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Genetic Markers in Circulating Tumour Cells as a Measure of the Metastatic Propensity of Uveal Melanoma

Master of Science (Human Biology)

Aaron Beasley

School of Medical and Health Sciences
Edith Cowan University
2017

Supervisors:
Doctor Elin Gray
Professor Melanie Ziman
Abstract

**Background:** Uveal melanoma (UM) is an extremely aggressive disease with approximately 50% of patients developing incurable metastatic disease. Therefore, accurate prognosis of a patient is necessary for closer follow up and the earlier implementation of systemic adjuvant therapies in those most likely to develop metastatic disease. Fortunately, UM can be classified into two distinct molecular classes based on clinically validated gene expression profiling, chromosomal aberrations and specific driver mutations, which accurately predict the metastatic propensity of the primary tumour. However, genetic testing currently requires biopsy of the eye which can lead to serious complications including permanent blindness. Therefore, an alternative source of primary tumour genetic material is needed to avoid these complications.

**Aims:** We proposed that circulating tumour cells (CTCs) are a viable source of tumour genetic material in which patient prognosis could be analysed. Firstly, we aimed to increase the sensitivity of an immunomagnetic enrichment protocol to capture CTCs. Secondly, we aimed to evaluate whole genome amplification methods for accurate single cells analysis to determine the genomic profile of UM cells. The combination of both aims would allow the use of UM CTCs for determining disease prognosis from an easily accessible blood sample.

**Methodologies:** **Aim 1 - To refine and evaluate methods for multi-marker immunomagnetic capture of UM CTCs.** A tissue microarray (TMA) was created from 1mm cores taken from archived primary UM tissue. Normal tissue and cutaneous melanoma were added as controls. The TMA was stained by immunohistochemistry (IHC) for melanoma, melanocyte, and stem cell markers. Stained tissue was assessed to determine intensity and coverage of staining. In addition to primary UM tissue, five UM cell lines were assessed for the same markers using flow cytometry and immunocytochemistry. Given their high level of staining of UM, 5HT2B, ABCB5, surface gp100 (BETEB), MCAM, and MCSP were coated to immunomagnetic beads and used to determine the retrieval rate of UM cell lines cells spiked into peripheral blood mononuclear cells at a known quantity. CTCs could be detected by immunofluorescent staining of MART1, gp100, and S100β.
**Aim 2 -** To develop methodologies for the detection of genetic markers of metastatic propensity using single UM cells. Single UM cell line cells plus respective bulk genomic DNA whole genome amplified and bulk genomic DNA were amplified using PicoPlex and Repli-G WGA kits to determine each kits’ respective viability of detecting CNVs using low-pass (0.01-0.1x) whole genome sequencing (WGS) on the IonPGM platform. Peripheral blood mononuclear cells (PBMCs) were used as negative controls. In addition, we tested if these methods allowed accurate CNV data after fixation, permeabilisation, and immunostaining. After ensuring cell processing had no significant effects on genomic profile of single cells, blood samples from patients were processed to isolate CTCs from PBMCs. Isolated CTCs were then whole genome amplified using PicoPlex and shallow sequenced using the IonPGM system.

**Results:** We validated several melanoma, melanocyte, and stem cell markers which have been previously shown to be expressed in cancer, cutaneous melanoma, or UM. We found that 5HT2B, and ABCB5, surface gp100 (BETEB), MCAM, and MCSP were highly expressed in primary UM tissue or UM cell lines and were able to immunomagnetically capture UM cell line cells. Concurrently, we validated the use of shallow (0.01x-0.1x depth) whole genome sequencing of single UM cells amplified using the PicoPlex WGA Kit and found that PicoPlex offered a robust method of amplifying single cells that have undergone immunomagnetic isolation, fixation, staining, and capture whilst retaining the original genetic profile of the parent cell line. Upon testing this in a patient, we found a gain of chromosome 8 which is an early event in UM tumourigenesis; aneuploidy of chromosome 8 is a genetic feature that may, with the aid of future studies, delineate patient metastatic risk.
Conclusion: Herein we validated several melanoma, melanocyte, and stem cell markers to increase the rate and number of CTCs captured from patients with primary UM. We then further validated the use of shallow (0.01-0.1x) WGS to detect CNVs in single cells amplified using PicoPlex. We have optimised and validated a pipeline, involving separation of CTCs from PBMCs via a multi-marker immunomagnetic captured method targeting 5HT2B, ABCB5, surface gp100 (BETEB), MCAM, and MCSP and detection via immunostaining of MART1, gp100, and S100β. Once isolated and immunostained, single CTCs are captured, whole genome amplified using PicoPlex, and sequenced on the IonPGM system to detect CNVs relevant to patient prognosis. This easier, more accessible way of detecting patient metastatic risk may enable patients to enrol in clinical trials, allow doctors to prescribe potential adjuvant therapies, and facilitate closer follow-up.
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:

[Signature]

Date:

[Date]
Acknowledgements

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To my dearest girlfriend, thank you for all your support and love through my Masters. My journey through this would not nearly have been as good as it was without you.
P.S. I owe you lots of dates.
Statement of Contribution of Others

Aaron Beasley conducted the experiments, analysis of results, and writing of papers included in the thesis. Timothy Isaacs and Fred Chen recruited patients into the study, and collected blood samples and clinical data. Tersia Vermeulen assisted with analysis of IHC results and supplied archived tumour for the TMA. Jacqueline Bentel processed UM samples for MLPA data, and assisted in the writing of Paper 1. James Freeman processed and counted CTCs. Michelle Pereira processed patient plasma and conducted ddPCR to detect ctDNA. Karen Shakespeare assisted in data collection. Bob Mirzai from Wendy Erber’s laboratory constructed the TMA from tissue provided, supplied control tissues and technical advice. Leslie Calapre assisted in IHC and ICC. Richard Allcock provided specialised technical advice, performed WGS and analysed derived data. Melanie Ziman was the secondary supervisor, provided overall supervision and reviewed the writing of the thesis. Elin Gray was the primary supervisor, provided general guidance throughout the Masters degree and review of the thesis.
List of Publications

1) Analysis of Circulating Tumour Cells in Early Stage Uveal Melanoma: Evaluation of Tumour Marker Expression to Increase Capture.
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Manuscript submitted

2) Next-Generation Sequencing of Single Uveal Melanoma Circulating Tumour Cells as an Alternative Method of Deriving Patient Prognostic Information
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3 Perth Retina, Subiaco, WA, Australia
4 Lions Eye Institute, Nedlands, WA, Australia
Manuscript in Preparation
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1.0 Introduction

Uveal melanoma (UM) is the most common intraocular malignancy in adults (McLaughlin et al., 2005). In Australia, it affects approximately 8.55 individuals per million per year (Vajdic et al., 2003). After detection of metastasis, 92% of patients die within two years. Moreover, approximately 50% of those diagnosed with localised UM develop incurable metastatic disease (Kujala et al., 2003). Although the treatment of localised UM has improved over the last 30 years, current treatment has little effect on the development of metastatic disease and survival rates have not changed (Singh et al., 2011).

To improve patient outcomes, detection of those with the highest risk of developing metastasis is necessary to implement early treatment interventions. Many cytogenetic features are able to accurately predict UM patient prognosis and propensity of the tumour to metastasise (Aalto et al., 2001; Harbour et al., 2010; Martin et al., 2013; Onken et al., 2004; van Raamsdonk et al., 2009; van Raamsdonk et al., 2010). However, current genetic analysis of the tumour requires direct biopsy of the eye, which can lead to serious complications such as retinal detachment, vitreous haemorrhage, and permanent blindness (Pereira et al., 2013). An alternative source of tumour genetic material can be found in circulating tumour cells (CTCs) which may allow analysis of the genetic determinants of metastatic propensity by means of a ‘liquid biopsy.’ However, current methods for the determination of these markers of metastatic disease in UM CTCs need to be developed and evaluated. Here we evaluated markers for UM CTC isolation and detection, as well as developed methods to determine genetic aberrations within single UM CTCs, to produce a robust protocol for determining the metastatic propensity of patients with primary UM tumours from a blood test.
2.0 Literature Review

2.1 Structure of the Eye and Uvea

The eyes are complex, fluid filled organs. They average 24mm in diameter (Martini et al., 2015) and resemble a small irregular spheroid. Three layers of tissue encase the fluid filled cavity; an outer fibrous layer (sclera), an intermediate vascular layer or uvea (iris, ciliary body, and choroid) and an inner layer (retina) (Figure 1).

The uveal or vascular layer is comprised of the iris, ciliary body, and choroid. This layer is responsible for the regulation of light entering the eye, a route for blood vessels that service the surrounding tissue, secretion and reabsorption of aqueous humour, and the control of lens shape (Martini et al., 2015). More importantly, the uveal layer contains melanocytes which dictate eye colour and absorption of light (Martini et al., 2015). The
malignant transformation of these melanocytes results in the formation of UM from either the anterior (iris) or posterior (ciliary body or choroid) uveal tract, with the majority arising in the choroid (COMS-20, 2003; McLaughlin et al., 2005).

### 2.2 Uveal Melanoma Epidemiology and Clinical Prognosis

#### 2.2.1 Epidemiology

UM is the most common intraocular malignancy in adults and the second most common form of melanoma (McLaughlin et al., 2005). The incidence of UM differs worldwide. In the United States, the incidence is around 5 cases per million (Singh et al., 2011). European incidence has a north-south gradient decreasing from >8 in northern countries to <2 cases per million in southern countries (Virgili et al., 2007). In a similar fashion to cutaneous melanoma, the incidence of UM in the Australian population is higher than the rest of the world with approximately 8.55 per million for UM (Vajdic et al., 2003). The age of diagnosis of UM ranges from 6-100 years, with a median age of 62 years (Singh et al., 2011). Interestingly, patients ≤20 years of age appear to have significantly higher incidence of iris UM compared to those aged ≥21 (Shields et al., 2012). The importance of gender in UM is contested in the literature; two studies showed higher incidences of UM in males compared to females (Singh et al., 2011; Virgili et al., 2007), while a third study did not note any significant difference between the genders (Shields et al., 2012). Lastly, Caucasian populations are predominantly affected in comparison to other races and ethnicities (Chang et al., 1998; Singh et al., 2011).

#### 2.2.2 Risk Factors for Primary Uveal Melanoma

There are several factors that lead to a predisposition in the development of UM, which can be divided into host and environmental factors. Host factors may include light eye colour, fair skin colour, and the inability to tan (Weis et al., 2006). Additionally, pre-existing naevi, the naevus of Ota (Shields et al., 2015; Singh et al., 1998; Singh et al., 2005; Sumich et al., 1998), and germline \textit{BAP1} mutations (Goldstein, 2011; Ismail et al., 2014) increase the risk of developing UM. Environmental factors include exposure to ultraviolet (UV) light from arc welding as a significant risk factor, while chronic UV and occupational sun exposure are marginal factors (Shah et al., 2005); latitude and leisurely sunlight exposure are not significant risk factors (Shah et al., 2005).
2.2.3 Prognosis: Clinical and Histopathological Features

At the time of initial diagnosis of UM, approximately 4% of patients have detectable metastasis (Finger et al., 2005). However, metastasis of UM occurs in approximately 50% of all patients (Jovanovic et al., 2013). UM metastasises via haematogenous dissemination, with the most common site of metastasis being the liver followed by the lungs and bone (COMS-15, 2001). Nevertheless, UM has been shown to metastasise to the brain, skin and any other location within the body (COMS-15, 2001). The median time from diagnosis of metastasis to death is <6 months, with the death rate of patients being 80% within one year, and 92% within two years after detection of metastasis (COMS-26, 2005).

Large tumour basal diameter (Figure 2A) (Kujala et al., 2003) and tumour thickness (apical height) (Figure 2B) (Shields et al., 2009) are associated with the development of metastatic disease and ultimately with poorer survival (Shields et al., 2007). Similarly, the presence of extraocular extensions has also been shown to correlate with increased mortality due to its association with increased tumour malignancy and more advanced disease (Coupland et al., 2008). However, Coupland et al. (2008) noted that the size of the extraocular extensions did not have any impact on mortality. Tumour size is incorporated into the COMS staging system (Appendix 8.5) and all of the above clinical features are incorporated into AJCC staging system (Appendix 8.6). These are the two most common methods of describing the stages of UM, and aid in the determination of patient prognosis (COMS-5, 1997; COMS-17, 2001; Kaliki et al., 2015; Shields et al., 2013). Patient age at time of diagnosis is another predictive feature, with >60 years being more closely associated with metastasis and death (Shields et al., 2012). Other clinical features of UM that predict prognosis of patients are ciliary body location, tumour colour, presence of subretinal fluid and intraocular haemorrhage (Shields et al., 2009).
Figure 2: Size and Thickness of Primary Uveal Melanoma Tumours and Their Association with Overall Survival. A) Cumulative incidence estimate of melanoma mortality based on largest basal diameter of the primary tumour. Adapted from (Kujala et al., 2003), and B) cumulative survival probability graph showing the effects of tumour apical height on survival. Adapted from (Shields et al., 2009).

Histopathological characteristics of the primary tumour provide information in regards to patient outcome and tumour metastatic propensity. Cell type is one distinct prognostic factor. There are two major types of cells involved in UM, spindle (Figure 3A and B) and epithelioid (Figure 3C). The loss of cohesion, larger and rounder nucleus, coarse chromatin, eosinophilic nucleolus, occasional multinucleated cells, and higher mitotic rate are all features that distinguish epithelioid cell type from spindle cell type (McLean et al., 2004). The presence of the epithelioid cell type by itself or in a mixed morphology (Figure 3D) has a significantly worse prognosis than that of the spindle cell type (Paul et al., 1962). In addition, the prognosis becomes poorer with increasing numbers of epithelioid cells per high power field (Seddon et al., 1987). Another predictor of prognosis is tumours with higher mitotic activity (McLean et al., 1977). Ki-67 is used to determine mitotic rate and it has been noted that high levels of Ki-67 in UM are associated with shorter survival (Karlsson et al., 1996).
Vascular features are also used in prognostication of patients. The intravascular presence of tumour cells is one histopathological prognostic parameter, with UM cell growth into vessels inside the tumour and vessels outside the tumour, or in both, are associated with a poorer survival than those without (Ly et al., 2010). Another vascular parameter useful in predicting the prognosis of patients with UM is the presence of vascular networks. Folberg et al. (1992; 1993) identified nine distinct vascular patterns with the presence of vascular networks, defined by three back to back vascular loops, correlating with the highest risk of metastatic death. Microvascular density has been also found associated with the presence of microvascular loops and networks, epithelioid cells, largest basal tumour diameter, and overall survival (Makitie et al., 1999).

Lastly, the presence of tumour infiltrating lymphocytes can be used to determine prognosis. M2 macrophages (CD68+, CD168+, and CD68+ CD168+) are the primary type of infiltrating macrophage in UM and have been shown to be increased drastically in tumours with monosomy of chromosome 3. M2 macrophages are associated with ciliary body involvement, increased microvascular density and significantly poorer prognosis for
survival (Bronkhorst et al., 2011). Additionally, class I and II HLA expression are also linked to the levels of immune cell infiltration, with lower levels of HLA expression correlating with lower levels of tumour infiltrate (de Waard-Siebinga et al., 1996).

The use of these clinical and histopathological markers as predictors of patient outcomes is problematic, due to the broad variability and no unified staging system (Werdich et al., 2013). Therefore, the use of genetic markers for the analysis of UM may provide a more accurate and objective prediction of the risk of metastatic disease.

2.3 Molecular Characteristics of Uveal Melanoma

2.3.1 Gene Expression

Comparison of gene expression signatures of primary tumour lesions from patients that developed metastasis with those that did not, demonstrated that UM can be organised into two distinct molecular classes - class I or class II tumours. Each class displays distinct clinical prognostic factors (Figure 4). The largest difference in expression comes from down-regulation of chromosome 3 genes and up-regulation of chromosome 8q genes (Onken et al., 2004). While class I expression reflects that of normal neural crest derived melanocytes, class II corresponds with an up-regulation of epithelial genes, causing these tumours to exhibit epithelial features such as polygonal cell morphology, up-regulation of epithelial cadherin (E-cadherin), colocalization of E-cadherin and β-catenin to the plasma membrane, and the formation of cell-cell adhesion and acinar structures (Onken et al., 2006). Conversion to this epithelial phenotype may be a precursor of a metastatic cell type (Onken et al., 2006).

These gene expression profiles were used to develop a clinically validated test called DescisionDx-UM, incorporating 12 class discriminating genes and three endogenous controls (Appendix 8.2) (Harbour and Chen, 2013). The test has a success rate of >97% and can be performed on tissue samples obtained by fine needle biopsy, resection, and enucleation (Field and Harbour, 2014). The result of this test classifies patients into class I or II categories, and guides the intensity of metastatic surveillance and can be used to determine whether the patient should be placed directly onto adjuvant systematic therapy (Field and Harbour, 2014).
Figure 4: Prognostic Differences between Class I and Class II Tumours. Survival probability between class I and class II gene expression in 50 UM patients. All deaths were caused by melanoma metastasis. Significant differences in survival between the classes of tumour were found ($P=0.01$). Adapted from (Onken et al., 2004).

Whilst the bulk of patients who develop metastasis harbour a class II gene expression profile, there are a subset of class I tumours that also give rise to metastasis. Therefore, class I tumours are subgrouped into ‘1a’ and ‘1b’ based on the different expression of epithelial cadherin (CDH1) and Ras-related protein Rab-31 (RAB31) (Field et al., 2016). Moreover, the presence of Preferentially Expressed Antigen in Melanoma (PRAME) mRNA is differentially expressed between these two subgroups of class I tumours, with 0% 5 year rate of metastasis of Class I^{Prame-} and 38% of Class I^{Prame+} (Field et al., 2016).

The use of gene expression profiling in primary UM can accurately predict the prognosis of patients, however, it requires biopsy of the primary tumour within the eye which can lead to complications such as vitreous haemorrhage, retinal detachment, and permanent blindness (Pereira et al., 2013).

### 2.3.2 Chromosomal Aberrations

UM displays a lack of genomic instability and aneuploidy compared to other cancers (Cross et al., 2003; Papadopoulos et al., 2002). Thus, chromosomal abnormalities found consistently in UM are thought to be drivers of tumourigenesis rather than unconnected random events. Specific chromosomal abnormalities, defining distinct prognostic and metastatic risks (Aalto et al., 2001), allow for a more accurate prognosis in comparison with current histopathological measures.
Monosomy of chromosome 3 is a common genetic aberration found in UM, occurring in approximately 50% of patients (Hoglund et al., 2004). In addition, monosomy 3 is able to predict patient prognosis (Figure 5A) (Prescher et al., 1996; Shields et al., 2011; White et al., 1998), as tumours with monosomy 3 have a significantly higher rate of metastasis when compared to tumours with disomy 3 (Prescher et al., 1996; Tschentscher et al., 2001). Moreover, monosomy of chromosome 3 is also associated with clinical and histopathological features such as large tumour diameter, epithelioid cell type, ciliary body involvement, vascular loops (Prescher et al., 1996; Scholes et al., 2003) and an inflammatory phenotype (Herwig and Grossniklaus, 2011; Maat et al., 2008).

