. Plasma cfDNA was isolated from 1-5 mL of plasma using QIAmp Circulating Nucleic Acid Kits (Qiagen, Hilden, Germany), as per the manufacturer's instructions. The recovered cfDNA was freezer-stored until ctDNA quantification.

4.3.6 ctDNA quantification

For the discovery cohort, the mutation target for ctDNA analysis was selected based on the mutation reported in each patient's molecular pathology result (*BRAF* mutant) or, if *BRAF* WT, obtained from next generation sequencing of tissue biopsy using a custom melanoma panel, as previously described by Calapre *et al.* [234]. Commercially available

and/or customised probes were used to analyse ctDNA by droplet digital PCR (ddPCR). To cover all patients, we used a total of twenty-two different hotspot sequence variants in 10 different genes (Table S4.2 and Table S4.3). Droplets were generated using an Automatic Droplet generator QX200 AutoDG (Bio-Rad) and analysed using the QuantaSoft analysis software version 1.7.4 (Bio-Rad). Amplifications were performed in 40 μ L reactions using previously described cycling conditions [14]. Quantification results were presented in copies of ctDNA per mL of plasma.

A cutoff of 20 copies/mL was used for comparison of our analyses with the results from different cohorts analysed in three laboratories. This is the minimum ctDNA concentration that could be reliably detected given that ctDNA was isolated from 1-5 mL of plasma, eluted in 30-60 μ L, an input of 5-8 μ L in the ddPCR reaction and using different copies per mL of plasma (copies/mL) as threshold based on specific assays (Table S4.2) [16, 217, 218, 300]. We confirmed the suitability of this cutoff value through ROC curve analysis using the discovery cohort data for prediction of 6-month PFS (Figure S4.1). Patients with more than 20 copies/mL were defined as having a high ctDNA level, while patients \leq 20 copies/mL were considered to have low ctDNA level.

4.3.7 Statistics

A comparison of the patients' characteristics between the discovery and the validation cohorts was performed using a Chi-Square or two-sided Fisher's exact test, with the frequencies, percentages and the *P*-values reported. Similarly, patient characteristics were compared by group using a Chi-Square or two-sided Fisher's exact test, reporting their corresponding *P*-values. A ROC curve was calculated to determine the best cutoff value to dichotomise ctDNA concentration to predict 6-month PFS. Multiple ctDNA cutoffs were calculated by averaging two consecutive ctDNA values. These values were then used to calculate survival HRs and 95% CI for each ctDNA cutoff following a previously described analysis [301].

Median PFS was calculated using the Kaplan-Meier method, and survival curves statistical significance was determined using the log-rank and Gehan-Breslow-Wilcoxon test, when indicated, to stress the importance of early events. Univariate and multivariate Cox regression analyses were performed for PFS and OS comparisons in the discovery

cohort, the validation cohort and ICI monotherapy or combination cohort. All statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software Inc.), SPSS version 25 (IBM) and R Studio (v.1.1.456). Results with $P < 0.05$ were considered statistically significant.

4.4 Results

4.4.1 Patient characteristics

The discovery cohort comprised of 125 plasma samples. Of these, 66 were from patients with *BRAF* mutant melanoma commencing first-line targeted therapy, and most patients received dabrafenib/trametinib (61/66, 92%; Figure 4.1 and Table 4.1). A total of 32 patients were treated with first-line ICI monotherapy or in combination (22 anti-PD-1 alone, 10 anti-CTLA-4 plus anti-PD-1 combination treatment) and 27 were treated with second-line ICI/s. Of the latter, 19 (70%) received a combination of BRAF±MEK inhibitors as first-line treatment, while 8 patients received ICI monotherapy as first line followed by combination ICI (Table 4.1 and Table S4.1). Overall, the discovery cohort included 2 *BRAF* mutant patients in the first-line ICI group (2/32, 6%) and 21 in the second-line ICI group (21/27, 78%).

4.4.2 Baseline ctDNA and progression-free survival

Consistent with previous studies, for patients treated with first-line targeted therapy, low plasma ctDNA level at baseline was predictive of longer PFS (median: 57 vs 29 weeks, HR 0.54, 95% CI 0.30-0.98, $P = 0.025$; Figure S4.2). We then evaluated the predictive value of baseline ctDNA levels in patients receiving first- or second-line ICI in the discovery cohort. No statistically significant differences between clinical patients' characteristics were associated with ctDNA levels (Fisher's exact test, Table S4.4). However, patients with low ctDNA levels prior to first-line treatment initiation ($N = 18$) had a significantly longer PFS, with undefined median, compared to patients with high levels of ctDNA (median PFS 8 weeks, HR 0.20, 95% CI 0.07-0.53, $P < 0.0001$; Figure 4.2A). The predictive value of ctDNA was significant across multiple cutoff values (Figure S4.3A). Multivariate Cox regression analysis controlling for age, sex, tumour stage, brain metastases and BRAF status confirmed that low ctDNA level at baseline was an independent predictor of longer PFS (HR 5.18, 95% CI 1.88-14.31, $P = 0.001$; Table S4.5).

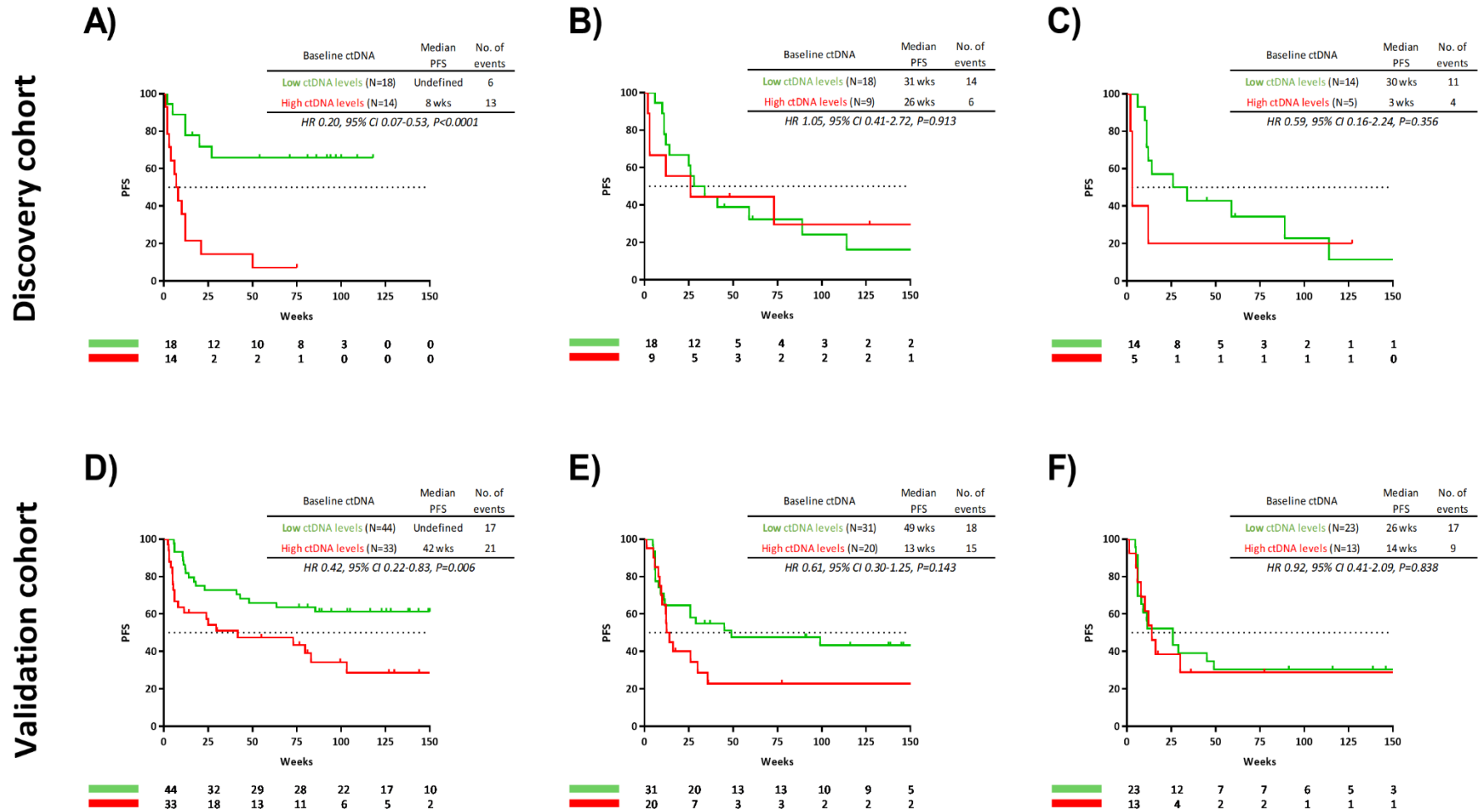


Figure 4.2. Kaplan-Meier curves for progression-free survival (PFS) of melanoma patients treated with ICI. Patients were stratified into those with low (green) or high (red) baseline ctDNA levels. Each graph denotes PFS outcomes in the discovery (A-C) or validation cohorts (D-F); for patients treated with ICI as first-line (A, D) or second-line treatment (B, E); or for BRAF mutant patients receiving ICI after failing first-line targeted therapy (C, F). Log-rank P-values, Hazard Ratio (HR) and 95% confidence intervals (CI) are indicated for each plot.

Analysis of patients receiving ICI as second-line ($N=27$) failed to demonstrate an association between low ctDNA and longer PFS (median PFS 31 vs 26 weeks, HR 1.05, 95% CI 0.41-2.72, $P=0.913$; Figure 4.2B) using 20 copies/mL as cutoff or any other value (Figure S4.3B). Similar results were observed when removing 5 patients with intracranial disease only (Figure S4.4A). As 19/27 (70%) of patients receiving second-line ICI, had BRAF inhibitors (with or without MEK inhibitors) as first-line treatment, we evaluated the PFS outcome of this sub-group of patients. Low baseline ctDNA in patients commencing ICI as second-line after failing therapy with BRAF \pm MEK inhibitors was not a predictor of longer PFS (median: 30 vs 3 weeks, HR 0.59, 95% CI 0.16-2.24, $P=0.356$; Figure 4.2C and Figure S4.3C), contrary to the first-line ICI setting. Nevertheless, this observation was derived from a small cohort (14 vs. 5) patients.

4.4.3 Validation cohort

Two independent melanoma patient cohorts receiving ICI/s in the first- or second-line setting were combined and used to validate our findings ($N=128$). This validation cohort comprised 77 patients treated with first-line ICI (37 anti-PD-1 monotherapy, 40 anti-CTLA-4 plus anti-PD-1 combination) and 51 patients treated with second-line ICI/s. Of these 51 patients, 36 (71%) were treated with first-line BRAF \pm MEK inhibitors (Table S4.1), while 14 (27%) were treated with ipilimumab and 1 (2%) was treated with ipilimumab plus nivolumab as first-line treatment. The cohort included 35 (35/77, 45%) *BRAF* mutant patients in the first-line ICI group and 38 (38/51, 75%) in the second-line ICI group. The validation cohort had a significantly higher number of *BRAF* mutant patients treated with first-line ICI than the discovery cohort (35/77, 45% vs 2/32, 6%, $P<0.0001$, respectively; Table S4.1). Similarly, there was a significantly higher number of patients treated with combination ICIs in the first-line setting than in the discovery cohort ($P=0.048$). No other statistical difference in patient characteristics was found between the validation and the discovery cohorts.

4.4.4 Baseline ctDNA predictive value in the validation cohort

Similar to the discovery cohort, patients with low baseline ctDNA levels prior to first-line ICI showed a significantly longer PFS than patients with high ctDNA levels (median PFS undefined vs 42 weeks, HR 0.42, 95% CI 0.22-0.83, $P=0.006$; Figure 4.2D). The predictive

value of ctDNA was significant across multiple cutoff values (Figure S4.3D). Multivariate Cox regression analysis controlling for age, sex, tumour stage, brain metastases, BRAF status and LDH confirmed that low ctDNA level at baseline prior to first-line ICI, was an independent predictor for longer PFS (HR 2.423, 95% CI 1.17-5.02, $P=0.017$; Table S4.6).

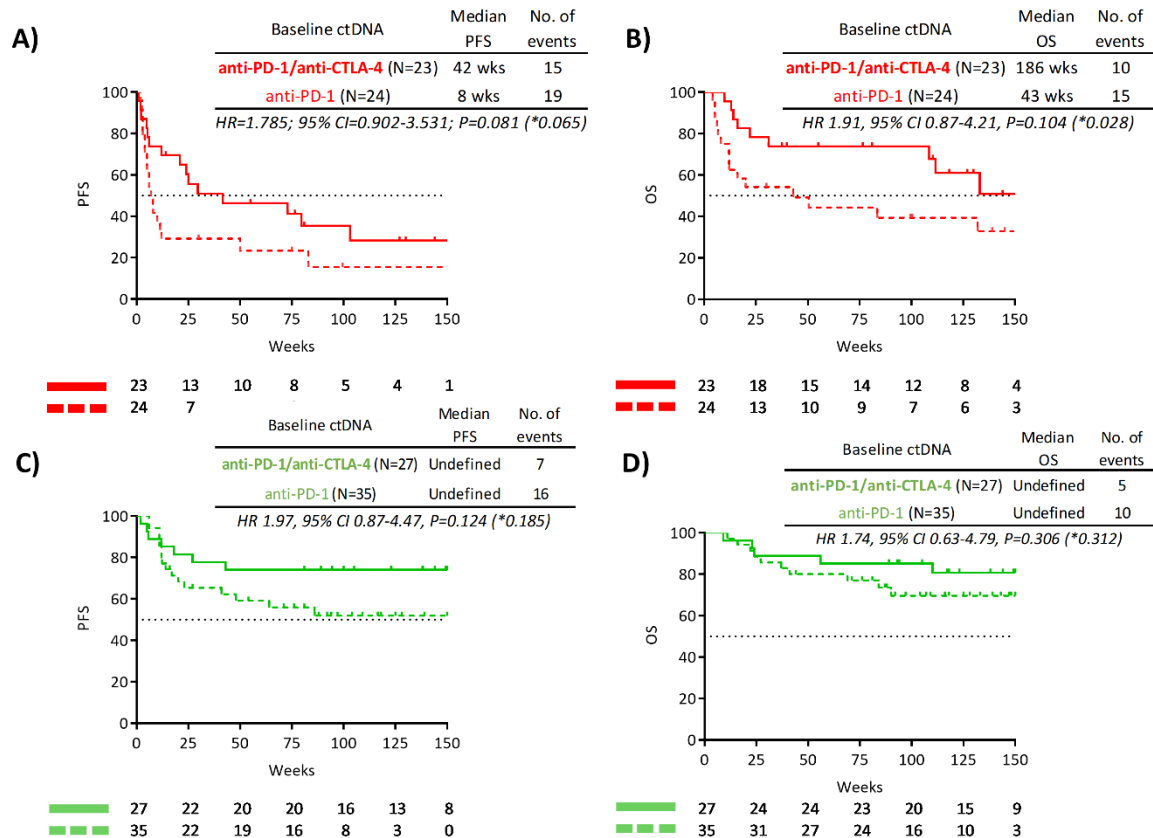


Figure 4.3. Kaplan-Meier plots comparing survival of patients receiving first-line single-anti-PD-1 and ICI combination. Patients were separated based on high (A-B, red) and low (C-D, green) baseline ctDNA levels, treated with anti-PD-1 alone (dashed line) or with combination anti-CTLA-4 plus anti-PD-1 (solid line). Graphs represent progression-free survival (PFS; A and C) and overall survival (OS; B and D). Log-rank P -values, Hazard Ratio (HR) and 95% confidence intervals (CI) are indicated for each plot. *Represents Gehan-Breslow-Wilcoxon P -values.

Similar to the discovery cohort, low ctDNA was not associated with longer PFS in the second-line setting in the validation cohort (median PFS 49 vs 13 weeks, HR 0.61, 95% CI 0.30-1.25, $P=0.143$; Figure 4.2E) using 20 copies/mL or below as cutoff value (Figure S4.3E). Notably, within the group with low ctDNA levels in the second-line ICI setting, a significantly higher number of patients had metastases in brain-only compared to the group with high ctDNA levels ($P=0.016$; Table S4.7). When removing from the analysis cases with intracranial disease only, patients with high ctDNA levels had decreased PFS (undefined vs 13 weeks, HR 0.41, 95% CI 0.18-0.93, $P=0.023$; Figure S4.4B).

For *BRAF* mutant patients who received ICI after failing first-line targeted therapies ($N=36$), low baseline ctDNA level did not predict longer PFS in the validation cohort (median PFS 26 vs 14 weeks, HR 0.92, 95% CI 0.41-2.09, $P=0.838$; Figure 4.2F and Figure S4.3F), as observed in the discovery cohort. This result was maintained when removing from the analysis patients with intracranial disease only (median PFS 26 vs 14 weeks, HR 0.70, 95% CI 0.27-1.78, $P=0.431$; Figure S4.4C).

4.4.5 Survival analysis in patients with high ctDNA treated with combination or single agent ICI in the first-line setting

Overall, ctDNA was found to be predictive of PFS in patients treated with first-line ICI. However, patients with high ctDNA levels in the validation cohort showed longer PFS compared to patients with similarly high baseline ctDNA levels in the discovery cohort (8 vs 42 weeks, red lines in Figure 4.2A and Figure 4.2D). It is important to note that in the discovery cohort, only 29% (4/14) of patients with high ctDNA levels at baseline were treated with combination anti-CTLA-4 plus anti-PD-1 therapy, compared to 58% (19/33) of patients in the validation cohort (Table S4.4 and Table S4.7).

To investigate whether the combination of ICIs is more effective in patients with high ctDNA, patients from both cohorts were combined and dichotomised according to whether they had high or low baseline ctDNA levels. Within these groups, survival outcomes were compared in patients who received single agent anti-PD-1 versus combination of ICIs. Comparison of patient characteristics revealed a larger proportion of *BRAF* mutant patients with low ctDNA levels received combination therapy ($P=0.003$; Table S4.8).

