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# Circulating tumour DNA: A non-invasive biomarker for melanoma

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# Circulating tumour DNA: A non-invasive biomarker for melanoma

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**Doctor of Philosophy**

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# ABSTRACT

Cutaneous melanoma accounts for 90% of all skin cancer deaths (Balch et al., 2010) and is responsible for 3.6% of deaths from cancer in Australia (Australian Institute of Health and Welfare, 2016). Whilst early detection and successful surgical removal of primary melanomas have improved survival rates (DeSantis et al., 2014), approximately 30% of these patients will have disease recurrence at some point in their lives (Soong et al., 1992; Soong et al., 1998). This is despite being considered disease free following treatment, which may have included surgical removal of the primary and/or its metastasis/es, radiation and/or systemic therapy. Whilst the risk of melanoma recurrence may correlate to some extent with the stage of the primary melanoma in terms of its size and thickness and whether it has metastasised (Shaw et al., 1987; Soong et al., 1992; Soong et al., 1998), recurrences occur even after thin melanomas (associated with low-risk for recurrence) that have been completely excised (Dalal et al., 2007; Jones et al., 2013; Leiter et al., 2012; Meier et al., 2002; Salama et al., 2013; Soong et al., 1998). Melanoma may recur at any point in time, even 10 or more years after a primary melanoma has been excised (Crowley et al., 1990; Dong et al., 2000; Hohnheiser et al., 2011; Kalady et al., 2003; Tsao et al., 1997). Recurrences may present in the same or in areas adjacent to the primary melanoma, however the majority of recurrences appear in lymph nodes or other organs, at which point the disease is among the most aggressive and treatment-resistant of all human cancers (Kenessey et al., 2012; Luke et al., 2017; Mocellin et al., 2013; Sanmamed et al., 2015; Ti'mar et al., 2013). In the metastatic setting, resective surgery of solitary metastases is associated with the most favourable outcome (Chua et al., 2010; Petersen et al., 2007; Sanki et al., 2009; Wasif et al., 2011), however systemic therapy options are dramatically improving survival of patients with unresectable metastases (Garbe et al., 2016). Overall, the greatest treatment efficacy is associated with a low disease burden at time of therapy (Hodi et al., 2010; Luke et al., 2017; McArthur et al., 2016; Sosman et al., 2011) and therefore early detection of melanoma recurrence is critical for improved survival.

To date, there are no reliable early markers of melanoma recurrence. Radiological imaging techniques and sentinel lymph node (SLN) biopsies (SLNB) are currently the methods employed to stage primary melanomas and detect metastases. Positron emission tomography (PET) with a labelled glucose analogue fluorine 18 fluorodeoxyglucose ($^{18}$F-FDG) combined with computed tomography (CT) scans (FDG-PET/CT), are used routinely to determine disease burden. These have limited sensitivity however for the detection of early stage melanoma micro-metastases (Meyers et al., 2009; Pfannenberg et al., 2015), thus cannot provide timely clinical evidence of disease recurrence (Belhocine et al., 2002; Hindié et al., 2011; Krug et al., 2008). Fluorine 18 fluorodeoxyglucose Positron Emission Tomography combined with Computed Tomography (FDG-PET/CT) may be used routinely for monitoring of melanoma patients at high risk of disease recurrence, but it is expensive (Gellén et al., 2015) and subjects patients to excessive radiation exposure (Rueth et al., 2015). Whilst routine SLNBs offer a survival advantage in monitoring recurrence in patients with >1.0mm thick melanomas (Faries et al., 2017; Morton et al., 2014), they are relatively invasive for routine monitoring (Agnese et al., 2003; Lens et al., 2002). Early stage melanoma patients who are considered disease free and are not at high risk for a recurrence, are not routinely assessed by SLNB, or PET/CT or LNB, but rather by physical examinations (Australian Cancer Network Melanoma Guidelines Revision Working Party, 2008). Thus, an additional monitoring regime that can be performed regularly and in conjunction with physical examinations could lead to timely interventions resulting in improved treatment options that will positively impact on the patient's quality of life and survival.

The detection and analysis of mutant specific circulating tumour DNA (ctDNA) is an emerging tool for detection of residual disease and for prognosis and monitoring of different cancers (Bettegowda et al., 2014; Dawson et al., 2013; Gray et al., 2015; Spindler et al., 2012). There is however, limited use of ctDNA for monitoring of residual disease and recurrence in clinically disease free patients

(Oshiro et al., 2015; Tie et al., 2016) and to date, this has not been assessed in melanoma. In melanoma, mainly V-raf murine sarcoma viral oncogene homolog B1 (*BRAF)* and to some extent, neuroblastoma RAS viral oncogene (*NRAS*) mutant ctDNA are utilised to monitor patients during therapy in the research setting (Ascierto et al., 2013a; Girotti et al., 2015; Gray et al., 2015; Sanmamed et al., 2015; Santiago-Walker et al., 2015). Notably, telomerase reverse transcriptase (*TERT)* promoter mutations are present in 50-70% of melanomas and confer a significantly poorer prognosis if found concurrently with *BRAF or NRAS* mutations relative to the occurrence of each mutation alone. Thus, the ability to monitor patients at all disease stages for the presence of *BRAF*, *NRAS* as well as *TERT* mutant ctDNA, would be advantageous even in *BRAF* and *NRAS* wild-type patients.

The overall aim of this thesis was to further develop existing tools that could regularly, inexpensively and non-invasively monitor melanoma patients for melanoma recurrence. Firstly, we focused on increasing the number of patients that could be monitored through ctDNA analysis. To do this we developed a new and innovative ddPCR *TERT* mutation assay and investigated its sensitivity alongside current assays in detecting mutations in melanoma tissue containing a small fraction of tumour cells. The significance of ctDNA for patient monitoring relative to current methods of clinical monitoring was then investigated in relation to melanoma recurrence. Finally, we conducted a retrospective analysis of ctDNA levels relative to metabolic tumour burden (MTB) derived from FDG-PET/CT to determine the lower limit of disease burden detectable by ctDNA using ddPCR.

In the first study of this thesis, a novel droplet digital PCR (ddPCR) assay for the concurrent detection of C228T and C250T *TERT* promoter mutations was designed and developed to display a lower limit of detection (LOD) of 0.17%. The assay was validated using 22 matched plasma and

tumour samples and showed a 68% concordance rate, with a sensitivity of 53% (95% CI, 27%-79%) and a specificity of 100% (95% CI, 59%-100%). Plasma samples from 56 metastatic melanoma patients and 56 healthy controls were tested for *TERT* promoter mutations confirming a specificity of 100% (95% CI, 94%-100%). Importantly, we not only detected *TERT* mutant specific ctDNA in 4 *BRAF* mutant cases, but this assay allowed ctDNA quantification in 11 *BRAF* wild-type cases, which allows for an increased number of patients to be monitored using ctDNA.

To monitor patients for recurrence using ctDNA, the mutational profile must first be determined from a patient's tumour. However, this may be difficult to obtain from tumours that have limited and/or low tumour cellularity and high heterogeneity, particularly when sourced from SLNB and fine needle aspiration biopsies of metastatic sites. Consequently, only limited, low-quality DNA may be isolated for use on different mutation detection platforms, each with varying analytical sensitivities. Limited previous studies focused predominantly on assessment of the *BRAF* V600 mutation (as the only actionable mutation), and, notably, in tumour samples with more than 50% cellularity. Given the prevalence of *TERT* promoter mutations which, together with *BRAF* and *NRAS* mutations provide prognostic significance, the ability to assess the presence of such mutations in patient tumours, at high sensitivity, would dramatically improve assessment of mutations. In the second study presented here, we evaluated the sensitivity of detection of *BRAF*, *NRAS* and *TERT* promoter mutations in 40 melanoma tissues, using ddPCR relative to Sanger sequencing and pyrosequencing. Tumour cellularity in our samples ranged from 5-50% (n=28) and 50-90% (n=12). Overall, ddPCR was the most sensitive, detecting one of the tested hotspot mutations in a total of 77.5% (31 of 40) of cases, including in 12.5% and 23% of samples deemed as wild-type by pyrosequencing and Sanger sequencing, respectively. The ddPCR sensitivity was particularly apparent among samples with less than 50% tumour cellularity. Therefore, implementation of ddPCR based assays could facilitate mutation detection of early stage tumours

and support research aimed at using ctDNA to improve early detection of residual disease and disease recurrence or progression.

In the third paper presented here, we assessed the sensitivity of ctDNA to detect disease recurrence. A cohort of 139 patients diagnosed with AJCC stages 0-III in the preceding 10 years were enrolled in the study between January 2015 and February 2017. A blood sample was collected at enrolment and on average 11 months thereafter. Patients were followed up for disease progression for a median time of 50.2 months. From the remaining cohort, three patients developed metastatic disease. The median follow-up from diagnosis of the primary tumour to stage IV disease was 34.4 months. The remaining patients had no clinical evidence of disease recurrence at last follow-up or at death from other causes. We analysed the primary tumour of 37 patients for mutations in *BRAF*, *NRAS* and *TERT*, and identified mutations in 30 patients (three patients with recurrence and 27 patients without recurrence). Using our proven, highly sensitive ddPCR tests we analysed *BRAF*, *NRAS* and *TERT* promoter mutated ctDNA in all available blood samples. Three serial plasma samples were available for each of the three patients who had recurred. CtDNA was detected at the time of radiological or biopsy confirmation of metastases in all three patients. Moreover, ctDNA was detectable in earlier plasma samples from one of the three patients; in this one patient, ctDNA was detected four months prior to clinical detection of gastric and ileum metastases by gastroscopy and biopsy. We detected no mutant specific ctDNA at any time point in the patients without recurrence. Whilst this data is limited because of the limited number of patients and the limited rates of recurrence in early disease stages (2.15%), it provides proof of concept that ctDNA may be a valuable tool to monitor early disease recurrence. Additionally, our assessments were limited by our knowledge of the level of sensitivity of the ctDNA analyses. There was therefore, a robust need to understand the correlation between ctDNA levels and the patient's tumour burden as assessed by metabolic activity using PET.

Given that the metabolic activities of tumours are measured routinely during clinical disease monitoring by assessment of FDG uptake using PET/CT (Larson et al., 1999), we hypothesised that if ctDNA levels correlate with metabolic tumour burden (MTB) derived from FDG-PET/CT scans in melanoma patients, we could determine the limit of detection (LOD) of ctDNA to signify disease recurrence which would indicate the limitations of ctDNA as a biomarker to identify low disease burden. Thus, the indications of ctDNA in the clinical setting will be more clearly identified OR, the need to improve the sensitivity of ctDNA is therefore apparent. Consequently, in the fourth paper of this thesis, we conducted a retrospective analysis of the ctDNA levels in 32 stage IV melanoma patients with active disease prior to systemic therapy. Corresponding FDG-PET/CT scans were examined and the MTB was determined from metabolic tumour volume (MTV) and tumour lesion glycolysis (TLG) (Larson et al., 1999; Winther-Larsen et al., 2017). Within this cohort of patients, ctDNA was detected in 72% of cases with the number of mutated copies per mL of plasma ranging from 1.6 to 52,440. A significant correlation between the MTB and allele frequency was found ($P<0.001$). Finally, ctDNA was not detectable in patients with a MTB value of less than 10 $cm^3$ and therefore we determined this as the lower LOD of ctDNA by ddPCR.

Overall, ctDNA tests were developed to monitor *TERT* promoter mutations in cell free DNA (cfDNA) in addition to those currently available for *BRAF* and *NRAS* therefore maximising the number of patients whose disease status can be monitored using ctDNA. We also demonstrated that ddPCR is a highly sensitive method for detection of *BRAF*, *NRAS* and *TERT* promoter mutations in tumour tissue. Using these tests, we identified a strong correlation between the level of ctDNA and metabolic tumour burden, suggesting, for the first time in melanoma, that ctDNA reflects melanoma disease burden. We also detected ctDNA in early stage melanoma patients that suffered disease recurrence. Prospective studies are now warranted to serially assess the amount of ctDNA after resective surgery to determine if the presence of ctDNA can detect residual disease, and whether

rising levels of ctDNA in the blood can detect disease recurrence earlier than current clinical methods. This will ultimately provide a sensitive method with which to monitor patients, to ensure timely, earlier interventions thereby improving melanoma survival rates.

# DECLARATION

I certify that this thesis does not, to the best of my knowledge and belief:

i. Incorporate without acknowledgement any material previously submitted for a degree or diploma in any institution of higher education;
ii. Contain any material previously published or written by another person except where due reference is made in the text of this thesis; or
iii. Contain any defamatory material.
iv. Contain any data that has not been collected in a manner consistent with ethics approval.

The Ethics Committee may refer any incidents involving requests for ethics approval after data collection to the relevant Faculty for action.

Signed: Date: 07 November 2017

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# LIST OF PUBLICATIONS

This thesis is submitted as a series of papers. As a result of the work performed for this thesis, one paper has been published, one paper is currently In Press and two papers have been submitted for publication. The papers are as follows:

Sensitive Droplet Digital PCR Method for Detection of *TERT* Promoter Mutations in Cell Free DNA from Patients with Metastatic Melanoma

Authors: Ashleigh C McEvoy, Leslie Calapre, Michelle R Pereira, Tindaro Giardina, Cleo Robinson, Muhammad A Khattak, Tarek M Meniawy, Antonia L Pritchard, Nicholas K Hayward, Benhur Amanuel, Michael Millward, Melanie Ziman, Elin S Gray

Published: Oncotarget, 2017, 8 (45), 78890-900. doi: 10.18632/oncotarget.20354.

Droplet Digital PCR for Mutation Detection in FFPE Melanoma Tissues: A Comparison with Sanger Sequencing and Pyrosequencing

Authors: Ashleigh C McEvoy, Benjamin A Wood, Nima M Ardakani, Michelle Pereira, Robert Pearce, Lester Cowell, Cleo Robinson, Fabienne Grieu-Iacopetta, Alexander J Spicer, Benhur Amanuel, Melanie Ziman, Elin S Gray

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## STATEMENT OF CONTRIBUTION TOWARDS PUBLICATIONS

For chapters 2-5, I contributed to the study design, experiment design, experimental work and data analysis. I drafted and edited each manuscript. I drafted and edited chapters 1, 6 and 7.

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# ABBREVIATIONS

| | |
|---|---|
| µL | Microliters |
| $^{18}$F-FDG | Fluorine 18 fluorodeoxyglucose |
| AJCC | American Joint Committee on Cancer |
| ARAF | V-raf murine sarcoma viral oncogene homolog A1 |
| *BRAF* | V-raf murine sarcoma viral oncogene homolog B1 |
| cfDNA | Cell free DNA |
| CDX | Circulating tumour cell-derived xenograph |
| CRAF | V-raf murine sarcoma viral oncogene homolog C1 |
| CT | Computed tomography |
| ctDNA | Circulating tumour DNA |
| CTLA-4 | Cytotoxic T-lymphocyte-associated antigen 4 |
| ddPCR | Droplet digital PCR |
| DNA | Deoxyribonucleic acid |
| dPCR | Digital PCR |
| ERK | Extracellular signal-regulated kinase |
| ETS | E26 transformation-specific |
| FDG-PET/CT | Fluorine 18 fluorodeoxyglucose Positron Emission Tomography combined with Computed Tomography |
| FFPE | Formalin fixed paraffin embedded |
| gDNA | Genomic DNA |
| H&E | Haematoxylin and eosin |
| *KRAS* | Kirsten rat sarcoma viral oncogene homolog |
| LDH | Lactate dehydrogenase |
| LOD | Limit of detection |

| | |
|---|---|
| LNB | Lymph node biopsy |
| LND | Lymph node dissection |
| NF1 | Neurofibromatosis type 1 |
| MAPK | Mitogen-activated kinase |
| MEK | Mitogen-activated protein kinase |
| miRNA | MicroRNAs |
| MTB | Metabolic tumour burden |
| MTV | Metabolic tumour volume |
| NCCN | National Comprehensive Cancer Network |
| ng | Nanograms |
| NGS | Next generation sequencing |
| *NRAS* | Neuroblastoma RAS viral oncogene |
| NS | Not significant |
| NSCLC | Non-small cell lung cancer |
| ORR | Objective response rate |
| OS | Overall survival |
| PD-1 | Programmed cell-death protein |
| PDX | Patient derived xenographs |
| PERCIST | PET Response Criteria in Solid Tumours |
| PET | Positron emission tomography |
| PFS | Progression free survival |
| *PIK3CA* | Phosphatidylinositol-4, 5-bisphosphate 3-kinase, catalytic subunit alpha |
| qPCR | Quantitative PCR |
| RAF | Rapid accelerated fibrosarcoma |
| RAS | Recurrent aphthous stomatitis |

| | |
|---|---|
| RNA | Ribonucleic acid |
| SE | Standard error |
| SLN | Sentinel lymph node |
| SLNB | Sentinel lymph node biopsy |
| SUV | Standardised uptake value |
| *TERT* | Telomerase reverse transcriptase |
| TLG | Tumour lesion glycolysis |
| TNM | Tumour / [lymph] nodes/ metastases |
| UV | Solar ultraviolet |
| WES | Whole exome sequencing |
| WGS | Whole genome sequencing |
| WT | Wild-type |

# CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Cutaneous melanoma (melanoma) is an aggressive skin cancer arising from transformed melanocytic cells in the basal layer of the epidermis. This transformation is the result of an accumulation of mutations in genes that would normally regulate cell division and proliferation, resulting in the uncontrolled growth of melanocytes (Dutton-Regester et al., 2012; Peyssonnaux et al., 2001; Ward et al., 2012). In Australia, although melanoma accounts for only 3.6% of all skin cancers (AIHW, 2013a), it is responsible for 90% of all skin cancer related deaths (Balch et al., 2010), presenting a considerable burden to public health (Rigel et al., 2000). A significant contributing factor to melanoma deaths is disease recurrence. Approximately 30% of patients experience recurrent melanoma despite having been disease free for a period of time, with 78% presenting as regional or distant metastases rather than local recurrences (Soong et al., 1992; Soong et al., 1998). Advances in systemic therapies for metastatic melanoma have resulted in increased median overall survival, from approximately nine months prior to 2011, to between 25.5 to 32 months in 2017 (Luke et al., 2017; Robert et al., 2017). Whilst two and three-year overall survival rates have been reported at 64% (Hodi et al., 2016) and 58%, respectively (Wolchok et al., 2017), timely treatment is fundamental to ensure enhanced response rates (Luke et al., 2017). Given the ongoing threat of lives lost from disease recurrence, routine monitoring and early detection of recurrence is of vital importance.

## 1.1 Melanoma Incidence and Mortality

In the last 50 years, melanoma incidence has increased steadily worldwide and is predicted to continue to rise (AIHW, 2012a). These increases have been particularly evident amongst fair skinned individuals, particularly in males above 60 years old (Akushevich et al., 2013; Garbe et al., 2009). In 2008 almost 200,000 new cases were diagnosed worldwide resulting in 46,000 deaths (Ferlay et al., 2010). The countries with the highest incidence and mortality rates are Australia and New Zealand (Baade et al., 2015; Sneyd et al., 2013). In Australia, melanoma is the third most

common cancer in both men and woman accounting for an estimated 12% in men and 9% in women of all newly diagnosed cancers (AIHW, 2017). In 2010, 11,405 new cases of melanoma were diagnosed in Australia and it is estimated that 14,000 and 17,570 new cases will be diagnosed in 2017 and 2020, respectively with an estimated 1,800 deaths in 2017 alone (Australian Institute of Health and Welfare, 2013b). In 2013, the incidence rate of melanoma amongst fair skinned people in Australia was 62 cases for men and 40 cases for women per 100,000 persons (AIHW, 2017). In the USA incidence rates range from 16.9 to 25.4 per 100,000 in women and men, respectively (Kohler et al., 2011). In Europe, in 2012, the estimated incidence was 11.0 and 11.4 per 100,000 in woman and men, respectively although wide variations were apparent between different geographical locations (Ferlay et al., 2013).

Whilst the incidence of melanoma continues to rise, five-year survival rates have increased from 40% in the 1940s (Rigel et al., 2000) to 85.8% and 90.7% for the periods 1982–1987 and 2006-2010, respectively (AIHW, 2012b). Such increases in survival rates are largely due to early detection and successful surgical removal of primary melanomas (DeSantis et al., 2014; Rigel et al., 2000). Despite these improvements, a significant percentage of early stage patients considered clinically disease free, later develop metastatic disease and die within 10 years of initial tumour resection (Balch et al., 2010). Once melanoma metastasises, it is among the most aggressive and treatment-resistant of all human cancers (Kenessey et al., 2012; Mocellin et al., 2013; Sanmamed et al., 2015; Ti'mar et al., 2013). As such there is a need for the development of techniques that identify patients at risk of disease recurrence as early as possible after resection of the primary tumour.

### 1.2 Classification and Staging of Primary Melanoma

Primary melanoma generally presents as one of four different histological subtypes: superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma and acral lentiginous

melanoma (Bastian, 2014). Each subtype has characteristic clinical features (Schaffer et al., 2000) and typical ages of occurrence (Chang et al., 1998). Within fair skinned populations, superficial spreading melanoma is the most common (59%), followed by nodular melanoma (21%), lentigo maligna melanoma (11%) and acral lentiginous melanoma (4%) (Garbe et al., 2009).