Figure 5: Worst Survival for Patients with Changes in Chromosome 3 and 8q. A) Monosomy 3 (n=43) is a significant prognostic feature when compared to disomy 3 (n=23) (P<0.0001) in patients. B) Patients with normal (n=14), gain, (n=24), or amplification (n=28) of 8q show significant differences in survival between the three categories (P<0.0001), between normal vs amplification (P<0.0001), between gain vs amplification (p=0.00125), however, normal vs gain was only trending toward significance (P=0.07). C) Lastly, monosomy 3 combined with 8q amplification (n=24) had significantly increased risk of death to metastasis (P=0.011) when compared to monosomy 3 combined with 8q gain (n=16) or monosomy 3 combined with normal 8q. Adapted from (Versluis et al., 2015).

Chromosome 8 is another non-random chromosomal aberration described in UM. Gains in copies of the long arm of chromosome 8 (G8q) can occur through isochromosome formation, unbalanced translocations, and gain of a complete chromosome 8, and are present in approximately 40% of UM cases (Hoglund et al., 2004), and are associated with reduced survival and reduced disease-free interval (Figure 5B) (Sisley et al., 1997). Aalto et al. (2001) found that 53% of metastasising primary tumours and 100% of metastases harboured gains in 8q. Additionally, deletions in 8p12-22, containing leucine
zipper tumour suppressor-1 (LZTS1), were also associated with quicker onset of metastasis, and thus higher metastatic efficiency (Onken et al., 2008). Karyotyping has shown that monosomy of chromosome 3 and gain of 8q correlate closely with metastasising UM, and tumours with a combination of both mutations exhibit a worse prognosis than those with each individual aberration alone (Figure 5C) (Dono et al., 2014; Versluis et al., 2015). Additionally, monosomy of chromosome 3 and gain in 8q in UM together with a large basal tumour diameter and extracellular matrix patterns show significantly reduced metastasis free survival (van Beek et al., 2014).

Chromosome 6 is another prognostically relevant marker in UM. Losses in 6q are associated with a poorer prognosis (Aalto et al., 2001). Inversely, gain in 6p is associated with a better prognosis (Damato et al., 2009; White et al., 1998). Additionally, the gain of 6p and monosomy of chromosome 3 appear to be mutually exclusive, with only 4% of cases showing monosomy of chromosome 3 and gain of 6p, although, no prognostic information was derived from this finding (Ehlers et al., 2008).

Loss of heterozygosity of chromosome 1p is an indicator of poor prognosis in tumours that also show concurrent monosomy of chromosome 3 (Hausler et al., 2005; Kilic et al., 2005). In particular, chromosome 1p36 primarily modified the survival of patients with monosomy of chromosome 3, with patients who harboured both losses having 7.8 times the risk of developing metastases (Kilic et al., 2005). Chromosome 1p loss also occurs in 40% of tumours with monosomy 3, and in 10% of tumours with disomy 3 (Hausler et al., 2005). Identification of genes on 1p31 showed reduced expression of Integrin Beta 3 Binding Protein (ITGB3BP), while expression of Phosphodiesterase 4B (PDE4B), and Interleukin 12 Receptor Subunit Beta 2 (IL12RB2) was absent in tumours with monosomy 3, indicating that these genes may influence progression of UM but are unlikely to be driver mutations (Hausler et al., 2005).

Chromosomal aberrations are powerful tools for the determination of metastatic propensity. A simple clinically validated test that is able to accurately determine copy number variations is the multiplex ligation-dependant probe analysis (MLPA), with results being highly predictive of patient survival (Damato et al., 2010; Lake et al., 2011; Vaarwater et al., 2012). However, as for gene expression profiling, biopsy of the primary tumour is required, and in addition to complications of the eye, various factors can affect
the assessment of chromosomal aberrations, particularly a tumour’s cellular heterogeneity and sampling errors, resulting in normal disomy 3 cells diluting tumour derived monosomy 3 cells (Damato et al., 2009). For example, fine needle aspiration biopsies may miss the most aggressive parts of a tumour due to the heterogeneous nature of tumour cells, impacting the results of chromosomal aberration prognostic testing techniques such as MLPA (Damato et al., 2009) or fluorescence in situ hybridisation (Chang et al., 2013; Mensink et al., 2009).

2.3.3 Genes Mutated in Uveal Melanoma

2.3.3.1 \textit{GNAQ} and \textit{GNA11}

Mutations in Guanine nucleotide-binding protein subunit α-Q (\textit{GNAQ}) and Guanine nucleotide-binding protein subunit α-11 (\textit{GNA11}) are common in UM (van Raamsdonk et al., 2010), which are found at 9q21.2 and 19p13.3 (Versluis et al., 2015). These genes encode similar Gqα subunits that consist of helical and catalytic GTPase domains (Figure 6) (Harbour and Chao, 2014).

Mutations to these genes cause the encoded receptor to be locked in an active state (van Raamsdonk et al., 2010). The constitutive activation of this receptor causes activation of the mitogen-activated protein kinase (MAPK) pathway (Bauer et al., 2009), a pathway found activated in cutaneous melanoma (Zuidervaart et al., 2005). The MAPK pathway is a key regulatory pathway that controls embryogenesis, cell differentiation, cell proliferation, and cell death (Pearson et al., 2001).

![Figure 6: GNAQ and GNA11 Protein Map and Location of Somatic Mutations in Uveal Melanoma. GNAQ and GNA11 protein domains. Commonly mutated sites in UM are indicated. Adapted from (Harbour and Chao, 2014).](image-url)
GNAQ and GNA11 somatic mutations appear to be mutually exclusive (Harbour and Chao, 2014), with 83% of UM harbouring mutations in either gene (van Raamsdonk et al., 2010). Two locations for driver mutations, within the switch regions, have been described (Figure 6) (van Raamsdonk et al., 2009; van Raamsdonk et al., 2010), with GNAQ harbouring Q209P, Q209L, Q209R (Bauer et al., 2009), or R183Q mutations (van Raamsdonk et al., 2010). Similarly, GNA11 harbours Q209L, Q209P, R183C, or R183H mutations (van Raamsdonk et al., 2010). The GNAQ and GNA11 Q209 mutations are found in approximately 45% and 32% of primary UM, and in 22% and 57% of UM metastases respectively (van Raamsdonk et al., 2010). R183 mutations in GNAQ or GNA11 are found in approximately 2% and 3% of primary UM respectively, and in 6% of metastases (van Raamsdonk et al., 2010).

Mutations in either GNAQ or GNA11 are found in all stages of UM, but show no correlation with patient survival (Koopmans et al., 2013). As further evidence for this lack of correlation, mutations in either gene can be found in benign blue naevi (van Raamsdonk et al., 2009). GNAQ and GNA11 mutations are thought to be early or initiating events in UM tumourigenesis, and are found in class I and II tumours (Harbour et al., 2013), supporting the fact that they alone are not responsible for metastasis.

2.3.3.2 PLCB4

Phospholipase C Beta 4 (PLCβ4) is located on chromosome 20p12. The encoded protein is responsible for the hydrolysis of phosphatidylinositol 4,5-bisphosphate into 1,4,5-inositol trisphosphate and 1,2-diacylglycerol, vital components of signal transduction in the light response in vertebrate retinas (Alvarez et al., 1995; Anderson and Brown, 1988). The synthesised 1,4,5-inositol trisphosphate is now free to diffuse and binds to its associated receptor causing increased intracellular Ca²⁺ levels, whilst 1,2-diacylglycerol remains associated to the membrane, and with increased Ca²⁺ levels causes the activation of protein kinase C (Lyon and Tesmer, 2013). This has been associated with cell proliferation and survival (Lyon and Tesmer, 2013), and is a downstream target of GNAQ/GNA11 (Johansson et al., 2016; Lyon and Tesmer, 2013).
Recently, deep sequencing of UM revealed recurrent mutations (D630Y) in \( PLC\beta 4 \)s highly conserved Y domain (Figure 7) responsible for transduction of extracellular signals in approximately 7% of samples. Mutant \( PLC\beta 4 \) were mutually exclusive to \( GNAQ \) and \( GNA11 \) mutations (Johansson et al., 2016).

**Figure 7**: PLC\( \beta 4 \) Protein Map and Location of Somatic Mutations in Uveal Melanoma. PLC\( \beta 4 \) protein domains with the location of the D630 driver mutation. Adapted from UniProt.

### 2.3.3.3 CYSLTR2

Cysteinyl leukotriene receptor 2 (\( CYSLTR2 \)) is found on chromosome 13q14. It is a seven transmembrane G protein coupled receptor, and known activator of GNAQ (Evans, 2002; Sakmar, 2002).

Recurrent mutations in \( CYSLTR2 \) were recently discovered in UM at codon L129 with a substitution of L129Q, located in helix 3 (H3) (Figure 8), which is part of the receptor that contacts the extracellular ligand, associates with other helixes, and interacts with the \( G\alpha \) subunit intracellularly causing its constitutive activation (Moore et al., 2016). In this same study, it was found that \( CYSLTR2 \) mutations followed the same mutual exclusivity pattern found in \( GNAQ, GNA11, \) and \( PLC\beta 4 \) and were mutated in approximately 3% of samples (Moore et al., 2016). Overall, mutations in \( GNAQ/GNA11, PLC\beta 4, \) and \( CYSLTR2 \) are mutually exclusive and are all confined to the same proliferative pathway, resulting in its constitutive activation (Figure 9).
Figure 8: CYSLTR2 Protein Map and Location of Somatic Mutations in Uveal Melanoma. CYSLTR2 protein domains and the location of L129Q somatic mutation. Adapted from UniProt.

Figure 9: Interactions between Four Common Genes with Recurrent Mutations. GNAQ/GNA11, PLCβ4, and CYSLTR2 protein interactions. Adapted from (Moore et al., 2016).

2.3.3.4 BAP1

Another commonly mutated gene in UM is BRCA1 associated protein 1 (BAP1) which encodes a nuclear ubiquitin carboxy-terminal hydrolase (UCH) (Jensen et al., 1998). The protein consists of UCH catalytic domain, BARD1 binding domain, HCF1 binding motif (HBM), BRCA1 binding domain (B), and nuclear localization sequences (NLS) (Figure 10) (Harbour and Chao, 2014).
UCHs are part of the deubiquitinating enzyme protein subfamily (Nishikawa et al., 2009). There are four known members of the UCH subfamily: UCH-L1, UCH-L3, UCH37, and BAP1 (Misaghi et al., 2009). Breast cancer 1 (BRCA1) is a tumour suppressor gene that when transcribed, helps mediate the repair of double strand DNA breaks, chromatin remodelling, cell cycle checkpoints, transcription, apoptosis, and centrosome duplication regulatory functions. BAP1 interacts with the RING finger domain of BRCA1, with the suggestion that BAP1’s carboxyl-terminus is bound to BRCA1 leaving the UCH catalytic domain free to interact with ubiquinated, or other ubiquitin like, substrates to remove them from the proteasome complex (Jensen et al., 1998). BAP1 binding to BRCA1 is shown to increase the cell growth suppressive qualities of BRCA1 (Nishikawa et al., 2009). In addition to binding to BRCA1, the BAP1 enzyme removes ubiquitin moieties from histone H2A and host cell factor 1 (HCF1) (Harbour and Chao, 2014). It is also noted that although ubiquitin can be used to direct proteins for degradation, BAP1 appears to also affect gene expression, cell cycle, and cellular identity (Eletr and Wilkinson, 2011; Harbour and Chao, 2014). The BAP1 protein is a tumour suppressor, and is found inactivated in other cancers (Misaghi et al., 2009). Most importantly, repair of double strand breaks is the mechanism by which BAP1 exerts its tumour suppressing activity (Yu et al., 2014).

Mutations affecting the protein encoded by BAP1 were found in 84% of class II metastasizing UM tumours, and cause premature protein termination or affect the ubiquitin carboxyl terminal hydrolase domain (Figure 10). Class I tumours were shown to rarely have BAP1 mutations (Harbour et al., 2010). BAP1 is found at 3p21.3 (Jensen et al., 1998), so the loss of chromosome 3 could explain the known relationship between
loss of heterozygosity (LOH) of chromosome 3 and metastasis of UM, with \textit{BAP1} undergoing inactivating mutations in one copy, and loss of the other copy by chromosomal deletions (Harbour and Chao, 2014).

\textbf{3.3.5 \textit{SF3B1}}

Splicing factor 3B, subunit 1 (\textit{SF3B1}) is located at 2q33.1 (Yang et al., 2013) and the protein consists of a U2AF2 interaction motif, PPP1R8 binding domain, SF3B14 interaction motif, and 22 non redundant HEAT domains (Figure 11) (Harbour and Chao, 2014; Wang et al., 1998). SF3B1 has been implicated more recently in UM. This gene has been shown to harbour driver mutations in its HEAT domains, consisting of single nucleotide point mutations primarily at amino acid R625 (Figure 11) (Harbour et al., 2013; Martin et al., 2013), encoding R625H (63%), R625C (27%), R625G (5%), and R625L (5%) mutations (Harbour et al., 2013).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure11.png}
\caption{SF3B1 Protein Map and Location of Somatic Mutations in Uveal Melanoma. \textit{SF3B1} protein domain with the mutation profile of driver mutations found in UM. The most common mutation site is noted by the large pin and less common mutation sites by the small bars. Adapted from (Harbour and Chao, 2014; Wang et al., 1998), and UniProt.}
\end{figure}

The mutations are usually heterozygous and are generally mutually exclusive with \textit{BAP1} mutations but occur with equal frequency in \textit{GNAQ} versus \textit{GNA11} mutated tumours. Mutations in \textit{SF3B1} are associated with class 1b tumours (Field et al., 2016), and disomy of chromosome 3 which is associated with a lower risk of metastasis (Tschentscher et al., 2001) and better survival rates (Prescher et al., 1996).
2.3.3.6 EIF1AX

Eukaryotic translation initiation factor 1A, X-linked (EIF1AX) is located at Xp22 (Martin et al., 2013). It encodes a protein responsible for the transfer of the Met-tRNA\textsubscript{f} to 40 S ribosomal subunits prior to mRNA binding to form the 40S pre-initiation complex (Chaudhuri et al., 1997). The protein consists of an N-terminal tail, oligonucleotide binding (OB) fold, and C-terminal tail (Figure 12) (Harbour and Chao, 2014).

Mutations in EIF1AX has been reported in cases of UM as hemizygous missense mutations in tumours with disomy of chromosome 3, with all mutations causing in-frame changes that affect the N-terminus of the protein (Figure 12) (Martin et al., 2013). In addition, tumours with EIF1AX mutations lacked SF3B1 mutations (Martin et al., 2013), indicating that these mutations are mutually exclusive. Furthermore, it has been shown that only mutant EIF1AX mRNA transcripts are expressed, pointing to epigenetic inactivation of the wild-type copy of EIF1AX (Harbour and Chao, 2014). As for SF3B1 mutations, EIF1AX is associated with class I tumours (Harbour and Chao, 2014), disomy of chromosome 3 (Martin et al., 2013), and better prognosis (Harbour and Chao, 2014).

**Figure 12: EIF1AX Protein Map and Location of Somatic Mutations in Uveal Melanoma.** EIF1AX protein domain with the mutation profile of driver mutations in UM. Small bars indicate small substitutions or deletions of 1-2 amino acids in the N-terminal tail. Adapted from (Harbour and Chao, 2014).

Almost 100% of UM harbour a hotspot mutation in either GNAQ, GNA11, PLC\textbeta{}4, or CYSLTR2. Although they offer no information regarding patient prognosis, they present a marker for use in methods such as droplet digital PCR (ddPCR), which is able to detect mutations found in cell-free and circulating tumour DNA (cfDNA and ctDNA respectively). Previous research has shown ctDNA correlates closely with tumour burden
(Ascierto et al., 2013; Sanmamed et al., 2015), this enables ctDNA to work as a surrogate biopsy for measuring tumour burden.

These key mutations and chromosomal aberrations assist not only with the diagnosis of UM, but also with prediction of metastatic propensity. Routine identification of these mutations may allow clinicians to determine the optimal treatment regimen for each individual patient. Examples of this are targeted therapies in cutaneous melanoma, with genetic testing of mutations in v-Raf murine sarcoma viral oncogene homolog B (BRAF) used in the determination of first-line treatment options (Spagnolo et al., 2015), and in UM many clinical trials are using genetic mutations as rational for targeted treatment therapies (Appendix 8.3).

2.4 Uveal Melanoma Treatments

Radiation or enucleation are the main treatment options for localised UM, with the majority of patients undergoing radiation treatment, saving enucleation for patients with large tumours in which radiation cannot reduce the tumour sufficiently (Yonekawa and Kim, 2012). Brachytherapy is the most common method used for treatment of choroidal melanoma (COMS-19, 2002); this method involves suturing a radioactive plaque onto the sclera, positioned behind the primary tumour for localised irradiation (Yonekawa and Kim, 2012).

Proton beam irradiation is the next most common treatment option, and unlike brachytherapy, the radiation is highly localised. Regression of tumours is shown by about six months, and regression continues following this time (Wilkes and Gragoudas, 1982). Recurrence rates are lower in patients receiving proton beam treatment (COMS-19, 2002). Radiation retinopathy leading to blindness is one complication of radiation therapy, where radiation causes endothelial damage and capillary occlusion, resulting in retinal haemorrhage, macular oedema, vascular sheathing, microaneurysms, retinal exudation, telangiectasia, retinal pigment epithelial atrophy, and cotton wool spots (COMS-30, 2009).
The use of systemic chemotherapies, to treat metastatic UM, appears to have no benefit. Combinations of lomustine, hydroxyurea, dacarbazine, Bacillus Calmette-Guerin vaccine, zinostatin, dibromodulcitol, dihydroxy anthracenedione, and aziridinyl benzoquinone have been trialled, however, no patients with metastatic ocular melanoma responded to treatment (Albert et al., 1996). As UM has a propensity to metastasise to the liver, several regional treatments of metastasis have been explored. These include liver resection (Mariani et al., 2009), chemo-embolisation (Sharma et al., 2008), and isolated hepatic perfusion (Ben-Shabat et al., 2015).

The use of therapies targeted to specific mutant proteins has been lacking until recently, due to unknown mutations in UM. However, this is now changing as a result of recent findings, with many targeted therapies entering clinical trials (Appendix 8.3) (Harbour and Chao, 2014). One example of recent advances in targeted therapies includes drugs targeting the Mitogen-activated protein kinase kinase (MEK) protein downstream of GNA11 and GNAQ (Harbour and Chao, 2014); a recent phase II trial using a MEK inhibitor extended progression free survival in patients by nine weeks when compared to standard temozolomide chemotherapy (Selumetinib, 2013). Using histone deacetylase inhibitors may be another potential therapeutic strategy. Recently, it was shown that histone deacetylase inhibitors in cells with loss of BAP1 induced morphological differentiation, cell cycle exit, and reversion to melanocytic gene expression profile in UM cell lines (Landreville et al., 2012).