Albeit not significant, a trend showing longer PFS was observed for patients with high baseline ctDNA treated with combination of ICIs when compared to those who received anti-PD-1 monotherapy (median: 42 vs 7.5 weeks, HR 1.79, 95% CI 0.90-3.53, $P=0.081$; Figure 4.3A). Similarly, patients treated with combination therapy showed longer OS (median: 186 vs 43 weeks, HR 1.91, 95% CI 0.87-4.21, $P=0.104$; Figure 4.3B). However, OS was significant when the Gehan-Breslow-Wilcoxon test was used ($P=0.028$), reflecting the difference in events occurring at the beginning of the curve. Patients with low ctDNA levels showed no differences in PFS or OS when treated with combination of ICIs versus

monotherapy (HR 1.97, 95% CI 0.87-4.47, $P=0.124$; Figure 4.3C and HR 1.74, 95%CI 0.63-4.79, $P=0.306$; Figure 4.3D, respectively). However, none of the groups reached median PFS or OS, and a limited number of events were recorded within the follow up time.

4.5 Discussion

Studies investigating the ability of baseline ctDNA to predict treatment outcome in melanoma patients undergoing ICI are scarce, mainly including *BRAF* mutant melanomas and do not differentiate between treatment lines [14, 212, 217]. In this study, we showed that baseline ctDNA in metastatic melanoma patients receiving first-line ICI is a strong predictor of clinical outcome, as shown in both the discovery and validation cohorts. In contrast, the predictive value of ctDNA is lost in second-line ICI, particularly in patients failing first-line targeted therapy. Overall, our results redefine the context of use of ctDNA as a predictive biomarker in melanoma patients receiving immunotherapy, confining its utility to the first-line treatment setting.

Currently, there is paucity of published data related to outcomes of patients who experience disease progression on BRAF inhibitor therapies and are treated with second-line ICI. A recent retrospective study demonstrated that while ICI first-line efficacy appears comparable to trial populations, ICI treatment of *BRAF* mutant patients failing targeted therapy demonstrated a significantly lower response [302-304], and indeed, any drug therapy has lower efficacy in the second-line setting [305-307]. Consistent with these findings, our results demonstrate the limitation of ctDNA as a predictive biomarker in the second-line setting. Mason et al. found that *BRAF* mutant patients refractory to first-line targeted therapy have a high proportion of brain metastases (47%) [302], a finding similar to that observed in the discovery and validation cohorts (37% and 58%, respectively). The brain is a common site of targeted therapy failure, often contributing to death [308]. Given that brain metastases have been reported to shed less ctDNA into the circulation [192, 300], the large proportion of disease progression within the brain, may contribute to the limited predictive value of ctDNA in this setting. In line with this, removal of patients with only intracranial metastases from the analysis improved the ctDNA predictive value in the validation cohort. However, ctDNA still failed to predict PFS for patients who received prior treatment with targeted therapies.

The predictive significance of ctDNA levels prior to commencing targeted therapies has been previously demonstrated, with the absence of detectable ctDNA correlating with longer survival in large cohorts of melanoma patients [12, 15, 187, 193]. Similarly, we found that baseline ctDNA level was a strong predictor of clinical outcome in melanoma patients in the first-line targeted therapy setting, showing that high ctDNA level is associated with poorer clinical outcomes.

The selection of first-line monotherapy over ICI combination is currently a complex decision and factors such as median tumour size, LDH levels, *BRAF* status, presence of brain metastases and comorbidities must be carefully considered [136, 309, 310]. We observed that in the validation cohort, more patients with high baseline ctDNA levels were treated with a combination of ICIs (19/33, 58%), while the patients in our discovery cohort were mainly treated with single agent ICI (10/14, 71%). In addition, the validation cohort had a significantly higher proportion of patients treated with a combination of ipilimumab and pembrolizumab as first-line therapy instead of ipilimumab plus nivolumab ($P=0.006$), as part of a clinical trial. Molecular and pre-clinical assessments of nivolumab and pembrolizumab suggest that these drugs could be interchanged and differences seen in clinical trials are likely related to patient populations rather than be drug-dependent [311]. Consequently, we showed that patients with high pre-treatment ctDNA levels tend to benefit from the combination of anti-CTLA-4 and anti-PD-1 as first-line therapy. Although ctDNA level correlates significantly with tumour burden [16, 235], multivariate analyses in previous studies have shown ctDNA to be an independent predictor of survival [217, 235]. In this context, our results suggest that more aggressive treatment will be particularly beneficial to those patients with high ctDNA levels.

Results from the Checkmate 067 study favours the use of ICI combination over anti-PD-1 monotherapy, showing that patients treated with a combination of ICIs had increased both response (58% vs 45%) and 5-year overall survival rates (52% vs 44%) compared with those treated with anti-PD-1 monotherapy. These differences were accentuated in patients with *BRAF* mutations, with an increased 5-year survival rate in the combination group (60% vs 46%) [136]. In addition, it has been described that in patients with PD-L1 negative tumours, the combination of PD-1 and CTLA-4 blockade was more effective than was either agent alone [109, 136]. Based on our results, elevated ctDNA may identify a group of melanoma patients that could benefit from ICI combination treatment. However,

our study was limited by the low number of patients with high ctDNA included in the survival analysis of single agent anti-PD-1 and combination of ICIs. Further prospective clinical trials are needed to confirm our observations and validate the use of ctDNA as a predictive biomarker for the treatment of melanoma patients.

4.6 Acknowledgements

We would like to thank the melanoma patients for their participation and support of the study. We also thank Aaron Beasley, Jamie Freeman, Paula van Miert, Mike Morici, Danielle Bartlett, and Pauline Zaenker for their help in the collection and processing of blood samples from patients and healthy controls. Furthermore, we extend our thanks to Dr Tindaro Giardina from PathWest for assistance with mutation profiling of tumours and Dr Johnny Lo for helping with part of the statistical analysis. Assistance from colleagues at Melanoma Institute Australia and the Ainsworth Foundation is also gratefully acknowledged.

4.7 Contribution of authors

G.M. performed experiments, conceptualised the study, edited the manuscript, realised data curation, analysis and visualisation. L.C. contributed to the design and supervised the study. M.R.P. and A.L.R. performed experiments and curated data. A.C.M. recruited participants and curated clinical information of patients. S-J.D., S.S., M.S.C., A.M., R.A.S., and G.L. contributed to the data analysis and revised the manuscript. J.L., H.R. and S.Q.W. provided data, contributed to the analysis and initial manuscript design. L.W., A.A., M.A.K., T.M.M. and M.M. provided patient and laboratory samples and reviewed the manuscript. C.R. and B.A. contributed to the data analysis and revised the manuscript. M.R.Z. provided funding and edited the manuscript. E.S.G. conceptualised, supervised and administered the study. All authors have reviewed and approved the final manuscript.

4.8 Supplementary Material

Table S4.1. Comparison of the patients' characteristics between the discovery cohort (N=59) and the validation cohort (N=128).

Variable	First-line ICI			P-value	Second-line ICI			P-value	ICI after Targeted Therapy			P-value
	Discovery N=32 (%)	Validation N=77 (%)	Total N=109 (%)		Discovery N=27 (%)	Validation N=51 (%)	Total N=78 (%)		Discovery N=19 (%)	Validation N=36 (%)	Total N=55 (%)	
Age												
≤65	12 (38)	38 (49)	50 (46)	0.296	14 (52)	30 (59)	44 (56)	0.634	8 (42)	21 (58)	29 (53)	0.273
>65	20 (62)	39 (51)	59 (54)		13 (48)	21 (41)	34 (44)		11 (58)	15 (42)	26 (47)	
Sex												
Female	7 (22)	25 (32)	32 (29)	0.357	7 (26)	20 (39)	27 (35)	0.319	5 (26)	15 (42)	20 (36)	0.378
Male	25 (78)	52 (68)	77 (71)		20 (74)	31 (61)	51 (65)		14 (74)	21 (58)	35 (64)	
AJCC Stage/M Classification												
IIIc/M1a/M1b	10 (32)	35 (45)	45 (41)	0.203	8 (30)	12 (24)	20 (26)	0.593	6 (32)	7 (19)	13 (24)	0.336
M1c/M1d	22 (68)	42 (55)	64 (59)		19 (70)	39 (76)	58 (74)		13 (68)	29 (81)	42 (76)	
Brain metastasis												
Yes	8 (25)	29 (38)	37 (34)	0.268	8 (30)	22 (43)	30 (38)	0.758	7 (37)	21 (58)	28 (51)	0.163
No	24 (75)	48 (62)	72 (66)		19 (70)	29 (57)	48 (62)		12 (63)	15 (42)	27 (49)	
Brain only metastasis												
Yes	2 (6)	1 (1)	3 (3)	0.206	5 (19)	8 (16)	13 (17)	0.758	5 (26)	8 (22)	13 (24)	0.749
No	30 (94)	76 (99)	106 (97)		22 (81)	43 (84)	65 (83)		14 (74)	28 (78)	42 (76)	
LDH levels												
Normal	8 (25)	55 (71)	63 (58)	-	10 (37)	35 (69)	45 (58)	-	6 (32)	24 (67)	30 (54)	-
Elevated	6 (19)	19 (25)	25 (23)		9 (33)	16 (31)	25 (32)		6 (32)	12 (33)	18 (33)	
N/A	18 (56)	3 (4)	21 (19)		8 (30)	8 (10)	7 (34)		7 (34)	7 (13)		
Treatment												
Nivolumab		8 (10)	8 (7)	*0.048	1 (3)	13 (25)	14 (18)	*0.424		8 (22)	8 (14)	*0.999
Pembrolizumab	22 (69)	29 (38)	51 (47)		17 (64)	26 (51)	43 (55)		12 (63)	16 (44)	28 (51)	
Ipilimumab/Nivolumab	10 (31)	15 (20)	25 (23)		9 (33)	8 (16)	17 (22)		7 (37)	5 (14)	12 (22)	
Ipilimumab/Pembrolizumab		25 (32)	25 (23)		4 (8)	4 (5)	7 (20)		7 (20)	7 (13)		
First-line therapy												
Vemurafenib						1 (2)	1 (1)		1 (3)	1 (2)		
Encorafenib/Binimetinib						1 (2)	1 (1)		1 (3)	1 (2)	-	
Dabrafenib/Trametinib					19 (70)	34 (67)	53 (68)	-	19 (100)	34 (94)	53 (96)	
Ipilimumab					6 (22)	14 (27)	20 (26)					
Pembrolizumab					2 (8)		2 (3)					
Ipilimumab/Nivolumab						1 (2)	1 (1)					
BRAF mutation status												
BRAF V600 Mutant	2 (6)	35 (45)	37 (34)	<0.0001	21 (78)	38 (75)	59 (76)	0.656	19 (100)	36 (100)	55 (100)	-
BRAF V600 WT	30 (94)	42 (55)	72 (66)		6 (22)	13 (25)	19 (24)					

*Analysis performed comparing anti-PD-1 monotherapy and anti-CTLA-4 plus anti-PD-1 combination therapy. N/A – not available.

Table S4.2. Specificity of ddPCR assays.

Assay	Healthy controls		Maximum false positive concentration (copies/mL)	Company
	Positive	Negative		
<i>BRAF V600E</i>	0	22	0	Life Technologies
<i>BRAF V600K</i>	0	23	0	IDT
<i>BRAF V600R</i>	0	24	0	IDT
<i>BRAF V600E2</i>	1	12	1	IDT
<i>BRAF K601E</i>	3	7	2	IDT
<i>BRAF L597Q</i>	0	16	0	Bio-Rad
<i>NRAS Q61K</i>	0	19	0	Bio-Rad
<i>NRAS Q61L</i>	3	9	7	Bio-Rad
<i>NRAS Q61R</i>	7	24	9	Bio-Rad
<i>NRAS G12D</i>	4	6	3	Bio-Rad
<i>NRAS G13D</i>	4	6	5	Bio-Rad
<i>TERT C228T/C250T Mult.</i>	6	50	10	IDT
<i>DPH3 C8T</i>	1	10	2	IDT
<i>GRM3 E538K</i>	0	10	0	Bio-Rad
<i>GRM3 S491L</i>	6	12	6	Bio-Rad
<i>FLT1 E011K</i>	3	7	9	Bio-Rad
<i>KIT L576P</i>	1	11	2	Bio-Rad
<i>NF1 P1851S</i>	3	11	3	Bio-Rad
<i>RAC1 P29S</i>	0	12	0	Bio-Rad
<i>TP53 R248W</i>	2	14	2	Bio-Rad
<i>TP53 R248Q</i>	6	8	2	Bio-Rad
<i>TP53 R158H</i>	1	9	2	Bio-Rad

Table S4.3. Mutation status of the melanoma patients included in the study.

Mutation status	Discovery Cohort			Validation Cohort	
	1st line ICI	2nd line ICI	1st line TT	1st line ICI	2nd line ICI
	N=32 (%)	N=27 (%)	N=66 (%)	N=77 (%)	N=51 (%)
BRAF					
<i>BRAF V600E</i>		13 (48)	50 (76)	24 (31)	27 (53)
<i>BRAF V600E2</i>			1 (2)		
<i>BRAF V600K</i>		5 (18)	12 (18)	11 (14)	9 (17)
<i>BRAF V600R</i>	1 (3)	2 (6)	3 (4)		2 (4)
<i>BRAF K601E</i>	1 (3)	1 (4)			
<i>BRAF L597Q</i>	1 (3)			1 (1)	
<i>BRAF L597S</i>					1 (2)
<i>BRAF L597R</i>				2 (3)	
<i>BRAF G466E</i>				1 (1)	
NRAS					
<i>NRAS Q61K</i>	8 (25)	1 (4)		12 (16)	4 (8)
<i>NRAS Q61L</i>	4 (13)			5 (7)	2 (4)
<i>NRAS Q61R</i>	3 (9)			13 (18)	4 (8)
<i>NRAS Q61H</i>				1 (1)	1 (2)
<i>NRAS G12D</i>	1 (3)				
<i>NRAS G13D</i>	1 (3)				
TERT					
<i>TERT Mult.</i>	1 (3)				
<i>TERT C228T</i>	1 (3)	1 (4)			
<i>TERT C250T</i>	1 (3)	1 (4)			
<i>TERT -125CC>TT</i>					1 (2)
GRM3					
<i>GRM3 E538K</i>	1 (3)				
<i>GRM3 S491L</i>	1 (3)				
FLT1					
<i>FLT1 E1011K</i>	1 (3)				
KIT					
<i>KIT L576P</i>	2 (7)			1 (1)	
<i>KIT K642E</i>				4 (5)	
NF1					
<i>NF1 P185S</i>		1 (4)			
RAC1					
<i>RAC1 P29S</i>	2 (7)			1 (1)	
TP53					
<i>TP53 R158H</i>	1 (3)				
<i>TP53 R248W</i>	1 (3)				
<i>TP53 R248Q</i>		1 (4)			
<i>TP53 Y220S</i>				1 (1)	
DPH3					
<i>DPH3 C8T</i>		1 (4)			

Table S4.4. Clinical characteristics at baseline of the melanoma patients included in the survival analysis of the discovery cohort.

Variable	First-line ICI			P-value	Second-line ICI			P-value	ICI after Targeted Therapy			P-value
	Low ctDNA N=18 (%)	High ctDNA N=14 (%)	Total N=32 (%)		Low ctDNA N=18 (%)	High ctDNA N=9 (%)	Total N=27 (%)		Low ctDNA N=14 (%)	High ctDNA N=5 (%)	Total N=19 (%)	
Age												
≤65	5 (28)	7 (50)	12 (38)	0.277	9 (50)	5 (56)	14 (52)	0.999	5 (36)	3 (60)	8 (42)	0.603
>65	13 (72)	7 (50)	20 (62)		9 (50)	4 (44)	13 (48)		9 (64)	2 (40)	11 (58)	
Sex												
Female	3 (17)	4 (29)	7 (22)	0.669	6 (33)	1 (12)	7 (26)	0.363	4 (29)	1 (20)	5 (26)	0.999
Male	15 (83)	10 (71)	25 (78)		12 (67)	8 (88)	20 (74)		10 (71)	4 (80)	14 (74)	
Melanoma Subtype												
Cutaneous	18 (100)	14 (100)	32 (100)	-	18 (100)	9 (100)	27 (100)	-	14 (100)	5 (100)	19 (100)	-
AJCC Stage/M Classification												
M1a/M1b	8 (44)	2 (14)	10 (32)	0.124	6 (33)	2 (24)	8 (30)	0.676	5 (36)	1 (20)	6 (32)	0.999
M1c/M1d	10 (56)	12 (86)	22 (68)		12 (67)	7 (76)	19 (70)		9 (64)	4 (80)	13 (68)	
ECOG												
0	11 (61)	4 (29)	15 (46)	0.087	11 (61)	3 (33)	14 (52)	0.237	8 (57)	2 (40)	10 (53)	0.629
1-3	7 (39)	10 (71)	17 (54)		7 (39)	6 (67)	13 (48)		6 (43)	3 (60)	9 (47)	
Brain metastasis												
Yes	5 (28)	3 (21)	8 (25)	0.999	6 (33)	2 (24)	8 (30)	0.676	6 (43)	1 (20)	7 (37)	0.603
No	13 (72)	11 (79)	24 (75)		12 (67)	7 (76)	19 (70)		8 (57)	4 (80)	12 (63)	
Brain only metastasis												
Yes	2 (11)		2 (6)	0.492	5 (28)		5 (19)	0.136	5 (36)		5 (26)	0.257
No	16 (89)	14 (100)	30 (94)		13 (72)	9 (100)	22 (81)		9 (64)	5 (100)	14 (74)	
LDH levels												
Normal	6 (33)	2 (14)	8 (25)	-	10 (55)		10 (37)	-	6 (43)		6 (32)	-
Elevated	1 (6)	5 (36)	6 (19)		3 (17)	6 (67)	9 (33)		3 (21)	3 (60)	6 (32)	
N/A	11 (61)	7 (50)	18 (56)		5 (28)	3 (33)	8 (30)		5 (36)	2 (40)	7 (34)	
Therapy												
Nivolumab				0.999	1 (6)		1 (3)	*0.193				0.106
Pembrolizumab	12 (67)	10 (71)	22 (69)		9 (50)	8 (89)	17 (64)		7 (50)	5 (100)	12 (63)	
Ipilimumab/Nivolumab	6 (33)	4 (29)	10 (31)		8 (44)	1 (11)	9 (33)		7 (50)		7 (37)	
Timing of blood draw												
During 1 st line Rx				0.629				0.629	8 (57)	2 (40)	10 (53)	0.629
After 1 st line Rx					6 (43)	3 (60)	9 (47)		6 (43)	3 (60)	9 (47)	
BRAF mutation status												
BRAF V600 Mutant	1 (6)	1 (7)	2 (6)	0.999	15 (83)	6 (67)	21 (78)	0.367	14 (100)	5 (100)	19 (100)	-
BRAF V600 WT	17 (94)	13 (93)	30 (94)		3 (17)	3 (33)	6 (22)					

*Analysis performed comparing anti-PD-1 monotherapy and anti-CTLA-4 plus anti-PD-1 combination therapy. N/A – not available.

