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The identification of diagnostic serological autoantibodies for Cutaneous Melanoma

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The Identification of Diagnostic Serological Autoantibodies for Cutaneous Melanoma

Research Thesis

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Abstract

Cutaneous melanoma is the most aggressive and drug resistant form of skin cancer and is responsible for most skin cancer related deaths in Australia and worldwide. Early detection of the cancer is the single most import factor in the prevention of disease progression. Therefore the identification and validation of highly sensitive and specific early stage diagnostic biomarkers for melanoma has been of great interest in recent years. Elevated levels of autoantibodies in the serum of early stage cancer patients have been associated with the presence of cancer of the breast, lung, ovary, colon, head and neck and prostate. Therefore, the presence of one or more serological autoantibody biomarkers in the blood of early stage melanoma patients may assist with diagnosis of the disease and may be developed as a routine diagnostic tool, such as a blood test, to aid the early detection of the cancer.

The aim of this project was to identify such diagnostic autoantibody biomarkers or panels of these markers, displaying high frequency in patients but none or low frequency in healthy volunteers, for the early detection of melanoma. The aim of the research was also to validate the identified markers in a larger cohort and to establish the optimal single marker or panel of biomarkers, displaying the highest sensitivity and specificity for melanoma.

To do this, we utilised serum samples from patients recently diagnosed with melanoma and age and gender matched healthy volunteers, who were confirmed as melanoma, cancer and autoimmune disease free. The samples were screened using a functional protein microarray, developed by our collaborators Oxford Gene Technology, Oxford, UK. The array contains 1627 human recombinant proteins of various tumour origin.

Our results showed that eight of the proteins attached to the microarray surface, exhibited high reactivity with melanoma patient sera and low or no reactivity with control sera and were therefore considered potential diagnostic biomarkers for the early detection of melanoma. Three of the microarray identified markers, namely SOX10, BRDG1 (STAP1) and SCYL3, were chosen for further investigation to validate the results obtained from the microarray in a larger cohort. We found that a combination of all autoantibody biomarkers had the highest diagnostic potential for early stage melanoma patients relative to healthy control samples. This finding coincides with recent studies which have found increased diagnostic potential in various cancers when a panel of multiple autoantibody biomarkers is utilised as opposed to individual markers alone. We also show here that two methodologies,

the functional protein microarray and an in-house indirect ELISA, show similar sensitivity and specificity scores for detection of melanoma autoantibodies in early stage melanoma regardless of whether individual biomarkers or combinations of these markers are used. Thus we confirm that these methods are appropriate for the identification and validation of diagnostic autoantibody biomarkers for early diagnosis of melanoma.

Interestingly, in our cohort, female patients displayed significantly higher levels of autoantibody markers than male patients. However, we found no statistically significant differences in patient autoantibody levels with regard to disease stage or age.

In conclusion, our findings correspond with the hypothesis upon which this research was based, that increased levels of autoantibodies in the serum of early stage melanoma patients are indicative of a positive disease status and may therefore be utilised to diagnose melanoma patients at an early stage.

Declaration

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

i.) incorporate without acknowledgement any material previously submitted for a degree or diploma in any institution of higher education;

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1. Introduction and Literature Review

Cutaneous melanoma (CM) is a highly treatment resistant, fatal skin cancer. It´s incidence accounts for only 4% of all skin cancers, however, melanoma is responsible for 80% of all recorded deaths from skin cancer (Lewis et al., 2005). Melanoma is the third most common cancer in all age groups, and it is the most commonly diagnosed cancer in the 15-39 year age group in Australia (Melanoma Institute Australia, 2013). It is also the leading cause of death in young males within the 15-39 year age group (Threlfall and Thompson, 2010). Worldwide, three million people are diagnosed with the cancer per annum (Kutting and Drexler, 2010). Australia has the highest incidence of melanoma in the world with Queensland followed by Western Australia being the states with the highest melanoma prevalence (Western Austalian Cancer Registry, 2010). In order to better manage the disease and to increase survival rates, there is a need for identification of diagnostic biomarkers for early stage disease, as late diagnosis drastically reduces 5-year survival rates. By contrast, patients diagnosed with early stage disease have a 5-year survival rate as high as 99% (Balch et al., 2009).

1.1 The origin of cutaneous melanoma

Melanoma is a malignant tumour of melanocytes, a subset of cells within the basal layer of the epidermis of the skin. Melanocytes also occur in the bulb of the hair follicle (Fitzpatrick, 1971; Haaka and Scott, 2001; Nordlund and Boissy, 2001). Melanocytes originate from neural crest cells and migrate to the basal layer of the epidermis during embryonic development (Chin, 2003). Normally situated at the border between the dermal and the epidermal layer of the skin (Figure 1), these cells are responsible for production of the pigment melanin. This pigment is responsible for the variety of human skin colour phenotypes (Rees, 2004). Furthermore, melanin pigment contributes to the natural defences of the human body, as it absorbs ultraviolet (UV) radiation from the sun (Hsu et al., 2002).

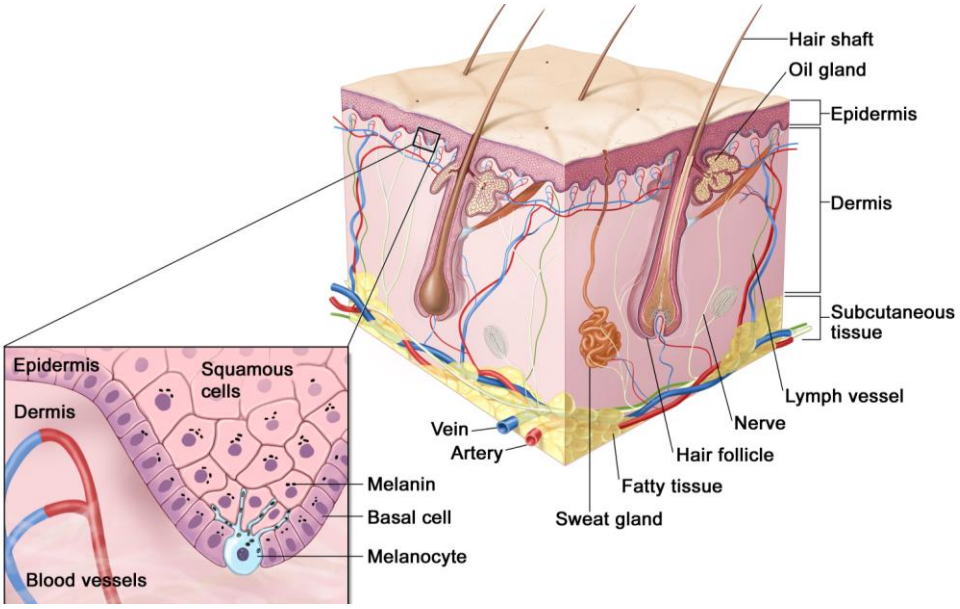


Figure 1: Location of melanocytes within the lower part of the epidermis of the skin. (Image from Masonic Cancer Centre, http://www.cancer.umn.edu)

Melanocytes prevent UV radiation damage to the DNA of the cells of the epidermis, by connecting to the keratinocytes via dendritic connections, forming a keratinocyte-melanocyte unit. Within each of these units, melanin pigment is transported from a singular melanocyte

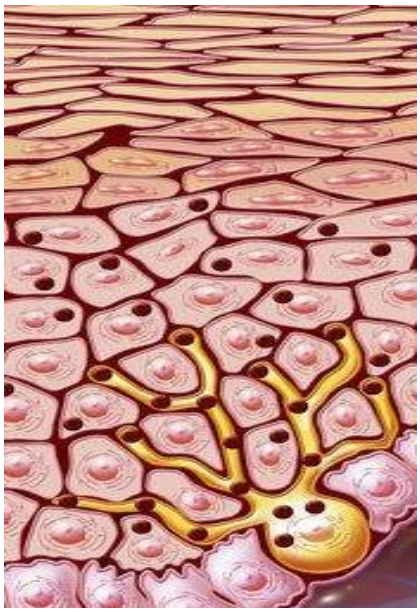


Figure 2: Melanocyte cellular extensions and melanin distribution. (Image from Science Photo Library, http://www.sciencephoto.com)

to up to 36 keratinocytes within the epidermal layer of the skin (Figure 2) (Chin, 2003).

In response to UV exposure, melanocytes proliferate resulting in an increase in the ratio of melanocytes to keratinocytes. This can result in the formation of a mole, or naevus (Patton et al., 2005). Melanocytes within a naevus eventually cease proliferation and become quiescent or senescent. However, melanocytes within a naevus may become activated due to mutations, with the result that there is uncontrolled replication of these cells (Patton et al., 2005). Although approximately 60% of melanomas arise from existing naevi, it should be noted that they can also arise *de novo* (Weatherhead et al., 2006).

There are two important stages of tumour growth within the epidermis; initially, uncontrolled proliferation of melanocytes results in radial, or superficial growth, whereas at later stages melanoma cells undergo a vertical growth phase. Once the melanoma growth phase reaches the vertical phase, melanoma cell growth is no longer under keratinocyte control, enabling melanoma cells to invade the dermis and therefore the circulatory and lymphatic systems (Postovit et al., 2007).

1.2 Major risk factors associated for cutaneous melanoma

UV radiation (UVA and UVB) has long been accepted as a major environmental cause of skin cancers including cutaneous melanoma (Leiter and Garbe, 2008; Mackie, 2005). Individuals who have a history of multiple incidents of severe sunburn during their lifetime and especially during their childhood, are said to have an increased risk of developing melanoma (Cho and Chang, 2010). Certain phenotypes such as paler skin, blue eyes, red hair and a large number of moles or freckles also increase the risk of developing melanoma (Duffy et al., 2007). The most common body sites to be diagnosed with melanoma are the back/buttock (37%), upper arm (21%), face (12%), neck (7%), forearm (7%), and lower leg/foot (7%) in males. In females, the most common body sites to be diagnosed with melanoma are the upper arm (23.1%), back/buttock (21.1%), lower leg/foot (17.3%), face (9.6%) and forearm (9.6%) (Youl et al., 2011).

The risk of developing melanoma is increased when eumelanin is decreased in individuals as a result of a single nucleotide polymorphism within a gene known as *melanocortin 1 receptor* (*MC1R*) (Rees, 2004). This leads to a decrease in the amount of melanin pigment being transported from melanocytes towards the keratinocytes and therefore a decrease in protection from UV radiation of the sun, creating a greater risk of developing DNA mutations

(Costin and Hearing, 2007). There is a decreased risk of malignant melanoma in populations with darker skin shades such as Native Americans, Hispanics and Asians as well as Africans (Czarnecki and Meehan, 2000). About 10% of all diagnosed melanomas are known to be caused by germline mutations in cyclin-dependent kinase 4 (*CDK4*) on chromosome 12, or the tumour suppressor gene, cyclin-dependent kinase inhibitor 2A (*CDKN2A*), on chromosome 9p21, indicating that family history is another risk factor for melanoma (Bataille, 2000; Chin, 2003; Nagore et al., 2000).

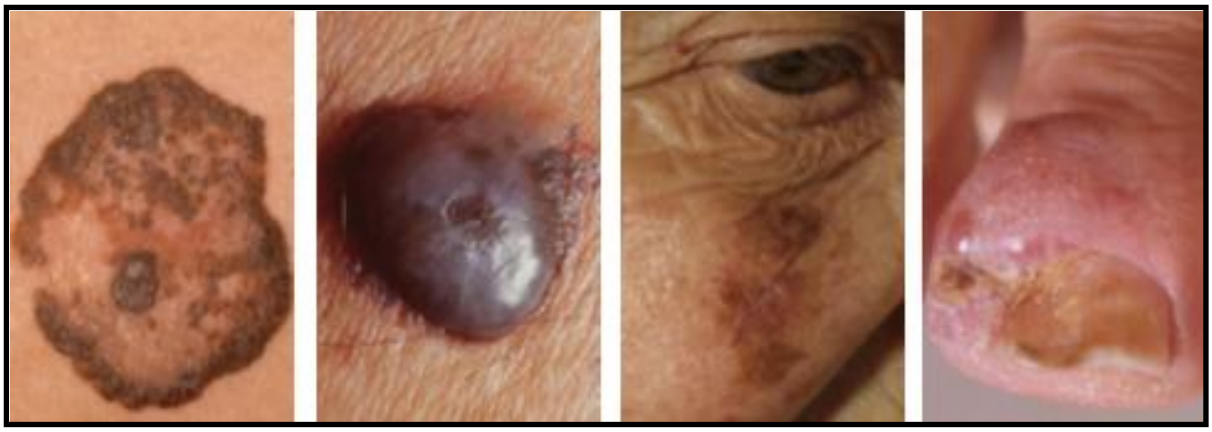
Familial melanoma is also caused by a polymorphism in *MITF* (Yokoyama et al., 2011), a master regulatory gene of melanocyte differentiation, proliferation and survival. *MITF* plays a role in cancer cell survival by increasing expression of *BCL2*, an anti-apoptotic gene (Banerjee, 2002; McGill and Horstmann, 2002). A germline variant, the E318K allele, results in an MITF protein with impaired sumoylation resulting in differentially regulated MITF targets, including BCL2 (Yokoyama et al., 2011).

Severe infections such as erysipelas have the potential to modify the course of melanoma significantly, in some cases leading to regression and potentially complete resolution of the cancer (Grange et al., 2002). Krone et al. (2005) showed that immune surveillance for melanoma is induced or enhanced by prior contact with pathogens such as those vaccinated with the Bacille Calmette-Guèrin (BCG) vaccine.

Epidemiological studies have shown that a deficiency in vitamin D increases the risk of cancers including melanoma (Chen et al., 2010; Field and Newton-Bishop, 2011; Gandini et al., 2005). Vitamin D has an anti-proliferative and pro-apoptotic effect on cancer cells; once bound to the vitamin D receptor, it will result in the transcription of genes which play a role in inhibition of MAPK signalling, apoptosis induction and cell-cycle inhibition. Therefore, people with vitamin D deficiency are at higher risk of developing melanoma (Krishnan et al., 2003).

1.3 Types of melanoma

Wallace Clark and his colleagues were the first to histologically classify melanoma into three different forms: superficial spreading, lentigo malignant and nodular (Clark Jr, 1967; Clark Jr et al., 1969). Dr. Richard Reed added a forth type which is known as acral lentiginous malignant melanoma (Reed, 1976).



Surface Spreading Melanoma | **Nodular Melanoma** | **Lentigo Maligna Melanoma** | **Acral Lentiginous Melanoma**

Figure 3: Types of melanoma (Image adapted from Hoek, 2006)

Superficial Spreading Melanoma (SSM) accounts for 50-70% of all melanomas with 75% of all SSM‟s arising *de novo* in the epidermis. SSM lesions typically show a wide variety of pigmentation and a pagetoid spread of cancerous melanoma cells within the epidermis (Demitsu et al., 2000).

Nodular Melanoma (NM) accounts for 15-35% of all melanomas. This type of melanoma is characterised by the absence of a radial growth phase but the lesion can be polypoid, nodular or pedunculated (Kiene et al., 1995; Plotnick et al., 1990).

Lentigo Maligna Melanoma (LMM) accounts for 5-15% of all melanomas and usually occurs on sun-exposed locations of the body such as the face and the skin of the upper body, particularly in elderly patients (Cohen, 1995). LMM is an *in situ* melanoma known as “Hutchinson Freckle”. Only 5% of all patients with a lentiga maligna lesion progress to develop lentigo maligna melanoma. This process may take several years (McKenna et al., 2006).