Ipilimumab immunotherapy has also recently been used in the treatment of UM. A recent phase II trial of ipilimumab in advanced UM patients showed median overall survival of 6.8 months compared to median overall survival of <6 months without treatment, with 47% of patients having stable disease, and none experiencing partial or complete response (COMS-26, 2005; Zimmer et al., 2015). A retrospective study of local treatments including ipilimumab, bevacizumab and kinase inhibitors was performed at the Mayo clinic. It was found that localised therapies were the only therapies to significantly improve survival, although, the patients in the trial had better prognostic markers at diagnosis of metastasis (Moser et al., 2015).
Ipilimumab, bevacizumab, and kinase inhibitors had a positive effect on survival, but did not reach statistical significance when used in patients with metastatic disease (Moser et al., 2015). Another immunotherapy involves targeting programmed death receptor 1 (PD-1) and programmed death ligand 1 (PD-L1). A recent trial evaluated the efficacy of PD-1 blockade in patients with metastatic UM. Out of 58 patients, only 2 (3.6%) had an objective response and 5 (9%) patients had stable disease for ≥6 months. The median progression free and overall survival were 2.6 and 7.6 months respectively (Algazi et al., 2016). The poor response to immunotherapies may indicate that UM lacks the immunogenicity found in other tumour types susceptible to immunotherapies. A recent study investigated the immunologic composition of UM metastasis, and found that the UM tumour infiltrating lymphocytes were CD4+ dominant, with discovery of a small subset of UM patients harbouring more antitumour infiltrating lymphocytes, comparable to patients with cutaneous melanoma, and correlating to the level of pigmentation found in the tumour using MRI (Algazi et al., 2016). Therefore, overall outcomes may improve in patients with this small subset of immunogenic UM, if detected and treated via immunotherapies.

Patient death occurs from metastasis of the primary tumour. In addition to the treatment information described above, previous reports have evaluated the effects of treatments for patients with metastatic disease and found no compelling evidence to suggest that treatment of patients with UM provides any survival benefit (Augsburger et al., 2009). Therefore, implementation of adjuvant therapies to prevent metastasis, or to manage progression of microscopic or macroscopic metastasis may be required to improve patient outcomes.

A literature review of historical randomised and non-randomised trials for adjuvant therapies (6 therapy types, from 1990-2009) revealed no difference in survival outcomes post adjuvant therapies. However, these trials predate routine genetic testing currently used to predict patient prognosis, and may not be targeting patients at a high risk of metastasis, and therefore may be statistically underpowered (Triozzi and Singh, 2014). Current adjuvant therapy trials pre-screen patients based on the genetic profile of the primary tumour, to identify those at a high risk of developing metastases. Review of clinicaltrials.gov reveals 4 active studies (Appendix 8.4) that attempt to prevent
metastasis, and one study that aims to prevent metastatic recurrence using tyrosine kinase inhibitors, cytotoxic chemicals or through biological interventions.

Another literature review suggests that other drugs may be also be useful as adjuvant therapies, particularly histone deacetylase inhibitors (Landreville et al., 2012), DNA methyltransferase inhibitors (Alcazar et al., 2012; Triozzi et al., 2012), MAPK inhibitors (Kirkwood et al., 2012), or MEK/tyrosine-protein kinase Met (MET) inhibitors (Chattopadhyay et al., 2014), due to their effects in either in vitro studies against UM cell lines or in pre-clinical trials. It is expected that some of these therapies will be tested in clinical trial in the near future. This underscores the need for methodologies to enable stratification of metastatic risk in the majority of patients with localised uveal melanoma.

2.5 Circulating Tumour Cells

2.5.1 Features, Identification, and Capture of Circulating Tumour Cells

Circulating tumour cells (CTCs) are tumour cells that have circulated into the peripheral blood of cancer patients, mediating metastatic dissemination. There are many studies evaluating the prognostic value of CTCs in patients with a variety of cancers. In metastatic prostate cancer (de Bono et al., 2008) for example, varying stages of small-cell lung cancer (Hou et al., 2012), metastatic colorectal cancer (Cohen et al., 2008), and metastatic breast cancer (Cristofanilli et al., 2004), higher levels of CTCs were associated with significantly reduced overall survival. Furthermore, CTCs are now recommended by the American Society of Clinical Oncology for the clinical staging of breast cancer (Harris et al., 2007).

As the presence of CTCs has proven useful in determination of prognosis in other cancers, investigation of CTCs in patients with UM may be a useful prognostic marker. Originally detection of CTCs via reverse transcriptase-polymerase chain reaction (RT-PCR) was a common method, targeting Tyrosinase (Boldin et al., 2005), MART1 (Schuster et al., 2007), and gp100 (Keilholz et al., 2004) mRNA in either a single or multi-marker approach. However, RT-PCR is an indirect measure of the presence of CTCs and cannot be used to concurrently demonstrate the presence of CTCs, nor does it provide genotypic and phenotypic information of the CTCs Moreover, RT-PCR cannot differentiate between CTCs and circulating RNA, and is prone to high background and nonspecific
amplification (Kowalewska et al., 2006; Paterlini-Brechot and Benali, 2007). Therefore, immunomagnetic methods of detecting CTCs were developed to allow for the isolation of intact cells.

By targeting melanoma-associated chondroitin sulphate proteoglycan (MCSP) conjugated to immunomagnetic beads, 1-5 CTCs were detected in 50 mL of peripheral blood in 19% (10/52) of patients with primary UM (Ulmer et al., 2008). The presence of the CTCs was associated with ciliary body invasion, advanced tumour stage, tumour basal diameter >14mm, and anterior tumour localization, which are all noted features in UM progression (Ulmer et al., 2008). Similarly, another study showed that 14% (13/94) of patients with primary UM have detectable CTCs prior to treatment (Suesskind et al., 2011). However, Suesskind et al. (2011) found that there was no association between the levels of CTCs and the propensity of the tumour to metastasise over a short median follow-up of 16 months.

The use of immunomagnetic beads coated with melanoma cell adhesion molecule (MCAM) for CTC isolation, followed by detection with MCSP immunostaining to confirm CTC status, increased the rate of CTC detection (50%) in primary UM patients, with more than 1 CTC captured per 7.5mL of blood (Bande et al., 2015). Interestingly, Band et al. (2015) noted that a higher level of CTCs (3/7.5mL) was found in a patient with a larger UM that harboured extrascleral extension and epithelioid pathology. Nevertheless, a single marker approach for CTC isolation is not sufficient for detecting CTCs in all patients.

To overcome the low sensitivity of a one marker approach, a dual immunomagnetic enrichment protocol was developed. Using two antibodies recognising the melanoma markers CD63 and glycoprotein 100 (gp100) (NKI/C3 and NKI/BETEB respectively) enabled the detection of CTCs in 94% (29/31) of patients with a median cell density of 3.5 cells per 10 mL of blood, with a cell range of 0-10.2 cells (Tura et al., 2014). In a follow-up study, 91% of patients (40/44) were found to have a median cell density of 2.4 per 10 mL of blood, with a range of 0-10.2 cells (Tura et al., 2016).

More recently, filtration based protocols have been described. As CTCs are generally larger than surrounding leukocytes (Mazzini et al., 2014) they are able to be collected via size based filtration. When this method was used, UM CTCs were identified in
approximately 50% (15/29) of patients with localised disease. The presence of CTCs did not associate with any clinical or pathological parameter, however, it was found that >10 CTCs per 10 mL of blood was a negative prognostic marker of disease-free and overall survival over a 24 month period (Mazzini et al., 2014). As this method filters on the basis of size, CTCs that are too small may be missed or normal cells that are clumped together may be kept.

The levels of CTCs in the peripheral blood have provided conflicting results in the determination of patient prognosis. Independent of the prognostic value of CTC quantification, genetic testing of well characterised prognostic markers in individual CTCs may provide more reliable information. However, the rate of CTC retrieval in patients requires improvements.

**Table 1: Additional Potential Markers for Use in Immunomagnetic Capture of Uveal Melanoma Circulating Tumour Cells**

<table>
<thead>
<tr>
<th>Name</th>
<th>Site (Potential Use)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP-binding cassette sub-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>family B member 5 (ABCB5)</td>
<td>Membrane (Capture)</td>
<td>In UM, ABCB5 is expressed in both pigmented and non-pigmented cells, with ABCB5 having preferential expression at the leading edge of the tumour (Thill et al., 2011). ABCB5 is expressed in cutaneous melanoma CTCs (Freeman et al., 2012; Gray et al., 2015a).</td>
</tr>
<tr>
<td>CD271 (NGFR)</td>
<td>Membrane (Capture)</td>
<td>In UM, 3D cell culture conditions that facilitated vascular mimicry patterns expression of CD271 was observed (Valyi-Nagy et al., 2012). CD271 is also expressed in cutaneous melanoma CTCs (Freeman et al., 2012; Gray et al., 2015a).</td>
</tr>
<tr>
<td>5-Hydroxytryptamine</td>
<td>Membrane (Capture)</td>
<td>In UM, expression profiling showed 5HT2B was significantly upregulated in UM liver metastases (Zhang et al., 2014) and is one of the class discriminating genes in the DecisionDx-UM test (Harbour and Chen, 2013). Its expression in cutaneous melanoma or associated CTCs is unknown.</td>
</tr>
<tr>
<td>Receptor activator of NF-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>κB (RANK)</td>
<td>Membrane (Capture)</td>
<td>In cutaneous melanoma, RANK is involved in migration and metastasis of tumour epithelial cells (Kupas et al., 2011), and is expressed in CTCs (Freeman et al., 2012; Gray et al., 2015a; Kupas et al., 2011). Its expression in UM is unknown.</td>
</tr>
<tr>
<td>Melanoma associated</td>
<td>Membrane (Capture)</td>
<td>MageA3 is expressed in approximately 50% of cutaneous melanoma (Vourc'h-Jourdain et al., 2009) and associated CTCs (Hoshimoto et al., 2012). It has not been tested in UM or UM CTCs.</td>
</tr>
<tr>
<td>Antigen 3 (MAGEA3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nestin</td>
<td>Intracellular (Quantification)</td>
<td>Cytoplasmic Nestin staining is positive in 76% of cells, predominantly in nonpigmented cells in UM (Thill et al., 2011), and in cutaneous melanoma CTCs (Fusi et al., 2011).</td>
</tr>
<tr>
<td>Melanoma antigen recognised</td>
<td>Intracellular (Quantification)</td>
<td>Mart1 is expressed by melanoma cells and melanocytes with high levels in early stage melanosomes and associates with differentiation and melanogenesis (Zhang et al., 2013). It is expressed in cutaneous melanoma CTCs (unpublished data), and in UM CTCs (Schuster et al., 2011).</td>
</tr>
<tr>
<td>by T cells (MART1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S100</td>
<td>Intracellular (Quantification)</td>
<td>S100 is detected in cells at all stages of liver metastasis in UM (Grossniklaus, 2013). It is also a melanogenesis marker.</td>
</tr>
</tbody>
</table>

A multi-marker immunomagnetic bead approach for CTC capture and collection has been described by our lab in cutaneous melanoma (Freeman et al., 2012). This approach to CTC collection provided a considerable improvement in the sensitivity of CTC collection, in comparison with single marker approach (Freeman et al., 2012). Current literature describing markers for immunomagnetic capture of UM is limited, with studies using either MCSP (Suesskind et al., 2011; Ulmer et al., 2008), MCAM (Bande et al., 2015; Bidard et al., 2014), or gp100 (Tura et al., 2014) for isolating CTCs, and two different markers for quantification by immunostaining of captured CTCs, including MCSP (Bidard et al., 2014; Suesskind et al., 2011; Tura et al., 2014; Ulmer et al., 2008), and gp100 (Tura et al., 2014).

Although MCSP has been shown to be expressed in 95% of primary UM (Li et al., 2003), and although the specificity of MCSP is high, to improve sensitivity, targeting of additional or other markers expressed in UM CTCs may be required to improve the low rate of capture (14-19%) (Suesskind et al., 2011; Ulmer et al., 2008). Furthermore, there are several other markers that may be useful (Table 1) in the immunomagnetic capture of UM CTCs due to their common expression in either cutaneous melanoma or UM.

### 2.5.2 Genetic Features of Circulating Tumour Cells

CTCs may constitute a source of tumour DNA reflecting the genetic landscape within the primary tumour (Heitzer et al., 2013a) and have recently been used to detect tumour specific mutations (Gasch et al., 2013; Heitzer et al., 2013a; Maheswaran et al., 2008). Single colorectal cancer CTCs were detected using an immunomagnetic method, then isolated by micromanipulation and amplified via whole genome amplification (WGA). The resultant WGA-DNA was sequenced and detectable mutations in Kirsten Rat Sarcoma Viral Oncogene Homolog (KRAS) and Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (PIK3CA) were identified in metastatic cases (Gasch et al., 2013). In a follow-up study, WGA-DNA of single CTCs was analysed for tumour specific copy number variations (analysed by comparative genomic hybridisation (CGH)) and driver mutations, analysed by ultra-deep sequencing, presently identified in both the primary tumour and metastases. In addition, mutations found initially only in CTCs were also discovered at subclonal level in both the primary tumour and metastases (Heitzer et al., 2013a). Furthermore, in this same study it was shown that the average copy number
profiles from 10 captured CTCs showed similarities between the solid tumours and the CTC copy numbers (Heitzer et al., 2013a).

Similar to the above, Ulmer et al. (2004) used MCSP to immunomagnetically capture CTCs from patients with cutaneous or uveal melanomas that had either localised or metastatic disease. A single UM CTC was isolated by micromanipulation and analysed by CGH. Genetic abnormalities associated with poor prognosis in UM such as monosomy of chromosome 3 was detected in this cell. More recently, CTCs were isolated from patients using gp100 and CD63 followed by fluorescence in-situ hybridisation to detect chromosome 3 copy numbers. It was found that in 10/11 cases tested, the status of chromosome 3 correlated with its status in the primary tumour (Tura et al., 2016).

Adding to the evidence that UM CTCs can act as a source of genetic material, it has been shown that genetic mutations within the primary are also present within metastases (Singh et al., 2009; Trolet et al., 2009); UM is a relatively stable malignancy (Cross et al., 2003), and as UM spreads by haematogenous dissemination (Tulley et al., 2004), CTCs released into the blood may harbour these mutations. As the genotype of UM can accurately predict the metastatic propensity of the primary tumour, detection and isolation of single CTCs could allow for an accessible and accurate method of prognostication.

In addition to CTCs, circulating tumour DNA (ctDNA) has been used to provide another means of non-invasive analysis of tumour characteristics (Bettegowda et al., 2014; Bidard et al., 2014; Dawson et al., 2013). These ctDNA manifests as a result of apoptosis and necrosis of tumour tissue (Alix-Panabieres et al., 2012), and should therefore encapsulate the genetic landscape of all tumours within the body. Due to a high proportion of recurrent hot spot mutations in GNAQ, GNA11, PLCβ4, and CYSLTR2, detection of ctDNA is easily possible in UM. Bidard et al., (2014) detected ctDNA in 84% UM patients with metastatic disease, with ctDNA levels correlating with miliary hepatic metastasis, metastasis volume and number of CTCs. Detectable ctDNA correlated with a poor prognosis (Bidard et al., 2014). However, the presence of ctDNA has not been yet evaluated in patients with localised UM.
3.0 Theoretical Framework

Given that approximately 50% of patients diagnosed with localised UM develop incurable metastatic disease (Jovanovic et al., 2013) and within two years, 92% of patients with detectable metastasis will die (COMS-26, 2005). It is therefore important to determine early whether a patient will develop metastatic disease. Armed with such knowledge, clinicians can evaluate adjuvant therapies at an earlier stage to prevent the development of metastases.

Histopathological features combined with molecular analysis are now able to accurately predict prognosis. Research into the expression of genes in UM has revealed two distinct classes with distinct/specific genetic aberrations and these are significantly associated with metastatic propensity (Figure 13). Current molecular prognostic testing, however, requires sampling of the primary tumour and due to the inherent complications of direct biopsy of the eye, such as vitreous haemorrhage, retinal detachment, and permanent blindness (Pereira et al., 2013), an alternative source of tumour DNA for genetic analysis is desirable. In this regard, CTCs may provide a suitable sample with which to assess the genetic characteristics of the tumour for determining the metastatic potential of the patient’s UM. However, the methods to carry out this analysis have yet to be rigorously evaluated.

Figure 13: Key Genetic Events in Uveal Melanoma Tumourigenesis. Diagram displaying the bifurcated progression pathway and a review of the key events in UM tumour formation and metastasis described on the literature review.
3.1 Hypothesis

CTCs are a suitable source of tumour DNA for the analysis of genetic markers of metastatic propensity in patients with UM.

3.2 Aims

The overall aim of this project is to refine and evaluate methods for analysing genetic characteristics that predict metastatic propensity using CTCs from patients with UM.

Aim 1: To refine and evaluate methods for multi-marker immunomagnetic capture of UM CTCs.

Aim 2: To develop methodologies for the detection of genetic markers of metastatic propensity using single UM cells.
4.0 Analysis of Circulating Tumour Cells in Early Stage Uveal Melanoma: Evaluation of Tumour Marker Expression to Increase Capture.

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Abstract

Despite excellent local control of the primary tumour approximately 50% of uveal melanoma (UM) patients will develop incurable metastatic disease. The stratification of UM patients into groups with better or worse prognosis is based on clinicopathological and molecular features and is critical for both patient management and for directing patients towards clinical trials. However, the classification of tumours is constrained by the invasiveness of the biopsy procedure and the limited availability of tissues when enucleation is not performed. Here we evaluate the feasibility of using circulating tumour DNA (ctDNA) and circulating tumour cells (CTCs) for molecular characterisation of UM in patients with localised disease. ctDNA was quantified using droplet digital PCR for detection of GNAQ and GNA11 Q209L mutations. Only 19% (5/27) of cases had detectable ctDNA. CTCs were immunocaptured from the blood of 23 primary UM patients by targeting the melanoma-associated chondroitin sulphate proteoglycan (MCSP). This resulted in the isolation of 1-37 CTCs from 15 (65%) patients, an inadequate efficiency for diagnostic use. As previous studies of cutaneous melanomas had indicated greater capture rates by targeting multiple rather than single markers, the expression of uveal and cutaneous melanoma specific markers was examined in UM primary tumours and cell lines. We found heterogeneous expression of common markers used for capturing CTCs in the tissue microarray (TMA) and cell lines. Markers such as MCSP, MCAM, or surface gp100 were heterogeneously expressed at a moderate to high level. In contrast 5HT2B and ABCB5 showed higher expression in tissue than in cell lines and MCSP was absent in the TMA but present in cell lines. This indicates that due to the heterogeneous nature primary UM, targeting of multiple surface markers and cytoplasmic markers would improve capture and detection of CTCs in patients. Immunomagnetic capture of UM cells using MCAM, MCSP and surface gp100 (BETEB) provided high recovery rates of UM cells. These findings suggest that combinations of antibodies would increase the efficacy of CTC capture in UM patients, allowing for molecular analysis to derive critical prognostic information.
Introduction

Uveal melanoma (UM) is the most common intraocular malignancy and the leading cause of death due to primary intraocular disease in adults (McLaughlin et al., 2005). Despite successful control of the primary tumour, incurable metastatic disease will ultimately develop in up to 50% of patients (Blum et al., 2016). Extensive analysis of primary UM has defined molecular features of the tumour cells that predict, with a high degree of accuracy, a patient’s risk for development of metastases. Biomarkers of poor prognosis include histopathological features of the tumour, loss of chromosome 3, gain in chromosome 6p and 8q (Damato et al., 2010), as well as the differential expression of marker genes panels that include well-characterised cancer-associated factors such as CDH1 (epithelial cadherin; E-cadherin), 5HT2B (5-hydroxytryptamine (serotonin) receptor 2B) and EIF1B (eukaryotic translation initiation factor 1B) (Harbour et al., 2013). Although distinct biomarker profiles have been validated for personalised patient management, invasive surgical procedures with significant risk of sight threatening complications are required in order to obtain sufficient tumour tissue for molecular analysis (Pereira et al., 2013). Routine implementation of less invasive strategies would enable early detection of metastasis and/or implementation of pre-emptive treatment strategies.