Superficial spreading melanoma predominantly arises on the trunk in men and on the lower extremities in women (MacKie et al., 2002). This form of melanoma has an association with a pre-existing naevus (Garbe et al., 2009) as well as exposure to UV rays and is more commonly found in young and middle-aged individuals (Black, 1988; Thomas et al., 2007). Nodular melanoma is notoriously invasive since it is rarely diagnosed in the early stages. It does not have a distinct intraepidermal growth pattern but comprises melanocytic nests within the epidermis and subcutaneous tissue. It can be found at any anatomical site (Menzies et al., 2013), and is more commonly found in elderly men with sun-damaged skin (Malvehy et al., 2012; Menzies et al., 2013). Lentigo maligna melanoma is typically seen in older people and occurs most commonly on the face, head and neck (Pralong et al., 2012; Reed et al., 2011). Although it is the least common of the four subtypes (Piliang, 2011), acral lentiginous melanoma is the most common subtype among Asians (Kim et al., 2014), Hispanics and dark skinned individuals (Wu et al., 2011). Lesions typically arise on skin surfaces not usually exposed to the sun such as the soles of the feet and palms of the hands (Pereda et al., 2013).

Currently the diagnostic process includes biopsy and histopathological assessment of the lesion to classify and stage the melanocytic lesion based on the American Joint Commission on Cancer (AJCC) guidelines. Staging of the tumour according to the TNM (tumour / [lymph] nodes/ metastases) staging system is based on histological features, including the thickness of the primary tumour, the mitotic rate and the level of ulceration, as well as on the presence or absence of metastases (Balch et al., 2009). The invasiveness and thickness of tumours have been

independently described by both Clark (1969) and Breslow (1970): The Clark classification is based on the anatomical level of invasion whilst the Breslow classification describes the vertical thickness of invasion in millimetres. Following the recommendations of the AJCC (Edge et al., 2010), the primary tumour staging is now based on the Breslow classification (Leilabadi et al., 2014). Additional histological features of the primary lesion, such as ulceration and the number of mitoses per square millimetre provide further prognostic and sub-staging information (T1-4). Depending on the T-sub stage, the thickness of the lesion is currently an important prognostic indicator. The N-stage refers to the pathological status of the lymph nodes, with the number of nodes involved and the distinction of micro or macro-metastasis being the measure of sub-stage. Macro-metastasis is defined as clinically palpable or radiologically visible lymph nodes and micro-metastasis is defined by the identification of tumour cells by conventional histopathology (Balch et al., 2009; Balch et al., 2011). The M-stage of the TNM staging system represents the metastatic stage, with sub-staging being determined by the anatomical site of the metastases together with lactate dehydrogenase (LDH) serum levels (Balch et al., 2009; Balch et al., 2011; Gershenwald et al., 2010; Piris et al., 2011).

**Table 1: American Joint Committee on Cancer: Staging of Cutaneous Melanoma**

| Patho-logical Staging | TNM | Tumour Thickness (mm) | Ulceration | Mitoses | No. of positive nodes | Nodal Mass | Sited Metastasis |
|---|---|---|---|---|---|---|---|
| Stage 0 | Tis | <1 | n/a | | 0 | - | - |
| Stage IA | T1a | ≤1.0 | No | <1/mm$^2$ | 0 | - | - |
| Stage IB | T1b | ≤1.0 | Yes | ≥1/mm$^2$ | 0 | - | - |
| | T2a | 1.01-2.0 | No | | 0 | - | - |
| Stage IIA | T2b | 1.01-2.0 | Yes | Any | 0 | - | - |
| | T3a | 2.01-4.0 | No | Any | 0 | - | - |
| Stage IIB | T3b | 2.01-4.0 | Yes | Any | 0 | - | - |
| | T4a | >4.0 | No | Any | 0 | - | - |
| Stage IIC | T4b | >4.0 | Yes | Any | 0 | - | - |
| Stage IIIA | N1a | Any | No | Any | 1 | Micro[a] | - |
| | N2a | Any | No | Any | 2-3 | Micro[a] | |
| Stage IIIB | N1a | Any | Yes | Any | 1 | Micro[a] | - |
| | N2a | Any | Yes | Any | 2-3 | Macro[b] | - |
| | N1b | Any | No | Any | 1 | Macro[b] | - |
| | N2b | Any | No | Any | 2-3 | Macro[b] | - |
| Stage IIIC | N1b | Any | Yes | Any | 1 | Micro[a] | - |
| | N2b | Any | Yes | Any | 2-3 | Macro[b] | - |
| | N3 | Any | Any | Any | 4 | Either | - |
| IV | M1a | Any | Any | Any | Any | Any | Distant skin, subcutaneous or distant lymph nodes |
| | M1b | Any | Any | Any | Any | Any | Lung metastases |
| | M1c | Any | Any | Any | Any | Any | All other visceral metastases or any metastates combined with elevated LDH* level |

[a] Micro-metastases: Diagnosed after sentinel lymph node biopsy and complete lymphadenectomy (if performed)

[b] Macro-metastases: Clinically detectable nodal metastases that exhibit either gross extra capsular extension or are confirmed by therapeutic lymphadenectomy

*Lactate dehydrogenase (LDH)

(Adapted from Balch et al., 2009 and 2011 and Aitken et al., 2008)

## 1.3 Primary Melanoma: Risk Factors

Although the cause of melanoma and associated risks are complex, one of the most recognised risk factors for primary melanoma is exposure to solar ultraviolet (UV) radiation (Caini et al., 2009; Chang et al., 2009; Gandini et al., 2005; Gilchrest et al., 1999; Hodis et al., 2012). Both intermittent intense and chronic cumulative amounts of sun exposure have been shown to play a role in the pathogenesis of melanoma (Gandini et al., 2005). Other factors which are associated with primary melanoma risk include, but are not limited to, phenotypic features such as fair skin, red hair, light eye colour, a high number of naevi, a family history and genetic predisposition (Rastrelli et al., 2014).

## 1.4 Genetics of Melanoma

Whilst all cancers are caused by somatic mutations, melanoma has the highest prevalence of somatic mutations, with the exception of non-melanoma skin cancer (Alexandrov et al., 2013). The landscape of genomic alterations in melanoma has been well described from primary and/or metastatic melanomas and is depicted in Figure 1 (Cancer Genome Atlas Network., 2015; Curtin et al., 2005; Hodis et al., 2012; Luke et al., 2017). Based on the pattern of the most prevalent and significantly mutated genes, four sub-types provide a framework for genomic classification; mutant *BRAF*, mutant *NRAS*, mutant neurofibromatosis type 1 (*NF1*) and triple wild-type (WT). The mutations commonly identified affect fundamental signalling pathways involved in cell growth, proliferation, cell-cycle control and restoration of DNA damage (Bosenberg et al., 2014; De Luca et al., 2012; Haluska et al., 2006; Hayward, 2003) and play a vital role in tumour formation and growth (Bosenberg et al., 2014; Keller et al., 2010; Romeo et al., 2013; Shaw et al., 2006).

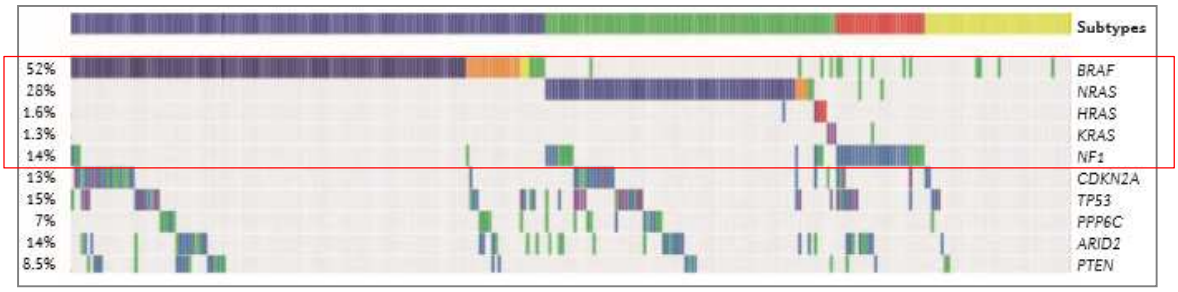


**Figure 1: Frequency and overlap of driver and tumour-suppressor genes associated with melanoma**

Categorisation of advanced-stage melanoma according to mutations in either BRAF, RAS, NF1 or triple negative (Red Box). Additional driver mutations are also found in other genes such as CDKN2A and PTEN. Adapted from Cancer Genome Atlas Network., 2015.

One of the most recognised pathways involved in melanoma initiation and progression is the mitogen-activated kinase (MAPK) pathway (Figure 2), regulated by receptor tyrosine kinases, G-protein-coupled receptors and cytokines. The rat sarcoma viral oncogene (RAS) protein, which is situated at the plasma membrane, is activated by the c-Kit receptor and in turn activates V-raf murine sarcoma viral oncogene homolog (RAF) A1 (ARAF), B1 (*BRAF*) and C1 (CRAF). This in turn phosphorylates and activates the mitogen-activated protein kinase (MEK). MEK activates the protein kinase extra-cellular-signal-regulated kinase (ERK) which activates transcription factors that result in gene transcription and therefore cell cycle regulation. This pathway is active in all cells including normal melanocytes (Yajima et al., 2012). In 90% of melanomas however, ERK is hyper-activated by mutated RAS and RAF proteins (Davies et al., 2002).

Mutations in *NRAS* have been identified in approximately 20% of melanomas (Griewank et al., 2013; Jakob et al., 2012; Yajima et al., 2012). The most common mutations in *NRAS* occur at codon 61 resulting in replacement of a glutamine residue by an arginine (Q61R) or lysine (Q61K) in the encoded protein (Exon 3) (Jakob et al., 2012; Platz et al., 2008). Other *NRAS* mutations observed in melanoma include genetic changes that result in 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/G12D). Under normal circumstances, the RAS oncoprotein induces MAPK/ERK phosphorylation to initiate cell proliferation and as such, mutated *NRAS* will result in constitutive activation of the MAPK signalling pathway resulting in increased cell proliferation and advancement of tumour growth.

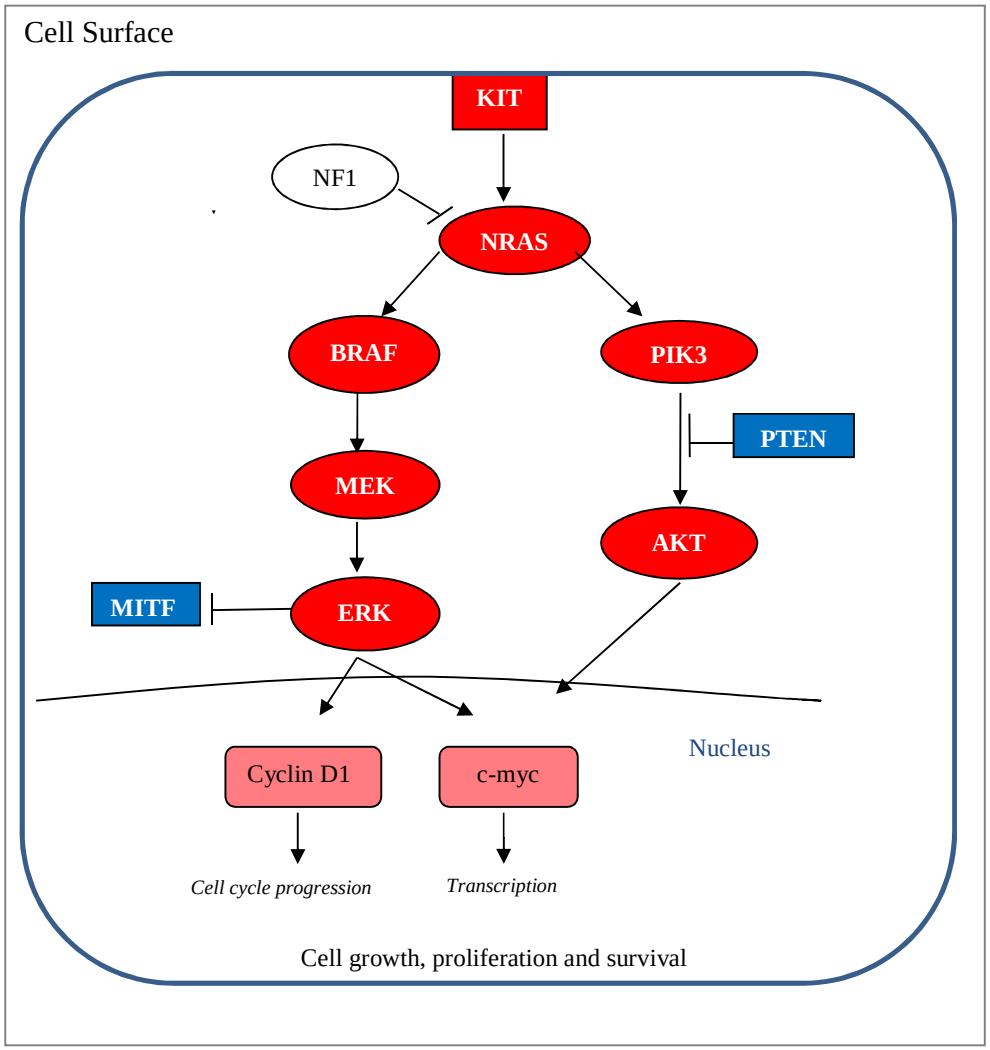


**Figure 2: MAPK signalling pathway in melanoma**

Proteins in red are affected by gain-of-function mutations and those in blue are affected by loss-of-function mutations in encoding genes. Adapted from Bastian (2014) and Bogenrieder et al. (2011).

Aberrant or constitutive activation of the RAF proteins, particularly BRAF in melanoma lead directly to abnormal differentiation, proliferation and inhibition of apoptosis in melanocytes. *BRAF* mutations are found in 50 to 60% of all melanomas (Boni et al., 2010; Hauschild et al., 2012; Ribas et al., 2011; Rubinstein et al., 2010b; Santarpia et al., 2012; Trunzer et al., 2013). A single base missense substitution in *BRAF* which is present on chromosome 7q34, (T to A at nucleotide 1,799) (GTG to GAG in exon 15), results in the substitution of a valine for a glutamic acid at codon 600

(V600E) in the encoded protein and this is the most common mutation, accounting for approximately 80% to 90% of *BRAF* mutations found in melanoma (Hauschild et al., 2012; Lovly et al., 2012; Wu et al., 2014). Interestingly *BRAF* V600E is also expressed in 80% of benign naevi and has been shown to drive senescence (Michaloglou et al., 2005; Pollock et al., 2003), contradicting the notion that such an early event would have an impact on tumour development (Long et al., 2011). Another mutation at codon 600 (nucleotides 1,798 and 1,799 where two bases GT are replaced by AA) encodes the *BRAF* V600K mutation, resulting in substitution of valine by lysine (Busam et al., 2013). This mutation is present in 5-12% of melanomas (Lovly et al., 2012; Rubinstein et al., 2010b). Interestingly, *BRAF* V600K mutated melanomas are present at a relatively higher frequency in Australia (Amanuel et al., 2012; Long et al., 2011) and are more prominent in melanoma patients ≥ 70 years (Menzies et al., 2012). Other mutations found in melanoma include but are not limited to V600R (valine - arginine), V600D (valine - aspartic acid), V600G (valine - glycine), V600M (valine - aspartic acid) and K601E (lysine - glutamic acid). Such mutations are however rare (Greaves et al., 2013).

*BRAF* codon 600 and *NRAS* codon 61 are the most commonly reported hotspot mutations detected in proto-oncogenes in melanoma. Notably, these same mutations detected in the primary tumour are almost always maintained in the corresponding metastases (Omholt et al., 2002; Omholt et al., 2003; Platz et al., 2008). Whilst *BRAF* and *NRAS* mutations are mostly mutually exclusive in melanoma (Davies et al., 2002; Omholt et al., 2002; Platz et al., 2008; Tsao et al., 2015), co-occurrence of these mutations has been reported in primary melanomas and in patients with acquired resistance to BRAF inhibitors (Edlundh-Rose et al., 2006; Goel et al., 2006; Gray et al., 2015; Jovanovic et al., 2010; Long et al., 2014a; Nagore et al., 2016a; Rizos et al., 2014). Generally, the frequencies of *BRAF* and *NRAS* mutations are different among the histological subtypes and sites of origin of melanoma (Lee et al., 2011).

On average among the four histological melanoma subgroups, the highest frequencies of *BRAF* mutations are found in superficial spreading, followed by nodular melanomas on intermittent sun exposed anatomical sites. In contrast, *NRAS* mutations are found more frequently in nodular followed by superficial spreading melanomas from continuously sun exposed anatomical sites (Ball et al., 1994; Edlundh-Rose et al., 2006; Lee et al., 2011; Saldanha et al., 2006). Furthermore, a significantly lower mean age at diagnosis has been registered among patients with *BRAF* mutated melanomas compared to *NRAS* mutations (Edlundh-Rose et al., 2006) and a more chronic pattern of UV exposure is evident in *NRAS* mutated melanomas (Devitt et al., 2011; Jakob et al., 2012). *NRAS* mutation status is also an independent predictor of shorter melanoma specific survival than *BRAF* mutant melanomas (Jakob et al., 2012).

*NF1* is the third most frequently mutated gene in melanoma and has been shown to be present in up to 46% of melanomas WT for *BRAF* and *RAS* mutations (Krauthammer et al., 2015). In contrast to *BRAF* and *NRAS*, *NF1* has no associated hotspot mutations, but rather aberrations occurring throughout the gene in the form of point mutations and small indels only (nonsense, frameshift, splice site and missense) (Hayward et al., 2017).

Another gene implicated in melanoma is the *TERT* gene. This gene encodes the catalytic subunit of telomerase which is a ribonucleioprotein responsible for maintaining telomere length (Counter et al., 1998). The replicating lifespan of most adult somatic cells is limited due to the silencing of *TERT*. In cancer however, *TERT* expression is reactivated which consequently permits replication immortality. Two cancer-specific hotspot mutations at chr5: 1,295,228 C>T and 1,295,250 C>T occur in the *TERT* promoter, hereafter termed C228T and C250T. These mutations result in a cytidine to thymidine transition in the *TERT* promoter region upstream of the ATG start site and result in the creation of novel E26 transformation-specific (ETS) transcription factor binding motifs (Figure 3). The presence of either one of these mutations has been reported in 33% and 85% of

primary and metastatic melanoma tissues, respectively (Horn et al., 2013). Moreover they are linked to fast growing melanomas and a poor prognosis (Griewank et al., 2014; Nagore et al., 2016b). Furthermore, 55% of melanoma cases harbour co-existing *TERT* promoter and *BRAF* or *NRAS* mutations, with co-existence related to poor disease-free survival (Nagore et al., 2016a).

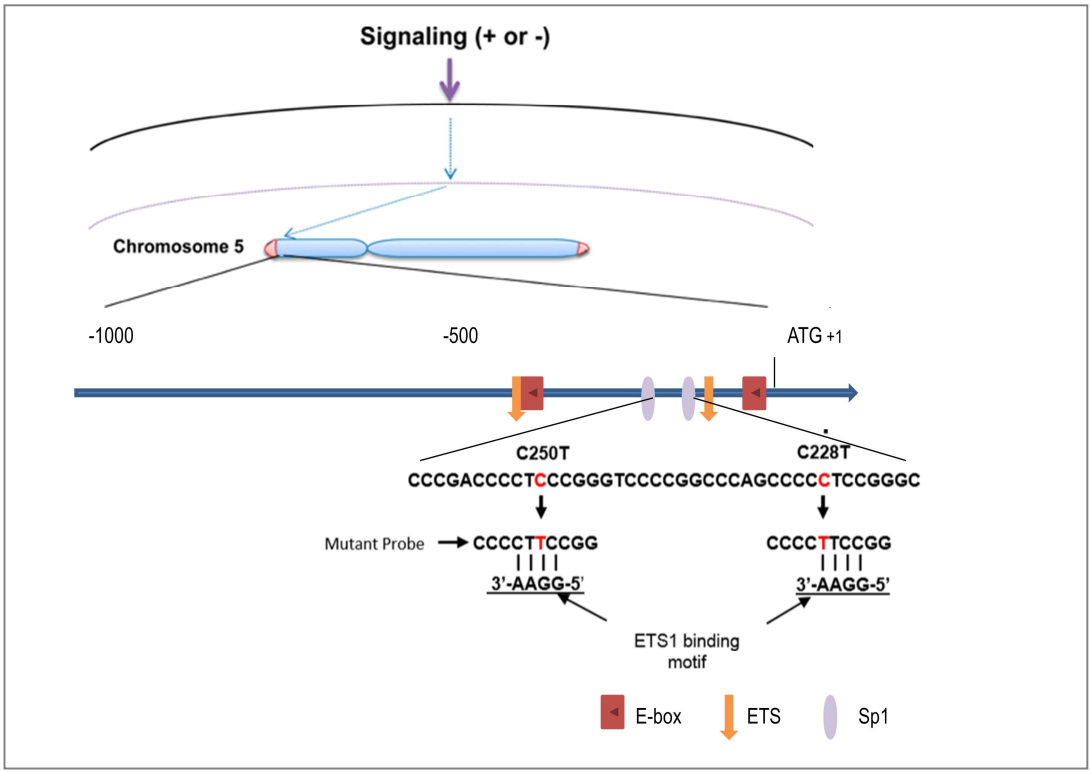


**Figure 3: Schematic illustration showing the *TERT* gene on chromosome 5 and its promoter (from ATG to-1000).**

Cancer specific *TERT* promoter mutations C250T and C228T at SP1 binding sites create ETS1 binding motifs adjacent to an E-box. Adapted from Liu et al., (2016).