Acral Lentiginous Melanoma (ALM) accounts for 5-10% of all melanomas and represents the most abundant melanoma type in people with dark skin colours or those of Japanese origin (Chen et al., 1999).

1.4 Melanoma staging methods

Melanoma is staged clinically according to the American Joint Committee on Cancer (AJCC) guidelines (Balch et al., 2009). These guidelines involve the classification of melanomas into stages based on information with respect to the primary **t**umour thickness, mitotic rate, the

presence of ulceration in the primary tumour and the presence of **m**etastasis to lymph **n**odes or distant sites. This is known as **TNM** staging.

Please refer to Table 1 and Figure 4 below for further details on the clinical characteristics of each TNM melanoma stage.

Table 1: TNM staging of melanoma. Adapted from (Balch et al., 2009)

Stage	Description
0	Primary tumour *in situ*
IA (local disease)	Primary tumour equal to or less than 1mm thick without ulceration and without evidence of metastasis
IB (local disease)	Primary tumour with a thickness of less than 1mm with ulceration but no evidence of metastasis OR primary tumour between 1 and 2mm thickness but no evidence of ulceration or metastasis
IIA (local disease)	Primary tumour between 1 and 2mm thickness with ulceration but no evidence of metastasis OR primary tumour with thickness between 2 and 4mm without ulceration and without evidence of metastasis
IIB (local disease)	Primary tumour between 2 and 4mm thickness with ulceration but no evidence of metastasis OR primary tumour with thickness greater than 4mm without ulceration and without evidence of metastasis
IIC (local disease)	Primary tumour greater than 4mm thick with ulceration but without evidence of metastasis
III (locoregional disease)	Primary tumour of any thickness with metastasis to one or several regional lymph nodes but without evidence of distant metastasis
IV (metastatic disease)	Primary tumour of any thickness with distant metastasis to other sites of the skin, subcutaneous tissue, lymph nodes, lungs and visceral organs

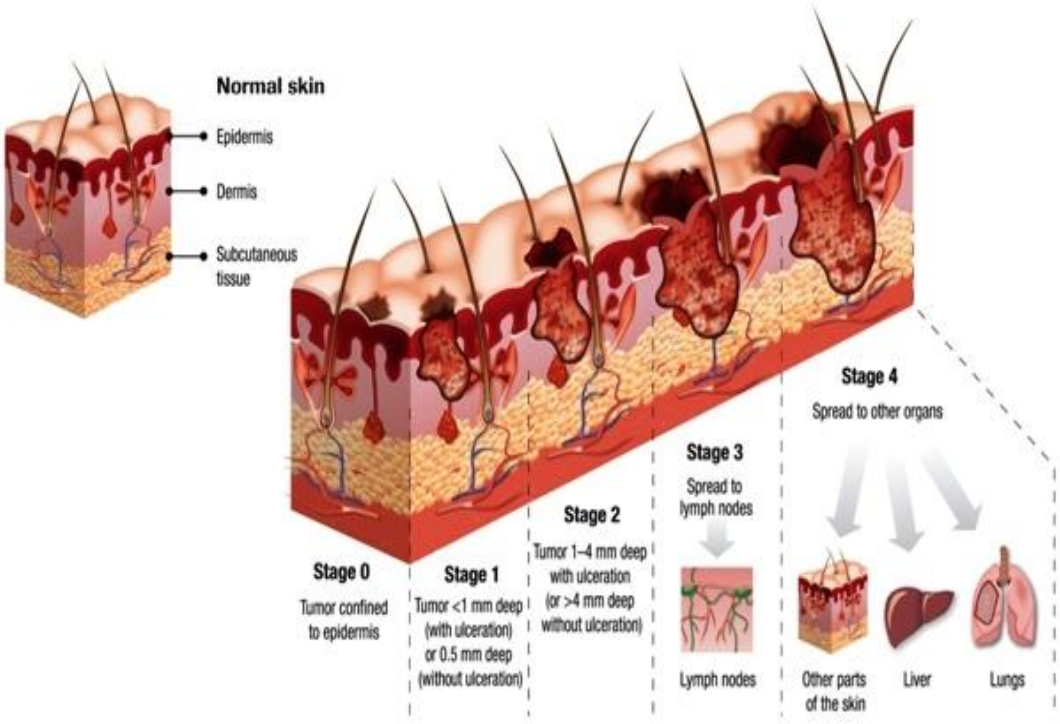


Figure 4: Melanoma stages (Image from melanoma.org.nz)

TNM stage IV melanoma patients may be further subdivided according to their levels of serum lactate dehydrogenase. Serum lactate dehydrogenase levels increase considerably with increasing tissue damage, thus providing a measure of prognosis in late stage patients (Agaruala, 2009; Balch et al., 2009).

The Breslow index is utilised in TNM staging of melanoma to determine the microstage of melanoma and indicates the measurement in millimetres of the vertical thickness (Table 2) of

Table 2: The Breslow system of tumour staging (Breslow, 1975)

Total thickness of tumour	Five year survival rate
0.00-0.76 mm	98-99%
0.76-1.49 mm	85%
1.50-2.49 mm	84%
4.00 mm	44%

the primary tumour (Breslow, 1969; Breslow, 1975). This measurement has a strong predictive value in the prognosis of patients with non-metastatic melanomas.

The Clark system (Clark et al., 1975) of melanoma microstaging is most commonly used by pathologists to grade a tumour according to depth of invasion of atypical cells or inflammatory infiltrates in relation to cutaneous histologic structures. The Clark level correlates prognosis to the anatomical level of local invasion (Table 3). Any primary melanoma tumour with a Clark level greater than II is considered an invasive tumour.

Table 3: Clark system of tumour staging (Clark et al., 1975)

Clark classification	Anatomical level of local invasion	Five year survival rate
Level I	Lesion involves only the epidermis = *in situ* melanoma	100-98%
Level II	Lesion invades the papillary dermis, but does not reach the papillary-reticular dermis	96-72%
Level III	Invasion fills and expands the papillary dermis, but does not penetrate the reticular dermis	90-46%
Level IV	Lesion invades into the reticular dermis, but does not penetrate into the subcutaneous tissue	67-31%
Level V	Invasion spreads through the reticular dermis into the subcutaneous tissue	48-12%

1.5 Diagnosis and prognosis of cutaneous melanoma

Initially, suspicious moles are examined using the ABCDE rule which determines a mole to be a potential melanoma if the mole is **a**symmetrical, has an irregular **b**order, is **c**oloured unevenly, has a **d**iameter greater than 6mm and changes occur over time, hence the mole is **e**volving (Abbasi et al., 2004).

Once a biopsy has been obtained, the collected tissue sample is analysed by cytological analysis and immunohistochemistry, using markers such as Human Melanoma Black 45 (HMB45), S100 calcium binding protein B (S100B) and Protein melan-A (MLANA), also known as melanoma antigen recognised by T-cells 1 (MART-1); to assess the presence of cutaneous melanoma (Mocellin et al., 2008; Pflugfelder et al., 2013). In order to increase the accuracy of these analytical tests, it is necessary to utilise a larger number of markers with a high sensitivity and specificity. In cases where immunohistochemistry results are indecisive, electron microscopy may be utilised (Lai et al., 1998). On occasion, there may be difficulty in diagnosing melanoma as the phenotype and genetic makeup can resemble the cells of other cancer types (Sheffield et al., 2002).

Patients with early stage melanoma (TNM stage I) have a high (up to 99%) 5-year survival rate after surgical removal of the primary tumour (Balch et al., 2009). Melanomas at early stages (TNM 0-IIC) that are treated with non-surgical therapies have a recurrence rate of up to 30% within the first 5 years after treatment (Zalaudek et al., 2003).

A poor prognosis and therefore high mortality rates are associated with metastatic melanoma stages III and IV (Ireland et al., 2011; Jack et al., 2006; Schatton et al., 2008). When distant metastases occur in non-visceral sites such as subcutaneous tissue, distant lymph nodes or other locations within the skin, the patient‟s 1-year survival rate may be as low as 54%. However, if the metastasis occurs in visceral sites, the 1-year survival rate is usually less than 35% (Gershenwald et al., 2008). Stage IV melanoma patients have a median survival as low as 7.5-8 months with a mortality rate of up to 80% (Mervic, 2012; Postovit et al., 2007).

The most accurate prognostic measures for melanoma to date are primary tumour thickness, the mitotic rate of primary tumours, the number of lymph node metastases, as well as the location of metastases, the nature of the lymph node metastases as either microscopic or macroscopic and elevated serum lactate dehydrogenase (Balch et al., 2009; Balch et al., 2010). Micrometastases are diagnosed via sentinel or elective lymphadenectomy, while macrometastases, defined as clinically detectable nodal or systemic metastases, can be defined by therapeutic lymphadenectomy or positron emission tomography (PET) scans (Marsden et al., 2010).

1.6 Melanoma tumour progression and metastasis

Studies have shown that metastasis of the primary melanoma tumour arises in up to 15% of melanoma patients with no evidence of melanoma tumour cells in their lymph nodes at the time of primary surgery (Balch et al., 2009). This indicates that melanoma cancer cells have the ability to spread not only via the lymphatic system but also throughout the circulatory system where they are then called circulating melanoma cells (Reid et al., 2013; Weight et al., 2006). The shedding of melanoma cells from the primary tumour and their invasion of the bloodstream is a prerequisite of melanoma metastasis (Figure 4) (Koyanagi et al., 2005; Mocellin et al., 2006). In fact, studies have shown that tumours shed millions of cells per day into the blood stream, however, only a few cells establish metastases that are clinically detectable (Figure 5) (Fidler et al., 2002; Jacob et al., 2007; Mitropapas et al., 2006; Palmieri et al., 2007; Reid et al., 2013).

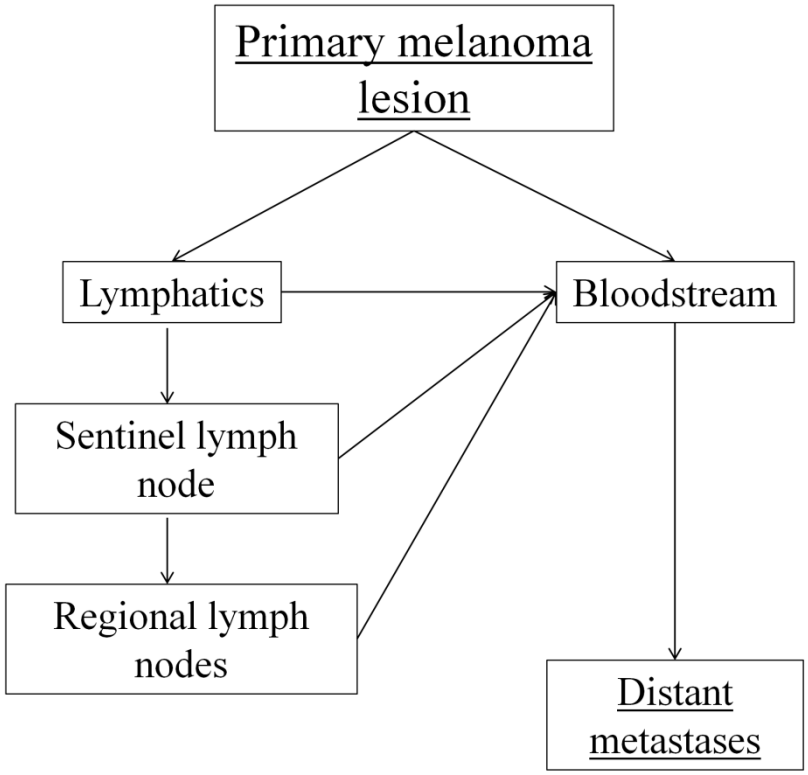


Figure 5: A diagram of the process of metastasis. (Figure adapted from Vereecken et al., 2011)

For secondary metastases to be formed, metastatic melanoma cells need to undergo the process of delamination, migration through tissues followed by cell adhesion at secondary

sites. An inverse correlation between circulating tumour cells and overall patient survival has been established for uveal melanoma (Ulmer et al., 2004) and other cancers such as metastatic breast cancer (Cristofanilli et al., 2004). Understanding the biology and the mechanism of metastasis provides new molecular targets and may help us to discover new biomarkers.

Since circulating melanoma cells are present in the blood of patients with melanoma stages I-IV (Freeman et al., 2012; Reid et al., 2013), detecting the presence of circulating tumour cells in peripheral blood of patients or the presence of specific serological autoantibody biomarkers against circulating melanoma cells or the primary tumour, can be used to diagnose the disease in early stage melanoma patients (Freeman et al., 2012; Hoon et al., 2000; Reid et al., 2013).

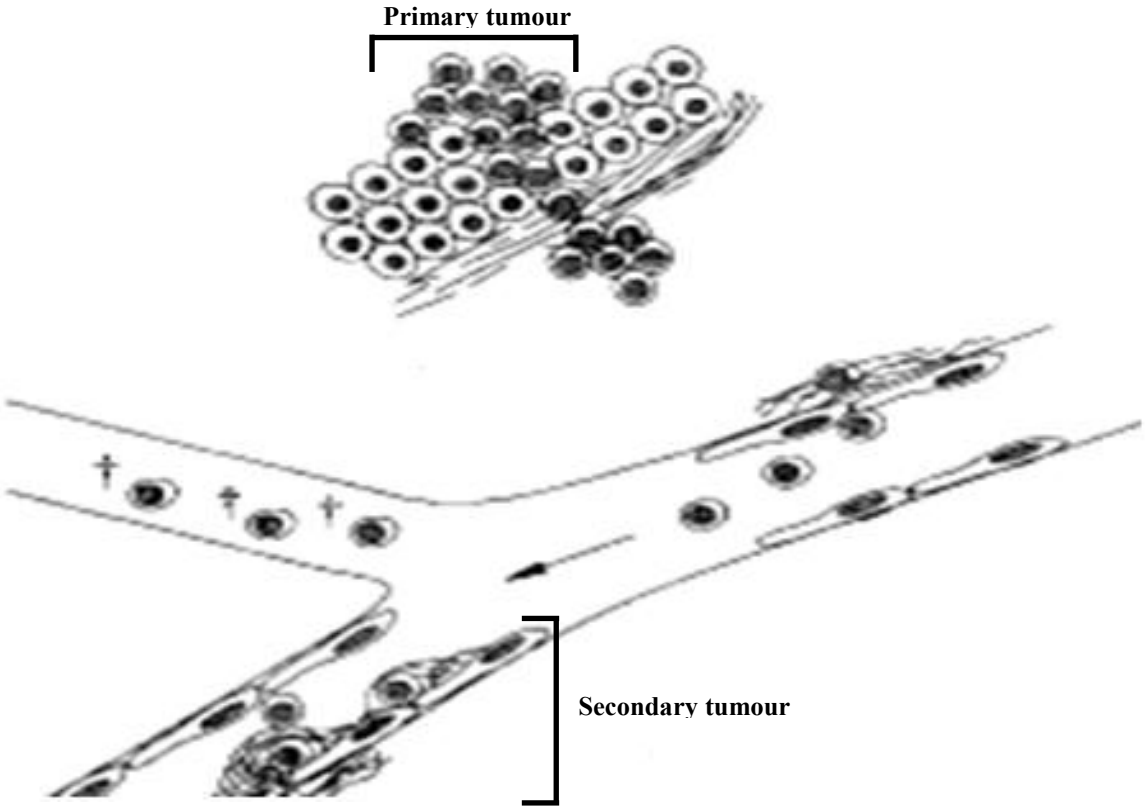


Figure 6: Diagram of tumour cell metastasis (Image adapted from Pantel et al., 1999).

