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Quantification of circulating melanoma cells

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Quantification of Circulating Melanoma Cells

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Abstract

Current prognostic techniques for Cutaneous Malignant Melanoma (CMM), a highly aggressive and drug resistant skin cancer, are inadequate at managing the disease and identifying early stage patients requiring treatment. It is thought that Circulating Tumour Cells (CTCs), which circulate in patient blood after being shed from solid tumours, may be useful in enhancing prognostic techniques and it has previously been shown in other malignancies that the presence of CTCs in patient blood is associated with poor prognosis. In CMM, CTCs can be detected through RT-PCR for melanoma associated markers, although this technique does not allow CTCs to be quantified.

In this study, melanoma CTCs were isolated from patient peripheral blood samples with a combination of MCSP, MCAM and ABCB5 antibody coupled immunomagnetic beads and were subsequently quantified. The immunomagnetic bead capture protocol was performed for 21 control and 33 patient blood samples ranging from clinical stage 0 to IV. Results showed a significant difference between the mean number of cells captured from control (mean=3.71) and patient (mean=24.45) blood samples ($p=0.01$). Furthermore, there was a significant relationship between the number of MCAM positive CTCs and disease stage ($r=0.486$, $p=0.004$), although there were no significant relationships between the number of MCSP positive, ABCB5 positive or total CTCs and disease stage. Overall, this study has identified MCAM positivity as a CTC marker associated with disease progression which may be useful in improving prognostic testing and disease management in melanoma patients.

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1. Introduction

Cutaneous Malignant Melanoma (CMM) is a highly aggressive skin cancer which is notoriously resistant to currently available cancer therapies. Although CMM only accounts for a small percentage of skin cancers, it is responsible for 80% of skin cancer related deaths (Lewis et al., 2005). Australia, along with New Zealand, North America and Northern Europe, has the highest incidence rate of melanoma worldwide (Villanueva & Herlyn, 2009). Disease outcome is largely determined by progression at the time of diagnosis. Patients with in-situ melanoma at time of detection have a very high probability of relapse free survival following surgical resection of the primary tumour, whereas those with metastatic disease at time of detection have poor prognosis. Currently available prognostic techniques, including primary tumour thickness and detection of metastasis in regional lymph nodes, have proved to be inadequate as 30% of patients with in situ melanoma (Koyanagi et al., 2005a) and 15% of patients with negative lymph nodes at the time of surgical intervention (Jack et al., 2006; Weight et al., 2006) go on to develop metastatic disease.

It is thought that the number of melanoma cells circulating in patient peripheral blood may be a useful prognostic measure although, at this time, circulating cells have not been effectively quantified in patients across all stages of disease. The presence of circulating melanoma cells can be detected by reverse-transcriptase polymerase chain reaction (RT-PCR) of melanoma associated markers in patient blood and results of previous studies have shown that detection of these markers correlates with poor prognosis (Arenberger et al., 2008; Hoon et al., 1995; Hoon et al., 2000; Koyanagi et al., 2005a; Lewis et al., 2005; Reynolds et al. 2003). This technique has demonstrated that circulating cells are present in peripheral blood of patients across clinical stages I to IV, even though there is no clinical evidence of metastasis in stage I and II patients (Hoon et al., 1995; Hoon et al., 2000; Koyanagi et al., 2005a). The fact that many stage 0, I and II patients later develop metastatic disease, even years after surgical intervention, suggests circulating tumour cells are involved in the formation of metastases and may therefore be useful in evaluating prognosis in early

stage patients. The major limitation of RT-PCR detection of circulating cell markers is that it does not allow circulating cells to be quantified.

In order to quantify circulating melanoma cells it is first necessary to isolate them from patient blood samples. Currently, the most promising method of isolating circulating melanoma cells is by immunomagnetic bead capture, where cells are targeted specifically by magnetic beads bound to melanoma antigen specific antibodies. This technique has been shown to be effective at isolating circulating melanoma cells (Faye et al., 2004; Kitago et al. 2009; Ulmer et al., 2004), although cells have not been able to be isolated in all tested patients. In this study, a combination of Melanoma-Associated Chondroitin Sulphate Proteoglycan (MCSP), Melanoma Cell Adhesion Molecule (MCAM) and ATP-Binding Cassette Subfamily B Member 5 (ABCB5) antibodies coupled to immunomagnetic beads were used for optimal isolation and subsequent quantification of circulating melanoma cells from patients of American Joint Committee on Cancer (AJCC) stages 0 to IV, in order to determine the relationship between the number of melanoma circulating cells and disease stage.

2. Literature Review

2.1. Cutaneous Malignant Melanoma

Cutaneous Malignant Melanoma is a highly aggressive and drug resistant form of skin cancer. Melanoma arises from melanocytes, the melanin pigment producing cells of the skin, which are of neuroectodermal origin (Florenes et al., 1994). During embryogenesis, pluripotent cells of the neural crest migrate to the skin, hair and eyes where they differentiate into melanocytes capable of producing melanin (Goding, 2007). Melanoma only accounts for 4% of skin cancers although it is responsible for 80% of skin cancer related deaths (Lewis et al., 2005). Australia, New Zealand, North America and Northern Europe have the highest incidence of melanoma worldwide (Villanueva & Herlyn, 2009). In Western Australia, melanoma has the highest incidence of any cancer in both males and females aged 15 to 39 (Threlfall & Thompson, 2009). Survival of the disease is largely dependent on progression at time of detection, particularly the extent of metastasis. Current prognostic techniques and treatments are ineffective at managing the disease and unless diagnosis and treatment occurs before the onset of metastasis, prognosis is extremely poor.

2.2. Melanoma Development

Melanomas can originate from existing naevi, circular areas of dark pigmentation on the skin, or can arise spontaneously (Tan, 2010). The most common cause of melanoma is excessive exposure to ultraviolet (UV) radiation, with both UVA and UVB wavelengths being implicated (Leiter & Garbe, 2008; Villanueva & Herlyn, 2009). Initially, melanocytes undergo proliferation resulting in the formation of benign naevi which may or may not progress to radial growth phase melanomas. Melanomas undergo two growth phases, a radial growth phase (RGP) where growth is superficial within the epidermis and a vertical growth phase (VGP) where the tumour increases in thickness invading deeper tissue layers (Fig 2.1.). Once the lesion has entered the vertical growth phase it becomes capable of metastasis as a result of invasion of subcutaneous tissue and entry into lymphatic and circulatory vessels (Fig 2.2.). Studies have shown that vertical growth phase cells are capable

of tumour formation when injected into immunodeficient mice (Villanueva & Herlyn, 2009).

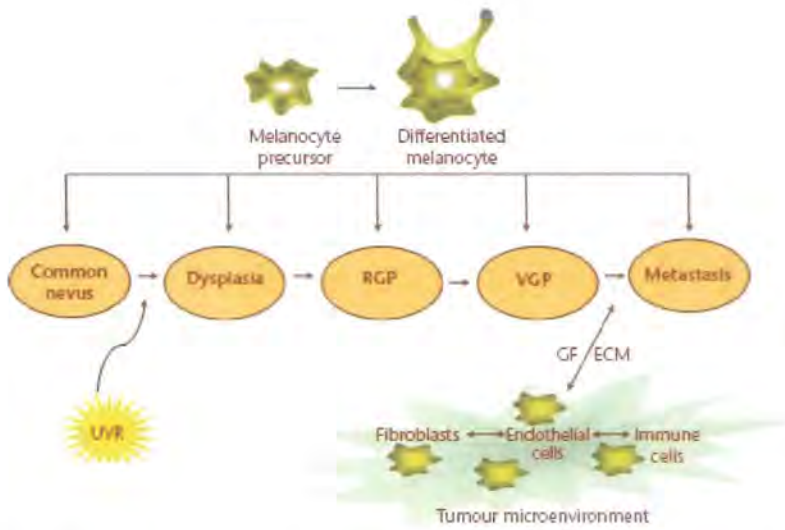


Figure 2.1. Typical pathway of melanoma development (Villanueva & Herlyn, 2009)

Primary tumour thickness is used as a prognostic indicator as increased thickness is associated with an increased number of metastases. Clinically, a tumour is regarded as thin if it is less than one millimetre in thickness, moderate if between one and four millimetres in thickness and thick if it is greater than four millimetres in thickness (Tan, 2010). Patients with a primary tumour thickness of less than 0.75mm have a 95% cure rate while patients with a primary tumour greater than 4mm in thickness have a cure rate of 20-25% (Breslow, 1970; Kerbel, 1990).



Figure 2.2. Diagram illustrating the ability of tumours to metastasise via the circulatory system (Pantel et al., 1999)

2.3. Mutations

The development of melanoma is multifactorial although, as mentioned previously, the major cause is excessive exposure to UV radiation. Melanocytes are normally under the control of adjacent keratinocytes which release chemical signals regulating proliferation and cell-cell adhesion, however, altered expression of regulating genes can result in uncontrolled proliferation and detachment of melanocytes (Haas et al., 2005). Approximately 10% of melanomas appear in genetically predisposed individuals, that is individuals with fair skin, increased freckling and red hair and a further 10% of cases are familial, appearing in individuals with a family history of the disease (Villanueva & Herlyn, 2009). Individuals with a large number of atypical or dysplastic nevi are also at greater risk of developing melanoma. There are a number of pathways and mutations associated with melanoma development. Genetic predisposition can be the result of polymorphisms in the Melanocortin-1 Receptor (MC1R) gene which are associated with red hair, fair skin and freckling (Villanueva & Herlyn, 2009). Inherited mutations in the CDKN2A and CDK4 genes are associated with familial melanoma, with CDKN2A mutations being the most frequent. CDK4 mutations are also regularly seen in sporadic melanomas where, like the majority of melanoma associated mutations, they develop spontaneously (Villanueva & Herlyn, 2009).

Other sporadic mutations commonly found in melanomas occur in oncogenes. Oncogenes, which in their normal form (proto-oncogenes) regulate cell proliferation, promote uncontrolled cell division contributing to tumour formation. The most common oncogene implicated in melanoma formation is BRAF, which is mutated in approximately 60% of naevi and melanomas (Davies et al., 2002). BRAF mutations cause constitutive activation of the MAPK pathway resulting in increased cell proliferation and survival contributing to melanoma formation (Sumimoto et al., 2006). Mutations in the NRAS oncogene, which are seen in 10-20% of melanomas, also activate the MAPK pathway (Marks & Neill, 2008, p. 149). BRAF and NRAS mutations are mutually exclusive suggesting they have similar roles in uncontrolled melanocyte proliferation leading to melanoma development (Villanueva & Herlyn, 2009).

2.4. Diagnosis and Treatment

Initial diagnosis of melanoma is made using the ABCDE system to determine whether a lesion is likely to be a melanoma. Using this system, a lesion may be a melanoma if it is asymmetrical, has an irregular border, uneven colouring, a diameter greater than 6mm and is evolving or changing over time (Tan, 2010) (Fig 2.3.). If a lesion is diagnosed as a melanoma it will be surgically resected with surrounding tissue in order to remove as many malignant cells as possible (Urist, 1996). The amount of surrounding tissue excised is based on the thickness of the tumour (Jack et al., 2006).



Figure 2.3. A typical example of superficial spreading melanoma depicting asymmetry, irregular border and uneven colouring (Ada County, n.d.).

If the patient has metastatic disease at the time of removal of the primary tumour prognosis is less favourable (Wascher et al., 2003). Usually if the tumour is greater than 0.75 millimetres in thickness or is ulcerated, the sentinel lymph node, which is the first node to receive drainage from a primary tumour and the most common first site of metastasis (Goto et al., 2008), will be examined for involvement and can be surgically removed if there is evidence of metastasis. If the sentinel node is metastatic, this process will be repeated in other regional nodes (Krag et al., 1995).

Surgical removal of secondary tumours is regularly undertaken in patients where metastasis is more distant than regional lymph nodes (Tan, 2010). If metastasis is extensive, chemotherapy, immunotherapy, interferon therapy or radiotherapy may be considered although melanoma is notoriously resistant to these treatments. Of these, the most successful therapies have proven to be immunotherapy with interleukin-2 and interferon therapy with interferon-alpha, however patient responses vary considerably and the toxicity of these treatments is high (Steen et al., 2008). The only chemotherapeutic agent currently approved for the treatment of metastatic melanoma by the US Food and Drug Administration is dacarbazine, which is often used in conjunction with other therapies (Blesa et al., 2009).

A number of targeted therapies have been trialed for use in the treatment of metastatic melanoma in order to specifically inhibit melanoma growth with the intent of increasing treatment efficacy while eluding the adverse effects of more general cancer therapies. The use of mutant *BRAF* inhibitors showed particular promise in this area (Shepherd et al., 2010) although initial success of trials applying this treatment have since been tempered by indications of resistance to this therapy (Fisher et al., 2010; Flaherty et al., 2010; Modjtahedi & Essapen, 2009).

2.5. Staging

It is important to accurately stage melanoma in order to determine prognosis and establish the best possible strategy for treating the disease. Staging of the disease is based on thickness of the primary tumour, the presence of ulceration and the extent of metastasis. The most widely used staging system for melanoma is that instigated by the American Joint Committee on Cancer (AJCC). Stages range from 0 to 4, where 0 describes a primary tumour in situ and 4 describes patients with extensive metastasis. Within these stages there are many sub-stages describing primary tumour thicknesses and specific sites and types of metastases; these are grouped into stages 0, 1A, 1B, 2A, 2B, 2C, 3 and 4 (American Joint Committee on Cancer, 2002) (Table 2.1.). Stage 1A melanoma is associated with a five-year survival rate of 95% whereas stage 4 melanoma is associated with a five-year survival rate of 7 to 19% depending on the number and location of metastases (Tan, 2010). Stage 4

patients can be further subdivided based on their serum lactate dehydrogenase (LDH) level.

Table 2.1. The AJCC Staging System for Melanoma (adapted from American Joint Committee on Cancer, 2002)

Stage	Description
0	Primary tumour in situ
1A	Primary tumour less than or equal to 1mm in thickness with no ulceration and no evidence of metastasis
1B	Primary tumour less than 1mm in thickness with ulceration but no evidence of metastasis OR primary tumour between 1 and 2mm in thickness with no ulceration and no evidence of metastasis
2A	Primary tumour between 1 and 2mm in thickness with ulceration but no evidence of metastasis OR primary tumour between 2 and 4 mm in thickness with no ulceration and no evidence of metastasis
2B	Primary tumour between 2 and 4mm in thickness with ulceration but no evidence of metastasis OR primary tumour greater than 4mm in thickness with no ulceration and no evidence of metastasis
2C	Primary tumour greater than 4mm in thickness with ulceration but no evidence of metastasis
3	Primary tumour any thickness with metastasis to one or more regional lymph nodes but no distant metastases
4	Primary tumour any thickness with distant metastasis to skin, subcutaneous tissue, lymph nodes, lungs and visceral organs

The LDH test allows the extent of metastasis to be determined. The test measures the level of serum lactate dehydrogenase (LDH) which increases in response to tissue damage, especially to the heart, lungs, liver, kidneys, brain and skeletal muscle resulting from invasion of tissues by metastatic tumours (Agarwala et al., 2009). LDH level is currently the strongest independent prognostic factor in advanced metastatic melanoma and therefore proves useful in the postoperative monitoring of patients as it is a good predictor of survival (Utikal et al., 2007). If serum LDH is elevated, the presence of metastases should be confirmed by CT, PET or MRI scans as elevated LDH is not exclusive to metastatic melanoma and can also result from muscle injury, liver disease and hypotension (Agarwala et al., 2009). It has been found that although elevated serum LDH is strongly associated with poor prognosis, there is a weak relationship between LDH level and tumour thickness. It is also the case that elevated LDH is not associated with specific sites of metastasis which shows that the LDH test should be performed irrespective of tumour size and location of metastases.

2.6. Melanoma Markers

To further optimise melanoma staging, there has been much recent research aiming to identify markers of disease progression. Current research is being undertaken to identify the expression of genes in metastatic cells which can be used to show residual disease in clinically disease-free patients. This will provide prognostic information in these patients revealing the likelihood of disease relapse (Hoon et al., 2000). The problem is that as more studies are undertaken, evidence shows that malignant melanoma is an extremely heterogeneous disease with patterns of gene expression varying considerably between cases and between cells within the same tumour (Hoon et al., 2000; Reynolds et al., 2003). However, although there is considerable variation within the disease, some genes are seen to be abnormally expressed in the majority of cases while others are implicated less frequently. Research continues into developing a reliable set of markers for use in profiling of the disease.

Other research has set out to establish which markers are associated with an aggressive phenotype with high metastatic potential as opposed to a less aggressive phenotype with low metastatic potential (Bittner et al., 2000; Ryu et al., 2007). Identification of these markers would allow the prognosis of early primary tumours to be evaluated independent of tumour thickness and before metastasis has occurred. Results to date have shown that the expression levels of many genes are altered in cells categorised as more aggressive compared with less aggressive cells. Many genes involved in cell cycle control, cell proliferation, DNA replication and repair and resistance to apoptosis are upregulated in more aggressive cells while many genes involved in cell adhesion and melanocyte differentiation are downregulated (Ryu et al., 2007). Unfortunately, results of these studies have been inconclusive due to the abundance of associated markers which vary considerably between cases resulting in a number of different phenotypes being associated with tumour aggressiveness (Seftor et al., 2002). It is also unclear whether primary tumour cells that appear to have an aggressive phenotype are the same cells responsible for the formation of secondary tumours in metastatic disease.

2.6.1. Types of Markers

Research has found an abundance of markers associated with melanoma. These markers may be normal melanocyte genes involved with normal melanocyte function, markers specific to melanoma which are not expressed in normal melanocytes or other tumours or non-specific markers involved with tumour growth and development which are expressed in a variety of cancer types (Medic et al., 2006). As previously mentioned, due to the heterogeneous nature of melanoma there are many markers associated with the disease with some being expressed more frequently than others. For this reason, only those markers detected in a large percentage of melanomas showing the greatest promise for use in improving diagnostic and prognostic techniques will be discussed.

2.6.1.1. Normal Melanocyte Markers

There are a number of markers associated with melanoma that are expressed in both normal melanocytes and melanoma cells. Tyrosinase is an enzyme involved in the production of melanin which is commonly used as a marker in melanoma diagnosis and prognosis (Osella-Abate et al., 2003; Proebstle et al., 2000; Schmidt et al., 2005). Although tyrosinase is expressed in normal melanocytes, its detection in patient blood suggests the presence of melanoma cells as normal melanocytes do not enter the circulatory system (Steen et al., 2008).

Microphthalmia-Associated Transcription Factor (MITF) is involved in the differentiation of melanocytes during melanocytic development (Loercher et al., 2005). It is also implicated in the proliferation and development of invasive behaviour of melanoma cells (Carreira et al., 2006; Garraway et al., 2005). Due to its role as a transcription factor, MITF regulates the expression of many other melanoma associated genes, such as MART-1 (Du et al., 2003).

Tyrosinase-Related Protein-1 (TYRP-1) and Tyrosinase-Related Protein-2 (TYRP-2) are both expressed in normal melanocytes and are involved in melanin synthesis (Urabe et al., 1993). These genes are also expressed in melanoma cells however their expression only provides prognostic information in more advanced stages of the disease (Takeuchi et al., 2004).

2.6.1.2. General Tumour Markers

A number of markers associated with melanoma are non-specific tumour markers expressed in a range of malignancies. Melanoma antigen gene A3 (MAGE-A3) is expressed in a variety of cancers including testicular cancer and melanoma, however it is not expressed in normal tissue with the exception of some reproductive cells such as male gametes and cells of the placenta (Koyanagi et al., 2005a). In melanoma, MAGE-A3 is more commonly expressed in early stages of the disease (Hoon et al., 2000; Koyanagi et al., 2005a).

S100 is a group of genes expressed in cells of neural crest origin and several S100 genes are expressed in neural crest derived tumours, which include melanoma (Schmidt et al., 2005). S100p is one S100 marker commonly used to detect metastasis in sentinel lymph nodes of melanoma patients (Goto et al., 2008). S100b is another S100 gene which is not frequently detected in early stage melanomas but is commonly detected in later stages of the disease where it corresponds with prognosis (Martenson et al., 2001).

2.6.1.3. Melanoma Specific Markers

Other markers are specific melanoma associated antigens which allow the differentiation of melanomas from other cutaneous malignancies. Melanoma Antigen Recognised by T cells-1 (MART-1), which also has the synonyms MLANA and Melan-A, is involved in the formation of melanosomes which are the melanin storing organelles contained in melanocytes (Du et al., 2003). MART-1 can be detected in peripheral blood of advanced stage patients suggesting its expression is related to disease progression and may be useful in predicting patient survival (Koyanagi et al., 2005a,b).

Melanoma-Associated Chondroitin Sulphate Proteoglycan (MCSP), also known as High-Molecular Weight Melanoma-Associated Antigen (HMW-MAA), is encoded by the CSPG4 gene (Wang et al., 2004). MCSP is expressed on the surface of melanoma cells and is implicated in cell adhesion, proliferation, migration and invasion (Yang et al., 2004; Yang et al., 2009). MCSP is uniformly and abundantly expressed in the majority of melanomas (Yang et al., 2004). It can further be detected in early metastatic cells during subcutaneous invasion (Wang et al., 2004) and is therefore a useful marker to target for detection of early metastasis. A study by Goto et al. (2008) showed that all sentinel lymph nodes in metastatic melanoma patients were positive for MCSP by immunohistochemical staining with MCSP antibody. The results also showed that MCSP was more specific and sensitive than MART-1 and S100p for detecting sentinel lymph node metastasis, both of which are commonly used for this purpose. Other research has shown MCSP is expressed on

the surface of circulating melanoma cells as circulating cells have been successfully captured using anti-MCSP bound immunomagnetic beads (Kitago et al. 2009; Ulmer et al., 2004).

Melanoma Cell Adhesion Molecule (MCAM), also known as MUC-18 and CD146, is a cell surface melanoma marker encoded by the MCAM gene (Wang et al., 2004). The sequence of MCAM is similar to the neural cell adhesion molecules of the immunoglobulin superfamily, which are involved in organogenesis during development (Lehmann et al., 1989). This suggests that MCAM is normally involved in intercellular adhesion and may also be developmentally regulated. It has been observed that MCAM is downregulated in normal melanocytes and the majority of early melanomas probably due to cell-cell contact with neighbouring keratinocytes (Shih et al., 1994). Low level expression is seen in early melanomas, which is thought to be due to escaped control of keratinocytes, and high level expression is observed in late stage melanomas due to constitutive activation of the MCAM gene. MCAM is constitutively expressed in 70% of metastatic melanomas with amplified expression associated with increased tumour thickness and aggressive invasive behaviour (Mangahas et al., 2004; Medic & Ziman, 2010). Studies have shown MCAM is frequently detected in multi-marker qRT-PCR assays of patient peripheral blood and its expression is strongly related to disease progression (Hoon et al., 2000).

2.7. Melanoma Stem Cells

Recent research has identified melanoma stem cells capable of self renewal and differentiation which may lead to tumourigenesis (Boiko et al., 2010; Sekulic et al., 2008). It is thought that proliferation of these highly resistant cells after treatment may result in disease relapse (Fig 2.4.). Similar cells have been identified in other malignancies and it is thought they may play a significant role in tumour maintenance in those tumours which are notoriously difficult to treat. It has been observed that normal stem cells show a great deal more resistance to toxic treatments such as chemotherapy and radiotherapy than their differentiated descendants (Sekulic et al., 2008). The idea that melanomas arise from tumorigenic stem cells is further hinted at

by the heterogenic nature of the disease which suggests tumours originate from cells capable of multi-lineage differentiation (Fang et al., 2005).

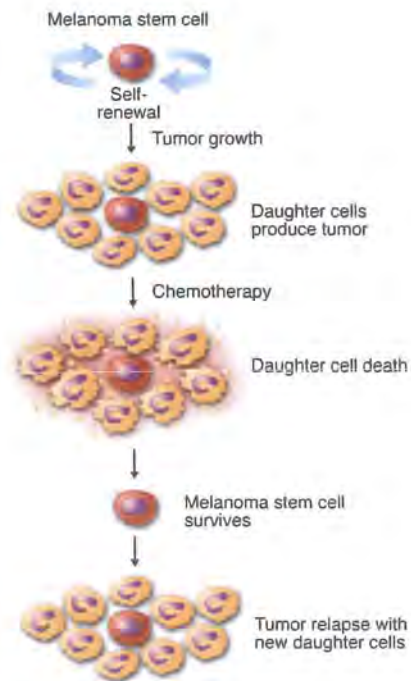


Figure 2.4. The proposed capability of melanoma stem cells for tumour maintenance and renewal (Sekulic et al., 2008)

A sub-population of melanoma stem cells express ATP-Binding Cassette Subfamily B Member 5 (ABCB5) on their surface. ABCB5 is actively involved in removing toxic substances, such as chemotherapeutic agents, from these cells (Chen et al., 2005). It has been shown that ABCB5 positive cells have greater tumorigenic capacity as well as a greater ability for renewal and differentiation than ABCB5 negative cells. The possible role of ABCB5 positive stem cells in tumour growth was shown by Schatton et al. (2008) who demonstrated inhibited tumour growth in mice that underwent treatment targeting ABCB5 positive cells. If the role of these stem cells in tumour formation is established and their presence among circulating cells is confirmed, approaches to treatment would be significantly influenced as these highly resistant cells would need to be targeted concurrently with the general tumour cell population in order to reduce the chance of disease relapse.