### 1.5 Recurrent Melanoma: Risk Factors and Prognosis

Approximately one third of all melanoma patients will experience a recurrence in their lifetime (Soong et al., 1998), despite having been disease free for a period of time, with 65% of these occurring within three years of surgical removal of the primary tumour (Geere et al., 2012). Furthermore, between 5 to 30% of patients diagnosed with AJCC stage I or II melanomas develop a

recurrence at some point in their lives (Balch et al., 2009; Salama et al., 2013; Turner et al., 2011). Survival outcomes from 17,000 melanoma patients from a variety of countries are shown in Table 2.

**Table 2: Survival Rates for Melanoma by TNM Staging**

| Stage | Five-year survival rate (%) | 10-year survival rate (%) |
|---|---|---|
| T1a | 95.3 | 87.9 |
| T1b | 90.9 | 83.1 |
| T2a | 89.0 | 79.2 |
| T2b | 77.4 | 64.4 |
| T3a | 78.7 | 63.8 |
| T3b | 63.0 | 50.8 |
| T4a | 67.4 | 53.9 |
| T4b | 45.1 | 32.3 |
| N1a | 69.5 | 63 |
| N2a | 63.6 | 56.9 |
| N1a | 52.8 | 37.8 |
| N2a | 49.6 | 35.9 |
| N1b | 59.0 | 47.7 |
| N2b | 46.3 | 39.2 |
| N1b | 29.0 | 24.4 |
| N2b | 24.0 | 15.0 |
| N3 | 26.7 | 18.4 |
| M1a | 18.8 | 15.7 |
| M1b | 6.7 | 2.5 |
| M1c | 9.5 | 6.0 |

(adapted from Balch et al., (2001) and Aitken et al., (2008))

Currently the AJCC sub-staging classification is the most accurate predictor of recurrence (Turner et al., 2011). The majority of primary melanomas (approximately 70%) are thin ≤ 1.0mm (Howlander et al., 2012), with a low metastatic propensity (Mays et al., 2010) yet 4 to 7% of these

patients die from metastatic disease (Bartlett et al., 2014; Gimotty et al., 2004; McKinnon et al., 2003). Given the high incidence of melanoma, this is a significant number of deaths.

Jones et al. (2013) followed 515 AJCC stage I-II melanoma patients in the United States of America (USA) for a median of 61 months to analyse the predictors and patterns of recurrence of melanoma in patients with early stage disease. Despite a negative SLNB, 83 from 515 patients (16%) experienced a recurrence. A deeper primary lesion (mean thickness, 2.7 vs. 1.8 mm, $p<0.01$) with ulceration (32.5% vs. 13.5%; $p<0.001$) was significantly associated with recurrence. Moreover, a recurrence was more likely to occur in patients with a primary lesion located in the head and neck region compared with all other locations combined (31.8% vs. 11.7%; $p<0.001$). They also showed that an older age at diagnosis (mean 57 years vs. 49 years) is significantly associated with a recurrence and that males are more prone to recurrence than females (Males 21% vs. Females 9%; $p<0.001$).

Whilst local and regional recurrences are associated with a higher five-year survival rate (by approximately 20%) than systemic recurrences (Reintgen et al., 1992), a local recurrence is correlated with systemic spread and may still harbour a poor prognosis (Meier et al., 2002). Meier (2002) and colleagues followed a cohort of 3001 patients in Germany diagnosed with a primary melanoma (stage I or II), for a median time of 10 years. Within this time frame, 15.5% of patients developed a recurrence. Follow-up was carried out at regular intervals; every three months for the first five years and thereafter at six monthly intervals. Recurrences were classified as satellite or in-transit (21.7%), regional lymph (50.2%) and distant metastasis (28.1%). Of those patients who were first diagnosed with a satellite or in-transit recurrence, 4.5% went on to develop regional lymph node metastases and 11% distant metastases. Of those patients who were first diagnosed with regional lymph metastases, 58.9% further progressed to distant metastases.

Later disease stages have a higher risk of recurrence and lower survival rates (Leiter et al., 2012; Romano et al., 2010). From follow-up data of 33,384 patients in Germany, Leiter at al. (2012) recorded 4,999 cases of recurrence (14.9%) after complete resection of a primary melanoma or a loco-regional metastasis. Stage III patients had a higher recurrence rate (51%) than stage II (39.5%) and stage I (7.1%). The follow-up period for this study was variable with the minimum surveillance period recorded as three months and the maximum as 10 years, with the median follow-up time to first recurrence being 44 months. The probability of recurrence free survival at one year was 98.4%, 86.2% and 68.3% and at three years was 95.3%, 71.8% and 52% for AJCC stages I, II and III, respectively. Within TNM stage III patients alone, later sub-stages have a higher risk of recurrence (48%, 71% and 85% for stages IIIA, IIIB and IIIC, respectively) and lower survival rates (20% for each stage IIIA and IIIB and 11% for stage IIIC) (Romano et al., 2010). Similarly, the maximum micro-metastasis size is associated with progression free survival (PFS) as shown by Baehner et al., (2011) who reported five-year PFS rates of 86.7% and 26.7% for patients with a maximum metastasis size of <0.6 mm and >5.5 mm, respectively.

In stage IV melanoma, Sosman et al. (2011) reported a 90% recurrence rate in a prospective multicentre study in the USA. They followed 64 completely resected stage IV melanoma patients for a median of five years. The median relapse-free survival was reported as five months despite treatment (local and/or systemic) and the median overall survival was reported as 21 months. Overall, survival at three years was 36% and at four years was 31%.

The prognosis for patients experiencing a recurrence is also dependant on the time of recurrence. Median survival rates are significantly lower for those with a recurrence in the first five years after primary tumour excision (59.8%), compared to those without a recurrence within five years (92.5%). Similarly, the survival rates are significantly lower for those with a recurrence in 10 years (38.9%) compared to those without a recurrence in the same time frame (84.3%) (Salama et al.,

2013). Moreover, the prognosis is dependent on the type of recurrence; five-year survival rates decline from 55% to 51% and 20% for local, regional node and systemic recurrence, respectively (Balch et al., 2010; Reintgen et al., 1992).

The majority of patients who develop a recurrence will do so within the first two years of initial diagnosis (Dong et al., 2000; Hohnheiser et al., 2011; Romano et al., 2010), however recurrences have been reported after 10 years (Dong et al., 2000; Hohnheiser et al., 2011). In a retrospective analysis of 9,223 stage I or II melanoma patients in the USA, Dong (2000) and colleagues showed 7% of patients developed a local recurrence. Within those that developed a local recurrence, more than half were evident within two years, 80% within five years, 5% after 10 years and 2% after 15 years. Similarly, Hohnheiser et al. (2011) showed that from a cohort of 2487 AJCC stage I, II and III melanoma patients, a recurrence was observed in 523 patients (21%), the majority of which occurred within two years, 81.6% within five years and 6.5% after 10 years.

In summary, approximately 5% to 30% of TNM early stage melanoma patients (stages I and II) experience disease recurrence within two to three years following excision of their tumour, half of which are reported as a regional recurrence where the disease presents in the lymph nodes after the patient has been disease free for a period of time. A quarter of recurrences are local, appearing within 2.5cm of the resected primary melanoma and another quarter are distant recurrences, where the disease presents in distant organs after the patient has been disease free for a period of time. The majority of recurrences are evident within 24 months after the initial diagnosis. In TNM stage III patients, 50% of patients will experience a recurrence, with the majority being evident within 12 months. In TNM stage IV patients, 90% will recur, with the median time to recurrence being five months. The majority of recurrences in TNM stage III and IV patients are systemic.

## 1.6 Systemic Therapies for Melanoma

Since the introduction of chemotherapeutic agents such as dacarbazine in 1975 and immunological therapies such as interferon-alpha in 1995 and interleukin-2 (IL-2) in 1998, the treatment of melanoma has changed dramatically (Luke et al., 2013). Dacarbazine has low response rates of only 5-15% and a median durability of six to 12 months (Chapman et al., 1999) and given its highly toxic nature, IL-2 is only suitable for selected fit patients (Atkins et al., 1999). Since the introduction in 2011 of small molecule inhibitors of BRAF or MEK and immunotherapy agents which target cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell-death protein (PD-1), the treatment landscape for melanoma has improved dramatically (Luke et al., 2017).

### 1.6.1 Targeted Therapies

Highly prevalent hot-spot mutations in V600 codon of *BRAF* have directed the development of BRAF inhibitors such as vemurafenib and dabrafenib. In a randomized phase III trial comparing vemurafenib to dacarbazine (Chapman et al., 2011), the objective response rate (ORR) was 48% for vemurafenib compared to 5% for dacarbazine and PFS and median overall survival (OS) reported in an extended follow-up study were 5.3 months versus 1.6 months and 13.3 months versus 10.0 months, respectively (McArthur et al., 2014). Whilst initial tumour regression is dramatic, long-term response is hindered by acquired resistance in many patients, with a number of mechanisms having been identified (Emery et al., 2009; Jiang et al., 2011; Wagle et al., 2011). For example, resistance to vemurafenib is mediated through the reactivation of the MAPK pathway as well as the activation of the AKT signalling pathway. An acquired mutation MEK1-C121S (downstream of BRAF) suppresses the MAPK pathway inhibitory activity of vemurafenib (Wagle et al., 2011). Additionally, mutually exclusive PDGFRβ upregulation or NRAS mutations are associated with acquired resistance (Nazarian et al., 2010).

Another BRAF inhibitor, dabrafenib was developed soon after vemurafenib and showed similar ORRs of 50% versus 6% and PFS of 5.1 months versus 2.7 months for dabrafenib when compared to dacarbazine (Hauschild et al., 2012). Whilst the PFS was longer for patients on dacarbazine in the dabrafenib/dacarbazine study compared to the vemurafenib/dacarbazine study, the dabrafenib/dacarbazine study was smaller with only 63 dacarbazine patients compared to 338 dacarbazine patients in the vemurafenib/dacarbazine study. Furthermore, with the primary endpoint of the dabrafenib/dacarbazine trial being PFS, dacarbazine patients were allowed to cross over to dabrafenib at time of progression. At data cut off (12 months from the start of the trial), only 14 patients remained in the dacarbazine group. Whilst this data suggests that dabrafenib treatment for *BRAF* V600 mutated melanoma provides a benefit in PFS, the small cohort raises concerns relating to reliability.

With the understanding of the downstream phosphorylation cascade causing a stepwise activation of MEK 1/2, a MEK inhibitor, trametinib was developed. In the phase III METRIC trial (Flaherty et al., 2012b), the median PFS was reported as 4.8 months and ORR of 22% for trametinib alone. ORR was then further improved when BRAF and MEK inhibitors were administered in combination. For the phase I/II study of dabrafenib in combination with trametinib an improved ORR of 76% was reported. Furthermore, an increased median PFS of 9.4 months (Flaherty et al., 2012a) and a median OS of 27.4 months were demonstrated (Flaherty et al., 2014). In phase III clinical trials, (Long et al., 2015; Robert et al., 2015) combined dabrafenib and trametinib showed similar ORR, PFS and OS to those observed in the phase I/II study. Similar results were also observed with the combination of vemurafenib and cobimetinib (a MEK1/2 inhibitor) (Ascierto et al., 2016; Larkin et al., 2014; McArthur et al., 2015; Ribas et al., 2014). Consequently, a combination of BRAF and MEK inhibitor therapy has become the standard of care for patients with *BRAF*-mutant melanoma (Luke et al., 2017).

Given that *RAS* mutations alone do not initiate oncogenic transformation and appear to require other co-operating genetic events (Fedorenko et al., 2013), the development of *NRAS* targeted therapy has remained elusive. The clinical benefit of MEK inhibitors for patients with *NRAS* Q61-mutant melanoma, although reserved (Ascierto et al., 2013b; Dummer et al., 2017), have resulted in regulatory approval of this drug for *NRAS* mutant patients (Luke et al., 2017). Furthermore, current evidence (Atefi et al., 2011; Ji et al., 2012; Kwong et al., 2012) suggests that MEK-inhibitor-based combination therapies are warranted (Luke et al., 2017). Additionally, with new insights into the high prevalence of *TERT* promoter mutations in melanoma and the identification and understanding that mutant *TERT* promoters cause reactivation of *TERT*, it is likely that development of *TERT* targeted therapy is imminent (Akıncılar et al., 2016).

### 1.6.2 Immunological Therapies

Immunological therapies such as interferon-alpha and IL-2 cytokines have historically been used to treat non-*BRAF* mutant metastatic melanoma patients, however they have shown modest response rates of 10-20% with substantial toxicity (Atkins et al., 1999; Coit et al., 2012; Kirkwood et al., 1996). The modern immunotherapy agents such as ipilimumab, pembrolizumab and nivolumab have undergone rapid clinical development and trials (Figure 5) (Luke et al., 2017). Ipilimumab is a human IgG1 monoclonal antibody that blocks the interaction of CTLA-4 with its ligands augmenting T-cell activation and proliferation (Yervoy ™, 2011). Pembrolizumab and nivolumab target PD-1 antibodies inhibiting T cell proliferation and cytokine production (Keytruda™, 2017; Opdivo™, 2017).

Since 2011, several clinical trials and research studies have included the use of monotherapy agents pembrolizumab, nivolumab, or ipilimumab and a combination of ipilimumab and nivolumab. In a large phase I study of 655 enrolled patients, single agent pembrolizumab was associated with an overall response rate (ORR) of 19% (Robert et al., 2016), whilst a phase I trial of concurrent

ipilimumab plus nivolumab was associated with an ORR of 40% (Wolchok et al., 2013). A phase III trial which studied pembrolizumab versus ipilimumab with the primary end points being PFS and OS, reported PFS rates of 47.3% and 46.4% for patients receiving different dosing regimens of pembrolizumab. PFS was 26.5% for patients in the ipilimumab group. After a minimum follow-up of 12 months for all patients, the OS rates were 74.1% and 68.4% for the different dosing regimes in the pembrolizumab group and 58.2% in the ipilimumab group. Furthermore, the anti-PD-1 antibody was associated with less high-grade toxicities than the CTLA-4 checkpoint inhibitor. From a multicentre trial whereby 945 patients underwent equal randomisation, combined nivolumab and ipilimumab was shown to exhibit the best OS at three years of 58%. This is relative to the OS when administered individually of 52% for nivolumab and 34% for ipilimumab. The progression free survival over this period was 39% in the combination therapy group, 32% in the nivolumab group and 10% in the ipilimumab group (Wolchok et al., 2017).

In summary, systemic treatments have dramatically improved patient outcome with the development of targeted and immunotherapy agents. The introduction of immunotherapy agents has provided a therapeutic option for patients who are not candidates for targeted therapy and provide an alternative option as second line therapy for *BRAF* positive patients.

### 1.7 Current Clinical Methods of Diagnosing Recurrence/Metastasis

Monitoring guidelines for melanoma are not uniform and differ from centre to centre as well as country to country (Leiter et al., 2012; Trotter et al., 2013). In Australia and New Zealand, monitoring guidelines are stage-specific (Australian Cancer Network Melanoma Guidelines Revision Working Party, 2008). Stage I melanoma patients should be monitored every six months for five years and thereafter annually, with a physical examination, including a full skin and lymph node examination. It is recommended that examinations of the skin and lymph nodes are conducted by a healthcare professional. Self-examinations are also recommended, and patients should be

properly educated on how to perform them. Stage II and III melanoma patients should be monitored every three to four months by physical examination (as for stage I patients) for the first five years and thereafter annually. Again, self-examinations are recommended. Radiological investigations are indicated for stages IIb, IIc to detect metastatic disease, using whole body PET or CT scans of the chest, abdomen and pelvis. There are however no guidelines to suggest how frequently these scans should be performed. Due to disseminated metastasis in stage III and IV patients, it is recommended that these patients be monitored on a case by case basis, although generally they are monitored by FDG-PET/CT three- monthly (Australian Cancer Network Melanoma Guidelines Revision Working Party, 2008).

#### 1.7.1 Sentinel Lymph Node Biopsy

In patients without palpable nodal involvement but with lesions >0.76mm thick and with a mitotic rate of >C1/mm$^2$, a SLNB may be performed to determine metastatic spread (Bartlett et al., 2014). SLNB provides important prognostic information and can identify patients with nodal metastases. Moreover, routine SLNB to monitor recurrence in patients with >1.0 mm thick melanomas offers a survival advantage (Faries et al., 2017; Morton et al., 2014), although the use of SLNB in all patients would be prohibitive financially (Agnese et al., 2003). Morton et al., (2014) evaluated the outcomes of 2001 TNM stage I or II melanoma patients and reported no significant treatment-related difference in the 10-year melanoma-specific survival rates between biopsy and observational groups: the mean (±standard error (SE)) survival rate for those with 1.2 to 3.5mm melanomas who underwent a SLNB was 81.4±1.5% and those who underwent nodal observation alone was 78.3±2.0% (p=0.18). The mean (±SE) survival rate in the biopsy group with >3.5mm melanomas was 64.4±4.6% compared to the observational group with a mean (±SE) survival rate of 58.9±4.1% (p=0.56). They did however report a significantly higher 10-year disease-free survival rate in the biopsy group compared to the observational group. The mean 10-year disease-free survival in the observation group with intermediate-thickness (1.2 to 3.5mm) melanomas was significantly lower

than in the group that underwent a SLNB (p=0.01) as was that for patients with thick melanomas (>3.5mm) (p=0.03). Similarly, Faries et al., (2017) have shown no benefit in melanoma-specific survival when comparing 1934 patients who underwent completion lymph-node dissection compared to 1755 patients who received nodal observation with ultra-sonography.

Dalal et al., (2007) analysed the patterns of recurrence and post-recurrence survival from a cohort of 1046 stage I and II patients with ≥ 1mm Breslow thickness who underwent a SLNB. Following a median follow-up of 36 months, 14.3% of SLN-negative patients experienced their first recurrence after a median time of 24 months, whereas 47% of SLN-positive patients experienced their first recurrence after a median time of 13 months. Postoperative follow-up was comprehensive with physical examinations being conducted between three to four months in the first 12 months, between three to six months for the following year and between six to 12 months thereafter. A complete blood count and serum LDH level were completed annually for the first two to three years of follow-up. Where clinically indicated, PET and CT scans were conducted. The pattern of recurrence was similar for both groups with approximately half of the first recurrence being systemic and one third as in-transit or local disease. Morton et al., (2014) also showed the pattern of first recurrence to be similar in both groups, although SLN negative patients experienced recurrent disease less frequently and far later than SLN positive patients. Whilst recurrences were more evident in the cohort with thicker melanomas (P<0.001), the pattern of recurrence was similar for both thin and thick melanomas which is consistent with other reports (Balch et al., 2000; Clary et al., 2001). In considering post-recurrence survival, the only independent indicator was the site of first recurrence, with systemic recurrence being associated with a shorter post-recurrence survival.

Bartlett et al., (2014), examined the role of SLNB in patients with thin melanomas (≤ 1.0mm). Within this cohort (n=781), they found SLN positivity to be low (3.7%) which is less than the complication rate associated with the procedure (Karakousis et al., 2007; McKinnon et al., 2003;

Murali et al., 2012; Wright et al., 2008). Whilst the presence of mitoses and Clark level was associated with SLN positivity in a multivariate analysis, the SLN positivity however was merely 0.7% in the absence of mitoses.

Only specific points in the node are tested in SLNB and since melanoma malignancy has a tendency to metastasise in small groups of cells or as single cells (Cook et al., 2008), it is likely that sites containing tumour cells may go undetected. By testing a set of three sections (each stained with S-100 protein, haematoxylin and eosin (H&E) and HMB-45) obtained from each of three specific points within the biopsy at 250μm intervals, the detection rate of SLN positivity was 71% in stage III melanoma patients (Spanknebel et al., 2005). Although the procedure is considered to be minimally invasive (Morton et al., 2014), it is prohibitively expensive for routine monitoring (Agnese et al., 2003; Lens et al., 2002), time consuming (Sabel et al., 2000) and is unlikely to be repeated often. Thus, SLNB is ineffective at diagnosing recurrence or disease progression in patients with thin melanomas, until such time that the disease has progressed, and the patient is categorised into a stage with a poorer prognosis (i.e. stage III). Despite a significantly shorter PFS and OS being evident with increasing microscopic tumour burden in LNs (Baehner et al., 2011), the impact on patient outcome of microscopic tumour burden in SLN cannot be ignored, therefore underscoring the need for better methods to detect recurrences from early stage disease.