1.7 Treatment options available for cutaneous melanoma

For primary or secondary metastatic tumours, surgical excision is the optimal treatment (Tan, 2010). For stage IV inoperable melanoma, a wide range of treatment options such as

immunotherapy, chemotherapy, interferon therapy or radiotherapy can also be considered; however, the use of these treatments has drastically decreased in recent times, as more specific treatments have been developed. To date, immunotherapies with interleukin-2 as well as interferon therapy with interferon-alpha have proven to be successful in some cases where metastasis is extensive. However, success rates are less than 10% (Steen et al., 2008).

Prior to 2011, the only chemotherapeutic agent approved for treatment of metastatic melanoma was the intravenous drug dacarbazine (Blesa, 2009). Specific targeted therapies for the treatment of late stage metastatic melanoma are now in clinical use to inhibit melanoma growth. *BRAF* inhibitors specifically target the V600E mutation which is found in greater than 50% of all melanomas. The mutation in *BRAF* results in activation of the mitogen-activated protein kinase (MAPK) pathway (Davies et al., 2002), responsible for cell regulatory processes including uncontrolled cell proliferation, increased motility, angiogenesis as well as abnormal prolonged cell survival (Tuveson et al., 2003). Treatment with these targeted therapies is successful in 78% of patients with dramatic reduction in tumour size (Bhatia et al., 2009; Laurent-Puig et al., 2009). However *BRAF* inhibitors may cause adverse side effects (Shepherd et al., 2010) and patients relapse after, on average, 7 months due to drug resistance (Solit and Rosen, 2011).

In 2011, a new drug, Ipilimumab, was approved by the US Food and Drug Administration. This drug can stimulate T-cells to enhance the immunological response towards cancer cells and increases patient survival rates from 6.4 months to 10 months (Wolchok et al., 2010b). However, this drug only has a response rate in 11% of patients and it can cause serious immune-related adverse events (Hodi et al., 2010; Wolchok et al., 2010a).

1.8 Biomarkers for early detection of cutaneous melanoma

There are two types of biomarkers: diagnostic biomarkers, which are used for screening tests, and prognostic biomarkers, which are used once the disease has been confirmed through diagnosis and which are used to predict a patient´s long term survival rates. Molecular tools such as DNA, proteins, peptides or mRNA can be measured in tumour tissue with specific qualitative and quantitative tools, and can be included as tumour biomarkers.

The research discussed in this document focuses on melanoma biomarkers found in the blood serum of melanoma patients. These biomarkers are referred to as serological biomarkers.

Serological biomarkers for cutaneous melanoma can be produced as part of an immunologic response towards the primary tumour and secreted into the bloodstream.

To date, serological biomarkers are generally not utilised to diagnose melanoma, but may prove valuable, particularly in occult melanoma cases where the primary tumour is not found (Kamposioras et al., 2011). Moreover, following the surgical excision of their primary tumour, melanoma patients generally do not receive further treatment and PET/ CT scans are currently the only way to monitor a limited number of patients with suspected advanced melanoma (Marsden et al., 2010).

In contrast to melanoma, prostate and ovarian cancers are commonly diagnosed using serological antigen tests. Prostate cancer screening tests, for example, are based on a single biomarker known as prostate specific antigen (PSA). PSA is produced in the prostate gland and may leak into the patient‟s blood stream (Pan and McCahy, 2011). However, although PSA serum levels are still used today, it has a specificity of less than fifty percent which results in frequent false positive results and therefore several unnecessary radiotherapy and surgical procedures (Zeliadt et al., 2011). Increased PSA serum levels may also arise due to non-cancerous conditions such as enlargement of the prostate, prostatitis and urinary tract infection (Pan and McCahy, 2011). In Australia, the College of General Practitioners does not recommend population screening using PSA (The Royal Australasian College of General Practitioners, 2009), whilst the Urological Society of Australia and New Zealand (USANZ) recommend access to the test as it helps in diagnosing many patients correctly and therefore increases patient survival rates. However, other test options such as a rectal examination may also aid in the diagnosis of the cancer (Urological Society of Australia and New Zealand, 2009).

There have been attempts to discover highly specific serological melanoma biomarkers, including LDH; however, their limitations do not support their clinical use as melanoma biomarkers for early stage melanoma. Therefore, the search for serological biomarkers is ongoing and more crucial than ever before, due to the increasing incidence of melanoma worldwide (Ferlay et al., 2004).

1.9 Serological biomarkers for melanoma

To date, researchers have predominately focused on investigating increased levels of various novel melanoma molecules in the blood to serve as potential biomarkers for the disease, but

have so far failed to identify a single biomarker or a combination of biomarkers with sufficiently high specificity and sensitivity for this cancer (Vereecken et al., 2011). Several molecules currently utilised, or currently under investigation as potential diagnostic or prognostic biomarkers for melanoma, include: c-reactive protein (CRP), S100-ß protein (S100B), melanoma inhibitory activity (MIA), growth and angiogenesis factors such as vascular endothelial growth factor (VEGF), galectin-3 (Gal-3), melanoma-associated antigens, CD44, intercellular adhesion molecule 1 (ICAM-1), melanin-related metabolites, matrix metalloproteinases (MMPs), cytokines, chemokines and their receptors and integrins, lactate dehydrogenase (LDH), as well as circulating melanoma cells (Pflugfelder et al., 2013).

The LDH enzyme has been considered the most appropriate prognostic serological marker of metastatic melanoma and is used in the American Joint Committee on Cancer (AJCC) staging system (Balch et al., 2009). As previously mentioned, elevated levels of LDH in serum of late stage melanoma patients are used to determine the level of metastatic load as its levels considerably increase with increasing tissue damage (Agaruala, 2009; Balch et al., 2010; Balch et al., 2001b). However, this enzyme is not specific for melanoma, nor is it suitable for disease diagnosis of early stage melanoma. Moreover, haemolysis and other factors in patients with hepatitis often result in false positive results (Agaruala, 2009; Balch et al., 2001a).

Molecules such as S100B are used during immunohistochemical analysis of excised melanoma tumour tissue but this marker is not specific for early stage melanoma when monitored in patient blood (Salama et al., 2008). To date, scientists have failed to show that these molecules have the appropriate specificity and sensitivity to serve as diagnostic tools for melanoma alone and the search for specific serological diagnostic melanoma biomarkers is ongoing.

1.10 Immunologic response to cancer

Although the exact mechanisms involved are still unknown, the immune system has evolved to achieve immune homeostasis in tissues through the delicate balance between pathogenic T-cell responses directed against tissue-specific antigens and the ability to inhibit these immune responses, avoiding the development of autoimmunity by discriminating autologous from non-autologous proteins (Rosenblum et al., 2011).

In response to foreign, infectious agents, the immune system mounts an immune response via a combination of adaptive and innate components. Initially, the detection of pathogen-associated molecular patterns (PAMPs) is sensed through either soluble patterns (PRRs) or cell-borne proteins, found on dendritic cells, macrophages and other immunologic cells and detected as non-autologous, initiating a strong immune response (Delves et al., 2011). Following their maturation and migration towards to the lymph nodes, self-antigen peptide-MHC complexes on antigen-presenting dendritic cells are recognised by T-cells (Rudensky et al., 2006). Upon the expression of a self-antigen, the thymus-derived regulatory T-cells, also known as T_{reg} cells, become activated, proliferate and differentiate into a more potent type of suppressor cells. These activated T-cells have been shown to mediate the resolution of organ-specific autoimmune responses in mice (Rosenblum et al., 2011). Once differentiated, these effector cells coordinate further macrophage activation, cytotoxic cell destruction and B-cell responses, resulting in the simultaneous activation of the innate and adaptive immune system to defend the body against foreign agents (Delves et al., 2011). Following the initial inflammatory response, T_{reg} cells have recently been found to be maintained within the affected tissue as „regulatory memory" cells which are primed cells responsible for the attenuation of subsequent autoimmune reactions (Rosenblum et al., 2011). Occasionally however, these regulatory mechanisms are evaded and the autoimmune response is maintained (Delves et al., 2011; Martini and Nath, 2009).

Immunologic responses towards cancers, including melanoma, are usually modest due to the tumour cell"s ability to recruit macrophages as well as other cells of the innate immune system to mask the tumour cell as autologous or "self", therefore supporting tumour survival and growth. Furthermore, tumour cells commonly lack PAMPs and contain very few other non-autologous determinants, resulting in tissue-resident dendritic cells remaining as immature cells which fail to migrate to the lymph nodes to present antigen in response to PAMP activation. This ultimately results in the failure of cells of the adaptive system to migrate into tissues which are most commonly occupied by tumour cells, further ensuring tumour survival (Rudensky et al., 2006). Inflammatory responses further enhance tumour growth due to the release of inflammatory mediators such as cytokines and chemokines by the tumour itself. These are able to recruit neutrophils and macrophages, as previously mentioned, which in turn produce more cytokines and other inflammatory mediators, promoting proliferation of the tumour as well as angiogenesis to provide a rich supply of

oxygen and nutrients and waste disposal required by rapidly proliferating tumour (Frank et al., 2011; Hammam et al., 2013).

Although the pathways involved in the production of autoantibodies and the immunologic recognition of non-autologous antigens are still elusive, research on prostate cancer has found an association between increased autoantibody levels in serum and prostate cancer development (McAndrew et al., 2012; Wang et al., 2005). This research utilised microarray technology to identify bromodomain-containing protein 2 (BRD2), eukaryotic translation initiation factor 4 gamma 1 (eIF4G1), ribosomal protein L22 (RPL22), ribosomal protein LBa (RPL13a), and hypothetical protein XP_373908 as the antigens most frequently bound to autoantibodies in prostate cancer patient serum. Except for hypothetical protein XP_373908, these structures were derived from intracellular proteins involved in regulating either transcription or translation and resembled autologous proteins in patients. However, their DNA sequences were not identical to the autologous proteins, which was suspected to have stimulated autoantibody production (Wang et al., 2005). The autoantibody signature was detected in only 5 of 14 serum samples from patients who had undergone prostatectomy and in 3 of 11 serum samples from patients with hormone-refractory disease, suggesting that the autoantibody profile is attenuated on removal of the "immunogen" or after treatment with antiandrogens, chemotherapeutic agents, or both (Wang et al., 2005).

Furthermore, small-cell lung cancer research has shown that a number of rare paraneoplastic neurologic autoimmune diseases (PNS) that damage neuronal tissues in a site remote from the tumour but are unrelated to metastasis (Schiller and Jones, 1993), are strongly associated with the cancer. Most patients harboured high levels of autoantibodies against neuronal proteins which are abnormally expressed in small-cell lung cancer tumours (Diesinger et al., 2002; Kazarian and Laird-Offringa, 2011). Interestingly, these autoantibodies can be found in a large number of small-cell lung cancer patients without symptoms of neurologic damage as well as some healthy volunteers, however in these cases the levels of the autoantibodies are very low, suggesting that high levels of the autoantibodies may have value for the diagnosis of small-cell lung cancer patients with PNS (Kazarian and Laird-Offringa, 2011). Currently, there are at least 20 identified small-cell lung cancer-PNS associated autoantibodies and their use in diagnostic tools may greatly assist in early detection as the neurological disease often precede the small-cell lung cancer tumour development (Kazarian and Laird-Offringa, 2011).

Additionally, a recent study has identified novel T-cell modulatory functions of the ATB-binding cassette sub-family B member 5 positive ($ABCB5^+$) melanoma subpopulations (Schatton et al., 2010). Blockage of ABCB5, an energy-dependent drug efflux p-glycoprotein pump situated in the cell membrane (Frank et al., 2003), results in increased intracellular drug accumulation which increases cytotoxicity, thereby improving melanoma chemotherapy success (Frank et al., 2005). Tumorigenic $ABCB5^+$ malignant melanoma initiating cells have been shown to possess the ability to inhibit interleukin 2 (IL-2)-dependent T-cell activation and to support the induction of $CD4^+CD25^+FoxP3^+$ regulatory T_{reg} cells, resulting in the suppression of the immune response. $ABCB5^+$ melanoma initiating cells have also been shown to have lower expression levels of the melanoma-associated antigens ML-IAP, MART-1, MAGE-A and NY-ESO-1, thereby evading an immunological response (Schatton et al., 2010).

1.11 Improved disease diagnosis through autoantibody detection

Recent studies have shown an increase in autoantibody production in early stages of several cancers (Gnjatic et al., 2009; Tan and Zhang, 2008) including cancer of the breast (Disis, 1994), lung (Diesinger et al., 2002), colon (Scanlan et al., 1998), ovary (Chatterjee, 2006) prostate (Wang et al., 2005) and head and neck cancer (Carey, 1983).

Tumour cells have the capacity to produce immune modulatory signals (Pietras and Östman, 2010), exploiting and potentially escaping immune regulatory mechanisms (Bronte and Mocellin, 2009; Kerkar and Restifo, 2012) to create an immune-suppressive and therefore tumour-promoting microenvironment which is now regarded as an active instead of passive participant in the survival and defence strategies of a tumour (Becker et al., 2013). Such a tumour-promoting microenvironment is achieved by inducing central and peripheral immunologic tolerance which is defined as “a state of unresponsiveness of the immune system to substances or tissue that have the capacity to elicit an immune response” (Murphy, 2012). Central tolerance is induced in the thymus and bone marrow and represents one of the main mechanisms that enable the immune system to discriminate autologous from non-autologous substances. Peripheral tolerance is induced in lymph nodes and other tissue and is important for the prevention of over reactivity of the immune system to environmental entities such as allergens. Central and peripheral tolerance of a tumour‟s microenvironment such as in melanoma may be induced by promoting T-helper 2 (Th2) responses which are regarded as rather tumour-promoting when compared to the Th1 responses, which are

regarded as tumour-inhibiting (Becker et al., 2013). Furthermore, the expression of the homo-tetrametric heme-containing cytosolic enzyme tryptophan 2.3-dioxygenase (TDO) has an inhibitory effect on anti-tumour immune responses, prolonging tumour survival (Pilotte et al., 2012). The resulting central and peripheral tolerance leads to an increase in autoantibody levels that would normally be removed by lymphocytes that have receptors that can bind strongly to autoantibodies and remove these by induction of apoptosis of any autoreactive cells or by induction of anergy of these cells (Murphy, 2012).

An alternate theory of the process of autoantibody production suggests that the precursors of antibody producing cells, B lymphocytes, must integrate with two signals from two different signal-detecting receptor proteins before antibody production and secretion into the bloodstream is facilitated (Vinuesa and Goodnow, 2002). This mechanism is thought to be the key for the immune system‟s ability to distinguish autologous from non-autologous molecules and therefore greatly reduces autoantibody production (Bretscher and Cohn, 1970). The theory suggests that any pathway, in which this two-signal control mechanism is overcome, will result in the production of antibodies against the body‟s own proteins, hence autoantibodies, and will therefore result in the presence of autoimmunity (Vinuesa and Goodnow, 2002). The first signal of this control mechanism originates from the direct binding of antigens to specific antigen receptors on cell-surface immunoglobulin molecules with variable antigen-binding sites on B cells. The second signal commonly originates from cytokine proteins as well as cell-surface growth factors produced by T helper cells, indicating that the production of circulating antibodies depends on T helper cells and B cells. Only if the B cells and T helper cells escape their tolerance to the same self-antigen simultaneously, autoantibody production can occur.