2.8. Circulating Tumour Cells

More recently, research has been conducted to measure markers of circulating melanoma cells in patient blood. The rationale of this is that previous studies have shown circulating tumour cells are often present in the circulation of cancer patients and are associated with tumour progression (Steen et al., 2008). It is estimated that on average, tumour cells circulate in the blood at a concentration of one per 10^6 to 10^7 normal cells (Krivacic et al., 2004). Due to the ability of circulating tumour cells to form metastases, their detection may identify patients with a greater risk of disease relapse significantly earlier than methodologies for the detection of secondary tumours or residual disease, which is currently undetectable at early stages (Reynolds et al., 2003). Because of this, it is thought that circulating melanoma cells may be a good measure of prognosis and may be able to guide treatment of the disease (Hoon et al., 2000; Steen et al., 2008; Ulmer et al., 2004). Current prognostic techniques, including measuring primary tumour thickness and sentinel lymph node examination, are unable to adequately guide treatment and it is thought that the detection of circulating melanoma cells may provide information that can be monitored to establish treatment strategies and determine their efficacy. Furthermore, these prognostic tests are unable to identify many early stage patients requiring treatment as a significant number of non-metastatic patients who are clinically disease free following surgery later develop metastatic disease. It has been suggested that approximately 30% of in situ patients (Koyanagi et al., 2005a) and 15% of patients with no evidence of lymph node metastasis (Jack et al., 2006; Weight et al., 2006) at the time of surgical intervention subsequently develop metastases. Quantifying circulating cells in early stage patients may therefore be useful in identifying those patients at risk of disease relapse who may benefit from some form of treatment.

2.8.1. qRT-PCR Detection of Markers

Early methods of detecting circulating tumour cells in patient blood involved the automated scanning of blood samples in order to identify labelled cells, however these methods were extremely time consuming. Quantitative Real-Time Polymerase

Chain Reaction (qRT-PCR) has allowed marker mRNA transcripts in circulating melanoma cells to be detected in patient peripheral blood samples suggesting the presence of circulating melanoma cells (Arenberger et al., 2008; Hoon et al., 1995; Hoon et al., 2000; Koyanagi et al., 2005a; Lewis et al., 2005; Reynolds et al. 2003). This technique can detect markers in extremely small quantities and far more rapidly than previous methods due to its sensitivity and efficiency. It has been shown that qRT-PCR can detect RNA from one malignant cell in 10^7 normal background cells which is sufficiently sensitive to detect low numbers of circulating tumour cells (Lewis et al., 2005). Furthermore, many studies have detected markers in peripheral blood of AJCC stage I and II patients who have no evidence of metastasis, suggesting the presence of circulating melanoma cells even in early stages of the disease (Hoon et al., 1995; Hoon et al., 2000; Koyanagi et al., 2005a).

It has been shown that circulating tumour cells can be detected in patient peripheral blood using a single marker qRT-PCR assay (Proebstle et al., 2000; Stoitchkov et al., 2001), although more recent studies have aimed to develop a suitable multi-marker assay to increase the sensitivity of the test and thus increase rates of detection. The reason for the requirement of a multi-marker assay is due to the heterogeneous nature of melanoma, which causes variability of marker expression between cells meaning not all cells will be detected by RT-PCR of a single mRNA transcript (Hoon et al., 2000). Studies employing multi-marker qRT-PCR assays have shown they offer a higher detection rate than single marker assays (Hoon et al., 1995; Koyanagi et al., 2005a; Reynolds et al. 2003), however a standard multi-marker assay has yet to be developed.

Research has shown, however, that there are many limitations to this technique. Due to the heterogeneous nature of melanoma, marker expression can vary considerably between patients and even within the same patient (Steen et al., 2008) resulting in the possibility of false negatives as the genes being targeted may not be expressed or may not be undergoing transcription at the time blood is obtained. One particular study showed that patients may be positive or negative for certain melanoma markers at different times of the day depending on the time blood samples were taken (Keilholz et al., 2004). Some studies have also reported a number of false

positives where control blood samples appear positive for particular melanoma markers. This could be due to inadequate specificity of the test, epithelial cell contamination of blood samples or simple issues of quality control (Steen et al., 2008). In addition to specificity issues, some studies have shown this technique to be inadequately sensitive, reporting detection of markers by qRT-PCR in fewer than 50% of stage IV patients with advanced metastatic disease (Mocellin et al., 2004).

2.8.2. Circulating Cells: Quantity vs. Phenotype

Although qRT-PCR shows circulating cells are present in patient blood, via the detection of melanoma markers, it does not allow them to be quantified. A suitable method allowing the quantification of circulating cells in patients at different clinical stages of melanoma would be valuable as it will help to reveal the relationship between CTC numbers and disease prognosis. At this point in time the influence of quantity and phenotype of circulating cells on prognosis is largely unknown with current research producing conflicting evidence. Some studies have shown that an increase in circulating cell numbers appears to correlate negatively with survival (Steen et al., 2008; Ulmer et al., 2004); although these studies are far from definitive as circulating cells were unable to be captured in many patients tested. Furthermore, some evidence suggests cells with tumourigenic potential, based on their phenotype, are rare (Boiko et al., 2010), whereas others argue that the majority of melanoma cells may be capable of tumour formation (Quintana et al., 2008). If an increase in circulating cell numbers is found to correlate with more advanced disease stage, circulating cell numbers could be used as a measure of disease progression to improve prognostic testing.

2.9. Immunomagnetic Bead Capture

Currently, immunomagnetic bead capture is one of the most promising methods of isolating circulating tumour cells from patient peripheral blood samples (Steen et al., 2008). Immunomagnetic beads, when coupled with antibody, allow for the isolation of specific cells within a sample as the antibody-bead ligand is allowed to bind to the cell type of interest which can then be isolated from the sample with the use of a magnet (Fig 2.5.).

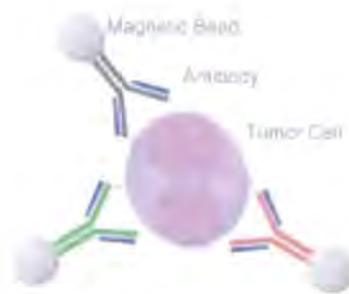


Figure 2.5. The principle of isolating CTCs through immunomagnetic enrichment. (Adnagen, n.d.) Magnetic beads are coupled with antibody which bind CTCs in patient peripheral blood. CTCs can subsequently be isolated from the sample using a magnet.

A number of studies have provided evidence showing isolation of melanoma cells from patient peripheral blood via immunomagnetic bead capture is successful (Faye et al., 2004; Kitago et al. 2009; Ulmer et al., 2004). In order for immunomagnetic beads to capture circulating tumour cells they must be able to bind a minimum of one cell per 10^6 background cells otherwise low numbers of circulating cells would not be able to be captured (Krivacic et al., 2004). The results of blood spiking experiments, where single melanoma cells have been added to control blood samples to demonstrate the detection limit of antibody-coupled beads, have shown that these beads are sensitive enough to bind low concentrations of circulating tumour cells. For example, a study by Faye et al. (2004) showed that anti-MCSP coupled immunomagnetic beads were able to bind a single melanoma cell in a sample containing 10^7 normal blood cells. A similar spiking experiment by Kitago et al. (2009) demonstrated their anti-MCSP beads were able to bind one melanoma cell in 5×10^6 blood cells. These results show that immunomagnetic bead capture is a sensitive enough method to isolate low quantities of circulating melanoma cells.

The CellSearch system uses EpCAM antibody bound immunomagnetic beads to isolate circulating tumour cells in a number of malignancies of epithelial origin (Steen et al., 2008). This system has been used successfully to isolate circulating cells from peripheral blood in breast cancer patients and was tested in stage III and IV melanoma patients as circulating melanoma cells are known to be positive for EpCAM. The results showed that circulating cell numbers appeared to correlate with survival as an increased number of circulating cells was associated with a decreased survival time. However, the anti-EpCAM beads were only able to capture circulating cells in some of the stage IV patients included in the study. As the marker used in this assay is not melanoma specific, the use of immunomagnetic beads bound to melanoma specific antibodies may be more successful.

Studies trialling immunomagnetic bead capture of circulating melanoma cells with anti-MCSP coupled beads have provided mixed results. One study was able to isolate circulating melanoma cells from 100% of stage IV patients tested (Kitago et al., 2009), while another was only able to detect circulating cells in 1% of patients ranging from stage I to IV; however this study defined captured cells as those bound to more than four beads which were detected by microscopy (Faye et al., 2004). A study by Ulmer et al. (2004) was able to isolate cells from patients of all AJCC stages with anti-MCSP beads, although cells were not detected in all patients; only 43% of stage IV patients were positive for circulating cells. Also, the number of cells captured in patients of each stage varied widely with considerable overlap, possibly due to heterogeneous expression, suggesting a combination of antibodies would be more effective at capturing cells. Since previous results have only been somewhat successful, different combinations of antibodies should be trialled to optimally capture circulating melanoma cells in patients at all stages of the disease in order to show the difference in quantity and phenotype of early and late stage cells.

2.9.1. Suitable Target Antigens

Due to the mixed success when a single marker was targeted in previous bead capture studies, as well as the fact that melanoma is extremely heterogeneous, a combination of antibodies was used in this study in order to maximise the chances of successfully isolating circulating melanoma cells in patients' at all stages of the disease. For an antibody to be suitable for capturing circulating cells it must have a number of characteristics. Firstly, the marker for which the antibody is specific must be expressed on the surface of melanoma cells. This is to ensure the antibody can recognise and bind to the marker which would not be possible if it was expressed within the cell. Antibodies should also target markers which are expressed in the majority of cells to ensure all circulating cells are captured when used in combination with other antibodies.

MCAM is constitutively expressed on the surface of the majority of advanced stage melanoma cells and its expression is strongly related to disease progression (Mangahas et al., 2004; Medic & Ziman, 2010; Shih et al., 1994). For this reason, MCAM is a suitable marker to target for the isolation of circulating melanoma cells in patients of more advanced stages of the disease. The fact that MCAM expression is associated with aggressive tumour behaviour also suggests that it is a suitable marker to target for the isolation of aggressive cells with high metastatic potential.

MCSP is expressed on the surface of early stage melanoma cells (Wang et al., 2004; Yang et al., 2004) and its expression continues throughout all stages of the disease (Faye et al., 2004; Goto et al., 2008; Kitago et al., 2009; Ulmer et al., 2004). MCSP is therefore a suitable cell surface marker to target for the isolation of early stage circulating cells as well as more advanced stage cells which do not express MCAM and therefore may have a less aggressive phenotype. As previously discussed, it has been suggested that although stage I and II patients do not have metastatic disease they may still have circulating tumour cells as peripheral blood of these patients has been shown to be positive for a number of melanoma markers by qRT-PCR (Hoon et al., 1995; Hoon et al., 2000; Koyanagi et al., 2005a).

Lastly, ABCB5 is expressed on the surface of a subset of melanoma stem cells which have greater tumourigenic capability than ABCB5 negative melanoma stem cells (Chen et al., 2005). ABCB5 antibody can therefore be used to isolate circulating ABCB5 positive melanoma stem cells which are thought to be implicated in disease relapse following treatment (Chen et al., 2005; Sekulic et al., 2008). Currently, no studies have shown melanoma stem cells in patient circulation although unpublished results from our laboratory have shown many patient blood samples are positive for ABCB5 by qRT-PCR.

2.9.2. Immunomagnetic Bead Capture in other Malignancies

The use of immunomagnetic bead capture to isolate circulating tumour cells in other malignancies, including those of the prostate and colon, has been investigated for some time. This technique is most commonly used however, and has provided the most success, in breast cancer patients. Many studies have provided evidence that this method is successful and have shown that the presence of circulating cells correlates with poor prognosis (Astrup et al., 2000; Cristofanilli et al., 2004; Raynor et al., 2009; Tewes et al., 2009). The use of this technique in breast cancer patients has proven to be both more sensitive and specific at detecting circulating cells than qRT-PCR (Bossolasco et al., 2002; Martin et al., 2001; Witzig et al., 2002) and metastatic cells circulating at 1 in 10^6 - 10^7 background cells can be consistently captured (Reuben et al., 2008). A study by Raynor et al. (2009) used immunomagnetic bead capture to isolate circulating cells from patient blood and subsequently used a multi-marker qRT-PCR assay on isolated cell RNA to detect tumour associated markers. The results of this study showed the number of patients positive for at least one marker increased with both disease stage and primary tumour thickness. Another study showed that patients positive for circulating cells had shorter overall survival than those classified as negative, where a patient was considered positive if they had five or more circulating cells per 7.5ml of blood (Cristofanilli et al., 2004). Quantification of circulating cells has also shown success at monitoring patient responses to treatment; specifically an increase in circulating cells at treatment completion predicts early relapse of the disease (Lobodasch et al., 2007; Pachmann et al., 2008; Pachmann et al., 2005).

The success of this method has led to its clinical use with the CellSearch system being approved by the US Food and Drug Administration for the detection of circulating cells in patients with metastatic carcinoma of the breast (Reuben et al., 2008). The reason for the greater success of this technique in breast cancer patients compared with melanoma patients is likely due to the trialling of different combinations of antibodies to capture circulating cells which has led to the development of many breast cancer tests, such as the AdnaTest (Adnagen), which target a combination of cell surface markers. Studies trialling this technique in melanoma patients have targeted single markers only going as far as testing a combination of clones of an antibody to isolate circulating cells (Kitago et al. 2009). Both carcinoma of the breast and melanoma are heterogeneous and for this reason the use of multiple antibodies proposed in this study may increase the success of this technique in melanoma.

Overall, the use of this technique in breast cancer patients has demonstrated circulating cells are an independent predictor of survival of greater value than standard prognostic measures (Reuben et al., 2008). This shows that isolation of circulating cells by immunomagnetic bead capture is useful for determining patient prognosis and with further investigation this technique has the potential to be similarly applied in the clinical testing of melanoma patients.

3. Summary and Theoretical Framework

The development of an immunomagnetic bead capture assay using antibodies able to effectively bind melanoma cells of all disease stages will provide a suitable method of isolating circulating melanoma cells. This technique will have advantages over the use of RT-PCR, which can only detect the presence of circulating cells, by allowing circulating cells to be captured and quantified. This will reveal the prognostic significance of circulating cell numbers which has not yet been determined. If it is found that circulating cell numbers correlate strongly with prognosis, i.e. circulating cell numbers increase with increased disease stage, this technique could be used routinely as a prognostic measure in combination with other available measures, such as primary tumour thickness. If this is the case, quantification of circulating cells could also be used as a measure of treatment efficacy. For example, the number of circulating melanoma cells in a patient undergoing therapy could be continually measured to determine whether the treatment is effective, i.e. whether it is reducing the number of circulating cells. This would allow the effectiveness of multiple treatments on individual patients to be determined.

As well as the quantification of circulating cells, this technique would allow phenotypic analysis of captured cells to be performed in further studies in order to characterise circulating cells of different disease stages. This would allow phenotypic differences between early and late stage cells to be determined, ultimately revealing the influence of circulating cell phenotype on prognosis.

In this study, MCSP, MCAM and ABCB5 antibody coupled immunomagnetic beads were used to isolate and subsequently quantify circulating melanoma cells from patient peripheral blood samples across all disease stages in order to determine a relationship between the number of circulating cells and disease stage.

4. Hypothesis and Aims

4.1. Hypothesis

The number of circulating melanoma cells in patient peripheral blood correlates with disease stage.

4.2. Aims

The overall aim of this research project is to isolate and quantify circulating melanoma cells in patient peripheral blood in order to determine the relationship between the number of CTCs and disease stage. This will be achieved by:

- 1) Checking for marker expression in melanoma cell line and tissue sections of different stages to determine the suitability of chosen antibodies to isolate circulating melanoma cells.
- 2) Manufacturing and testing MCSP, MCAM and ABCB5 antibody coupled immunomagnetic beads.
- 3) Isolating circulating melanoma cells from patient peripheral blood samples.
- 4) Quantifying total circulating cells relative to disease stage.
- 5) Quantifying MCSP, MCAM and ABCB5 positive circulating cells relative to disease stage.

5. Materials and Methods

5.1. Antibodies

5.1.1. MCAM

The MCAM antibody used for cell immunofluorescence and immunomagnetic bead coupling was mouse monoclonal anti-human CD146, isotype IgG1 kappa, at a concentration of 0.5mg/ml, sourced from BD Biosciences (San Diego, USA). The MCAM antibody used for tissue immunohistochemistry was rabbit monoclonal anti-human CD146, isotype IgG, sourced from Abcam (Cambridge, USA).

5.1.2. MCSP

The anti-MCSP antibody used for cell immunohistochemistry, tissue immunohistochemistry and immunomagnetic bead coupling was mouse monoclonal anti-human MCSP clone number 9.2.27, isotype IgG2a, at a concentration of 0.5mg/ml, sourced from BD Biosciences.

5.1.3. ABCB5

The anti-ABCB5 antibody used for immunomagnetic bead coupling was rabbit polyclonal anti-human ABCB5 at a concentration of 0.31mg/ml, sourced from Rockland Immunochemicals. The anti-ABCB5 antibody used for flow-cytometry was mouse monoclonal anti-human ABCB5, clone number 3C21D12, received from Markus Frank, Brigham and Women's Hospital, Harvard Medical School (Boston, USA).

5.2. Cell lines

The A2058 melanoma cell line was used to demonstrate marker expression in melanoma cells as well as for experimental testing of antibody-coupled immunomagnetic beads. This highly invasive malignant melanoma cell line was established from a lymph node metastasis of a 43 year old male. HEK293 cells (Human Embryonic Kidney 293 cells) were used as a negative control in the testing of marker expression and antibody-coupled beads.

5.3. Blood Samples

5.3.1. Patient Samples

This study was conducted according to the National Statement on Ethical Conduct in Research Involving Humans, approved by the Edith Cowan University Human Research Ethics Committee. Patients with clinical disease stages 0 - IV were recruited by five Perth based clinicians. Six stage 0 patients, ten stage I patients, eight stage II patients, three stage III patients and six stage IV patients were included in the study. Patients were diagnosed and staged based on clinical and pathological data using the AJCC staging system for melanoma (Table 2.1). Patients with metastatic disease were treated as per standard clinical protocols. Participants signed informed consent with the clinician in accordance with protocols safeguarding patient rights and were provided with a clinical trial form for blood collection. Approximately 4ml of blood was drawn by phlebotomists into EDTA tubes, stored refrigerated at pathology centres and collected when possible. A clinical registrar recorded patient clinical data in an excel spreadsheet kept separate from laboratory results.

5.3.2. Control Samples

Control blood for use in experimental testing of immunomagnetic beads was my

own or sourced from volunteers and was drawn by a phlebotomist into EDTA tubes and used immediately. There were 21 control blood samples used for comparison with patient samples, which were obtained from healthy volunteers with no previous history of melanoma and drawn by a phlebotomist into EDTA tubes and processed within a few hours. The first few millilitres of all blood samples collected for this study were discarded in order to avoid epithelial cell contamination.

5.4. Marker Expression in A2058 Melanoma Cell Line

The expression of MCAM and MCSP in A2058 melanoma cells was demonstrated by immunofluorescence. A2058 cells and HEK293 cells, to be used as a negative control, were plated onto sterile coverslips at a density of 5×10^4 cells per coverslip and allowed to adhere overnight. The media was removed by aspiration and the coverslips were washed in PBS. The PBS was then aspirated and the cells were fixed by a 10 minute room temperature incubation in 4% paraformaldehyde. The cells were washed twice with PBS and permeabilised by incubation in 0.2% Triton X-100 in PBS for 5 minutes at room temperature. The coverslips were then washed three times in 0.2% Triton X-100 in PBS over 5 minutes.

The coverslips were incubated at room temperature for 60 minutes with the primary antibody solution. Antibodies were diluted 1 in 500 in 0.2% Triton X-100 in PBS containing 3% BSA. The coverslips were then washed three times in 0.2% Triton X-100 in PBS over 5 minutes. They were then incubated at room temperature for 20 minutes with the secondary antibody (anti-mouse IgG conjugated to Alexa Fluor 488), also diluted 1 in 500 in 0.2% Triton X-100 in PBS containing 3% BSA. Coverslips were again washed three times in 0.2% Triton X-100 in PBS over 5 minutes, drained well and placed onto a drop of mounting medium (ProLong Gold antifade reagent, containing DAPI for nuclear staining from Invitrogen (Oslo, Norway)) on a microscope slide. The slides were allowed

to air dry before viewing by fluorescence microscopy with an epifluorescent Olympus BX51 microscope equipped with an Olympus DP71 camera.

The demonstration of ABCB5 expression in these cells was attempted using the above protocol however staining was negative raising questions regarding the suitability of this particular ABCB5 antibody for immunofluorescence. Instead, flow-cytometry was used in order to demonstrate ABCB5 expression in A2058 cells.

5.5. Flow Cytometry

A2058 cells were harvested with media containing EDTA (2mM EDTA in DMEM media) and resuspended in 10ml of DMEM media containing 10% FBS. 0.5ml was counted on the Vi-Cell-XR Cell Viability Analyser (Beckman Coulter, Brea, USA) and the appropriate volume containing approximately 500,000 cells was dispensed into each of 4 tubes. The cells were pelleted at 2000rpm for 5 minutes and the media was removed. The pellets were washed in 500µl of MACS buffer (2mM EDTA, 0.5% BSA in PBS, pH 7.2) (Miltenyi, Bergisch Gladbach, Germany), pelleted again and the supernatants were aspirated to remove any remaining media. The pellets were then resuspended in 500µl of MACS buffer. Two tubes were incubated with primary antibody, one with ABCB5 antibody (1/500) and another with MCSP antibody conjugated to PE (1/500), for 20 minutes at room temperature. The cells were then pelleted, the supernatant was aspirated and the pellet was resuspended in 500µl of MACS buffer. The tube containing ABCB5 antibody and one tube without a primary were both incubated with secondary antibody (anti-mouse IgG conjugated to Alexa Fluor 488) for 20 minutes at room temperature. The tubes were then centrifuged at 2000rpm for 5 minutes, the supernatant was removed and the pellets were resuspended in 500µl of MACS buffer. These samples were then run on a Gallios Flow Cytometer (Beckman Coulter). Cells were separated by side and forward scatter

and antibody staining was detected by analysis of fluorescence emission of cells where positive cells had increased fluorescence as a result of antibody conjugated fluorochromes.

5.6. Marker Expression in Paraffin Embedded Melanoma Tissue Sections

The expression of MCSP and MCAM in sections of early and late stage melanoma tissue was demonstrated by immunofluorescence. Three sections each of in situ, invasive primary and metastatic melanomas were stained with MCSP and MCAM antibodies in order to determine the extent of expression of these genes in different stages of the disease. Demonstration of ABCB5 expression was not attempted in tissue sections due to the extremely limited quantity of antibody available and the inability to demonstrate ABCB5 expression in A2058 cells by immunofluorescence.

5.6.1. MCAM Staining

Paraffin embedded tissue sections on glass slides were dewaxed by incubation at 58⁰C for 20 minutes followed by a series of wash steps through xylene, ethanol and distilled water. Antigen retrieval was performed with EDTA/Tris buffer, pH 8.0, by three 5 minute cycles in a microwave set at 50% power (approximately 750 watts). Slides were then cooled in a cold water bath for 20 minutes, briefly dried and marked with a pap pen. Slides were then washed with PBS three times for 5 minutes. Protein blocking was performed by 1 hour incubation at room temperature with 10% normal goat serum (NGS) in PBST (0.2% Triton X-100 in PBS, pH7.4). The protein blocking solution was poured off and the slides were then incubated with primary antibody solution (rabbit anti-human MCAM diluted 1 in 500 in PBST containing 1% NGS) for 1 hour at room temperature. Following this, slides were kept overnight in PBS at 4⁰C.

Slides were allowed to reach room temperature and were washed with PBS three

times for 5 minutes. Sections were then incubated with secondary antibody solution (anti-rabbit IgG conjugated to Alexa Fluor 488 diluted 1 in 500 in PBST containing 1% NGS and Hoechst 1 in 50,000) for 1 hour at room temperature. PBS was pipetted onto the slides to remove the antibody solution and slides were subsequently washed in PBS for 1 hour, changing the PBS every 10 minutes. Slides were then mounted while still wet with a drop of Fluorsave Reagent (Calbiochem, San Diego, USA) and were allowed to dry before viewing by fluorescence microscopy with a drop of ProLong Gold anti-fade reagent containing DAPI (Invitrogen), as described in 5.8. This method was previously described in Medic and Ziman (2010).