Given that metastasis in UM arises from haematogenous dissemination, investigation of circulating tumour cells (CTCs) and circulating tumour DNA (ctDNA) could provide a unique opportunity for genetic analysis of the patient’s tumour through a simple and safe blood test. The high proportion of recurrent hot spot mutations in GNAQ and GNA11 allows the detection of ctDNA in UM by droplet digital PCR (Versluis et al., 2015). Bidard et al. (2014), detected ctDNA in 84% UM patients with metastatic disease, with ctDNA levels correlating with miliary hepatic metastasis, metastasis volume and number of CTCs. Detectable ctDNA correlated with a poor prognosis (Bidard et al., 2014). However, the presence of ctDNA is yet to be evaluated in patients with localised UM. On the other hand, circulating tumour cells (CTCs) have been previously detected early stage UM (Suesskind et al., 2011; Ulmer et al., 2008). CTCs have shown to constitute a source of tumour DNA that reflects the genetic landscape of the primary tumour (Gasch et al., 2013; Heitzer et al., 2013a; Maheswaran et al., 2008). Thus, it would be possible that CTCs can be used to detect tumour specific mutations and chromosomal copy number
variations that predict the risk of metastasis for individual UM patients. To achieve this, CTCs need to be efficiently isolated in early stage UM cases.

Current methods for isolating UM CTCs involve immunomagnetic capture and size based filtration, however the well-documented heterogeneous nature of UM cells is likely to complicate the successful isolation of CTCs from all patients. Immunomagnetic capture of UM CTCs by targeting the melanoma-associated chondroitin sulphate proteoglycan (MCSP (also known as CSPG4, NG2)) protein was shown to successfully detect CTCs in patients with primary disease, detecting CTCs in 19% (1-5 CTCs, median = 2.5 CTCs, per 50 mL of whole blood) (Ulmer et al., 2008) and 14% (1-8 CTCs, median = 1, per 50 mL of whole blood) of patients (Suesskind et al., 2011). Bidard et al. detected UM CTCs in only 30% of patients with metastatic disease using the CellSearch system (Janssen Diagnostics) which targets the melanoma marker MCAM (melanoma cell adhesion molecule) and stains for MCSP (Bidard et al., 2014). However, a method using dual marker enrichment protocol targeting CD63 (NKI/C3) and glycoprotein 100 (gp100, NKI/BETEB), allowed for the detection of CTCs in 94% of patients with primary UM (Tura et al., 2014). Thus, a multi-marker approach may be key to enriching the capture of CTCs from the majority, if not all, UM patients. In fact, our previous study in metastatic melanoma showed that targeting multiple melanoma specific membrane proteins resulted in the enrichment of a larger number of CTCs (Freeman et al., 2012).

Here we initially evaluated the blood of primary UM patients for both: the number of CTCs immunomagnetically captured using a single marker (MCSP) and the level of plasma ctDNA. To enable greater efficacy and accuracy of capture of CTCs from UM patients, we then systematically analysed the expression of several markers in a primary UM tumour microarray and in five UM cell lines. Furthermore, we tested these markers alone and in combination for their capacity to immunomagnetically capture UM cells spiked into peripheral blood.
Materials and Methods

Patients

UM patients from the Lions Eye Institute and Royal Perth Hospital, Perth, Western Australia were enrolled in the study between March 2014 and August 2016. UM was diagnosed by clinical and ultrasound examination performed by a specialist ophthalmologist to evaluate the size and location of the intraocular tumour including the presence of ciliary body involvement. Written informed consent was obtained from all patients under approved Human Research Ethics Committee protocols from Edith Cowan University (No. 11543) and Sir Charles Gardner Hospital (No. 2013-246), Western Australia. Peripheral blood samples were taken prior to radiation plaque insertion or enucleation. For CTC quantification, blood was collected in Vacutainer K2 EDTA tubes (BD Biosciences), stored at 4°C, and processed within 24 hours. Plasma was isolated by double centrifugation at 1600g for 10 min and stored at -80°C.

Antibody-Bead Coupling

Antibodies (BD Biosciences, Table S1) were covalently bound to magnetic beads using a Dynabead Antibody Coupling Kit (Invitrogen) following manufacturer’s instructions. 10µg of antibody was used per mg of Dynabeads.

Circulating Tumour Cell Capture and Quantification

Peripheral blood mononuclear cells (PBMCs) were isolated from blood by density gradient centrifugation over Ficoll-Paque (GE Healthcare) and resuspended in 1mL MACS buffer (0.5% bovine serum albumin (BSA), 2mM EDTA in PBS, pH 7.2) prior to the addition of 5µL of MCSP coated immunomagnetic beads. Cells and beads were incubated at 4°C for one hour with rotation. Using a DynaMag-2 magnet (Life Technologies), bead-captured cells were washed 3 times with MACS buffer, and then fixed with 4% paraformaldehyde for 10 minutes at room temperature (RT). Cells were washed once in PBS, incubated in PBS containing 10% normal donkey serum (NDS), 1% BSA, and 0.2% Triton-X100 (TX-100) for 15 minutes then incubated for 1 hour at RT with anti-MART1/gp100/S100β and with anti-CD45-PE antibodies diluted in PBS/1% NDS/1% BSA/0.1% TX-100. After incubation, cells were washed in PBS/1% BSA/0.1% TX-100, and incubated in 1:500 donkey anti-rabbit Alexa Fluor IgG 488 (Abcam) for 30 minutes at RT and placed on a magnet for 2 minutes. The resulting pellets were washed
3 times with PBS/1%BSA/0.1%TX-100, resuspended in PBS, then mounted using Prolong Gold Anti-Fade reagent with DAPI (Life Technologies). Slides were stored at 4°C, visualised and scanned using the Eclipse Ti-E inverted fluorescent microscope (Nikon®). Stained cells were analysed using the NIS-Elements Analysis software, version 4.2 (Nikon®, Japan). CTCs were defined as nucleated cells (DAPI positive) that were positively stained for gp100, MART1 and S100β, and negatively stained for CD45.

**Circulating Tumour DNA Quantification**

Cell free DNA (cfDNA) was extracted from 1-5 mL of plasma using a QIAamp Circulating Nucleic Acid kit (Qiagen) according to the manufacturer’s instructions and stored at -80°C. ctDNA was quantified using droplet digital PCR using PrimePCR ddPCR Mutation Assays (BioRad) for GNAQ Q209L and GNA11 Q209L. These assays are also able to detect GNAQ/GNA11 Q209P mutations (Versluis et al., 2015). Droplets were generated using an Automated Droplet Generator (BioRad), amplified using a C1000 Touch Thermal Cycler (BioRad) and analysed using a QX200 system (Bio-Rad). A mutation-positive control, a healthy (wild-type) control and a no template control were included in each run. QuantaSoft version 1.6.6 analysis software (Bio-Rad) was used for data acquisition and analysis. Only tests providing more than 10,000 droplets were used for analysis. The number of copies of mutated DNA per 20 µL reaction was extrapolated to calculate copies per mL. Samples derived from the plasma of healthy individuals were used to determine the specificity of each assay (Table S2).

**UM Specimens and Tissue Microarray Construction**

Formalin-fixed, paraffin-embedded (FFPE) blocks from 10 primary choroidal UM treated by enucleation between 2012-2014 at Royal Perth Hospital, Western Australia, were used to construct the tissue microarray (TMA). A waiver of consent was obtained for all archived tissue blocks under approved Human Research Ethics Committee protocols from Edith Cowan University (No. 12593), Western Australia. The TMA was generated using the TMA Master Tissue Microarrayer (3DHistech). Duplicate (8 patients) and quadruplicate (2 patients) 1 mm cores were taken from areas with high tumour content designated by a pathologist. Non-UM control tissues were obtained from FFPE cutaneous melanoma and normal tonsil, liver, lung, breast, and skin.
Multiplex Ligation-Dependant Probe Amplification for Detection of Copy Number Variants

To determine chromosomal copy number variations (CNVs), DNA was extracted from FFPE UM specimens by proteinase K digestion and purification through spin columns (Qiagen). For MLPA analysis, 50-120ng DNA was analysed using a SALSA P027-C1 UM probemix kit according to the manufacturer’s instructions (MRC Holland, Amsterdam, Netherlands). Samples were separated by capillary electrophoresis on an ABI-3730XL DNA Analyzer (Applied Biosystems) and analysed using Coffalyser software (MRC Holland) to determine copy number changes on chromosomes 1p, 3, 6 and 8.

Immunohistochemistry

Immunohistochemistry was performed on 4 µm sections cut from the FFPE TMA block. Sections were deparaffinised in xylene followed by rehydration in graded ethanol for 3 minutes each then washed in running dH2O for 1 minute. Antigen retrieval was performed in an 850W microwave oven for 15 minutes on 100% power in sodium citrate pH 6.0 buffer (gp100, MART1) or EDTA pH 8.0 buffer (MCAM, Nestin, ABCB5, RANK, 5HT2B, S100β, MCSP). Slides were then cooled for 8 minutes in running dH2O, permeabilised in 0.025% TX-100 in TBS (50 mM Tris-Cl, 150 mM NaCl, pH 7.6) for 20 minutes then immunostained using an EnVision+ Dual Link System-HRP (DAB+) (Dako) according to the manufacturer’s instructions. Briefly, slides were incubated with Endogenous Enzyme Block for 10 minutes, rinsed 3 times for 5 minutes in TBS/0.025% TX-100 then incubated overnight at 4°C with primary antibody diluted in TBS/1% BSA (Table S1). The following day, slides were washed 5 times for 5 minutes in TBS/0.025% TX-100, incubated with Labelled Polymer-HRP for 30 minutes, rinsed 3 times for 5 minutes in TBS/0.025% TX-100, incubated for 5 minutes with Substrate Chromogen, and then rinsed in dH2O. Slides were counterstained with Harris hematoxylin (Sigma) for 8 minutes, rinsed in running dH2O for 1 minute, blued in 0.2% ammonia water for 2 minutes and mounted with ProLong Gold Antifade Mountant (Thermo Fisher Scientific). Immunostaining for each marker was evaluated by two independent observers as follows: negative (0), weak (1), moderate (2) or strong (3).
Cell lines

The cell lines MM28, MP38, MP46, MP65 and MP41 exhibiting genetic profiles typical of clinical UM were kindly donated by Prof Roman-Roman from the Institut Curie, France (Amirouchene-Angelozzi et al., 2014). Cells were cultured in RPMI 1640 medium supplemented with 20% foetal bovine serum (FBS) at 37°C in a humidified 5% CO₂ incubator.

Flow Cytometry

MM28, MP38, MP41, MP46, and MP65 cells were harvested by incubation in 5mM EDTA in RPMI 1640, resuspended then washed 3 times in FACS buffer (PBS with 0.1% BSA, 25mM HEPES, 1mM EDTA, pH 7.0), incubated with primary antibody (Table S1) for 30 minutes at 4°C and washed 3 times in FACS buffer. Cells were then incubated with secondary antibody Alexa Fluor 488 conjugated donkey anti-rabbit or anti-mouse IgG (Abcam) diluted 1:500 in FACS buffer for 15 minutes at RT and washed 3 times in FACS buffer prior to flow cytometric analysis on a Gallios Flow Cytometer (Beckman Coulter) and analysed with the Kaluza software package (Beckman Coulter).

Immunocytochemistry

UM cell lines were fixed in PBS/4% paraformaldehyde for 10 minutes, washed 3 times for 5 minutes in PBS, blocked in PBS/1% BSA/10% NDS, then incubated overnight at 4°C with primary antibody (Table S1) diluted in PBS/1% BSA/1% NDS and washed 5 times for 5 minutes in PBS. Cells were then incubated for 1 hour at RT with Alexa Fluor 488 conjugated donkey anti-rabbit or anti-mouse IgG (Abcam, Table S1) diluted 1:500 in PBS/1% BSA/1% NDS, washed 3 times for 5 minutes in PBS, mounted with ProLong Gold Antifade Mountant plus DAPI (Thermo Fisher Scientific) and analysed using an Olympus BX51 microscope equipped with an Olympus DP71 camera and DP Manager Software.

Retrieval of UM Cells Spiked into Peripheral Blood Mononuclear Cells

UM cell lines were labelled with 2 µM CellTracker Red CMTPX Dye (Life Technologies) by incubation at 37°C for 30 mins and harvested using 5 mM EDTA/RPMI 1640 (Gibco). Only cells with more than 90% viability were used. Following harvesting,
50 cells (BETEB, MCAM, and MCSP) were manually captured and spiked into PBMCs, equivalent to 4 mL blood, resuspended in MACS buffer. For 5HT2B and ABCB5 capture 1000 cells were spiked by dilution. 1 µL of antibody-bead conjugate was added to the cells and incubated for 1 hour at 4°C with gentle rotation. Enriched cells were then placed on a DynaMag-2 magnet (Invitrogen), washed 3 times with MACS buffer, fixed in 4% PFA/PBS, washed once in PBS, and mounted using Prolong Gold with DAPI Antifade Mounting Medium (Invitrogen). Slides were left for 24 hrs before scanning the entire area for DAPI/CMTPX positivity. Cells that had a blue labelled nucleus, with red cytoplasm were determined to be spiked UM cells whereas blue labelled nuclei without red cytoplasm were classified as WBCs.

Results

Quantification of Circulating Tumour DNA (ctDNA) and Circulating Tumour Cells (CTCs) in Patients with Localised UM Patients

To determine whether ctDNA was detectable in patients with localised UM, plasma samples from 27 patients (clinical characteristics detailed in Table 1) were analysed for the presence of the UM associated mutations GNAQ Q209L/P and GNA11 Q209L/P. We did not gain previous knowledge of the mutational status of patient matched tumours. Instead all patient bloods were tested for these mutations as they have been reported to occur in approximately 77% of UMs (van Raamsdonk et al., 2010). We detected ctDNA in 5 of the 27 patients tested (19%, range 2-28.5 copies), 2 cases had a GNAQ Q209L mutation and 3 had the GNA11 Q209L variant. Of note, all five cases with detectable ctDNA also had CTCs (Figure 1b). On those with detectable mutations, ctDNA levels were associated with tumour size (largest basal/apical diameter) (Figure 1c and d).

To determine the UM CTC detection rate using a single target, CTCs were isolated from blood samples donated by 23 UM patients using immunomagnetic capture targeting MCSP. True CTCs were identified in this population by positive staining for MART1/gp100/S100β and negative staining for CD45 (Figure 1a). A total of 15 (65%) individuals had at least 1 CTC in 8 mL of blood, with a range of 1-37 CTCs detected, while 12 (52%) patients had 2 or more detectable CTCs. Only single cells, rather than clusters, were detected in all cases. The presence or quantity of CTCs captured using MCSP did not correlate with the tumour basal or apical size (Figure e and f). Among
the 10 cases that underwent tumour biopsy no significant difference was found between the number of CTCs and monosomy or loss of chromosome 3 in the tumour (Figure 1g).

**Analysis of Marker Expression in primary UM tumours**

Due to our moderate rate of CTC capture in patients with a single marker, MCSP, we performed a literature search to determine potential cell surface markers that could be used in combination to improve the detection rate and the number of CTCs captured and intracellular markers that could improve CTC identification.

The markers chosen included previously described targets for immunocapture or identification of UM CTCs, such MCAM (Bidard et al., 2014), glycoprotein 100 (gp100) (Hoashi et al., 2005; Tura et al., 2014; Tura et al., 2016), melanoma antigen recognised by T cells 1 (MART1) (Zhang et al., 2013), and S100 calcium binding protein β (S100β) (Harpio and Einarsson, 2004). Other molecules selected included 5-hydroxytryptamine receptor 2B (5HT2B), a membrane protein previously shown to be upregulated in class II UMs (high metastatic risk) (Onken et al., 2004) and liver metastasis (Zhang et al., 2014). ATP-binding cassette sub-family B member 5 (ABCB5) previously found to be enriched in cutaneous melanoma CTCs (Gray et al., 2015a; Schatton et al., 2008), and reported to be localised at the leading edge of UMs (Thill et al., 2011), receptor activator of nuclear factor κβ (RANK), which is involved in migration and metastasis of cutaneous melanoma (Kupas et al., 2011) and found in cutaneous melanoma CTCs (Gray et al., 2015a), and Nestin, a cancer stem cell marker (Neradil and Veselska, 2015), found primarily in non-pigmented cells in UM (Thill et al., 2011), were also assessed for expression in UM tumour tissue.

Marker expression was assessed in a tumour microarray (detailed clinical characteristics Table 2) according to the intensity of immunohistochemistry staining (Figure 2a and b). All tumour cores were assessable, with the exception of tumour specimen PUM7, where only 3 of 4 cores could be scored. Representative images of positively stained cores for each of the markers are shown (Figure 2c). Duplicate cores were generally consistent in their staining intensity and the average intensity score per protein was calculated relative to the mean intensity score of all tumours that expressed the protein. For most cases, where strong staining for an individual marker was observed, that marker was homogeneously expressed in the tumour (Figure 2c). An exception was S100β where
only a proportion of tumour cells exhibited strong expression, with positively stained cells often clustered in small areas (Figure 2c). All tumours expressed gp100 and MART1, with an average expression intensity of 1.4±0.4 and 2.5±0.7 respectively (Table 3). Other intracellular markers, S100β and Nestin were expressed at similar intensity but in a lower proportion of tumours (70% and 60%, respectively). Interestingly, ABCB5 was strongly expressed in 90% of tumours (average expression intensity 2.7±0.5). Of note, in addition to its well-characterised membranous location (Lutz et al., 2016), ABCB5 was also localised within the cytoplasm of UM cells (Figure 2c). Other markers such as 5HT2B (1.1±0.2), MCAM (1.6±0.5) and RANK (1.3±0.4) exhibited moderate levels of predominantly membrane-associated expression and were expressed in a lower proportion of tumour samples (Table 3). Interestingly, 5HT2B and MCAM expression coincide in various tumours (PUM5, 6, 10 and 9) and within areas of the tumours (Figure 2c). Lastly, MCSP was not detected in any of the UM specimens, but was strongly expressed in the cutaneous melanomas used as positive controls (Figure 2a).

Analysis of Marker Expression in Primary and Metastatic Cell Lines

We further evaluated the expression of marker proteins in cell lines derived from primary (MP38, MP41, MP46, and MP65) and metastatic (MM28) UM using flow cytometry and immunocytochemistry. Flow cytometric analysis of UM cell lines revealed high levels of expression of cell surface gp100 (BETEB), MCAM and MCSP, with differential marker expression between cell lines (Figure 3). For example, MCSP, a biomarker commonly used to capture CTCs was expressed in all cell lines except for MP41. In contrast, 5HT2B and ABCB5 were expressed in only a small proportion of the cells within each cell line, apart from MP38, which exhibited low but homogeneous staining for 5HT2B (Figure 3).