Table S4.5. Multivariate Cox proportional-hazards regression analysis for associations between ctDNA levels and PFS in patients receiving first-line ICI in the discovery cohort (N=32).

Variables	First-line ICIs			
	Univariate		Multivariate	
	<i>HR (95% CI)</i>	<i>P-value</i>	<i>HR (95% CI)</i>	<i>P-value</i>
Age (≤65 vs. >65)	0.84 (0.34 - 2.09)	0.705	1.06 (0.39 - 2.85)	0.906
Sex (female vs. male)	0.54 (0.20 - 1.42)	0.209	0.65 (0.23 - 1.81)	0.405
AJCC Stage (M1a/b vs. M1c/d)	4.05 (1.17 - 14.02)	0.027	2.59 (0.67 - 10.12)	0.170
Brain metastasis (no vs. yes)	2.31 (0.89 - 5.92)	0.082	1.84 (0.61 - 5.58)	0.283
<i>BRAF</i> mutation status (mut vs. WT)	1.33 (0.18 - 10.02)	0.779	1.23 (0.14 - 10.47)	0.851
ctDNA levels (low vs. high)	5.70 (2.11 - 15.38)	<0.001	5.18 (1.88 - 14.31)	0.001

Table S4.6. Multivariate Cox proportional-hazards regression analysis for associations between ctDNA levels and PFS in patients receiving first-line ICIs in the validation cohort (N=77).

Variables	First-line ICI			
	Univariate		Multivariate	
	<i>HR (95% CI)</i>	<i>P-value</i>	<i>HR (95% CI)</i>	<i>P-value</i>
Age (≤65 vs. >65)	1.08 (0.57 - 2.03)	0.824	0.72 (0.33 - 1.59)	0.419
Sex (female vs. male)	1.27 (0.63 - 2.55)	0.512	1.28 (0.58 - 2.81)	0.545
AJCC Stage (IIIc/M1a/b vs. M1c/d)	1.44 (0.75 - 2.76)	0.272	1.58 (0.62 - 3.03)	0.341
LDH levels (normal vs. elevated)	1.13 (0.53 - 2.41)	0.752	0.74 (0.32 - 1.71)	0.484
Brain metastasis (no vs. yes)	1.24 (0.65 - 2.38)	0.514	0.94 (0.38 - 2.32)	0.889
<i>BRAF</i> mutation status (mut vs. WT)	1.33 (0.69 - 2.54)	0.397	1.52 (0.72 - 3.23)	0.273
ctDNA levels (low vs. high)	2.39 (1.26 - 4.55)	0.008	2.42 (1.17 - 5.02)	0.017

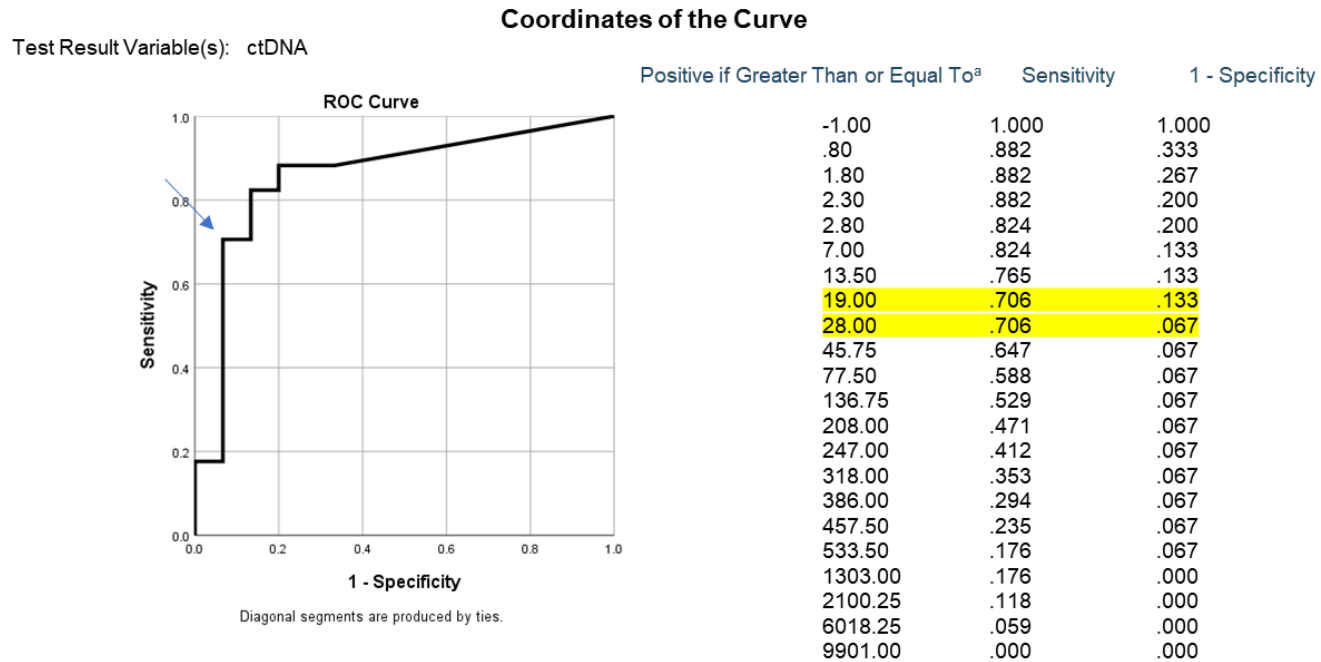
Table S4.7. Clinical characteristics at baseline of the melanoma patients included in the survival analysis of the validation cohort.

Variable	First-line ICI			P-value	Second-line ICI			P-value	ICI after Targeted Therapy			P-value
	Low ctDNA N=44 (%)	High ctDNA N=33 (%)	Total N=77 (%)		Low ctDNA N=31 (%)	High ctDNA N=20 (%)	Total N=51 (%)		Low ctDNA N=23 (%)	High ctDNA N=13 (%)	Total N=36 (%)	
Age												
≤65	23 (51)	15 (45)	38 (49)	0.647	18 (58)	12 (60)	30 (59)	0.999	14 (61)	7 (54)	21 (58)	0.736
>65	21 (49)	18 (55)	39 (51)		13 (42)	8 (40)	21 (41)		9 (39)	6 (46)	15 (42)	
Sex												
Female	16 (36)	9 (27)	25 (32)	0.466	11 (35)	9 (45)	20 (39)	0.565	8 (35)	7 (54)	15 (42)	0.310
Male	28 (64)	24 (73)	52 (68)		20 (65)	11 (55)	31 (61)		15 (65)	6 (46)	21 (58)	
Melanoma Subtype												
Cutaneous	33 (76)	27 (82)	60 (78)	-	26 (84)	12 (60)	38 (75)	-	19 (82)	8 (62)	27 (74)	-
Acral	1 (2)	1 (3)	2 (3)	-	1 (3)	2 (10)	3 (6)	-	1 (5)	1 (7)	2 (6)	-
Mucosal	1 (2)		1 (1)	-				-				-
N/A	9 (20)	5 (15)	14 (18)	-	4 (13)	6 (30)	10 (19)	-	3 (13)	4 (31)	7 (20)	-
AJCC Stage/M Classification												
IIIc/M1a/M1b	24 (55)	11 (33)	35 (45)	0.071	8 (26)	4 (20)	12 (24)	0.743	5 (22)	2 (15)	7 (19)	0.999
M1c/M1d	20 (45)	22 (67)	42 (55)		23 (74)	16 (80)	39 (76)		18 (78)	11 (85)	29 (81)	
ECOG												
0	26 (59)	16 (48)	42 (55)	-	16 (52)	4 (20)	20 (39)	-	10 (43)	1 (7)	11 (30)	-
1-3	11 (25)	14 (42)	25 (32)	-	9 (29)	10 (50)	19 (37)	-	8 (35)	7 (54)	15 (42)	-
N/A	7 (16)	3 (10)	10 (13)	-	6 (19)	6 (30)	12 (24)	-	5 (22)	5 (39)	10 (28)	-
Brain metastasis												
Yes	13 (30)	16 (48)	29 (38)	0.102	16 (52)	6 (30)	22 (43)	0.157	16 (70)	5 (38)	21 (58)	0.089
No	31 (70)	17 (52)	48 (62)		15 (48)	14 (70)	29 (57)		7 (30)	8 (62)	15 (42)	
Brain only metastasis												
Yes	1 (2)		1 (1)	0.999	8 (26)		8 (16)	0.016	8 (35)		8 (22)	0.032
No	43 (98)	33 (100)	76 (99)		23 (74)	20 (100)	43 (84)		15 (65)	13 (100)	28 (78)	
LDH levels												
Normal	38 (86)	17 (52)	55 (71)	0.002	24 (77)	11 (55)	35 (69)	0.126	18 (78)	6 (46)	24 (67)	0.071
Elevated	5 (11)	14 (42)	19 (25)		7 (23)	9 (45)	16 (31)		5 (22)	7 (54)	12 (33)	
N/A	1 (3)	2 (6)	3 (4)	-				-				-
Therapy												
Nivolumab	3 (7)	5 (15)	8 (10)	*0.491	11 (35)	2 (10)	13 (25)	*0.999	7 (30)	1 (7)	8 (22)	*0.720
Pembrolizumab	20 (45)	9 (27)	29 (38)		13 (42)	13 (65)	26 (51)		9 (39)	7 (55)	16 (44)	
Ipilimumab/Nivolumab	6 (14)	9 (27)	15 (20)		4 (13)	4 (20)	8 (16)		4 (17)	1 (7)	5 (14)	
Ipilimumab/Pembrolizumab	15 (34)	10 (31)	25 (32)		3 (10)	1 (5)	4 (8)		3 (13)	4 (31)	7 (20)	
Timing of blood draw												
During 1 st line Rx									6 (26)	3 (23)	9 (25)	0.999
After 1 st line Rx									17 (74)	10 (77)	27 (75)	
BRAF mutation status												
BRAF V600 Mutant	20 (45)	15 (45)	35 (45)	0.999	24 (77)	14 (70)	38 (75)	0.743	23 (100)	13 (100)	36 (100)	-
BRAF V600 WT	24 (55)	18 (55)	42 (55)		7 (23)	6 (30)	13 (25)					

*Analysis performed comparing anti-PD-1 monotherapy and anti-CTLA-4 plus anti-PD-1 combination therapy. N/A – not available.

Table S4.8. Clinical characteristics at baseline of the melanoma patients included in Figure 4.3.

Variable	High ctDNA			P-value	Low ctDNA			P-value
	Combination Immunotherapy N=23 (%)	Anti-PD-1 monotherapy N=24 (%)	Total N=47 (%)		Combination Immunotherapy N=27 (%)	Anti-PD-1 monotherapy N=35 (%)	Total N=62 (%)	
Age								
≤65	10 (43)	9 (37.5)	19 (40)	0.770	16 (59)	12 (34)	28 (45)	0.721
>65	13 (57)	15 (62.5)	28 (60)		11 (41)	23 (66)	34 (55)	
Sex								
Female	9 (39)	4 (17)	13 (28)	0.110	11 (41)	8 (23)	19 (31)	0.169
Male	14 (61)	20 (83)	34 (72)		16 (59)	27 (77)	43 (69)	
AJCC Stage/M Classification								
IIIc/M1a/M1b	7 (30)	6 (25)	13 (28)	0.751	13 (48)	19 (54)	32 (52)	0.798
M1c/M1d	16 (70)	18 (75)	34 (72)		14 (52)	16 (46)	30 (48)	
Brain metastasis								
Yes	10 (43)	9 (37.5)	19 (40)	0.770	10 (37)	8 (23)	18 (29)	0.267
No	13 (57)	15 (62.5)	28 (60)		17 (63)	27 (77)	44 (71)	
Brain only metastasis								
Yes				-	2 (7)	1 (3)	3 (5)	0.575
No	23 (100)	24 (100)	47 (100)		25 (93)	34 (97)	59 (95)	
LDH levels								
Normal	10 (43)	9 (38)	19 (40)	-	20 (74)	24 (69)	44 (71)	-
Elevated	12 (52)	7 (29)	19 (40)		4 (15)	2 (5)	6 (10)	
N/A	1 (5)	8 (33)	9 (20)		3 (11)	9 (26)	12 (19)	
BRAF mutation status								
BRAF V600 Mutant	11 (48)	5 (21)	16 (34)	0.068	15 (56)	6 (17)	21 (34)	0.003
BRAF V600 WT	12 (52)	19 (79)	31 (66)		12 (44)	29 (83)	41 (66)	



The test result variable(s): ctDNA has at least one tie between the positive actual state group and the negative actual state group.

a. The smallest cutoff value is the minimum observed test value minus 1, and the largest cutoff value is the maximum observed test value plus 1. All the other cutoff values are the averages of two consecutive ordered observed test values.

Figure S4.1. ROC curve for ctDNA values in the first-line ICI for the discovery cohort. Analysis was performed using 6 months PFS as read out. The table indicates the sensitivity and the specificity associated to each cutoff value. Highlighted in yellow and indicated by the arrow is the cutoff values surrounding 20 copies per mL. Data was analysed and plotted using SPSS.

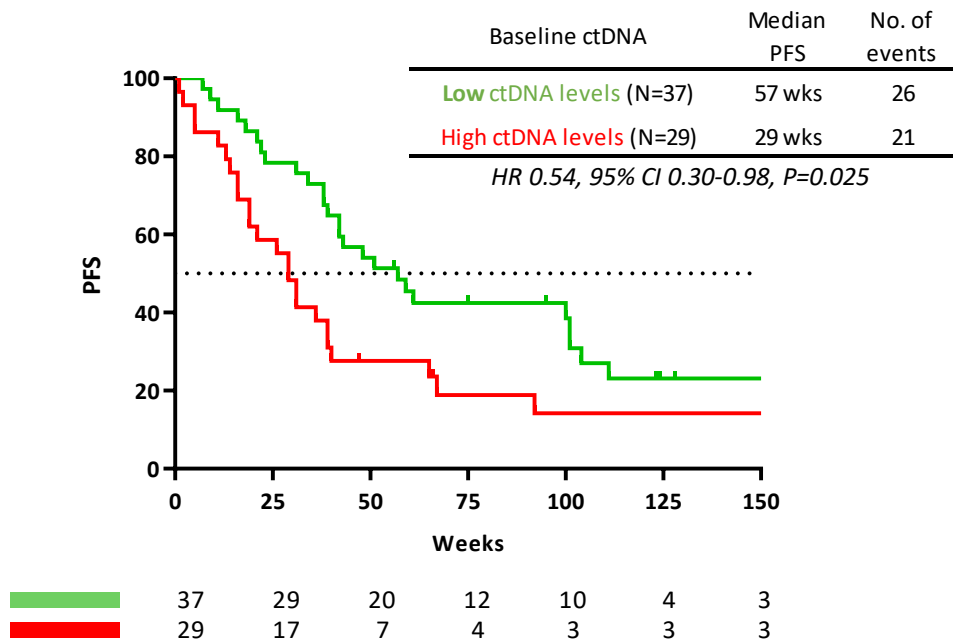


Figure S4.2. Baseline ctDNA levels relative to survival in first-line targeted therapy. Progression-free survival (PFS) curve of melanoma patients with low and high ctDNA levels in the study cohort. Significant Cox regression P -values, Hazard Ratio (HR) and 95% confidence intervals (CI) are indicated in the plot.