#### 1.7.2 Positron Emission Tomography / Computed Tomography Scans

FDG-PET/CT provides valuable information on the location and metabolic activity of suspicious cancerous lesions through real-time whole-body imaging (Gellén et al., 2015). CT alone provides information on the locality of any lesions within internal organs, whilst PET alone provides detail on normal and abnormal tissue metabolism. Being a radio-labelled glucose analogue, $^{18}$F-FDG accumulates in tissues with high glucose utilisation, thereby revealing the metabolic activity of tumour cells. The principal advantage of radionuclide imaging is the high tumour to non-tumour

contrast providing functional information about tumours (Bai et al., 2013). A combination of PET and CT technologies in oncology helps to identify and localise functional abnormalities (Blodgett et al., 2007) and is commonly used to diagnose systemic metastasis, being highly effective (up to 90%) in detecting and differentiating distant metastases in melanoma (Friedman et al., 2004; Larson et al., 2006; Rodriguez Rivera et al., 2014). FDG-PET/CT is also used routinely to assess therapy response in melanoma and other cancers (Juweid et al., 2006; Ott et al., 2006; Schwarz et al., 2005; Strobel et al., 2008).

The diagnostic performance of FDG-PET/CT to monitor patients was assessed in a retrospective study of 250 stage II and III melanoma patients. Reinhardt et al. (2006) used FDG-PET/CT, PET alone and CT alone to assess metastatic disease, recurrence and treatment evaluation. With regards to nodal staging of melanoma, the differences between efficacy of FDG-PET/CT, PET alone and CT alone, were marginal. They found however, that there were significant differences between the technologies when restaging to diagnose metastatic disease and for treatment evaluation. FDG-PET/CT was superior at 97.2%, PET alone at 92.8% and CT alone showing accuracy of 78.8% for assessment of nodal and metastatic disease, which is considerably superior to detection rates using SLNB.

Such radiologic assessments in stage I and II melanoma have not been well regarded as they fail to provide clinical evidence of disease recurrence or prognostic information (Belhocine et al., 2002; Hindié et al., 2011; Krug et al., 2008). In a prospective study that compared SNB to PET imaging in stages I, II and III cutaneous melanoma patients, Wagner et al., (1999) reported sensitivity and specificity of 94.4% and 100%, respectively for detecting occult lymph node metastases by SNB compared to 16.7% sensitivity and 95.8% specificity using FDG-PET/CT. Conversely, Danielsen et al., (2016) assessed the utility of FDG-PET/CT in detecting melanoma metastasis of newly-diagnosed high-risk primary melanoma patients. As part of their initial staging, 32 of 167 patients

had a positive scan, with FDG-PET/CT detecting regional metastatic disease in 18 clinically node-negative patients. Whilst the yield of positive PET/CT scans was relatively high, it is important to note that the patient cohort was selected for their high-risk of metastatic disease. Additionally, there has been much disagreement regarding the cost-effectiveness and utility of FDG-PET/CT in the initial staging of early stage melanoma patients (Bastiaannet et al., 2012; Bastiaannet et al., 2009; Haddad et al., 2013; Wagner et al., 1999; Wagner et al., 2005; Wagner et al., 2011). In summary, because these imaging techniques are unable to detect micro-metastases (Meyers et al., 2009; Pfannenberg et al., 2015), are associated with high costs (Gellén et al., 2015) and additional radiation exposure (Rueth et al., 2015), they are used less commonly for routine monitoring of patients with early stage melanoma.

#### 1.7.2.1 Quantitative Analysis of FDG-PET/CT Scans

Whilst qualitative visual interpretation of FDG-PET/CT scans is the most commonly used assessment and is highly effective (Juweid et al., 2007; Wahl et al., 2009), quantitative assessment of FDG-PET/CT images is the most accurate measure of disease burden (Bai et al., 2013). The fundamental basis of PET scanning uses the positron decay of a variety of isotopes to provide a positive image relative to a background rate of $^{18}$F-FDG uptake in normal tissue. A radioactive nucleus produces a positron which travels a short distance until it reaches an electron. The resultant mass is then converted into two 511 keV photons which travel in opposite directions. Small scintillation crystals (detectors) record the decay. Reconstruction algorithms are then used to compute the tracer distribution image (Bai et al., 2013). The first quantitative measurement values were calculated by the administered radiotracer dose per gram of tissue however the results were dependant on the size of the patient (MTV) (Woodard et al., 1975). More recently, the standardised uptake value (SUV) is used which takes into account the size of the patient (in terms of body weight, surface area or lean body mass) with a calculation based on the decay-corrected tumour activity concentration and the amount of radiotracer administered (Strauss et al., 1991). Whole-

body MTB assessed by SUV from FDG-PET/CT scans will predict a more accurate patient outcome than tumour uptake alone (MTV) (Bai et al., 2013). Consequently volume based PET parameters which measure MTV, calculated semi-automatically by the software, have now been introduced (Winther-Larsen et al., 2017). MTV multiplied by the mean SUV of each delineated lesion provides the TLG, which when added together for all evaluable lesions, provides the overall MTB at any one time within a patient (Larson et al., 1999). Furthermore, such quantitative analysis has been shown to be more effective than qualitative analysis in distinguishing between ineffective and effective treatment in the early stages of systemic therapy (Lin et al., 2007; Wahl et al., 2009). In a study assessing 92 patients with newly diagnosed diffuse large B-cell lymphoma, Lin et al., (2007) showed that 15% of patients who were considered positive for ongoing tumour presence, on visual analysis, were in fact good responders and thus had reduced tumour size, which was evident when quantitatively analysed for metabolic rate. Similarly, quantitative assessment of tumour uptake and changes in tumour uptake, have been shown to predict survival in oesophageal and non-small-cell lung cancer NSCLC (Sasaki et al., 2005; Yanagawa et al., 2012). Whilst quantitative measurements of FDG-PET/CT have recently been described (Bai et al., 2013; Lin et al., 2007; Sasaki et al., 2005; Winther-Larsen et al., 2017; Yanagawa et al., 2012), they have not yet been reported in melanoma.

### 1.8 Alternative Methods of Detecting Residual Disease and Disease Progression in Melanoma

Measuring cellular components, shed from tumour cells into the blood stream, such as microRNAs (miRNA), circulating tumour cells (CTCs) or ctDNA, commonly referred to as a "liquid biopsy", have been used more recently to provide some insight into the level of disease burden, therefore providing some understanding of an individual's prognosis (Gray et al., 2015; Khoja et al., 2014; Klinac et al., 2014; Kopreski et al., 1999; Stark et al., 2015). Table 3 shows a comparison of these three "liquid biopsy" methods.

**Table 3: Comparison of "liquid biopsy methods"**

| Method | Source | Strength | Limitation |
|---|---|---|---|
| miRNA | Plasma, urine, saliva | Non-invasive<br>Highly sensitive | Low specificity<br>Not tumour specific |
| CTCs | Peripheral blood | Non-invasive<br>Approved by FDA in clinical practice (CELLSEARCH) | Very rare events<br>Difficult to detect<br>Low sensitivity<br>Low specificity<br>Requires enrichment step (size or immunomagnetic) |
| ctDNA | Serum or plasma | Non-invasive<br>Heightened abundance<br>Highly sensitive<br>Highly specific | Cancer-specific aberrations must first be determined for PCR based assays.<br>Large background of wild-type DNA |

#### 1.8.1 Circulating microRNAs (miRNAs)

miRNAs are non-coding, small (approximately 22 nucleotides) RNAs which regulate gene expression. Primarily contained in micro-vesicles or exosomes, or bound to the miRNA-mediated silencing complex (AG02) (Allegra et al., 2012; De Guire et al., 2013), they have been shown to be released by tumour cells into the circulation (Mitchell et al., 2008) and as such are considered potentially valuable as a prognostic biomarker for melanoma recurrence (Fleming et al., 2015; Stark et al., 2015). In a retrospective analysis, Fleming et al., (2015) reported that a 4-miRNA signature panel could distinguish between recurrent and non-recurrent melanoma cases. Having classified patients into high and low risk recurring groups, they observed sensitivities of 80.9% and 84.6% and the specificity was 60.1% and 66.1%, respectively. The negative predictive value for the two cohorts was 87.4% and 90.2%, respectively. Recently, Stark et al., (2015) detected the presence of melanoma (relative to controls) using a 7-miRNA panel (MELmiR-7) with 93% sensitivity and 82% specificity when at least 4 miRNAs were expressed. Additionally, they were able to better

characterise the OS of melanoma patients using this melanoma specific panel, compared to serological markers such LDH and S100B. Due to the limited availability of serially collected samples to detect recurrence however, there were no markers associated with time to recurrence and as such further studies will be required to strengthen this data.

Contradictory to the abovementioned studies, it has been shown that miRNAs may not always be shed directly from the tumour, with more recent studies showing a discrepancy between circulating miRNA levels in serum and miRNA levels in tumour tissue (Selth et al., 2013; Wulfken et al., 2011).

### 1.8.2 Circulating Tumour Cells (CTCs)

A cancerous tumour is the result of an over proliferation of cells during which time some cells may separate from the tumour and are consequently transported through the lymphatic system or bloodstream. These intact cells that are shed from a primary tumour or its metastases found circulating in the peripheral blood of patients, are referred to as circulating tumour cells (CTCs) (Fernandez et al., 2014). These appear to persist for a short period of time in the circulation as evidenced by analysis of prostate cancer patients who had detectable CTCs prior to surgery, but 24 hours after surgical resection had no evidence of CTCs (Stott et al., 2010). Although CTC quantification has been correlated with overall survival in metastatic melanoma patients (Khoja et al., 2014; Khoja et al., 2013) and are a valuable monitoring tool to evaluate treatment efficacy in melanoma patients (Gray et al., 2015; Klinac et al., 2014), they commonly occur at very low concentrations such that one tumour cell is found in a background of $1x10^6$ blood cells. In metastatic cancer patients, there are generally fewer than 10 CTCs per 1mL of blood (which normally contains $1x10^9$ red blood cells and $1x10^6$ white blood cells) (Haber et al., 2014). Consequently, the detection of CTCs and their characterisation necessitate exceptionally sensitive

and specific analytical methods (Pantel et al., 2013) that can sift through substantial numbers of blood cells without damaging or loosing CTCs and then identify the CTCs using immunophenotyping, cytopathology or molecular genetics (Haber et al., 2014).

Regardless, CTCs have been shown to be a key indicator of metastatic disease, disease recurrence, overall survival and treatment response in a number of different metastatic cancers (Cristofanilli et al., 2004; Cristofanilli et al., 2005; Danila et al., 2007; de Bono et al., 2008; Giuliano et al., 2011; Hayes et al., 2006; Hou et al., 2012; Krebs et al., 2011; Liu et al., 2009; Miller et al., 2010).

Few studies have focused on CTCs in patients with non-metastatic or early stage cancer (Lucci et al., 2012; Uen et al., 2008), however few have been in the melanoma setting (Freeman et al., 2012; Gray et al., 2015). Furthermore, none have monitored CTC levels over time in an attempt to diagnose disease recurrence. In breast cancer, Lucci (2012) and colleagues established a significant correlation between the number of CTCs and disease stage using the CellSearch® platform. Of the 302 patients, 89% were staged as TNM stage I or II. Whilst ≥2 CTCs predicted a worse overall survival, they also showed that ≥1 CTC per 7.5ml of blood is an independent predictor of relapse or death in chemotherapy naive patients with non-metastatic breast cancer and the hazard ratio for disease progression increased with the increasing number of CTCs in 7.5ml of blood.

Similarly, during a follow-up period of 44 months, Uen (2008) and colleagues identified postoperative relapse in 29% of their colorectal cancer patients and 28% of their colon cancer patients using CTCs. They used a membrane array method to detect CTCs and found that the persistent presence of CTCs (1 day prior to surgery and 1 week post-surgery) in TNM stage I to III colorectal (n=438) and colon (n=282) cancer patients was significantly correlated with a shorter relapse-free survival period (Uen et al., 2008). Additionally, patients with detectable levels of CTCs prior to surgery and undetectable levels of CTCs three months post-surgery were found to

have a longer relapse free survival period. The sensitivity level for this detection method was set at 5 CTCs per 1ml of blood.

By contrast, Thalgott and colleagues (2013) reported that there was no significant difference in the number of CTCs present in healthy controls and prostate cancer patients. Amongst the prostate cancer patients, they demonstrated that more than or equal to three CTCs was associated with a shorter overall survival rate than more than three CTCs isolated using the CellSearch® system. The low detection rate of CTCs (5%) may however be due to the selective targeting of only 1 marker (EpCAM) which is inherent to the CellSearch® System. Additionally, the length of time between blood draw and processing (96 hours) may have an effect on the CTC counts; CTC counts have only been shown to be stable for up to 72 hours with the CellSearch® platform (Riethdorf et al., 2007).

#### 1.8.3 Circulating Tumour DNA (ctDNA)

It is well established that during cellular turnover, or other forms of cell death, fragments of DNA, cfDNA, are shed into the bloodstream (Stroun et al., 2001). Under normal circumstances, necrotic or apoptotic cells are cleared by infiltrating phagocytes, resulting in relatively low levels of cfDNA in the blood stream of healthy individuals (Crowley et al., 2013). However, in certain conditions, such as following exhaustive exercise, myocardial infarction or surgery where inflammation or tissue injury result, cfDNA levels can be considerably increased (Antonatos et al., 2006; Beiter et al., 2011; Chang et al., 2003; Haber et al., 2014). In the case of myocardial infarctions, tissue injury as a result of prolonged ischemia ultimately leads to necrosis and thus the potential for cfDNA to be released into the circulation (Chang et al., 2003). Intense exercise has been shown to be associated with an inflammatory response (Fehrenbach et al., 2000) and metabolic muscular damage (Brancaccio et al., 2010), which may induce the release of intracellular contents such as cfDNA after the cellular membrane has been damaged as a result of necrosis.

Due to increased cell death and necrosis in cancer, increased levels of cfDNA are present in the blood of cancer patients (Delgado et al., 2013; Diaz et al., 2014; Hashad et al., 2012; No et al., 2012; Park et al., 2012) but these levels can vary widely, depending on the stage of disease (Perkins et al., 2012). In cancer patients, a small fraction (between <0.1% to 10%) of total cfDNA is purely tumour derived (Diehl et al., 2008; Haber et al., 2014). This is referred to as ctDNA (Jen et al., 2000) and emanates from either a primary tumour, metastasis or CTCs (Haber et al., 2014) (Figure 4) and can be identified through the detection of cancer specific mutations.

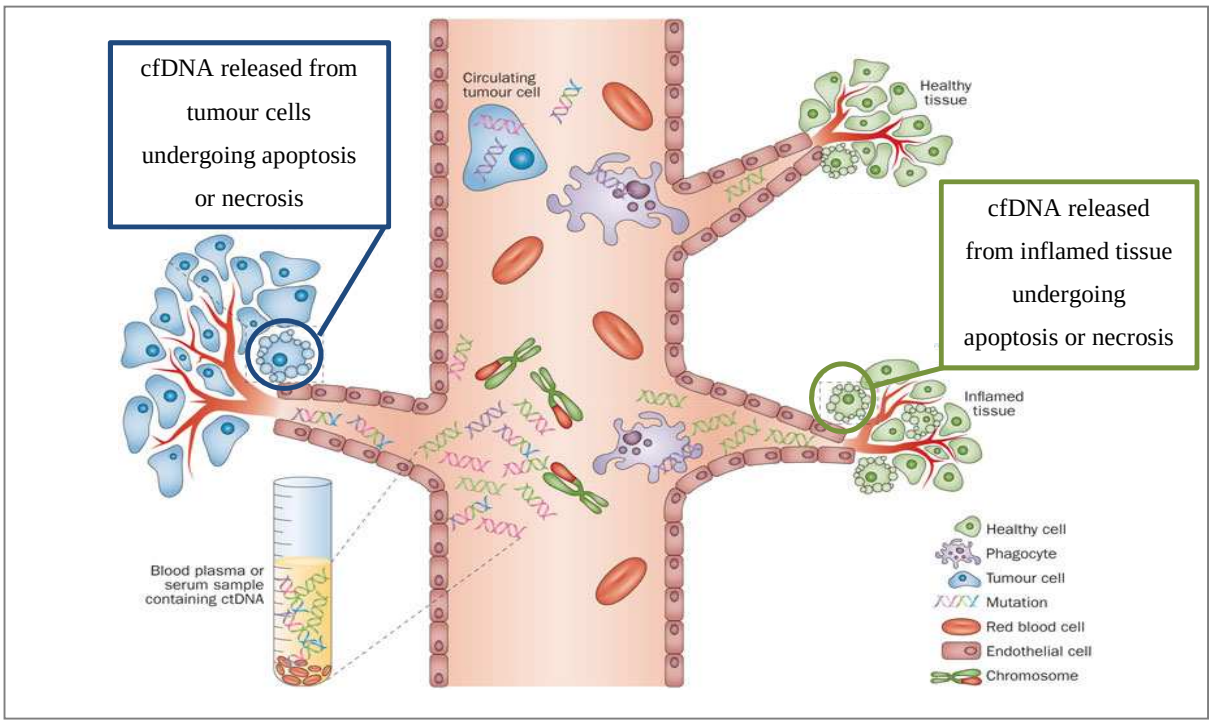


**Figure 4: Release and collection of circulating tumour DNA from blood**

CfDNA is released from cells undergoing apoptosis or necrosis in diseased, inflamed or healthy tissue and can be extracted from plasma. Tumour specific genetic aberrations such as point mutations (DNA strands represented by consecutive purple, red, green and blue), copy number variations (red segment of chromosomes) and structural reorganisation (green and red DNA strands can then be quantified). Abbreviations: cfDNA, cell free DNA; ctDNA, circulating tumour DNA. Adapted from Crowley et al., 2013.

CtDNA has a relatively short half-life (ranging from 16 minutes (Lo et al., 1999) to approximately two hours (Diaz et al., 2014{Diehl, 2008 #2127)) which allows for real-time monitoring of tumour

changes. The detection and quantification of tumour-specific genetic aberrations in ctDNA therefore have many potential clinical applications which have been demonstrated at various stages of lung, breast and colorectal cancers from early diagnosis (Bettegowda et al., 2014; Newman et al., 2014) to detection of residual disease (Garcia-Murillas et al., 2015; Tie et al., 2016), and can be utilised for prognosis determination (Bettegowda et al., 2014; Dawson et al., 2013) and monitoring response to therapy (Dawson et al., 2013; Sundaresan et al., 2016; Wang et al., 2017; Zheng et al., 2016). Studies have shown that ctDNA can serve as a surrogate marker for tumour burden, with ctDNA levels corresponding with clinical course in several cancers, including melanoma, breast, lung and colorectal (Abbosh et al., 2017; Dawson et al., 2013; Diehl et al., 2008; Garcia-Murillas et al., 2015; Gray et al., 2015; Lipson et al., 2014; Murtaza et al., 2013; Sanmamed et al., 2015). Levels of ctDNA decline after successful pharmacological therapy and rapidly increase with disease progression (Dawson et al., 2013; Diehl et al., 2008; Forshew et al., 2012; Girotti et al., 2015; Gray et al., 2015). The presence of mutant-specific ctDNA strongly correlates with overall survival prior to treatment in stage IV melanoma patients (Gonzalez-Cao et al., 2015; Gray et al., 2015; Knol et al., 2016; Lee et al., 2017a; Sanmamed et al., 2015; Santiago-Walker et al., 2015) and may also prove to be a valuable monitoring tool at various disease stages in melanoma. Importantly however, Bettegowda et al. (2014) have shown that the detection of ctDNA may depend on the site of metastases as tumours confined to the central nervous system and those with mucinous features (eg. Brain, bone marrow) often present with undetectable ctDNA. This suggests that the blood-brain barrier and mucin could pose as physical obstacles which prevent ctDNA from entering the circulation (Bettegowda et al., 2014).

In a seminal study of 223 patients with localised colorectal, gastrooesophageal, pancreatic and breast cancer, with no clinical or radiographic evidence of distant metastases, Bettegowda et al. (2014) detected ctDNA in 55% of cases using the BEAMing platform to measure a subset of mutations at known ‘hotspots’ of Kirsten rat sarcoma viral oncogene homolog (*KRAS*), *NRAS*,

Phosphatidylinositol-4, 5-bisphosphate 3-kinase, catalytic subunit alpha (*PIK3CA*), and *BRAF* genes. The number of patients with detectable ctDNA and the ctDNA concentration correlated with TNM stage; ctDNA was detected in 47%, 55% and 69% of patients with any cancer tested at stages I, II and III, respectively.

Research in colorectal patients has shown that following complete resection, ctDNA levels drop sharply, although in many cases they remain detectable (Diehl et al., 2008). In patients with no detectable ctDNA levels post-surgery, patients remained disease free, whilst all but one of the patients with disease recurrence had detectable ctDNA levels. More recently, Tie et al., (2016) demonstrated that the presence of ctDNA after resection of stage II colon cancer, provides direct evidence of residual disease and identifies patients at very high risk of recurrence, who may require chemotherapy to prevent recurrence. Based on these results, a randomized, ctDNA-driven clinical trial in stage II colon cancer (DYNAMIC) has been recently initiated.