5.6.2. MCSP Staining

The above method proved unsatisfactory for MCSP staining so a suitable protocol was optimised specifically for this antibody. Optimal results for MCSP staining were achieved by following the same protocol as described in 5.6.1., although primary antibody concentration and incubation time was altered. Primary antibody (mouse anti-human MCSP) was diluted 1 in 20, in PBST containing 1% NGS and incubated overnight at 4⁰C. The remaining portion of the protocol was identical to that described in 5.6.1.

For both MCAM and MCSP staining, positive and negative controls were used to confirm positive staining within tissue sections. Paraffin-embedded A2058 cells were used as a positive control and sections stained with secondary antibody only were used as negative controls.

5.7. Antibody-Bead Coupling

MCAM, MCSP and ABCB5 antibodies were bound to immunomagnetic beads using the Dynabeads Antibody Coupling Kit (Invitrogen). This kit contained

Dynabeads M-270 Epoxy beads, coupling solutions ('C1' and 'C2'), wash buffers ('LB' and 'HB') and storage buffer ('SB').

It was decided to use 10µg of antibody per mg of beads for optimal antibody-bead coupling, based on manufacturer's suggestions. Three volumes of Dynabead M-270 Epoxy beads were weighed out, one for each antibody, and were washed by vortexing in 1ml of C1 solution. The washed beads were then collected by a magnet and the supernatant was removed. The appropriate volumes of C1, as indicated in the kit instructions, were added to the beads along with the corresponding volumes of each antibody and the resulting solutions were mixed by vortexing. The appropriate volumes of C2 were added and the solutions were again mixed by vortexing. The antibody-bead solutions were incubated on a roller at 37⁰C overnight to allow binding. The antibody coupled beads were collected on a magnet and the supernatant was removed. The beads then underwent a series of washes described in the manufacturer's instructions and were finally resuspended in storage buffer and stored at 4⁰C until use.

5.8. Testing Antibody-Coupled Beads on A2058 Cells

A2058 cells were harvested with accutase and resuspended in 10ml DMEM media containing 10% FBS. 0.5ml was counted on the Vi-Cell-XR Cell Viability Analyser (Beckman Coulter) and the appropriate volume containing approximately 500,000 cells was added to each of 3 tubes. 1µl of beads was added to each tube, one bead type per tube, and tubes were then incubated for 30 minutes at 4⁰C on a roller to allow binding. Tubes were placed on a magnet to collect the bead bound cells and the supernatant was removed. The beads were washed 3 times in 1ml MACS buffer (2mM EDTA, 0.5% BSA in PBS, pH 7.2), resuspended in 100µl MACS buffer and pipetted onto coverslips on a magnet. The liquid was aspirated and the coverslips were allowed to dry before being mounted onto microscope slides with a drop of ProLong Gold anti-fade reagent containing DAPI (Invitrogen).

5.9. Bead Capacity

A2058 and HEK293 cells were harvested with accutase and counted on the Vi-Cell-XR Cell Viability Analyser (Beckman Coulter). The volume containing approximately 500,000 cells was calculated for each cell type and added to 6 tubes, 3 containing A2058 cells and 3 containing HEK293 cells. The cells were pelleted at 2000rpm for 5 minutes, the supernatants were removed and the pellets were washed in 1ml MACS buffer (2mM EDTA, 0.5% BSA in PBS, pH 7.2). The solutions were then centrifuged again, the supernatants were discarded and the pellets were resuspended in 1ml MACS buffer. 1µl of beads was then added to each tube, each bead type being added to one tube of A2058 and one tube of HEK293 cells. The tubes were then incubated on a roller for 30 minutes at 4°C.

Tubes were placed on a magnet to collect the beads and washed 5 times with MACS buffer. The beads were resuspended in 1ml MACS buffer and were analysed on the Vi-Cell-XR Cell Viability Analyser in order to count the number of cells bound to the beads.

5.10. Preliminary Patient Test

Red blood cell (RBC) lysis buffer (140mM NH₄Cl, 17mM Tris, pH 7.65) was added to a patient blood sample to a total volume of 50ml and incubated at 37°C until the solution became clear, indicating complete lysis. The solution was centrifuged at 300g for 5 minutes at 4°C and the supernatant was discarded. The pellet was washed in RBC lysis buffer, centrifuged again, the supernatant was discarded and the pellet was resuspended in 3ml MACS buffer. This solution was split into 3 tubes and 1µl of 1/10 diluted beads was added, a different bead type for each tube. The tubes were incubated on a roller for 30 minutes at 4°C to allow binding and subsequently placed on a magnet to collect the beads. The beads were washed 5 times with MACS buffer, resuspended in 100µl of MACS buffer

and mounted onto microscope slides with a drop of ProLong Gold anti-fade reagent containing DAPI (Invitrogen) as described in 5.8.

5.11. Control Blood- 30 Minute versus Overnight Incubation

A control blood sample was incubated at 37⁰C with RBC lysis buffer until lysis was complete. The solution was centrifuged at 300g for 5 minutes at 4⁰C to pellet the remaining cells and the supernatant was discarded. The pellet was washed in RBC lysis buffer, re-pelleted and the supernatant was discarded. The pellet was resuspended in 6 ml MACS buffer and split into 6 eppendorf tubes. 1µl of 1/10 diluted beads was added to each tube, with each bead type being added to two tubes. One set of tubes was incubated on a roller for 30 minutes at 4⁰C, while the second set of tubes was incubated on a roller at 4⁰C overnight.

Tubes were subsequently placed on a magnet to collect the beads and washed five times with MACS buffer. The beads were resuspended in 100µl MACS buffer, pipetted onto coverslips on a magnet, the wash buffer was aspirated and the beads were mounted onto microscope slides.

5.12. Trypan Blue Staining of Captured Cells

RBC lysis of a patient blood sample was performed as described in 5.10. The solution was centrifuged at 300g for 5 minutes at 4⁰C and the supernatant was removed. The pellet was washed in RBC lysis buffer, centrifuged again and the supernatant was discarded once more. The pellet was resuspended in 1ml MACS buffer and 1µl of 1/10 diluted MCAM beads was added. The sample was incubated on a roller at 4⁰C overnight to allow binding. The tube was placed on a magnet and the beads were washed 5 times with MACS buffer. The beads were resuspended in 100µl of MACS buffer, mixed with 0.1% trypan blue and mounted on a microscope slide with a drop of ProLong Gold anti-fade reagent containing

DAPI (Invitrogen), as described in 5.8.

5.13. MACS versus Triton X-100 Wash

A2058 and HEK293 cells were harvested with accutase and counted on the Vi-Cell-XR Cell Viability Analyser (Beckman Coulter). The volume of approximately 10^6 cells was calculated for each cell type and added to an eppendorf tube. $1\mu\text{l}$ of MCAM beads was added to each tube and the tubes were incubated on a roller for 30 minutes at 4°C to allow binding. Each tube was then split into two and placed on a magnet. One set of tubes containing A2058 and HEK293 cells were washed 5 times with MACS buffer (2mM EDTA, 0.5% BSA in PBS, pH 7.2), while the other set of tubes were washed 5 times with 0.2% Triton X-100 in PBS. Samples were then mounted onto microscope slides with a drop of ProLong Gold anti-fade reagent containing DAPI (Invitrogen), as described in 5.8.

5.14. Blood Spiking

A2058 cells were harvested with EDTA media (2mM EDTA in DMEM media) and resuspended in 10ml of DMEM media containing 10% FBS. 1ml of cells was diluted 1/10 in a petri dish for spiking into blood. RBC lysis of control blood performed as described in 5.10. The cells were pelleted at 300g for 5 minutes at 4°C and the supernatant was discarded. The pellet was resuspended in MACS buffer and 0.5ml was analysed on the Vi-Cell-XR Cell Viability Analyser (Beckman Coulter) to determine cell count and viability. The remainder of the resuspended blood cells were split equally into eppendorf tubes. The desired number of A2058 cells were pipetted up from the petri dish under a microscope using a micropipette and spiked into the eppendorf tubes containing control blood cells. 1, 10, 30, 50 and 100 cell spikes were performed and repeated four times. Once A2058 cells had been spiked into blood cells, $1\mu\text{l}$ of 1/10 diluted MCSP beads was added and tubes were incubated on a roller at 4°C overnight.

The tubes were placed on a magnet to collect the beads, the supernatant was removed and the beads were washed 5 times in MACS buffer containing 0.2% Triton-X 100. The beads were resuspended in 100µl MACS buffer with 0.2% Triton-X 100 and mounted on microscope slides with a drop of ProLong Gold anti-fade reagent containing DAPI (Invitrogen), as described in 5.8. Cells were then counted by fluorescence microscopy with an epifluorescent Olympus BX51 microscope equipped with an Olympus DP71 camera to determine the number of spiked cells retrieved following incubation with the beads.

5.15. Non-Specific Binding in Fresh versus 4 Day Old Blood

Two control blood samples were taken from a healthy volunteer and one was processed immediately while the other was refrigerated and processed after 4 days. RBC lysis of samples was performed as described in 5.10. The remaining cells were pelleted at 300g for 5 minutes at 4⁰C and the supernatant was discarded. The pellet was washed in RBC lysis buffer, re-centrifuged and the supernatant was again removed. The pellets were resuspended in 3.5ml MACS buffer, 0.5ml was analysed on the Vi-Cell-XR Cell Viability Analyser (Beckman Coulter) to determine cell count and viability and the remaining 3ml was split into 3 tubes. 1µl of 1/10 diluted beads was added to each tube, one bead type per tube, and the tubes were incubated on a roller at 4⁰C overnight. Tubes were placed on a magnet and washed 5 times with 0.2% Triton X-100 in MACS buffer. The beads were resuspended in 100µl of the wash buffer, mounted onto microscope slides with a drop of ProLong Gold anti-fade reagent containing DAPI (Invitrogen), as described in 5.8., and viewed by fluorescence microscopy with an Olympus BX51 microscope equipped with an Olympus DP71 camera.

5.16. Isolation of Circulating Melanoma Cells from Patient Blood

To isolate melanoma cells from patient blood samples, firstly RBC lysis was performed as described in 5.10. The solution was pelleted at 300g for 5 minutes

at 4⁰C and the supernatant was discarded. The pellet was washed in RBC lysis buffer and re-pelleted to remove any remaining unlysed red blood cells. The pellet was resuspended in 3.5ml of MACS buffer (2mM EDTA, 0.5% BSA in PBS, pH 7.2) and mixed thoroughly to remove any cell clumps and homogenise the solution. 0.5ml of this sample was analysed on the Vi-Cell-XR Cell Viability Analyser (Beckman Coulter) to determine cell count and viability. The remaining 3ml was split into 3 eppendorf tubes and 1µl of 1/10 diluted beads were added, one bead type per tube. The blood cells and beads, suspended in 1ml MACS buffer, were incubated overnight on a roller at 4⁰C.

Tubes were then placed on a magnet to collect the beads and any bead-bound cells and the supernatant, containing unbound cells, was removed. The beads were then washed five times with 500µl of MACS buffer containing 0.2% Triton X-100 to remove any non-specifically bound cells. The beads were resuspended in 100µl MACS buffer containing 0.2% Triton-X 100 and were pipetted onto coverslips on a magnet before being mounted onto microscope slides with a drop of ProLong Gold anti-fade reagent containing DAPI (Invitrogen), as described in 5.8. Slides were left overnight to allow for full penetration of DAPI for optimal nuclear staining before being scanned by fluorescence microscopy using an Olympus BX51 microscope equipped with an Olympus DP71 camera in order to count the number of cells bound to each bead type. This protocol was performed for 21 control blood samples and 33 patient blood samples.

5.17. Statistical Analysis

Results obtained after performing immunomagnetic bead capture on patient and control samples were analysed using Predictive Analytics Software (PASW) (IBM, Somers, New York, USA), in order to demonstrate any relationships between CTC numbers and disease stage.

Firstly, the total numbers of captured cells in patient and control groups were compared using an independent samples t-test in order to illustrate the difference

between the mean numbers of captured cells in these two groups. A boxplot was generated in order to show the difference in the distribution of total captured cells between patient and control groups.

The relationship between the total cell count and the total captured cells of patient samples was analysed using the Pearson product-moment correlation coefficient. This analysis was performed in order to demonstrate the relationship between these two continuous variables to show whether total cell count was a confounding variable and therefore influenced the number of cells captured by immunomagnetic isolation.

The relationships between total captured cells, MCSP cells, MCAM cells, ABCB5 cells and disease stage were determined using Spearman's rank order correlation coefficient for non-parametric data. This test is useful for demonstrating correlation using ordinal, or ranked, variables, such as disease stage. These analyses were performed to show any correlations between these variables in order to demonstrate whether disease stage is a predictor of circulating cell numbers and thus support or refute the hypothesis that the number of circulating melanoma cells in patient peripheral blood correlates with disease stage. Patients were also split into non-metastatic (consisting of stage 0, I and II) and metastatic (consisting of stage III and IV) groups, in order to demonstrate the difference between the mean number of total captured cells in metastatic and non-metastatic patients using an independent samples t-test.

Patient 29, who had a total of 232 captured cells, was an extreme outlier and for this reason, statistical analyses were performed including and excluding this patient. It was found that the inclusion of this patient did not have a significant effect on the outcome and thus, all results are shown with this patient included.

6. Results

In this study, an assay for the isolation of circulating melanoma cells from patient peripheral blood samples by immunomagnetic bead capture was developed. First, target markers were chosen based on current literature and demonstration of their expression in melanoma cells and tissue by immunofluorescence. Antibody-coupled immunomagnetic beads were then manufactured and tested on control and melanoma cell lines in order to optimise the bead capture protocol. Finally, this protocol was performed for 21 control and 33 patient blood samples and the results were analysed to determine any relationships between the number of captured cells and disease stage.

6.1. Demonstration of Chosen Antigen Expression in Melanoma Cell Line by Immunofluorescence

Immunohistochemistry was performed to demonstrate MCSP, MCAM and ABCB5 expression in A2058 melanoma cells. Demonstration of expression of these markers in a metastatic melanoma cell line will provide evidence suggesting these markers will also be expressed in melanoma CTCs and will therefore be useful target markers for capturing CTCs from patient blood. Alexa Fluor 488 (A488), conjugated to anti-mouse IgG (for MCSP and MCAM antibodies) and anti-rabbit IgG (for ABCB5 antibody) were used as secondary antibodies to detect positive staining in the cells by fluorescence microscopy. HEK293 cells, as well as A2058 cells with secondary antibody only, were used as negative controls and therefore should be unstained in order to demonstrate that melanoma cells were stained specifically as a result of antibodies binding to target markers on their cell surface.

Fig.6.1.A shows A2058 cells stained with MCSP antibody and anti-mouse IgG conjugated to A488. All cells were positively stained as a result of IgG-A488 binding to the cell surface via anti-MCSP, confirming MCSP expression in all

A2058 melanoma cells. In contrast, no staining was observed in HEK293 cells (Fig.6.1.B) indicating MCSP is not expressed in these cells and further confirming the specificity of MCSP for A2058 melanoma cells. Therefore, MCSP is a useful antigen to target for immunomagnetic isolation of circulating melanoma cells.

Fig.6.1.C shows A2058 cells stained with MCAM antibody and anti-mouse IgG conjugated to A488. Again all cells were strongly positively stained whereas HEK293 cells were MCAM negative (Fig.6.1.D). This indicates that the MCAM antibody also binds specifically to A2058 melanoma cells and suggests MCAM is also a useful antigen to target for capturing circulating melanoma cells from patient blood. These results show that all A2058 melanoma cells are MCSP and MCAM positive, however this is not the case in all melanoma cells. MCSP is constitutively expressed in the majority of melanomas across all disease stages (Yang et al., 2004) whereas MCAM expression is associated with an aggressive, invasive phenotype and is linked with later stage disease (Mangahas et al., 2004). For this reason it is not expected that all circulating cells will be both MCSP and MCAM positive although it should be the case that all MCAM positive cells are also MSCP positive. Accordingly, a greater number of MCSP positive circulating cells than MCAM positive circulating cells are expected to be observed.

No non-specific staining was observed in the negative control where cells were stained only with A488 conjugated IgG without primary antibody (Fig.6.1.G). This confirms that the immunofluorescence observed in A2058 cells stained for either MCSP or MCAM was specific.

Attempts to demonstrate ABCB5 expression in A2058 melanoma cells by immunofluorescence with ABCB5 antibody was unsuccessful (Fig.6.1.E). This negative result is presumably due to the lack of suitability of this specific ABCB5 antibody for immunohistochemistry, an application for which it has not been tested (Rockland Immunochemicals, 2010). The alternative method of flow

cytometry was used to demonstrate ABCB5 expression in A2058 cells as reported below.

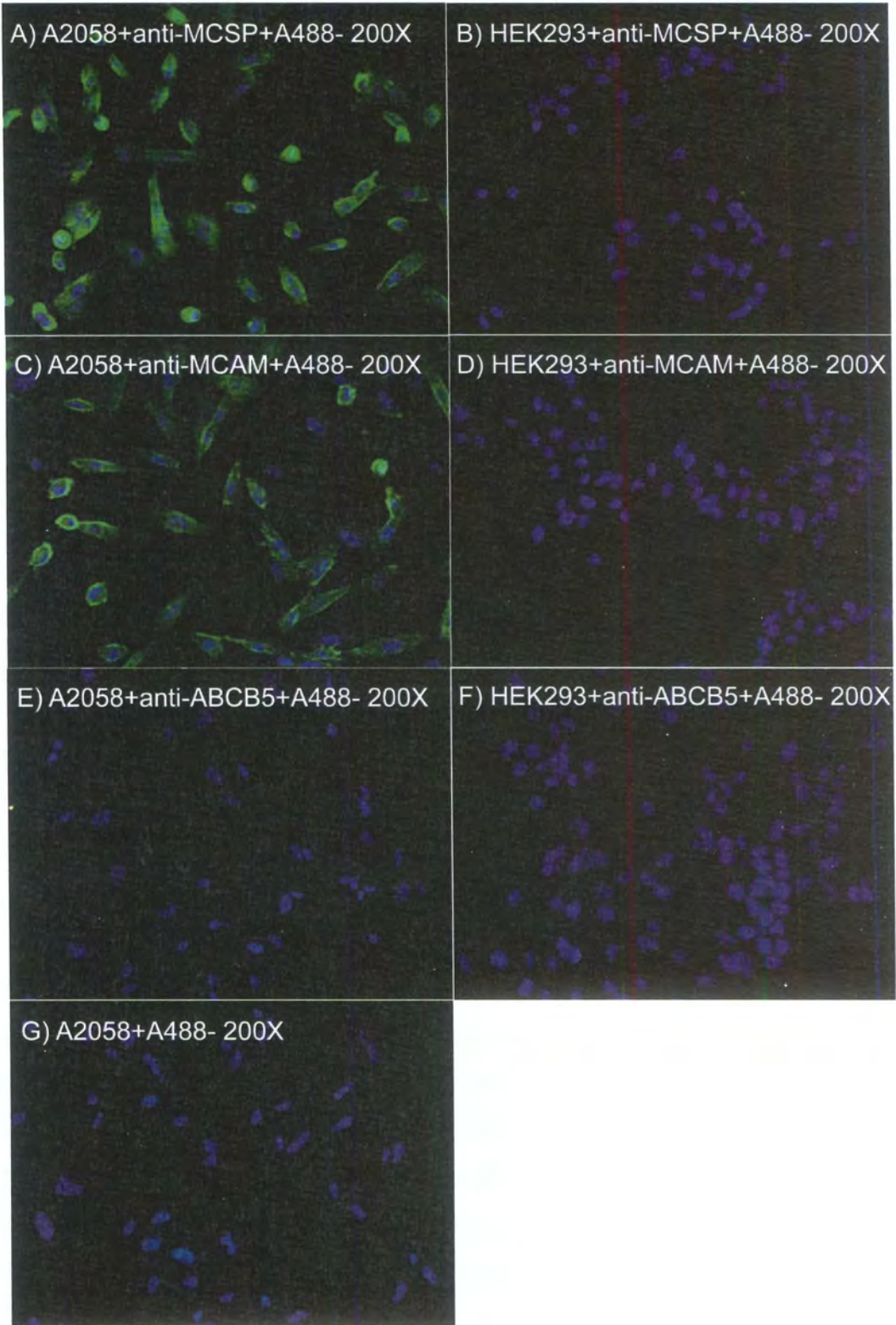


Figure 6.1. Immunofluorescence of A2058 melanoma cell line with MCSP, MCAM and ABCB5 antibodies. HEK293 cells were used as a negative control for each antibody.

6.2. Demonstration of ABCB5 Expression in Melanoma Cell Line by Flow Cytometry

ABCB5 expression in A2058 melanoma cells was unable to be demonstrated by immunofluorescence, as illustrated in Fig. 6.1.E. For this reason, flow cytometry was performed with ABCB5 primary antibody and A488 conjugated secondary antibody to determine the percentage of ABCB5 expressing A2058 cells. Again, HEK293 cells and A2058 cells with only secondary antibody were used as negative controls to demonstrate ABCB5 staining was specific due to ABCB5 antibody binding to ABCB5 on the surface of A2058 cells.

Fig.6.2.A shows the peak fluorescence emission of A2058 cells without antibody, which has been adjusted to sit entirely within the first decade of the graph in order to observe any increased fluorescence in samples containing A2058 cells bound to antibody. All graphs were gated by placing a boundary at the first decade to separate cells within the first decade (unstained cells) from those with increased fluorescence. This was performed to demonstrate the percentage of cells which had increased fluorescence, and were therefore considered positive, compared with those contained in the first decade of the graph. Analysis of results following gating of the graphs revealed that 0.44% of unstained A2058 cells had increased fluorescence in Fluorescence Channel 1 (FL1) and 0.35% of cells had increased fluorescence in Fluorescence Channel 2 (FL2) (Fig.6.2.A).

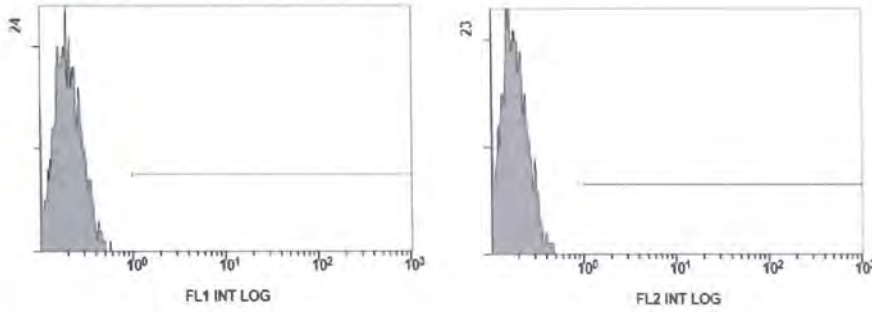
Fig.6.2.B shows the fluorescence emission of A2058 cells bound to MCSP antibody, which was used as a positive control in this experiment. The MCSP antibody was conjugated to the fluorochrome PE which produces a peak emission at 575nm and should be seen in FL2. When A2058 cells were stained with MCSP antibody and analysed by flow cytometry, an increased fluorescence was observed in FL2 as the bulk of the cell population was visible outside of the first decade of the graph, demonstrating that the cells were bound to the MCSP antibody. Gating the graph to determine the proportion of cells outside the first

decade revealed that 99.56% of cells were MCSP positive shown by an increase in relative fluorescence.

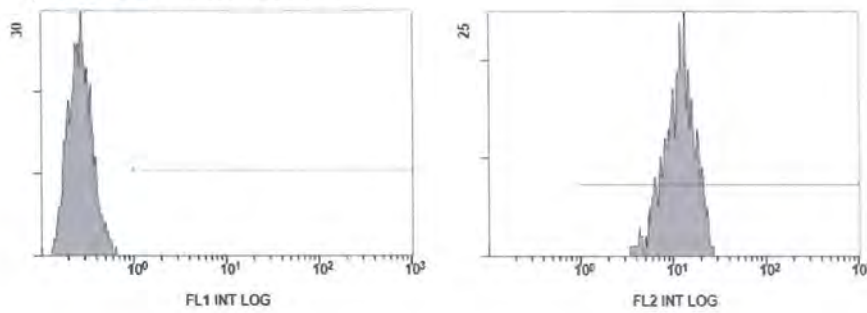
To identify the number of A2058 cells positive for ABCB5, cells were incubated with ABCB5 antibody and secondary antibody, conjugated to Alexa Fluor 488 (A488) before being analysed by flow cytometry. A488 produces a peak emission at 519nm which should be observed in FL1. As shown in Fig.6.2.C, there is a slight shift of the cell population in FL1 which suggests a small number of A2058 cells have increased fluorescence at 519nm and are therefore positive for ABCB5. Gating of this graph to demonstrate the proportion of cells with increased fluorescence revealed that 5.05% of these cells appeared outside of the first decade of the graph in FL1, which shows that a small population of A2058 cells are ABCB5 positive. It was expected that only a small percentage of A2058 cells would be ABCB5 positive as this marker is only expressed on a subset of melanoma stem cells (Chen et al., 2005).