We also assessed, by immunocytochemistry, the expression of intracellular markers which can be used for identification of CTCs and found that the melanocyte markers gp100 and MART1 were uniformly expressed in all cell lines. Nestin and S100β were expressed in a more heterogeneous pattern, with Nestin exhibiting high expression in MM28, MP38, and MP41 whilst MP46 and MP65 had medium levels of expression, and S100β only expressed in a subset of cells in each cell line (Figure 4).
Immunomagnetic Isolation of UM Cell Lines

To determine if 5HT2B, ABCB5, gp100 (BETEB), MCAM, and MCSP were suitable for capturing UM CTCs, we spiked cells into the equivalent of 4 mL of isolated PBMCs. We found that immunomagnetic capture and enrichment of UM cells using individual markers could capture large proportion of the spiked cells; BETEB 32±2/50 (64%); MCAM (51%) and MCSP 33±3/50 (66%) (Figure 5). However, targeting 5HT2B and ABCB5 captured a very low percentage of spiked cells 5HT2B 5±1/1000 (0.5%); ABCB5 3.6±0.5/1000 (0.36%), consistent with the low frequency of positive cells for these markers (Figure 3). When all beads were combined a similar level of cell retrieval 59±9.5/100 (59%) was found when compared to individual beads targeting MCAM, BETEB or MCSP (Figure 5).

Discussion

Prognostication and routine monitoring of UM patients constitute essential components of disease management that are hampered by the limited availability of primary tumour tissue, resulting from the preferred use of brachytherapy with sight-conserving surgery, and a lack of sensitive validated tests to detect minimal residual disease or early disease recurrence. As up to 50% of UM patients will develop metastatic tumours following short or very long latency periods (Kujala et al., 2003), the ongoing monitoring of patients should ideally involve relatively non-invasive procedures that minimise the high cumulative radiation exposure associated with repeated scans. Analysis of CTCs, both to stratify UMs into low or high risk categories and to monitor disease recurrence or progression potentially fulfils these criteria, pending the development of robust CTC capture and test methods.

As shown in this study, ctDNA is not commonly detectable in blood of patients with localised UM. In contrast, most metastatic UM cases we have tested have detectable ctDNA (data not shown), consistent with the report by Bidard et al. (Bidard et al., 2014). Thus, ctDNA monitoring using sensitive methodologies like ddPCR might be useful for early detection of metastatic disease. In addition to GNAQ and GNA11 mutations, other ‘hotspot’ mutations have been identified in primary UM, such as PLCβ4 D630Y (7%) and CYSLTR L129Q (3%) (Johansson et al., 2016; Moore et al., 2016). The inclusion of
assays to test for these mutations would increase the utility of ctDNA for monitoring of disease burden.

To enrich for CTCs, a single marker has been used in patients with UM and this has typically resulted in low numbers of patients having detectable CTCs. Previous reports using MCSP to capture CTCs attained a detection rate of 14-19% in UM patients with non-metastatic disease, with 1-5 CTCs detected in 50 mL of blood (Suesskind et al., 2011; Ulmer et al., 2008). Although when we used the same single marker (MCSP) to capture CTCs, our method of immunomagnetic isolation achieved a slightly higher detection rate than previous reports, with 1-37 CTCs detected in 65% of cases, a result that may be accounted for by differences in the isolation and detection methods employed, such as immunostaining with multiple well expressed melanoma markers, and direct conjugation of antibodies to immunomagnetic beads. In other studies, that used different markers to capture CTCs, diverse rates of detection have been reported. For example, a study using CellSearch® (MCAM based capture) achieved a detection rate of 50% in patients with non-metastatic disease, with 1-3 CTCs detected in 7.5 mL of blood (Bande et al., 2015). More recently, Tura et al., (2014) showed that by targeting multiple markers NKI/C3 and NKI/BETEB (CD63 and gp100) a CTC capture rate of 94% was able to be achieved in patients with non-metastatic disease, with 1-10 CTCs detected in 10 mL of blood. Consistent with these results, our laboratory has previously shown that a multi-marker immunomagnetic enrichment protocol captured a higher number of cutaneous melanoma CTCs by targeting four markers (Freeman et al., 2012).

To investigate potential markers for use for CTC capture, a panel comprising, 5HT2B, ABCB5, surface-gp100 (BETEB), MCAM, and MCSP was chosen based on the known roles of these factors in UM (Bande et al., 2015; Harbour and Chen, 2013; Thill et al., 2011; Tura et al., 2014; Ulmer et al., 2008). Surprisingly, MCSP, which we and others have successfully used to capture CTCs, was not expressed in any of the primary human UM tumour specimens although it was strongly expressed in all but one of the UM cell lines. Similar results of MCSP variable expression have been previously reported on other UM cell lines (Cools-Lartigue et al., 2008). MCSP is highly expressed in cutaneous melanomas, and although its expression is not well-characterised in UM, a previous study has described its expression in approximately 95% (18/19) of primary UM tumours (Campoli et al., 2004; Li et al., 2003). Reasons for the apparent lack of concordance
between MCSP immunohistochemistry results and both our successful use of MCSP antibodies to capture UM CTCs and the previous report of Li et al. (2003) are unknown. However, it is interesting to note that the MCSP antibody clone (9.2.27) used in our CTC capture protocol and in the study of Li et al (2003) were identical and differed from the antibody clone used for immunohistochemical detection of MCSP. Another explanation for our lack of apparent immunostaining of MCSP in our UM specimens may be due to improper storage conditions or length of storage (Ramos-Vara et al., 2014; Xie et al., 2011). It is unknown the specific effects of these conditions on MCSP. However, we should note that other markers were positively stained in the same tumour samples.

Of the other markers tested, the well-characterised melanoma antigens, MART1 and gp100 were expressed in all our UM tumours, supporting previous studies of their detection in UM specimens and their potential inclusion in marker panels to capture UM CTCs (de Vries et al., 1998). Similarly, S100β was widely expressed but in a lesser proportion (70%) of our UM specimens, while MCAM, which was previously reported to be expressed in all of a cohort of 35 specimens (Beutel et al., 2009), was expressed in just 4 of our 10 UM specimens. TMAs provide a useful means to evaluate biomarker expression, however the relatively small areas of tumour tissue that are able to be evaluated in 1mm cores, may result in underestimation of antigen expression if the immunohistochemical staining pattern is heterogeneous. For example, heterogeneous MCAM expression in UM described in a previous report (Beutel et al., 2009) may have contributed to the lower proportion of MCAM-expressing tumours identified in the present study.

A previous study has shown that 5HT2B expression is strongly associated with class II UM (Onken et al., 2010) and that its expression is significantly upregulated in UM liver metastases (Zhang et al., 2014). Although the role of 5HT2B in metastatic progression of UM or promotion of metastatic tumour growth is unknown, inclusion of 5HT2B in biomarker panels for CTC isolation may facilitate capture of class II UM cells.

An interesting finding was that ABCB5 was expressed at elevated levels in a high proportion (90%) of UM specimens in a predominantly cytoplasmic localisation. This contrasts with the sporadic expression of ABCB5 in cutaneous melanoma tumours (Frank et al., 2005; Gray et al., 2015a). ABCB5, is a cancer stem cell marker (Schatton et al.,
2008) over-expressed in cutaneous melanoma CTCs (Gray et al., 2015a; Ma et al., 2010). As class II UM significantly correlates with the gene expression profiles of primitive ectodermal and neural stem cells (Chang et al., 2008), use of ABCB5 in biomarker panels to detect UM CTCs may select for cells with an increased propensity for metastasis development.

Biomarker expression was also examined in UM cell lines, which carry chromosomal losses and gains typical of human UM specimens (Aalto et al., 2001; Damato et al., 2010). Several of the markers, notably MCAM, MCSP and gp100 (BETEB) were expressed in most UM cell lines and in the large majority of cells in those cell lines. On the other hand, 5HT2B and ABCB5, which was highly expressed in human UM tissue, was weakly and sporadically expressed in the UM cell lines analysed. Discordance on antigen expression between tumour tissue and cell lines could be attributed to environmental differences, and selection or adaptation to in vitro growing conditions (Kamalidehghan et al., 2012).

5HT2B, ABCB5, surface gp100 (BETEB), MCAM, and MCSP were also tested for their ability to isolate spiked UM cell line cells into PBMCs. We found that BETEB, MCAM, and MCSP individually retrieved ~60% of UM of spiked cells, while 5HT2B and ABCB5 only managed to retrieve an extremely low number (>1%) of cells. This may be due to a combination of factors such as low expression level on UM cells or the affinity of antibodies used. However, both markers, although capturing only a small fraction of the spiked cells, had little non-specific binding to PBMCs. When each marker was combined, the retrieval rate was similar to the capture rates of BETEB, MCAM, and MCSP individually, which may indicate that as cell lines express high levels of each marker, the difference in binding capacity is minimal. The benefit of multiple markers is targeting the wide phenotypic differences in patients, where if for example, a patient lacks MCSP expression then the remaining markers will continue to ensure CTCs are isolated in these patients. Future studies will determine the detection rate yield by using this marker combination to capture CTCs in localised UM patients.

Previous reports have identified that the numbers of CTCs found in patients with localised UM do not appear to correlate with prognosis or survival outcomes (Suesskind et al., 2011; Tura et al., 2014). Similarly, CTC numbers did not correlate with known tissue prognostic markers, such as monosomy of chromosome 3 and tumour size in the small
number of cases in the present study. Therefore, our aim is not to enumerate CTCs as prognostic marker, but to capture CTCs in most patients to provide sufficient cells to analyse the genetics of the parental tumour. CTCs as a liquid biopsy may present an alternative source of tumour genetic material able to be used to investigate markers of patient prognosis, and therefore attempting to capture CTCs in almost all patients is worthy of investigation. In this regard, CTCs have been shown to constitute a source of tumour genetic material which represents that within the primary tumour (Heitzer et al., 2013a). Tura et al. (2016) showed that chromosome 3 loss, a marker of poor prognosis could be determined by fluorescence in-situ hybridization in single CTCs and that these results matched the primary tumour in 10/11 cases.

In summary, the inaccessibility of primary UM and the strong likelihood of metastatic progression of the disease following only local control of tumours, presents major challenges for pathology in the provision of prognostic marker profiles and for monitoring of progression of the disease. These issues may be overcome using liquid biopsies to analyse CTCs and ctDNA, however optimisation of methods will be required to allow findings to be translated into clinical practice. A necessary first step in this process is the determination and characterisation of biomarker panels for CTC recognition, with the present study identifying membrane biomarkers that are widely expressed in UM and which may be combined to maximize CTC capture in UM patients.

**Acknowledgements**

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<table>
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<th>Sex (M/F)</th>
<th>CTC Count</th>
<th>ctDNA Copies</th>
<th>ctDNA Mutation</th>
<th>Eye</th>
<th>Location</th>
<th>Cell Morphology (Cellular Classification)</th>
<th>Genetic Features</th>
<th>Tumour Size (Apex) (mm)</th>
<th>Largest Basal Tumour Diameter (mm)</th>
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Dash (-) where data was unavailable.
Table 2: Clinical, Genotypic, and Histological Characteristics of Patient Tissue Samples Used on the Tissue Micro Array

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<th>ID</th>
<th>Age</th>
<th>Cell Morphology (Callender)</th>
<th>TMA Cell Morphology (Callender)</th>
<th>Tumour Size Basal x Height</th>
<th>Location</th>
<th>Metastatic Disease: Interval Time (Months)</th>
<th>MLPA</th>
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<td>No convincing evidence of chromosomal loss or gain**</td>
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<td>PUM4</td>
<td>47</td>
<td>Mixed</td>
<td>Epithelioid / Mixed</td>
<td>11 x 12 mm</td>
<td>Right choroidal</td>
<td>Yes: -3</td>
<td>loss 3p, 3q, gain 8q</td>
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<td>59</td>
<td>Mixed</td>
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<td>PUM6</td>
<td>31</td>
<td>Mixed (epithelioid cells &lt;10%)</td>
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<td>Yes: 12</td>
<td>8q gain, some evidence of 3p loss, but not strong</td>
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<td>Spindle B</td>
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<td>No convincing evidence of chromosomal loss or gain</td>
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<td>PUM8</td>
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<td>11 x 12 mm</td>
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<td>Unknown</td>
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<td>Yes: 3</td>
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<td>79</td>
<td>Mixed</td>
<td>Epithelioid</td>
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<td>No: 19</td>
<td>No convincing evidence of chromosomal loss or gain**</td>
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* Age at enucleation
** Poorer quality DNA
Table 3: Average Marker Intensity Score

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<th>S100β</th>
<th>Nestin</th>
<th>5HT2B</th>
<th>MCAM</th>
<th>RANK</th>
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<td>1</td>
<td>2</td>
<td>1.5</td>
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<td>PUM1</td>
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<td>0</td>
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0 – not expressed, 1 – low expression, 2 – moderate expression, 3 – high expression
## Supplementary Table 2: Antibody Information

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<th>Use</th>
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<td>IHC, ICC, FACS</td>
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<td>Abcam</td>
<td>ab34165</td>
<td>ICC, FACS</td>
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</tr>
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<td>ab18102</td>
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<td>1:100</td>
</tr>
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<td>ab13918</td>
<td>IHC</td>
<td>1:100</td>
</tr>
<tr>
<td>S100</td>
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<td>ICC, FACS</td>
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Immunohistochemistry (IHC), Immunocytochemistry (ICC), Flow Cytometry (FACS)
Figure Legends

**Figure 1: Circulating Tumour Cell and Circulating Tumour DNA Quantification in Primary Uveal Melanoma.** a) Example of a UM CTC found in patient blood. Green fluorescence (AF488, Mel) indicates staining with a mix of antibodies against the melanoma markers MART1, gp100 and S100β; red fluorescence (PE) indicates CD45 positivity and blue fluorescence (DAPI) indicates the presence of a nucleus. CTCs were identified as green and blue positive and red negative cells. Graphs illustrate CTC count vs b) basal median diameter (n=20) or c) tumour size as apical height (n=22). d) Comparison of CTC counts in UM patients with and without monosomy in Chr3 (n=8). e) Graphs illustrate ctDNA copies/mL vs CTC count in 8 mL of blood (n=23). Blue dots indicate the presence of the GNAQ Q209L mutation and red dots indicate the GNA11 Q209L mutation. Graphs indicate ctDNA copies/mL vs f) basal median diameter (n=23) or g) tumour size as apical height (n=25).

**Figure 2: Immunohistochemistry of Uveal Melanoma.** TMA staining demonstrating a) Examples of how tumours were scored for protein localisation. Left shows strong membranous staining and right shows strong cytoplasmic staining. Representative image of positive MCSP membranous staining. b) Examples of the criteria used to measure staining intensity, from 1 indicating weak staining to 3, the most intense staining. A tissue staining negatively by immunohistochemistry (0), is also shown (left), with intrinsic melanin pigment. c) Shows a typical positive staining pattern for each of the markers analysed. Dark granular black spots are melanin deposits. All images taken at 200x magnification. Scale Bar = 200 µM.

**Figure 3: Expression of Markers in Cell Lines by Flow Cytometry.** Flow cytometric analysis of primary (MP38, MP41, MP46, and MP65) and metastatic (MM28) cell lines. Grey profiles represent negative controls using either rabbit or mouse IgGs depending on the primary antibody host

**Figure 4: Immunocytochemical Analysis of Uveal Melanoma Cell Lines.** Intracellular markers and isotype controls. All images taken at 400x magnification. Scale bar denoting 100 µm.
Figure 5: Recovery of Spiked Uveal Melanoma Cells from the Mononuclear Blood Cell Fraction. 50 cells were spiked into PBMC equivalent to 4 ml of blood. MP38 cells were used to test beads conjugated to antibodies targeting 5HT2B, MP41 were used for BETEB and MCAM, MP46 were used for ABCB5 and all beads, and MP65 cells were used for MCSP. Data represent the mean ± SD of three independent experiments.
Figure 1
Figure 2
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</table>

Figure 3
Figure 4
Figure 5
5.0 Next-Generation Sequencing of Single Uveal Melanoma Circulating Tumour Cells as an Alternative Method of Deriving Patient Prognostic Information

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Key Words:
Uveal melanoma, next-generation sequencing, whole genome amplification, circulating tumour cells
Abstract

Metastatic risk can be easily defined by mutations in chromosomes 1, 3, 6, and 8 in uveal melanoma (UM). Previous research has indicated that circulating tumour cells (CTCs) are detectable in virtually all patients, irrespective of the propensity of their tumour to metastasise. Herein we tested the ability to accurately determine copy number alterations after isolation and detection of single CTCs. We found that immunomagnetic enrichment, fixation, permeabilisation, and immunostaining caused no significant alteration to CNV detected within single UM cells after whole genome amplification using PicoPlex whole genome amplification kit followed by low-pass whole genome sequencing (WGS) using the Ion Torrent PGM system. Once optimised, we validated then used this protocol to detect a gain of chromosome 8 in a single patient with UM, a classically poor prognostic feature. Therefore, we show here that CTCs offer a non-invasive method to acquire tumour genetic material with which to assess UM patient prognosis.
Introduction

Uveal melanoma (UM) has a strong propensity to metastasize, with approximately 50% of all patients developing incurable metastatic disease. Patients at a high metastatic risk can be identified by genetic analysis of the primary tumour by gene expression analysis (Onken et al., 2010), detection of distinct chromosomal copy number variations (CNV) (Damato et al., 2010), or genetic mutations (Harbour and Chao, 2014).

Although powerful for determining patient metastatic potential and prognosis, these molecular tools require the acquisition of tumour material from the eye which can lead to several complications, including blindness (Pereira et al., 2013). Fortunately, there may be another suitable source of tumour genetic material from which patient prognosis can be derived. Circulating tumour cells (CTCs) are cells shed from a tumour and carried around the body via the cardiovascular or lymphatic system. CTCs have previously been shown to harbour similar genetic profiles to patient primary tumours (Heitzer et al., 2013a), and can be used to detect tumour specific mutations in other cancers (Gasch et al., 2013; Heitzer et al., 2013a; Maheswaran et al., 2008). In UM a single CTC has been amplified and analysed by array comparative genomic hybridisation showing abnormalities associated with poor prognosis in UM (Ulmer et al., 2004), and more recently prognosis has been derived from CTCs in patients using a modified fluorescent in-situ hybridisation technique to detect CNVs of chromosome 3. (Tura et al., 2016).

As the genotype of UM can predict the metastatic propensity of the primary tumour, the detection and isolation of single CTCs may allow for an accessible and accurate method of prognostication. However, there are several methodological hurdles that need to be overcome to produce a cost-effective assay for whole genome CNV analysis. Firstly, the processing of patient blood samples to isolate and detect patient CTCs may affect the genetic material of the cell. Secondly, as single cells do not harbour enough DNA for any meaningful genetic analysis they must undergo whole genome amplification which may also lead to discrepancies in the analysis of the genetic profile of the cell. Herein we assessed the effects of processing single UM cells (isolation, detection, capture, whole genome amplification, and low-pass whole genome sequencing) to determine the viability of CTCs as an alternative, less invasive method of accurately predicting patient prognosis.
Materials and Methods

Cell Culture

The cell lines MP38, MP41, and MP65 exhibiting genetic profiles typical of clinical UM were kindly donated by Prof Roman-Roman from the Institut Curie, France (Amirouchene-Angelozzi et al., 2014). Cells were cultured in RPMI 1640 medium supplemented with 20% foetal bovine serum (FBS), 100 units/mL Penicillin and 100 μg/mL Streptomycin (Gibco) at 37°C in a humidified 5% CO₂ incubator.

Antibody-Bead Coupling

CD146 (MCAM, P1H12, BD Biosciences, 550314), melanoma associated chondroitin sulphate (MCSP, 9.2.27, BD Biosciences, 554275), and surface gp100 (NKI/BETEB, Abcam, ab34165) were covalently bound to magnetic beads using a Dynabead Antibody Coupling Kit (Invitrogen) following manufacturer’s instructions. 10µg of antibody was used per mg of Dynabeads.