For some important classes of microorganisms, however, the second signal can originate not from T helper cells but from a specific component of the pathogen itself (Vinuesa and Goodnow, 2002). Lipopolysaccharide is one such T-cell independent antigen and forms a major part of gram-negative bacteria cell walls. The unique lipid group of the lipopolysaccharide is recognised by the toll-like receptor 4 (TLR4) cell-surface receptor present on all B cells (Poltorak, 1998). High concentrations of lipopolysaccharide stimulate the proliferation of all B cells, however, at low concentrations, the simultaneous recognition of the lipopolysaccharide by antigen receptors and TLR4 causes the production of a signal which facilitates the production of circulating autoantibodies specifically by bacteria- specific B cells (Cooke, 1994).

The immune response towards tumour-associated antigens (TAAs) presented in early stages of carcinogenesis, is thought to occur in response to cancer immunosurveillance, the process by which the immune system recognises and destroys invading pathogens as well as host cells that have become cancerous (Anderson and LaBaer, 2005; Caron et al., 2007; Finn, 2005). It has also been suggested that genetic, hormonal and environmental influences may play a part in triggering autoimmunity.

Immunologic processes causing autoantibody production are believed to be generated by the immune system in response to mutations, degradation, over-expression of proteins and/or the release of proteins from damaged tissue, such as the increased release of VEGF at tumour sites to enhance angiogenesis, or the release of cytokines and chemokines to cause inflammation at the tumour site which further enhances tumour growth (Chen et al., 2005; Järås and Anderson, 2011; Kazarian and Laird-Offringa, 2011; Scanlan et al., 2004). Autoantibody production is also believed to be caused by mis-presentation or mis-folding of proteins which may be recognised by the immune system leading to autoantibody production and therefore, tumour-associated antigens (TAAs) or proteins that have undergone alternate post-translational modifications (PTMs) may be recognised as non-autologous (Anderson and LaBaer, 2005; Caron et al., 2007; Salazar and Disis, 2003), i.e. their phosphorylation, glycosylation, oxidation or proteolytic cleavage could generate a neo-epitope or enhance self-epitope presentation and affinity to the major histocompatibility complex or T-cell receptor, inducing an immune response (Hanash, 2003). A neo-epitope is an epitope which is located within an unexposed region of the protein, preventing any interaction between the molecule and antibodies or lymphocytes, therefore avoiding the induction of an immune response against the molecule. The neo-epitope may only cause an immune response or tolerance when it´s structure is exposed by a conformational change or stereochemical alteration of the protein structure (Genovese et al., 2012).

The benefits of the diagnostic use of autoantibody production include improved disease diagnosis in early stage cancer patients as the presence of autoantibodies may precede disease symptoms (Tan and Zhang, 2008). Autoantibody detection may also offer high specificity and sensitivity to improve accuracy of diagnostic tools as they are highly accessible, and are easily obtained from the blood of patients so can be utilised for routine monitoring of patients. Furthermore, unlike most other serological proteins in early stage patients, autoantibodies are stable and easily purified from serum (McAndrew et al., 2012).

1.12 Methodology of autoantibody detection

To advance the discovery of novel combinations of autoantibody biomarkers, techniques which allow the simultaneous screening of multiple biomarkers are required. Examples of such methodologies include serological analysis of tumour antigens by recombinant cDNA expression cloning (SEREX), phage display, serological proteome analysis (SERPA), multiple affinity protein profiling (MAPPing) or protein microarrays. For a comprehensive overview and comparison of methodologies and associated processes used to detect multiple autoantibodies simultaneously, please refer to (Zaenker and Ziman, 2013). A brief overview of these methods will be provided here. Please also refer to Appendix 8.1 to view a summary figure of methods commonly used to detect autoantibodies.

1.12.1 Serological analysis of tumour antigens by recombinant cDNA expression cloning (SEREX)

SEREX, the serological analysis of tumour antigens by recombinant cDNA expression cloning, was first developed in 1995 (Sahin et al., 1997; Sahin et al., 1995). This technique utilises antibody reactivity with autologous cancer patient sera to identify immunogenic tumour proteins (Caron et al., 2007; Gunawardana and Diamandis, 2007). The cDNA expression library utilised in this methodology is constructed from tumour specimens of interest and then cloned into λ-phage expression vectors which are used to transfect *Escherichia coli.* The resulting recombinant proteins are then transferred onto a nitrocellulose membrane which is incubated with diluted patient sera. Clones which are reactive with high-titer IgG antibodies are identified using an enzyme conjugated secondary antibody specific for human IgG. The cDNA clone is sequenced and the autoantigen identified. The major advantage of using SEREX is the fact that it allows the identification of tumour-associated antigens from *in vivo* material. Another advantage of this technology is that it allows the identification of several tumour-specific antigens in one experiment. Furthermore, both the tumour-specific antigen and its coding cDNA are present in the same plaque when immunoscreening is performed which allows the subsequent sequencing of matched cDNA immediately. The disadvantage of SEREX is the high likelihood of false-positive results. Secondly, the use of tumour tissue from a single cancer patient followed by screening with autologous patient sera limits identification of tumour-associated antigens to that patient. Moreover, this complex methodology does not detect alternate tumour-associated post-translational modifications of antigens (Caron et al., 2007). Patients may also exhibit

autoimmunity to autologous proteins and therefore irrelevant non-cancer-associated proteins may be detected. Furthermore, parallel analysis with healthy donor sera as controls cannot be performed easily.

1.12.2 Phage display

Alternatively, a cDNA phage display library is constructed directly from tumour tissue or a cancer cell line derived from patient tumour material (Mintz et al., 2003). Phage clones which bind to cancer sera are identified through a differential biopanning approach (Chatterjee et al., 2009). Alternatively, a more cost-effective method is to construct the cDNA phage display library by expressing the phage proteins fused to the antigens on the surface of bacteriophages. The phage display method has the advantage of allowing the simultaneous screening of a large number of antigens against the sera of cancer patients relative to serum of healthy individuals (Mintz et al., 2003; Wang et al., 2005). The phage-display method has a higher throughput value than the SEREX method but again, antigens with alternate post-translational modifications cannot be detected using the phage-display method (Anderson and LaBaer, 2005; Fossa et al., 2004).

1.12.3 Protein microarray

The protein array methods are advantageous in that they require only minute amounts of patient sera (Robinson et al., 2003) while enabling the simultaneous screening of large numbers of antigens in a single test (Balboni et al., 2006; Bouwman et al., 2003; Davies et al., 2005; Kijanka and Murphy, 2009; Madoz-Gurpide et al., 2008; Sartain et al., 2006). In this methodology, purified or recombinant as well as synthetic proteins are used. Alternatively, fractured proteins of tumour origin are spotted onto the microarray platform. Arrays are then incubated with patient and control sera (Anderson and LaBaer, 2005; Caron et al., 2007; Madoz-Gurpide et al., 2001; Qiu et al., 2004). The array platform can be either two dimensional (such as nitrocellulose membranes, microtitre plates or glass slides) or three dimensional (such as nanoparticles or beads). While protein microarray methods are commonly used to analyse recombinant proteins expressed from *Escherichia coli* cells, other host expression systems such as yeast and insect cells have been used to produce libraries presenting proteins with the correct post-translational modifications. The disadvantage associated with this method is the requirement for high quality protein synthesis (Megliorino et al., 2005). Furthermore, studies utilising protein microarrays are time restricted due to the short shelf-life of protein arrays (Anderson and LaBaer, 2005; Ramachandran et al., 2004).

1.12.4 Reverse-capture microarray

In this method, the antibodies reacting with specific proteins are spotted onto the microarray. Similar to the protein microarray, the reverse-capture microarray is incubated with tumour lysate and serum proteins and the microarrays with captured proteins are then further incubated with sera from patients and controls. The autoantibodies are detected with fluorescent-labelled secondary antibody (Ehrlich et al., 2006; Ehrlich et al., 2008; Qin et al., 2006). The advantage of the utilisation of "reverse-capture" microarray technology is the elimination of the need for recombinant proteins and allows the instant identification of cancer-specific autoantibodies. However, only known antigens and their commercially available antibodies can be analysed and immunoreactivity with post-translationally modified antigens cannot be differentiated unless antibodies which bind exclusively to these antigens are commercially available.

1.12.5 Serological proteome analysis (SERPA)

Serological proteome analysis (SERPA) (Klade et al., 2001) is also known as PROTEOMEX. This technique is very useful for detection of tumour-associated antigens since it incorporates an effective separation of a complex mixture of proteins based on their isoelectric points and molecular weights through 2D electrophoresis and western blotting followed by identification by mass spectrometry (Anderson and LaBaer, 2005; Chung et al., 2004; Nakanishi et al., 2006). Proteins from the tumour tissue of interest are transferred onto a nitrocellulose membrane and immobilised. The sera from cancer patients and controls are separately screened using the immobilised proteins. The appropriate immunoreactive profiles are compared and the cancer-associated antigenic spots are identified by mass spectrometry. Similar to the SEREX technique, the advantage of the SERPA technique is the use of *in vivo* derived tumour-associated antigens. Furthermore, the SERPA technique allows for the identification of tumour-specific post-translational modifications and isoforms but is limited in terms of the identification of low-abundance and transmembrane tumour-associated antigens (Balboni et al., 2006; Canelle et al., 2005; Caron et al., 2007). SERPA also enables the easy parallel analysis of tumour proteins with healthy donor sera as controls and avoids the time-consuming construction of cDNA libraries, enabling this methodology to be completed within a few hours compared to several days for SEREX and phage-display technology. However, due to the way that western blots are prepared, SERPA can only be used to detect linear epitopes (Casiano et al., 2006).

1.12.6 Multiple affinity protein profiling (MAPPing)

The MAPPing methodology incorporates 2D immunoaffinity chromatography which is followed by the identification of tumour-associated antigens by tandem mass spectrometry analysis (Hardouin et al., 2007). In the first phase of the initial immunoaffinity chromatography, lysate from cancer cell lines or tumour tissue containing nonspecific tumour-associated antigens is bound to IgG that was obtained from healthy controls in an immunoaffinity column. The flow-through fraction is then subjected to 2D immunoaffinity in a column that contains IgG from cancer patients and columns can be utilised in parallel (Caron et al., 2005). The tumour antigens which are captured in the patient columns are eluted and digested for identification by nano-liquid chromatography mass spectrometry. MAPPing ensures that the tumour antigens are maintained in a solution which allows the potential identification of structural epitopes. The disadvantages associated with this method include the restriction of the tumour antigen identification to antibody interactions with a low dissociation rate constant. Furthermore, immunoprecipitation using these affinity columns limits the detection of tumour antigens in more complex protein solutions, such as cell lysate.

1.13 Oxford Gene Technology functional protein microarray

The optimal technology, for multiplexing a vast number of antigens on one microarray, is the Oxford Gene Technology (OGT) array. It requires minimal volumes of serum sample per assay and hundreds of antigens can be analysed simultaneously. OGT functional protein array is therefore an ideal platform for autoantibody biomarker discovery in early stage melanoma patients, as the array enables the simultaneous assay of a large number of antigens due to its ideal spotting density and requires as little as 5µl of undiluted serum sample to achieve this.

Importantly, to maintain the conformation of all epitopes of the spotted proteins and therefore ensure highly specific autoantibody binding to these, the proteins are immobilised in their natural, functional three-dimensional structure (Tan and Zhang, 2008). Production of multiplex protein microarrays in other formats often results in denatured proteins when they are immobilised on the glass slide of the microarray, thus non-specific binding may occur if the proteins hydrophobic core is exposed and false negatives may result when the discontinuous epitopes are destroyed. To avoid such situations, Oxford Gene Technology attaches a unique biotin carboxyl carrier protein tag (BCCP) to each individual protein on the functional protein array and attaches the proteins to the glass surface of the microarray via biotinylation (Koopmann et al., 2005). As demonstrated in Figure 7, this special BCCP tag

ensures that the correct three-dimensional functional protein structure and its epitopes and hydrophobic core are maintained and it lifts the protein a certain distance away from the array glass slide to ensure optimal binding (Butler et al., 1997; Houen and Koch, 1997; Yoshida et al., 2004).

The OGT functional protein microarray utilised for this research consisted of a carefully selected set of 1627 proteins, including kinases, signalling proteins, transcription factors and secreted proteins. The proteins were chosen using the serological analysis of recombinant cDNA expression libraries (SEREX). SEREX, as described in section 3.5.1, involves, in this case, the use of diluted serum from cancer patients being used to screen for antibodies that react against proteins expressed from cDNA libraries generated from cancer tissue. This strategy of antigen selection is based upon the assumption that anti-tumour antibodies are indicative of T- helper cells specific for such an antigen and therefore this method of protein selection is highly specific for appropriate cancer related proteins, further ensuring the accuracy of the microarray test (Stempfer et al., 2010).

The assay platform also allows the simultaneous testing of patient and control samples on one microchip. The comparison of patient and control samples, processed under the same conditions allows for exact comparison between the two groups.

To avoid technical error, every protein is applied to the microarray in quadruplicate. The replicates of the proteins on the array therefore allow repeated measurement of autoantibody binding.

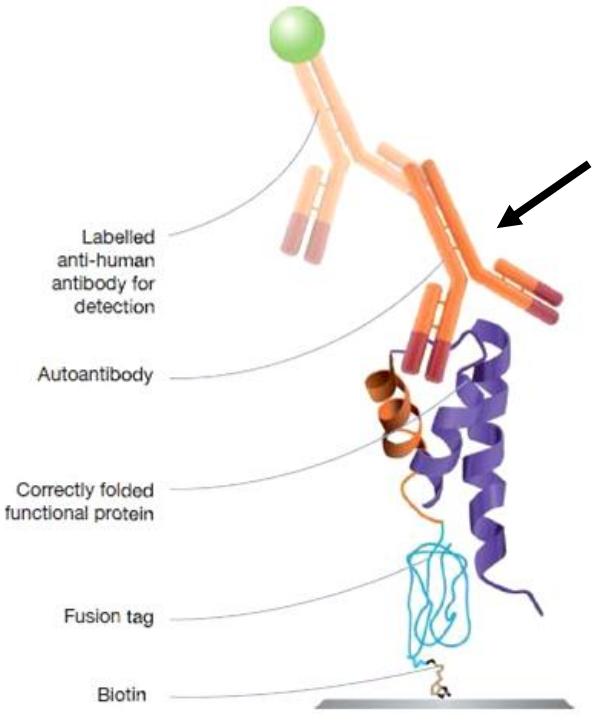


Figure 7: BCCP tag conserving the proteins natural three dimensional structure. (Image adapted from McAndrew et al., 2012)

A previous study has successfully utilised the Oxford gene technology functional protein microarray to identify autoantibodies (Wang et al., 2005). This study on prostate cancer, with a cohort of 73 participants, identified 15 potential autoantibody biomarkers for prostate cancer (McAndrew et al., 2012). A combination of these biomarkers showed both a sensitivity and specificity of greater than 90% for prostate cancer. Out of this panel, 8 of the 15 potential prostate cancer autoantibody biomarkers had previously been associated with the cancer, indicating the validity of this technology, and highlighted the ability of the array to identify novel autoantibody biomarkers (McAndrew et al., 2012). By comparison, the single biomarker PSA, currently utilised in prostate cancer screening has a sensitivity of 86% and a specificity of 33% (Harvey et al., 2009). The findings of this prostate cancer OGT study are currently being further substantiated in a larger cohort of 1800 participants (McAndrew et al., 2012). We therefore hypothesised, that the OGT array could be utilised to similarly identify autoantibodies specific for melanoma.