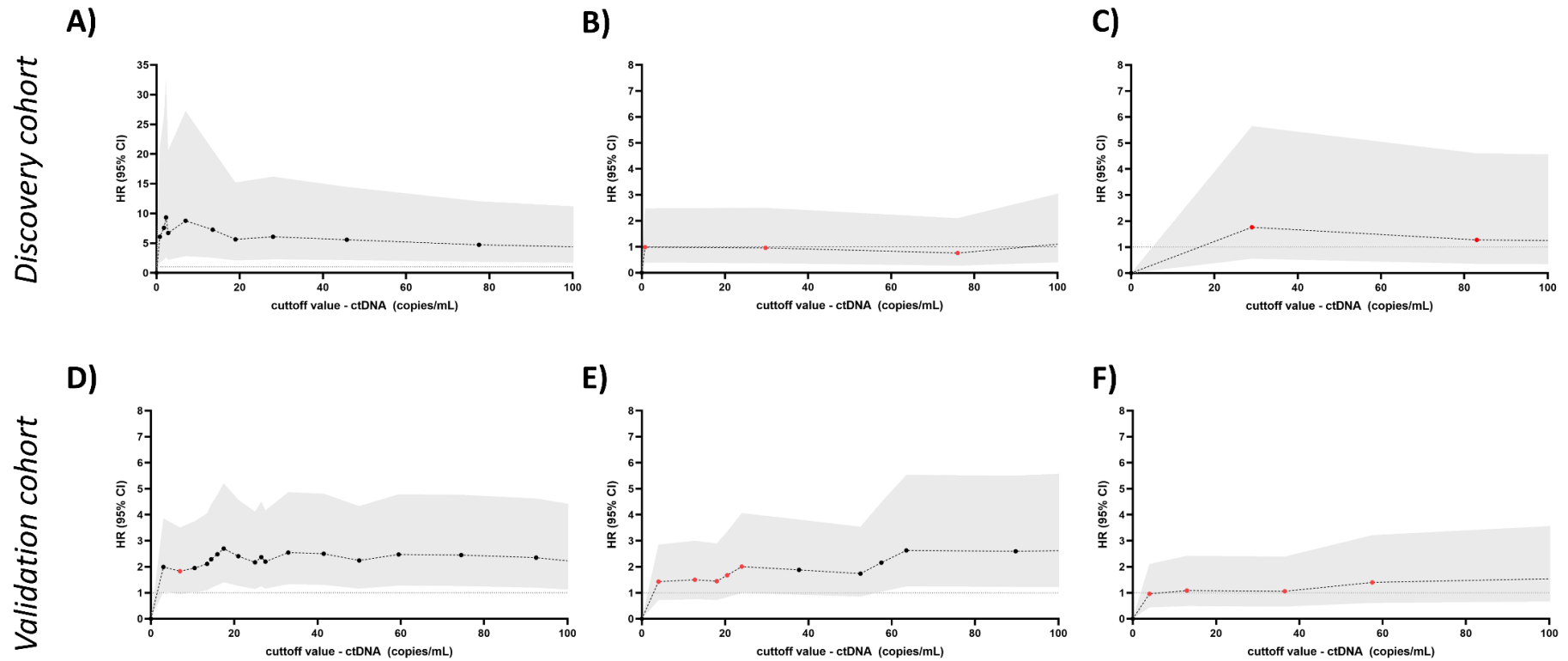


Figure S4.3. Cutoff optimisation by correlation with survival and ctDNA data. **A-F**, Relation between various ctDNA cutoffs, progression-free survival (PFS) and hazard ratio (HR) value, including 95% CI (grey shade). Each dot represents a possible ctDNA cutoff, and red dots denote non-predictive cutoffs ($P > 0.05$), in the discovery (**A-C**) or validation cohorts (**D-F**). Each graph denotes survival HR for ctDNA cutoffs in patients treated with first-line ICI baselines (**A, D**), second-line ICI baselines (**B, E**) or only *BRAF* mutant patients receiving ICI that were treated with first-line targeted therapy (**C, F**).

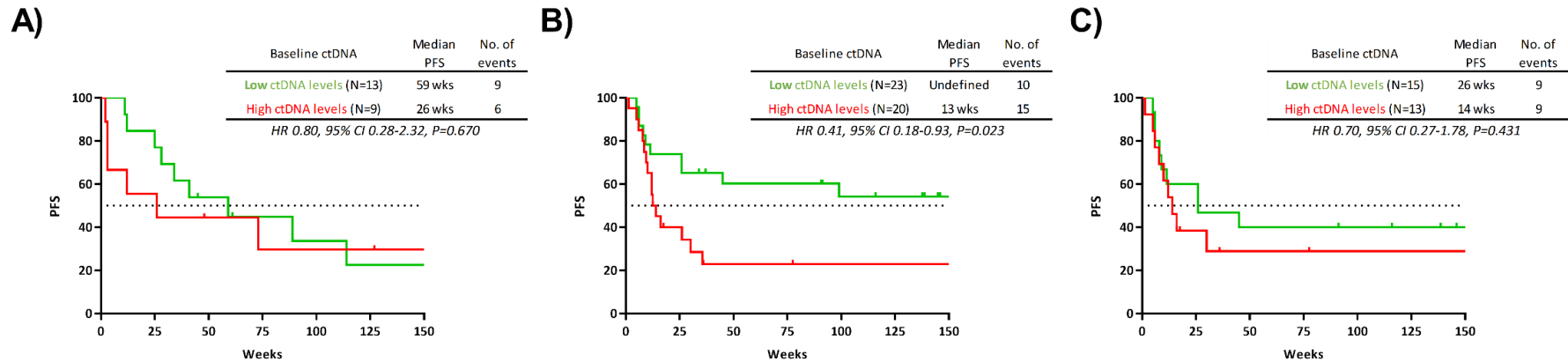


Figure S4.4. Kaplan-Meier curves for progression-free survival (PFS) of second-line melanoma patients without intracranial disease only treated with ICI. Patients were stratified into those with low (green) or high (red) baseline ctDNA levels. Each graph denotes PFS outcomes in the discovery (**A**) or validation cohorts (**B, C**); for patients treated with ICI as second-line treatment (**A, B**); or for *BRAF* mutant patients receiving ICI after failing first-line targeted therapy (**C**). Log-rank *P*-values, Hazard Ratio (HR) and 95% confidence intervals (CI) are indicated for each plot.

CHAPTER 5. THE PROGNOSTIC IMPACT OF CIRCULATING TUMOUR DNA IN MELANOMA PATIENTS TREATED WITH SYSTEMIC THERAPIES: BEYOND *BRAF* MUTANT DETECTION

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5.1 Abstract

In this study, we evaluated the predictive value of circulating tumour DNA (ctDNA) to inform therapeutic outcomes in metastatic melanoma patients receiving systemic therapies. We analysed 142 plasma samples from metastatic melanoma patients prior to commencement of systemic therapy: 70 were treated with BRAF/MEK inhibitors and 72 with immunotherapies. Patient specific droplet digital polymerase chain reaction assays were designed for ctDNA detection. Plasma ctDNA was detected in 56% of patients prior to first-line anti-PD1 and/or anti-CTLA-4 treatment. The detection rate in the immunotherapy cohort was comparably lower than those with BRAF inhibitors (76%, $P=0.0149$). Decreasing ctDNA levels within 12 weeks of treatment was strongly concordant with treatment response (Cohen's $k=0.798$, $P<0.001$) and predictive of longer progression free survival. Notably, a slower kinetic of ctDNA decline was observed in patients treated with immunotherapy compared to those on BRAF/MEK inhibitors. Whole exome sequencing of ctDNA was also conducted in 9 patients commencing anti-PD-1 therapy to derive tumour mutational burden (TMB) and neoepitope load measurements. The results showed a trend of high TMB and neoepitope load in responders compared to non-responders. Overall, our data suggest that changes in ctDNA can serve as an early indicator of therapy outcomes in metastatic melanoma patients treated with systemic therapies and therefore may serve as a tool to guide treatment decisions.

Keywords: circulating tumour DNA (ctDNA), melanoma, *BRAF*, response, targeted therapy, immunotherapy, neoantigen load, tumour mutational burden.

5.2 Introduction

In recent years, improved knowledge of melanoma pathogenesis has led to the development of BRAF and MEK inhibitors that target tumours carrying *BRAF* oncogenic mutations, accounting for 40%-50% of all melanoma cases. Similarly, antibody-mediated blockade of immune checkpoints, particularly the cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and the programmed cell-death protein 1 (PD-1), have markedly improved patient outcome in the last 5 years [116, 129, 152, 226, 312, 313]. However, a significant number of patients do not achieve sustained benefit from either targeted therapy or immunotherapy [129, 152, 226, 312, 313]. The most appropriate treatment sequence or therapy combinations that can maximise patient outcomes remains controversial [297, 314]. Predictive biomarkers of therapy response that can be assessed prior to initiation of treatment and early during therapy are critical to guide clinical management of metastatic melanoma.

Analysis of tumour specific cell-free DNA (cfDNA) has been previously reported to be a reliable companion diagnostic biomarker in oncology [192, 275, 315, 316]. In melanoma, circulating tumour DNA (ctDNA) is a potential non-invasive alternative to tumour tissue biopsy for molecular profiling and longitudinal disease monitoring in the metastatic setting [317]. In addition, baseline ctDNA levels and subsequent decline with treatment have been indicated as an early predictor of tumour response and clinical benefit [14, 193, 217]. To confirm the utility of ctDNA as a clinical biomarker, its ability to monitor and/or predict treatment response and clinical outcome requires further validation in a large cohort of melanoma patients, especially in those treated with immunotherapy.

In melanoma, *BRAF* mutant ctDNA has been found to be a robust biomarker for disease burden and tumour status of patients prior to and during targeted treatment [14, 16, 234, 235]. However, many patients receiving immunotherapy, are *BRAF* wild-type (WT). Thus, the detection rate of ctDNA and the value of ctDNA-based longitudinal monitoring in non-*BRAF* melanoma patients need to be specifically assessed.

Mutations, genetic rearrangements, insertions and deletions can encode novel, cancer-specific neoantigens. Activation of T-cells is initiated by the recognition of novel peptides presented by human leukocyte antigens (HLA) complex. A high tumour mutational burden (TMB) was associated with better survival outcomes in non-small cell lung

cancer (NSCLC) [318-322], melanoma [323, 324] and other cancers. Nonetheless, the predictive value of tissue-derived TMB for immunotherapy response needs further scrutiny and standardisation [325-327]. In this context, ctDNA has the potential to capture the mutational profile of all existing metastases [16]. However, whether this biomarker presents as an easily accessible and suitable tumour source for whole exome mutational load analysis and TMB measurement or neoepitope predictions in melanoma needs to be further defined.

In this study, we aimed to ascertain the clinical utility of ctDNA to inform treatment response and survival in metastatic melanoma patients receiving systemic therapy. We compared ctDNA levels, detection rates, decay kinetics and predictive value between patients treated with immune checkpoint inhibitors and targeted therapies. We also explored whether ctDNA can be used for estimating tumour mutational and neoepitope load, to predict response to immune checkpoint inhibiting therapies.

5.3 Materials and methods

5.3.1 Patients

We analysed a total of 142 plasma samples collected prior to commencing systemic therapy and 227 follow-up samples collected within 24 weeks of treatment initiation from 118 metastatic melanoma patients enrolled in the study between 2013-2018 at Sir Charles Gairdner Hospital (SCGH) and Fiona Stanley Hospital (FSH) in Perth, Western Australia. A subset of 24 patients were considered as baseline for their first- and second-line therapy. Additional details of study design and patient inclusion or exclusion criteria in the different analyses can be found in Fig. S2. Approval by the Human Research Ethics Committee protocols from Edith Cowan University (No. 11543 and No. 18957) and Sir Charles Gairdner Hospital (No. 2013-246) was granted for this prospective study. Written informed consent was obtained from each subject prior to sample collection and analysis. Experiments were performed in accordance with institutional and national guidelines and regulations. Patients were clinically monitored with median follow-up duration of 113 weeks (range: 28-286 weeks). Patient characteristics and clinical parameters are summarised in Table S5.4.

5.3.2 Treatment response and disease progression assessment

Tumour disease responses were assessed radiologically by computed tomography (CT) and/or ¹⁸F-labeled fluorodeoxyglucose positron emission tomography (FDG-PET) scans at two to three monthly intervals. Patients were defined as responders if they had significant reduction in tumour size by the RECIST 1.1 on CT or FDG-PET scan as per the treating clinician, or presented a durable stable disease lasting more than 6 months. Progression-free survival (PFS) was defined as the time interval between the start of therapy and the date of first clinical progression. Overall survival (OS) was defined as the time interval between the start of therapy and death. Additionally, metastatic melanoma patients were stratified into four M-subcategories at baseline based on the location of the metastases [296].

5.3.3 Plasma samples preparation and cfDNA extractions

Blood samples were collected prior to initiation of treatment and during subsequent follow-ups, into EDTA vacutainer or Cell-Free DNA BCT® (Streck, La Vista, NE) tubes. Within 24 hours of blood collection, plasma was separated by centrifugation at 300 g for 20 minutes, followed by a second centrifugation at 4700 g for 10 minutes. All isolated plasma was stored at -80°C until extraction. Plasma cfDNA was isolated from 1-5 mL of plasma using QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. The recovered cfDNA was eluted in 40 µL AVE buffer (Qiagen, Hilden, Germany) and stored at -80°C until ctDNA quantification by droplet digital PCR (ddPCR).

5.3.4 Tissue analysis

Mutational profile of *BRAF* WT patients were identified from tissue biopsies as previously described by Calapre *et al.* [234] A custom targeted next generation sequencing panel of 30 melanoma-associated genes (Illumina, San Diego, CA, USA) with 950 amplicons and an Illumina MiSeq instrument were used to identify mutational targets for ctDNA analysis in *BRAF* WT patients. Genomic variants were annotated using the Illumina Variant Studio 3.0 software (Illumina). Mutational targets were selected based on the criteria previously described [234].

5.3.5 Plasma ctDNA analysis

Commercially available and/or customised probes were used to analyse ctDNA by ddPCR. Droplets were generated using an Automatic Droplet generator QX200 AutoDG (Bio-Rad, Hercules, CA). Amplifications were performed in 40 μ L reactions using cycling conditions previously described [14]. Twenty-six different mutation variants in 10 different genes were utilised. Customised primers and probes for *TERT* and *DPH3* promoter mutation analyses were performed as previously reported by McEvoy *et al.* [257] and Calapre *et al.* [234], respectively. Limit of blank for the ctDNA assays was determined using normal plasma samples from at least 10 healthy controls. Levels of ctDNA were defined based on the level of false positive droplets as previously specified in Calapre *et al.* [234] or are detailed in Table S5.5. Samples yielding copies/mL of plasma equal or below the maximum false positive concentration were deemed ctDNA negative.

5.3.6 Whole exome sequencing

The concentrations of cfDNA used for WES ranged from 1 to 7 ng/ μ L of cfDNA, with ctDNA fraction >7% abundance. Whole exome sequencing (WES) was carried out using the Exome-seq Agilent V6 capture Kit (Agilent) by Novogene (Hong Kong, China). Sequence reads were aligned against human reference genome (hg19) using the Burrows-Wheeler aligner (BWA). Duplicate reads were marked with Picard Tools, reads were realigned against known indels and base qualities recalibrated using Genome Analysis Toolkit. As BWA assumes a unimodal distribution of fragment size, we adjusted the Proper Pair bit in read pairs following the approach in BWA but the fragment sizes were fitted against a mixture of two Gaussian models.

Somatic variants were identified with an in-house tool using the statistical framework described by Li *et al.* [328] We used a model assuming diploid germline and calculated the phred-scaled likelihoods for possible genotypes. The tumour sample was modelled as a mixture of tumour and normal cell DNA and likelihoods were calculated for an array of different variant allele frequencies. The constrained log-likelihood ratio (CLR) was calculated and variants with CLR score of <70 were excluded from further analysis. Identified variants were further annotated using ANNOVAR.

5.3.7 Neoepitope Load Prediction

To predict neoantigens formed by the somatic variants, we used pVACseq v4.0.9 [329] with epitope lengths 8-11 and NetMHCpan binding predictions [330].

5.3.8 Statistics

Differences between ctDNA levels were estimated by unpaired t-test from the log transformed data. Paired t-test was used to evaluate difference between ctDNA levels at first and second-line treatment in *BRAF* mutant patients. Differences between the detection rates were assessed using two-sided Fisher's exact test. PFS and OS were estimated using the Kaplan-Meier method, and differences were evaluated using Mantel-Cox tests. Concordance between the clinical response and the ctDNA kinetics was calculated using the Cohen kappa measure with 95% CI from 1000 bias-adjusted and accelerated bootstrap (BCa) replications. Statistical difference between baseline and follow-up ctDNA levels from same individuals were assessed by the Poisson test, using the minimum and maximum values plus total droplet counts as analytical variables. Frequencies and percentages by group along with their corresponding *P*-values of the chi-squared test are reported in Table S5.1. Multivariate Cox regression analysis was performed for PFS and OS in Table S5.2. The unpaired two-tailed t-test was used to compare mutational and neoepitope load between patients that were responders or non-responders to anti-PD-1 immunotherapy. Pearson correlation was used to determine correlation between mutational burden and neoepitope load. All statistical analyses were performed using R version 5.2, GraphPad Prism version 5 and SPSSv22.0. Results were considered statistically significant at $P < 0.05$.

5.4 Results

5.4.1 Plasma ctDNA detection in melanoma patients commencing systemic therapy

We first evaluated the rate of ctDNA detection in 142 plasma samples collected prior to treatment initiation (Fig. 1).