Single Cell Isolation and Processing

Cell Line: UM cell line cells were incubated in serum-depleted media for 16-18 hours to synchronise the cell cycle of all cells (Supplementary Figure 1), then at this point cells were harvested using 5mM EDTA in RPMI 1640 media. Three cells were captured and subjected to WGA. The rest of the cells were placed into 1 mL MACS buffer and 5 µL Dyna-Beads coated with anti-CD146 antibody was added, followed by a 1 hr incubation at 4°C on a roller. Cells were then placed on a magnet for 2 minutes, and washed with magnetic activated cell sorting buffer (MACS, 0.5% BSA/2mM EDTA/PBS, pH 7.2) 3 times. Cells were resuspended in 4% paraformaldehyde in PBS for 10 minutes, placed on a magnet for 2 minutes and washed in PBS 3 times. Three cells were captured at this point and subjected to WGA. The rest of the cells were blocked and permeabilised using 3% BSA/10% normal donkey serum/0.2% Triton-X 100/PBS for 15 minutes, washed once in 1% BSA/0.1% triton-X 100/PBS, and incubated with primary antibody (MART1, gp100, S100, CD45) diluted in 1% BSA/0.1% triton-X 100/PBS for 1 hr, followed by incubation with secondary antibody donkey anti-rabbit Alexa Fluor 488 (Abcam) and 10 µg/mL Hoechst 33342 (Thermo Scientific) for 30 minutes. Cells were washed 3 times in PBS. Cell suspension was placed on a glass microscope slide and analysed by fluorescent
microscopy. Single cells were picked using the MMI CellEctor (Molecular Machines) and placed into 0.2 mL PCR tubes (Eppendorf).

**PBMC:** Healthy donor blood was subjected to density gradient centrifugation via Ficoll-Paque to separate peripheral blood mononuclear cells. After 3 washes in 50 mL PBS cells were resuspended in 1mL PBS. Some cells were captured at this point and subjected to WGA. The remainder of the cells were processed as described for the UM cell line protocol above.

**Whole Genome Amplification**

Whole genome amplification (WGA) was performed using the PicoPlex WGA Kit (Rubicon Genomics) and the Repli-G Single Cell Kit (Qiagen) to manufacturer’s specifications following isolation of single cells. 1 ng of genomic DNA (gDNA) from a healthy donor, and from MP38, MP41, and MP65 cell lines was also amplified. WGA-DNA was purified using the Qiaquick PCR Purification Kit (Qiagen) and quantified using the Qubit dsDNA BR Assay Kit (Invitrogen). Whole Genome Amplified DNA was run on the High Sensitivity D1000 ScreenTape Assay (Agilent) to ensure that the majority of WGA product centred around 400bp in size. WGA-DNA was also subjected to multiplex PCR covering 100, 200, 300, and 400bp amplicons in the *GAPDH* gene as described previously (van Beers et al., 2006). WGA-DNA was regarded as high quality if the majority of WGA-DNA fragments were approximately 400bp in size and the multiplex PCR produced 4 amplicons.

**Low-Pass Whole Genome Sequencing**

One hundred nanograms of DNA was used to construct 200bp libraries made using the Ion Xpress Plus Fragment Library Kit (Life Technologies) and barcoded using the Ion Xpress Barcode Adapters 1-96 Kit (Life Technologies). Somatic mutations and CNVs were analysed using the Ion Reporter software (Life Technologies).

**Ethics and Patient Blood Samples**

All procedures have been approved by Human Research Ethics Committees at Edith Cowan University (No. 11543) and Sir Charles Gardner Hospital (No. 2013-246), and Royal Perth Hospital, Western Australia. Patient blood samples were obtained from
Royal Perth Hospital, Western Australia. The patient was diagnosed as per guidelines by
the American Joint Committee on Cancer. Patient blood samples were taken prior to
commencement of treatment. Blood was drawn by phlebotomists into BD Vacutainer K2
EDTA tubes (BD Biosciences) and processed within 4 hours.

**Patient Sample Processing**

The patient is a 59-year-old female diagnosed with choroidal UM. Peripheral blood was
drawn prior to radiation plaque therapy. After harvesting plasma, PBMCs were isolated
by density gradient centrifugation using Ficoll-Paque (GE Healthcare). Separated PBMCs
were resuspended in 1mL MACS buffer, and 2µL of Dyna-Beads coated with anti-surface
gp100, MCAM, and MCSP, was added. Cells were fixed in 4% paraformaldehyde and
immunostained as described above for UM cell lines, and analysed by fluorescent
microscopy. Cells that were green (MART1/gp100/S100β positive), not red (CD45
negative), and had a blue nucleus (Hoechst 33342) were deemed to be CTCs, and cells
that were red with blue nucleus were classified as PBMCs. Single CTCs and PBMCs were
captured using the MMI CellEctor (Molecular Machines) and deposited in 0.2 mL PCR
tubes in 2 µL volumes. Captured cells were whole genome amplified by PicoPlex and
sequenced as described above in Whole Genome Amplification and Sequencing.
Results

We selected 3 UM cell lines with multiple chromosomal CNVs (Amirouchene-Angelozzi et al., 2014), MP38, MP41, and MP65 to evaluate our methodology. Single cells were picked and subjected to WGA, i) prior to any processing, ii) after fixation using paraformaldehyde, and iii) after permeabilisation and immunostaining.

Quality Control of Whole Genome Amplified DNA

To ensure that the WGA produced high quality DNA suitable for accurate CNV analysis, WGA-DNA was analysed using TapeStation and multiplex PCR. WGA-DNA was determined to be suitable for low-pass WGS if the multiplex PCR produced 4 amplicons (Figure 1a) and if the TapeStation showed the majority of WGA-DNA to be approximately 400bp in length (Figure 1b).

Effects of the Whole Genome Amplification Process on Downstream Copy Number Variants

Firstly, we wished to ascertain whether the WGA process itself led to any significant biases during sequencing. We sequenced bulk gDNA, 1ng of gDNA subjected to WGA, and 3 single cell WGA-DNA products. Here we showed that when compared to the bulk gDNA, the gDNA-WGA (PicoPlex only) and single cell WGA-DNA from both PicoPlex (Figure 2) and Repli-G (Figure 3) do not differ significantly. Small differences were observed, e.g. the lack of a gain in 6p and 14p in MP38 cell 3 (Figure 2a), possibly due to the stochastic effect of cell culture. However, Repli-G has more noticeable spread on the copy number per bins within the chromosomes, reducing confidence in the CNV call. For example, more CNV errors were observed in Repli-G MP41 Cell 2 than in any of the PicoPlex analysed cells where the CNVs observed resembled the known values (Amirouchene-Angelozzi et al., 2014). Therefore, the process of WGA by PicoPlex introduced little bias at a WGS depth of 0.01-0.1x.

Effects of Cell Processing on Whole Genome Amplification and Copy Number Variants

Next, we wanted to test the effects of processing of cells, for the identification of CTCs in patients. To determine these effects, we analysed WGA-DNA of cells that had been
fixed, or fixed, permeabilised, and stained using both Repli-G and PicoPlex WGA methods. Repli-G did not amplify samples that had been fixed. Compared to the genomic DNA (Figure 4a), fixation in 4% paraformaldehyde did not significantly alter the PicoPlex WGA-DNA (Figure 4b). However, the full process of fixation, permeabilisation, and staining did cause some alterations to the chromosomal CNV, such as a gain in the entirety of chromosome 2 and variance in chromosome 12q, however these cells continued to correctly display the correct prognostic information, concordant with the gDNA alterations found in chromosomes 1, 6, and 8 (Figure 4b-d).

**Copy Number Variations Detected in Patient Samples**

To demonstrate the clinical application of the methodology described, we isolated CTCs from a UM patient prior to radiation plaque insertion. We detected one CTC in this patient and isolated this for further analysis. We found in a single CTC from this one patient (Figure 5), a gain of chromosome 8, a regularly amplified chromosome in poor prognostic UM.
Discussion

The majority of UM CTC studies utilise detection and quantification of CTCs to try and determine prognosis but have generally found no significance between the levels of CTCs and patient prognosis (Suesskind et al., 2011; Tura et al., 2014). We have shown similar results in our previous studies (not published). Approximately 50% of patients develop metastasis, and most patients have CTCs irrespective of the propensity of their tumour to metastasise, indicating the presence of CTCs is not the determining factor in the production of metastasis. Therefore, the ability to detect the genomic features in these CTCs may offer a greater perspective into patient metastatic risk and prognosis over quantification of CTCs. This study illustrates that immunomagnetic capture and detection of single UM cells followed by WGA and low-pass WGS (>0.01-0.1X) can accurately represent the genomic landscape of the primary tumour.

The notable differences we found in UM cells that were immunomagnetically captured, fixed, permeabilised, and stained prior to amplification with PicoPlex relative to known chromosomal arrangements (Amirouchene-Angelozzi et al., 2014), may be partially explained by the use of a later passage of MP41 compared to the initial experiments on fresh cells, as single PBMCs consistently retain their balanced karyotype. Thus, PicoPlex is a suitable choice of WGA that is able to retain the genetic profile of single cells, and is suitable for detection of CNVs associated with prognosis in UM.

The critical step in the success of using single CTCs as a surrogate for primary tissue to determine prognosis, is the choice of WGA kit. Previous studies have assessed the limitations and benefits of many different kits (Gawad et al., 2016; Huang et al., 2015; Zong et al., 2012), but we found that PicoPlex was able to amplify single cell derived DNA with only minor alterations regardless of chemical fixation via covalent cross-linking. Moreover, PicoPlex has an intermediate false positive/negative and high uniformity of coverage (Gawad et al., 2016), allowing for greater success in measuring CNVs (de Bourcy et al., 2014).

PicoPlex, when compared to the multiple displacement amplification (MDA) method such as Repli-G, produces small (~400bp) fragments. This prevents the use of some probe based detection methods for downstream analysis such as ddPCR or Ampliseq kits to detect somatic alterations in the amplified DNA as amplification sites may not be covered
by previously designed primers whereas detection of SNPs relevant to UM was possible in all successful cells amplified using the MDA method (Figure S2).

Due to the infrequent nature of fine-needle biopsies, comparison between the tumour and CTC genotypes was not possible for the single patient assessed here. Future studies would be required to confirm the relationship between genetic profile of the primary tumour and disseminated CTCs. However, our single patient CTC displayed a gain of chromosome 8 which has been regarded as an early event in tumourigenesis of UM (Ehlers et al., 2008; Hoglund et al., 2004), and may indicate the dissemination of CTCs is also an early event of UM metastasis in this patient. Tura et al (2016) revealed significant concordance between the status of chromosome 3 in the primary tumour and CTCs. Furthermore, methods that employ WGA may be able to further stratify patients with class I tumour phenotype into the more recently described class Ia or Ib owing to mutations that generally segregate into these classes (Field et al., 2016) enabling a finer assessment of patient metastatic propensity over the classical class I/II. Additionally, a small portion of metastasising UM are disomy 3. WGA-DNA may be useful in determining mutations associated with disomy 3 metastatic UM (Lake et al., 2010) or isodisomy 3 via allelic frequency. However, this assessment was beyond the scope of this study.

Previous studies have used array comparative hybridization (Ulmer et al., 2004) or fluorescence in-situ hybridisation (FISH) (Tura et al., 2016) to detect changes relevant to patient prognosis. We utilised low-pass WGS due to its effectiveness in determining chromosomal arm level alterations across the whole genome, whereas aCGH is expensive and FISH is limited to a single or a couple of loci, and orientation of cells can cause under- or overestimation of changes to CTC genomic profiles. Therefore, the use of low-pass WGS as a method of predicting CNV alterations may be more beneficial due to its lower cost, higher resolution and the lack of requirement for the correct orientation of CTCs on a slide. Nevertheless, WGS for each patient also requires amplification of a WBC as a control to properly compare CNVs in each patient, which does raise the cost per patient.

Currently, the multiplex ligation-dependent probe amplification (MLPA) technique is used to assess the copy number status of chromosomes 1p, 3, 6, and 8. The cost of each MLPA test is approximately $30 (Australian dollars). Multiple positive controls and normal DNA controls need to be run at the same time. Further increases in cost are derived
from running only 1-2 samples at a time given the low frequency of the disease and the need for a fast turnaround time. Further increases in cost are derived from running only 1-2 samples at a time given the low frequency of the disease and the need for a fast turnaround time. The cost of our methodology is higher, at approximately $190 (Australian dollars) a sample, noting that multiple CTCs are taken per patient, including a single PBMC. Although the cost of our methodology is high on a per sample basis, it reduces the reliance on a trained surgeon to acquire tissue, prevents any cost if there are complications from surgery, reduces morbidity, and provides more information about the genotype of the patient. Although beyond the scope of this study, WGA-DNA could possibly be used to detect mutations in genes that can stratify patients into more precise prognostic categories if required, or can be used to detect isodisomy of chromosomes using allelic fraction.

In summary, our study demonstrates that isolation, WGA, and low-pass WGS harbours little artefacts or aberrations and therefore the cells isolated may provide an easier and safer alternative ‘liquid biopsy’ of the patient primary tumour. Insights into the genomic characteristics of primary tumours provides crucial prognostic information that can be used to refer patients to clinical trials, allows more routine monitoring for metastatic disease, and provides opportunities for the use of potential adjuvant therapies to prevent metastatic disease.

**Acknowledgements**

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Figure Legends

**Figure 1: Quality Control of Whole Genome Amplified DNA.** a) 1 and 20 - genomic DNA ladder; 1 – positive PCR control; 3-5, 7-9, 11, 13, 15-18 – high quality WGA-DNA; 6, 10, 14 – poor quality WGA-DNA.; and 19, PCR negative control. New WGA product was made to replace the DNA in lanes 6, 10, and 14 to ensure all 4 amplicons were present before sequencing. b) Left – High quality WGA-DNA with the majority of fragments centred around 400bp in length; right – poor quality WGA-DNA with the majority of DNA fragments lower than 400bp in size.

**Figure 2: Effects of PicoPlex Whole Genome Amplification on the Genomic Landscape of Uveal Melanoma Cells.** UM cell lines MP38, MP41, and MP65 plus a healthy donor had gDNA extracted and sequenced along with gDNA amplified using PicoPlex and Picoplex WGA-DNA from 3 single cells from the respective cell line / donor, a) MP38, b) MP41, c) MP65, and d) a healthy donor. The whole genome amplified genomic DNA and individual cells all show concordance with the respective genomic DNA. Minor changes to cell line genomic profiles, such as gain in 6p and 14p in MP38 PicoPlex Cell 3 when compared to the gDNA, may be the result of the stochastic effects of cell culture rather than artefacts from the kit.

**Figure 4: Repli-G Whole Genome Amplification on the Genomic Landscape of Uveal Melanoma Cells.** UM cell line MP41 had genomic DNA extracted and sequenced a) along with DNA amplified from single cells b-d) by Repli-G. The DNA product produced was sequenced alongside MP41 genomic DNA. Each of the 3 cells displays concordance with the sequenced genomic DNA, however the sequenced cells appear to have more alterations in CNV between bins when compared to their PicoPlex alternatives (Figure 3) such as aberrant gains/losses in chromosomes 4p, 5, 8, 16, 19, and 20 in MP41 Repli-G Cell 2 (Figure 6c).

**Figure 5: Assessment of Cell Processing on Genomic Quality.** To test whether processing the cells would affect the genomic profile of the cell after amplification with PicoPlex we sequenced a) MP41 gDNA, b) 1 fixed MP41 cell, and c-f) 3 fixed, permeabilised, and stained MP41 cells and 1 healthy donor PBMC. We found that after immunomagnetic isolation, fixation in 4% paraformaldehyde caused no aberrations to the genomic profile of the cell, and that fixation, permeabilisation, and staining had minor
alterations however the prognostic information was still clearly visible, however, there were alterations in chromosomes such as 2 and 12.

Figure 6: Genomic Profile of a Single Circulating Tumour Cell Captured in a Patient with Primary Uveal Melanoma. **a)** Example of CTC captured. CTCs are heavily coated in immunomagnetic beads and fluoresce green, whereas PBMCs have few beads and fluoresce red. Image taken at 200x magnification. **b)** A single CTC found in a patient with primary UM was found after immunomagnetic isolation, fixation, permeabilisation, and staining. The CTC was captured and amplified using PicoPlex WGA and underwent low-pass WGS. The resultant genomic profile indicated a distinct gain of chromosome 8 whilst the other chromosomes remained diploid, with several other chromosomes appearing to trend toward a detectable gain.

Supplementary Figure 1: Cell Synchronisation by Serum Depletion: Histograms displaying cell cycle gating and per cent of cells in each cell cycle phase. “MP41 Control” shows ~ 12.2% in S phase whilst “MP41 Starved” with serum depletion only had ~5.2% of cells in S phase.

Supplementary Figure 2: Representative results of ddPCR histograms. GNA11 Q209L assay was tested in samples amplified by **a)** Repli-G and **b-c)** PicoPlex. Blue dots represent mutant DNA positive drops, green dots represent wildtype DNA positive drops, orange dots represent both positive for mutant and wild type DNA, and black dots represent empty droplets. Loci are more consistently amplified in Repli-G samples **a)** than PicoPlex samples **b-c)**.
Figure 1
Figure 2a

a)

MP38 gDNA

MP38 gDNA Whole Genome Amplified

MP38 PicoPlex Cell 1

MP38 PicoPlex Cell 2

MP38 PicoPlex Cell 3
b)

MP41 gDNA

MP41 gDNA Whole Genome Amplified

MP41 PicoPlex Cell 1

MP41 PicoPlex Cell 2

MP41 PicoPlex Cell 3

Figure 2b
c)

MP65 gDNA

MP65 gDNA Whole Genome Amplified

MP65 PicoPlex Cell 1

MP65 PicoPlex Cell 2

MP65 PicoPlex Cell 3

Figure 2c
d) 

Control gDNA

Control gDNA Amplified

Control SC PBMC

Figure 2d
Figure 3

a) MP41 gDNA

b) MP41 Repli-G Cell 1

c) MP41 Repli-G Cell 2

d) MP41 Repli-G Cell 3
a) MP41 gDNA

b) MP41 Fixed and Amplified

c) MP41 Fixed, Permeablised, Stained and Amplified 1

d) MP41 Fixed, Permeablised, Stained and Amplified 2

e) MP41 Fixed, Permeablised, Stained and Amplified 3

f) Control PBMC Fixed, Permeabilised, Stained, and Amplified

Figure 4
b)

Patient #1 CTC 1

Figure 5
Supplementary Data

Supplementary Method

Cell Cycle Synchronisation

Prior to isolating and capturing single cells from UM cell lines for WGA, their cell cycles were synchronised to reduce the probability of capturing cells in S-phase, which can lead to the detection of DNA imbalances. For this, cells were cultured with serum-depleted RPMI 1640 for 16-18 hours at 37°C in a humidified 5% CO₂ incubator. To evaluate that cells were synchronised we compared MP41 cells that were cultured in RPMI 1640 medium containing FBS and MP41 cells that were cultured in serum-depleted RPMI 1640 medium. MP41 cells were harvested using 5mM EDTA in RPMI 1640, washed twice with PBS, and fixed using 70% Ethanol in distilled water. Cells were incubated with propidium iodide and RNase A (Dako) for 10 minutes, washed 3 times in FACS buffer (PBS with 0.1% BSA, 25mM HEPES, 1mM EDTA, pH 7.0) prior to flow cytometric analysis on a Gallios Flow Cytometer (Beckman Coulter) and analysed with the Kaluza software package (Beckman Coulter).
Supplementary Figure 1
6.0 General Discussion

The ability to predict the metastatic risk of patients with primary UM is essential for delineation of UM patients at higher risk, to provide personalised monitoring strategies and for the testing and implementation of adjuvant therapies to prevent metastatic disease. Unfortunately, despite the availability of molecular profiling methods for accurate prediction of metastatic risk, tumour biopsy is only occasionally performed. Vision threatening complications associated with acquisition of tumour tissue through an invasive procedure, combined with the current lack of effective therapies precludes many patients from consenting to the test. Implementation of an alternative, safer method of predicting patient prognosis is paramount to enable adjuvant therapy clinical trials.