Other studies for the identification of highly sensitive and specific biomarkers or panels of biomarkers for colorectal cancer and systemic lupus erythematosus (SLE), utilising the OGT functional microarray platform, are awaiting publication (J. Poole-Johnson, OGT, personal communication, May 2013).

Traditional methods for the characterisation of autoantibodies such as enzyme-linked immunosorbent assay (ELISA) are commonly used in laboratories to detect autoantibodies. These however require large volumes of sample and substantial amounts of purified antigen. This methodology can also be very time consuming. The sample volume may increase even further when ELISA is used to profile for multiple antigens. Therefore, ELISAs are not recommended for highly specific screening of hundreds or in some cases thousands of antigens and the need for an improved assay methodology with equal or greater performance than ELISAs such as the OGT microarray, is required. ELISAs are however, extremely useful for confirming the presence of individual autoantibodies in serum, once identified by more sensitive screening methods. We therefore hypothesised, that an indirect ELISA will aid in the validation of the results obtained from the microarray in a larger cohort.

2. Theoretical Framework

Melanoma continues to be an aggressive and drug resistant skin cancer (Lewis et al., 2005). Late diagnosis of the primary tumour often results in invasive tumours in which melanoma cells start to infiltrate the lymphatic and circulatory system resulting in metastasis and a dramatic drop in the patient´s survival due to a lack of effective treatments. Once the cancerous cells are detected by the patient‟s immune system, autoantibodies against the cancer are thought to be produced due to differences in expression levels, mutations, protein release from damaged tissue and mis-folding or mis-presentation of proteins. Levels of such potential autoantibody biomarkers in the serum of early stage melanoma patients may be utilised to diagnose melanoma and form the basis for a diagnostic blood test, as the levels of autoantibodies may precede the occurrence of disease symptoms (Gnjatic et al., 2009).

High levels of autoantibodies have been associated with other cancers such as cancers of the breast (Disis, 1994), lung (Diesinger et al., 2002), colon (Scanlan et al., 1998), ovary (Chatterjee, 2006), head and neck (Carey, 1983) and prostate (Wang et al., 2005). Clinical diagnosis of prostate cancer has traditionally utilised a single biomarker, prostate specific antigen (PSA), (Zeliadt et al., 2011). However, this has a low sensitivity of 52% and a specificity of up to 79% (Xie et al., 2011). More recent studies, utilising the OGT functional protein microarray, have identified 15 potential autoantibody biomarkers for prostate cancer and the use of combinations of these markers displayed greater sensitivity and specificity than PSA alone (Harvey et al., 2009; McAndrew et al., 2012). Therefore in this study the aim was to use OGT to identify highly specific and sensitive serological diagnostic autoantibody biomarkers for the early diagnosis of cutaneous melanoma. Furthermore, this study aimed also to validate identified biomarkers in a larger cohort.

2.1 Hypothesis

Increased levels of autoantibodies in the serum of early stage (TNM 0 and I) melanoma patients are indicative of a positive disease status and therefore may be utilised to assist with diagnosis of melanoma patients at early disease stages.

2.2 Aims

Aim 1: *To identify melanoma specific serological autoantibodies that can be used as diagnostic biomarkers for early stage (TNM 0 and I) cutaneous melanoma.*

To do this, serum from 40 participants, including 20 TNM stage 0 and I melanoma patients and 20 age and gender matched healthy volunteers, was tested against 1627 antigens on an OGT function protein microarray.

Aim 2: *To confirm the expression of the potential autoantibody biomarkers by ELISA in a larger population of melanoma patients.*

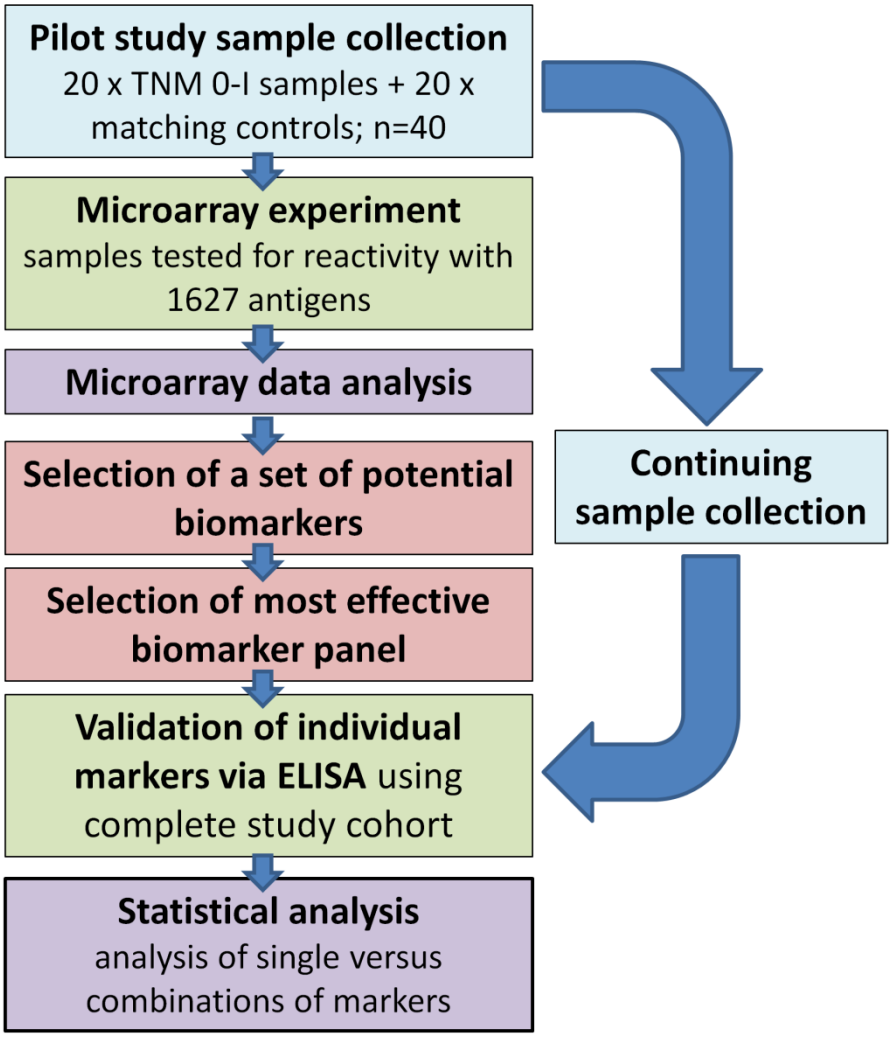
This was achieved by testing the initial pilot study cohort as well as new samples against 3 identified biomarkers, namely SOX10, BRDG1 (STAP1) and SCYL3, using an indirect enzyme-linked immunosorbent assay (n = 104, 113 and 114 respectively).

Aim 3: *To statistically analyse the efficacy of single compared to combinations of melanoma autoantibodies to act as diagnostic biomarkers.*

This was achieved by utilising the statistical package SPSS Statistics 19 (IBM) to perform parametric t-tests and one-way ANOVAs as well as nonparametric Mann-Whitney U and Kruskal-Wallis tests, dependent on the nature of data distribution and normality, to determine statistically significant differences between patient and control samples when tested against any of the three autoantibody markers mentioned in Aim 2. Pearson chi-square test and Fishers exact test were used to examine statistical significance of marker expression incidence between patients and controls. Calculations of sensitivity, specificity and positive and negative predictive value of individual markers versus marker combinations from the microarray and enzyme-linked immunosorbent assays were performed for investigation of diagnostic efficacy.

3. Methods and Materials

3.1 Experimental design flow chart



3.2 Participant recruitment

3.2.1 Pilot study cohort recruitment

Participants of the pilot study consisted of patients (n=20) recently diagnosed with melanoma and age and gender matched healthy volunteers (n=20). Controls were confirmed as melanoma, cancer and autoimmune disease free.

Healthy volunteers were recruited by the principal researcher through the display and electronic circulation of a healthy volunteer recruitment flyer (appendix 8.2) which was approved by the Human Research Ethics Committee of Edith Cowan University (No.8482).

Only early stage melanoma patients (TNM stage 0 and I) were recruited. Patients were diagnosed as melanoma positive by pathological examination of excised lesions. All patients were staged according to guidelines of the American Joint Committee on Cancer (AJCC) (section 1.4). All patients were recruited by collaborating clinicians. All patient blood samples were taken at the time of tumour excision. Patients at other melanoma stages were not recruited to ensure that the small pilot study cohort represented, as closely as possible, early stage melanoma.

All participants received an information letter, which was also approved by the Human Research Ethics Committee of Edith Cowan University (No. 8482), outlining the parameters of the study and all were required to sign a consent form to participate in the study (appendix 8.3). With consent from the participant, clinicians supplied patient clinical data to the principal researcher. Patient samples were de-identified upon arrival at the research facility and randomly assigned a study number. All personal details were kept confidential in a locked facility and were not utilised for any other studies.

3.2.2 Complete study cohort recruitment

For confirmation of markers, a larger cohort was utilised. Participants of this complete/ final research study cohort consisted of up to 60 patients of various disease stages recently diagnosed with melanoma and up to 54 healthy volunteers, confirmed as melanoma, cancer and autoimmune disease free. Healthy volunteers were recruited by the principal researcher through the display and electronic circulation of a healthy volunteer recruitment flyer (appendix 8.2) which was approved by the Human Research Ethics Committee of Edith Cowan University (No.8482) and an appointment was arranged for the single blood sample

collection to be performed by a trained phlebotomist in a phlebotomy approved room at the research facility at Edith Cowan University.

For this cohort, patients of all melanoma cancer stages were recruited. Patients were diagnosed as melanoma positive by pathological examination of excised lesions and staged according to guidelines of the American Joint Committee on Cancer (AJCC) (section 1.4). As described in section 3.2.1, with consent from the participant, clinicians supplied patient clinical data to the principal researcher. Patient samples were de-identified upon arrival at the research facility and randomly assigned a study number. All personal details were kept confidential in a locked facility and were not and may not be utilised for any other purposes or any other studies.

3.3 Sample collection

Once the participants provided consent to participate in the study, a single blood sample was collected. For patients, the blood sample was collected at the time of tumour excision or within 1 month of the initial diagnosis of melanoma.

A small volume of blood (8.5ml) was collected from patients and controls by a trained phlebotomist at pathology centres or at Edith Cowan University by venous puncture into a BD vacutainer serum separation tube (SST).

3.4 Serum extraction and storage

All blood samples were processed within 24 hours. Serum was extracted by centrifugation at 1600g for 10 minutes. Using a sterile micropipette, up to 10 aliquots of 100µl of serum were pipetted into each of 10 UV-treated, sterile, 1.7ml microcentrifuge tubes. The microcentrifuge tubes were labelled with the participants study number, the date of serum extraction and the number of the aliquot and were then stored at -80°C until shipped for the pilot study microarray experiment or until utilisation for the ELISA experiments.

3.5 Microarray technology – Pilot Study

Once sample collection of the pilot study cohort and serum extraction was complete for all patients (n=20) and controls (n=20), two 100µl aliquots of each serum sample were sent to Oxford University, Oxford, UK where the patented OGT functional protein microarray was utilised to identify serological biomarkers highly expressed in patient samples but absent or present in very low levels in healthy volunteer samples.

3.5.1 Functional protein microarray construction

The protein microarray was constructed by Oxford Gene Technology (OGT) as follows: Briefly, the SEREX mammalian gene collection database was used to identify tumour associated factors. This was achieved by isolating mRNA from various cancer cells, including melanoma, to construct a cDNA library which was then packaged into a bacteriophage. The phages containing the cancer cDNA were then applied to a dish containing an *Escherichia coli* (*E. coli*) lawn and the bacterial culture was infected with the tumour cDNA under conditions that permitted expression of the tumour-derived proteins. Replica "lifts" of the bacterial lawn were made using nitrocellulose membranes and these were then probed with diluted antisera from various cancer patients. Bacterial colonies that were expressing tumour-derived proteins were detected by antibodies within the patient serum and could then be identified by isolating the phage from the relevant colony and sequencing the tumour cDNA contained within the bacteriophage (Stempfer et al., 2010). This method was used to obtain the 1627 proteins utilised on the microarray. A full list of these genes/proteins is available upon request.

The full-length open reading frames of the identified 1627 target genes were cloned in-frame together with a sequence which encoded the c-terminal *E. coli* biotin carboxyl carrier protein (BCCP) fused to the myc epitope in a baculovirus transfer vector. The reading frame and sequence were then sequence verified. The recombinant baculoviruses were generated, amplified and expressed in S*f*9 cells which were originally grown in suspension using standard methods adapted for 24-well deep well plates (Chambers et al., 2004; Zhao et al., 2003). Recombinant protein expression was analysed for protein integrity and biotinylation by Western Blot and appropriate cells harbouring the recombinant proteins were lysed. The lysates were spotted onto a streptavidin-coated glass slide (Schott Nexterion, Jena, Germany) in quadruplicate using a 300µm solid pin equipped QArray2 Microarrayer processor (Genetix, New Milton, UK). This process was followed by a 2 hour incubation period to allow the biotinylated proteins to bind to the straptavidin-coated glass surface. After incubation, non-bound proteins were washed away and the slide was blocked with bovine serum albimin (BSA). Following this step, the glass slide was referred to as a cancer antigen array which was stored at -20°C where it is stable for a period of up to 12 months (Gnjatic et al., 2009).

Control proteins were also included on the microarray to ensure microarray quality control. These included the BCCP tag, namely BCCP, BCCP-myc, β-galactosidase-BCCP-myc and β-galactosidase-BCCP, as well as Cy3/ Cy5-labeled biotinylated BSA marker, biotinylated IgG and IgM dilution series, unlabeled biotinylated BSA and buffer only control spots (Gnjatic et al., 2009).

3.5.2 Array profiling assay

Following the construction of the functional protein array, the serum samples collected for this study were applied to the array followed by the addition of fluorescent labelled anti-human antibody IgG for detection. Wherever an antibody or autoantibody within the serum sample interacted with its specific protein antigen, a plate reader detected fluorescence of the bound fluorescently-labelled IgG within the appropriate microarray well. The location of each protein on the microarray is known and therefore each protein bound by a specific autoantibody could be identified. The intensity of fluorescence was proportional to the amount of autoantibody-protein interaction (Gnjatic et al., 2009; McAndrew et al., 2012).

To achieve this result, the array was incubated in Quadriperm dishes (Greiner BioOne, Stonehouse, UK) and placed on a horizontal shaker at 50 rpm for a period of 2 hours at room temperature and individual sera samples were applied after being diluted 1:200 in 2ml buffer (0.1% Triton X100 (v/v), 0.1% BSA (w/v) in PBS). After several washes, the anti-human IgG was diluted 1:1000 in assay buffer and labelled with a fluorescent tag (Dako Cytomation) by incubation for 2 hours at room temperature according to the manufacturer´s recommended protocols. Following this, the plate was washed again and dried by centrifugation. The arrays were scanned at 10µm resolution using a microarray scanner (Axon 4200AL with GenePix Pro Software, Molecular Devices, Sunnyvale, CA 94089) and fluorescence of labelled IgG was detected according to the manufacturer‟s instructions. Images taken were saved as 16-bit tiff files and analysis was performed using GenePix software (Gnjatic et al., 2009). Local background was subtracted automatically and the median fluorescence units (rfu) for each microarray spot were recorded.