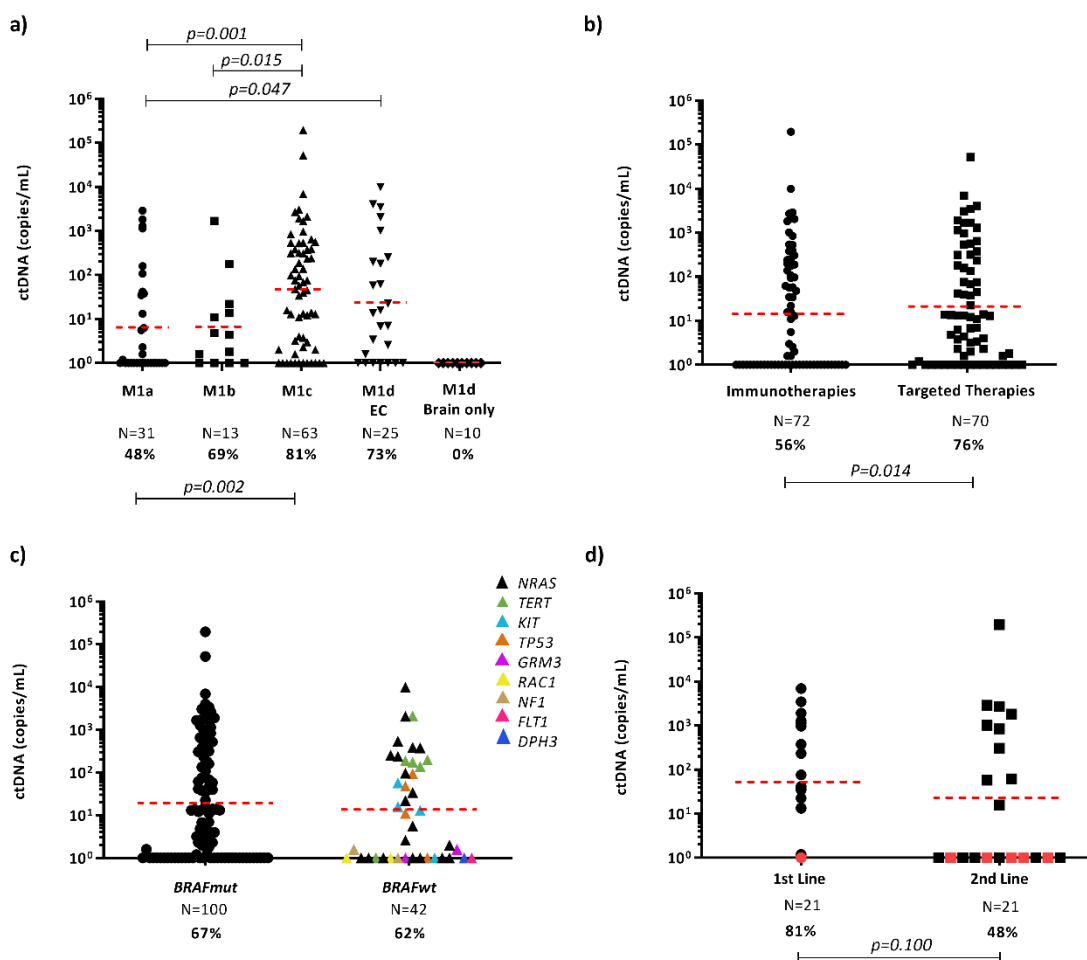


Figure 5.1. ctDNA quantification in melanoma patients prior to commencing systemic therapy. **(a)** Plasma ctDNA levels (copies/mL of plasma) in melanoma samples (N=144), stratified by M status. M1d cases were further subdivided into those with extracranial (EC) and those with brain only metastases. Percentages denote the frequency of patients with detectable ctDNA. The geometric mean of ctDNA concentrations is indicated for each group by a dashed red line. Unpaired t-test *P*-values of the log-transformed ctDNA levels are indicated. **(b)** Dot plot diagram showing ctDNA at baseline in patients treated with immunotherapy (IT) and targeted therapy (TT). **(c)** ctDNA detection in patients with *BRAF*, *NRAS*, or *BRAF/NRAS* wild-type tumours commencing first-line treatment. **(d)** ctDNA detection at first-line and second-line treatment in *BRAF* mutant patients. Red dots represent patients with intracranial disease only at time of starting therapy.

The ctDNA detection rate was 65% overall, but patients with one or more prominent visceral metastases (M1c), particularly in the liver, bone and lung, had significantly higher ctDNA detection rates when compared to those with M1a disease ($P=0.002$; Figure 5.1A). Similarly, median ctDNA levels were significantly higher in M1c patients compared with M1a ($P=0.001$) or M1b ($P=0.015$). In addition, ctDNA levels in patients with M1d and extracranial disease were significantly higher compared with M1a disease ($P=0.047$).

Notably, none of the ten M1d patients with brain only metastases had detectable ctDNA (Figure 5.1A), indicating that ctDNA levels are influenced by the site of metastases.

5.4.2 Baseline ctDNA detection prior systemic treatments

We then compared the ctDNA detection rates in plasma collected prior to commencing treatment with immune checkpoint inhibitors or targeted agents. We observed reduced ctDNA concentrations and a significantly lower detection rate in patients receiving immunotherapy when compared to those receiving BRAFi±MEKi (56% vs 76%; $P=0.014$; Figure 5.1B).

Due to the difference in ctDNA detection rates between the targeted therapy and immunotherapy groups, we evaluated whether the mutational target used for ctDNA analysis influenced these results. Comparison of the detection rate of ctDNA between mutational targets demonstrated no significant difference between ctDNA levels and detection rate (geometric mean: 19.2 copies/mL, 67/100, 67%) in patients with *BRAF* versus patients with other melanoma associated mutations (geometric mean: 13.7 copies/mL, 26/42, 62%; Figure 5.1C).

To determine if ctDNA levels are influenced by the line of therapy, we compared ctDNA levels in 21 *BRAF* mutant patients that received first-line targeted therapy and second-line immunotherapy (Figure 5.1D). This sequence of treatment is commonly used for *BRAF* mutant melanoma in Australia. Despite not showing statistical significance, ctDNA detection rate was lower in patients commencing second-line treatment (81% vs 48%, $P=0.100$). This result is likely influenced by the effectiveness of regular radiological monitoring in identifying disease progression at low tumour burden.

5.4.3 Longitudinal ctDNA monitoring for prediction of response

We further investigated whether ctDNA positivity at baseline and early during the treatment course were correlated with treatment response. A total of 84 patients with longitudinal blood collections within 12 weeks of treatment were included and stratified according to treatment, that is, targeted therapy (N=47) vs immunotherapy (N=37), and divided into three groups depending on the ctDNA profile during the first 12 weeks of treatment (Figure 5.2A). Similar to that shown by Lee *et al.* [217], Group A consisted of

patients with undetectable ctDNA levels at baseline and during 12 weeks of therapy or non-significant ctDNA changes. Group B had detectable baseline ctDNA that became undetectable or significantly reduced during treatment and group C includes patients that were either ctDNA positive or negative at baseline with static or significantly increased levels during the first 12 weeks of therapy. Overall, groups A and B represented patients that showed a biological response, evidenced by undetectable or a significant reduction in ctDNA levels, and group C was comprised of patients that did not show a biological response, that is, detectable or non-significant reduction in ctDNA levels.

An 86% observed agreement was found between the best clinical response within 6 months from treatment initiation and the biological response offered by longitudinal ctDNA monitoring (72/84). Notably, a subset of seven patients without objective response or unequivocal disease progression, who were treated with either immunotherapies (N=3) or targeted therapies (N=4), had a biological response. A strong agreement was found between the biological and the clinical response (Figure 5.2B; $\kappa=0.798$, 95% CI 0.570 to 0.958, N=77, $P<0.001$), when these seven patients were excluded from the analysis. Discordance was observed in five patients (5/77, 6%), with three patients noted to have no detectable or significant decrease in ctDNA levels despite having clinical progression (PD) in subcutaneous lesions (Patient #170.2 and 755), lymph nodes (755), muscle (755 and 486) and brain (170.2). The PD lesions observed in patient 170.2 were in the subcutaneous tissue and brain. By contrast, two patients (538 and 493) were found to have a clinical response to pembrolizumab and dabrafenib/trametinib, respectively, but no biological response was observed.

We next compared the biological ctDNA response with longitudinal blood collection for a period of 24 weeks after starting treatment. In this cohort, most patients treated with anti-CTLA-4 and anti-CTLA-4/PD-1 did not show radiological response to therapy, and their ctDNA levels remained high (Figure S5.1A and S5.1B). By contrast, 17 of the 21 (81%) patients receiving anti-PD-1 immunotherapy had a partial response (PR) or complete response (CR; Figure S5.1C). The clinical response rate in the targeted therapy cohort was also high (41/47, 87%) but a number of these patients (10/41, 24%) developed resistance and relapsed within the first 24 weeks of therapy, with 9 of them demonstrating rebounding ctDNA levels (Figure S5.1D).

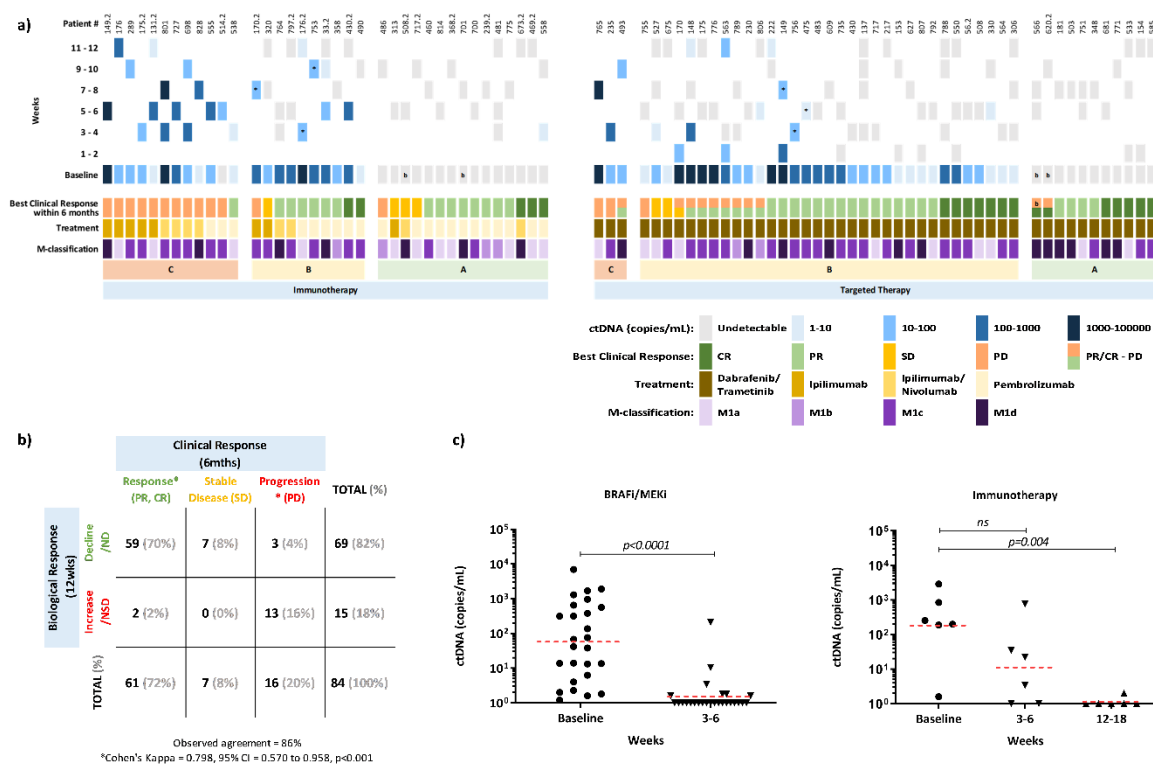


Figure 5.2. ctDNA levels early during treatment relative to clinical response. **(a)** Columns represent each patient, best clinical response, treatment type, and longitudinal quantitative ctDNA results. Patients treated with immunotherapy or targeted therapy were stratified into three profile groups: A=undetectable ctDNA at baseline and during treatment with biological response, B=detectable ctDNA at baseline that became undetectable during treatment or had a significant biological response and C=detectable/undetectable ctDNA at baseline that remained or became detectable during therapy without significant biological response. *Significant Biological Response. **b** Presence of only intracranial malignant disease at baseline or at PD. **(b)** Concordance between best clinical response at 6 months and biological ctDNA response within the 12 weeks of treatment. Patients categorised as clinically responders (PR/CR, N=61), patients with stable disease (SD, N=7), and patients with disease progression (PD, N=16) and, ctDNA responders (Group A and B; N=69) or non-responders (Group C; N=15) based on their biological ctDNA response over the first 12 weeks of treatment. Abbreviations: ND=Not detectable; NSD=Non-significant decrease. **(c)** Plasma ctDNA levels at baseline and follow-up in patients that responded to targeted therapy (N=26) and to immunotherapy (N=6). *P*-values of paired *t*-tests are indicated. The geometric mean ctDNA concentration is indicated for each group by a dashed red line.

We analysed patients with objective clinical response that had detectable ctDNA at baseline and assessable follow-up samples. Within these groups, ctDNA dropped significantly by 3-6 weeks in the targeted therapy cohort (N=26, $P<0.0001$; Figure 5.2C). In contrast, most patients (67%) who responded to immunotherapy had detectable ctDNA levels at first follow-up and, only had the significant drop to undetectable levels on their second follow-up by 12-18 weeks ($P=0.004$, Figure 5.2C).

5.4.4 Longitudinal ctDNA monitoring for prediction of survival

We evaluated whether the ctDNA changes during the first 12 weeks of treatment (groups A, B or C) had prognostic value in patients treated with immunotherapy. For the survival analysis, patients receiving single-agent immunotherapy ipilimumab (N=8) were excluded due to their poor response rate and rapid transition into anti-PD-1, which may confound survival analysis. Clinical characteristics across the three groups were similar for age, sex, tumour stage, the prevalence of brain metastases and prior lines of treatment (Table S5.1).

In patients receiving immunotherapy, groups A and B had significantly longer progression-free survival (PFS) and overall survival (OS) compared to group C (Figure 5.3A and 5.3B). Median PFS for groups B and C was 73 and 5 weeks respectively, but was not reached for group A. The hazard ratio (HR) was 0.052 (95% CI=0.010 to 0.275, $P=0.0005$) for group A and 0.176 (95% CI=0.041 to 0.750, $P=0.019$) for group B when compared with group C. There was no statistical difference in the PFS of groups A and B ($P>0.05$). Median OS for group B and C were 150 and 24 weeks respectively but was not reached for group A (Figure 5.3B). The HR was 0.081 (95% CI=0.014 to 0.454, $P=0.004$) for group A and 0.190 (95% CI=0.395 to 0.922, $P=0.039$) for group B when compared with group C. There was no statistical difference in the OS of groups A and B (HR=0.705, 95% CI=0.134 to 3.693, $P>0.05$).

In a multivariate Cox regression model, ctDNA kinetics in group C was found to be an independent predictor of shorter PFS (HR=16.9, 95% CI=2.68 to 106.25, $P=0.003$) and OS (HR=22.48, 95% CI=2.75 to 183.69, $P=0.004$; Table S5.2). Notwithstanding the low number of samples in Group C (N=3), in patients treated with targeted therapy (Figure 5.3C), group A had longer PFS when compared to compared to group B and C. Median PFS was 100, 39 and 5 weeks for group A, B and C, respectively. When compared with group A, the hazard HR was 0.458 (95% CI=0.215 to 0.977, $P=0.043$) for group B and 0.001 (95% CI= 4.548×10^{-5} to 0.027, $P<0.0001$) for group C. Despite the differences in the PFS between these groups, there was no difference between their median OS (Figure 5.3D, $P>0.05$). A multivariate Cox regression model, found that ctDNA kinetics in group B is an independent predictor of decreased PFS (HR=6.65, 95% CI=1.80 to 24.56, $P=0.004$) and OS (HR=10.51, 95% CI=1.78 to 62.04, $P=0.009$; Table S5.2). Due to the low number of

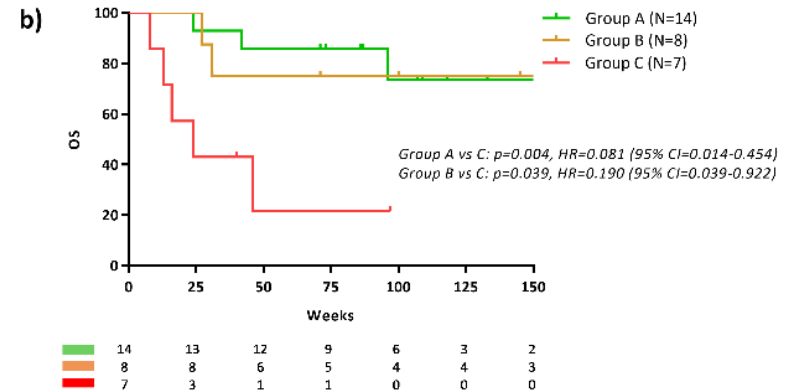
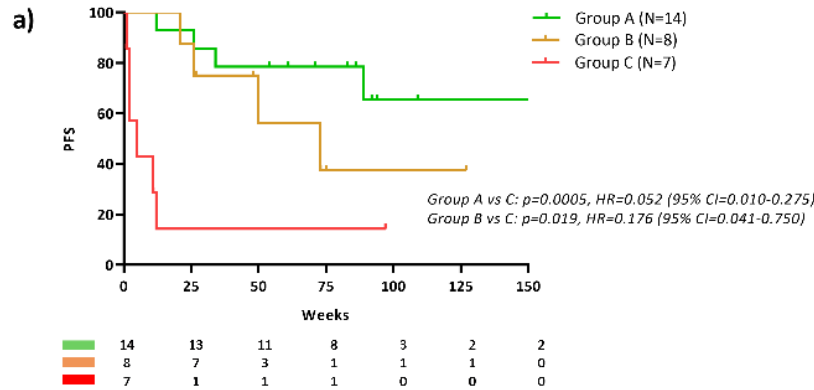
samples in group C (N=3), these patients were excluded from the analysis. Interestingly, the prediction significance of ctDNA kinetics was attributed to the presence of patients with visceral plus brain and brain only patients in the analysis. When excluding group C patients from the multivariate analysis, ctDNA kinetics between groups was not found to be an independent predictor of PFS.

5.4.5 Measuring mutational burden using ctDNA

Plasma ctDNA analysis constitutes an attractive approach for real-time assessment of tumour mutational profile and alleviates caveats associated with tissue biopsies including tumour heterogeneity. Here, we determined the feasibility of quantifying mutational load in patient-derived cfDNA. We screened melanoma patients treated with anti-PD-1 inhibitor as a first- or second-line treatment, with a ctDNA fraction of more than 7% abundance by ddPCR. Nine patients were selected and dichotomised according to their best clinical response to therapy, with responders noted as having either a partial response, a complete response or prolonged stable disease. Non-responders are those without clinical or objective response and who had progressive disease within 6 months of treatment initiation. Clinical characteristics for these patients are described in Table S5.3.

Mutational data were obtained from the nine patients, with the number of mutations ranging from 1-58 per Mb of DNA (Table 5.1). While patients that responded to anti-PD-1 inhibitor had higher tumour mutational burden (TMB) compared to non-responders (mean: 21 vs 6 per Mb), the difference was not statistically significant (Figure 5.4A). Nonetheless, our results may be confounded by the small sample size analysed for TMB.