Through this study, we aimed to establish a methodology to make use of CTCs as an accessible source of tumour material for genetic profiling and prognosis of UM (Figure 14). First, we evaluated melanocyte, melanoma, and stem cell markers on a primary UM TMA and 5 UM cell lines to find markers that were highly expressed. After determining 5HT2B, ABCB5, gp100, MCAM, and MCSP were suitable extracellular markers for targeting UM cells, we tested their rate of capture in UM cell lines. We discover that certain markers provided a low (5HT2B and ACBC5) and others a moderate (gp100, MCAM, and MCSP) capture rate. Furthermore, we used UM cell lines to validate that the methods of processing the cells for detection of CTCs followed by WGA does not cause changes to the genomic profile of the cell, when assessed by WGS. Once these criteria had been established we determined that single CTC isolation was an effective means of determining CNVs found in UM.

Figure 14: Pipeline of Circulating Tumour Cell Analysis in Patients. Pipeline of utilising CTCs as an alternative to a biopsy. Blood is drawn prior to radiation plaque insertion, followed by isolation and capture of CTCs, followed by WGA and WGS of captured CTCs WGS data is analysed to give a whole genome CNV report.
Capture and identification of UM CTCs poses significant challenges. Previous literature indicates that single markers such as MCSP (Suesskind et al., 2011) and MCAM (Bande et al., 2015) alone are not sufficient for a viable alternative to tissue biopsy. More recently, Tura et al (2014) demonstrated that by using two markers, CD63 and surface gp100, that they could capture a median density of 3.5 CTCs with a range of 0-10.2 cells per 10 mL of blood in 93.5% of patients with primary UM. This result demonstrated that a multi-marker immunomagnetic enrichment protocol was capable of significantly improving CTC capture in patients. Using this knowledge, we added surface gp100 (NKI/BETEB) to the multi-marker capture of CTCs. Conversely, although CD63 is a highly important protein in melanogenesis (van Niel et al., 2011), and highly expressed in UM (Cools-Lartigue et al., 2008), we opted to avoid CD63 due to its high expression on non-melanoma cells (Human Protein Atlas available from www.proteinatlas.org, (Uhlen et al., 2015)) and microvesicles (Andreu and Yáñez-Mó, 2014), which may lead to higher background of non-melanoma cells/microvesicles captured. This could confound the analysis of single CTCs by contaminating them with other sources of extracellular DNA. Therefore, we targeted markers that are primarily expressed in melanocytes, melanoma, and stem cells to avoid this issue. Our preliminary data suggests that our multi-marker panel, comprising of 5HT2B, ABCB5, surface gp100, MCAM, and MCSP is suitable for capturing CTCs in patients, although analysis of a large cohort of patients using our multi-marker panel is required to fully determine the capture rate.

Although we expect that our multi-marker approach will increase CTC capture rates, one noticeable limitation of analysing CTCs to predict patient prognosis, is that these may not be found in all patients. It is important to note that implementation of a CTC based test does not preclude patients from having the biopsy. The blood test should be performed prior to plaque insertion and if no CTCs are captured from the patient’s blood sample, there is still the option of a biopsy at the time of surgery. Therefore, our proposed pipeline of analysing single CTCs supplements the current standard of care rather than offering a replacement.
If CTCs are not found in the sample patients can opt for a biopsy which provides several options for analysis, after extraction of the DNA; i) the biopsy can be whole genome amplified and sequenced for detection of CNVs across the genome such as the example shown in Figure 15, ii) MLPA can be performed to detect CNVs in chromosomes 1p, 3, 6, and 8, or lastly, iii) RNA can be extracted and gene expression profiling can be used to segregate patients into class Ia, Ib, or II (Figure 15). Moreover, DNA amplified could in future studies be used to detect mutations in BAP1, SF3B1, or EIF1AX to finer segregate patient prognosis.

**Figure 15: Proposed Circulating Tumour Cell ‘Liquid Biopsy’ Pipeline.** Proposed pipeline of CTCs for prediction of patient prognosis. After blood collection and isolation of CTCs, WGA and WGS would be performed to detect CNV changes associated with prognosis. If no CTCs are found, patients are still able to request a biopsy at time of radiation plaque insertion. DNA or RNA would be extracted, followed by either i) WGA and WGS, or ii) MLPA to detect CNV changes associated with prognosis, or iii) gene expression analysis of RNA to segregate patients into class Ia, Ib, or II.

Peripheral venous blood is a highly accessible, minimally invasive, and safe method of acquiring blood used for ‘liquid biopsies.’ However, recently it has been shown that arterial blood may harbour more CTCs than venous blood, with CTCs detected in 100% of metastatic UM patients (Terai et al., 2015). Generally, arterial blood is more difficult to acquire, and more invasive for the patient, but the overall benefit may be greater than the risk, especially when compared to the intraocular biopsy. Interestingly, Terai et al. (2015) used the CellSearch system for detection of CTCs which employs MCAM based capture and MCSP detection. This may indicate that similarly to current findings in venous blood (Tura et al., 2014; Tura et al., 2016), a multi-marker immunomagnetic panel might have a greater benefit in arterial blood by increasing the number of CTCs detected in each patient.
Whilst CTCs constitute a source of genetic material for use in predicting patient prognosis through CNVs, there has been no correlation between the numbers of CTCs quantified and patient outcomes (Suesskind et al., 2011; Tura et al., 2014). In contrast, whilst we have shown ctDNA is rarely detectable in patients with primary UM, previous studies have detected ctDNA in 84% of patients with metastatic disease (Bidard et al., 2014). Therefore, tracking ctDNA in patients with tumours at a high risk of metastasizing may provide early indications that metastatic lesions have arisen, most likely within the liver (COMS-15, 2001). In other cancers, ctDNA has been used to measure tumour mutational load (Murtaza et al., 2013), residual disease (Diehl et al., 2008), resistance to therapy (Gray et al., 2015b), tumour burden (Bettegowda et al., 2014), tumour relapse (Garcia-Murillas et al., 2015), and CNVs (Heitzer et al., 2013b). Fortunately, the majority of UM have hot spot mutations in GNAQ, GNA11, PLCβ4, or CYSLTR2 which are easily detectable using technologies such as ddPCR. This combination of all these markers could allow for analysis of ctDNA without prior knowledge of the mutational status of the tumours in the majority of cases as approximately 94% of patients have a mutation in these hotspots (Moore et al., 2016). However, a negative result always will have to be regarded as negative only for the tested mutations and not the lack of ctDNA. Nevertheless, the use of these mutations enables tracking of ctDNA regardless of whether a patient’s primary or metastatic lesions were biopsied. Further studies are required to ascertain whether ctDNA can be used as an early indicator of metastatic disease and that it is comparable or superior to current tests such as positron emission tomography or magnetic resonance imaging.

Recent data has shown that fine-needle biopsies via transcorneal, transscleral, or transvitreal have a true positive rate of 92% when using cellularity to diagnose UM. Moreover, prognostication using FISH could only be performed on 84.6% of fine needle biopsies due to the lack of cells (Singh et al., 2016). Factors that improved prognostic yield were enucleation, larger tumour size, and distance from fovea. Additionally, patients who required transvitreal or transcorneal biopsy had a greater rate of failure when compared to transscleral (68.8%, 87.5%, and 98.6% respectively) (Singh et al., 2016). It is currently unknown the failure rate of using CTCs as a minimally invasive liquid biopsy, but we envisage it to be used as an alternative to tumour biopsy. If the liquid biopsy
produces inconclusive results an intraocular biopsy can still be performed at the time of radiation plaque insertion.

Mutations in \textit{BAP1}, \textit{SF3B1}, and \textit{EIF1AX} can further stratify patients into more refined prognostic classes (Field et al., 2016). Detection of these mutations in PicoPlex WGA proved to be challenging, due to the size of PicoPlex product (~400bp), and that targeted or whole exome sequencing requires primers to synthesise fragments for sequencing and these may not overlap correctly in the small DNA fragments produced by PicoPlex to produce an amplicon. Our preliminary data in testing targeted sequencing of a PicoPlex WGA-DNA using Ampliseq gave extremely erroneous results. Furthermore, primerless whole genome sequencing at a suitable depth to analyse mutations is currently prohibitively expensive. Fortunately, sequencing costs are continually decreasing, according to the National Institute of Health (NIH), and further refinement of high throughput sequencing may further reduce the per-genome cost of sequencing.

Overall, the primary objective of a less invasive method of determining patient prognosis is to reduce the complications associated with biopsy, and to encourage more patients to determine their prognosis. If patient prognosis has been determined the implementation of neo-adjuvant therapies, adjuvant therapies, closer follow-up, or even entry into clinical trials can occur. Current clinical trials into adjuvant therapies, targeted therapies, and immunotherapies all require prior information on a patient’s risk of developing metastatic disease (Appendix 8.3 & 8.4). Recent clinical trials show slight benefits to patients utilising targeted therapies (Selumetinib, 2013), whereas immunotherapies generally have little to no effect (Algazi et al., 2016; Moser et al., 2015). Adjuvant therapy has previously been reported to have survival benefits in various cancers, such as breast (Goss et al., 2005) and colon (Ribic et al., 2003; Schmoll et al., 2015) cancers. Although there is no evidence that current adjuvant therapies for UM have any impact on survival of patients with high risk of developing metastasis, current and future trials of different drugs may find a survival benefit or preferably preclude metastatic disease in patients, and therefore it would be beneficial for all patients to know their prognosis so that they may partake in clinical trials, or therapies which may provide them with improved quality of life.
6.1 Limitations

A limitation of this study is the sample cohort. As UM is a rare disease, in an area with a low population (approximately 1.8 million people in Perth, Western Australia) it is difficult to gain the appropriate numbers for in-depth analysis. A multicentre study from various locations in Australia would be crucial for determining the concordance between the genetic characteristics of CTCs and their correlation with the primary tumour and patient prognosis.

Similarly, due to the timeframe of a Masters project, and first having to validate all the methods used to analyse single CTCs, we were only able to capture 1 CTC in a single patient. To further validate whether CTCs can represent the primary tissue and thus the risk of metastatic risk in patients, further CTC analysis in a larger cohort of patients is required.

In addition, the use of a TMA for the primary tissue may have caused an under or over representation of the immunopositivity of proteins, due to the 1 mm cores. Although care was taken to acquire tissue in histologically distinct areas of the tumour, the overall small size of the cores gives a small snapshot of the tumour. For example, if a marker is highly expressed on the tumour invading front, it may be under represented in our study, as the edges of the tumours were avoided. This is however, offset by using multiple cores per tumour in different areas. TMA’s are routinely used due to their benefits of screening a higher number of patients more efficiently and we found the TMA to be of significant benefit for assessment of markers.

Whilst we have shown in cell lines PicoPlex introduces only minor changes to ploidy across the genome, if these changes occur in chromosomes relating to metastatic risk it could alter the prognostic call. This would introduce false positive results. Fortunately, our testing indicates that the amplification method is quite robust and unless the reaction failed, the resultant WGA-DNA harboured a match to the original material. It is also important to note an additional limitation to our study was that the amplification of cells unfixed, fixed, and fixed-permeabilised and stained was performed with different passages of MP41 cells and the changes could be due to the effects of cell culture. By attempting to capture more than 1 CTC per patient we aim to obtain consistent chromosomal CNV across various cells to remove any random CNV introduced as an
artefact. Nevertheless, in our preliminary data, sequenced PBMCs from patients have all displayed the correct ploidy. Further studies will provide a larger cohort of sequenced WGA cells and provide a better indication of the false positive rate.

6.2 Future Directions

Following on from this study we will focus on determining whether CTCs can predict patient prognosis as do current methods such as gene expression profiling or MLPA, however these require tissue biopsies. To assess CTC concordance with tissue analysis, a prospectively designed study would be carried out, where peripheral blood samples and matched biopsies would be taken and genotyped prior to therapy of the primary tumour, and patients would be followed over a >3-year period.

In addition, future studies will involve pre-clinical mouse models where dissemination of CTCs from the primary tumour will be modulated with various drug combinations to try and disrupt metastases forming in the liver. These experiments could provide clear answers of the potential benefit of adjuvant therapy to prevent metastatic UM in at risk patients.

6.3 Conclusion

Our study validated 5HT2B, ABCB5, gp100, MCAM, and MCSP as highly expressed in UM and suitable candidates for capture of CTCs in patients with primary UM. Furthermore, we validated the use of PicoPlex WGA and low-pass WGS as an effective means of whole genome CNV analysis of CTCs that have been immunomagnetically enriched from PBMCs, fixed, permeabilised, and immunostained. Using this pipeline of a multi-marker immunomagnetic enrichment protocol, we were able to determine in a single patient a gain of the chromosome 8, a poor prognostic feature in UM.

It is clear that our study has established an effective means of utilising CTCs to detect chromosomal aberrations associated with metastatic risk in UM. Future studies will validate this methodology in which CTCs can be used as an alternative source of tumour genetic material from which patient prognosis can be derived, therefore potentially reducing complications associated with biopsy of the eye, while providing more patients at a high risk of developing metastases the option of closer follow-up, and possible adjuvant therapy options.
7.0 References


therapy in receptor-positive breast cancer: updated findings from NCIC CTG MA.17. *Journal of the National Cancer Institute* 97:1262-1271.


Karlsson, M., B. Boeryd, J. Carstensen, B. Franlund, B. Gustafsson, B. Kagedal, X.F.
Sun, and S. Wingren. 1996. Correlations of Ki-67 and PCNA to DNA ploidy, S-
phase fraction and survival in uveal melanoma. European Journal of Cancer
32A:357-362.

detection of circulating tumor cells in cutaneous and ocular melanoma and
quality assessment by real-time reverse transcriptase-polymerase chain reaction.

Kilic, E., N.C. Naus, W. van Gils, C.C. Klaver, M.E. van Til, M.M. Verbiest, T. Stijnen,
Concurrent loss of chromosome arm 1p and chromosome 3 predicts a decreased
disease-free survival in uveal melanoma patients. Investigative ophthalmology &
visual science 46:2253-2257.

Kirkwood, J.M., L. Bastholt, C. Robert, J. Sosman, J. Larkin, P. Hersey, M. Middleton,
M. Cantarini, V. Zazulina, K. Kemsley, and R. Dummer. 2012. Phase II, Open-
Label, Randomized Trial of the MEK1/2 Inhibitor Selumetinib as Monotherapy
versus Temozolomide in Patients with Advanced Melanoma. Clinical cancer
research : an official journal of the American Association for Cancer Research
18:555-567.

2013. Patient survival in uveal melanoma is not affected by oncogenic mutations

Kowalewska, M., M. Chechlinska, S. Markowicz, P. Kober, and R. Nowak. 2006. The
relevance of RT-PCR detection of disseminated tumour cells is hampered by the
expression of markers regarded as tumour-specific in activated lymphocytes.

Kujala, E., T. Makitie, and T. Kivela. 2003. Very long-term prognosis of patients with
malignant uveal melanoma. Investigative ophthalmology & visual science
44:4651-4659.

Kupas, V., C. Weishaupt, D. Siepmann, M.L. Kaserer, M. Eickelmann, D. Metze, T.A.
Luger, S. Beissert, and K. Loser. 2011. RANK is expressed in metastatic
melanoma and highly upregulated on melanoma-initiating cells. *Journal of Investigative Dermatology* 131:944-955.


inverse SD of nucleolar area as prognostic factors. *Archives of ophthalmology* 105:801-806.


8.0 Appendices

8.1 Study Ethics Information and Consent Form

PARTICIPANT INFORMATION SHEET

Identification of markers for diagnosis and prognosis of cancer

Prof Mel Ziman, Prof Michael Millward, A/Professor Fred Chen, Dr Tim Isaacs, Dr Chris Quirk, Dr Adnan Khattak, Dr Lester Cowell, Dr Graham Potter, Dr David Prentice, Mr Mark Lee, Prof Robert Pearce, Ms Anna Reid, Mr James Freeman, Dr Elin Gray, Ms Pauline Zaenker, Ms Kit Dufall, Dr Johan Poole-Johnson, Dr Arif Anwar, Mrs Ashleigh McEvoy, Dr Henry Law, Dr Carlos Aya-Bonilla, Mr Aaron Beasley.

Please take time to read the following information carefully and discuss it with your friends, family and general practitioner if you wish. Ask us any question if some part of the information is not clear to you or if you would like more information. Please do this before you sign this consent form.

Who is funding this study?

NHMRC, Cancer Research Trust, Edith Cowan University

Contact persons:

Should you have questions about the study you may contact:

Prof. Mel Ziman Phone No. 6304 3640 Mobile: 0419929851

All study participants will be provided with a copy of the Information Sheet and Consent Form. They may keep the information sheet for their personal records.
You may decide to be in the study or not take part at all. If you do decide to take part in this study, you may stop at any time. However, before you decide, it is important that you understand why this research is being done and what it will involve.

Whatever your decision, this decision will not lead to any penalty or affect your regular medical care or any benefit to which you are otherwise entitled.

The following information sheet will explain the study and will include details such as:

- Why this trial might be suitable for you;
- The possible risks (side-effects) and benefits of the new test;
- The type, frequency and risks of any medical tests or procedures required by the trial;
- The nature of your participation including how many visits you will make to the hospital
- Your rights and responsibilities
- Who is funding this study
What is the purpose of the study?

This study is a research project in which we are investigating circulating tumour cells (CTCs), gene expression (RNA), cell free DNA and proteins in the peripheral blood of patients with cancer relative to these markers in tumour tissue and in control bloods. It is anticipated that changes in cellular, genetic or protein markers in the blood of patients are indicative of tumour presence (diagnostic) or may provide information on patient outcome or tumour progression (prognostic) or response to treatment.

We will isolate and quantify circulating tumour cells and genetic material (DNA and RNA), as well as proteins and sugars from the blood of all participants. We will characterise the cells, genes and proteins and sugars for markers of cancer.

If we find that there are circulating tumour cells in your blood, we may ask your consent to isolate and grow the isolated circulating tumour cells and use them for laboratory experiments. We may ask your consent to access a small amount of your archival tumour tissue stored at Pathology Centres or fresh tissue at the time of surgical removal or biopsy for routine pathological diagnosis. We only require a small portion of your tumour that is additional to that required for pathological diagnosis. Tumour tissue may include both primary and metastatic tissue samples where appropriate. DNA from your circulating cells and tumour tissue will be compared for mutation analysis. We will also isolate proteins from your blood for analysis relative to control samples.

1500 participants will be invited to participate.

Why is this study suitable to me?

You have been invited to participate in this study because you have been diagnosed with cancer.

How long will I be in this study?