3.6 Statistical analysis of microarray results

For the statistical analysis of the microarray raw data, the median rfu values of the quadruplicate spots for each of the 1627 proteins were averaged within each array, followed by the proportional adjustment and normalisation of the average rfu values for all antigens

according to the mean of IgG values obtained from all IgG controls and standard fluorescence markers on the array. The adjusted values for each antigen were averaged across replicate arrays and expressed as a percentage of interquartile differences per array (Gnjatic et al., 2009). This was calculated through the application of the formula:

$$\text{(Observed value)} - \text{(25th percentile)} \;/\; \text{(75th percentile)} - \text{(25}^{\text{th}}\text{ percentile)}$$

All percentage values were then normalised using a standard quantile normalisation matrix (Bolstad et al., 2003) in which all percentage values of each array are ranked and replaced by the average of percentages for antigens with the same rank on all arrays. This resulted in data distribution with identical median and quartile values, allowing interarray comparisons (Gnjatic et al., 2009). Following the normalisation of the array data, the interquartile difference was calculated for each antigen across all arrays, to establish a cut-off value to enable the identification of autoantibodies showing significant seroreactivity. This was achieved by calculating the average of all expression and fluorescence readings across the microarray slide and defining every sample with a fold change of 2 or more above the calculated average as a sample positive for the marker tested. In other words, for an autoantibody to be defined as positive and therefore a potential serological biomarker for early stage melanoma patients, the level of fluorescence of the particular autoantibody-protein interaction needed to have values greater than 2-2.5 times the interquartile difference above the 75th percentile (Gnjatic et al., 2009). All autoantibodies detected that applied to these conditions were considered as a panel for further investigations.

Only application of serum to the OGT functional protein array and preliminary statistical analysis of the microarray generated data were performed by OGT staff. The collection of pathological and healthy sera and the remainder of the project and statistical analysis were performed by myself, the MSc candidate.

In order to minimise the likelihood of false positive and negative results and to prove the reliability of the serological biomarker, the marker must exhibit sufficient specificity and sensitivity to melanoma tumours and should be absent or present in significantly lower quantities in healthy volunteers. The specificity of such a biomarker is determined by the proportion of healthy individuals that test negative for the marker, and the sensitivity of the biomarker refers to the proportion of patients with confirmed disease testing positive for the

marker (Vereecken et al., 2011). To test this, an ROC curve analysis was performed and the area under the curve (AUC) was regarded as representation of sensitivity and specificity scores. Two sample proportional statistical analysis and ROC curve analysis of sensitivity and specificity of individual markers was performed utilising the statistical software STATA® in order to establish if there was a significant relationship between the occurrence of the selected specific autoantibodies and melanoma disease occurrence and therefore diagnosis. Logistic regression analysis was not able to be performed due to "missing observations" in tests with 100% success, such as where all test subjects or participants were detected by a combination of markers or none of the participants were detected by a biomarker panel. Furthermore, all data was checked for normal distribution by performing a normalisation test in SPSS. For data with normal distribution, a t-test or one way ANOVA was performed. Alternatively, a Mann-Whitney U test or Kruskal-Wallis test was performed for data sets that did not exhibit normal distribution of data. Pearson chi-square test and Fishers exact test were used to examine statistical significance of positive marker incidence in the patient and control cohort.

3.7 Confirmation of microarray results using enzyme-linked immunosorbent assay (ELISA)

To confirm these markers as effective diagnostic melanoma markers, 3 markers, namely SOX10, BRDG1 (STAP1) and SCYL3, were chosen for further investigation. SOX10 and BRDG1 (STAP1) were chosen as these markers displayed the highest frequency in patients (30%) of those markers absent in the microarray control samples (Table 11). SCYL3 was chosen for further study as it displayed the highest percentage difference of frequency in patients versus controls in the microarray samples (45%) (Table 11).

As there were no commercially available ELISA kits for the three markers, an „in-house" indirect ELISA was constructed (Engvall and Perlman, 1971) as described in the following sections. For the indirect ELISA, the appropriate antigen was bound to the well. Thereafter, patient serum sample was applied to the well, resulting in the formation of an antigen-antibody binding complex detected by a secondary HRP-labelled anti-human IgG antibody (Figure 8).

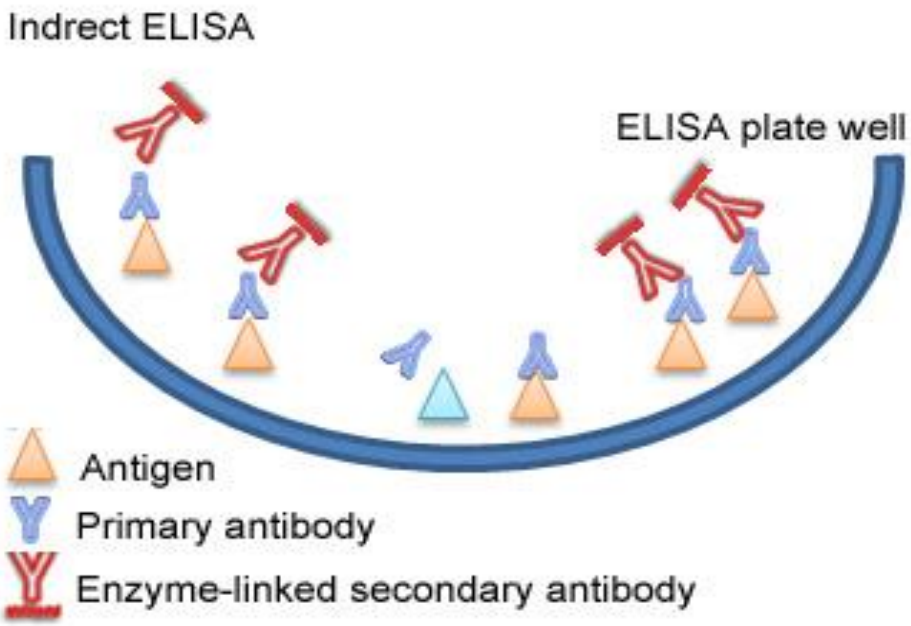


Figure 8: Indirect ELISA principle (Image from www.elisa-antibody.com**)**

3.7.1 SOX10 ELISA optimisation

To confirm that the indirect ELISA was an appropriate method to validate the microarray pilot study results and to optimise sample and antigen concentrations, we chose SOX10 for the majority of the optimisation tests as it was readily available commercially. For the first SOX10 optimisation step, a set of four patient samples B2, B9, B11 and B16 and three control samples CC1, CC2 and CC4 were chosen.

The indirect ELISA for this optimisation step was performed as follows: The required number of U16 Maxisorp Nunc immune modules plate segments (Nunc, Thermo Scientific) was assembled into the plate frame. SOX10 full-length human recombinant protein (Abvova) was purchased at a concentration of 0.04µg/µl and diluted in 50mM carbonate buffer pH 9.6 to a final concentration of 0.5µg/ml, 1µg/ml and 2µg/ml respectively to assess the optimal concentration for the final ELISA. 50µl per well of each of the solutions containing the three protein coating concentrations were loaded into their appropriate well in the appropriate columns (Figure 9). The plate was then covered with an adhesive film for microplates (Sealplate) and the plate was left to incubate overnight on an orbital shaker at a speed of 150 rpm at 4°C.

	Sample	2ug/ul SOX10				1ug/ul SOX10				0.5ug/ul SOX10			
Patient serum samples	B2	neat sample	1 in 5 dilution	1 in 100 dilution	1 in 10000 dilution	neat sample	1 in 5 dilution	1 in 100 dilution	1 in 10000 dilution	neat sample	1 in 5 dilution	1 in 100 dilution	1 in 10000 dilution
	B9												
	B11												
	B16												
Control serum samples	CC1												
	CC2												
	CC4												
	Blank												

Figure 9: SOX10 optimisation ELISA 1 template

After 24 hours, the plate was washed 3 times with 300µl 1xPBS at pH 7.4 per well using a plate washer (BioRad plate washer, model 1575) and 100µl of 3% PBS/ Bovine Serum Albumin (BSA) („Bovostar" by BOVOGEN Biologicals) blocking solution was loaded into each well. The plate was then covered again and left to incubate on an orbital shaker at 150 rpm at room temperature for 1 hour.

Following this incubation, plates were washed 3 times with 300µl 1xPBS per well and 50µl of patient or control serum was loaded into the appropriate well (Figure 9). To assess the optimal dilution of the serum sample applied to the assay, the samples were loaded onto the plate in 4 different dilutions. The first column of each third of the 96-well ELISA plate was coated with 50µl of neat or undiluted sera. The second column within each part of the ELISA plate coated with a different protein coating concentration, had 50µl of 1 in 5 diluted sera added to each well. The third column in each section of the ELISA plate was loaded with 50µl of 1 in 100 diluted sera per well and the fourth and final column contained the same volume of sera per well but at a 1 in 10000 dilution. Blanks contained 50µl of 0.5% PBS/BSA pH 7.4 buffer only (Figure 9). The plates were then resealed and left to incubate for 2 hours on an orbital shaker at 4°C.

Following the sample incubation, plates were washed 3 times with 300µl 1x PBST (0.04% tween at pH 7.4) per well and the rabbit anti-human secondary IgG antibody linked to Horseradish Peroxidase (Thermo Scientific) was diluted 1 in 10000 by adding 5ul of secondary antibody stock to 50ml PBST at pH 7.4. 50µl of this dilution was then added to each well on the plate using a multichannel pipette. The plates were then resealed and incubated for 1 hour on an orbital shaker at room temperature.

The plate was re-washed with 300µl 1x PBST (0.04% tween at pH 7.4) per well. Thereafter, 50µl of a mixed solution of 6mL TMB Peroxidase Substrate and 6mL Peroxidase Substrate Solution B (KPL Ltd.), at room temperature, was loaded into each well. The plate was then covered with a light protective box and the assay was left to develop at room temperature for approximately 30 minutes or until a dark blue colour was observed in each well indicating the reaction of the TMP solution with Horseradish Peroxidise linked to the secondary antibody.

The reaction was stopped by adding 50µl of 1M H_3PO_4 phosphoric acid to each well and plates were then read at A450 for chemiluminescence using the FLUOstar OPTIMA (BMG Labtech) and results were analysed (section 3.8).

The next optimisation experiment was aimed at establishing the optimal sample dilution factor. To test for this, six pilot study patients samples, B16, B2, B9, B11, B5 and B8, shown to be positive for SOX10 in the microarray experiment (Appendix 3.5), were utilised. These samples had a broad range of rfu values (high to low) in the microarray experiment. Five control samples, 3 from the microarray experiment, shown to have no or low reactivity with SOX10) and 2 new control samples were also included in this experiment.

This optimisation step followed the same indirect ELISA protocol described for optimisation step 1, except that the entire 96-well plate was coated at the same antigen/protein coating concentration of 1µg/ml, and serum was diluted 1 in 5, 1 in 10, 1 in 40 and 1 in 200 in 0.5% PBS/BSA buffer and added onto the 96-well plate in duplicate (Table 4).

Other optimisation experiments, such as changes in development time and serum sample

Table 4: SOX10 optimisation ELISA 2 template

	Patient sample number (SOX10 microarray fold-change)						**Control sample number**					
Sample dilution	**B16 (3.9)**	**B2 (3.5)**	**B9 (3.4)**	**B11 (2.4)**	**B5 (2.2)**	**B8 (2.0)**	**CC1**	**CC2**	**CC4**	**CC33**	**CC35**	**CC37**
1 in 5												
1 in 5												
1 in 10												
1 in 10												
1 in 20												
1 in 20												
1 in 40												
1 in 40												

concentration, were performed and their relevant results are discussed further in the following sections.

3.7.2 BRDG1 (STAP1) and SCYL3 optimisation

The ELISA optimisation experiments performed for the remaining two markers BRDG1 (STAP1) and SCYL3 mirror the optimisation experiments described for SOX10 (section 3.7.1) STAP1 full-length human recombinant protein (Abvova) provided at a concentration of 0.27µg/µl and SCYL3 full-length human recombinant protein (Abnova) provided at a concentration of 0.1µg/µl were diluted in 50mM carbonate buffer pH 9.6 to a final concentration of 0.5µg/ml, 1µg/ml and 2µg/ml respectively for the first optimisation step and to 1µg/ml for any following optimisation step.

The results of these optimisation experiments for the BRDG1 and SCYL3 are described in section 3.8.3 and 3.8.4.

3.8 ELISA optimisation results

As detailed above, optimisation experiments were performed to establish optimal antigen coating concentration, patient serum sample dilution, dilution of secondary antibody and development time for each of the three individual proteins tested.

3.8.1 SOX10 optimisation ELISA 1 results

For the first SOX10 optimisation step, a set of four patient samples, B2, B9, B11 and B16, which displayed the highest fold change above the average rfu values for the marker SOX10 (Appendix 8.5) and three control samples were chosen. According to the microarray data, none of the pilot study control samples tested positive for SOX10 (Appendix 8.5). Therefore, the control samples CC1, CC2 and CC4, from the pilot study cohort tested on the microarray, were randomly selected for utilisation in this optimisation step.

The following table over the page (Table 5) shows the results of SOX10 optimisation ELISA trial 1 alongside the p-values for each sera dilution tested against the three coating protein concentrations:

Table 5: SOX10 ELISA optimisation trial 1 results

	Dilutions											
sample number	**neat**	**1in5**	**1in100**	**1in10000**	**neat**	**1in5**	**1in100**	**1in10000**	**neat**	**1in5**	**1in100**	**1in10000**
B2	0.632	0.561	0.245	0.038	0.761	0.284	0.09	0.03	0.66	0.228	0.09	0.041
B9	1.265	0.938	0.249	0.035	1.513	1.243	0.245	0.027	1.518	1.181	0.284	0.04
B11	1.593	0.446	0.182	0.033	1.802	0.347	0.15	0.049	1.759	0.371	0.15	0.033
B16	1.878	1.402	0.215	0.037	1.956	1.269	0.17	0.027	2.071	1.135	0.136	0.046
CC1	1.584	0.448	0.138	0.025	1.622	0.352	0.114	0.032	1.46	0.38	0.104	0.028
CC2	0.853	0.312	0.096	0.039	0.789	0.268	0.087	0.027	0.779	0.25	0.078	0.036
CC4	1.876	0.365	0.148	0.037	1.972	0.274	0.107	0.037	1.895	0.242	0.114	0.037
Blank	0.042	0.031	0.04	0.039	0.044	0.033	0.037	0.03	0.036	0.044	0.048	0.035
	2ug/ml protein concentration				**1ug/ml protein concentration**				**0.5ug/ml protein concentration**			
	p= 0.986	*p*= 0.059	*p*= 0.737	*p*= 0.035	*p*= 0.751	*p*= 0.000	*p*= 0.213	*p*= 0.252	*p*= 0.935	*p*= 0.000	*p*= 0.166	*p*= 0.906
	p-value (patients versus controls)											

As seen in Table 5, a statistically significant difference between patient and control samples can be observed at a coating protein concentration of 2µg/ml at a sample dilution of 1 in 10000. The next best conditions were the 1 in 5 serum sample dilution and 1 in 100 dilution at protein concentrations 1µg/ml and 0.5 µg/ml. Nevertheless sample size may have great effect on the p-value and increased sample sizes may prove other conditions to be optimal.