Immunotherapy



Targeted therapy

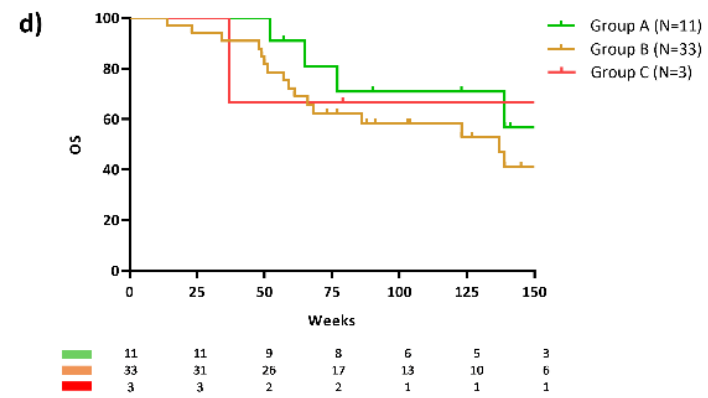
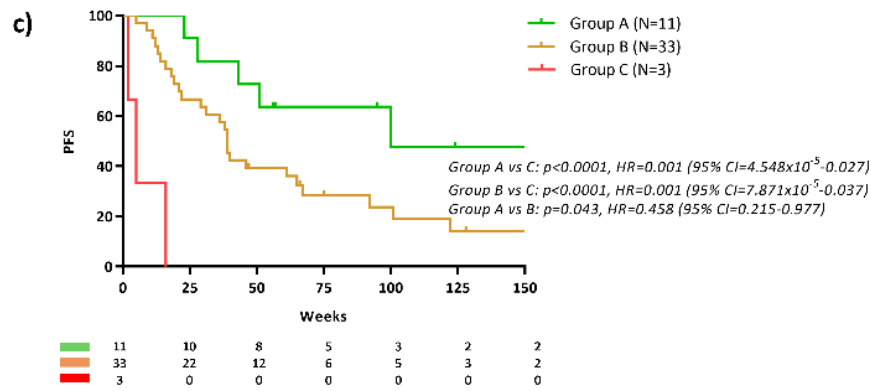


Figure 5.3. ctDNA levels early during treatment relative to survival. Progression free survival (PFS) and overall survival (OS) curves for patients treated with immunotherapy (**a, b**) or targeted therapy (**c, d**) stratified into the three previously detailed profile groups A, B and C. Cox regression *P*-values, Hazard Ratio (HR) and 95% confidence intervals (CI) are indicated for each plot.

Table 5.1. Mutational burden and predicted neoepitope load of nine melanoma patients.

Response Classification	Sample ID	Mutational Burden (per Mb)	Predicted Neoantigens IC50<500nM	Predicted Neoantigens IC50<50nM
Responders (R)	MP0104	22	1362	236
	MP0105	2	131	32
	MP0201	4	432	86
	MP0302	58	1516	259
	MP0303	22	429	57
Non-Responders (NR)	MP0102	10	439	105
	MP0103	1	114	25
	MP0301	1	25	3
	MP0304	14	469	75

As mutational burden alone did not explain clinical benefit from anti-PD-1 inhibitors, we hypothesised that the presence of specific tumour neoantigens might explain the varied dichotomised patients that are likely to benefit from this immunotherapy. To identify these neoepitopes, the HLA-I phenotype of each patient were identified and the bioinformatics pipeline for pVACSeq (<https://github.com/griffithlab/pVAC-Seq>) was used for neoepitope prediction.

The number of predicted neoepitopes with a binding affinity of IC50<500nM ranged from 25-1516 and was higher in responders (mean = 774) versus non-responders (mean = 262) to immunotherapy (Figure 5.4B). The number of predicted neoepitopes with strong binding affinity (IC50<50nM) ranged from 3-259 and was similarly higher in responders compared to non-responders (mean = 134 vs 52, Figure 5.4C). However, the difference in the number of neoepitopes in these two groups was again not significant ($P>0.05$). Nonetheless, the number of neoepitopes correlated with the mutational burden (Figure 5.4D-E). Overall, there was a trend that high neoepitope load was associated with response to anti-PD-1 treatments. Nevertheless, three of the five responders had neoepitope loads in the same range as the non-responders, indicating that at a singular patient level, this parameter alone cannot be used for treatment decisions.

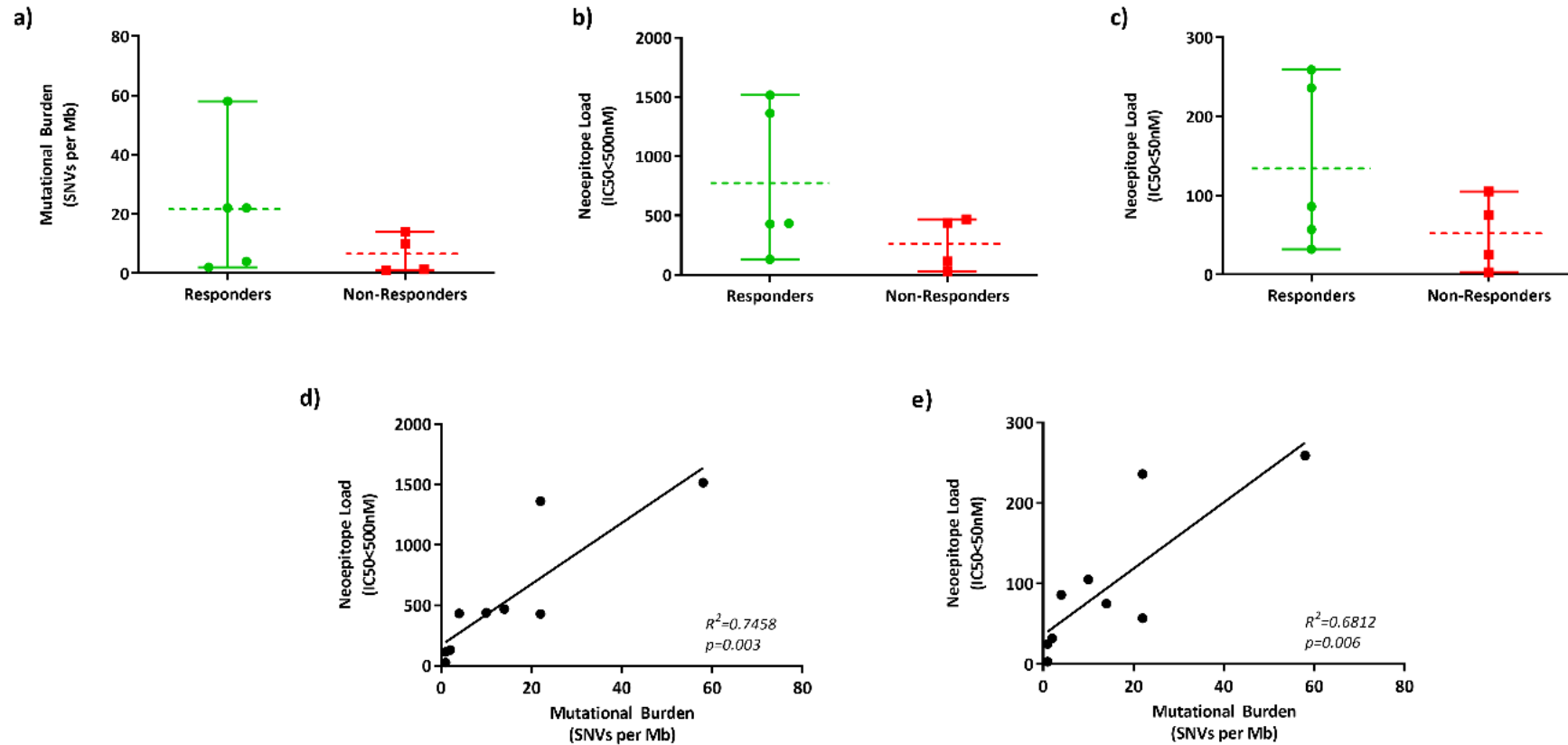


Figure 5.4. ctDNA as a tumour source for mutational burden analysis. **(a)** Vertical scatter plot of the difference in the mutational burden (number of single-nucleotide variants (SNVs per Mb of DNA) in responders (green) and non-responders (red) to anti-PD-1 blockade. Graphs indicating the difference in the number of low - IC50<500nM **(b)** and high - IC50<50nM **(c)** affinity neoantigens in melanoma patients treated with immunotherapy. Correlation between the mutational burden and neopeptide loads at IC50<500nM **(d)** and IC50<50nM **(e)**.

5.5 Discussion

The prognostic value of ctDNA in melanoma patients has been previously shown by number of studies [13, 16, 187, 217, 331]. In this study, we found ctDNA detectability at baseline and during treatment course to be a strong predictor of clinical outcome. In particular, we showed that high levels of ctDNA at baseline and throughout the first 12 weeks of treatment were indicative of poor survival outcome in melanoma patients receiving first-line immune checkpoint inhibitors as well as on those receiving targeted therapies. Moreover, patients with undetectable ctDNA at baseline, who remained ctDNA negative during treatment, have a longer time to progression irrespective of treatment. Notably, detectability of ctDNA and its resolution during treatment was also associated with good clinical outcome in patients treated with immunotherapy and targeted therapies. In addition, we describe for the first time a different ctDNA pattern of response in targeted therapy and in immunotherapy.

Overall, our findings underscore the suitability of ctDNA as a prognostic biomarker for the currently available treatments of melanoma patients. Our findings indicate that ctDNA is most informative as an early indicator of clinical response. In fact, we found a significant concordance between baseline ctDNA levels and response to first-line immunotherapy and targeted therapy. The decline in ctDNA levels was found to be highly concordant with the radiological response to treatment, while increasing ctDNA levels was correlated with disease progression. These results are supported by previous findings [217] and further demonstrated the ability of ctDNA to accurately reflect disease status of patients, making it a valuable surrogate or companion biomarker for patient surveillance during treatment.

Interestingly, we found a low response rate amidst patients treated with anti-PD-1 plus anti-CTLA-4, in contrast with that observed in clinical trials [109, 129]. The patients in our combined immunotherapy cohort had extensive brain metastases and/or widespread disease, which may have reduced the response rates. Moreover, very few patients in our cohort were treated with combined immunotherapy, and therefore the response rates observed here may not necessarily reflect that of previous studies.

We also want to highlight the difference in the rate of ctDNA decay between patients treated with targeted therapy and immunotherapy. In this study, we observed a delayed

velocity of ctDNA decay in patients that respond to immunotherapy compared to patients undergoing targeted therapy. This data reflects the time interval necessary to unleash an immune response to cancer [332], which needs to be taken as an important consideration when monitoring response to different types of treatment through a liquid biopsy. The current treatment approach for melanoma is based on evaluating disease progression, followed by treatment modification to potentially improve patient outcomes and discontinue ineffective therapy. Our data suggest that an observation period may be required prior to conclusive evaluation of therapeutic benefits to immunotherapy and treatment modification decisions.

While ctDNA was found to be a reliable prognostic and surveillance biomarker, it is not without limitations. A significant roadblock for ctDNA analysis in this study was the low detection rate of ctDNA prior to anti-PD-1 and/or anti-CTLA-4 treatment compared with targeted therapy. As indicated above, most patients with detectable ctDNA have prominent visceral metastases, particularly to the liver. The variation of tumour cell turnover at different metastatic sites may have an impact on the detectability of ctDNA. In addition, the low detection rate may have been affected by the specificity of the assay used for ctDNA analysis. Aside from our in-house *BRAF* assays, which have been previously reported to have high specificity and sensitivity [263], assays for other mutations have a lower limit of detection due to noise [234]. Differences in assay threshold may also affect the detection rate of ctDNA in melanoma patients treated with immune-checkpoint inhibitors. Thus, the site of metastases and the assay specificity of the mutational target for ctDNA analysis appears to highly influence the variation in the detection rate observed in this study.

Previous studies have demonstrated the predictive value of tissue-derived mutational and neoepitope load for immunotherapy response in NSCLC [318, 319] and melanoma [323]. In this study, we also explored the potential utility of ctDNA for mutational and neoepitope load analysis in melanoma. Gandara *et al.* [322] demonstrated the utility of blood tumour mutational burden as a clinically-actionable biomarker for anti-PD-L1 in NSCLC. Similarly, our exploratory analysis also demonstrated that whole exome sequence (WES)-defined molecular analysis for clarifying tumour mutational burden in ctDNA is possible. In our cohort, mutational load was unable to discriminate between responders and non-responders to anti-PD-1 inhibitor. Nonetheless, we observed a trend showing

high neoepitope load in patients that achieved clinical benefit to anti-PD-1 blockade. The small sample size was not sufficient to discriminate between responders and non-responders to immunotherapy. These findings may be confounded by the small sample size mostly consisting of patients with high levels of ctDNA (>7% frequency abundance). WES analysis imposed the need to select for patients with high ctDNA fraction, which excluded most samples in our cohort. On the other hand, mutational burden derived from targeted sequencing has been previously shown to be sufficient for stratifying responders and non-responders to immunotherapy [322]. Thus, a targeted approach, with the addition of unique molecular identifiers (UMI), may be more fitting for ctDNA mutational burden analysis, as it will be able to control for PCR errors and allow interrogation of variants at low allelic fraction (<1%).

In conclusion, ctDNA has significant clinical value as a biomarker of prognosis and therapeutic response for melanoma. Nonetheless, limitations inherent to ctDNA analysis need to be clearly defined and thoroughly addressed prior to its implementation in the clinic.

5.6 Acknowledgements

We would like to thank the melanoma patients for their participation and support of the study. We also thank Aaron Beasley, Jamie Freeman, Paula van Miert, Mike Morici, Danielle Bartlett and Pauline Zaenker for their help in the collection and processing of blood samples from patients and healthy controls. Furthermore, we extend our thanks to Dr Tindaro Giardina from PathWest for helping in the mutational profiling of tumours. We thank Johnny Lo for his advice on the statistical analysis. We also thank A/Prof Mark Watson and Dr Abha Chopra from the Institute for Immunology and Infectious Diseases for their help with the HLA typing.

5.7 Contribution of authors

Conceptualization, L.C, E.S.G. and G.M.; methodology, G.M., L.C. and E.S.G.; formal analysis, G.M., L.C., P.A.J., N.K.H., C.R. and B.A.; resources, L.W., M.A.K., M.M and T.M.M.; data curation, G.M., M.R.P., A.L.R. and A.C.M.; writing-original draft preparation, L.C. and G.M.; writing-review and editing, LC, G.M., L.W., M.A.K. and T.M.M.; visualization, G.M., L.C. and E.S.G.; supervision, L.C., E.S.G. and M.Z.; project administration, L.C., E.S.G. and M.Z.;

funding acquisition, M.Z., E.G. and L.C. All authors have read and agreed to the published version of the manuscript.