A2058 cells with secondary anti-mouse IgG, conjugated to A488 alone, without primary antibody was used as a negative control (Fig.6.2.D.). Gating of FL1 following analysis by flow cytometry revealed that only 0.52% of cells had increased fluorescence when no primary antibody was present. This negative control confirms that ABCB5 is expressed in approximately 5% of A2058 cells (Fig.6.2.C) and highlights the fact that ABCB5 expression is limited to melanoma stem cell populations, or at least rare melanoma cells, as opposed to MCSP and MCAM expression which was observed in all cells of the A2058 melanoma cell line (Fig.6.1.A and Fig.6.1.C respectively).

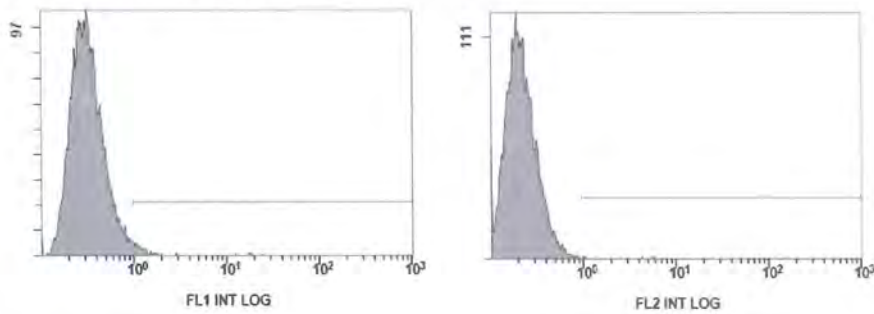
A) A2058



B) A2058+anti-MCSP-PE



C) A2058+anti-ABCB5+A488



D) A2058+A488

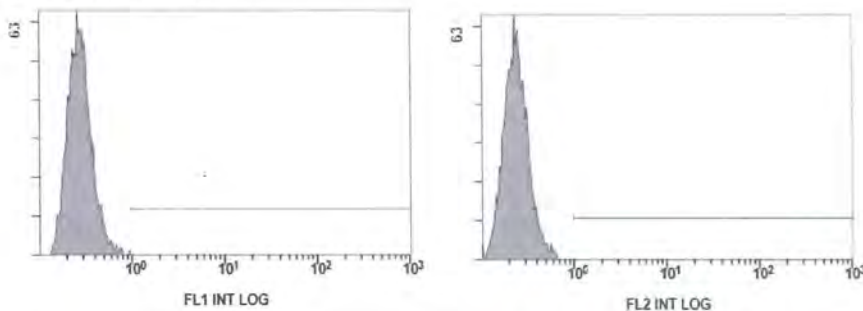


Figure 6.2. Analysis of ABCB5 expression in A2058 melanoma cells by flow cytometry. The peak emission of A2058 cells alone was adjusted to sit within the first decade of the graph and graphs were then gated (shown as the horizontal line beginning at 10^0) to separate cells with increased fluorescence from those within the first decade of the graph in order to reveal the percentage of cells with increased fluorescence in each sample. Cells appearing outside the first decade of the graph as a result of increased fluorescence were considered positive.

6.3. Demonstration of MCAM and MCSP Expression in Melanoma Tissue Sections by Immunofluorescence

Paraffin-embedded melanoma tissue sections were stained with MCAM and MCSP antibodies to demonstrate expression of these markers in melanoma tumours by immunofluorescence. ABCB5 staining was not performed due to the inability to stain A2058 melanoma cells with ABCB5 antibody by immunofluorescence. Three sections each of in situ, invasive primary and metastatic melanoma were stained in order to demonstrate the relative expression of these markers in melanoma tissue of different disease stages. The idea of this was to illustrate marker expression relative to disease progression in order to demonstrate the suitability of MCSP and MCAM as antigens to target in the isolation of melanoma CTCs and to provide an idea of the number of MCSP and MCAM positive circulating cells expected relative to disease stage. Specific staining of tissue sections was confirmed by staining of paraffin-embedded A2058 cells as a positive control, as A2058 cells were shown to be MCAM and MCSP positive (Fig.6.1.), and staining of sections with secondary antibody only, with no primary antibody, as a negative control to observe background fluorescence and therefore confirm true staining.

Fig.6.3.A and Fig.6.4.A show a section of in-situ melanoma of the superficial spreading type, which was excised from a patient's left arm. Within the lesion there were one or two small nests of invasive cells, although invasion was confined to the superficial epidermis, as described in the patient's histopathology report. Staining with MCAM antibody and Alexa Fluor 488 (A488), conjugated to anti-mouse IgG (Fig.6.3.A), revealed small clusters of strongly positive cells sparsely distributed within the lower epidermis. Staining with MCSP antibody and A488 (Fig.6.4.A) revealed small clusters of weakly stained cells as well as weakly stained individual cells scattered throughout the epidermis.

Fig.6.3.B and Fig.6.4.B show a different section of the same superficial spreading in-situ melanoma seen in Fig.6.3.A and Fig.6.4.A. Staining with MCAM antibody and A488 (Fig.6.3.B) revealed small clusters of strongly positive cells distributed throughout the epidermis. Staining with MCSP antibody and A488 (Fig.6.4.B) also showed small clusters of positively stained cells within the epidermis although staining was weaker.

Fig.6.3.C and Fig.6.4.C show a section of superficial spreading in-situ melanoma that has developed within a dysplastic naevus which was excised from the right shoulder of a patient. Staining with MCAM antibody and A488 (Fig.6.3.C) revealed a very large area of MCAM expressing cells throughout a large portion of the epidermis of the section, which may indicate that many cells within this tumour have an aggressive phenotype although it was excised while in-situ. Smaller clusters of positive cells, as well as individual positive cells, were also seen spread throughout the epidermis. Staining with MCSP antibody and A488 (Fig.6.4.C) similarly demonstrated small clusters of MCSP positive cells as well as positively stained individual cells distributed throughout the epidermis of this lesion.

Fig.6.3.D and Fig.6.4.D show a section of invasive, ulcerated, nodular, melanoma excised from the back of a patient. The tumour is 4.77mm in depth from the stratum granulosum, or granular layer, of the epidermis and the patient was diagnosed histologically to be at clinical disease stage III at the time of excision of the primary tumour. Staining with MCAM antibody and A488 (Fig.6.3.D) revealed small areas of positively stained cells spread throughout the epidermis and dermis. Staining with MCSP and A488 antibodies (Fig.6.4.D) similarly revealed a few individual cells positive for MCSP distributed throughout the epidermis and dermis.

Fig.6.3.E and Fig.6.4.E show a section of invasive superficial spreading melanoma excised from a patient's brow. The tumour measures 1.03mm in depth from the stratum granulosum and the patient was diagnosed as having stage II

disease at the time of tumour excision. Staining with MCAM and A488 antibodies (Fig.6.3.E) revealed small clusters of positive cells along with individual positive cells scattered throughout the epidermis and dermis. Staining with MCSP and A488 antibodies (Fig.6.4.E) revealed small clusters of weakly stained cells predominantly within the dermis.

Fig.6.3.F and Fig.6.4.F show a section of invasive superficial spreading melanoma excised from the left back of a patient. The tumour measured 1.74mm in depth from the stratum granulosum and the patient was diagnosed as having stage II disease at the time of primary tumour removal. Staining for MCAM and A488 (Fig.6.3.F) revealed medium to large areas of positively stained cells, along with small positive clusters of cells, distributed throughout the epidermis and dermis. Staining for MCSP and A488 (Fig.6.4.F) revealed small clusters of positive cells distributed along the lower epidermis. Staining of these cells was slightly stronger than previous MCSP staining although more patchy.

Fig.6.3.G and Fig.6.4.G show a section of a melanoma metastasis excised from the skin above the right scapula of a patient. The epidermis in these sections appeared normal in structure whereas the dermis appeared highly disorganised. Staining with MCAM and A488 antibodies (Fig.6.3.G) revealed very little MCAM expression in the epidermis whereas the majority of cells of the dermis were MCAM positive. Staining for MCSP and A488 (Fig.6.4.G) revealed small clusters of MCSP positive cells and individual positively stained cells scattered throughout the dermis.

Fig.6.3.H and Fig.6.4.H show a section of a melanoma metastasis excised from the skin of a patient's right arm. Structure of the epidermis in these sections appeared normal whereas the dermis appeared more disorganised and unstructured. Staining for MCAM and A488 (Fig.6.3.H) demonstrated that the majority of cells of the dermis were MCAM positive while the cells of the

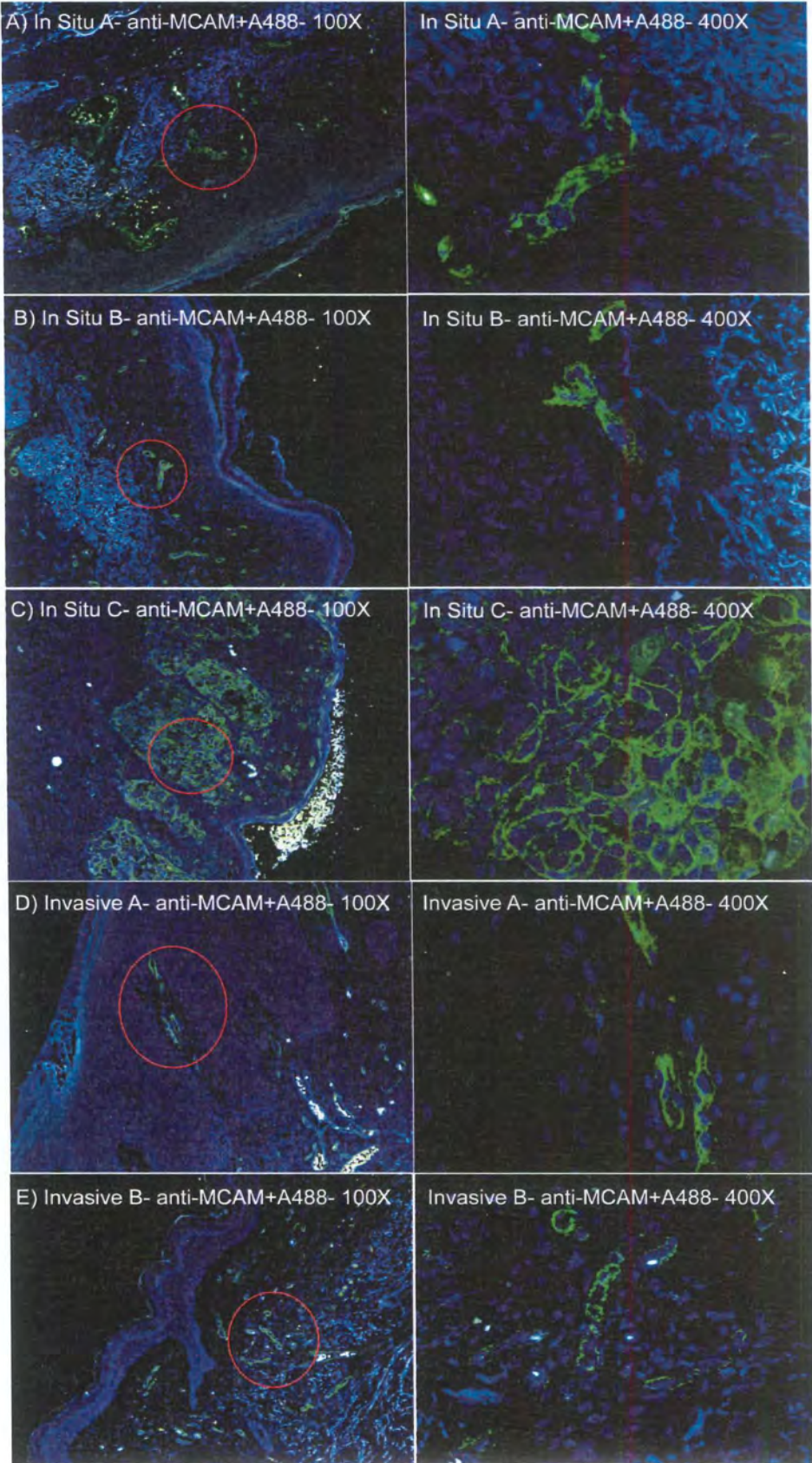
epidermis were negative. Staining for MCSP and A488 (Fig.6.4.H) showed small to medium areas of positively stained cells throughout the dermis.

Fig.6.3.I and Fig.6.4.I show a section of a melanoma metastasis with a lymph node replaced by malignant melanoma cells. The tissue structure appeared highly disorganised with densely packed cells of varying morphology. Staining for MCAM and A488 (Fig.6.3.I) revealed that the entire section was positive for MCAM. Staining for MCSP and A488 (Fig.6.4.I) revealed medium to large areas of positive cells distributed throughout the tissue although staining was weak.

Overall, there was an observed increase in the number of positively stained cells as disease severity increased as the majority of cells in metastatic tissue were positively stained compared with few positive cells in early stage tissue. This finding suggests disease progression may be associated with an increase in melanoma CTCs, especially since the number of MCAM positive cells, which are associated with aggressive, invasive behaviour, increased with disease severity. The demonstration of expression of these markers in all tissue sections from in situ to metastatic disease also supports the proposal that these markers are useful target antigens for the isolation of melanoma CTCs by immunomagnetic bead capture.

It is important to note that the MCSP antibody used in this experiment produced inferior results to the MCAM antibody as there was more MCAM staining observed than MCSP staining and MCSP staining was significantly weaker. MCSP is generally noted in the literature to be expressed on the majority of melanoma cells (Yang et al., 2004); however this was not observed in this experiment. The less than optimal results obtained for MCSP staining are thought to be due to difficulties with this particular antibody when used for immunohistochemistry. Although results were improved by multiple experiments trialling different antibody concentrations and antigen retrieval methods, results were not as clear or consistent as those achieved using the MCAM antibody.

Optimisation of these results would require additional MCSP antibodies to be trialled.



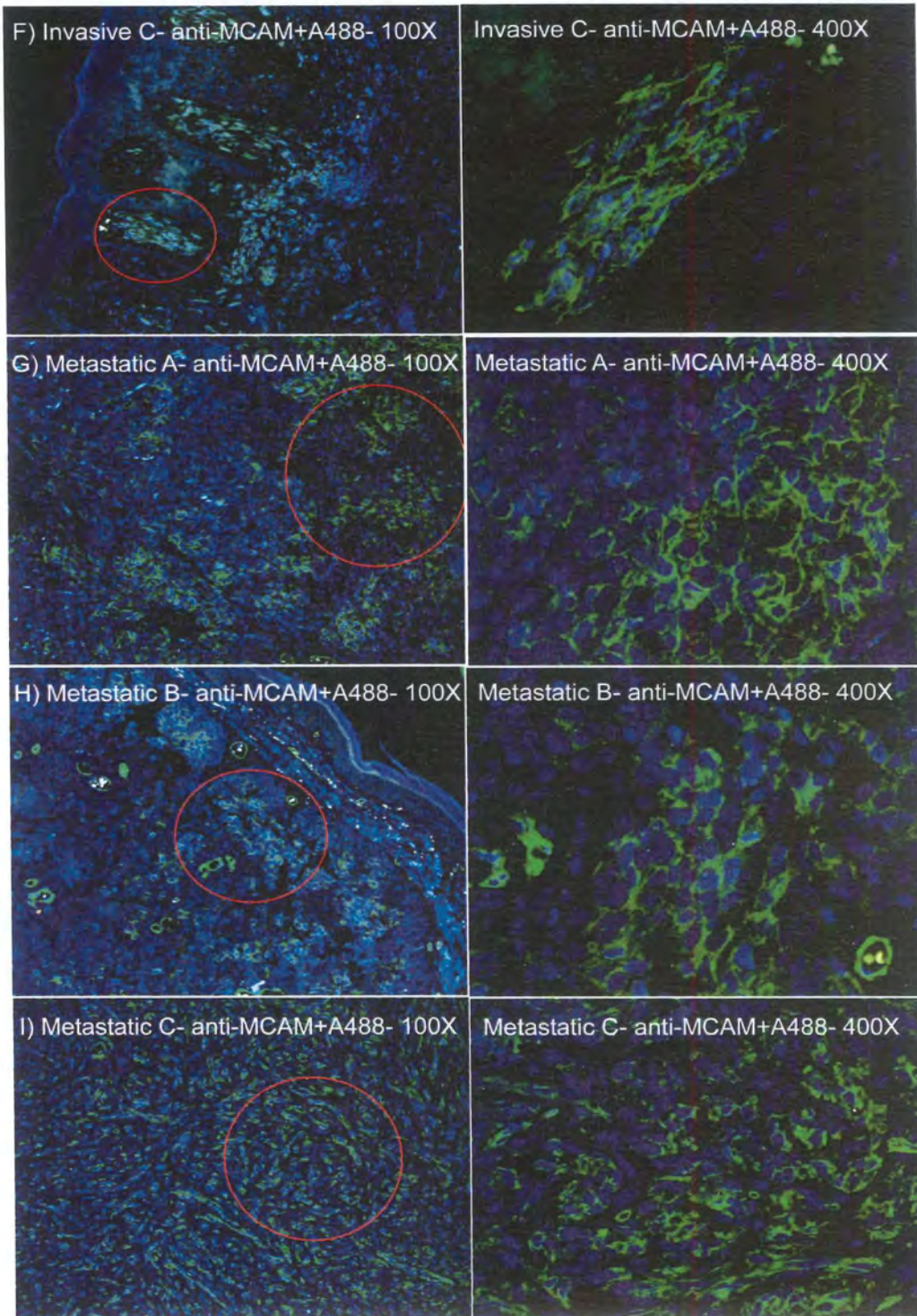
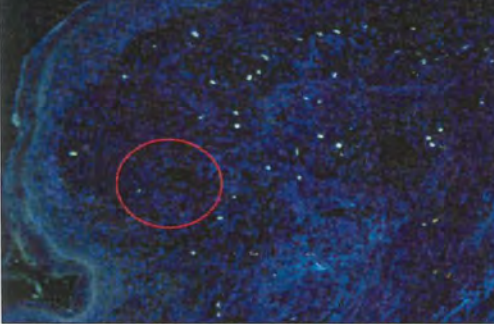
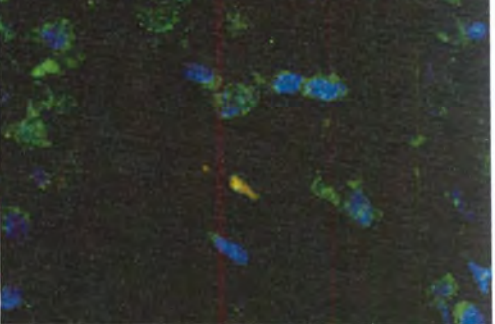


Figure 6.3. Immunohistochemistry of paraffin embedded melanoma tissue sections, stained with MCAM antibody and secondary anti-mouse IgG conjugated to A488. Red circles in the 100X magnified images illustrate the positively stained area shown at 400X magnification.

A) In Situ A- anti-MCSP+A488- 100X



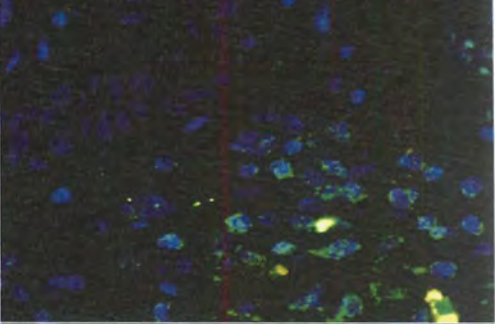
In Situ A- anti-MCSP+A488- 400X



B) In Situ B- anti-MCSP+A488- 100X



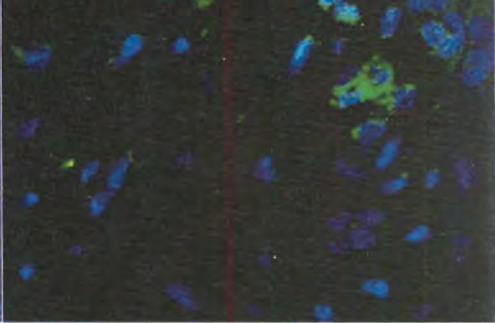
In Situ B- anti-MCSP+A488- 400X



C) In Situ C- anti-MCSP+A488- 100X



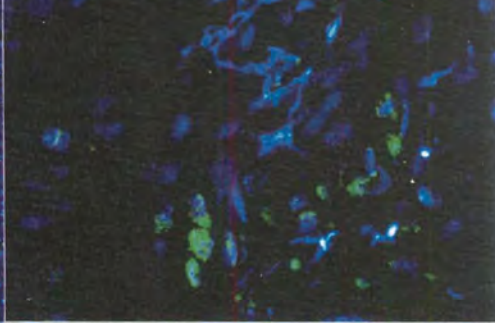
In Situ C- anti-MCSP+A488- 400X



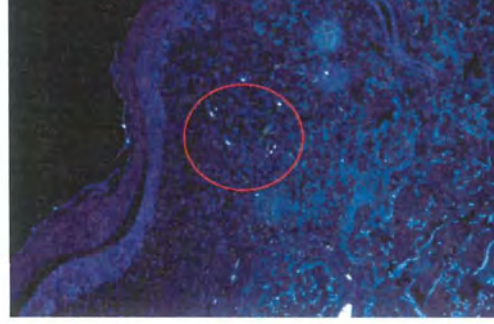
D) Invasive A- anti-MCSP+A488- 100X



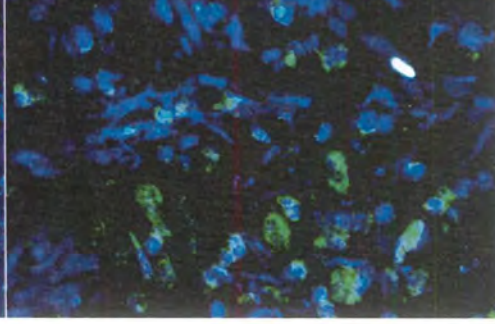
Invasive A- anti-MCSP+A488- 400X



E) Invasive B- anti-MCSP+A488- 100X



Invasive B- anti-MCSP+A488- 400X



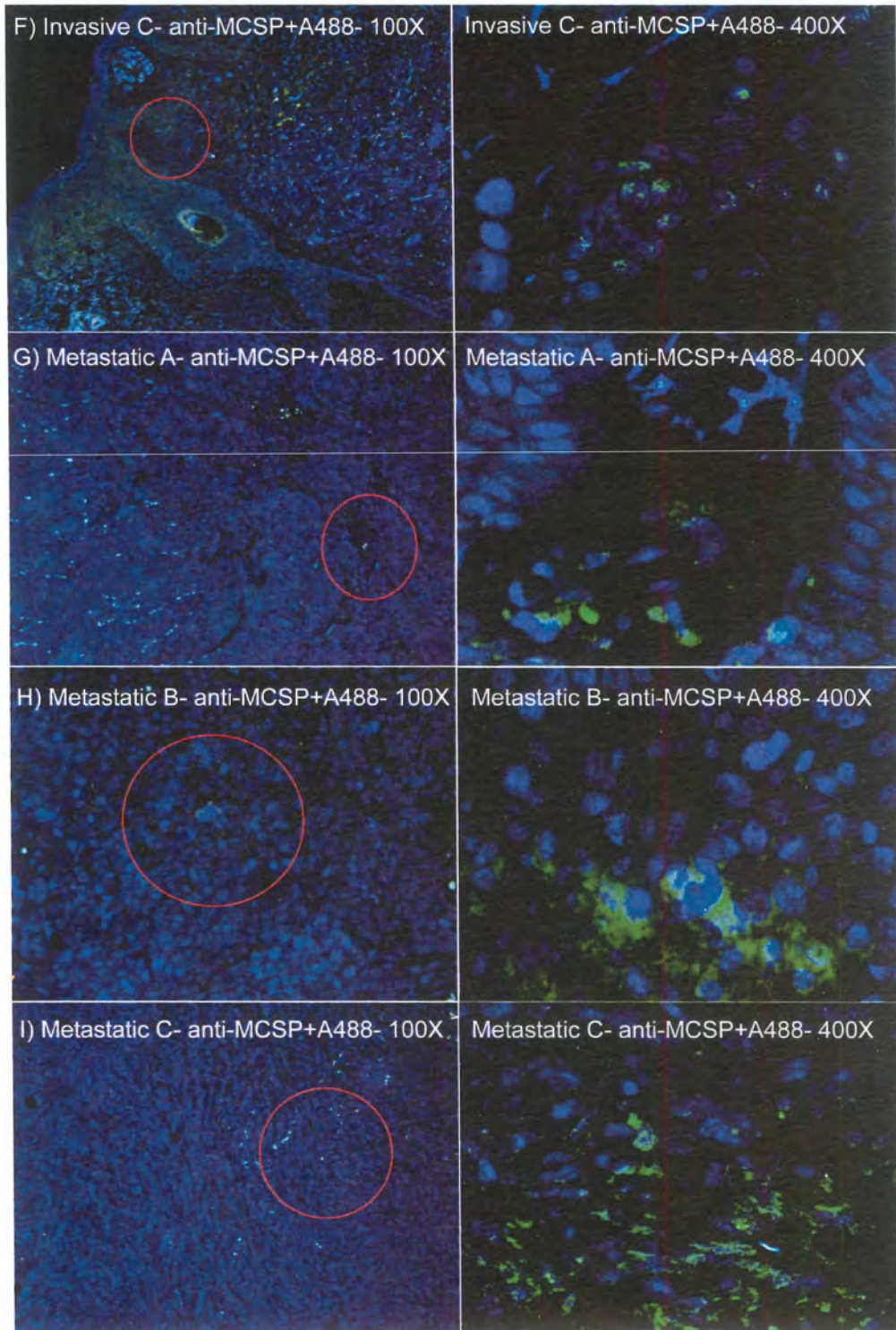


Figure 6.4. Immunohistochemistry of paraffin embedded melanoma tissue sections, stained with MCSP antibody and secondary anti-mouse IgG conjugated to A488. Red circles in the 100X magnified images illustrate the positively stained area shown at 400X magnification.