Few ctDNA studies have focused on early stage cancer (Beaver et al., 2014; Bettegowda et al., 2014; Oshiro et al., 2015; Phallen et al., 2017; Sozzi et al., 2001). From these studies, only one study has monitored ctDNA levels over time and found that ctDNA quantification and characterisation are suitable for detecting recurrence (Sozzi et al., 2001). CtDNA quantification and analysis in 84 patients with non-small cell lung cancer (stages I - III of which 16.6% were stage Ia, 38% were stage Ib, 17.8% were stage II and 27.3% were stage III), showed that ctDNA was measurable in 96% of patients at baseline. A decline in ctDNA levels was evident in relapse-free individuals when assessed in serial blood samples taken within one to six months post-surgery. Conversely, in 4 patients who experienced metastasis, local recurrence, or a new primary tumour within two years following surgery, there was a two to 20-fold increase in ctDNA in their second or third blood sample taken between seven and 23 months after surgery.

Similarly, Oshiro et al. (2015) showed that ctDNA levels were able to predict breast cancer recurrence more frequently in *PIK3CA* mutant ctDNA positive patients (n=25) than in ctDNA negative patients (n=85). The study used a digital PCR (dPCR) assay, with serum from 110 *PIK3CA* mutant breast cancer patients (TNM stage I to III) collected prior to primary surgery. Recurrence free survival was significantly lower in ctDNA positive patients compared to ctDNA negative patients (P=0.0029). Furthermore, they found that the greater the number of mutant copies (copy number range was 13-2500 copies/ml), the greater the trend towards a recurrence.

The ability to detect ctDNA in early-stage patients was assessed in a prospective study of 29 early-stages (TNM stage I and II) breast cancer patients (Beaver et al., 2014). In this study pre and post-surgery blood samples were tested as opposed to serial monitoring until a recurrence occurred. The authors demonstrated that high fidelity PCR followed by ddPCR enabled ctDNA with *PIK3CA* mutations to be detected in blood samples from 13 patients' pre-surgery, with 93.3% sensitivity and 100% specificity. Post-surgical blood samples were collected between 8 and 72 days after surgery from 10 of these *PIK3CA* positive patients and detectable levels were found in 5 patients despite these patients having no clinical evidence of disease. Of the patients with detectable mutant ctDNA levels post-surgery, 40% (n=2) experienced a recurrence within 36 months.

Using ctDNA at a single post-surgical time-point or with serial follow-up plasma samples, Garcia-Murillas et al., (2015) were able to predict, with high accuracy, metastatic relapse in patients with early stage breast cancer. Moreover, an increased sensitivity for predicting a relapse was evident, with a median lead-time of 7.9 months when mutation tracking in serial samples was used. The study focused on detecting ctDNA in plasma of 55 patients after curative surgical treatment. Notably, the patients in this series had no regular imaging scans during follow-up which the authors suggest would be required for future prospective studies.

Other tumour biomarkers such as miRNA and CTCs have provided limited sensitivity and specificity and as such are unlikely to meet clinical requirements (Yong et al., 2014; Bettegowda et al., 2014). The specificity of miRNAs has been reported as low as 60.1% (Fleming et al., 2015) which is considerably lower than the specificity of ctDNA at 100% (Gray et al., 2015). Similarly, the detection rate of ctDNA in breast cancer patients reached 97%, whereas CTC detection rates were only 78% (Dawson et al., 2013). In a study conducted by Bettegowda and colleagues (2014), 13 of 16 cancer patients were ctDNA positive whilst only 3 of 16 patients were CTC positive. Additionally, in patients that tested positive for both CTCs and ctDNA, the ctDNA value was 50-fold more than the CTC value. Together these studies suggest that ctDNA is more sensitive than miRNA and CTCs.

While several studies have determined ctDNA as a marker of recurrence and it has been suggested as a surrogate marker for tumour burden, the level of sensitivity of ctDNA relative to quantitatively assessed MTB has not been assessed, until recently. Winther-Larsen (2017) and colleagues studied the correlation between ctDNA and MTB in NSCLC. In this study, TLG was calculated for all lesions where the SUV was at least 1.5 times the mean liver SUV. The MTB was calculated as the sum of all TLG which correlated significantly with the frequency abundance of mutated cfDNA determined by next generation sequencing (NGS) (Ion AmpliSeq Colon and Lung cancel panel v2). In a subset of 24 patients, with positive ctDNA, a significant correlation was observed between the allele frequency of ctDNA and MTB (P=0.001). Additionally, a significantly shorter median overall survival was recorded in patients with a positive ctDNA result relative to those with a negative ctDNA result. Whilst ctDNA detection in this cohort was not performed by ddPCR, this study suggests for the first time, that ctDNA could be used as an indirect measure of tumour burden and as a complimentary modality to functional imaging. Understanding the extent to which ctDNA correlates with MTB in melanoma would indicate the level of sensitivity of ctDNA and provide

evidence that a simple blood collection could facilitate more frequent disease assessment and as such should be considered a priority in melanoma research.

#### 1.8.3.1 Methods for Detection of ctDNA

There are a number of different techniques available for detecting low levels of tumour associated genetic aberrations in cfDNA, including next generation sequencing (NGS) (Dawson et al., 2013; Forshew et al., 2012; Winther-Larsen et al., 2017), whole genome sequencing (WGS) including tagged amplicon deep sequencing (Dawson et al., 2013), allele-specific PCR and digital PCR (dPCR), (Diehl et al., 2008; Gray et al., 2015; Sanmamed et al., 2015; Yung et al., 2009). Whilst each has its own advantages and disadvantages, costs, availability of tumour samples, the quality and quantity of available DNA, practicality, sensitivity and specificity are significant factors affecting the relative success of each detection method. The analysis of ctDNA utilises various amplification and sequencing methods which can be separated into two groups based on whether the objective is to examine all genes in an untargeted approach or monitor specific genes or mutations in a targeted approach.

Whole exome sequencing (WES) and WGS are untargeted approaches to measuring ctDNA where a disease specific mutation is not evident. In this case, all genes are interrogated which involves high costs, requires high quality DNA and extensive data analysis which must be performed by a bioinformatician (Chan et al., 2013; Crowley et al., 2013). Where a disease is predominantly characterised by mutational hotspots (as is the case in melanoma), the need for WGS is not always necessary, however such sequencing approaches may be useful in initial mutation discovery for subsequent use with more sensitive target approaches.

Various targeted sequencing methods have been reported at varying levels of sensitivity, such as the Ion AmpliSeq panel (Ion Torrent Technology) at 0.5% (Rothe et al., 2014), tagged amplicon deep

sequencing (Tam-Seq) at 2% (Forshew et al., 2012) and CAPP-Seq, at allele frequencies as low as 0.02% (Newman et al., 2014).

Digital PCR (dPCR) is another targeted approach for measuring ctDNA however it offers a more robust sensitivity (Li et al., 2006). dPCR is based on a concept of limiting dilutions, where the DNA of interest is separated within oil-water droplets and then each droplet is amplified using PCR, followed by quantification using poisson distribution (Sykes et al., 1992). Briefly, PCR uses DNA polymerase to synthesise new strands of target DNA, bounded by primers at the ends of the sequence to be amplified, resulting in amplification of that particular region of DNA into billions of copies (Hue-Roye et al., 2008). Although the sensitivity of dPCR platforms allows for the detection of low frequency mutations which cannot be quantified by quantitative PCR (qPCR), pre-identification of gene targets is necessary. The two most widely used dPCR platforms are digital droplet PCR (ddPCR) and BEAMing (beads, emulsion, amplification, magnetics) PCR. Briefly, the BEAMing platform is composed of 4 components; beading, emulsification, amplification, and magnetics. DNA molecules are bound to magnetic beads coated with primers and the reaction is separated into droplets containing one molecule of DNA and one magnetic bead. Following PCR amplification, the resulting bead-DNA complex is separated using a magnet. The DNA is denatured and allowed to hybridize with fluorescent probes specific to each template and measured using flow cytometry. This method has been shown to detect mutated alleles in a background of wild-type alleles with a sensitivity of 0.01% (Li et al., 2006).

Once a mutation is identified in patient tissue, cfDNA isolated from patient blood can be tested for the relevant mutant-specific ctDNA using the QX200 ddPCR system (Bio-Rad). DdPCR allows detection of a single mutant allele in amongst 10,000 wild type alleles (Crowley et al., 2013; Richardson et al., 2012). Moreover, this technique allows the quantification of normal and mutant

DNA in any given plasma sample (Higgins et al., 2011; Richardson et al., 2012) at sensitivities as low as 0.001% (Hindson et al., 2011; Reid et al., 2015).

DdPCR initially involves the separation of DNA together with a reaction mixture into approximately 20,000 droplets (Figure 5) separated by emulsions of oil, water and stabilizing chemicals. A PCR reaction then takes place in each droplet in separate reaction chambers containing 0-5 molecules of target DNA. Amplified DNA in each droplet is assessed for target DNA (Figure 6). Positive droplets display increased fluorescence over negative droplets. QuantaSoft analysis software (Bio-Rad) measures the number of positive and negative droplets in a sample for each fluorophore. The fraction of positive droplets, fitted to a Poisson distribution, enables the target DNA molecules to be quantified in units of copies/µL (Bio-Rad Laboratories, 2017; Sanmamed et al., 2015). The software allows visualisation of the data in a variety of formats including a 2-D plot of droplet fluorescence (Figure 7).

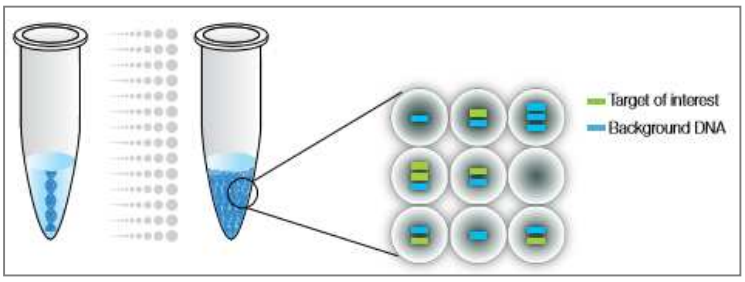


**Figure 5: DdPCR droplet partitioning**

A single PCR sample is partitioned into approximately 20,000 droplets each with 0-5 template copies (Bio-Rad Laboratories, 2017).

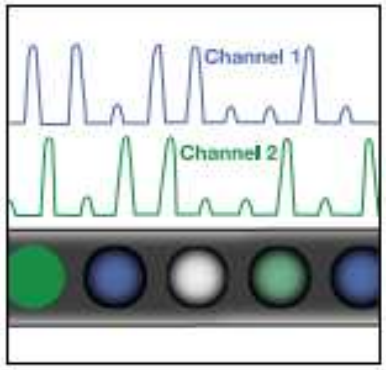


**Figure 6: DdPCR reading using a two-colour fluorescence system**

The fluorescence measurements are used to count the number of positive and negative droplets in each sample (Bio-Rad Laboratories, 2017).

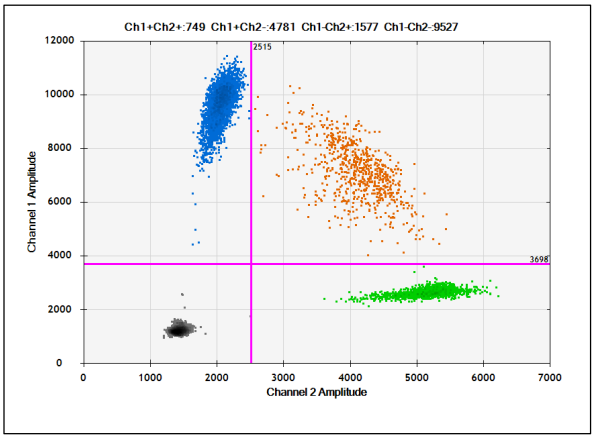


**Figure 7: QuantasSoft 2-D plot of droplet fluorescence**

Channel 1 fluorescence (FAM) is plotted versus Channel 2 fluorescence (VIC or HEX) for each droplet. The droplets are clustered into four colours with each colour representing the positivity of the fluorescence: Blue – positive/positive, orange – positive/negative, green – negative/negative (wild-type), black – empty.

Due to 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 (Olmedillas-López et al., 2017). From studies assessing the utility of ctDNA as a biomarker of disease status in stage IV melanoma, the prognostic sensitivity of ddPCR appears similar to that of the BEAMing method,

with positive identification of ctDNA in late stage melanoma patients ranging from 73% to 89% (Ascierto et al., 2013a; Bettegowda et al., 2014; Chang et al., 2016; Gray et al., 2015; Sanmamed et al., 2015). This is not surprising considering that both platforms amplify DNA templates within water-oil droplets before quantification. Importantly, a 100% agreement in the detection rate of *BRAF* V600 mutations in stage IV melanoma patients has been shown between ddPCR and BEAMing (Janku et al., 2015). Although ddPCR and BEAMing generate comparable results, ddPCR was selected for the purposes of this study given availability of the methodology and instrumentation in our laboratory.

Given the sensitivity of ddPCR to detect mutant-specific ctDNA in metastatic patients has not yet reached 100%, and the lower limit of tumour burden detectable by ddPCR, as well as the recent development of platforms to quantitively calculate MTB from PET/CT scans (as discussed earlier), a correlation between ctDNA levels and MTB would determine the efficacy of ctDNA in measuring disease burden. This will provide us with the lowest limit of disease burden that ctDNA can detect.

#### 1.8.3.2 CtDNA in Melanoma

Few studies have addressed the value of ctDNA quantification in melanoma relative to disease status. Rather the focus of ctDNA in melanoma has resided in the metastatic setting specifically with regards to measurements of treatment response and resistance (Ascierto et al., 2013a; Chang et al., 2016; Girotti et al., 2015; Gray et al., 2015; Lipson et al., 2014; Sanmamed et al., 2015; Santiago-Walker et al., 2015; Tsao et al., 2015; Wong et al., 2017).

Gray et al. (2015) have shown that mutant-specific ctDNA levels can be used to track treatment response. In patients with advanced metastatic melanoma, ctDNA was measured in 48 patients prior to treatment with either targeted therapies or immunotherapies. Tumour-specific ctDNA was detectable by ddPCR in 73% of patients and response to therapy was significantly associated with

lower baseline levels of ctDNA (median 21.2 copies per ml) compared to non-responders with median levels of 225 copies per ml (P=0.048). PFS of longer than six months was also significantly associated with a lower median ctDNA level of 10.5 copies per ml, compared to 152.5 copies per ml for those with PFS of less than six months (P=0.019). Prominently, mutant-specific ctDNA levels dropped according to response to treatment and was detectable prior to or at the time of progressive disease evidenced by radiological imaging. *NRAS* mutations have previously been detected in patients with acquired resistance to *BRAF* inhibitors (Long et al., 2014a; Rizos et al., 2014; Shi et al., 2014). Similarly, circulating *NRAS* mutations were detected in 43% of patients who developed acquired resistance to *BRAF* inhibitors, despite no mutated *NRAS* ctDNA being detectable prior to the commencement of treatment (Gray et al., 2015).

Similarly, Girotti et al., (2015) have shown by serial analysis (using next-generation sequencing) from 101 patients, that mutant-specific ctDNA tracks the genomic evolution of cutaneous, acral, mucosal or uveal melanoma in response to therapy and provides early evidence of acquired drug resistance. Furthermore, ctDNA usually revealed the disease change prior to imaging.

More recently, Wong et al., (2017) showed ctDNA as a complementary modality to radiological imaging to provide real-time monitoring of tumour burden as well as genomic changes throughout treatment in metastatic melanoma patients. The study included serial analysis of FDG-PET/CT scans and ctDNA by next-generation sequencing and dPCR from 52 patients undergoing systemic therapy. Mutant *NRAS, BRAF* and *TERT* ctDNA levels correlated with metabolic disease burden as quantified on FDG-PET/CT scans by adapting the PET Response Criteria in Solid Tumours (PERCIST) recommendations (Wahl et al., 2009). Interestingly, cerebral and subcutaneous disease sites were not depicted well in the plasma (Wong et al., 2017). As with previously mentioned studies by Gray et al. (2015) and Girotti et al. (2015), Wong et al. (2017) showed that early changes in ctDNA were indicators of treatment response and PFS. Moreover, the inclusion of FDG-PET/CT

scans in the Wong et al., study, has shown the extent to which ctDNA reflects changes in metabolic disease burden. Additionally, the authors demonstrated that ctDNA comprehensively captures the genomic heterogeneity across multiple disease sites by comparing multiregional biopsy specimens at autopsy with the genomic configuration of plasma.

### 1.9 Rationale for this Study

Early stage melanoma patients are never safe from the possibility of disease recurrence and yet there are no effective means of detecting disease recurrence until the disease has progressed to levels that are associated with a poor prognosis. The convenience of a blood sample that could facilitate frequent assessment of disease presence and progression would be particularly advantageous for melanoma patients. Additionally, the detection limit of ctDNA in patients' needs to be identified and correlated with current imaging techniques so that we can classify the limit of sensitivity of ddPCR in clinical samples and confirm the level of disease burden that can be detected by ctDNA analysis. A correlation between MTB measured by FDG-PET/CT and levels of ctDNA in melanoma patients would underscore the role of ctDNA as a non-invasive, complimentary method to FDG-PET/CT for real-time monitoring of tumour burden and hence the detection of disease recurrence.

Mutant-specific ctDNA is an emerging tool for detection of residual disease and for the prognosis and monitoring of different cancers (Bettegowda et al., 2014; Dawson et al., 2013; Garcia-Murillas et al., 2015; Newman et al., 2014; Tie et al., 2016). To date, the focus of ctDNA measurements has primarily been on *BRAF* and more recently *NRAS* mutations. Given new insights into the high prevalence of *TERT* promoter mutations in melanoma, the likely development of *TERT* targeted therapies (Akıncılar et al., 2016), and the potential for using ctDNA to detect disease recurrence, a sensitive method for detection of *TERT* promoter mutations in melanoma tumour tissue and cfDNA is warranted.

Before ctDNA levels can be tested and monitored, the patient's specific mutation profile must first be identified from either the primary or metastatic tumour. This however can be challenging, particularly where tumours contain limited and often low-quality DNA, with low cellularity and high tumour heterogeneity. Different methods of mutation detection tolerate different degrees of DNA quality (Chen et al., 2015). Although micro or macro-dissection and careful selection of tumour tissue is commonly employed when using primary or bulky metastatic tumour tissues, in specimens such as SLNB and fine needle aspiration biopsies of metastatic sites, tumour cellularity can be extremely low and macro-dissection can be challenging or impossible (Chen et al., 2015). This highlights the need to determine the most sensitive method of detecting mutations in melanoma tissue biopsies for improved patient treatment decisions, using only a small number of tumour cells.

It is evident that ctDNA can be detected in the blood of both early and late stage cancer patients. Moreover, the presence of ctDNA after surgery is associated with disease recurrence or progression in many cancers and may therefore provide a useful marker of early disease spread in melanoma particularly when FDG-PET/CT and SLNB have little efficacy. Ultimately, a method that is non-invasive, can be performed at regular intervals and provides early evidence of disease progression or recurrence, when a chance of a favourable outcome is highest, will reduce the burden of disease and increase overall survival. As a blood-based biomarker, ctDNA offers a non-invasive and easily accessible method of providing a real-time snap shot of tumour burden, however the ctDNA content is known to differ in various tumour types and stages, and may vary between patients (Schwarzenbach et al., 2011). Patients with early stage melanoma, although at a far reduced risk of recurrence than stage IV patients, currently have no effective surveillance strategy available to them that can detect disease progression or recurrence until the patient has progressed to stage III or IV disease, at which time their prognosis becomes poor. CtDNA may serve as a useful monitoring tool for melanoma patients having undergone tumour resection and at risk of disease recurrence,

providing earlier evidence of disease progression than current methodologies and therefore needs to be investigated.

### 1.10 Aims

The overarching aim of this project was to develop a tool that could regularly, inexpensively and non-invasively monitor early stage melanoma patients for melanoma recurrence.

The specific aims of the project were:

**Aim 1**: To develop a ddPCR probe-based assay to simultaneously detect multiple *TERT* promoter mutations in melanoma tumours and plasma cfDNA to allow for an increased number of patients who could be monitored through ctDNA analysis.

**Aim 2**: To compare the sensitivity and specificity of ddPCR relative to other commonly utilised methods to detect mutations in melanoma tissue containing a small fraction of tumour cells.

**Aim 3:** Investigate the presence of *BRAF, NRAS* and/or *TERT* promoter mutant ctDNA in early stage melanoma patients using ddPCR to determine if ctDNA will serve as a prognostic biomarker for melanoma recurrence.

**Aim 4:** Use comparative analysis to assess the correlation between ctDNA levels and MTB derived from $^{18}$F-FDG FDG-PET/CT to determine the efficacy of ctDNA in measuring disease burden as a complementary modality to $^{18}$F-FDG FDG-PET/CT.

# CHAPTER 2: SENSITIVE DROPLET DIGITAL PCR METHOD FOR DETECTION OF *TERT* PROMOTER MUTATIONS IN CELL FREE DNA FROM PATIENTS WITH METASTATIC MELANOMA

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## 2.1 Abstract

**Background**

Currently mainly *BRAF* mutant ctDNA is utilized to monitor patients with melanoma. *TERT* promoter mutations are common in various cancers and found in up to 70% of melanomas, including half of *BRAF* wild-type cases. Therefore, a sensitive method for detection of *TERT* promoter mutations would increase the number of patients who could be monitored through ctDNA analysis.