Neat samples did not display any statistical significance between patient and control samples and were therefore not selected for further investigation. The 1 in 10000 dilution showed significance between the two groups of samples at antigen plate coating concentration 2µg/ml ($p=0.035$), however, the raw data values were too low and almost matched the value obtained from the well containing the blank. Therefore, this dilution was also excluded from further investigation. As illustrated in Table 5, the 1 in 5 dilution of sera showed significant differences between patient and control samples under both the 1µg/ml and 0.5µg/ml antigen coating conditions ($p=0.000$). It was therefore concluded that dilution factors equal to or above 1 in 5 are optimal for utilisation in a SOX10 ELISA. Antigen coating concentration 1µg/ml was then chosen to be utilised in further SOX10 ELISA based experiments as it is the concentration most consistent with similar studies (Desmetz et al., 2008; Gnjatic et al., 2009).

3.8.2 SOX10 optimisation ELISA 2 results

Table 6 demonstrates the results obtained from the second SOX10 optimisation trial which was aimed at establishing the optimal sample dilution to be utilised in the final SOX10 ELISA. In this experiment, pilot study patient samples B16, B2, B9, B11, B5 and B8 were tested for reactivity with the SOX10 antigen. The order, in which the samples are listed here, mirrors the order in which the samples were added to the 96-well ELISA plate (table 4 and 6) and also represents, in a descending order, their fold-change above the average rfu values on the microarray. Control samples CC1, CC2, CC4 and CC33 were used in this optimisation ELISA as these samples showed no or very low reactivity with the SOX10 antigen on the microarray (Appendix 8.5). Additional control samples CC35 and CC37 were also included, not previously tested on the microarray.

As indicated in Table 6, a statistically significant difference in reactivity between patient and control samples was found at increasing higher dilutions. Lower dilution factors 1 in 5 and 1 in 10, did not display any significant difference between patients and controls. Therefore, a dilution 1 in 200 was found to be optimal for the SOX10 ELISA as it showed the highest

statistical significance between patient and control samples fluorescence readings. Samples were also diluted 1 in 200 in the microarray experiment.

Additional optimisation experiments included the determination of the optimal SOX10 assay development time. To ascertain this, the secondary antibody solution was applied to only one of the duplicates and pH 7.4 PBST (0.4% tween) buffer only was applied to the other well. The reaction was stopped and the time of assay development noted when the wells containing the buffer only started to stain light blue, indicating background signal of the assay. For SOX10, the optimal assay development time was established as 25 minutes, as this time provided the lowest background signal versus the highest detection signal and a coefficient of variation (CV) below 10% was regarded as an indication low interplate variability.

Table 6: SOX10 optimisation ELISA 2 results

	Patient sample number (SOX10 microarray fold-change)						Control sample number						p-value (patients versus controls)
Sample dilution	**B16 (3.9)**	**B2 (3.5)**	**B9 (3.4)**	**B11 (2.4)**	**B5 (2.2)**	**B8 (2.0)**	**CC1**	**CC2**	**CC4**	**CC33**	**CC35**	**CC37**	
1in5	1.053	0.424	0.901	0.474	0.270	0.797	0.413	0.246	0.309	1.260	0.324	0.338	0.200
1in5	1.050	0.421	0.926	0.453	0.237	0.710	0.380	0.240	0.326	1.375	0.313	0.337	
1in10	0.681	0.393	0.852	0.392	0.220	0.701	0.296	0.197	0.270	0.466	0.280	0.304	0.078
1in10	0.529	0.359	0.506	0.355	0.224	0.671	0.295	0.200	0.280	0.725	0.297	0.304	
1in40	0.353	0.243	0.514	0.293	0.158	0.466	0.213	0.145	0.190	0.201	0.200	0.192	0.035
1in40	0.328	0.252	0.360	0.318	0.187	0.532	0.218	0.174	0.217	0.218	0.239	0.107	
1in200	0.404	0.215	0.287	0.695	0.116	0.097	0.174	0.219	0.186	0.115	0.158	0.174	0.028
1in200	0.368	0.225	0.297	0.655	0.121	0.102	0.176	0.117	0.196	0.112	0.165	0.163	

3.8.3 BRDG1 (STAP1) optimisation ELISA results

Table 7 illustrates the results obtained from the BRDG1 (STAP1) optimisation experiment which was aimed at establishing the optimal antigen coating concentration, sample dilution and assay development time to be utilised in the final BRDG1 ELISA. In this experiment, pilot study patient samples B17, B12, B7 and B22 were tested for reactivity with the BRDG1 (STAP1) antigen. These samples displayed the highest rfu fold change across the microarray experiment for this marker (Appendix 8.5). Furthermore sample B17, which displayed the highest fold-change on the microarray was added in duplicate to establish the optimal development time for the BRDG1 (STAP1) assay. Since BRDG1 did not show any reactivity with the control study cohort in the microarray experiment, the control samples CC20, CC23 and CC32 were randomly selected for inclusion in the experiment.

As displayed in Table 7, the optimal antigen coating concentration showing a statistically significant difference between patients and control samples was 1µg/ml. Therefore, this concentration was chosen to be utilised for further BRDG1 optimisation as well as for the final ELISA. The optimal BRDG1 assay development time was timed to be 45 mins and the optimal sera dilution was found to be 1 in 200.

Table 7: BRDG1 (STAP1) optimisation ELISA 1 results

	Dilutions											
sample number	**1in5**	**1in50**	**1in100**	**1in200**	**1in5**	**1in50**	**1in100**	**1in200**	**1in5**	**1in50**	**1in100**	**1in200**
B17 (without sec. Ab.)	0.033	0.031	0.038	0.030	0.030	0.034	0.032	0.035	0.037	0.031	0.037	0.030
B17 (with sec. Ab.)	0.565	0.206	0.120	0.102	0.502	0.118	0.118	0.098	0.451	0.162	0.116	0.065
B12	0.472	0.233	0.113	0.075	0.508	0.173	0.137	0.068	0.536	0.196	0.127	0.048
B7	1.371	0.145	0.073	0.062	0.317	0.120	0.123	0.057	0.311	0.122	0.296	0.053
B22	0.22	0.095	0.064	0.121	0.313	0.093	0.053	0.114	0.253	0.094	0.068	0.098
CC20	0.284	0.100	0.070	0.057	0.281	0.094	0.073	0.043	0.349	0.098	0.09	0.037
CC23	0.328	0.140	0.094	0.068	0.426	0.152	0.104	0.042	0.518	0.119	0.096	0.032
CC32	0.342	0.120	0.076	0.063	0.392	0.133	0.106	0.039	0.402	0.120	0.086	0.035
	2ug/ul				**1ug/ul**				**0.5ug/ul**			
	0.099	0.057	0.010	0.027	0.047	0.890	0.329	0.008	0.229	0.062	0.087	0.121
	p-value (patients versus controls)											

3.8.4 SCYL3 optimisation ELISA results:

Table 8 illustrates the results obtained from the SCYL3 optimisation trial which was aimed at establishing the optimal antigen coating concentration, sample dilution and assay development time to be utilised in the final SCYL3 ELISA. In this experiment, pilot study patient samples B6, B5, B19 and B10 were tested for reactivity with the SCYL3 antigen. These samples displayed the highest rfu fold change across the microarray experiment for the marker. Furthermore sample B6, which displayed the highest fold-change on the microarray (Appendix 8.5) was added in duplicate to establish the optimal development time of the SCYL3 assay. The control samples utilised in this experiment were CC6, CC14 and CC21. These samples were tested against this marker on the microarray but did not show positivity for the marker and should therefore show no or very low reactivity with the SCYL3 antigen coated onto the well.

As displayed in Table 8, the optimal antigen coating concentrations showing a statistically significant difference between patients and control samples were 1µg/ml and 0.5µg/ml. The 1µg/ml concentration was chosen to be utilised for further SCYL3 optimisation as well as for the final ELISA. The optimal SCYL3 assay development time was 35 mins and further optimisation ELISAs showed that a 1 in 200 dilution was also the optimal sample dilution.

Table 8: SCYL3 optimisation ELISA trial 1 results

	Dilutions											
sample number	**1in50**	**1in100**	**1in200**	**1in400**	**1in50**	**1in100**	**1in200**	**1in400**	**1in50**	**1in100**	**1in200**	**1in400**
B6 (without secondary antibody)	0.026	0.024	0.044	0.027	0.027	0.036	0.036	0.044	0.027	0.029	0.034	0.034
B6 (with secondary antibody)	0.367	0.237	0.145	0.087	0.228	0.163	0.099	0.079	0.175	0.14	0.092	0.067
B5	0.166	0.114	0.089	0.062	0.132	0.092	0.074	0.064	0.125	0.094	0.069	0.057
B19	0.185	0.116	0.087	0.074	0.151	0.117	0.080	0.083	0.141	0.093	0.080	0.067
B10	0.278	0.163	0.108	0.063	0.167	0.121	0.083	0.058	0.155	0.113	0.083	0.061
CC6	0.299	0.194	0.112	0.080	0.184	0.170	0.094	0.081	0.231	0.153	0.094	0.074
CC14	0.164	0.117	0.093	0.079	0.133	0.114	0.079	0.072	0.126	0.119	0.132	0.069
CC21	0.422	0.265	0.175	0.128	0.300	0.225	0.152	0.111	0.313	0.204	0.17	0.117
	2ug/ml				**1ug/ml**				**0.5ug/ml**			
	0.751	0.791	0.333	0.071	0.204	0.403	0.043	0.265	0.106	0.313	0.152	0.016
	p-value (patients versus controls)											

3.8.5 Final ELISA protocol

After optimisation of ELISAs for all three markers, SOX10, BRDG1 (STAP1) and SCYL3, a final ELISA protocol was devised and is described below:

The required number of U16 Maxisorp Nunc immune modules plate segments (Nunc, Thermo Scientific) was assembled into the plate frames. The full-length human recombinant protein stock solution of each marker was diluted in 50mM pH 9.6 carbonate buffer to a final concentration of 1µg/ml. 50µl per well of each of the protein coating buffer concentration solutions were loaded into the 96-wells per plate. The plates were then covered with an adhesive film for microplates (Sealplate) and the plates were left to incubate overnight on an orbital shaker at a speed of 150 rpm at 4°C.

After 24 hours, the plates were washed 3 times with 300µl 1xPBS at pH 7.4 per well using a plate washer (BioRad plate washer, model 1575) and 100µl of 3% PBS/ Bovine Serum Albumin (BSA) („Bovostar" by BOVOGEN Biologicals) blocking solution was loaded into each well. The plates were then covered again and left to incubate on an orbital shaker at 150 rpm at room temperature for 1 hour.

Following this incubation, plates were washed 3 times with 300µl 1xPBS per well and 50µl of 1 in 200 diluted patient and control serum, diluted with 0.5% pH 7.4 PBS/BSA buffer, was loaded into the appropriate well. A blank containing 50µl of 0.5% PBS/BSA pH 7.4 buffer only was also added to each plate along with 5 standard points.

The standard solution for each marker was made by combining 100µl of each pilot study patient sample that displayed positivity for SOX10, BRDG1 (STAP1) and SCYL3 (section 8.6) on the microarray. A serial 1 in 2 dilution was then performed with addition of the diluent 0.5% pH 7.4 PBS/BSA buffer and the top 5 dilutions were added to each plate as a standard curve. Results of the standard curve from each ELISA plate were used to calculate interplate variability, as shown in the results section.

The plates were then resealed and left to incubate for 2 hours on an orbital shaker at 4°C.

Following the sample incubation, plates were washed with 300µl 1x PBST (0.04% tween at pH 7.4) per well and the rabbit anti-human secondary IgG antibody linked to Horseradish Peroxidase (Thermo Scientific) was diluted 1 in 10000 by adding 5ul of secondary antibody stock to 50ml PBST at pH 7.4. 50µl of this dilution was then added to each well on the plate

using a multichannel pipette. The plates were then resealed and incubated for 1 hour on an orbital shaker at room temperature.

The plates were re-washed with 300µl 1x PBST (0.04% tween at pH 7.4) per well. Thereafter, 50µl of a mixed solution of 6mL TMB Peroxidase Substrate and 6mL Peroxidase Substrate Solution B (KPL Ltd.), at room temperature, was loaded into each well. The plates were then covered with a light protective box and the assay was left to develop at room temperature until a dark blue colour was observed; 25 minutes for SOX10, 45 minutes for BRDG1 (STAP1) and for 35 minutes SCYL3.

The reaction was stopped by adding 50µl of 1M H_3PO_4 phosphoric acid to each well and plates were then read for chemiluminescence at A450 using the FLUOstar OPTIMA (BMG Labtech).

3.8.6 Statistical analysis of final ELISA results

Upon collection of enzyme-linked immunosorbent assay results, data was tested for normality through utilisation of the „explore descriptive statistics" feature of the statistical package SPSS Statistics 19 (IBM). Data was numerically tested for normalisation through application of the Shapiro-Wilk test and significance greater than 0.05 was considered an indication of normally distributed data. To test for statistically significant differences in rfu values between patient and control samples, nonparametric Mann-Whitney U tests were performed using SPSS where data was abnormally distributed. For data that was normally distributed, a t-test was utilised. A nonparametric analysis was conducted if at least one groups displayed abnormal distribution of data. When only one value was available to be utilised for such comparisons, as in situations where only one sample represented a group ie.: only one control sample showed positivity for a marker, the one-way ANOVA nonparametric alternative Kruskal-Wallis test was performed instead of the Mann-Whitney U test or a one-way ANOVA was performed for normally distributed data, as both the Mann-Whitney U and t-test require a minimum of two values or two samples per group. Pearson chi-square test and Fishers exact test were used to examine statistical significance of positive marker incidence in the patient and control cohort. Box-plot graphs were constructed for the majority of comparative statistical analyses. The statistical package SPSS Statistics 19 (IBM) was further utilised to search for significant differences in incidence between age groups, gender and disease stages in patients positive for the markers. Due to the abnormal distribution of the majority of the data obtained in this research study, a nonparametric Kruskal-Wallis test was

performed to search for such differences between groups and any result displaying $p<0.05$ was defined as statistically significant. The sensitivity of a marker or combination of markers was determined by the percentage of patients diagnosed as true positives for the marker or a combination of markers. The specificity of a marker or combination of markers was denoted as the percentage of healthy volunteers or controls being diagnosed as true negatives or melanoma free and the positive and negative predictive values of a marker or combination of markers was denoted as the proportions of positive and negative results of a marker or a combination of markers. The term positive predictive value (PPV) is defined as the number of true positives divided by the number of true positives plus the number of false positives. Or in other words, the PPV equals the number of true positives divided by the total number of positive tests. The term negative predictive value (NPV) is defined as the number of true negatives divided by the number of true negatives plus the number of false negatives. Or in other words, the NPV equals the number of true negatives divided by the total number of negative tests (Fletcher and Suzanne, 2005). Finally the raw data results from both methodologies, the microarray and ELISA experiments, were compared for statistical significance between rfu values for the microarray and chemiluminescence values for the ELISA, through utilisation of the Mann-Whitney U test.

4. Results

4.1 Pilot study cohort

The pilot study cohort (n=40) consisted of twenty patients recently diagnosed with melanoma and twenty age and gender matched healthy volunteers, confirmed as melanoma, cancer and autoimmune disease free. Of the twenty patient samples, sixteen were male patients and four were female patients. The number of male and female control samples matched those of the patient cohort. Out of the twenty patient samples, five samples originated from patients with TNM stage 1 melanoma of which three were male samples and two were female samples. The remaining fifteen samples originated from TNM stage 0 *in situ* melanoma patients of which thirteen were male samples and two were female samples. Below is a summary of the average age of the pilot study participant with respect to their gender (Table 9). The age difference investigated here represented normally distributed data and therefore an independent t-test was utilised to calculate potential significant differences between the average age of male and female patients and controls. In the pilot study cohort, there was no significant difference in demographics between patients and controls (Table 9).