6.4. Testing the ability of Antibody-Coupled Immunomagnetic Beads to Bind Melanoma Cells

A2058 cells were incubated with anti-MCSP, anti-MCAM and anti-ABCB5 coupled immunomagnetic beads in order to test their ability to bind A2058 melanoma cells, which have previously been shown to express these markers by immunofluorescence and flow cytometry (Fig.6.1 and Fig.6.2. respectively). If the beads bind these cells successfully then they should also be able to bind circulating melanoma cells expressing these markers. The binding of A2058 cells to each bead type was observed by fluorescence microscopy.

Fig.6.5.A shows anti-MCSP coupled immunomagnetic beads bound to A2058 cells. This image shows that anti-MCSP coupled beads (red) are successful at binding A2058 cells, which are MCSP positive, as indicated in Fig. 6.1.A. The ability of anti-MCSP coupled beads to bind A2058 cells implies that similar results should also be obtained when they are used to bind MCSP positive circulating melanoma cells from patient blood.

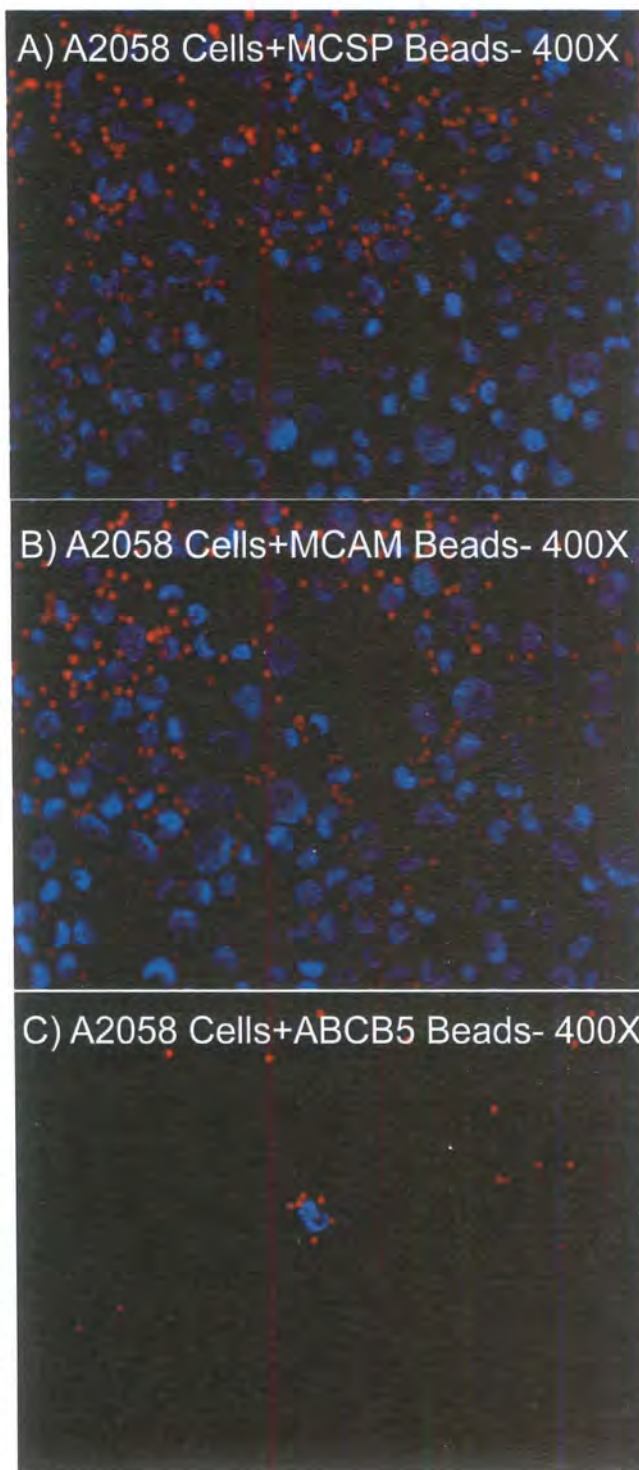


Figure 6.5. Testing anti-MCSP, anti-MCAM and anti-ABCB5 coupled immunomagnetic beads on A2058 melanoma cells.

The result of this test also demonstrated that 1 μ l of anti-MCSP coupled beads was able to bind many thousand A2058 cells.

Fig.6.5.B shows anti-MCAM coupled immunomagnetic beads bound to A2058 cells. This image shows that anti-MCAM beads also bind A2058 cells successfully, which are MCAM positive (Fig. 6.1.C.), and should therefore be able to bind circulating MCAM positive cells from patient blood. The result of this test demonstrates 1 μ l of anti-MCAM beads is also capable of binding thousands of A2058 cells.

Fig.6.5.C shows anti-ABCB5 coupled beads bound to A2058 cells. This result demonstrates that anti-ABCB5 beads were able to bind only a small percentage of A2058 cells. Similar results were observed with flow cytometry (Fig.6.2.C.), suggesting only a small sub-population of A2058 cells are ABCB5 positive which are likely to be stem or rare melanoma cells. This suggests that the anti-ABCB5 beads bind ABCB5 positive cells specifically and should therefore be successful at isolating ABCB5 positive circulating melanoma stem cells from patient blood.

6.5. Testing the Binding Capacity of Antibody-Coupled immunomagnetic Beads by Cell Counting

A2058 melanoma cells were incubated with anti-MCSP, anti-MCAM and anti-ABCB5 beads and counted on the Vi-Cell-XR Cell Viability Analyser (Beckman Coulter) in order to demonstrate the binding capacity of 1µl of each bead type, i.e. the amount of cells 1µl of beads is capable of binding. HEK293 cells were used as a negative control to demonstrate the amount of non-specific binding resulting from incubation with each bead type.

Table 6.1.demonstrates the binding capacity of 1µl of anti-MCSP, anti-MCAM and anti-ABCB5 coupled immunomagnetic beads when incubated with 5×10^5 A2058 and 5×10^5 HEK293 cells. The cell count was generated using the Vi-Cell-XR Cell Viability Analyser, which counts the number of cells in a representative portion of the sample to determine the approximate number of cells per ml of sample.

The results show that 1µl of each bead type is capable of binding many thousand A2058 cells, although the number of cells bound to anti-ABCB5 beads seems high compared to previous results. This test also demonstrated that all three bead types exhibited a high level of non-specific binding as each bead type bound approximately 50,000 HEK293 cells which are negative for MCSP, MCAM and ABCB5 (Fig. 6.1.). This high level of non-specific binding of the beads was possibly due to conjugated antibodies or the beads themselves attaching to the surface of cells other than through an antibody-antigen bond. This non-specific binding would need to be overcome before isolating circulating cells from patient blood to ensure captured cells have been bound specifically based on expression of a particular marker.

Table 6.1. The binding capacity of 1µl anti-MCSP, anti-MCAM and anti-ABCB5 coupled immunomagnetic beads as determined by the Vi-Cell-XR Cell Viability Analyser (Beckman Coulter).

Sample	Cells
A2058 + MCSP Beads	0.14 x10 ⁶
A2058 + MCAM Beads	0.067 x10 ⁶
A2058 + ABCB5 Beads	0.073 x10 ⁶
HEK293 + MCSP Beads	0.046 x10 ⁶
HEK293 + MCAM Beads	0.052 x10 ⁶
HEK293 + ABCB5 Beads	0.058 x10 ⁶

6.6. Testing Antibody-Coupled Immunomagnetic Beads on a Preliminary Patient Sample

A patient blood sample was tested to assess the number of cells captured by each bead type. Based on the high binding capacity of 1µl of beads demonstrated in Table 6.1., it was decided to use 1µl of 1/10 diluted beads which should be sufficient to capture all circulating cells within any patient sample tested and save on the amount of beads used. Due to the large amount of unbound cells observed in the patient sample relative to bead-bound cells, and because the total number of cells (both bound and unbound) was far greater than expected, it was decided to count only bead-bound cells. Unbound cells were ignored as it was presumed they were non-specifically bound leukocytes which

had survived washing. This presumption was supported by the substantial amount of non-specific binding to HEK293 cells previously observed (Table 6.1.).

The results also show a relatively high number of bead-bound cells captured by anti-MCAM beads compared to cells captured by anti-MCSP beads (Table 6.2). This was not expected as MSCP should be expressed in any cells which are MCAM positive as MCSP is expressed in the vast majority of melanoma cells (Yang et al., 2004). Due to this result, and given the relatively high number of anti-ABCB5 bead bound cells, it was thought that many cells had bound non-specifically to the beads. This was further suggested by the large number of unbound cells in the sample which were thought to be non-specifically isolated leukocytes.

Table 6.2. The number of bead-bound cells captured by immunomagnetic isolation in a preliminary patient blood sample.

Sample	Bead Bound Cells
MCSP Beads	5
MCAM Beads	126
ABCB5 Beads	26

6.7. Comparison of Non-Specific Binding of Beads between 30 Minute and Overnight Incubation

In order to determine an optimal incubation time that ensures the maximum isolation of circulating melanoma cells from patient blood without increasing the amount of non-specific binding, an experiment was conducted using a control

blood sample to observe the difference in the number of cells bound non-specifically by each bead type between a 30 minute and overnight incubation. Therefore, the idea of this experiment was to determine whether an increased incubation time increases the amount of non-specific binding of the beads. Again, only bead-bound cells were counted due to the large number of unbound cells observed in the samples, presumably due to non-specific binding. The results showed that an overnight incubation did not increase the amount of non-specific binding (Table 6.3.), suggesting an overnight incubation could be used for patient samples. The number of bound cells in each sample was however greater than expected in a control blood sample further suggesting a problem with non-specific binding of leukocytes to the beads.

Table 6.3. Comparison of the number of bead-bound cells captured from control blood with immunomagnetic beads for 30 minute and overnight incubation.

Sample	Bead Bound Cells	
	<i>30 mins</i>	<i>Overnight</i>
MCSP Beads	10	2
MCAM Beads	4	8
ABCB5 Beads	5	2
Total Cells	19	12

6.8. Demonstration of Captured Cell Viability by Trypan Blue Staining

In this experiment, a patient blood sample was incubated overnight with anti-MCAM coupled immunomagnetic beads, washed with MACS buffer (2mM EDTA, 0.5% BSA in PBS, pH 7.2) and stained with 0.1% trypan blue solution in order to

determine whether cells captured by immunomagnetic beads were viable. Trypan blue does not stain viable cells as it is unable to pass through the intact cell membrane of a live cell. On the other hand, trypan blue does pass through the cell membrane of a dead cell and therefore selectively stains dead cells blue. Following washing of the beads and mixing bead-bound cells with trypan blue solution, the viability of captured cells was observed by microscopy. Fig.6.6. shows cells captured by anti-MCAM coupled beads are live as there is an absence of blue staining within the cells. It was observed that all captured cells were viable as all were unstained.



Figure 6.6. Trypan blue stain of cells captured by anti-MCAM immunomagnetic beads from patient blood. The absence of blue cellular staining indicates captured cells are viable.

6.9. Comparison of MACS Buffer and Triton X-100 Washing to reduce Non-Specific Binding of Beads

Previous results have demonstrated a relatively high level of non-specific binding by the immunomagnetic beads when either HEK293 control cells (Table 6.1.) or control blood samples (Table 6.3.) were utilised. In order to reduce non-specific binding, a more stringent washing buffer containing 0.2% Triton X-100 was tested. Previous results had shown that the highest level of non-specific binding was produced by anti-MCAM coupled beads (Table 6.2., other results not shown). In this experiment, anti-MCAM beads were incubated with both A2058 and HEK293 cells. Following incubation, both samples were split into two; one washed with MACS buffer and the other with 0.2% Triton X-100 in PBS in order to compare the amount of non-specific binding between the samples. Following

washing, the beads were mounted onto microscope slides and viewed by microscopy in order to determine whether washing with 0.2% Triton X-100 in PBS reduced the amount of non-specific binding of anti-MCAM beads to HEK293 cells.

Fig.6.7.A demonstrates the binding of A2058 cells by anti-MCAM coupled beads following washing with MACS buffer. Fig.6.7.B shows A2058 cells are still bound to the beads following washing with 0.2% Triton X-100, however the cells have clumped together making it difficult to distinguish individual cells and therefore count them. However, based on the number and size of the clumps, as seen in Fig.6.7.B, it was estimated that the number of cells in the sample was similar to the number of cells observed following washing with MACS buffer (Fig.6.7.A). This demonstrated that the Triton X-100 has not caused specifically bound A2058 cells to be removed from the beads during washing. Due to the fact that 5×10^5 cells were used in protocol optimisation experiments, it was thought that clumping would not be an issue when quantifying cells from patient blood following Triton X-100 washing, as far lower numbers of captured cells were expected.

Fig.6.7.C shows a large number of HEK293 cells non-specifically bound to anti-MCAM beads which were not removed by washing with MACS buffer. On the other hand, there were no HEK293 cells observed following washing with 0.2% Triton X-100 in PBS (Fig.6.7.D) demonstrating the effectiveness of Triton X-100 at removing non-specifically bound HEK293 cells from anti-MCAM beads. As a result of this experiment, MACS buffer containing 0.2% Triton X-100 was produced to be used for washing patient and control samples following overnight incubation with the beads in the final optimised protocol.

Due to the low number of captured cells observed in further testing with 0.2% Triton X-100 washing, with very few unbound cells seen, it was decided to count all cells in the sample rather than bead-bound cells only. It was thought that the

very few unbound cells remaining in the sample would initially have been bead-bound in order to be isolated from the sample and survive stringent washing with 0.2% Triton X-100 in MACS buffer. It may have been the case in the few cells that were not bead-bound during viewing by microscopy that the bead-cell bond was broken following mounting the cells onto a microscope slide and leaving overnight for optimal nuclear staining with DAPI. This was suggested as unbound cells were usually observed extremely closely to at least one bead.

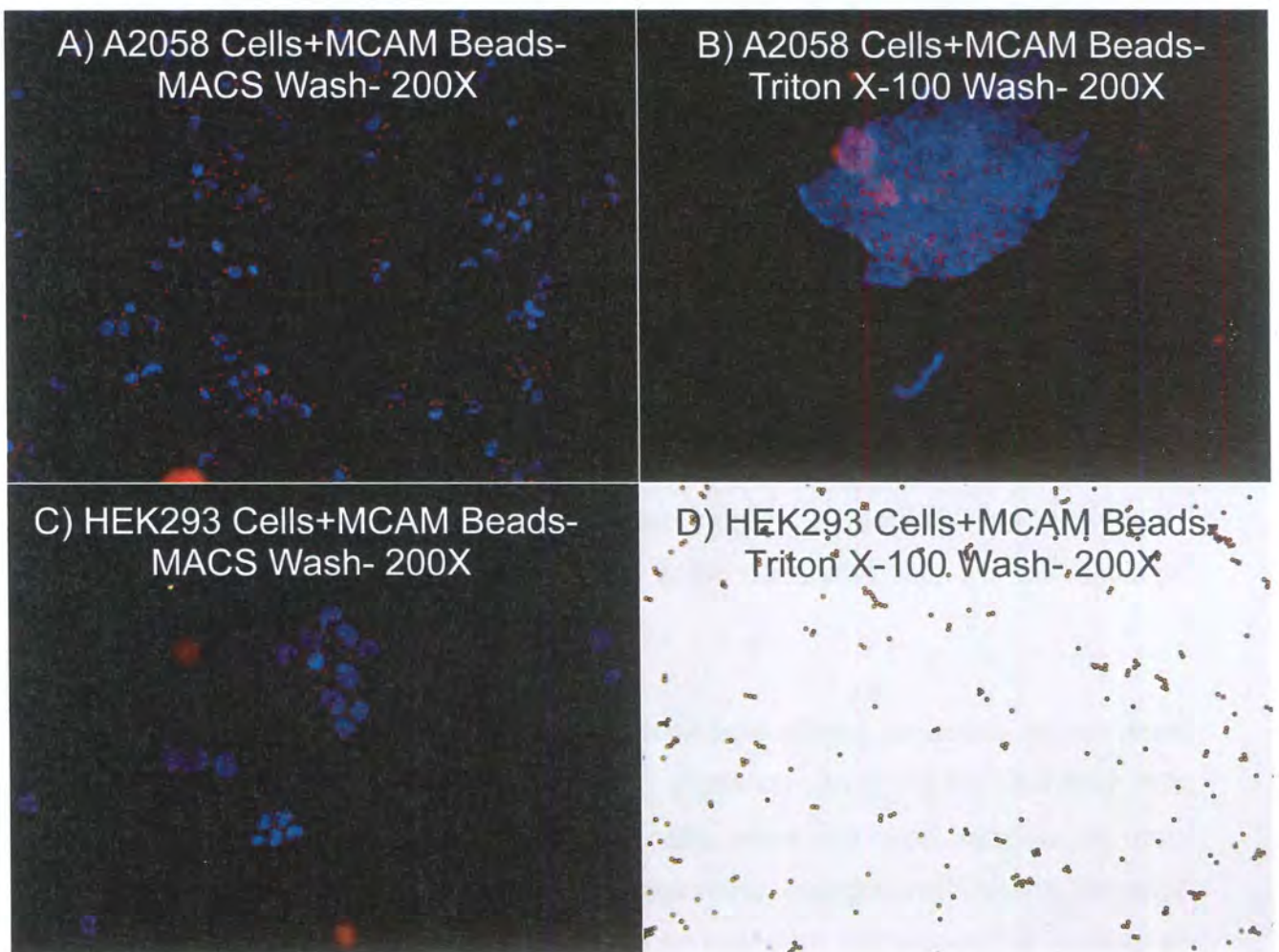


Figure 6.7. Comparison of non-specific binding of HEK293 cells by anti-MCAM coupled beads between MACS buffer and 0.2% Triton X-100/PBS washing. 6.6.D was photographed by light microscopy rather than fluorescence microscopy for a clearer image of the unbound beads as no cells were present.

6.10. Demonstration of Assay Sensitivity and Specificity by Spiking Melanoma Cells into Control Blood

In this experiment, increasing numbers of A2058 cells were spiked into control blood samples and incubated with anti-MCSP coupled beads overnight. Following washing and mounting the beads onto microscope slides, the number of cells in each sample was counted by microscopy in order to determine the sensitivity and specificity of the bead capture assay. The purpose of this was to determine the proportion of spiked cells that could be captured by the beads which may be comparable to the percentage of CTCs captured from a patient blood sample. Due to the variability in the number of cells retrieved with each spike, the experiment was replicated a number of times in order to determine an average number of cells retrieved for each number of cells spiked.

Spiking with 0 cells demonstrated that there is a small amount of non-specific binding in the assay (Table 6.4); although it has been significantly reduced since Triton X-100 washing has been introduced. Due to the presence of some non-specific background binding it cannot be determined whether a single A2058 cell was retrieved in the single cell spikes. In actuality, a decrease in the average number of cells retrieved in a single cell spike compared with a 0 cell spike is seen, although this is not significant.

The mean values show that as the number of cells spiked increases, so too does the number of cells retrieved (Table 6.4.). However, it cannot be said from this experiment exactly how many A2058 cells were retrieved relative to non-specifically bound cells. Nonetheless, assuming background binding remains constant throughout the samples, there is an apparent increase in the number of A2058 cells retrieved as the number of A2058 cells spiked is augmented.

Although the number of cells retrieved increases with an increase in spiked cells, it can also be seen that as the number of cells spiked increases, the number of

cells retrieved becomes more variable. The explanation for this is that an increase in cell clumping associated with an increased number of spiked cells was observed. Many of these clumps appeared as a fluorescent blue smear where individual cells could not be distinguished and therefore could not be counted. The result of this was that frequently there were more cells retrieved by the beads than could be recorded as the exact number of cells could not be determined.

Table 6.4. The number of cells retrieved after spiking control blood with an increasing number of A2058 cells and incubating with anti-MCSP coupled immunomagnetic beads.

<i>Approximate Number of Cells Spiked</i>	<i>Cells Captured</i>				
	<i>Spike 1</i>	<i>Spike 2</i>	<i>Spike 3</i>	<i>Spike 4</i>	<i>Mean</i>
0	4	3	5	5	4.25
1	3	4	6	2	3.75
10	9	14	10	6	9.75
30	13	8	21	23	16.25
50	42	31	29	40	35.5
100	117	61	19	46	60.75

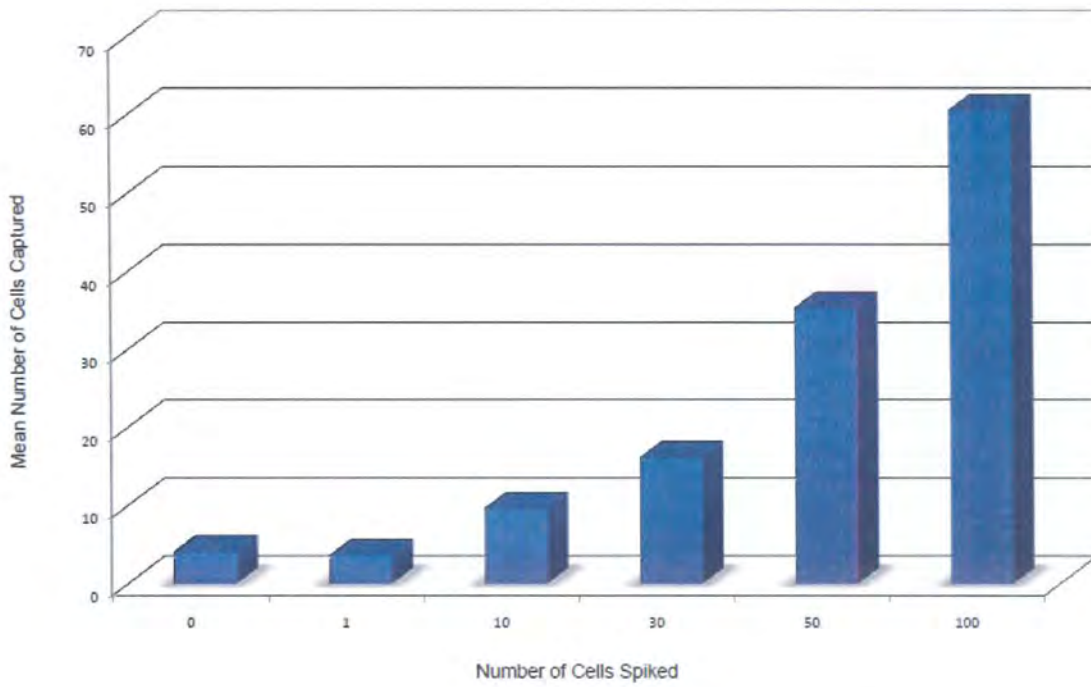


Figure 6.8. A graphical representation of the mean number of cells captured after spiking control blood with an increasing number of A2058 cells and incubating with anti-MCSP coupled immunomagnetic beads.

6.11. Comparison of Non-Specific Binding in Fresh and Four-Day-Old Control Blood

The majority of patient blood samples included in this study were not fresh, instead averaging 3 days old. For this reason, an experiment was performed with control blood in order to determine whether increased age of the sample resulted in increased non-specific binding. In this experiment, two blood samples were taken from the same volunteer to be processed at different times. The first sample was processed within hours of it being taken while the second sample was processed after being refrigerated for 4 days. The results in Table 6.5 demonstrate that ageing the blood sample did not result in increased non-specific binding which suggests results obtained from older patient samples are as valid as those obtained from fresh samples.

Table 6.5. Comparison of the number of non-specifically bound cells in fresh and 4 day old control blood.