**Methods**

A ddPCR assay was designed for the concurrent detection of chr5:1,295,228 C>T and chr5:1,295,250 C>T *TERT* promoter mutations. The assay was validated using 39 melanoma cell lines and 22 matched plasma and tumour samples. In addition, plasma samples from 56 metastatic melanoma patients and 56 healthy controls were tested for *TERT* promoter mutations.

**Results**

The established ddPCR assay detected *TERT* promoter mutations with a lower LOD of 0.17%. Total concordance was demonstrated between ddPCR and Sanger sequencing in all cell lines except one, where a second mutation within the probe binding site. tissue was 68% (15/22), with a sensitivity of 53% (95% CI, 27%-79%) and a specificity of 100% (95% CI, 59%-100%). A significantly longer PFS (p=0.028) was evident in ctDNA negative patients. Of significant importance, our *TERT* promoter mutations ddPCR assay allowed detection of ctDNA in 11 *BRAF* wild-type cases.

**Conclusions**

The *TERT* promoter mutation ddPCR assay offers a sensitive test for molecular analysis of melanoma tumours and ctDNA, which has the potential to be applied to other cancers.

*TERT* encodes the catalytic subunit of telomerase, a ribonucleoprotein responsible for maintaining telomere length of chromosomes which play an integral role in cell immortality. Using linkage analysis and high-throughput sequencing, Horn et al. (2013) reported somatic mutations in 74% of metastatic melanoma human cell lines, 85% of metastatic melanoma tumour tissues and 33% of primary melanomas. These mutations are the result of a cytidine to thymidine transition in the promoter of the *TERT* gene, at chromosome 5: 1,295,228 C>T and 1,295,250 C>T, hereafter termed C228T and C250T. These mutations create a putative consensus ETS (E26 transformation-specific) /ternary complex factor binding motif (GGAA/T), which is associated with an increase in *TERT* expression (Horn et al., 2013; Kumar et al., 2014). The presence of these mutations in cutaneous melanoma is associated with fast growing melanomas (Nagore et al., 2016b) and poor prognosis (Griewank et al., 2014). The co-existence of *TERT* promoter mutations with *BRAF* or *NRAS* mutations (in 55% of cases) is associated with poor disease-free and melanoma-specific survival (Nagore et al., 2016a). *TERT* promoter mutations occur frequently in a number of other cancers: 80–90% of glioblastoma multiforme, 60% of hepatocellular carcinoma, 60% of bladder cancer, 70% of basal cell carcinoma, 50% of cutaneous squamous cell carcinoma and up to 30% of thyroid cancers (Borah et al., 2015; Killela et al., 2013; Liu et al., 2014; Liu et al., 2013b; Nault et al., 2013; Vinagre et al., 2013) and are associated with aggressive disease in thyroid carcinoma (Yin et al., 2016), glioblastoma (Huse, 2014), neuroblastoma (Simon et al., 2015) and renal cell carcinoma (Wang et al., 2014). Therefore, it is of significant clinical benefit to develop a non-invasive and sensitive test that determines the *TERT* promoter mutation status in cancer patients.

Molecular profiling of tumours to aid cancer prognosis and to identify actionable therapeutic targets has become routine practice in clinical oncology. Whilst tumour tissue samples are typically used for mutation analysis, access to the tumour for biopsy, and the quality and quantity of the sample may hinder detection, particularly when methods with limited sensitivity are employed. Commonly used methods include Sanger sequencing, melting curve analysis and pyrosequencing which have

limits of sensitivity of 15%-20%, 10% and 5%, respectively (Tsiatis et al., 2010). More recently, tumour related aberrations have been determined in plasma cfDNA (Ascierto et al., 2013a; Bettegowda et al., 2014; Chang et al., 2016; Diehl et al., 2008; Heitzer et al., 2015; Tsao et al., 2015). This is referred to as "liquid biopsy", a relatively non-invasive test that can be performed regularly and provides information from the sum of all tumours at any one-time point. It is, therefore, a valuable biomarker for monitoring disease progression and response to therapy (Gray et al., 2015; Heitzer et al., 2015).

Whilst a variety of methods have been used to detect mutations from ctDNA, Hindson et al. (2011), have shown ddPCR to be a highly sensitive platform, enabling absolute quantitation of mutant *BRAF* down to 0.001% allelic fraction. Various studies have since shown the utility of testing mutant *BRAF* in plasma of melanoma patients using ddPCR (Ascierto et al., 2013a; Girotti et al., 2015; Gray et al., 2015; Sanmamed et al., 2015; Santiago-Walker et al., 2015). In particular, our laboratory has demonstrated that ctDNA analysis allows tracking of patient response to therapy and resistance acquisition (Gray et al., 2015). Given the high prevalence of the *TERT* promoter mutations C228T and C250T in cutaneous melanoma (Heidenreich et al., 2014; Nagore et al., 2016a), their addition to existing tests for detection of mutant *BRAF* and *NRAS* will allow monitoring of most melanoma patients using ddPCR. Furthermore, it has been shown that concurrence of mutations in the *TERT* promoter with *BRAF* or *NRAS* mutations predispose patients to fast growing and aggressive disease, thus detection of multiple mutations including mutant *TERT* could serve as a prognostic marker.

We report here on the development of a ddPCR probe-based assay to simultaneously detect the *TERT* promoter mutations C250T and C228T. One probe binds the wild-type sequence overlapping position C228, while a second probe binds the mutant sequence resulting from C228T or C250T mutations, as both mutations reconstitute the putative ETS binding site (Figure 1). First, we tested

the concordance of this assay for the detection of *TERT* promoter mutations in 39 melanoma cell lines relative to Sanger sequencing, and in 22 plasma samples relative to patient matched tumour tissue. We also determined the sensitivity and specificity of this assay for the detection of *TERT* promoter mutations using plasma derived cfDNA from 56 melanoma patients and 56 healthy controls.

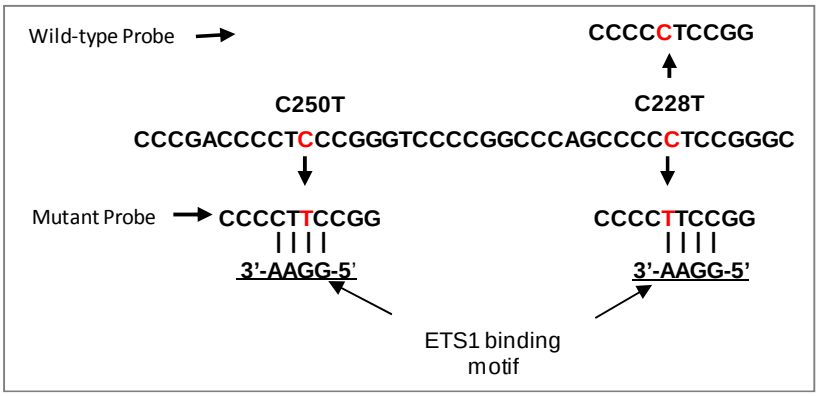



**Figure 1: Location of ddPCR assay probes relative to ETS1 binding motifs generated by the C228T and C250T *TERT* promoter mutations.** Probes for the identification of wild-type and mutant sequences are indicated. Both mutant sites are detected by the same probe.

### 2.3 Results

The designed primer sets were tested for amplification of the genomic region of interest by end-point PCR. Amplification conditions were optimised by testing a range of annealing temperatures (55-61ºC). As shown in Figure 2A, the primers failed to amplify the required fragment in the absence of Q-solution (Qiagen). Optimal amplification was achieved in the presence of Q-solution between 61-64ºC (Figure 2B). The PCR fragment obtained was subjected to Sanger sequencing to confirm its specificity.

Next, droplet digital PCRs were performed at a gradient of annealing temperatures from 52$^{o}$C to 65$^{o}$C for the detection of the C228T mutation in gDNA from 1205Lu cells (Figure 2C and D) and the C250T mutation in gDNA from UACC62 cells (Figure 2E and F). Optimal droplet segregation

was observed at 57$^{o}$C. Hereafter all ddPCR assays were performed with an annealing/extension temperature of 57$^{o}$C.

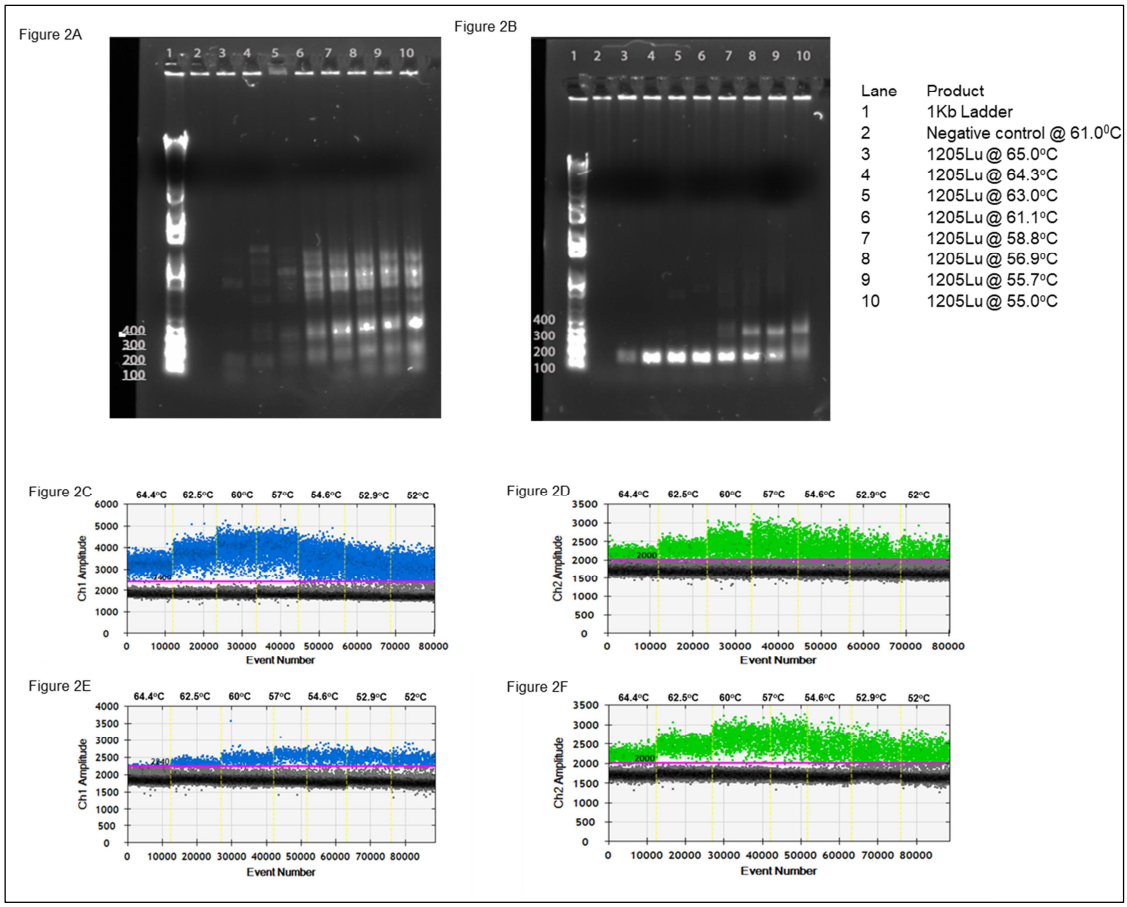



**Figure 2: Optimization of ddPCR for detection of *TERT* promoter mutations.** PCR fragments from cell line 1205Lu amplified at varying temperatures without (A) and with (B) "Q solution". gDNA of cell lines 1205Lu-C228T (C and D) and UACC62-C250T (E and F) were used as template for the *TERT* ddPCR at varying annealing temperatures. FAM signal from mutant probe binding to C228T (C) or C250T (E). HEX signal from binding of wild-type probe (D and F).

To evaluate the quantitative linearity and the LOD of the ddPCR assay, serial dilutions of mutant gDNA from cell lines 1205Lu (C228T mutant) and UACC62 (C250T mutant) were mixed in a background of wild-type human gDNA to achieve a final concentration of gDNA of 20 ng/µL (Figure 3), with each dilution tested in 8 replicates. At 0% mutant DNA, we identified that a maximum of two false positive droplets were observed in some of the 8 replicates, with an average of 0.068 ± 0.049%. Therefore, the lower LOD was defined at 0.17%, the percentage false positives detectable at two standard deviations over mean background (Armbruster et al., 2008).

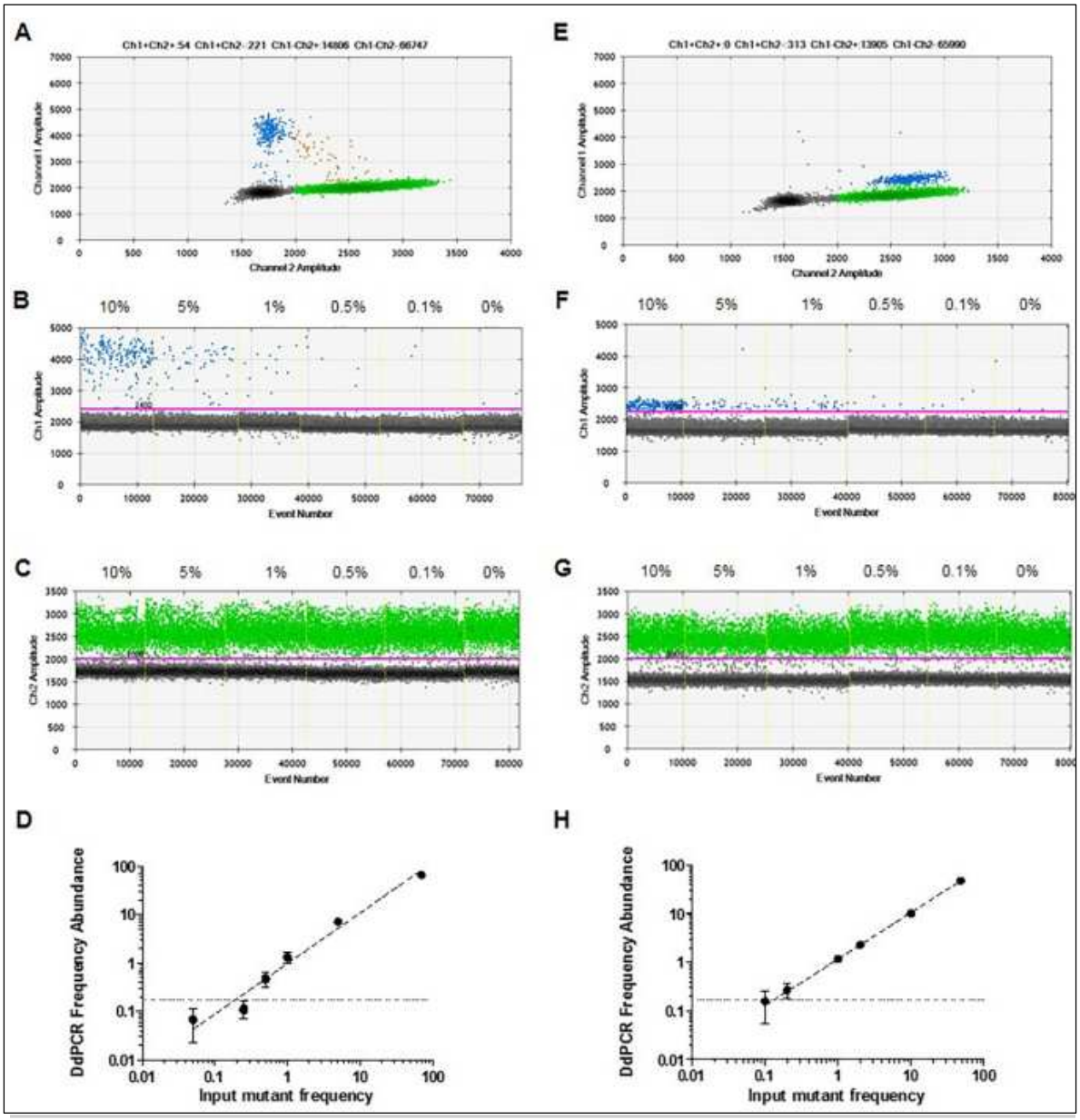



**Figure 3: Detection of *TERT* promoter mutations in the presence of homologous wild-type DNA.** Serial dilutions of DNA from mutant cell lines 1205Lu – C228T (A-D) and UACC62 – C250T (E-H) were prepared in a constant background of wild-type human genomic DNA. 2D plots of ddPCR read out at 10% of mutant DNA (A and E). 1D plots indicating mutant (B and F) and wild-type (C ad G) DNA detection. Analytical sensitivity (LOD) of the assay (D and H). Obtained frequency abundances and standard deviations were plotted versus expected mutant frequencies based on input. The LOD, defined as two SD over the mean frequency abundance obtained at 0% when only wild-type DNA was used as input, was indicated as dashed lines in both graphs.

**Table 1: Validation of C228T and C250T *TERT* promoter mutation detection in melanoma cell lines**

| Cell Line | Sanger Sequencing | ddPCR |
|---|---|---|
| C024 | wt | wt |
| C055 | wt | wt |
| C092 | wt | wt |
| C096 | wt | wt |
| HGA | wt | wt |
| C022 | C228T | C228T |
| C037 | C228T | C228T |
| C058 | C228T | C228T |
| D41 | C228T | C228T |
| MM409 | C228T | C228T |
| D22 | C228T | C228T |
| MM473 | C228T | C228T |
| A06 | C228T[a] | C228T[a] |
| C076 | C228T[a] | C228T[a] |
| MM455 | C228T[a] | C228T[a] |
| 1205Lu | C228T[a] | C228T[a] |
| A15 | C250T | C250T |
| A14 | C250T | C250T |
| C002 | C250T | C250T |
| MM537 | C250T | C250T |
| SKMEL13 | C250T | C250T |
| MM386 | C250T | C250T |
| D01 | C250T | C250T |
| MM229 | C250T[a] | C250T[a] |
| MM253 | C250T[a] | C250T[a] |
| MM266 | C250T[a] | C250T[a] |
| C001 | C250T[a] | C250T[a] |
| C045 | C250T[a] | C250T[a] |
| D40 | C250T[a] | C250T[a] |
| UACC62 | C250T[a] | C250T[a] |
| MM396 | C227T/C228T | wt |
| A07 | C227T/C228T | wt |
| C054 | C227T/C228T | wt |
| C062 | C227T/C228T | wt |
| C057 | C241T/C242T | wt |
| C108 | C241T/C242T | wt |
| D28 | C241T/C242T | wt |
| SKMEL5 | C241T/C242T | wt |
| C021 | C250T[b] | wt |

[a]Homozygous

[b]C021 carried an additional C253T polymorphism.

To validate the assay, we tested 39 cell lines with known *TERT* promoter mutant or wild-type status (Table 1). We confirmed detection of the C228T and/or the C250T *TERT* promoter

mutation in only those cell lines identified as positive for these two mutations, while those previously identified as wild-type showed no positivity for *TERT* DNA mutations by ddPCR. Cell lines that harboured an alternative *TERT* mutation other than C228T or C250T showed as wild-type in our assay. In addition, the C250T mutation was not detected in cell line C021, due to the presence of a C253T single nucleotide polymorphism in the probe binding site (Supplementary Figure 1). Simultaneous C250T and C253T mutations have been reported in 2% of melanoma cells lines (Horn et al., 2013).

Tumour tissue samples from 22 stage IV (AJCC) metastatic melanoma patients were tested for C228T and C250T *TERT* promoter mutations by ddPCR using the *TERT* assay (Table 2). As reported in the literature (Griewank et al., 2014; Hayward et al., 2017; Heidenreich et al., 2014), most tumour tissues tested harboured at least one of these mutations (68%, n=15); 11 harboured the C228T mutation and 4 harboured the C250T mutation. No tissue samples were found to contain both *TERT* promoter mutations.

**Table 2: Detection of *TERT* promoter mutations in ctDNA and paired tumour tissue.**

| | **Tumour Tissue** | | |
|---|---|---|---|
| **Plasma ctDNA** | + | - | **Total** |
| + | 8 | 0 | 8 |
| - | 7 | 7 | 14 |
| **Total** | 15 | 7 | 22 |

Plasma derived cfDNA from these 22 patients were also tested for *TERT* promoter mutations. These plasma samples were collected from patients with active metastatic disease prior to any systemic therapeutic intervention. Overall, the concordance between tumour tissue and plasma testing was 68% (15/22). No patient was positive for a *TERT* promoter mutation in plasma and negative in its corresponding tumour tissue (100% specificity). Of 15 plasmas from patients with confirmed *TERT* promoter positive tumours, eight were identified as positive for the same

mutation, whereas seven cases were positive in the tissue but negative in the plasma sample (Table 2). Thus, the sensitivity of our *TERT* C228T/C250T mutation detection in plasma was estimated as 53% (95% CI 27%-79%). In a cox regression analysis, patients with detectable ctDNA at baseline (n=8) had a significantly shorter PFS compared to patients who had no detectable ctDNA (n=7) (*p*=0.028, Hazard ratio: 4.48 (CI, 1.18-17.06) (Figure 4a).