5.8 Supplementary Material

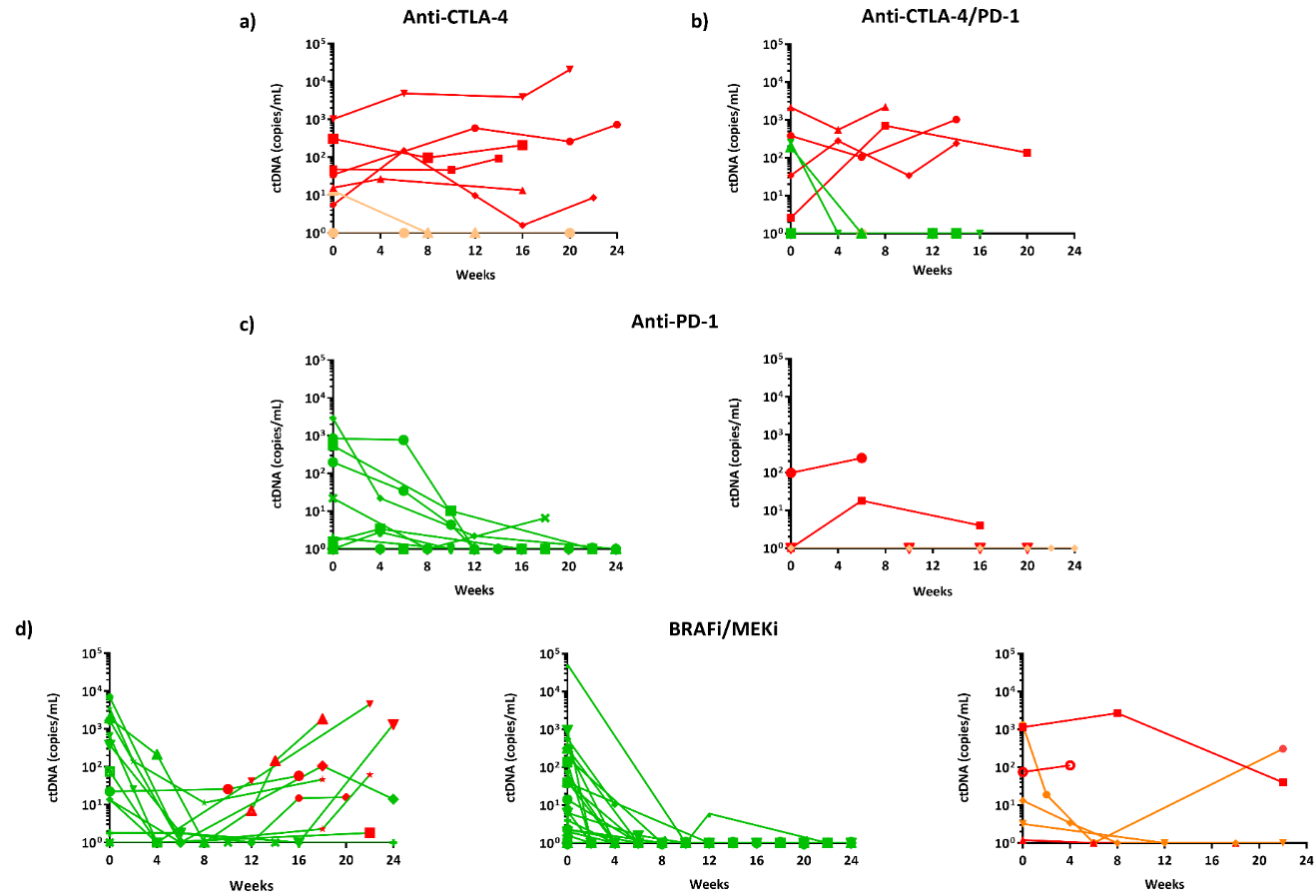
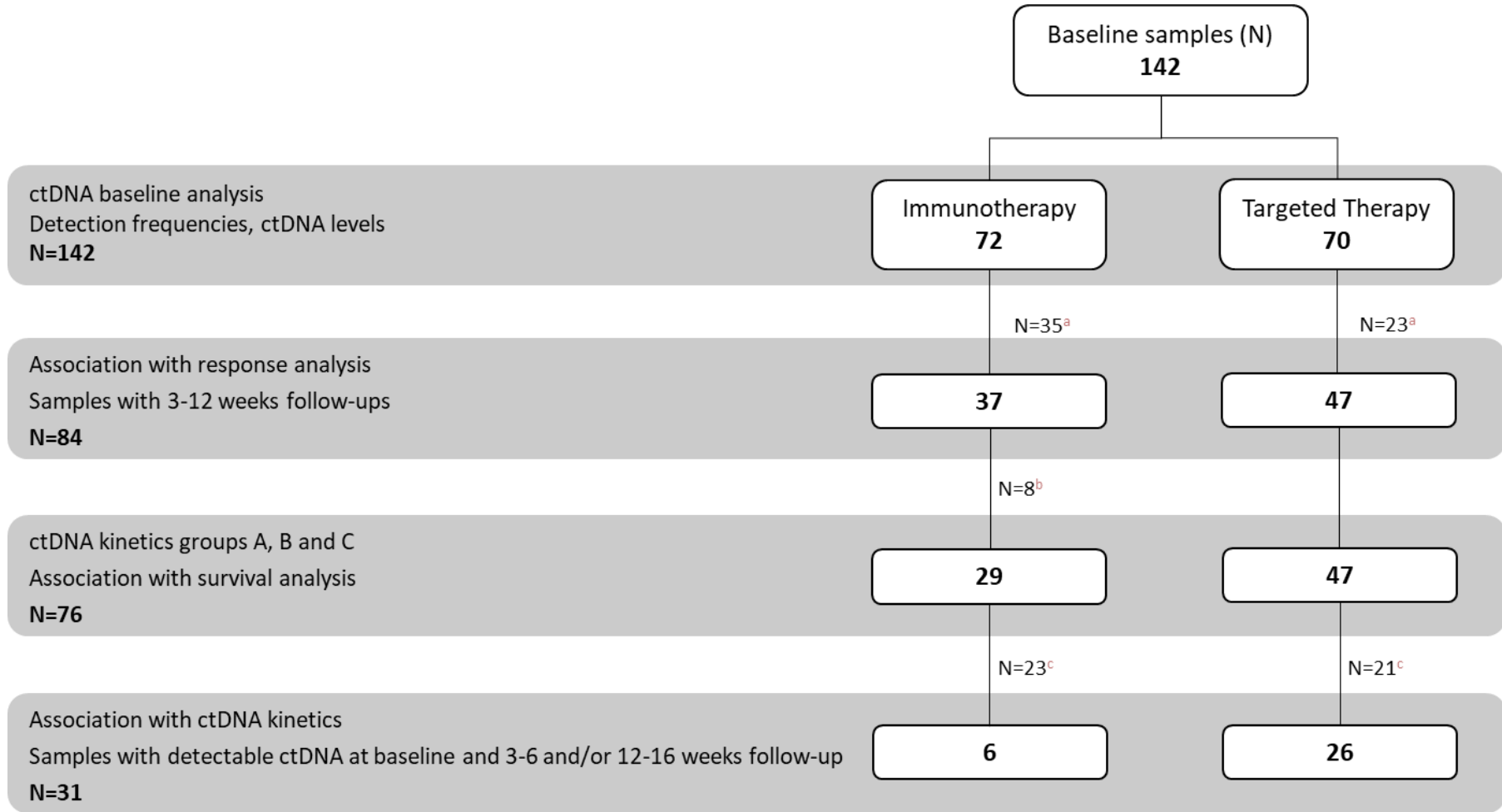


Figure S5.1. Kinetics of ctDNA decay. Time course of biological response for patients undergoing first or second-line treatment with (a) anti-CTLA-4 (N=8), (b) anti-CTLA-4 plus anti-PD-1 (N=8), (c) anti-PD-1 (N=21) or (d) targeted therapy (BRAFi/MEKi) (N=47). Solid lines in green, orange and red denotes treatment responders, stable disease and non-responders, respectively. Solid lines in green with red symbol represents patients that developed resistance to targeted therapy.



^a Exclusion of samples without follow-up within 12 weeks of therapy. ^b Exclusion of ipilimumab cases. ^c Exclusion of samples without detectable ctDNA at baseline and assessable 3-6 and/or 12-16 week follow-ups.

Figure S5.2. Flow chart showing group of samples included in the analyses.

Table S5.1. Clinical characteristics at baseline of the melanoma patients categorised in Groups A, B, and C included in the survival analysis (N=76).

Variable	Immunotherapy				P-value (A/B/C)	Targeted Therapy				P-value (A/B/C)
	Group A N=14 (%)	Group B N=8 (%)	Group C N=7 (%)	Total N=29 (%)		Group A N=11 (%)	Group B N=33 (%)	Group C N=3 (%)	Total N=47 (%)	
Age										
≤65	5 (36)	6 (75)	3 (43)	14 (48)	0.196	5 (45)	18 (55)	2 (67)	25 (53)	0.775
>65	9 (64)	2 (25)	4 (57)	15 (52)		6 (55)	15 (45)	1 (33)	22 (47)	
Gender										
Female	3 (21)	1 (12)	4 (57)	8 (28)	0.120	5 (45)	10 (30)	1 (33)	16 (34)	0.655
Male	11 (79)	7 (88)	3 (43)	21 (72)		6 (55)	23 (70)	2 (67)	37 (66)	
M Classification										
M1a/M1b	9 (67)	2 (25)	1 (14)	12 (41)	0.049	2 (18)	9 (27)	1 (33)	12 (26)	0.794
M1c/M1d	5 (36)	6 (75)	6 (86)	17 (59)		9 (82)	24 (73)	2 (67)	35 (74)	
Brain metastasis										
Yes	3 (21)	2 (25)	1 (14)	6 (21)	0.873	6 (55)	5 (15)	1 (33)	12 (26)	0.032
No	11 (79)	6 (75)	6 (86)	23 (79)		5 (45)	28 (85)	2 (67)	36 (74)	
Brain only metastasis										
Yes	2 (14)			2 (7)	N/A	2 (18)			2 (4)	N/A
No	12 (86)	8 (100)	7 (100)	27 (93)		9 (82)	33 (100)	3 (100)	45 (96)	
ECOG status										
0	9 (64)	3 (37.5)	3 (43)	15 (52)	N/A	8 (73)	16 (48)	2 (67)	26 (55)	N/A
1,2,3	3 (22)	3 (37.5)	3 (43)	9 (31)		1 (9)	5 (15)		6 (13)	
Not available	2 (14)	2 (25)	1 (14)	5 (17)		2 (18)	12 (36)	1 (33)	15 (32)	
LDH levels										
Normal	7 (50)	2 (25)	1 (14)	10 (34)	N/A	8 (73)	13 (39)		21 (44)	N/A
Abnormal	1 (7)	4 (50)	3 (43)	8 (28)			13 (39)	1 (33)	14 (30)	
Not available	6 (43)	2 (25)	3 (43)	11 (38)		3 (27)	7 (22)	2 (67)	12 (26)	
Prior lines of therapy										
Yes	6 (43)	4 (50)	1 (14)	11 (38)	0.316	1 (6)	2 (6)		3 (6)	N/A
No	8 (57)	4 (50)	6 (86)	18 (62)		10 (94)	31 (94)	3 (100)	44 (94)	
BRAF mutational status										
BRAF Mutant	6 (43)	2 (25)	1 (14)	9 (31)	0.373	11 (100)	33 (100)	3 (100)	47 (100)	N/A
BRAF WT	8 (57)	6 (75)	6 (86)	20 (69)						

N/A: not applicable.

Table S5.2. Multivariate Cox proportional-hazards regression analysis for associations between ctDNA levels and survival.

<i>Variables</i>	<i>Group A/B/C Immunotherapy</i>				<i>Group A/B Targeted Therapy</i>			
	<i>Progression free survival</i>		<i>Overall survival</i>		<i>Progression free survival</i>		<i>Overall survival</i>	
	<i>HR (95% CI)</i>	<i>P-value</i>	<i>HR (95% CI)</i>	<i>P-value</i>	<i>HR (95% CI)</i>	<i>P-value</i>	<i>HR (95% CI)</i>	<i>P-value</i>
Age (≤65 vs. >65)	0.34 (0.73 - 1.61)	0.175	0.51 (0.11 - 2.48)	0.407	1.41 (0.64 - 3.05)	0.388	1.82 (0.68 - 4.87)	0.228
Gender (female vs. male)	0.87 (0.21 - 3.69)	0.851	10.02 (1.27 - 78.71)	0.028	1.17 (0.52 - 2.62)	0.694	0.87 (0.31 - 2.39)	0.792
M Classification (M1a/b vs. M1c/d)	0.95 (0.19 - 4.62)	0.956	5.94 (0.63 - 55.78)	0.119	0.80 (0.32 - 2.02)	0.650	1.24 (0.37 - 4.19)	0.720
Brain metastasis (yes vs. no)	3.76 (0.71 - 19.85)	0.118	1.95 (0.33 - 11.49)	0.459	3.05 (1.09 - 8.54)	0.034	5.25 (1.34 - 20.50)	0.017
Brain only metastasis (yes vs. no)	-	-	-	-	10.04 (1.27 - 79.27)	0.029	14.20 (1.48 - 136.34)	0.021
Prior lines of therapy (yes vs. no)	0.84 (0.08 - 8.05)	0.881	0.12 (0.004 - 3.73)	0.228	0.64 (0.15 - 2.67)	0.542	0.23 (0.02 - 2.07)	0.191
<i>BRAF</i> mutational status (mut vs. WT)	1.45 (0.13 - 15.48)	0.758	6.32 (0.21 - 189.11)	0.287	-	-	-	-
ctDNA levels								
- ctDNA kinetics group								
Group A vs.	1		1		1		1	
Group B	1.91 (0.32 - 11.22)	0.471	0.98 (0.13 - 7.17)	0.991	6.65 (1.80 - 24.56)	0.004	10.51 (1.78 - 62.04)	0.009
Group C	16.90 (2.68 - 106.25)	0.003	22.48 (2.75 - 183.69)	0.004	-	-	-	-

Table S5.3. Clinical characteristics of melanoma patients in this pilot cohort.

Sample ID	BRAF Status	Treatment	Best Clinical Response	Previous Immunotherapy	Start of Treatment	Treatment Completion or Latest Clinic	Length of Treatment (wks)	Abdomen	Adrenal	Brain	Bone	Liver	Oropharynx	Lung	Lymph Node	Mesentery	Pancreas	Pelvic Region	Sub-cutaneous
MP0104	BRAF Mut	Pembrolizumab	PR	Yes (Ipilimumab)	25/08/2014	29/08/2016	105								x				
MP0105	BRAF WT	Pembrolizumab	CR	Yes (Ipilimumab)	10/02/2015	29/12/2017	150			x				x					x
MP0201	BRAF Mut	Pembrolizumab	CR	No (Radiation Therapy)	5/08/2016	27/02/2018	82				x	x		x					
MP0303	BRAF Mut	Pembrolizumab	PR	No	15/09/2017	Ongoing	90 ^a				x	x							
MP0302	BRAF Mut	Pembrolizumab	SD	No	13/12/2017	31/05/2018	24		x					x	x				
MP0102	BRAF WT	Pembrolizumab	PD	No	10/12/2015	12/02/2016	9						x		x	x			
MP0103	BRAF Mut	Pembrolizumab	PD	No	13/05/2016	4/07/2016	7				x	x			x				
MP0301	BRAF WT	Pembrolizumab	PD	No	11/06/2015	18/09/2015	14												
MP0304	BRAF WT	Pembrolizumab	PD	No	03/04/2017	25/05/2017	7	x		x	x								

^a Weeks in treatment as of 31/08/2019

Table S5.4. Demographic, clinicopathologic, and treatment characteristics of included samples.

Variable	All samples N=142	Immunotherapy N=72 (%)	Targeted Therapy N=70 (%)
Age			
20-49		11 (15)	20 (29)
50-69		38 (53)	31 (44)
70-99		23 (32)	19 (27)
Sex			
Female		18 (25)	25 (36)
Male		54 (75)	45 (64)
M Classification			
M1a		16 (22)	15 (21)
M1b		7 (10)	6 (9)
M1c		31 (43)	32 (46)
M1d		18 (25)	17 (24)
Mutational Status			
<i>BRAF</i> Mutant		30 (42)	70 (100)
<i>NRAS</i> Mutant		20 (28)	
Others		22 (30)	
Treatment			
Anti-PD1 inhibitor			
Pembrolizumab		40 (56)	
Nivolumab		1 (1)	
Anti-CTLA-4 inhibitor			
Ipilimumab		12 (17)	
Anti-PD-1 plus anti-CTLA-4 inhibitor			
Ipilimumab/Nivolumab		19 (26)	
BRAF_i			
Vemurafenib			4 (6)
Dabrafenib			1 (1.5)
BRAF_i plus MEK_i			
Dabrafenib/Trametinib			64 (91)
Vemurafenib/Cobimetinib			1 (1.5)

Table S5.5. Specificity of ddPCR assays.

Assay	Healthy controls		Maximum false positive concentration (copies/mL)
	Positive	Negative	
<i>BRAF V600E2</i>	1	12	1
<i>BRAF K601E</i>	3	7	2
<i>BRAF L597Q</i>	0	16	0
<i>NRAS Q61R</i>	7	24	9
<i>NRAS G12D</i>	4	6	3
<i>NRAS G13D</i>	4	6	5
<i>GRM3 E538K</i>	0	10	0
<i>GRM3 S491L</i>	6	12	6
<i>FLT1 E011K</i>	3	7	9
<i>TP53 R158H</i>	1	9	2

CHAPTER 6. PLASMA CTDNA FAILS TO RELIABLY DETECT CLINICAL PROGRESSION IN METASTATIC MELANOMA PATIENTS

Publication details

This chapter is a manuscript which has been submitted for peer-review to a journal and it is aimed at a general clinical audience.

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Chapter 6 is not available in this version of the thesis.

CHAPTER 7. GENERAL DISCUSSION AND FUTURE DIRECTIONS

Plasma ctDNA is currently considered a promising biomarker of disease status for metastatic cancer [275, 316, 358]. In this context, the studies in this thesis evaluated the validity of ctDNA analysis across the whole spectrum of metastatic melanoma management: i) molecular profiling prior to therapy initiation to aid targeted therapy selection (Chapter 3), ii) prognostic and predictive potential of baseline ctDNA levels (Chapter 4), iii) monitoring response to treatment (Chapter 5) and iv) detection of disease progression (Chapter 6) (Figure 7.1).

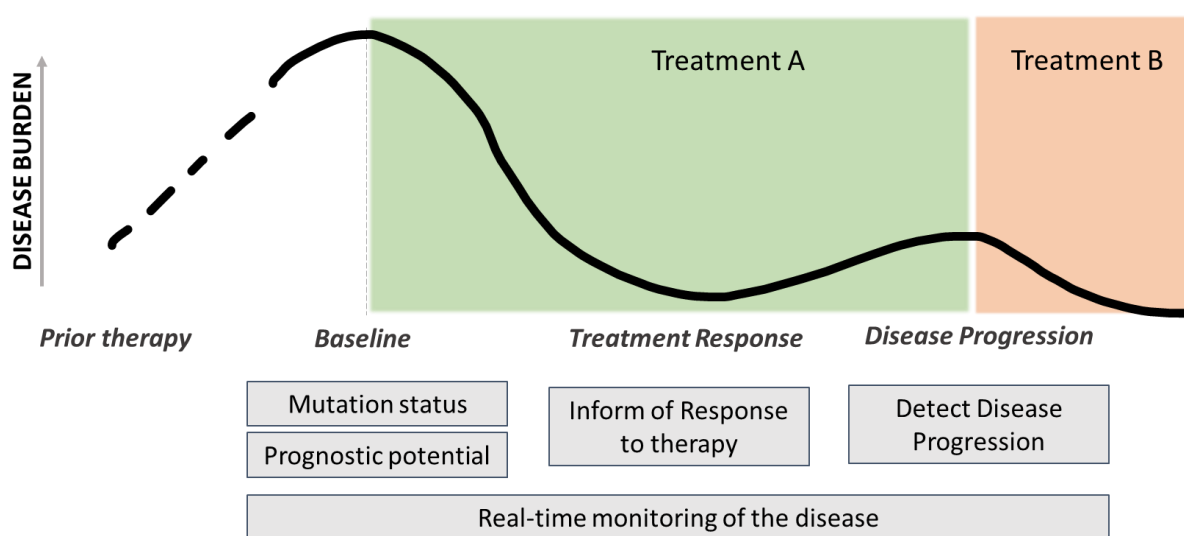


Figure 7.1. Applications of ctDNA analysis during metastatic melanoma management.

Detection of ctDNA requires highly sensitive and specific assays, especially in patients with low tumour burden. In melanoma, multiple studies have relied on a single mutation targeted approach for ctDNA analysis using droplet-based PCR systems, particularly ddPCR and BEAMing. Both methods generally target a single mutation at a time and have been shown to have a substantially high analytical sensitivity compared to other ctDNA detection assays [359]. Direct comparison of ddPCR and BEAMing indicated a high agreement ($\kappa = 0.87$; 95% CI, 0.81–0.93) [360]. Therefore, the use of ddPCR for ctDNA detection through these studies is in line with current best practice.