Sample	Cells	
	<i>Fresh Blood</i>	<i>4 Day Old Blood</i>
MCSP Beads	0	1
MCAM Beads	4	1
ABCB5 Beads	0	0
Total Cells	4	2

6.12. Results of Immunomagnetic Bead Capture in Control Samples

Table 6.6. shows the number of cells captured from control blood samples following immunomagnetic cell sorting with anti-MCSP, anti-MCAM and anti-ABCB5 coupled beads. All samples were processed within hours of being taken and analysis with the Vi-Cell-XR Cell Viability Analyser (Beckman Coulter) demonstrated that cell counts were relatively high and reasonably consistent. Furthermore, all samples had a high viability.

Very few cells were captured in most samples demonstrating that the amount of non-specific binding seen in the assay is low; however some samples produced a relatively large number of captured cells as a result of cell clumping. Cell clumping was seen predominantly in samples 2, 13, 14 and 20 and explains the larger number of cells captured from these samples. This cell clumping did not appear to be related to total cell count, as demonstrated in Table 6.6. Also, there did not appear to be an association between clumping and a particular bead type. The reason for cell clumping in some samples was unclear. It may have resulted from washing with Triton X-100, as this caused A2058 cells to clump (Fig. 6.7.B.), or it may have been the case that some blood samples had a tendency to clump more than others as a result of the composition of cells within the sample which may have been altered, for example in response to injury or infection. The reason for cell clumps being captured by the magnetic beads was also unclear. This could have been due to beads becoming lodged in cell clumps during incubation or a single bead bound cell clumping with other unbound cells. Overall however, there was a significant difference observed between the number of cells captured in control samples versus patient samples, as described in 6.14.1.

Table 6.6. Results of the immunomagnetic bead capture protocol performed for 21 control blood samples showing the total number of cells captured from each sample as well as the number of cells captured by each bead type.

Sample	Age of Sample	Total Cell Count (x10 ⁶ /ml)	Viability (%)	MCSP Cells	MCAM Cells	ABCB5 Cells	Total Cells
1	<1 day	4.86	98.9	1	0	0	1
2	<1 day	3.83	99.2	0	0	8	8
3	<1 day	6.51	99.0	0	0	0	0
4	<1 day	2.27	98.8	0	1	1	2
5	<1 day	4.71	99.0	0	1	0	1
6	<1 day	6.21	98.9	0	0	1	1
7	<1 day	3.68	97.9	0	0	0	0
8	<1 day	3.27	98.2	1	0	0	1
9	<1 day	4.50	97.8	0	1	0	1
10	<1 day	4.47	99.1	1	0	0	1
11	<1 day	2.27	99.2	0	2	0	2
12	<1 day	3.09	98.7	3	0	1	4
13	<1 day	4.30	97.7	12	3	1	16
14	<1 day	3.11	98.7	0	0	10	10
15	<1 day	5.04	98.4	0	0	1	1
16	<1 day	2.51	99.1	0	0	1	1
17	<1 day	3.92	96.4	1	2	0	3
18	<1 day	2.83	97.4	1	1	0	2
19	<1 day	2.92	98.5	1	0	1	2
20	<1 day	2.26	94.7	14	3	0	17
21	<1 day	4.29	98.0	3	0	1	4

6.13. Results of Immunomagnetic Bead Capture in Patient Samples

Table 6.7. shows the number of cells captured from patient samples following incubation with anti-MCSP, anti-MCAM and anti-ABCB5 immunomagnetic beads. Total cell counts in these samples, as determined by the Vi-Cell-XR Cell Viability Analyser (Beckman Coulter), were more variable than those of control patients. This was possibly due to increased age range of the samples, compared with control samples, which were all processed within hours of collection, or an increased number of leukocytes in some patient samples as a result of a heightened immune response to their disease. Although the majority of patient samples were not fresh, ranging from 0 to 9 days old, all samples had a high viability, as determined by the Vi-Cell-XR Cell Viability Analyser, suggesting the majority, if not all, melanoma CTCs within the sample were viable. Patient samples included in this study, presented in table 6.7, have been arranged in order of stage (0-IV) and available clinical information (date of diagnosis of current primary tumour and information regarding disease recurrence) has been included in the table for comparison with the number of captured cells.

Table 6.7. Immunomagnetic bead capture results for 33 patient blood samples showing the number of cells captured by each bead type and available clinical data.

Sample	Age of Sample	Total Cell Count (x10 ⁶ /ml)	Viability (%)	MCSP Cells	MCAM Cells	ABCB5 Cells	Total Cells	Disease Stage	Date of Diagnosis of Current Primary Tumour (month/year)	Melanoma Recurrence	Treatment (other than primary removal)
1	4 days	1.11	97.1	9	2	3	14	0	01/2009	None	None
2	4 days	0.58	95.8	4	0	3	7	0	02/2008	None	None
3	3 days	1.24	97.2	7	3	2	12	0	10/1999	None	None
4	4 days	0.34	95.7	0	1	0	1	0	05/1996	None	None
5	4 days	0.90	97.7	3	2	1	6	0	10/2004	2 primaries diagnosed around same time	None
6	4 days	1.49	97.3	55	4	5	64	0	08/2008	2 primaries diagnosed around same time	None
7	1 day	1.84	98.4	13	3	5	21	1	05/2009	First primary removed 09/2008	None
8	1 day	1.12	91.4	21	1	14	36	1	07/2010	Recurrent disease- first primary removed 10/1999, 2 in-situ tumours removed 07/2010	None
9	1 day	1.89	97.1	15	0	2	17	1	08/2010	Recurrent disease- first primary removed 10/2006	None
10	4 days	0.85	96.3	35	3	0	38	1	07/2010	None	None
11	3 days	0.83	96.5	3	0	2	5	1	10/1994	None	None

Sample	Age of Sample	Total Cell Count (x10 ⁶ /ml)	Viability (%)	MCSP Cells	MCAM Cells	ABCB5 Cells	Total Cells	Disease Stage	Date of Diagnosis of Current Primary Tumour (month/year)	Melanoma Recurrence	Treatment (other than primary removal)
12	4 days	1.20	97.8	4	0	0	4	1	09/2010	2 primaries diagnosed around same time	None
13	2 days	2.07	98.3	14	0	1	15	1	07/2010	None	None
14	3 days	1.13	96.1	3	1	1	5	1	12/1997	None	None
15	7 days	1.64	96.1	20	3	7	30	1	05/2009	First primary removed 08/2008	None
16	6 days	0.78	93.9	0	0	0	0	1	08/2009	None	None
17	<1 day	2.51	98.0	2	1	0	3	2	06/2006	None	None
18	3 days	2.95	70.7	10	1	5	16	2	02/2008	Recurrent primary tumour. Diagnosed with invasive and in-situ tumours at same time.	None
19	4 days	0.73	96.7	8	1	0	9	2	02/2008	None	None
20	3 days	1.39	92.0	3	0	0	3	2	01/2004	Initial invasive primary removed, second in-situ tumour diagnosed months later.	None
21	9 days	0.77	94.0	26	3	9	38	2	02/2005	None	None
22	<1day	5.82	98.1	8	7	1	16	2	03/2010	None	None

Sample	Age of Sample	Total Cell Count (x10 ⁶ /ml)	Viability (%)	MCSP Cells	MCAM Cells	ABCB5 Cells	Total Cells	Disease Stage	Date of Diagnosis of Current Primary Tumour (month/year)	Melanoma Recurrence	Treatment (other than primary removal)
23	1 day	2.28	96.8	1	0	0	1	2	05/2009	Recurrent primary tumour	None
24	2 days	2.42	95.3	6	3	1	10	2	2005	None	None
25	6 days	2.63	97.3	26	3	6	35	3	07/2007	Nodal metastasis 02/08, 03/2009 new primary	None
26	4 days	1.26	93.2	4	4	2	10	3	04/2005	None	None
27	<1 day	10.44	98.8	43	7	17	67	3	2010	In-situ melanoma removed in 1996. Metastatic melanoma diagnosed 04/2010	Radiotherapy
28	<1 day	5.36	99.1	2	7	2	11	4	11/2002	9 secondary tumours removed 11/2009	Radiotherapy and immunotherapy
29	2 days	1.73	95.3	148	12	72	232	4	N/A	Stage IV diagnosis 12/2003. 9 secondary tumours removed between 12/2003 and 09/2008	Vaccine (radiotherapy in 2005)
30	4 days	1.54	95.1	4	3	0	7	4	08/2009	Groin metastasis removed 09/2009	None
31	2 days	0.88	95.3	16	9	9	34	4	08/1996	2010- femoral lymph node and multiple vertebral metastases removed	BRAF trial
32	2 days	8.30	98.3	6	2	2	10	4	11/2002	02/2006 lung metastasis removed, 02/2008 two subcutaneous metastases in abdominal wall removed.	None
33	1 day	2.93	98.7	10	13	7	30	4	2005	Multiple metastases removed, most recent 06/2010	Immunotherapy

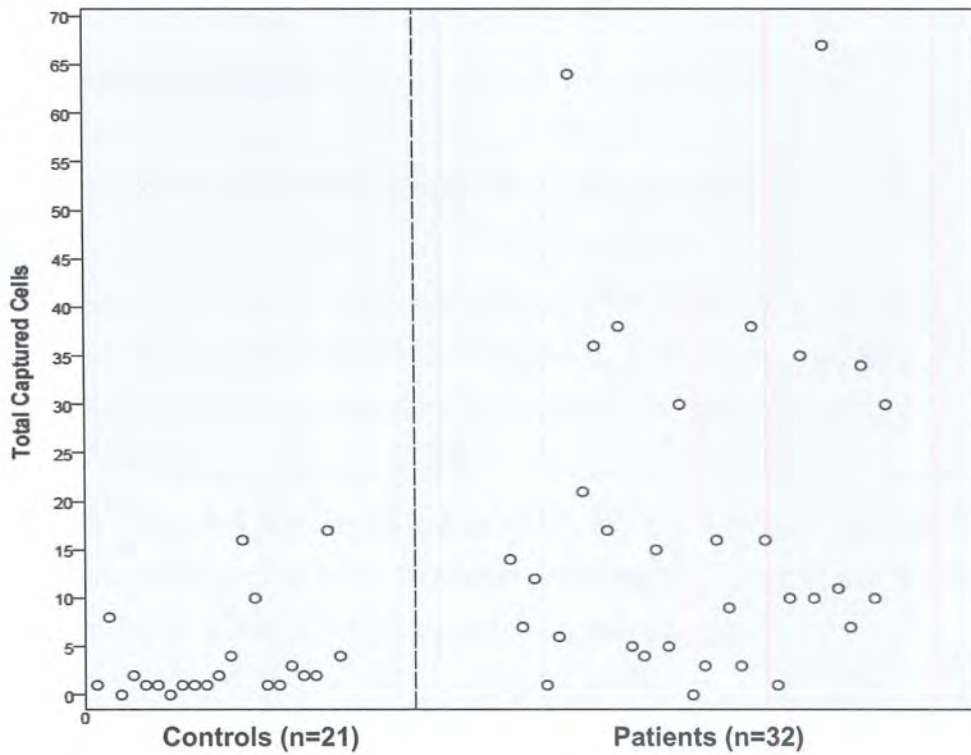


Figure 6.9. Scatterplot illustrating the total number of cells captured in 21 control and 32 patient samples. Patient 29 was not plotted, due to being an extreme outlier, in order to observe the difference in cell numbers in the remaining samples more clearly.

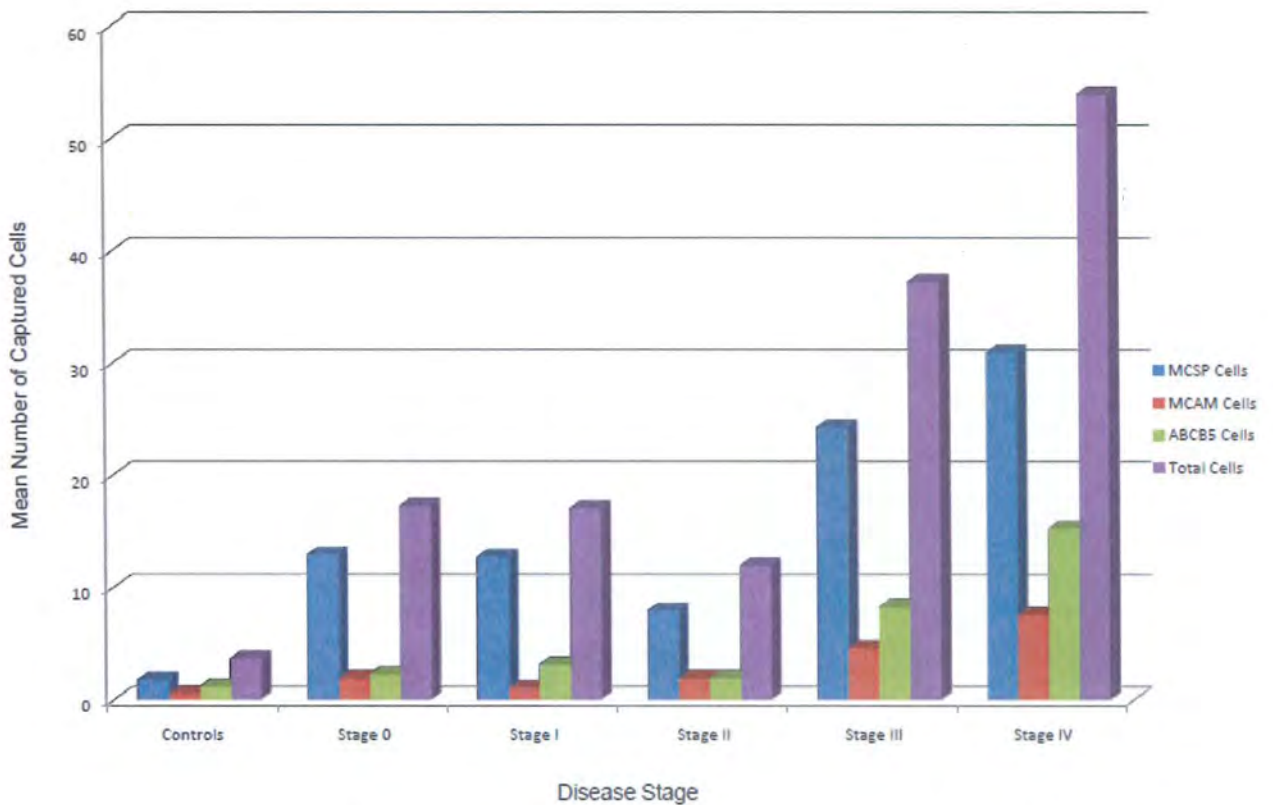


Figure 6.10. A graphical representation of the mean number of MCSP, MCAM, ABCB5 and total captured cells for controls and patients relative to disease stage.

6.14. Statistical Analysis

6.14.1. Total Captured Cells- Controls versus Patients

An independent samples t-test was conducted to compare the total number of cells captured from patient and control groups. There was a significant difference in the number of cells captured from patient samples (mean=24.45, standard deviation=40.92) and control samples (mean=3.71, standard deviation=4.92), where $t(33.44)=2.88$ and $p=0.01$. Fig. 6.11. shows a boxplot comparison of the total number of captured cells between patients and controls demonstrating the significant difference in distribution between the two groups.

Comparison of the Distribution of Total Captured Cells between Control and Patient Samples

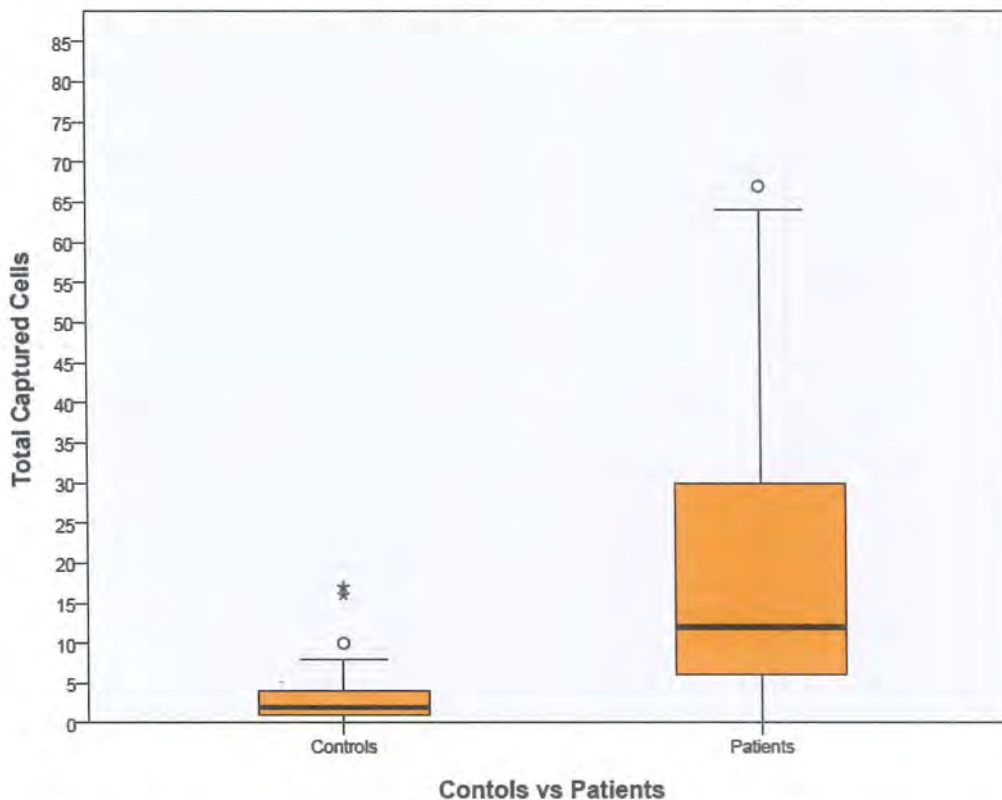


Figure 6.11. Boxplot comparing the distribution of the total number of cells captured in 21 control and 33 patient blood samples. Outliers are represented by circles and extreme outliers are represented by stars. Patient 29 was an extreme outlier with 232 cells although their score is not visible as a result of the limited y-axis scale.

6.14.2. The Relationship between Total Cell Count and Total Captured Cells

It was thought that the total number of cells in any given patient sample may have an influence on the total number of cells captured from the sample as there is a degree of non-specific binding, which may be proportional to the number of cells in the sample. The relationship between the total cell count of the patient samples (as determined by analysis with the Vi-Cell-XR Cell Viability Analyser) and the total number of captured cells was investigated using Pearson product-moment correlation coefficient. There was an insignificant, extremely weak positive correlation between these variables ($r=0.096$, $n=33$, $p=0.59$) (Fig. 6.12.), demonstrating that there is no relationship between the total number of cells in a sample and the number of cells captured from the sample by immunomagnetic isolation. This eliminates total cell count as a confounding factor.

The Relationship between Total Cell Count and Total Captured Cells

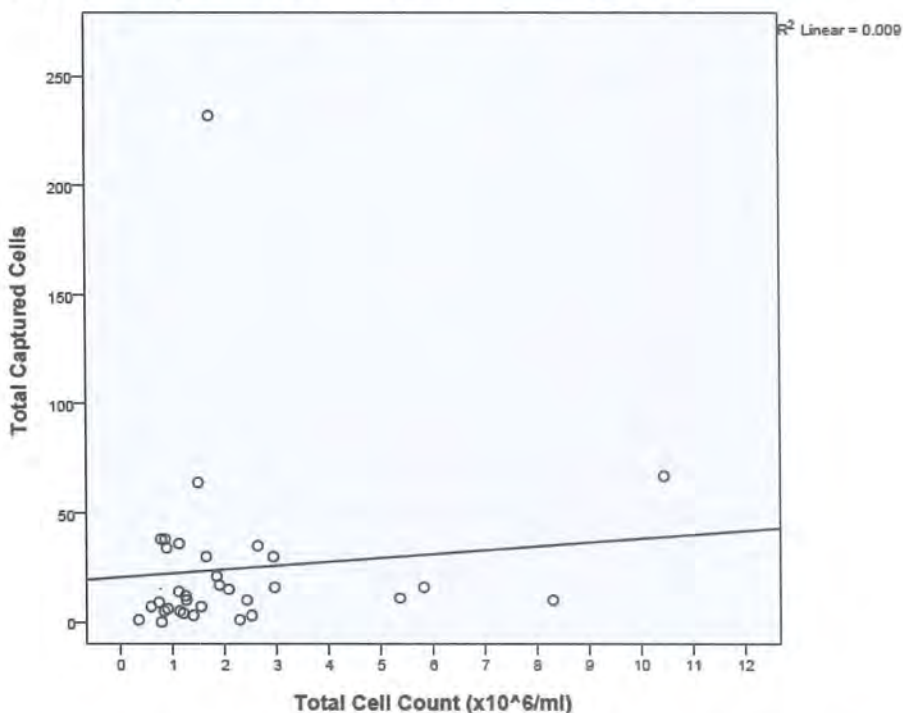


Figure 6.12. Scatterplot of Total Cell Count vs Total Captured Cells for all 33 patient samples included in this study. The fit line has been added to the graph to demonstrate the extremely weak positive relationship between the two variables ($r=0.096$) which was insignificant ($p=0.59$).

6.14.3. Total Captured Cells versus Disease Stage

The relationship between total captured cells and disease stage in patient samples was investigated using Spearman's rank order correlation coefficient. There was an insignificant, weak positive correlation between these variables ($r=0.205$, $n=33$, $p=0.25$) (Fig. 6.13.), demonstrating that there is no true relationship between the total number of captured cells and disease stage within the sample population. A partial correlation between these variables was also performed controlling for total cell count, which had no effect on the outcome (not reported). This supports the results illustrated in Fig. 6.12., suggesting total cell count has no confounding effect on the total number of captured cells.

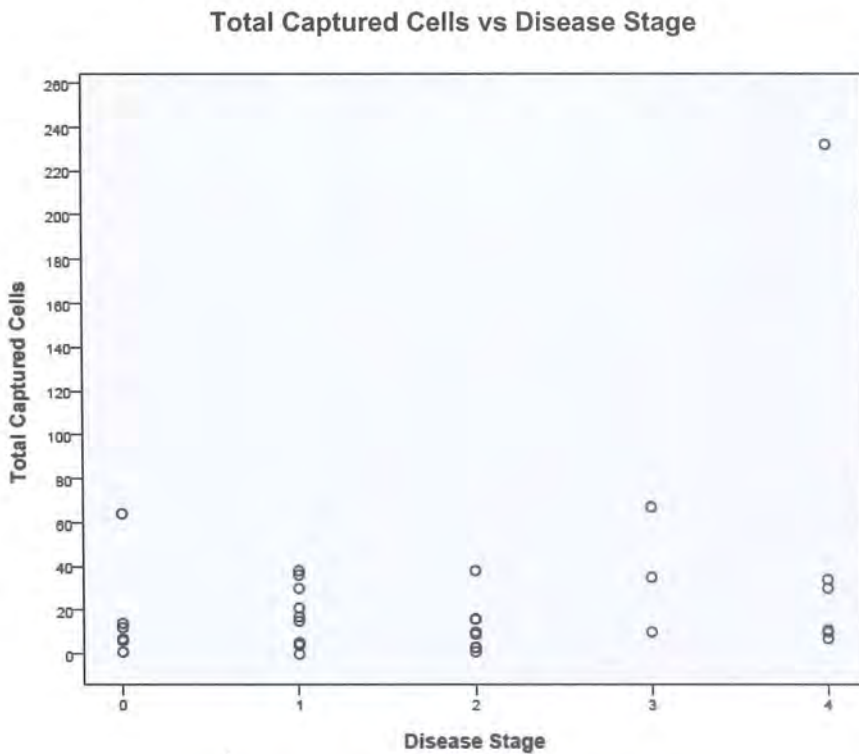


Figure 6.13. Scatterplot of total captured cells vs disease stage for all 33 patient samples included in this study.

Patient samples were also divided into two groups, metastatic and non-metastatic, in order to analyse the difference in the mean number of total captured cells between these two groups by an independent samples t-test. Group 1 was made up of all non-metastatic patient samples (stages 0, I and II, n=24) while group 2 was made up of all metastatic patient samples (stages III and IV, n=9). The results showed there was a difference in the number of cells captured from metastatic patient samples (mean=48.44, standard deviation=71.45) and non-metastatic patient samples (mean=15.46, standard deviation=15.61), where $t(8.288)=1.373$. However, this difference was insignificant ($p=0.21$) due to the low sample sizes of the groups, especially the metastatic sample (n=9), and the inclusion of patient 29, an extreme outlier. Fig. 6.14. shows a boxplot comparison of the total number of captured cells between metastatic and non-metastatic patients demonstrating the difference in distribution of total captured cell numbers between the two groups.