The study will be conducted over a five year period. As a patient you will be asked to provide a blood sample at the time of surgical removal of your tumour or at the commencement of additional therapy and at follow-up.

As a patient you may also be asked to provide consent for us to access a small amount of
your tumour tissue which has been stored at Pathology Centres after being used for diagnosis. Alternately you may be asked to provide a small sample of fresh tumour tissue at time of removal or biopsy of your tumour.

**What will happen if I decide to be in this study?**

If you agree to participate you will be asked to provide blood samples at the time of surgical removal of your tumour or before and during treatment, where appropriate. You will be asked to provide additional blood samples at the time of routine clinical follow-up visits to your clinician.

The amount of blood required for this study is small (approximately 40 ml per visit). Blood will be drawn into 8 x 4 ml EDTA blood tubes and 1 x 4 ml SST tube, for isolation of the circulating cells and for analysing genes, proteins and sugars in your blood.

Generally you will not be contacted between visits. If you require general information about the research at any time then you are able to contact the researchers should you wish to do so. Contact details are provided in the information sheet.

Your blood will be tested relative to blood from other participants. Your samples are only identifiable by a coded number, and the researchers performing the tests will not know which samples are yours.

Your samples will be stored in locked freezers in secure research laboratories during the research study and will be discarded five years after completion of the study or upon your written request.

**Are there any reasons I should not be in this study?**

The clinical staff collaborating in this study will discuss the research with you in detail and will ensure that this trial is both safe and appropriate for you.

**What are the costs to me?**

There will be no additional costs over and above your visits to the doctor. Blood will be taken at the hospital or private clinical practice or at Edith Cowan University when and where you visit your doctor for treatment and follow-up visits. Your tumour tissue may be required for comparison with your blood sample and your tissue will be accessed
through clinicians and pathology centres and will be extra tissue that is not required for diagnosis.

**What are the possible benefits of taking part?**

The results of these studies may be of interest to you and your family and you may decide whether or not the information may be disclosed to your family. You will be provided with the results of the project in general. However, the research will not provide you with any detailed information about your health or genetic diseases in general.

Donation of your sample may assist researchers to provide a more detailed and specific diagnosis of cancer now and in the future and to assist with improvements in treatment of cancer.

**How will my safety be ensured?**

In this study, the samples that you provide are blood samples and you may also be asked to provide permission to access a small quantity of your tissue sample(s). There is very little risk to you as only a small volume of blood is required for the test and the tissue has already been removed during surgery. However please do not hesitate to contact the study coordinator or your doctor in relation to any adverse effects you think you are experiencing. If the effects are severe enough, the doctor may stop your participation in the study.

The study may produce abnormal results in which case your clinician will be notified and additional clinical tests will be performed if your doctor feels it is in your best medical interest. When you stop participation in the study you will be clinically assessed as you were at the beginning of the study.

**What alternatives do I have to going on this study?**

This study does not affect your treatment. Your treatment will continue in the same manner whether you decide to participate in the study or not.

You may wish to discuss with your doctor or the researchers how the test will benefit patient treatment now or in the future.
What are the possible side effects, risks and discomforts of taking part?

In this study, only a small volume of blood is taken (40 ml), so there is very little risk to you in this procedure. You may suffer a small amount of discomfort when you donate the blood sample, like the feeling of a pin prick or bruising.

The likelihood of side effects from donating blood is small, around 1 in 100. However should you suffer any side effects please tell your doctor immediately about any new or unusual symptoms that you get.

What if new information comes along during the study?

Sometimes new information becomes available as a study progresses. You will be told about any information that could be important to you and to your decision to continue in the study. If you then want to continue in the trial, you may be asked to sign a revised consent form.

Stopping the study early:

Sometimes a trial needs to be stopped early because of safety concerns, because the trial is not effective enough, or for other reasons. If this occurs, the reasons will be explained to you and your treatment will continue as it would have without the test. Your treatment will not be influenced by the test in any way.

What happens at the end of the study?

At the end of the study your visits to your doctor will continue and your treatment will not be affected by the outcome of the research.

What if something goes wrong?

You will receive the best medical care available during and after the test, but because these are still relatively new tests, unexpected results may be obtained. In the unlikely event of risks to your health being identified then you will be provided with the necessary care.
Medical treatment will be provided at no cost to you for research-related harm. The term “research-related harm” means both physical and mental injury caused by the procedures required by the trial.

Your participation in this study does not prejudice any right to compensation which you may have under statute or common law.

**Will my taking part in this study be kept confidential?**

The researchers will need to collect personal data about you, which may be sensitive, such as your relevant health information. This includes clinical records relating to the diagnosis and treatment of your cancer. The researchers may also need to get some of your health information from other health service providers, e.g. another hospital, pathology laboratory, radiographer, GP or other medical specialist.

Any personal or health information will be kept private and confidential. It will be stored securely and only authorised persons, who understand it must be kept confidential, will have access to it. Your study details will be given a number so that your identity will not be apparent. The trial records will be kept at The School of Medical Sciences at Edith Cowan University during the study and in a locked archive for at least 10 years from the time the study is closed, and will be destroyed by incineration thereafter.

Authorised representatives of the researchers, the investigating doctors, the Hospital or University Human Research Ethics Committees, Research Governance and other regulatory bodies may require access to your study records for study procedures and/or for data analysis. Your sample may be sent to people in other states or other countries for analysis, however your sample will be identified by a sample number only, and your name and personal details will not be provided. In all cases when dealing with your sample, personal or health information, researchers are required to comply with privacy laws that protect you.

The result of the research will be made available to other doctors through medical journals or meetings, but you will not be identifiable in these communications. By taking part in this study you agree not to restrict the use of any data even if you withdraw. Your rights under any applicable data protection laws are not affected. Your sample will be destroyed upon written request if you withdraw from the study.
Will I find out the results of the study?

The value of the research is not known at this time. You will be notified of the results of the research in general terms at your request and the outcomes of the research as a whole may be provided to you upon completion of the project.

Who has reviewed the study?

The Sir Charles Gairdner Hospital Human Research Ethics Committee and the Edith Cowan University Human Research Ethics Committee have reviewed this study and have given approval for conducting this research trial. In doing so this study conforms to the principles set out by the National Statement on Ethical Conduct in Research involving Humans and according to the Good Clinical Practice Guidelines.

If you have any concerns or complaints and wish to talk to an independent person, please contact:

Research Ethics Officer	Phone: 6304 2170	Email: research.ethics@ecu.edu.au
CONSENT FORM

Identification of markers for diagnosis and prognosis of cancer

Prof Mel Ziman, Prof. Michael Millward, A/Professor Fred Chen, Dr Tim Isaacs, Dr Chris Quirk, Dr Adnan Khattak, Dr Lester Cowell, Dr Graham Potter, Dr David Prentice, Mr Mark Lee, Mr Robert Pearce, Ms. Anna Reid, Mr. James Freeman, Dr Elin Gray, Ms. Pauline Zaenker, Ms Kit Dufall, Dr Johan Poole-Johnson, Dr Arif, Anwar, Ms Ashleigh McEvoy, Mr Aaron Beasley.

Participant Name:_________________________________________

Date of Birth: _______________

Address: ------------------------------   Phone Number:----------------------------------

1. I have been given clear information (verbal and written) about this study and have been given time to consider whether I want to take part.

2. I have been told about the possible advantages and risks of taking part in the study and I understand what I am being asked to do.
3. I have been able to have a member of my family or a friend with me while I was told about the study. I have been able to ask questions and all questions have been answered satisfactorily.

4. I know that I do not have to take part in the study and that I can withdraw at any time during the study without affecting my future medical care. My participation in the study does not affect any right to compensation, which I may have under statute or common law.

5. I agree to take part in this research study and for the data obtained to be published provided my name or other identifying information is not used.

6. I provide consent for my medical history to be made available to the researchers.

7. I provide consent for the researchers to access a small sample of blood (40mls) and if necessary, my tumour tissue that is additional to that required for diagnosis.

8. I provide consent for my circulating tumour cells isolated from my peripheral blood to be used in laboratory experiments.

9. I understand that my sample and associated data may be used for future cancer research by researchers at Edith Cowan University in collaboration with researchers from other universities.

10. I consent to blood and tissue samples being taken and donate that blood and tissue absolutely for testing and research into cancer and related health areas.
11. I understand that access to my blood donation and tissue sample for research will only
be released where the research project that wishes to use my blood donation and/or
tissue sample has been approved by an Ethics Committee

If you are unclear about anything you have read in the Participant Information Sheet or
this Consent Form, please speak to your doctor before signing this Consent Form.

Name of Participant    Signature of Participant                                Date
____________________  ____________________  __________

Name of Investigator   Signature of Investigator
Date
____________________  ____________________  __________

The Sir Charles Gairdner Hospital and Edith Cowan University Human Research Ethics
Committees have given ethics approval for this study. If you have any concerns you can
contact the Chief Investigator, Prof Mel Ziman: Phone (08) 63043640

All study participants will be provided with a copy of the Information Sheet and
Consent Form for their personal records.
## 8.2 DecisionDx-UM Genes

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene</th>
<th>Regulation in class II</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDH1</td>
<td>E-cadherin</td>
<td>Up</td>
</tr>
<tr>
<td>ECM1</td>
<td>Extracellular matrix protein 1</td>
<td>Up</td>
</tr>
<tr>
<td>E1F1B</td>
<td>Eukaryotic translation initiation factor 1B</td>
<td>Down</td>
</tr>
<tr>
<td>FXR1</td>
<td>Fragile X mental retardation autosomal homolog 1</td>
<td>Down</td>
</tr>
<tr>
<td>HTR2B</td>
<td>5-hydroxytryptamine (serotonin) receptor 2B</td>
<td>Up</td>
</tr>
<tr>
<td>ID2</td>
<td>Inhibitor of DNA binding 2</td>
<td>Down</td>
</tr>
<tr>
<td>LMCD1</td>
<td>LIM and cysteine-rich domains 1</td>
<td>Down</td>
</tr>
<tr>
<td>LTA4H</td>
<td>Leukotriene A4 hydrolase</td>
<td>Down</td>
</tr>
<tr>
<td>MTUS1</td>
<td>Microtubule-associated tumor suppressor 1</td>
<td>Down</td>
</tr>
<tr>
<td>RAB31</td>
<td>RAB31, member RAS oncogene family</td>
<td>Up</td>
</tr>
<tr>
<td>ROBO1</td>
<td>Roundabout, axon guidance receptor 1</td>
<td>Down</td>
</tr>
<tr>
<td>SATB1</td>
<td>SATB homeobox 1</td>
<td>Down</td>
</tr>
<tr>
<td>MRPS21</td>
<td>Mitochondrial ribosomal protein S21</td>
<td>Normal (Control)</td>
</tr>
<tr>
<td>RBM23</td>
<td>RNA binding motif protein 23</td>
<td>Normal (Control)</td>
</tr>
<tr>
<td>SAP130</td>
<td>Sin3A-associated protein</td>
<td>Normal (Control)</td>
</tr>
</tbody>
</table>
### 8.3 Clinical Trials of Targeted Therapies for UM

<table>
<thead>
<tr>
<th>ClinicalTrials.gov Identifier</th>
<th>Compound Tested</th>
<th>Target</th>
<th>Mutation Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT01430416</td>
<td>AEB071</td>
<td>PKC</td>
<td>GNAQ/11</td>
</tr>
<tr>
<td>NCT01801358</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCT01551459</td>
<td>Sunitinib</td>
<td>MEK</td>
<td>GNAQ/11</td>
</tr>
<tr>
<td>NCT01005472</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCT01252251</td>
<td>Everolimus</td>
<td>mTOR</td>
<td>GNAQ/11</td>
</tr>
<tr>
<td>NCT01587352</td>
<td>Vorinostat</td>
<td>HDAC</td>
<td>BAP1</td>
</tr>
<tr>
<td>NCT00121225</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCT01377025</td>
<td>Sorafenib</td>
<td>RAF-kinases</td>
<td>GNAQ/11</td>
</tr>
<tr>
<td>NCT01893099</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCT00329641</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCT01801358</td>
<td>MEK162</td>
<td>MEK</td>
<td>GNAQ/11</td>
</tr>
<tr>
<td>NCT01143402</td>
<td>Selumetinib</td>
<td>MEK</td>
<td>GNAQ/11</td>
</tr>
<tr>
<td>NCT01835145</td>
<td>Cabozantinib</td>
<td>MEK, KIT</td>
<td>GNAQ/11</td>
</tr>
<tr>
<td>NCT00104884</td>
<td>Romidepsin</td>
<td>HDAC</td>
<td>BAP1</td>
</tr>
</tbody>
</table>
### 8.4 Adjuvant Therapy Clinical Trials for High Risk Patients

<table>
<thead>
<tr>
<th>Clinicaltrials.gov Identifier</th>
<th>Compound Tested</th>
<th>Primary Outcome Measure</th>
<th>Secondary Outcome Measures</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT02843386</td>
<td>Fotemustin</td>
<td>Metastasis free survival [3 yrs]</td>
<td>Overall survival [3yrs] Safety</td>
</tr>
<tr>
<td>NCT02068586</td>
<td>Sunitinib</td>
<td>Overall survival [2 yrs] Relapse free survival [2yrs]</td>
<td>Tolerability</td>
</tr>
<tr>
<td>NCT01983748</td>
<td>Autologous dendritic cells loaded with autologous tumour RNA</td>
<td>Prolongation of disease free survival [36 mths]</td>
<td>Prolongation of overall survival [36 mths]</td>
</tr>
<tr>
<td>NCT01100528</td>
<td>Recombinant interferon alfa-2b Dacarbazine Laboratory biomarker analysis</td>
<td>Disease free survival</td>
<td>Safety Relationship between plasma biomarkers of immune function and tumour invasion and clinical outcome</td>
</tr>
</tbody>
</table>
### 8.5 COMS Staging System

<table>
<thead>
<tr>
<th>Measure</th>
<th>Small</th>
<th>Medium</th>
<th>Large</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apical Height</td>
<td>1.0-3.0 mm</td>
<td>3.1-8.0 mm</td>
<td>&gt;0.8 mm</td>
</tr>
<tr>
<td>Basal Diameter</td>
<td>5.0-16.0 mm</td>
<td>≤16.0 mm</td>
<td>&gt;16.0 mm when apical height ≥2 mm</td>
</tr>
</tbody>
</table>

(COMS-5, 1997; COMS-17, 2001)
### 8.6 AJCC Staging System

<table>
<thead>
<tr>
<th>Location</th>
<th>Tumour Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iris</td>
<td>T1</td>
<td>Limited to iris</td>
</tr>
<tr>
<td>Iris</td>
<td>T1a</td>
<td>Limited to iris and &lt;3 clock hours in size</td>
</tr>
<tr>
<td>Iris</td>
<td>T1b</td>
<td>Limited to iris and &gt;3 clock hours in size</td>
</tr>
<tr>
<td>Iris</td>
<td>T1c</td>
<td>Limited to iris with secondary glaucoma</td>
</tr>
<tr>
<td>Iris</td>
<td>T2</td>
<td>Extending into ciliary body, choroid or both</td>
</tr>
<tr>
<td>Iris</td>
<td>T2a</td>
<td>Confluent with or extending into ciliary body, choroid or both and has secondary glaucoma</td>
</tr>
<tr>
<td>Iris</td>
<td>T3</td>
<td>Scleral extension</td>
</tr>
<tr>
<td>Iris</td>
<td>T3a</td>
<td>Confluent with or extending into ciliary body, choroid or both with scleral extension</td>
</tr>
<tr>
<td>Iris</td>
<td>T4</td>
<td>Extrascleral extension</td>
</tr>
<tr>
<td>Iris</td>
<td>T4a</td>
<td>Extrascleral extension ≤5 mm in diameter</td>
</tr>
<tr>
<td>Iris</td>
<td>T4b</td>
<td>Extrascleral extension ≥5 mm in diameter</td>
</tr>
<tr>
<td>Iris</td>
<td>T5</td>
<td>Tumour size category 1</td>
</tr>
<tr>
<td>Iris</td>
<td>T1a</td>
<td>No ciliary body involvement or extraocular extension</td>
</tr>
<tr>
<td>Iris</td>
<td>T1b</td>
<td>Ciliary body involvement</td>
</tr>
<tr>
<td>Iris</td>
<td>T1c</td>
<td>No ciliary body involvement, extraocular extension ≤5 mm in diameter</td>
</tr>
<tr>
<td>Iris</td>
<td>T1d</td>
<td>Ciliary body involvement, extraocular extension ≥5 mm in diameter</td>
</tr>
<tr>
<td>Iris</td>
<td>T2</td>
<td>Tumour size category 2</td>
</tr>
<tr>
<td>Iris</td>
<td>T2a</td>
<td>No ciliary body involvement or extraocular extension</td>
</tr>
<tr>
<td>Iris</td>
<td>T2b</td>
<td>Ciliary body involvement</td>
</tr>
<tr>
<td>Iris</td>
<td>T2c</td>
<td>No ciliary body involvement, extraocular extension ≤5 mm in diameter</td>
</tr>
<tr>
<td>Iris</td>
<td>T2d</td>
<td>Ciliary body involvement, extraocular extension ≥5 mm in diameter</td>
</tr>
<tr>
<td>Iris</td>
<td>T3</td>
<td>Tumour size category 3</td>
</tr>
<tr>
<td>Iris</td>
<td>T3a</td>
<td>No ciliary body involvement or extraocular extension</td>
</tr>
<tr>
<td>Iris</td>
<td>T3b</td>
<td>Ciliary body involvement</td>
</tr>
<tr>
<td>Iris</td>
<td>T3c</td>
<td>No ciliary body involvement, extraocular extension ≤5 mm in diameter</td>
</tr>
<tr>
<td>Iris</td>
<td>T3d</td>
<td>Ciliary body involvement, extraocular extension ≥5 mm in diameter</td>
</tr>
<tr>
<td>Iris</td>
<td>T4</td>
<td>Tumour size category 4</td>
</tr>
<tr>
<td>Iris</td>
<td>T4a</td>
<td>No ciliary body involvement or extraocular extension</td>
</tr>
<tr>
<td>Iris</td>
<td>T4b</td>
<td>Ciliary body involvement</td>
</tr>
<tr>
<td>Iris</td>
<td>T4c</td>
<td>No ciliary body involvement, extraocular extension ≤5 mm in diameter</td>
</tr>
<tr>
<td>Iris</td>
<td>T4d</td>
<td>Ciliary body involvement, extraocular extension ≥5 mm in diameter</td>
</tr>
<tr>
<td>Iris</td>
<td>T4e</td>
<td>Any tumour size category with extraocular extension ≥5 mm in diameter</td>
</tr>
<tr>
<td>Iris</td>
<td>N0</td>
<td>No regional lymph node metastasis</td>
</tr>
<tr>
<td>Iris</td>
<td>N1</td>
<td>Regional lymph node metastasis</td>
</tr>
<tr>
<td>Iris</td>
<td>M0</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>Iris</td>
<td>M1</td>
<td>Distant metastasis</td>
</tr>
<tr>
<td>Iris</td>
<td>M1a</td>
<td>Diameter of largest metastasis is ≤3 cm</td>
</tr>
<tr>
<td>Iris</td>
<td>M1b</td>
<td>Diameter of largest metastasis is 3.1-8 cm</td>
</tr>
<tr>
<td>Iris</td>
<td>M1c</td>
<td>Diameter of largest metastasis is ≥8 cm</td>
</tr>
</tbody>
</table>

(Edge et al., 2010)