To further demonstrate the detection rate of *TERT* ctDNA in metastatic melanoma we tested 56 plasma samples from randomly selected stage IV (AJCC) melanoma patients (mean age 65 years, ranging from 35 to 85 years) with known *BRAF* but unknown *TERT* mutational status and compared this to 56 plasma samples from healthy individuals (mean age 51 years, ranging from 24 to 81 years). The *TERT* ddPCR assay detected a statistically significant difference in the copies of mutant *TERT* ctDNA in plasma from metastatic melanoma patients relative to those from healthy controls (p=0.006, Figure 4B). We found *TERT* mutant DNA in 11 of 38 *BRAF* wild-type and in four of the 18 *BRAF* V600E/K patients. The number of *TERT* promoter copies per mL of plasma detected in the melanoma patient cohort varied from 11.2 to 176 copies per mL (Figure 4B). No *TERT* promoter mutant DNA was detected in any of the 56 healthy control plasmas. Based on these results the assay specificity was estimated as 100% (95% CI 94%-100%).

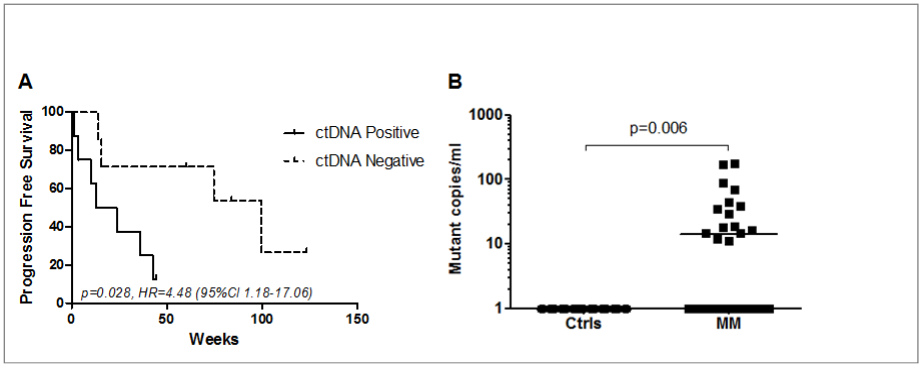



**Figure 4: Detection of *TERT* promoter mutations in plasma.** A. Kaplan-Meier plots of PFS probabilities of patients with detectable (n=8) and undetectable (n=7) ctDNA levels at baseline. Cox regression p-value, Hazard ratio (HR) and confidence interval (CI) are indicated. B. Copies of mutant DNA per mL of plasma were significantly higher in metastatic melanoma patients (MM) (N=56) compared to healthy controls (Ctrls) (N=56). P=0.006, Mann-Whitney U-test.

## 2.4 Discussion

Here we describe and validate a method to detect the two most common *TERT* promoter mutations found in melanoma tumours using ddPCR. *TERT* promoter mutations occur in melanoma as frequently as (Griewank et al., 2014), or more frequently (Nagore et al., 2016a) than *BRAF* mutations, and yet mainly *BRAF* mutant-specific cfDNA is being used to monitor melanoma patients for response to therapy and disease progression (Santiago-Walker et al., 2015). The inclusion of *TERT* promoter mutations within ctDNA for monitoring would increase the number of patients for whom ctDNA could be used to determine disease status, particularly amongst *BRAF* and *NRAS* wild-type melanoma patients. This will enable large studies on the clinical utility of ctDNA monitoring to provide evidence of the efficacy of this marker for determining disease progression, to inform cessation of ineffective therapies (Girotti et al., 2015; Gray et al., 2015) and to guide alternative therapy.

Our assay allowed for detection of mutant *TERT* in biologically relevant samples, such as FFPE tumour DNA and plasma of metastatic melanoma patients at high specificity. Using cell line derived DNA, we optimized the assay to detect as little as 0.17% mutant *TERT* DNA in dilutions

of wild-type DNA. This is significantly lower than limits of detection reported for other mutation detection platforms such as allele-specific PCR at 1% (De Castro et al., 2012) and pyrosequencing at 5%, melting curve analysis at 10% and Sanger sequencing at 20% (Tsiatis et al., 2010). While we and others have shown ddPCR to detect *BRAF* mutant fraction as low as 0.001% (Hindson et al., 2011; Reid et al., 2015), we were unable to achieve this sensitivity with the *TERT* assay developed here, possibly due to the highly GC rich area of the promoter region of this gene, resulting in background signal and limited segregation of positive and negative droplets. In fact, during the development of this assay, multiple primers, probes and amplification conditions were tested without success. The conditions detailed here, including the addition of LNA at the specific nucleotides and the use of Q-solution in the amplification mix, were indispensable for successful amplification.

We validated the assay in terms of accuracy and reliability by showing 97.4% concordance with the genotype of 39 melanoma cell lines. Of the cell lines analysed that harboured either a C228T or C250T mutation, 14 were heterozygous and nine homozygous. A major limitation of our assay is that it cannot detect other *TERT* promoter mutations and it can be affected by SNPs within the probe binding sites. This was apparent by the results obtained from nine cell lines with known *TERT* promoter dinucleotide mutations C227T/C228T and C241T/C242T, which have been reported to exist in 5.2% and 10.4% of primary melanomas, respectively (Horn et al., 2013). Similarly, a negative result was reported for cell line CO12 which harbours a C253T SNP on the probe binding site. Further development of ddPCR assays to detect these other *TERT* promoter mutations (Nagore et al., 2016a) would ensure that a maximum number of patients could be monitored. In addition and given that SNPs in this region can also affect patient prognosis (Nagore et al., 2016a), germline sequence analysis should be performed complementary to the analysis of *TERT* promoter somatic mutations.

It is notable that all patients with *TERT* promoter mutations in plasma had corresponding mutations in matched tumour tissue and as such no false positive plasma samples were detected. High concordance between mutational profiles in plasma ctDNA and matched tumour tissue have been reported in several studies from patients with melanoma (Ascierto et al., 2013a; Sanmamed et al., 2015; Santiago-Walker et al., 2015), breast cancer (Bettegowda et al., 2014; Dawson et al., 2013; Higgins et al., 2012), non-small cell lung cancer (Narayan et al., 2012; Newman et al., 2014) and colorectal cancer (Bettegowda et al., 2014; Diehl et al., 2008; Misale et al., 2012). In our study, seven patients with *TERT* promoter positive tumours had no detectable *TERT* promoter mutations in matched plasma samples. This is similar to the findings by Lee et al, (2017a) who detected ctDNA in 53% of patients prior to treatment initiation. The lack of detectable ctDNA in a subset of patients may be explained by the pathophysiology of the tumour or its metastasis, as ctDNA concentration has been correlated with tumour size (Kamat et al., 2006; Lee et al., 2017a; Thierry et al., 2010), metastatic spread or disease burden (Lee et al., 2017a; Parkinson et al., 2016; Sanmamed et al., 2015), tumour vascularisation (Thierry et al., 2016) and site of metastasis (Bettegowda et al., 2014). A retrospective analysis of PFS in this group of patients revealed a significant difference between patients with negative and positive ctDNA results. This further supports previous findings that low or undetectable level of ctDNA is a predictor of long term treatment benefit (Ascierto et al., 2013a; Gray et al., 2015; Lee et al., 2017a; Sanmamed et al., 2015; Santiago-Walker et al., 2015).

Previous studies have reported detection rates for *BRAF* V600E mutations in plasma of metastatic patients at 76 to 84.3% (Sanmamed et al., 2015; Santiago-Walker et al., 2015) and for *BRAF* V600K at 81 to 89% (Ascierto et al., 2013a; Santiago-Walker et al., 2015). In other cancers, Bettegowda et al. (2014) identified mutant ctDNA in 75% of patients with a variety of cancers including ovarian, breast, bladder, gastrooesophageal and colorectal cancers. Considering our detection rates of *TERT* promoter mutations in ctDNA are lower (53%) than

these reports, it would be necessary for this investigation to be conducted in a larger cohort controlling for tumour burden, metastatic sites and mutation variety. Nevertheless, our *TERT* promoter mutation assay allowed ctDNA detection in 11 of 38 *BRAF* wild-type tumours. Thus, our assay may facilitate ctDNA monitoring on *BRAF* wild-type cases, most of which will receive immunotherapy as a first line of treatment.

Nagore and colleagues (2016a) have shown that melanoma patients harbouring these specific *TERT* promoter mutations, in combination with *BRAF*/*NRAS* mutations within their tumour tissue, have a significantly shorter disease free survival than patients without this combination. In fact, Li et al., (2016) have shown that *TERT* promoter mutations are key downstream targets of the RAS-ERK pathway for malignant progression of *BRAF* mutant melanomas. Furthermore, Akincilar et al. (2016) have shown that *TERT* transcription is driven by mediation of long-range chromatin interaction and enrichment of active histone marks through the recruitment of GABPA to mutant *TERT* promoters, specifically C228T and C250T. These authors have consequently suggested that inhibitors could be designed to hinder *TERT* transcription in cancer cells with these mutations. As such, routine genetic testing of melanoma patients for *TERT* promoter mutations in addition to mutant *BRAF* and *NRAS* would be clinically beneficial.

*TERT* promoter mutations have been identified in numerous other cancers such as thyroid, bladder, hepatocellular cancer and malignant glioblastoma [6-8]. Consequently, the assay described here may allow ctDNA monitoring in multiple other malignancies. However, the assay would require validation for each of these cancers.

In conclusion, we report on the development of a ddPCR assay for the detection of two common *TERT* promoter mutations in cell lines, tumour tissue and ctDNA. Our results suggest that the

*TERT* ddPCR assay could prove useful as a companion diagnostic to predict treatment benefit and to monitor response in melanoma patients and could be extended to other malignancies.

## 2.5 Materials and Methods

### Ethics

This study was approved by the Human Ethics Committees at Edith Cowan University (No. 11543) and Sir Charles Gardner Hospital (No.2013-246).

### Genomic DNA extraction

gDNA) with known *TERT* promoter mutations was obtained from melanoma cell lines 1205Lu (Wistar Institute) and UACC62 (National Cancer Institute) to be used as positive controls. In addition, gDNA was extracted from 39 melanoma cell lines from the QIMR Berghofer Medical Research Institute (Dutten-Regester et al., 2012). Wild-type gDNA was obtained from the white blood cell pellets collected from 4 mL whole blood from one healthy control. DNA was isolated using the QIAamp DNA Mini Kit (Qiagen, Australia) as per the manufacturer's instructions. gDNA was eluted in AE buffer (Qiagen) and stored at 4$^{o}$C until further processing.

### Plasma sample preparation

Blood samples were collected from AJCC stage IV melanoma patients, prior to initiation of any systemic therapy, into EDTA vacutainer tubes and stored at 4$^{o}$C. Plasma was separated within 24 hours by centrifugation at 1600 g for 10 minutes, followed by a second centrifugation at 2000 g for 10 minutes, and then stored at -80$^{o}$C until extraction.

### DNA extraction from plasma

cfDNA was isolated from 5 mL of plasma from healthy donors and AJCC stage IV metastatic melanoma patients using the QIAamp Circulating Nucleic Acid Kit (Qiagen) as per the

manufacturer's instructions. cfDNA was eluted in 40 µl AVE buffer (Qiagen) and stored at -80$^{o}$C until ctDNA quantification.

**DNA extraction from FFPE tissue**

Following review and macro-dissection by an experienced pathologist, gDNA was extracted from 10 x 5µm unstained sections of FFPE tissue using the QIAamp DNA mini kit (Qiagen) as per the manufacturer's instructions. Only FFPE tissues stored at room temperature, for less than seven years were used. The DNA concentration and purity were determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and Qubit 2.0 Fluorometer (Life Technologies, USA) instruments.

**PCR**

The following primers were used to amplify a 163bp product incorporating both hotspot mutations (C228T and C250T) in the *TERT* promoter region: 5'- AGCGCTGCCTGAAACTCG -3' (forward) and 5'- CCTGCCCCTTCACCTTCCAG -3' (reverse). Primers were synthesised by GeneWorks (Thebarton, SA, Australia). For optimization of the PCR amplification of *TERT* promoter mutations, we first performed end point PCRs containing, 1 x ddPCR supermix (Bio-Rad), 900 nM of each primer and 50 ng of template gDNA, with and without 1 x Q solution (Qiagen). Amplifications were performed using the following cycling conditions: 1 cycle of 95°C for 15 minutes, 40 cycles of 95°C for 30 seconds and a range of temperatures from 55°C to 65°C for 30 seconds, followed by 68°C for 30 seconds and 1 cycle of 68°C for 10 minutes. PCR products of 163bp were detected by gel electrophoresis on a 1% agarose gel in Tris-acetate-EDTA (TAE) buffer containing SYBR® Safe DNA Gel Stain (Life Technologies).

**Droplet digital PCR**

A probe was designed to detect both C228T and C250T mutation as both mutations result in the same sequencing string (Figure 1). Due to the short size of the probe, Locked Nucleic Acid

(LNA) bases were introduced on the bases indicated with a "+" (*TERT* Mut: /56-FAM/CCC+C+T+T+CCGG/3IABkFQ/). A second probe was designed to recognize the C228 loci, also containing LNA bases, (*TERT* WT, /5HEX/CCCC+C+T+CCGG/3IABkFQ/). Probes were custom synthesized by Integrated DNA Technologies (IDT). Amplifications were performed in a 20 µL reaction containing 1 x ddPCR Supermix for Probes (No dUTP, Bio-Rad), 1x Q solution (Qiagen), 250 nM of each probe and 900 nM of each primer plus template.

Droplets were generated using the Automatic Droplet generator QX200 AutoDG (Bio-Rad). Amplifications were performed using the following cycling conditions: 1 cycle of 95°C (2.5C/s ramp) for 10 minutes, 40 cycles of 94°C (2.5C/s ramp) for 30 seconds and 57°C for 1 minute, followed by 1 cycle of 98°C (2.5C/s ramp) for 10 minutes. Annealing/extension temperature was optimized using temperature gradients from 52$^{o}$C to 65$^{o}$C. The sample was held at 4$^{o}$C until further processing. Droplets were analysed through a QX200 droplet reader (Bio-Rad). QuantaSoft analysis software (Bio-Rad) was used to acquire and analyse data.

To evaluate the LOD of our *TERT* ddPCR assay, gDNA from cell lines 1205Lu (C228T) or UACC62 (C250T) were serially diluted into normal human DNA obtained from white blood cells of healthy controls to achieve from 100% to 0% mutant alleles. Each dilution was tested in a series of eight repetitions all completed in one run.

Cell lines with known C228T and C250T *TERT* promoter mutations, as well as cell lines wild-type for both mutations (as determined by Sanger sequencing) were used to validate the assay. The reaction mix was prepared as above using 50 ng of gDNA as template.

For plasma ctDNA analysis, 5 µL of cfDNA (maximum template volume possible) was added per reaction irrespective of the cfDNA concentration. Each run included a non-template control,

gDNA from a healthy control and gDNA from the cell lines containing the *TERT* mutations: 1205Lu (C228T) and UACC62 (C250T). Only samples with more than two positive droplets were considered positive. The number of mutated DNA copies per 20 µl reaction was extrapolated to calculate copies per mL using the following equation:

Copies/mL of plasma = C*EV/TV/PV

PV = Volume of plasma used for cfDNA extraction (ml)

EV = Volume in which cfDNA was eluted (µl)

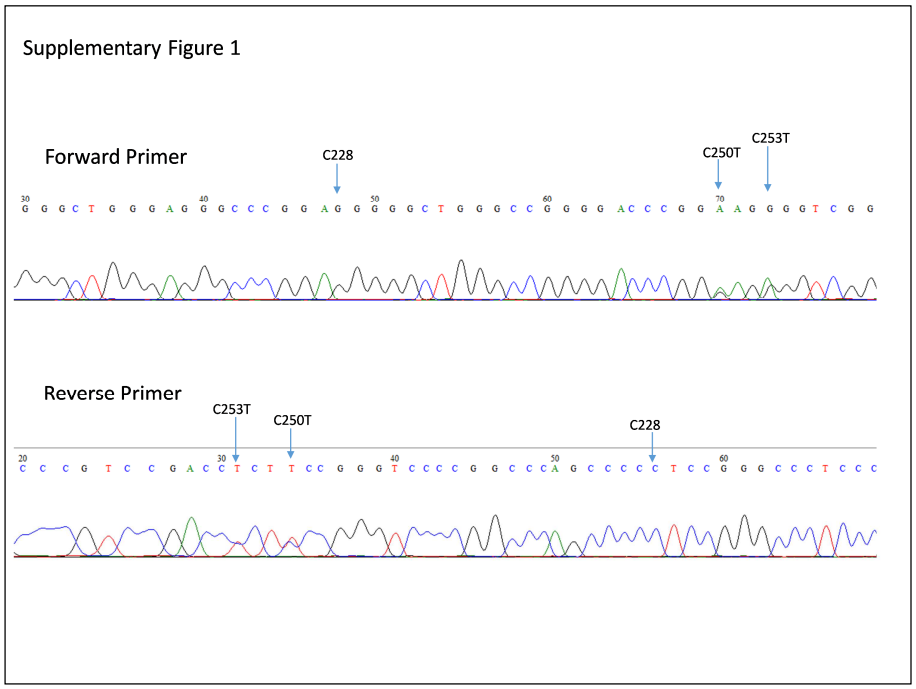
TV = Volume of cfDNA added to the PCR reaction (µl)

C = copies/20µl (data derived from QuantaSoft).

**Statistical analysis**

Sensitivity and specificity of the assay was calculated using a contingency table analysed using a Fisher's exact test. Comparison between ctDNA concentrations in patient and control samples were performed using the non-parametric Mann-Whitney *U*-test. A Cox proportional hazards regression analysis was performed to examine association of ctDNA detection with PFS. Statistical analyses were performed using Statistical Package for Social Sciences for Window version 22 (SPSS, Chicago, IL) and plotted using GraphPad Prism version 5.

## 2.6 Supplementary Materials



## CHAPTER 3: DROPLET DIGITAL PCR FOR MUTATION DETECTION IN FORMALIN FIXED PARAFFIN-EMBEDDED MELANOMA TISSUES: COMPARISON WITH SANGER SEQUENCING AND PYROSEQUENCING

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## CHAPTER 4: MONITORING MELANOMA PROGRESSION WITH CIRCULATING TUMOUR DNA: A PROOF OF CONCEPT FROM THREE CASE STUDIES

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## CHAPTER 5: CORRELATION BETWEEN CIRCULATING TUMOUR DNA AND METABOLIC TUMOUR BURDEN IN METASTATIC MELANOMA PATIENTS

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# CHAPTER 6: GENERAL DISCUSSION AND FUTURE DIRECTIONS

Cutaneous melanoma accounts for 90% of all skin cancer deaths (Balch et al., 2010) and a large proportion of these deaths are as a result of disease recurrence (Soong et al., 1992; Soong et al., 1998) despite the patient being considered disease free following treatment. Although the risk of recurrence largely correlates with thickness and invasion of the primary tumour (Shaw et al., 1987; Soong et al., 1992; Soong et al., 1998), recurrences have also occurred 10 or more years after thin melanomas have been completely excised (Crowley et al., 1990; Dalal et al., 2007; Dong et al., 2000; Hohnheiser et al., 2011; Jones et al., 2013; Kalady et al., 2003; Leiter et al., 2012; Meier et al., 2002; Salama et al., 2013; Soong et al., 1998; Tsao et al., 1997). Importantly, the majority of recurrences appear in lymph nodes or other organs, at which point the disease is among the most aggressive and treatment-resistant of human cancers (Kenessey et al., 2012; Mocellin et al., 2013; Sanmamed et al., 2015; Ti'mar et al., 2013). Given that the greatest treatment efficacy is associated with a low disease burden (Hodi et al., 2010; Luke et al., 2017; McArthur et al., 2016; Sosman et al., 2011), early detection of melanoma recurrence is critical for improved survival. Surveillance strategies for patients with thin melanomas however are limited to physical examinations alone (Australian Cancer Network Melanoma Guidelines Revision Working Party, 2008) and therefore an additional surveillance approach that can be performed regularly and in conjunction with physical examinations could lead to timely detection of disease recurrence allowing for earlier interventions. CtDNA represents a new generation of biomarkers for detection of residual disease and monitoring of different malignancies (Bettegowda et al., 2014; Dawson et al., 2013; Gray et al., 2015; Spindler et al., 2012). As ctDNA has not been assessed as a marker for recurrence in clinically disease-free melanoma patients, our central aim was to develop a tool that could regularly, inexpensively and non-invasively monitor early stage melanoma patients for melanoma recurrence.

We addressed the central aim in four stages. The first was to develop a new assay to detect *TERT* mutations in ctDNA and tumour samples, the second to compare the sensitivity and specificity of ddPCR relative to other commonly utilised methods of detecting mutations in melanoma tissue containing a small fraction of tumour cells. The third stage investigated the presence of *BRAF*, *NRAS* and/or *TERT* promoter mutant ctDNA in early stage melanoma patients using ddPCR to determine if ctDNA will serve as a prognostic biomarker for melanoma recurrence. The final stage involved comparative analysis to assess the correlation between ctDNA levels and MTB derived $^{18}$F-FDG FDG-PET/CT to determine the efficacy of ctDNA in measuring disease burden as a complimentary modality to $^{18}$F-FDG FDG-PET/CT. With regards to the ddPCR *TERT* assay and the correlation between ctDNA and MTB, the work presented in this thesis is novel.