Comparison of the Distribution of Total Captured Cells between Metastatic and Non-Metastatic Patient Samples

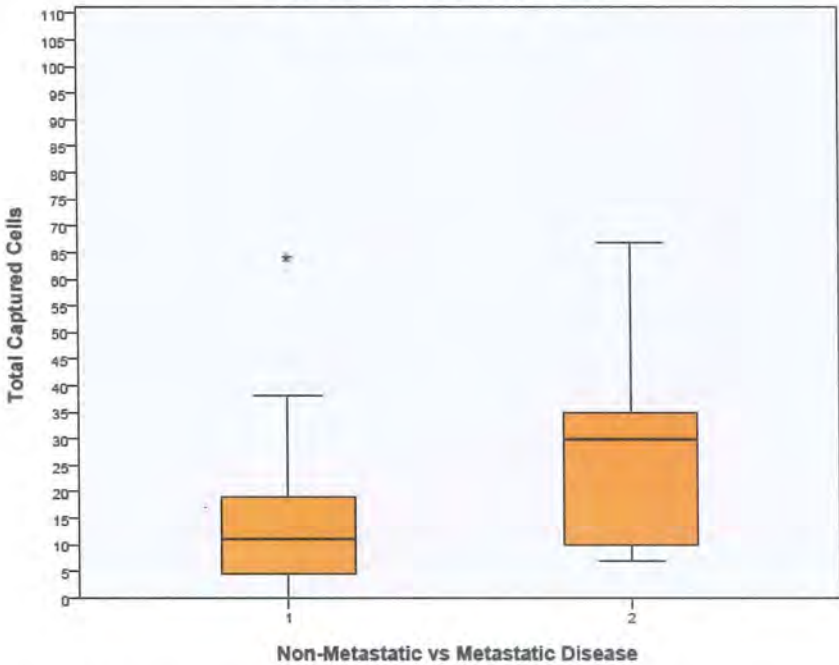


Figure 6.14. Boxplot comparing the distribution of the total number of cells captured in non-metastatic (group 1) and metastatic (group 2) patient samples. Extreme outliers are represented by stars although, again, patient 29 is not visible on the graph as a result of the limited y-axis scale.

6.14.4. MCSP Captured Cells versus Disease Stage

The relationship between the number of cells captured with MCSP beads and disease stage in patient samples was investigated using Spearman's rank order correlation coefficient. There was an insignificant, weak positive correlation between these variables ($r=0.116$, $n=33$, $p=0.521$), demonstrating that there is no true relationship between the number of cells captured with MCSP beads and disease stage within the sample population (Fig.6.15.).

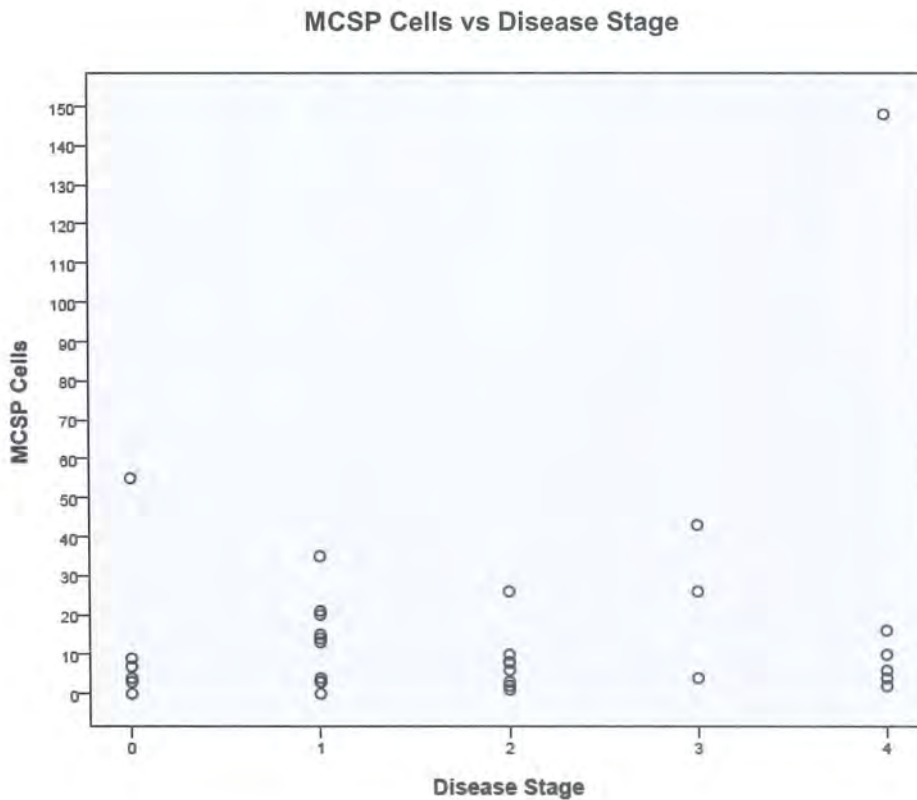


Figure 6.15. Scatterplot of captured MCSP cells versus disease stage for all 33 patient samples included in this study.

6.14.5. MCAM Captured Cells versus Disease Stage

The relationship between the number of cells captured with MCAM beads and disease stage in patient samples was investigated using Spearman's rank order correlation coefficient. There was a significant, medium positive correlation between these variables ($r=0.486$, $n=33$, $p=0.004$), demonstrating that there is a significant relationship between the number of cells captured with MCAM beads and disease stage (Fig. 6.16.).

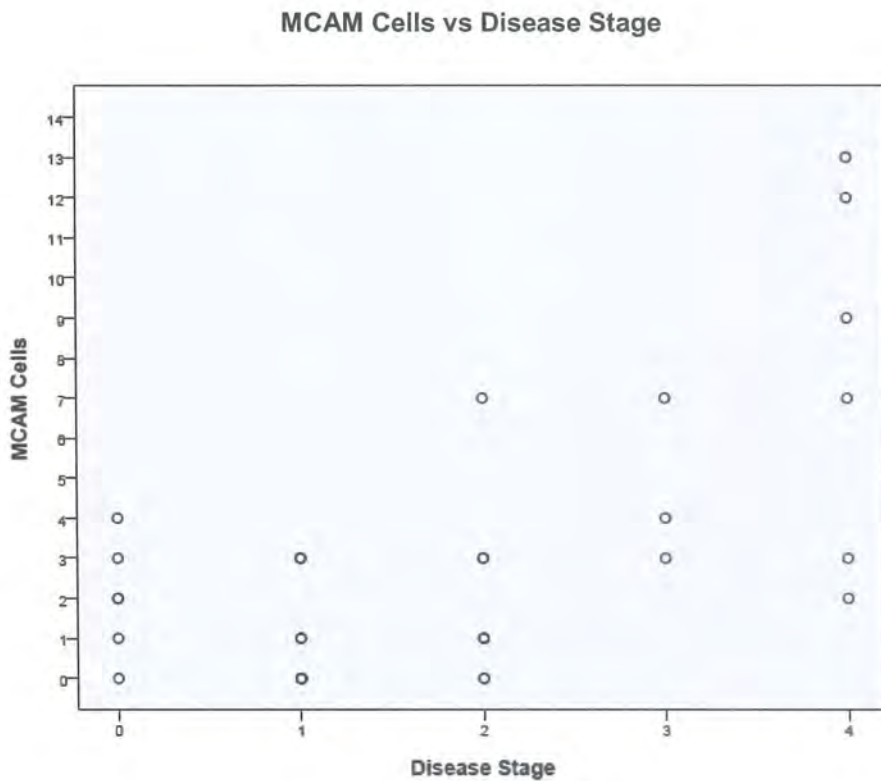


Figure 6.16. Scatterplot of captured MCAM cells versus disease stage for all 33 patient samples included in this study.

6.14.6. ABCB5 Captured Cells versus Disease Stage

The relationship between the number of cells captured with ABCB5 beads and disease stage in patient samples was investigated using Spearman's rank order correlation coefficient. There was an insignificant, weak positive correlation between these variables ($r=0.190$, $n=33$, $p=0.289$), demonstrating that there is no real relationship between the number of cells captured with ABCB5 beads and disease stage within the sample population (Fig. 6.17.).

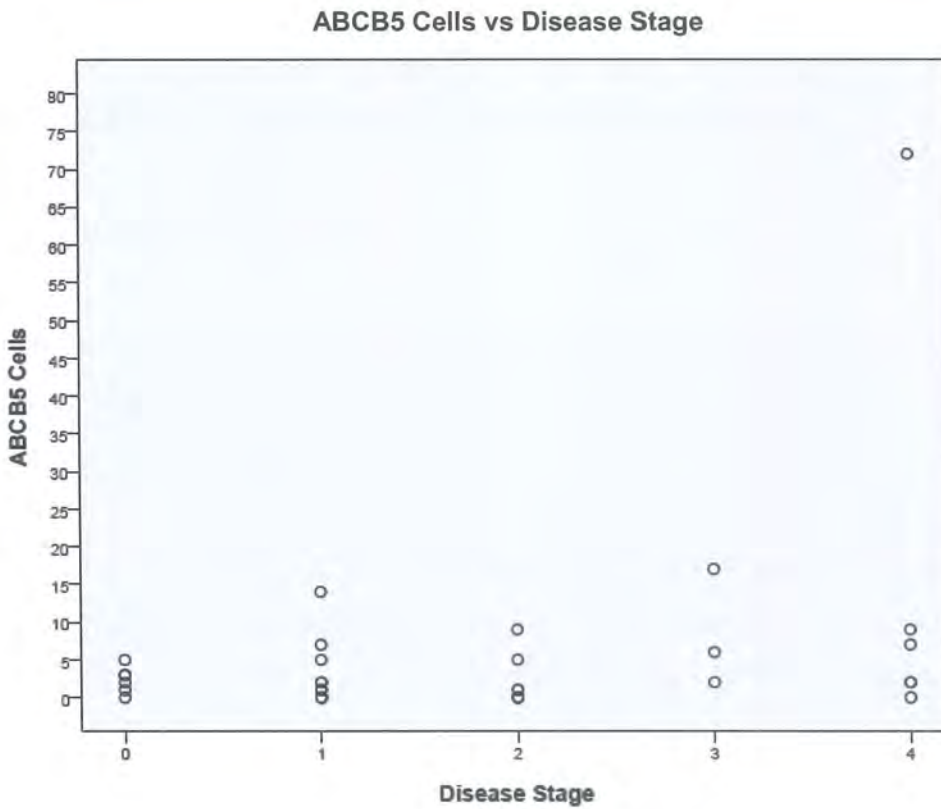


Figure 6.17. Scatterplot of captured ABCB5 cells versus disease stage for all 33 patient samples included in this study.

7. Discussion

The aim of this study was to quantify CTCs in peripheral blood samples from Cutaneous Malignant Melanoma patients in order to determine the relationship between the number of CTCs and clinical disease stage. Immunomagnetic beads coupled with MCSP, MCAM and ABCB5 antibodies were used to target three different melanoma cell surface antigens for optimal isolation of melanoma CTCs for subsequent quantification. The use of these three antibodies also demonstrated differences in circulating cell phenotype relative to disease stage as a result of expression of different target markers. Results of this study contribute to the development of a prognostic blood test that will aid in improving disease management and staging. In addition, quantification of circulating melanoma cells may prove useful in measuring treatment efficacy and identifying early stage patients requiring treatment.

7.1. Total Captured Cells

The total number of captured cells for each patient blood sample was defined as the sum of the number of cells captured with each bead type. This may not be a true representation of the total number of CTCs within whole blood samples as antibody-coupled beads would have only captured CTCs expressing the target antigen and therefore would not have captured other CTCs negative for that particular marker. It is also the case that cell counts for each marker may overlap as cells captured with anti-MCAM beads may also be MCSP positive and vice versa. For these reasons, total captured cell numbers are approximate values used to give some idea of CTC quantity. In order to produce a more accurate representation of total CTC numbers in future experiments, all bead types should be added to blood samples in combination.

7.2. Controls versus Patients

There was a small amount of non-specific binding observed in control samples in this study; however, quantification of captured cells demonstrated that there was a statistically significant difference in the total number of cells

isolated from control and patient samples. If it is assumed that the amount of non-specific binding remains relatively constant in all blood samples, this demonstrates that the cell capture assay employed in this study was successful at binding circulating melanoma cells. Boxplot analysis of the distribution of total captured cells between control and patient groups also illustrated that the number of cells captured in patients was both larger and more variable than in control samples, which exhibited consistently low levels of non-specific binding (Fig. 6.11.). Given that the number of cells captured from patient samples was significantly higher than the number of cells captured from control samples, it is clear that CTCs were successfully isolated from patient samples.

Other CTC isolation studies have also demonstrated low level non-specific background binding. For example, a study by Stott et al. (2010) isolated CTCs from metastatic prostate cancer patients and stained captured cells with Prostate Specific Antigen (PSA), a highly specific prostate cell marker, and CD45, a leukocyte marker, in order to determine which cells were specifically bound CTCs versus non-specifically bound leukocytes. It was found that there was a degree of non-specific background binding to leukocytes in control samples, ranging from 0 to 8 cells (mean=3) (Stott et al., 2010). This was similar to the amount of non-specific binding observed in this study, where the number of cells captured in control samples ranged from 0 to 17 (mean=3.71).

7.3. Captured Cells versus Disease Stage

Melanoma is an extremely heterogeneous disease and therefore CTCs are likely to have a variety of different phenotypes. For this reason a number of antigens were targeted in order to capture as many CTCs as possible from patient blood samples. Interestingly, there was only a moderate increase in the total number of captured cells relative to disease stage. Overall, it was observed that the number of CTCs isolated with anti-MCSP beads was greater than those isolated with anti-MCAM or anti-ABCB5 beads regardless of disease stage, although similar numbers of MCSP positive cells were captured across all disease stages. In contrast, although the numbers of

captured MCAM positive CTCs were small, they significantly correlated with a more metastatic phenotype and therefore provided insight into the difference in CTC phenotype relative to disease stage.

7.3.1. MCAM Cells versus Disease Stage

Analysis of the correlation between the number of cells captured with anti-MCAM coupled beads and disease stage revealed a significant relationship between these variables ($r=0.486$, $p=0.004$) when assessed by Spearman's rank order correlation coefficient. This result indicates that as disease stage increases so too does the quantity of MCAM positive cells, although the amount of MCAM cells in all samples was relatively low compared with the number of MCSP cells. This is the first study to demonstrate a correlation between melanoma CTC phenotype and disease stage. This finding is however supported by current literature which details that MCAM expression in melanoma primary tumours increases with disease severity and is associated with aggressive and invasive tumour behavior (Mangahas et al., 2004; Medic & Ziman, 2010). Due to the aggressive and invasive phenotype of MCAM positive cells, it may be the case that these cells have increased tumour-forming capability. Circulating MCAM positive cells may therefore play a significant role in the extravasation of CTCs and subsequent invasion of tissue sites around the body to form of metastases. The fact that the patients with the three highest quantities of captured MCAM positive cells (patients 33, 29 and 31) are all stage IV patients and have all had multiple metastases supports this idea.

7.3.2. MCSP Cells versus Disease Stage

Interestingly, in contrast to results obtained for MCAM positive CTCs, analysis of the correlation between MCSP positive captured cells and disease stage demonstrated a weak positive relationship when assessed by Spearman's rank order correlation coefficient ($r=0.116$), however this was not statistically significant ($p=0.52$). MCSP is constitutively expressed in the majority of melanomas and is not related to disease progression (Yang et al., 2004);

however an increase in the number of MCSP positive CTCs was expected in metastatic patients compared with non-metastatic patients. In previous studies using anti-MCSP coupled immunomagnetic beads to isolate CTCs from melanoma patient blood samples, a correlation between CTC positivity, where a sample was positive if CTCs were isolated, and disease stage has been shown (Ulmer et al., 2004). Although MCSP positive CTCs were quantified in this study, the relationship between CTC quantity and disease stage was not determined as CTCs were only detected in 26% of samples tested.

Increased numbers of CTCs are expected to be found in later stages of disease when cells of the primary tumour are more invasive or metastatic and are therefore able to escape the primary tumour and enter circulation. However, the results presented here suggest that in fact this may not be the case. In support of this finding, several reports indicate that a large number of cells are simply sloughed off the tumour regardless of stage (Bockhorn et al., 2007). However, a stronger relationship between MCSP positive cells and disease stage may be shown with an increased sample size.

7.3.3. ABCB5 Cells versus Disease Stage

Similarly, statistical analysis using Spearman's rank order correlation coefficient showed a weak positive correlation between cells captured with ABCB5 beads and disease stage ($r=0.190$), however this was not statistically significant ($p=0.29$). Like captured MCAM cells, the number of captured ABCB5 cells was low compared with MCSP cells, often comparable to the amount of background binding observed in control samples. This is the first study to date detailing numbers of circulating melanoma stem cells in patient peripheral blood. The number of melanoma stem cells in circulation relative to non-stem circulating cells appears low although similar to expected values based on demonstration of ABCB5 expression in the A2058 melanoma cell line, which suggested ABCB5 was associated with rare melanoma cells (6.2.). An interesting point to note is that the patients with the three highest numbers of ABCB5 positive cells (patients 8, 27 and 29), although all of different

disease stages, have all had the disease for many years; patient 8 was diagnosed in 1999, patient 27 in 1996 and patient 29 was diagnosed with stage IV disease in 2003. Furthermore, all three of these patients have recurrent disease. It may be the case that the large number of captured ABCB5 cells in these patients is related to disease recurrence, as the proposed role of ABCB5 positive melanoma stem cells is in tumour maintenance and renewal (Boiko et al., 2010; Sekulic et al., 2008).

7.3.4. Total Captured Cells versus Disease Stage

It is interesting to note the number of captured cells in patients with early stage, non-metastatic disease. This is an important finding demonstrating that CTCs are indeed present at early stages and may account for the significant number of patients with early stage disease who suffer recurrence, sometimes years after removal of the primary tumour (Jack et al., 2006; Koyanagi et al., 2005a; Weight et al., 2006).

Analysis of the correlation between total captured cells in patient samples and disease stage showed that there was no significant relationship. There was a weak positive correlation between these variables ($r=0.205$) and there was a difference in the mean number of total captured cells between non-metastatic (stage 0, I and II) and metastatic (stage III and IV) patients (Fig. 6.10.). However, these findings were not statistically significant ($p=0.25$ and $p=0.21$ respectively), possibly due to the small sample size. If more patient samples were included in the study, especially metastatic patient samples, these relationships may have approached significance as it was expected to see a significant increase in the number of CTCs at least between non-metastatic and metastatic patients, if not across all disease stages. Again, however, it may be the case that cells are sloughed from the primary tumour and enter circulation regardless of an invasive phenotype (Bockhorn et al., 2007), resulting in similar numbers of CTCs across all disease stages. If this is the case then it may be the phenotype of CTCs that significantly differs relative to disease stage rather than the number of cells, as suggested by the significant increase in MCAM positive cells relative to disease stage as reported in 7.2.1.

7.4. Numbers of Captured Cells

It has been suggested that CTCs circulate in patient blood at an approximate concentration of 1 in 10^6 to 10^7 blood cells (Krivacic et al., 2004). In the patient samples used in the study, the mean total cell count was 2.21×10^6 /ml in a 3.5ml sample, which equates to 7.74×10^6 cells, although this was post red blood cell lysis. The mean number of cells captured from these patient samples, containing 7.74×10^6 total cells on average, was 24.45. When taking into account the presence of non-specific background binding and the fact that only leukocytes were counted and not erythrocytes, resulting in a far lower total cell count, the number of captured cells observed is reasonably close to the number of cells expected based on the approximate concentration of CTCs in the blood of cancer patients.

Later stage metastatic patients may occasionally show far greater numbers of CTCs, such as the case with patient sample 29, and overall showed a greater range in the number of total captured cells compared with non-metastatic patients (Fig.6.14.). This variation in the number of CTCs captured in metastatic patients has been confirmed in other studies, for example, a study by Ulmer et al. (2004) captured between 1 and 456 CTCs in stage III patients (median=2) and between 1 and 2410 CTCs in stage IV patients (median=5).

This variation in the number of CTCs seen in metastatic patients has also been observed in other malignancies. For example, Stott et al. (2010) quantified CTCs in metastatic prostate cancer patients using an antibody coated chip which binds CTCs during mixing of patient blood. The results of this study showed that the number of CTCs in metastatic prostate cancer patients ranged from 12 to 3167 per ml of blood. The variation in the number of CTCs demonstrated in this study and by Stott et al. and Ulmer et al. may reflect variations in patient immune responses, disease history or individual responses to treatment.

7.5. The Influence of Patient Melanoma Recurrence on the Number of Captured Cells

Table 6.7. shows all available information regarding the history of melanoma, including date of diagnosis of the most recent primary tumour and clinical history, for all 33 patients included in this study. This information may go some way to explaining the number of captured cells in some patients, although trends are difficult to determine as a result of huge variation in time since diagnosis and disease history within the small sample population.

Despite the absence of trends, there are some interesting results, where the total number of captured cells seems to be at least somewhat explained by the patient's history of the disease. Patient 4 was diagnosed with in situ melanoma in 1996 and since excision of the tumour, there has been no disease recurrence reported. The total number of cells captured in this patient was 1, which is less than the mean number of cells captured from control samples (mean=3.71), suggesting that this patient has no CTCs, which is expected since the patient has been disease free for over 14 years. Patient 29 has stage IV disease and had the largest number of total captured cells of any patient included in this study (232). This patient has had stage IV disease since 2003 and had nine separate secondary tumours removed in the five years following stage IV diagnosis. This large number of metastases along with the length of time this patient has had late stage disease may explain the large number of cells captured from this patient.

Although it appears that some extreme high and low numbers of cells can be explained by clinical history of the disease in some patients, there are other radical values which cannot be explained from the available information. For example, patient 6, a stage 0 patient with no disease recurrence had a large number of captured cells (64). Instances such as this make it difficult to determine any apparent relationships between the number of captured cells and clinical history of the disease. However, the large number of CTCs in this patient may indicate a risk of disease recurrence since primary tumour removal occurred in 2008 (Table 6.7.).

7.6. The Influence of Treatment on the Number of Captured Cells

From the data available for patients included in this study, it is difficult to say whether treatment type has an influence on the number of circulating cells since only five patients were undergoing treatment at the time blood samples were taken (Table 6.7). Because of this and the fact that each of these five individuals is undergoing a different type, or combination of treatments, it is impossible to determine any trends in the results. Instead it can only be speculated as to whether the treatment any of these patients is undergoing has had an effect on the number of cells captured by immunomagnetic isolation.

Patient 28, with stage IV disease, is currently undergoing a combination of radiotherapy and immunotherapy to combat their serious disease which has resulted in multiple metastases in various locations. The total number of cells captured from this patient's blood sample was 11, which is relatively low. It is possible that this combination of treatments is having some effect lessening the number of CTCs in this patient although due to limited information, this is difficult to determine. It was also noted that this patient sample had a relatively large amount of clumping, which resulted in a count lower than the actual number of cells present, as many cells could not be distinguished within these clumps.

Patient 29, also with stage IV disease, has received a melanoma vaccine, as other more standard cancer therapies have been unsuccessful at treating their serious disease. This patient had by far the highest total number of captured cells which may indicate that their treatment is ineffective at reducing the number of CTCs, and may therefore be ineffective at reducing the risk of metastasis formation. In contrast, patient 33, who recently had multiple metastases removed, had a relatively low number of total captured cells following adjuvant therapy, indicating that their treatment may have been successful at reducing CTC numbers. These examples suggest that the number of CTCs captured from patient blood samples may be indicative of patient treatment success, although it is difficult to determine due to the small

number of patients who were undergoing treatment, or had recently undergone treatment other than surgery, at the time of blood collection.

7.7. Limitations of the Study

7.7.1. Non-Specific Binding

A few problems arose with the final protocol used for isolating CTCs which impacted on the results of this study. Firstly there was an issue with non-specific binding of the beads resulting in cells being isolated from control blood samples. The initial high level of non-specific binding observed was reduced with the addition of 0.2% Triton X-100 to the washing buffer; however a low level of background binding remained. It is unclear whether this non-specific binding was due to the antibody conjugated to the beads binding non-specifically, control blood cells aberrantly expressing target markers, as a result of being *ex vivo*, or Fc receptors on the surface of immune cells binding antibody. There are a several products available that could be tested in future experiments in order to ensure these problems are not occurring. Firstly there are blood collection tubes designed to inhibit abnormal gene expression in blood cells, thus reducing the chance of them binding to antibody as a result of atypical marker expression. For example, Cyto-Chex Blood Collection Tubes (Streck, Omaha, USA) contain a cell preservative which maintains the integrity of blood cell marker expression for up to 7 days which would inhibit aberrant expression of melanoma markers in these cells. There are also a number of Fc receptor blockers available, which inhibit antibodies binding to Fc receptors on the surface of cells such as B cells, monocytes, and macrophages in order to reduce non-specific binding of antibodies to these cells. Melanoma cells, like the majority of tumour cells, are also Fc receptor expressing cells and therefore Fc receptor blocking would ensure immunomagnetic beads are binding to melanoma cells as a result of the coupled antibody binding to its target marker rather than to Fc receptors on the cell surface.