Given that a large proportion of melanomas carry hotspot mutations in *BRAF* or *NRAS* [74], the use of single mutation target has been exploited for tracking disease burden over

time. For individuals that do not harbour these common variants, sequencing of tumour samples is necessary to identify mutational targets for ctDNA analysis by ddPCR [234].

In Chapter 2, we described an optimal and reproducible cfDNA extraction [249-251] and testing protocol that enables sensitive and specific detection and quantification of ctDNA from plasma samples. This ddPCR assay has been the primary approach used in the studies comprising this thesis.

In addition to the sensitivity of each assays, it is also apparent that pre-analytical factors can significantly affect ctDNA detection rate [243, 244, 355, 361]. Importantly, ctDNA studies should report on the conditions in plasma separation and storage, transport and cfDNA extraction to allow comparison to be made between studies. To provide robust and comparable results, Chapter 2 outlines the use of optimal blood collection tubes [246] and the most efficient column-based cfDNA extraction method, previously vetted by the CANCER-ID consortium study. The study compared the extraction efficiency of 6 different cfDNA extraction kits from a total of 17 independent laboratories and concluded that the QIAamp Circulating Nucleic Acid kit showed the highest recovery, independent of the blood collection tube used [247].

Using a multiplex *BRAF* assay for ddPCR, the study in Chapter 3 demonstrated a high degree of concordance between standard-of-care tumour-based *BRAF* mutation profiling and plasma-based testing. The positive agreement was found to be 78% and an overall agreement of 88%, supporting the results of multiple other studies [216, 218, 224, 225, 362]. Nevertheless, it is important to note that undetectable mutant *BRAF* ctDNA, without prior knowledge of the patients' *BRAF* status, does not preclude the possibility that the variant is either absent in the tumour or that there was an insufficient amount of ctDNA present in the specimen. This has been recognised as a major limitation for the use of ctDNA for tumour genotyping [276]. Accordingly, while the FDA and the EMA approved the ctDNA test for detection of the *EGFR* p.T790M resistance mediating mutation in (NSCLC), undetectable ctDNA results have to be followed by the use of a tissue biopsy to confirm the absence of the mutation [276]. In line with this recommendation, if *BRAF* genotyping is to be pursued through ctDNA profiling, a negative result will need confirmation on a tumour sample before a patient can be deemed *BRAF*-mutant negative and potential exclusion from *BRAF* targeted therapies.

Chapter 3 also presents an attempt to model the potential economic benefit of *BRAF* profiling through cfDNA testing. We calculated that savings of at least \$36,238 per annum could be incurred if *BRAF* cfDNA testing was performed instead of tumour biopsy analyses, representing a reduction of 22% on the overall expense of *BRAF* testing. These results support the inclusion of ctDNA analysis to establish tumour genotype prior to treatment with targeted therapies is to be considered.

The main advantage of cfDNA analysis is that it offers an alternative route for assessing *BRAF* status in cases where metastases are widespread and tissue biopsy is not possible or does not yield sufficient material for testing. Additionally, the high prevalence of activating *BRAF* mutations in colorectal, thyroid, and ovarian cancers [289], as well as clinical trials with targeted therapies [290, 291], enable its use beyond melanoma. Moreover, *BRAF* mutation tests may become relevant to other cancer types that receive regulatory approval for the use of BRAF inhibitor therapy.

Despite the encouraging results presented in this thesis, obtaining a tissue sample for molecular profiling is not typically a major obstacle in metastatic melanoma, as biopsies are commonly performed to confirm the disease. Therefore, although ctDNA testing for *BRAF* detection is clinically valid, in practice it will only be useful and cost-effective in a minority of cases. For example, for cases where a tumour biopsy will involve a very invasive procedure, the patient has significant comorbidities and there is a need for a rapid tumour turnaround for treatment decision [219]. Similarly, it is not uncommon that FNA procedures yield insufficient or unsuitable tumour material for genotyping, and in such cases, a rapid and minimally invasive ctDNA testing may provide critical information to inform treatment course.

Limited economic studies have investigated the use of this liquid biopsy in oncology and most of them have shown only a moderate to no economic benefit [363-365]. These results underscore the reality that the implementation of ctDNA analysis into clinical practice necessitates thorough cost-effectiveness calculations to further support its clinical utility [366]. The economic benefit must be related to each specific context of use for ctDNA analysis in the management of cancer, i.e. prognostication for early intervention, genotyping for treatment selection or disease monitoring. Notwithstanding the cost associated with performing a liquid biopsy needs to be outweighed by the

benefit, which for most applications would be ultimately an improvement in survival. However, survival benefits also depend on the efficacy of the associated therapeutic intervention. Thus, it is only through careful study designs and/or large clinical trials that incorporate ctDNA results to inform treatment decisions, that the health economic benefit of liquid biopsies can be accurately assessed and their regulatory approval can move forward.

Currently, treatment selection and monitoring a patient's disease course during therapy are considered important priorities for melanoma management. It is in this context that a liquid biopsy may have the biggest impact. Once the mutation profile has been identified, first-line selection treatment by the medical professionals involves accounting for multiple factors such as median tumour size, LDH levels, *BRAF* status, presence of brain metastases and comorbidities that must be carefully considered [136, 309, 310].

In Chapter 4, we analysed the ability of ctDNA to assist with treatment selection and patient stratification. Similar to previous studies, we found that high ctDNA levels are associated with poorer clinical outcomes [12, 15, 187, 193], while a low baseline ctDNA level correlated with tumour shrinkage on radiology [213] and longer PFS in melanoma patients treated with immunotherapies [14]. By contrast, Lee et al. showed that a decline in ctDNA level during treatment, but not low concentration at baseline, was predictive of longer PFS and OS [217]. This may be due to the inclusion of a large proportion of patients receiving immunotherapy in the second-line setting.

Indeed, we found that the predictive value of ctDNA was lost in the second-line treatment setting, particularly in patients failing first-line targeted therapy. Importantly, these findings were validated using an independent cohort of patients with samples collected and analysed in laboratories of collaborators in Sydney and Melbourne. Diverse factors arising at disease progression including the presence of brain metastases and the decreased response to second-line therapy may limit the prognostic value of ctDNA in pre-treated patients. Therefore, the prognostic potential of ctDNA must be taken with caution in this population. Future studies evaluating ctDNA detectability in relation to previous systemic therapy and anatomical tumour location are also necessary. Moreover, there is a need to understand the origin of ctDNA and the cellular dynamics that govern ctDNA production and shedding [367, 368].

A novel finding of the study described in Chapter 4, was the observation that patients with high pre-treatment ctDNA levels had better outcomes if treated with the combination of anti-CTLA-4 and anti-PD-1 as first-line therapy. Although ctDNA levels correlate significantly with tumour burden [16, 235], multivariate analyses in previous studies have shown ctDNA to be a stronger and independent predictor of survival than tumour burden [201, 211, 212, 217, 235]. In this context, our results suggest that more aggressive combination treatment will be particularly beneficial to those patients with high ctDNA levels. On the other hand, patients with low or undetectable ctDNA may be treated with anti-PD-1 monotherapy, which is associated with less adverse events [369, 370]. However, we should note that the observation regarding patients with high ctDNA levels benefiting from combination immunotherapy, was the result of a post-hoc analysis combining both cohorts. Therefore the study was not appropriately designed or powered to strongly reaffirm this conclusion. A prospective clinical trial is needed to confirm our observations and validate the use of ctDNA to guide immunotherapy selection.

In Chapter 5, the study defines that decreasing ctDNA levels early during treatment is a strong predictor of clinical outcome. In particular, high levels of ctDNA prior to treatment and early during therapy were indicative of poor survival outcome, while patients with undetectable ctDNA at baseline had a longer time to progression irrespective of treatment. These results are supported by previous findings [214, 215, 217, 371] in patients treated with immunotherapy and further demonstrated the capacity of ctDNA to accurately monitor treatment response and predict clinical outcome in a large cohort of melanoma patients. These findings underscore the suitability of ctDNA as a prognostic biomarker for the currently available treatments of melanoma patients. Moreover, the ability of undetectable or decreasing ctDNA levels to accurately distinguish between true progression or pseudoprogression has also been investigated [218]. This ambiguous pattern of response, described as an initial increase in size of tumour lesions followed by a delayed partial response, can hinder accurate report of response in patients treated with immunotherapies [372]. In this setting, ctDNA can provide critical clinical information.

The need of ctDNA analysis beyond BRAF and NRAS mutations becomes critical for melanoma subtypes other than cutaneous since they are known to carry different driver mutations. For example, acral and mucosal melanomas are known to carry large

proportions of *KIT* and *NF1* mutations [373], which are not restricted to singular hotspots. In addition to point mutations, acral and mucosal melanomas are commonly associated with copy number alterations. A study monitoring copy number alterations in acral and mucosal cfDNA, found that plasma copy number ratio was correlated with clinical response or progression [374]. Uveal melanomas carry mutually exclusive activating mutations in *GNAQ*, *GNA11*, *PLC β 4*, and *CYSLTR2*, accounting for more than 90% of all uveal patients [375-377]. Various studies have utilised these ctDNA mutations to track uveal melanoma evolution and monitor treatment response [378, 379].

The increased number of therapies available to treat melanoma requires the stratification of patients into appropriate treatments, stressing the importance of regular monitoring for timely detection of disease progression and adjustment of treatment. Current clinical surveillance and monitoring are performed by regular blood tests and expensive imaging technologies, which are utilised for efficient detection of progressive metastatic disease. The results presented in Chapter 6 revealed that ctDNA was not reliable for the detection of disease progression when compared with imaging technologies. We found that in 47% of the cases, ctDNA failed to detect disease progression. Our results are, in contrast with other studies indicating a high ctDNA detection rate at the time of progression (51% and 100%) and that an increasing ctDNA level is pre-emptive of clinical progression, with a calculated lead time of 25 and 21 weeks [224, 225]. Furthermore, Váraljai *et al.* described that in 86% of the 36 melanoma patients that did not show response to systemic treatment, ctDNA increase preceded radiological progression with an average lead-time window of 3.5 months [347].

A few factors that could explain the lower ctDNA detection rate at progression reported in this thesis must be mentioned. Firstly, the studies defined above are comprised of smaller cohorts that used BEAMing technology for ctDNA detection [224, 225]. Nevertheless, previous studies indicated that BEAMing has the same level of sensitivity as ddPCR [360]. Secondly, and possibly the critical point, previous studies have compared ctDNA to CT scan-based progression by RECIST. On the other hand, PET scans are the modality of choice for disease monitoring in Australia and the one used in our studies. Of note, published studies have shown that CT scans are less sensitive than PET scans for detection of metastatic lesions [380, 381]. Thirdly, the ability of ctDNA to detect progressive disease should be described by a statistically significant increase in ctDNA

copies. While this point is controversial and some researchers have suggested to perform a t-test of the triplicate results, we chose to do a Poisson test, in line with the nature of a ddPCR quantification [382]. Herbreteau *et al.* [216] was the first study to calculate the significance of changes in ctDNA levels using Poisson analysis. Similarly, we have developed a method in Chapter 4 and Chapter 6 that quantifies significant changes in ctDNA levels. Ultimately, this study highlights the need to develop specific criteria when evaluating response or progression by changes in ctDNA levels.

Previous studies by our group and others have shown that ctDNA correlates with tumour size [12, 14, 209, 210] and more strongly with MTB [235]. However, we observed that ctDNA levels did not correlate with MTB at the time of progression compared to the analysis at baseline (Figure 6.4). This finding may support the observation in Chapter 4 that ctDNA levels at the time of starting second-line treatment, i.e. at progressive disease to first-line therapy, fail to accurately provide prognostic information. Notably, both studies were evaluated in the context of targeted therapies as first-line treatment, underscoring the need of additional studies investigating how the pathological state of the tumour and resistance mechanisms to MAPK inhibition may affect ctDNA release. In this context, melanomas might undergo metabolic reprogramming at the time of progression to therapy [383] and important changes in the microenvironment of the tumour lesions might reduce the shedding of ctDNA into the bloodstream.

Across all the studies in this thesis, we highlighted an important limitation of ctDNA analysis: the lack of or limited ctDNA shedding in patients with disease restricted to the brain. This represents a critical issue, as current studies have shown that a high proportion of *BRAF* mutant patients that progress to first-line targeted therapy present with brain metastases [302], with 20% of patients presenting only intracranial progression [384], often contributing to death [308, 385]. Given that brain metastases have been reported to shed less ctDNA into the bloodstream [192, 300], the large proportion of disease progression within the brain may contribute to the limited predictive value of baseline ctDNA and the low ctDNA detection rate in patients with intracranial disease only. Notably, in the case of brain metastatic lesions, ctDNA can be found in the cerebrospinal fluid providing an alternative source for liquid biopsies [282, 344]. However, the complexity and invasiveness of lumbar puncture can be a significant challenge for its routine use in the clinical environment.

Most studies of ctDNA in melanoma have mainly monitored singular specific mutations using highly sensitive targeted assays. However, the use of a limited number of variants in these studies may have hampered our ability to capture the full genetic heterogeneity present in melanoma tumours. Moreover, it is critical to notice that promoter mutation targets, such as *TERT*, have been observed to be under-represented in cfDNA fractions [16, 257]. Therefore, the analysis of multiple mutations in the same sample could significantly increase ctDNA detection levels. The recent development of next-generation sequencing panels that target melanoma-specific mutations has previously been shown to derive a reliable concordance of mutations between tumour tissue and blood samples [234, 386]. The continuous development of these assays will continue to support the suitability of ctDNA analysis for genetic profiling, especially in patients that do not carry a primary *BRAF* mutation.

A major limitation on our approach for ctDNA analysis is the use of targeted assays, commonly restricted to one mutation per patient. The use of these assays is important when identifying variants associated with response to drugs, such as *BRAF* in melanoma [74] or *EGFR* in non-small cell lung cancer [387]. The common use of this oncogenic driver mutation has been described as reliable and stable in ctDNA, with relapsing tumours maintaining their reliance on these key oncogenic drivers [388]. Moreover, we employed a tumour-informed approach to determine a key mutation to track disease in patients with *BRAF* WT melanomas. However, it is possible that factors such as clonality and oncogenic dependence may affect their frequency abundance in the tumours and therefore their concentration in plasma. For example, the development of clonal evolution with the rise of secondary mutations that mediate resistance is affected by treatment with targeted agents [13] and cfDNA can accurately reveal different sub-clonal responses to therapy [389] and escape mechanisms such as *BRAF* amplification [234].

The analysis of multiple variants will not only enable a more accurate disease assessment but also increase our ability to detect tumoral DNA. Recently, the development of genomic technologies has enabled the testing and monitoring of multiple tumour-specific mutations in a single assay.

The MassArray System, which utilises mass spectrometry to accurately measure PCR-derived amplicons, is also emerging as a viable alternative approach for multiplexing

different mutational targets for ctDNA detection and monitoring [188, 234]. Nevertheless, each of the targets remains constrained to single mutational changes. On the other hand, targeted sequencing can be used to study small regions, such as, individual exons, or a larger number of loci, expanding our ability to detect multiple genes of interest [194-197].

Analysis of ctDNA using targeted NGS requires incorporation of UMIs, which enable error suppression and detection of low frequency mutations [390]. Nevertheless, the high cfDNA input required, overall cost and lack of standardisation of the analysis pipelines associated with these technologies, limit their use to the research settings. Current studies are exploring the use of these assays in various tumour types and future cost reductions and validations will enable its implementation in ctDNA detection [391]. Beyond mutational analysis on ctDNA, the development of tests such as CancerSEEK that combine protein with genetic biomarkers has the potential to increase sensitivity [392], and may significantly increase the detection rate of residual disease and pathological progression. Furthermore, others studies have reported on multiparametric assays that include supplementary cancer biomarkers, such as metabolites, mRNA transcripts, miRNAs or methylated DNA sequences, can increase the detection rate and help to accurately identify malignancy from a single blood sample [236].

Altogether, ctDNA analysis has a unique place in the management of melanoma. This minimally invasive modality can rapidly determine the mutation status prior to treatment initiation, serve as a prognostic biomarker in the first-line therapy setting and indicate response early during therapy. However, here we show that this liquid biopsy only detects disease progression in half of the patients when compared to sensitive imaging technologies and has no prognostic value in the second-line setting.

Studies such as the ones presented in this thesis are important to inform design and support the development of randomised ctDNA-based interventional clinical trials. Currently, multiple clinical trials aim at validating the role of ctDNA as a predictive biomarker across different tumour types and status of disease [393]. Notably, the MELCIRC trial (NCT02862743) is currently investigating the concordance between mutations in ctDNA and tumour tissue, as well as the prognostic impact of the ctDNA genetic profile on survival. Importantly, the phase II CActUS (NCT03808441) trial aims

to evaluate whether ctDNA can aid effective switching from targeted to immune therapy based on ctDNA decline, early during treatment.

Interest in ctDNA has been increasing during the last decade, especially in the oncology setting. However, there is a crucial need to increase the consistency between the protocols used in handling the sample and the techniques used for analysis and reporting the results. The research presented here stresses the need to develop a uniform and standard template for plasma isolation and storage, ctDNA analysis and interpretation of results. Such protocol will allow for the easy integration of this biomarker into clinical trials by providing clear rules on the optimal pre-analytical steps and ctDNA quantification platforms. In addition, strict collection windows should be applied in order to make head-to-head comparison between ctDNA and imaging techniques possible. The use of ctDNA in melanoma should follow the path of other malignancies such as the recent ctDNA applications in colorectal cancer whitepaper [394]. Ultimately, the growing body of work and evidence in the expanding field will clear the way for implementing ctDNA analysis into routine clinical management [395] and improve cancer care through precision oncology.

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