In the first part of this study, we developed a ddPCR assay for the concurrent detection of C228T and C250T *TERT* promoter mutations in tumour tissue and cfDNA. As a result of the assay development, we were able to detect *TERT* mutant DNA in 11 of 38 *BRAF* wild-type which allows for an increased number of patients who can be monitored with ctDNA particularly amongst *BRAF* and *NRAS* wild-type patients. Specifically, our assay may facilitate ctDNA monitoring of patients who may receive immunotherapy as a first line of treatment. Furthermore, it is likely that *TERT* transcription inhibitors will soon be developed (Akıncılar et al., 2016) with the recent discovery that *TERT* transcription is driven by mediation of long-range chromatin interaction and enrichment of active histone marks through the recruitment of GABPA to *TERT* promoters. The newly developed *TERT* detection assay will be highly beneficial as it will not only aid in determining (from tumour tissue) which patients can be treated with these inhibitors but will allow for ctDNA analysis to track patient response to that therapy as indicated for *BRAF* and *NRAS* (Gray et al., 2015). Furthermore, the ability to monitor *BRAF* and *NRAS* wild-type patients will increase the number of patients that can be monitored with ctDNA.

The detection of mutant *TERT* however does not only increase the number of patients that can be monitored but can also be used for prognostic purposes. Patients who harbour C228T and C250T *TERT* promoter mutations are associated with a poor prognosis (Griewank et al., 2014; Nagore et al., 2016b). In line with this, we showed significant differences in PFS probabilities of patients with detectable (n=8) and undetectable (n=7) *TERT* mutant ctDNA levels at baseline. This further supports previous findings that low or undetectable levels of ctDNA is a predictor of long term treatment benefit (Ascierto et al., 2013a; Gray et al., 2015; Lee et al., 2017a; Sanmamed et al., 2015; Santiago-Walker et al., 2015).

We have now shown that the detection of mutant *TERT* in tumour tissue and plasma provides useful clinical information. This will enable large studies on the clinical utility of ctDNA monitoring to provide evidence of the efficacy of this marker for determining disease progression, to inform cessation of ineffective therapies and to guide alternative therapy. (Girotti et al., 2015; Gray et al., 2015)

Whilst we fulfilled our aim to develop an assay to detect C228T and C250T mutations, one obvious limitation to our assay is that it cannot detect other *TERT* promoter mutations and it can be affected by SNPs within the probe binding sites. Hence further development of the assay to detect other *TERT* promoter mutations (Nagore et al., 2016a) would further increase the number of patients who could be monitored.

Monitoring patients for recurrence using ctDNA requires knowledge of the patients' mutational profile. Whilst there are a variety of methodologies available for mutation profiling, each detection method tolerates different degrees of DNA quality. In many instances, genetic analysis is performed on limited and often low-quality DNA, from tumours with low tumour cellularity and high tumour heterogeneity. This is particularly apparent when tumours are sourced from

SLNB and fine needle aspiration biopsies of metastatic sites and as such we set out to determine the sensitivity of ddPCR on tumours of varying cellularity.

In the second publication, we evaluated the relative sensitivities of Sanger sequencing, pyrosequencing and ddPCR in detecting common mutations in *BRAF*, *NRAS* and *TERT* promoter from FFPE tumour with a range of tumour cellularity. Although Sanger sequencing and pyrosequencing are the most commonly employed methods of *BRAF* and *NRAS* mutation detection in molecular pathology laboratories, ddPCR was the most sensitive method, detecting at least one mutation in 77.5% (31 of 40) of cases, including in 12.5% and 23% of samples deemed as wild-type for all three mutant genes by pyrosequencing and Sanger sequencing, respectively. Importantly, the ddPCR sensitivity was particularly apparent among samples with less than 50% tumour cellularity. This suggests that ddPCR may further provide the opportunity for assessing the tumour mutational profile in more patients, particularly where macro-dissection is not possible, such as sentinel lymph node biopsies and fine needle aspiration biopsies of metastatic sites.

DdPCR is not routinely used in molecular pathology laboratories, possibly due to it being a relatively new methodology, however the platform has received much attention in a short space of time, particularly in melanoma research (Ashida et al., 2017; Chang et al., 2016; Gray et al., 2015; Hindson et al., 2013; Huang et al., 2016; Knol et al., 2016; Reid et al., 2015; Sanmamed et al., 2015; Schreuer et al., 2016; Tsao et al., 2015). Considering the multiple advantages that have been associated with ddPCR including absolute quantification, lower susceptibility to PCR inhibitors which affect amplification efficiency, resilience to differences in sample quality particularly at low concentrations, increased precision and high reproducibility between runs (Dingle et al., 2013; Hindson et al., 2013; Zhao et al., 2016), it was anticipated that ddPCR would be the most sensitive of the three methodologies. Importantly and as expected, the results

showed ddPCR to be a highly suitable platform for identifying mutations at a very low variant allele frequency. Thus, ddPCR offers research laboratories an alternative option where the tools and expertise to perform macro-dissection are not available. Additionally, ddPCR offers an alternative option in the clinical setting when macro-dissection is not possible, such as sentinel lymph node biopsies and fine needle aspiration biopsies of metastatic sites. The ability of ddPCR to detect mutations from a tumour containing only a small fraction of neoplastic cells, may further provide the opportunity for assessing prognosis, recurrence, metastasis and response to therapy in more patients.

Conversely, ddPCR has disadvantages which must be considered. This platform is limited to specific mutations and does not provide sequencing information, which Sanger sequencing and pyrosequencing are able to do. Consequently, the use of ddPCR may require multiple tests before a mutation is identified. This would not only require more DNA, which may be limited, but also additional hands-on time and therefore cost. Therefore, before such testing can be implemented into the clinical environment, multiplex ddPCR assays, similar to our TERT assay, that can test a specific exon or region will need to be developed to ensure minimal cost, hand-on time and DNA input is required.

Finally, given its ease of use and sensitivity, implementation of ddPCR based assays could facilitate mutation detection in early stage tumours and support the clinical use of ctDNA to improve early detection of residual disease and disease recurrence or progression. Thus, for the inclusion of such testing to be introduced into the clinic, standardisation of technical approaches and storage conditions will be required.

Following on from this we investigated whether ctDNA detection by ddPCR could be used to detect disease recurrence. We conducted retrospective surveillance using mutant-specific

ctDNA for three patients with cutaneous melanoma who experienced disease recurrence at distant sites and 27 patients who had no clinical evidence of disease recurrence. In patients with no evidence of disease recurrence, ctDNA was not detectable at any time point. CtDNA was however detected at the time of radiological or biopsy confirmation of metastases in all three patients who presented with disease recurrence. Moreover, in one case, plasma ctDNA detected recurrence four months prior to clinical evidence of disease recurrence. We acknowledge that the patient had not been having regular PET/CT scans and therefore cannot confirm if PET/CT would have detected disease recurrence at a similar time point to that of ctDNA, however it must be emphasised that the patient suffered from severe claustrophobia and as such ctDNA testing (had it been completed in real-time) would have prompted more thorough investigations 4 months prior to the gastroscopy. Of particular importance, Chapter 4 supports previous studies that have shown that recurrences occur even after surgical removal of thin melanomas. This highlights the need for an additional monitoring regime that can be performed regularly and potentially lead to timely interventions that will result in improved treatment options ultimately having a positive impact on the patient's quality of life and survival.

As expected almost half of this patient cohort harboured a *TERT* promoter mutation and thus the *TERT* detection assay developed as part of this thesis, was utilised to determine their ctDNA levels. *TERT* mutant ctDNA however, was not detectable in those patients with no evidence of disease recurrence, nor in the one patient who presented with disease recurrence and who harboured a C228T *TERT* promoter mutation. Considering *NRAS* mutant ctDNA was detectable at low allele frequency in this patient, we considered that the level of tumour burden is perhaps too small to detect the mutant *TERT* ctDNA, given that *NRAS* mutant ctDNA was detected at only 1.4 copies/mL of blood.

CtDNA has been detected in early stage breast (Beaver et al., 2014) and colon (Tie et al., 2016) cancer patients following resective surgery, indicating the presence of minimal residual disease. This provides evidence that ctDNA can be used to identify patients at risk of disease recurrence. Given that no mutant-specific ctDNA was detected in any of our patients at baseline, we would suggest that using our current methodology, ctDNA cannot be used to evaluate minimal residual disease in melanoma. Possible reasons for our inability to detect residual disease earlier, is that unlike Tie et al., (2016), who extracted cfDNA from 10 mL of plasma we only used 5 mL of plasma. Considering that DNA amounts vary between 0.1 ng and 100 ng DNA per mL of plasma (Chiu et al., 2006; Chun et al., 2006; Fatouros et al., 2006; Lázár et al., 2006; Rhodes et al., 2006; Schmidt et al., 2005), cfDNA yield will vary dependant on the volume of plasma in the first instance. Thus, to increase the sensitivity of the assays for detection of minimal residual disease in melanoma to guide the management of the disease, we suggest increasing the amount of plasma from which cfDNA is extracted. Additionally, a pre-amplification step of the target DNA, prior to ddPCR (Kiselinova et al., 2014; Pasternak et al., 2008) may further increase the sensitivity.

Notwithstanding this, our study provides a proof of concept and attests to the feasibility of mutant-specific ctDNA analysis for early stage melanoma patients at risk of disease recurrence. Furthermore, ctDNA surveillance provides an alternative method of monitoring patients without the additional risk of radiation exposure, invasiveness and cost. With the added advantage of a simple blood test being minimally invasive, ctDNA surveillance could be conducted far more regularly than PET/CT scans. Thus, our detection of disease recurrence at the time of clinical confirmation of disease recurrence suggests that ctDNA can be used to detect disease progression. CtDNA may therefore be assessed routinely to provide an increased window of opportunity for intervention that is likely to positively impact on the patient's survival. It is important to know therefore, what the lowest level of disease burden is detectable by ctDNA.

It has been proposed that, in the main, ctDNA levels correlate with disease burden assessed by qualitative PET/CT assessment (Girotti et al., 2015; Gray et al., 2015; Janku et al., 2015; Knol et al., 2016; Santiago-Walker et al., 2015; Tsao et al., 2015; Xi et al., 2016), with low or undetectable levels of ctDNA having been shown as a predictor of long term treatment benefit (Ascierto et al., 2013a; Gray et al., 2015; Lee et al., 2017a; Sanmamed et al., 2015; Santiago-Walker et al., 2015). Furthermore, the diagnostic sensitivity of ddPCR to detect mutant-specific ctDNA in metastatic patients is between 73% to 89% (Ascierto et al., 2013a; Gray et al., 2015; Santiago-Walker et al., 2015). Given that ctDNA is not detectable in all metastatic melanoma patients and based on our findings in the previous study, it was apparent that there was a robust need to understand the limit of detecting ctDNA in terms of tumour burden if ctDNA is to be used for routine monitoring of melanoma patients.

Considering quantitative assessment of FDG-PET/CT images can now be used to determine disease burden (Bai et al., 2013), we hypothesised that if ctDNA levels correlate with MTB derived from FDG-PET/CT scans in melanoma patients, we could determine the LOD of ctDNA as a potential surrogate to signify disease recurrence. We therefore conducted a retrospective analysis of ctDNA levels and MTB of 32 metastatic melanoma patients and found a significant correlation between the number of mutated copies per mL of plasma and the MTB in $cm^3$ measured by FDG-PET/CT, supporting the hypothesis that ctDNA is suitable as a surrogate indicator of tumour burden and aggressiveness.

To our knowledge this is the first correlative analysis in melanoma, to compare the level of ctDNA with MTB calculated from the sum of TLG for all evaluable lesions. In comparison to other radiologic imaging techniques, FDG-PET/CT has been hailed as the most superior in the management of melanoma (Akcali et al., 2007; Holder Jr et al., 1998; Reinhardt et al., 2006) due to its high sensitivity and specificity. As such, determining the limit at which ctDNA can be

detected relative to MTB provides evidence of the diagnostic sensitivity of our assays to detect mutant-specific ctDNA, which is pertinent to the detection of recurrence. Whilst our results exhibit a strong correlation between the number of mutated copies per mL of plasma and MTB, thus measuring both tumour burden and aggressiveness, it also illustrates its limitations in detecting low disease burden, which may have implications for detecting disease recurrence, particularly in patients with early stage melanoma. Even though approximately 80% of melanoma patients will harbour *BRAF*, *NRAS* and/or *TERT* mutations and our specific focus on these mutations throughout this project, none of the patients in our retrospective cohort harboured a *TERT* promoter mutation. Regardless however, we were able to determine the limit of disease burden that our current assay can detect. We are confident from the results from two cases (one with a single lung lesion and the other with nodal and liver metastases), that our threshold of ctDNA detection is currently at an MTB value of 10. Of particular importance is the ability of our assays to detect nodal disease; early evidence of such would provide the greatest window of opportunity for successful intervention. Interestingly, Wong et al., (2017) have shown that nodal involvement often displays high levels of ctDNA. Our results directly contradict this finding; however, it is pertinent to point out that the cohort in the Wong study (although no exact disclosure as to the number of nodes involved) had a higher disease burden across the board than we did in our study. Wong reported a median of 1,112 copies/mL of plasma (range 63-97,000) compared to our median of 38 copies/mL of plasma (range 1.6-52,440 copies/mL) suggesting that the disease burden in our patients was lower than those in the Wong study.

In our cohort, only three patients presented with nodal disease alone, none of whom had detectable ctDNA. A few caveats however are worth mentioning; two cases had MTB below our threshold of 10 and in two cases, cfDNA was extracted from only 1mL of plasma which is likely to reduce our capacity to detect ctDNA (Sherwood et al., 2016). Similarly, we were unable to detect ctDNA in the patient who presented with nodal and brain metastases and in the one patient

who presented with brain only metastases. Given that low or undetectable ctDNA levels have been commonly observed in patients presenting with brain only metastases (Bettegowda et al., 2014; De Mattos-Arruda et al., 2015; Wong et al., 2017), our results are not surprising. Stage III patients have a significantly better overall prognosis than stage IV patients (Aitken et al., 2008; Baade et al., 2015; Harries et al., 2016), and that the majority (50.2%) of first recurrences are seen in regional lymph nodes with 58.9% of these cases further progressing to distant metastases (Meier et al., 2002), therefore it is imperative to detect nodal recurrences in a timely manner. Interestingly, there is minimal evidence of detecting ctDNA in pre-surgery stage III melanoma patients (Wong et al., 2017), although ctDNA has been commonly reported in early stage lung (Sozzi et al., 2001), breast (Beaver et al., 2014) and colon (Tie et al., 2016) cancers. Thus, further studies are required to determine if in fact, ctDNA can be regularly detected in stage III melanoma patients as an early measure of clinically detectable disease.

Unfortunately, a limitation of this study was the number of patients where cfDNA had been extracted from only 1mL of plasma. As previously mentioned, the volume of plasma in the first instance affects the cfDNA and consequently the ctDNA yield (Sherwood et al., 2016). Whilst this is not relevant in the majority of cases with a high MTB, it introduces a bias that we cannot ignore. A total of 37.5% (n=12) of our samples were extracted from 1mL of plasma with 25% of these (n=3) having no detectable ctDNA. Of the 9 cases with detectable ctDNA, multiple metastatic disease sites were involved. Of the three cases with no detectable ctDNA, two cases involved nodal disease alone (a caveat previously discussed) however one case had a high MTB with bone metastases alone. Future studies will almost certainly require a prospective nature to ensure that a consistent volume of plasma is used for cfDNA extraction, thus removing any potential confounding affects.

In summary, the final stage of this thesis has directly compared the level of ctDNA with MTB to determine the lowest level of disease burden that our current assays can detect with ctDNA. Our measurements of MTB incorporate both tumour burden and tumour activity which therefore provides an overall perspective of the disease status of a patient. We have provided evidence of a significant correlation between ctDNA and MTB in treatment naïve patients which suggests that quantification of ctDNA between scans may provide a minimally invasive option with which to detect changes in disease burden in melanoma. Whilst detection of ctDNA in patients with an MTB≤10 will necessitate further improvements in the technology, we have defined the limit in global tumour burden for which ctDNA can be detected in blood.

Furthermore, we have observed a significantly shorter PFS in those patients with detectable ctDNA. This underscores the clinical utility of ctDNA analysis to be conducted at regular intervals between standard FDG PET/CT imaging, or between physical examinations (as would be the case for patients not having routine function imaging), to ultimately provide clinicians with more frequent windows of opportunity where interventions can be implemented timeously.

The results presented in this thesis provide a proof of principle for the potential application of personalised ctDNA monitoring for melanoma recurrence. Firstly the developments of a novel assay to detect *TERT* mutant ctDNA by ddPCR has allowed for an increased number of patients that may be monitored with ctDNA. This is exemplified by our detection of *TERT* mutations in *BRAF* WT patients and the detection of trackable mutations in tumour samples with minimal cellularity. Finally, the detection of ctDNA in patients presenting with disease recurrence has been further explored by determining the lower limit of disease burden that our current assays can detect.

# CHAPTER 7: CONCLUSION

As evidenced by our work in this thesis and others (Crowley et al., 1990; Dong et al., 2000; Hohnheiser et al., 2011; Kalady et al., 2003; Tsao et al., 1997); (Balch et al., 2009; Brauer et al., 2010; Faries et al., 2013), the risk of melanoma recurrence is never entirely removed and melanoma may recur at any point in time. As such, our central aim of this project was to develop a tool that could be used regularly, inexpensively and non-invasively to monitor early stage melanoma patients for melanoma recurrence.

To do this we developed a novel assay to detect the two most common *TERT* promoter mutations found in melanoma tumours using ddPCR. *TERT* promoter mutations occur in melanoma as frequently as (Griewank et al., 2014), or more frequently (Nagore et al., 2016a) than *BRAF* mutations, and yet mainly *BRAF* mutant-specific cfDNA is being used to monitor melanoma patients for response to therapy and disease progression (Santiago-Walker et al., 2015). The inclusion of *TERT* promoter mutations within ctDNA for monitoring would increase the number of patients to 70-80% for whom ctDNA could be used to determine disease status, particularly amongst *BRAF* and *NRAS* wild-type melanoma patients. This will enable large studies on the clinical utility of ctDNA monitoring to provide evidence of the efficacy of this marker for determining disease progression, to inform cessation of ineffective therapies (Girotti et al., 2015; Gray et al., 2015) and to guide alternative therapy. With the *TERT* assay designed, we demonstrated the superior sensitivity of ddPCR to detect *BRAF*, *NRAS* and *TERT* promoter mutations in tumour tissue, even at low tumour content., offering research laboratories an alternative option where macro-dissection is unavailable to them and an alternative option in the clinical setting when macro-dissection is not possible, such as sentinel lymph node biopsies and fine needle aspiration biopsies of metastatic sites.

Furthermore, we detected ctDNA in early stage melanoma patients suffering with disease recurrence and, we recognised a strong correlation between the level of ctDNA and MTB. Overall these results underscore that ctDNA closely reflects disease burden and aggressiveness of melanoma. Further increasing the sensitivity and specificity of our ddPCR assays will improve the ability of ctDNA to detect a lower burden of disease.

Finally, recurrent melanoma is an issue of significant public health importance and there is a great need for a way in which melanoma patients can be regularly, inexpensively and non-invasively monitored, in order to improve survival. Here we present a new and innovative ddPCR *TERT* mutation assay and validate circulating tumour DNA as a biomarker for the early detection of recurrent melanoma. Our results serve as a strong rationale in support of large prospective studies/clinical trials that will validate the clinical utility of ctDNA monitoring in melanoma patients.

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# Appendix 1

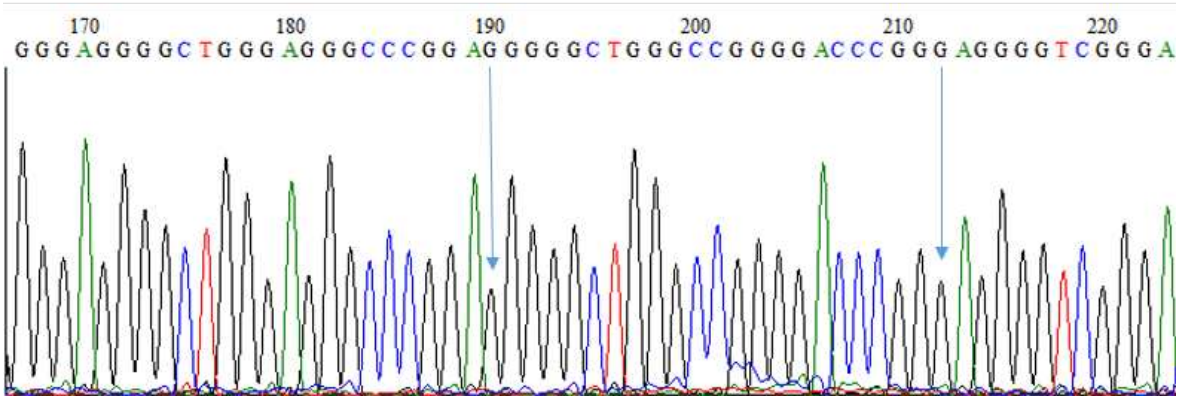


Figure 1: Sanger sequencing chromatogram from cell line HGA