7.7.2. Cell Clumping

There was also an issue with clumping in samples, which resulted in clumps of cells forming during overnight incubation, which were removed by washing of the beads, or smaller clumps of cells forming, which survived washing and were seen during microscopy. Formation of cell clumps during incubation may have resulted in CTCs being lost during washing if they became a part of the clumps, whereas the presence of smaller clumps were a problem during cell counting by microscopy. These clumps often appeared as smears in which very few or no cells could be identified. This often resulted in a cell count less than the actual number of cells in the sample as only cells identified without doubt were counted. It is unsure whether these were clumps of melanoma cells, clumps of blood cells or a mixture of both. They could have been isolated from the rest of the sample as a result of magnetic beads becoming entangled in a cell clump or unbound cells clumping with bead bound cells resulting in clumps becoming bound by magnetic separation. Clumping was not observed in all blood samples and it did not appear to be related to either total cell count or the age of the sample. This suggests that some samples had a tendency to clump more than others possibly due to the cell composition of the sample.

7.7.3. Captured Cell Numbers in Early Disease Stages

Results of this study demonstrate that CTCs are indeed present at early clinical disease stages, which may account for the significant number of patients with early stage disease who suffer recurrence. In some cases however, the number of cells captured in early stage, non-metastatic patients was much larger than expected. This could have been due to increased non-specific binding in some patient samples, however, if it can be assumed that the amount of background non-specific binding is reasonably constant, and is similar to the amount of non-specific binding observed in other studies (Stott et al., 2010), there may be another explanation for this. It doesn't appear that a large number of captured cells in early stage patients, for example patients 6, 8 and 10, was related to the total cell count of the patient sample, which

suggests it may be the cell composition of the sample that has resulted in an increased number of bound cells. As discussed, there may be an issue with bead bound antibodies binding to Fc receptors on the surface of leukocytes. It could be the case therefore that if a patient has a heightened immune response to their tumour, which results in an increase in Fc receptor expressing cells, there may be an increase in non-specific binding. Of course it would also be case that metastatic patients would have a heightened immune response to their disease, although a large number of captured cells observed in early stage patients seems unusual and is less likely to be a large number of CTCs. Alternately, it may be the case that large numbers of cells enter circulation even at early disease stages although the immune system perhaps destroys the majority of these cells. Early stage patients with a large number of CTCs may therefore have a weakened immune response and large CTC numbers may indicate the possibility of disease relapse. This idea is supported by the fact that early stage patients with a relatively high number of captured cells generally had more primary tumours removed (Table 6.7.), indicating a large number of cells may correlate with a risk for disease recurrence. To confirm this, patients would need to be followed for several years to show an association between CTC numbers and disease recurrence.

7.8. Suggested Future Experiments

7.8.1. Staining Captured Cells

Cells captured by immunomagnetic isolation should be stained with melanoma markers In order to confirm that they are in fact melanoma cells and not non-specifically bound leukocytes. Staining with the same antibodies used to isolate the cells from patient blood will show whether the cells are positive for these markers in order to confirm that the beads are binding cells specifically, via their conjugated antibody. Furthermore, staining these cells with other melanoma specific markers would demonstrate that they are melanoma cells rather than other cells that may be aberrantly expressing the markers that have been targeted in their isolation. Due to the fact that in this study there was a low level of non-specific binding, which has also been

observed in other studies (Stott et al., 2010), staining captured cells is necessary to reveal the proportion of cells specifically bound versus non-specifically bound in order to demonstrate the specificity of the assay. Stott et al. (2010) in fact used immunostaining to produce a true CTC count in metastatic prostate cancer patients by staining with PSA (Prostate Specific Antigen) to confirm CTCs and CD45 (a leukocyte marker) to subtract non-specifically bound leukocytes from CTC counts.

Here we tested immunohistochemical and immunofluorescence staining using a number of different protocols and antibodies although results were largely unsuccessful. A major issue with staining captured cells was that the magnetic beads themselves strongly fluoresced under all wavelengths of the fluorescence microscope. This made it difficult for weak staining to be observed under any wavelength as sufficient exposure resulted in the fluorescence of the beads overpowering that of the cells. When staining was weak, this problem was exacerbated by fixing the cells with paraformaldehyde as it resulted in increased fluorescence of the beads.

Incubating A2058 cells with anti-MCAM beads, fixing them with 2% paraformaldehyde and staining with anti-MCSP and anti-mouse IgG conjugated to Alexa Fluor 546 produced the most successful staining. In this protocol, cells were washed only after antibody incubation steps. The issue with fixing the cells before washing was that all non-specifically bound cells became fixed to the beads and could not be removed by washing. This was discovered when carrying out this method with HEK293 control cells which remained bound to the beads following washing, although they were unstained.

In an attempt to overcome this problem, captured cells were washed 5 times with 0.2% Triton-X 100 in MACS buffer, as per usual, before fixing with 2% paraformaldehyde and subsequently following the staining protocol. When this method was tested the cells did not stain, possibly because Triton X-100 had removed the cell surface proteins during washing. This explanation is supported by the fact that incubating cells with immunomagnetic beads

overnight in MACS buffer containing 0.2% Triton X-100, instead of straight MACS buffer, resulted in no cells binding to the beads. In order to overcome this problem, new washing buffers should be tested. It was also found following this protocol, upon viewing by microscopy, that many cells had become unbound from the beads and consequently were lost from the sample. This was possibly due to over-washing the sample, as a result of performing the standard washing protocol and subsequently washing the cells following fixing and again after each antibody incubation step. Simply reducing the total number of wash steps in the protocol may solve this problem otherwise a different washing buffer may be required.

Due to the fact that strong staining was only seen following fixing the cells, which also fixed non-specifically bound cells, this method was not suitable for staining cells isolated from patient samples. This is because a true representation of the amount of specifically bound cells versus non-specifically bound cells would not be shown.

Staining the cells within the patient's total blood, following RBC lysis, before incubation with the beads was also tried. However it was found that washing and re-pelleting the cells in between antibody incubation steps resulted in loss of the cell pellet likely due to cell lysis. Problems with these methods need to be overcome in subsequent experiments in order to develop a sufficient protocol for staining isolated melanoma cells to sufficiently demonstrate that the circulating cell isolation protocol is specific and correctly captures circulating melanoma cells from blood.

7.8.2. RT-PCR of Captured Cell RNA

Similarly to staining captured cells, RT-PCR could also be used to detect the expression of melanoma markers in cells isolated by immunomagnetic beads. Using primers for the markers used to isolate circulating cells (MCSP, MCAM and ABCB5) would confirm that captured cells had bound specifically to the beads as a result of expression of these markers, while amplification of other melanoma specific markers will confirm that isolated cells are melanoma cells.

This technique has been performed in other studies which have isolated melanoma CTCs from patient blood by immunomagnetic beads in order to confirm that captured cells are in fact melanoma cells and to test for expression of specific markers in these cells (Kitago et al., 2009).

The Dynabeads mRNA Direct kit (Invitrogen) was used to isolate mRNA from cells captured by immunomagnetic isolation for use in RT-PCR. This kit allows mRNA to be isolated from cells and tissue of various types with the use of magnetic beads covalently coupled to oligo (dT)₂₅ which binds to the polyA tail of mRNA molecules following cell lysis. This mRNA can subsequently be used to manufacture cDNA which can be used in a PCR reaction to amplify melanoma specific markers. Once mRNA had been isolated from captured cells using the kit and had been converted to cDNA in a reverse transcriptase reaction, a real-time PCR reaction was performed with primers for GAPDH, a housekeeping gene constitutively expressed in all cell types. The reason for this was to show whether enough RNA had been isolated from the cells to allow PCR to be performed. The result of this real time reaction showed a small peak on the graph indicating GAPDH was present, although it took more than 40 cycles to appear which indicated that the amount of RNA isolated from the cells was extremely low; too low to provide any tangible results. This result was unsuccessful possibly due to the small amount of RNA contained in the samples, which consisted of very few cells. However it is feasible that this method would perform better following protocol optimisation to successfully isolate RNA from small cell numbers. There are other methods of RNA isolation suitable for small quantities of RNA that may also be useful for this application and should therefore be tested, for example RNA isolation with TRIzol reagent (Invitrogen).

7.9. Other Methods of Isolating Circulating Cells

Recent studies have explored the use of methods other than immunomagnetic bead capture for the isolation of CTCs. Isolating CTCs from patient blood in order to characterise or quantify them is a relatively new area of research and as such, there are a number of studies being undertaken to

develop an optimal method of isolating these cells specifically and efficiently. As previously discussed, a study by Stott et al. (2010) has implemented an antibody coated chip that passively mixes patient blood allowing CTCs to come into contact with antibody bound to the chip which binds these cells and isolates them from whole blood. This study was able to capture CTCs in 93% of metastatic prostate cancer patients. Subsequent immunofluorescent staining confirmed bound cells were CTCs and eliminated false positives by staining with CD45, a leukocyte marker.

A study by Kojima et al. (2009) used Green Fluorescent Protein (GFP) expressing attenuated adenovirus specific for telomerase to infect viable CTCs which could be detected as a result of GFP expression in infected cells following viral replication. CTCs were detected by automated scanning for GFP by fluorescence microscopy. Telomerase is expressed in many cancer cell types and results in maintenance of telomere lengths allowing these cells to divide without limit rendering them immortal. This study infected blood samples from patients with a variety of telomerase expressing malignancies with the GFP expressing virus and it was found that GFP expression could be detected in all viable CTCs 48 hours after infection.

A study by De Giorgi et al. (2010) isolated CTCs from melanoma patients by filtration as melanoma CTCs are larger than other blood cells ($>16\mu\text{m}$). Captured cells were confirmed as melanoma cells by performing RT-PCR to detect tyrosinase expression. Results of this study showed that CTCs were only captured in 62.5% of metastatic melanoma patients suggesting this technique may be less sensitive than immunomagnetic bead capture and other methods of capturing CTCs. However, no CTCs were captured in controls or stage 0 patients suggesting it may be more specific than other methods of CTC isolation.

Another study employed density gradient centrifugation, a technique used to separate cell types or cell components based on differences in density, in order to separate CTCs from whole blood in oesophageal carcinoma patients (Hoffmann et al., 2010). RT-PCR was then performed to check captured cells

for survivin (SVV) expression, a marker that varies in expression in response to treatment in gastrointestinal malignancies, in order to confirm they were CTCs. SVV was detected in 77% of patients showing that this technique was reasonably successful. These novel techniques, along with immunomagnetic bead capture, show potential for optimal CTC isolation and therefore need to be explored further and tested in melanoma patients in order to determine which method is the most sensitive and specific for isolating circulating melanoma cells from patient peripheral blood.

7.10. Characterising CTCs

As well as allowing CTCs to be quantified, the use of immunomagnetic beads to isolate CTCs from patient blood would also allow characterisation studies to be performed in order to analyse the phenotype of these cells. The main idea of characterising these cells is to demonstrate differences in phenotype between early stage and late stage cells and to characterise an aggressive phenotype with high metastatic potential compared with a non-aggressive phenotype with low metastatic potential. Determining a series of markers associated with metastasis and aggressive behaviour would allow CTCs isolated from patient blood to be tested for these markers by RT-PCR in order to determine the patient's risk of metastasis and disease progression and thus improve prognosis. Characterising a patient's disease based on CTC marker expression would also allow specific treatment strategies to be implemented with the aim of targeting particular cell types in order to more effectively combat the disease.

7.11. Conclusion

In this study, it has been shown for the first time that the phenotype of melanoma CTCs can be differentiated since a metastatic circulating cell phenotype was identified which correlated with disease stage. A statistically significant relationship was demonstrated between the number of cells isolated with anti-MCAM coupled beads and disease stage. MCAM expression is associated with an aggressive and invasive phenotype with the

potential for metastasis and therefore quantification of MCAM positive CTCs may be useful in monitoring disease progression and assessing the requirement for treatment in early stage patients before the development of metastatic disease. This would be a significant clinical tool since currently there is no method of identifying early stage patients requiring treatment following surgical removal of their primary tumour, as although they are clinically residual disease free, many of these patients go on to develop metastatic disease (Jack et al., 2006; Koyanagi et al., 2005a; Weight et al., 2006).

Results of this study also indicate that there is no significant association between the total number of captured cells and disease stage. While future studies with larger patient populations may change this finding, it may indeed be the case that variation in melanoma CTCs is in the cell phenotype rather than the number of cells and this too is a significant finding.

There were a number of issues that arose with the development of this cell capture protocol, such as non-specific binding and cell clumping, which need to be overcome in future experiments in order to increase the sensitivity and specificity of the assay. The use of immunofluorescent staining or RT-PCR to demonstrate marker expression in captured cells also needs to be explored further to verify that captured cells are in fact melanoma cells. Once these issues have been addressed and the protocol has been optimised, the effectiveness of immunomagnetic bead capture to isolate CTCs in melanoma patients should be compared with other recently developed techniques which have been utilised for the isolation of CTCs in other malignancies. Development of an optimal strategy for the isolation of circulating melanoma cells will allow the relationship between CTCs and disease outcome to be further explored with the aim of improving prognosis and disease management.

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9. Appendices

Appendix A

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Development of a Blood Test for Cutaneous Malignant Melanoma

— Subject Information Sheet —

Purpose

This study will investigate genes, proteins and cells that are abnormally present in the peripheral blood of patients with Cutaneous Malignant Melanoma or other forms of skin cancer but are not present in normal healthy individuals. It is anticipated that genes and proteins may be used as markers to identify cancer cells in the blood of patients as well as provide some indication of tumour progression.

Background

The genes and proteins that play a role in formation of normal skin cells are for the most part, well known. A different set of genes and proteins may be switched on or genes and proteins that are present in normal skin cells may be present in high levels in melanoma tumour cells but not in other skin cells. The reason for the expression of these genes and proteins in melanoma cells and their role in tumour development and progression is unknown. In order to provide more accurate diagnosis and treatments, it is important for medical scientists to understand the signals that lead to skin cancer formation and progression. Using genetic and protein markers, scientists are developing a blood test that can detect migrating melanoma cells in peripheral blood. Ultimately the test will be available for use as in routine diagnostic check ups.

Procedure

Your participation requires you to donate a small sample of blood. The blood will be taken by a phlebotomist or your doctor who is working in collaboration with the researchers and may be providing clinically relevant details relating to the diagnosis of your disease.

As a patient with Cutaneous Malignant Melanoma you will be asked to provide a small quantity (17 ml) of blood at the time of surgical removal of your tumour, or at routine follow up clinical visits six months, one year, two years and three years after surgery.

If you are a patient with other forms of skin cancer or a volunteer providing your blood for controls, then you will only be required to provide your blood on a single occasion.

You may be asked to provide consent for the use of your tissue which has been stored at pathology laboratories. The tissue required for this study will only be archival tissue no longer required for diagnostic or treatment purposes. You will not be asked to undergo any further procedures, merely to provide consent for the use of extraneous tissue.

Risks

In this study, the sample that you provide is a blood sample and there is very little risk to you in this procedure as only a small volume of blood is required for the test. You may also be asked to provide access to your archival tissue samples at no risk to yourself

Benefits

Individual:

There are NO individual benefits from this study at this stage. However an examination of your skin for skin lesions is provided free of charge to you. As the research is at an early stage and no definite conclusions can be drawn from the results of the blood test at this stage, results will be provided to your clinician upon request. However, should an abnormal result be obtained, you or your clinician will be informed and you will be assisted to make the necessary decisions about any changes to your treatment in accordance with the NHMRC guidelines for Human Research.

Community:

The results of this study may provide information about the role of genes, proteins and cells in tumour development and progression. The results may also help identify genes and proteins involved in tumour metastasis and therefore may at a future date, be used to assist with diagnosis and treatment of skin cancer, and malignant melanoma in particular.

Confidentiality of Data

Personal details will not be available to all researchers. Only the chief investigator will have your medical details and your name or patient number. Laboratory researchers will have your medical details only and your sample will be given a code number to protect your identity. Therefore you cannot be identified by laboratory researchers but collective results may be published. Your personal details may be obtained from medical records by your clinician only.

Subject Rights

Participation in this research is voluntary and you are free to withdraw from the study at any time and for any reason, without prejudice in any way.

The project has been approved by the Human Research Ethics Committee at Edith Cowan University (Project No. 2932) and by the Human Research Ethics Committee at Sir Charles Gairdner Hospital (Project No. 2007-123). These Human Research Ethics Committees are fully constituted ethics committees and follows guidelines laid down by the National Health and Medical Research Committee of Australia.

If you have any questions or require any further information about the research project, please contact:

Mel Ziman on telephone number **6304 5171**

If you have any concerns or complaints about the research project and wish to talk to an independent person, you may contact:

Research Ethics Officer

Edith Cowan University

100 Joondalup Drive

JOONDALUP WA 6027

Phone: (08) 6304 2170

Email: research.ethics@ecu.edu.au

Appendix B

PARTICIPANT INFORMATION SHEET

Development of a blood test for Cutaneous Malignant Melanoma

Research on the use of a blood test to monitor Melanoma progression

**A/Prof Mel Ziman, Dr Mark Brown, Prof. Michael Millward, Mr. Mark Lee,
A/Prof Robert Pearce, Dr Prasad Kumarasinghe, Dr. Judith Cole,
Mr James Freeman, Ms Tamara Esmaili.**

Please take time to read the following information carefully and discuss it with your friends, family and clinician 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 and where will it be conducted?

Edith Cowan University

Contact persons:

Should you have questions about the study you may contact:

A/Prof. Mel Ziman Phone No. 6304 5171 Mobile: 0419929851(after hours:)

All study participants will be provided with a copy of the Information Sheet and Consent Form 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 abnormalities in gene and protein expression in the peripheral blood of patients with Cutaneous Malignant Melanoma relative to gene expression in patients with other forms of skin cancer and to normal healthy individuals. It is anticipated that these genes and proteins may be used as markers to identify melanoma cells in the blood of patients as well as provide some indication of tumour progression.
Over 900 participants will be involved.

Why is this study suitable to me?

You have been invited to participate in this study because you have been diagnosed with Cutaneous Malignant Melanoma, or you have been invited to participate because you have been diagnosed with another form of skin cancer or a mole or you are asked to participate as a normal healthy volunteer.

How long will I be in this study?

If you agree to participate you will be asked to undergo a skin examination by a practicing clinician (either a dermatologist or a plastic surgeon). This service will be provided free of charge if you are a healthy volunteer.

You will then be asked to provide a blood sample.

As a patient the blood sample will be taken before surgical removal of your tumour. If you are invited to participate as a patient with Cutaneous Malignant Melanoma you will be asked to provide additional blood samples at the time of routine clinical follow up visits to your clinician at 6 months, one year, two years and even three years after surgery.

Clinical Visits	Dates
Before Surgical Removal of Tumour	
6 Month clinical follow up	
1 Year clinical follow up	
2 Year clinical follow up	
3 Year clinical follow up	

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

- The study will be conducted over a five year period. If you are a healthy volunteer you will be asked to undergo a free skin examination and provide one blood sample at a time that is suitable for you.
- If you are a patient with a skin lesion you will be asked to provide one blood sample at the time of surgical removal of your lesion.
- If you are a patient with Cutaneous Malignant Melanoma you will be required to give a blood sample five times over the course of three years.
- The amount of blood required for this study is small (only 17 ml per visit). Blood will be drawn into two different types of blood tubes.
- 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.

- Your blood will be tested relative to blood from other participants and those of healthy volunteers. Because your sample is only identifiable by a coded number, the researchers performing the tests will not know which sample is yours.
- samples will be stored in the locked freezers during the melanoma research study conducted at Edith Cowan University and will then be discarded upon 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 these 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 when and where you visit your doctor for treatment and follow up visits.

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. However, the research will not at this stage provide you with any detailed information about your health or genetic diseases now or in the future. Donation of your sample may assist researchers to provide a more detailed and specific diagnosis of melanoma in the future.

How will my safety be ensured?

In this study, the sample that you provide is a blood sample and there is very little risk to you in this procedure as only a small volume of blood is required for the test. 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. You may also be asked to provide access to archival tissue samples that are stored in pathology laboratories where they were used for clinical diagnosis. Should this be the case only archival tissue samples will be used and no fresh tissue is required and you will not be required to undergo any further surgical procedures.

The clinician may find a lesion on your skin or the blood study may produce abnormal results in which case you and your clinician will be notified and additional clinical tests will be performed if your doctor feels it is in your best medical interest.

What alternatives do I have to going on this study?

Your participation is voluntary. This study does not affect your treatment or your health. 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 in the future even though there is no immediate benefit to your own treatment if you participate in the study.

What are the possible side effects, risks and discomforts of taking part?

In this study, only a small volume of blood is taken (17 ml) at each scheduled visit 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. If archival tissue samples are used there is no additional risk to you.

The likelihood of side effects from donating blood are 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 about a blood test 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 product or 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. The researchers may also need to get some of your health information from other health service providers, eg 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 Exercise Biomedical and Health Science at Edith Cowan University during the study and in a locked archive for at least 5 years and for a maximum of 15 years from the time the study is closed, and may be destroyed at any time thereafter.

Authorised representatives of the researchers, the investigating doctors, or University Human Research Ethics Committees, and other regulatory bodies may require access to your study records to verify study procedures and/or data. In all cases when dealing with your information, these people 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.

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 Edith Cowan University Human Research Ethics Committee and the Sir Charles Gairdner Hospital Human Research Ethics Committee have reviewed this study and have given approval for the conduct of 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.

The Edith Cowan University and Sir Charles Gairdner Human Research Ethics Committees have given ethics approval for the conduct of this project.

If you have any questions or require any further information about the research project, please contact:

Mel Ziman on telephone number **6304 5171**

If you have any concerns or complaints about the research project and wish to talk to an independent person, you may contact:

Research Ethics Officer
Edith Cowan University
100 Joondalup Drive
JOONDALUP WA 6027
Phone: (08) 6304 2170
Email: research.ethics@ecu.edu.au

Or the secretary of the Sir Charles Gairdner Hospital Human Research Ethics Committee on telephone No. (08) 9346.2999

All study participants will be provided with a copy of the Information Sheet and Consent Form for their personal records.

CONSENT FORM

Development of a Blood Test for Cutaneous Malignant Melanoma

Investigators: A/Prof Mel Ziman, Dr Mark Brown, Prof. Michael Millward, , A/Prof. Robert Pearce, Mr. Mark Lee, Dr Prasad Kumarasinghe, Dr. Peter Heenan, Dr Judy Cole, Mr James Freeman, Ms Tammy Esmaili

Participant Name: _____

Date of Birth: _____

Address: _____

Phone Number: _____

Email: _____

Name and address of GP _____

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

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

Appendix C

List of Abbreviations

µl- Microlitre
A488- Alexa-Fluor 488
ABCB5- ATP-Binding Cassette Subfamily B, Member 5
AJCC- American Joint Committee on Cancer
BSA- Bovine Serum Albumin
CDKN2A- Cyclin-Dependent Kinase Inhibitor 2A
CDK4- Cyclin-Dependent Kinase 4
cDNA- Complementary Deoxyribonucleic Acid
CMM- Cutaneous Malignant Melanoma
CT- Computed Tomography
CTC- Circulating Tumour Cell
DAPI- 4',6-diamidino-2-phenylindole
DMEM- Dulbecco's Modified Eagle Medium
DNA- Deoxyribonucleic Acid
EDTA- Ethylenediaminetetraacetic Acid
EpCAM- Epithelial Cell Adhesion Molecule
FBS- Foetal Bovine Serum
Fc- Fragment Crystallizable
FL1- Fluorescence Channel 1
FL2- Fluorescence Channel 2
GAPDH- Glyceraldehyde 3-phosphate dehydrogenase
GFP- Green Fluorescent Protein
HEK 293- Human Embryonic Kidney 293
IgG- Immunoglobulin G
LDH- Lactate Dehydrogenase
MAGE-A3- Melanoma Antigen Gene A3
MAPK- Mitogen-Activated Protein Kinase
MART-1- Melanoma Antigen Recognised by T-Cells-1
MC1R- Melanocortin-1 Receptor
MCAM- Melanoma Cell Adhesion Molecule
MCSP- Melanoma Associated Chondroitin Sulphate Proteoglycan
MITF- Microphthalmia-Associated Transcription Factor
MRI- Magnetic Resonance Imaging
mRNA- Messenger Ribonucleic Acid
NGS- Normal Goat Serum
PBS- Phosphate Buffered Saline
PET- Positron Emission Tomography
PSA- Prostate Specific Antigen
qRT-PCR- Quantitative Reverse-Transcriptase Polymerase Chain Reaction

RBC- Red Blood Cell

RGP- Radial Growth Phase

RNA- Ribonucleic Acid

RT-PCR- Reverse-Transcriptase Polymerase Chain Reaction

SVV- Survivin

TYRP-1- Tyrosinase-Related Protein-1

TYRP-2- Tyrosinase-Related Protein-2

UV- Ultraviolet

VGP- Vertical Growth Phase