Table 9: Average ages in years of pilot study cohort with respect to gender

	Patient (years)	**Control (years)**	**p-value**
Male	58.31	55.88	0.926
Female	56.25	54	0.973

A full list of patient clinical data including stage, primary tumour location, previous history of melanoma and other diseases as well as blood sample collection time relative to disease diagnosis is attached in Appendix section 8.4.

4.2 Microarray results (OGT)

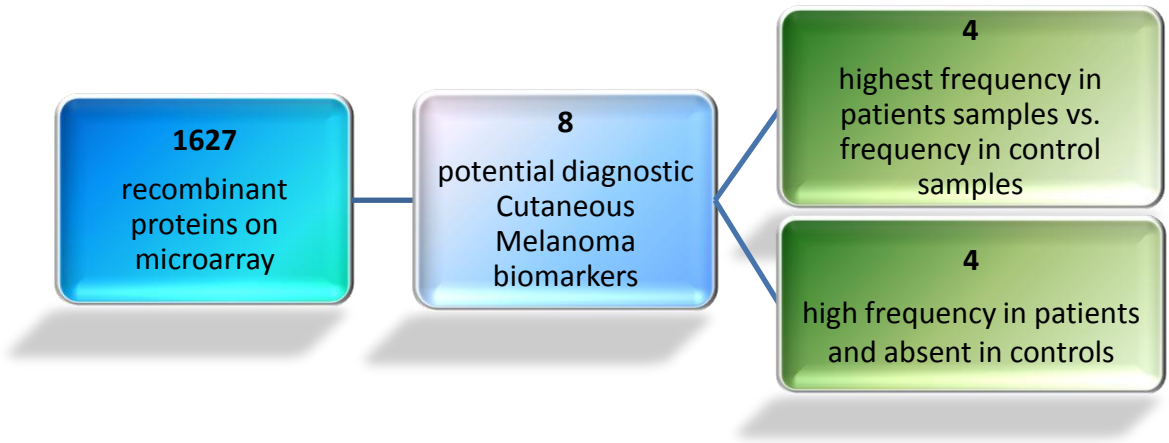


Figure 10: Eight markers were identified by the microarray platform as potential CM diagnostic biomarkers

Sera from all pilot study participants (n=40) were analysed on the Oxford Gene Technology (OGT) functional protein microarray for expression of autoantibodies specific to melanoma. Out of 1627 various human recombinant proteins attached to the functional protein microarray, eight were identified as potential diagnostic cutaneous melanoma biomarkers due to their reactivity with patient sera and low or no reactivity with control sera. Of these eight markers, four had the highest frequency in patient versus control samples and the remaining four displayed a high frequency in patient samples and were absent in control samples (Figure 10).

Figure 11 below illustrates the rfu fold change values in each pilot study sample relative to the 4 markers identified with the highest frequency in patient versus control samples. Any sample displaying a fold-change greater than 2 (marked by a red line) was regarded as positive for the marker tested.

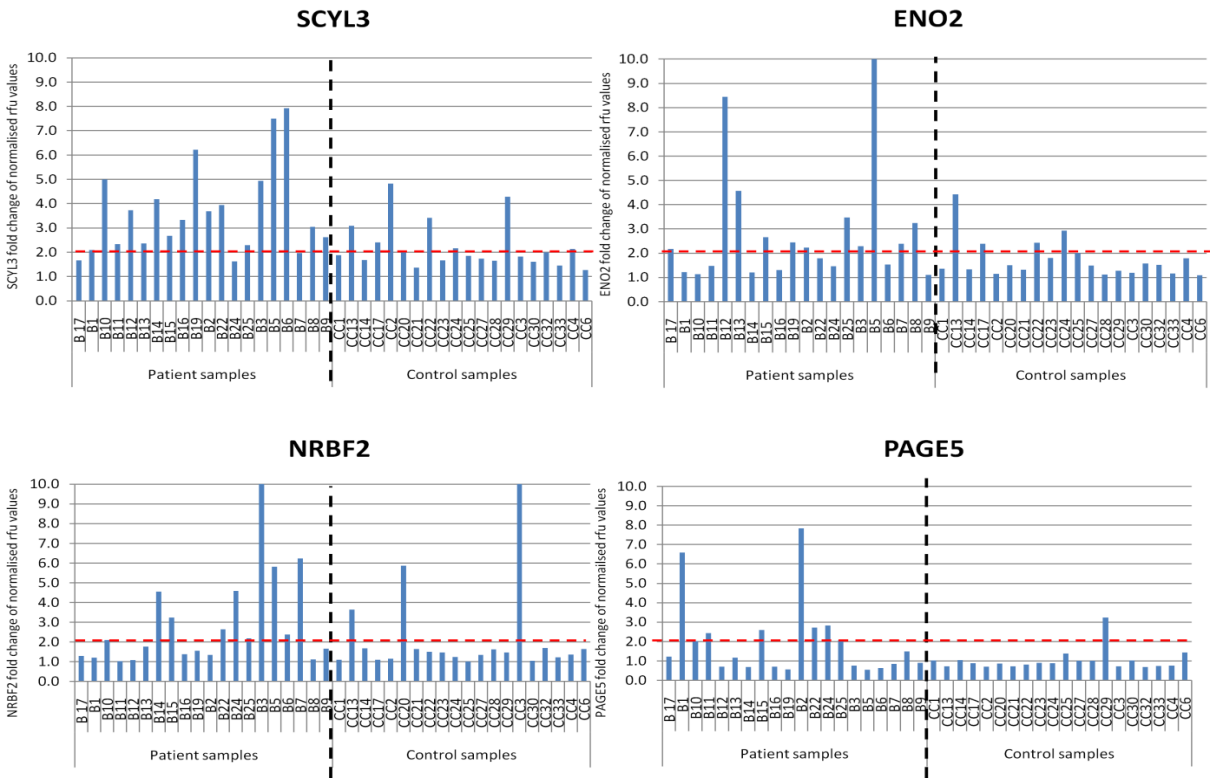


Figure 11: Pilot study sample rfu values for SCYL3, ENO2, NRBF2 and PAGE5

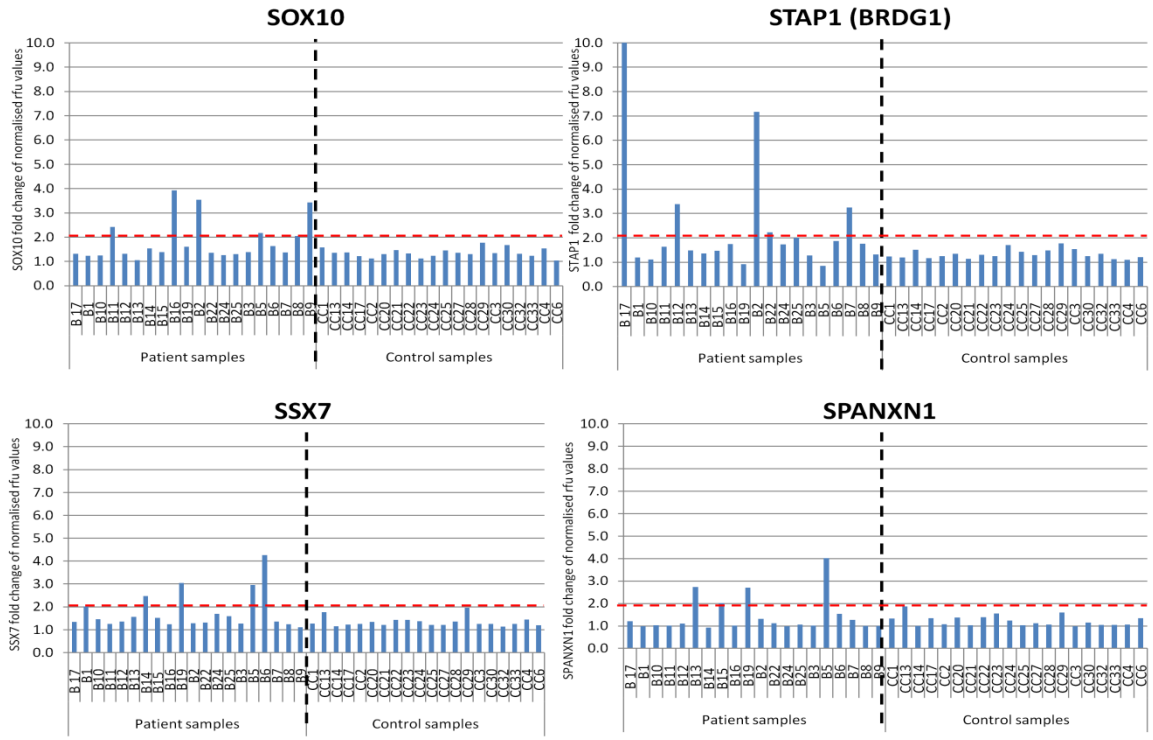


Figure 12: Pilot study rfu values for SOX10, STAP1, SSX7 and SPANXN1

Figure 12 on the previous page illustrates the rfu fold change value in each pilot study sample relative to the 4 markers that displayed a high frequency in patient samples and were absent in control samples.

Table 10 summarises the results for all the identified markers. For each marker, the mean rfu fold change (>2) in samples and controls above the overall background median rfu value for the array is given.

For SCYL3 there was a statistically significant difference between microarray fluorescence fold change values in patients versus control samples tested on the OGT microarray (p=0.001). The markers STAP1 and SSX7 also displayed statistically significant differences in patient and control fold change values (p=0.012 and p=0.033 respectively).

Table 10: Mean rfu fold change in patients and controls for each potential marker

Marker	rfu value in patients (mean ± Std Dev)	rfu value in controls (mean ± Std Dev)	Difference (p-value*)
SCYL3	3.65± 1.84	2.22 ± 0.96	0.001
ENO2	2.85 ± 2.53	1.74 ± 0.80	0.098
NRBF2	2.87 ± 2.36	2.17 ± 2.28	0.126
PAGE5	1.96 ± 1.96	1.02 ± 0.56	0.292
SOX10	1.82 ± 0.84	1.35 ± 0.20	0.063
STAP1	2.49 ± 2.63	1.32 ± 0.19	0.012
SSX7	1.77 ± 0.82	1.34 ± 0.21	0.033
SPANXN1	1.45 ± 0.81	1.23 ± 0.24	0.543
*p-value obtained by Mann-Whitney U analysis; p<0.05 was regarded as significant			

Table 11 and figure 13 below summarise the frequency with which the eight biomarkers were present in patient and control sera:

Table 11: Frequency of 8 microarray identified markers in patient versus control samples

Protein Name	Frequency in patients (n = 20)	Frequency in controls (n = 20)	Difference (n)	Frequency in patients (%)	Frequency in controls (%)	Difference (%)
SCYL3	17.0	8.0	9.0	85.0	40.0	45.0
ENO2	11.0	4.0	7.0	55.0	20.0	35.0
NRBF2	10.0	3.0	7.0	50.0	15.0	35.0
PAGE5	8.0	1.0	7.0	40.0	5.0	35.0
SOX10	6.0	0.0	6.0	30.0	0.0	30.0
STAP1	6.0	0.0	6.0	30.0	0.0	30.0
SSX7	5.0	0.0	5.0	25.0	0.0	25.0
SPANXN1	4.0	0.0	4.0	20.0	0.0	20.0

The autoantibody biomarkers with highest frequency in patient samples relative to their frequency in control samples are displayed in the top four rows of Table 11, while the autoantibody biomarkers identified with high frequency in patients but no positivity in control sera are shown in the bottom four rows if table 11 (grey). SCYL3 exhibits the highest percentage difference in frequency between patient and control sera (45%). Moreover, this marker has a 10% higher frequency of positivity in the patient versus control sera relative to markers ENO2, NRBF2 and PAGE5 which exhibit 35% difference in frequency between patients and controls. The difference in frequency of positivity between patient and control sera was 30% for SOX10 and BRDG1 and 25% and 20% respectively for SSX7 and SPANX1.

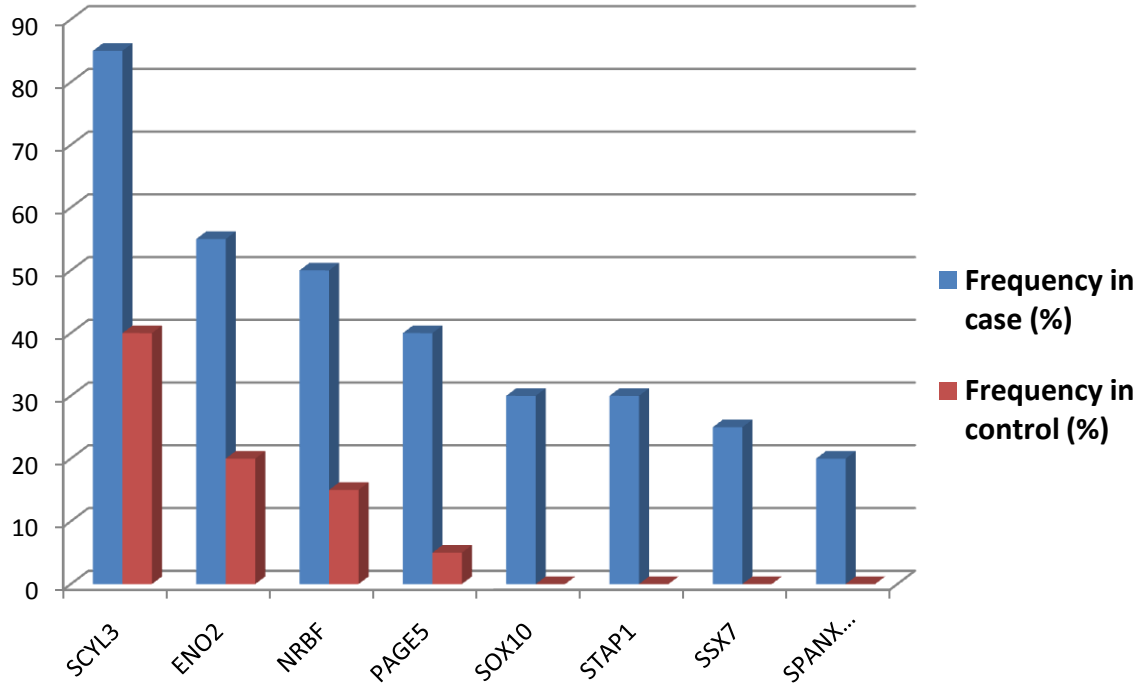


Figure 13: Frequency of the 8 biomarkers in patients versus controls

Figure 13 provides graphical representation of the ability of the potential biomarkers to identify melanoma autoantibodies in patient versus control samples. SCYL3 diagnosed 85% of the patient cohort as melanoma autoantibodies, while ENO2, NRBF2 and PAGE5 were positive in 55%, 50% and 40% of patients respectively. SOX10 and STAP1, SSX7 and SPANXN1 were positive in 30%, 25% and 20% of the patients respectively; however, according to these preliminary results, their specificity when utilised in a clinical setting would be as high as 100% since none of the control samples showed positivity for any of these 4 markers. PAGE5 incorrectly diagnosed one control sample as melanoma positive and diagnosed 40% of patient samples correctly. However, this resulted in a frequency difference between patient and control samples of 35%.

For cross-referencing purposes, section 8.5 in the appendix contains tables that illustrate the samples positive for the 8 potential biomarkers identified on the OGT microarray. The sample number, participant age, gender and rfu fold change for the appropriate marker are provided in this section as are the mean and range for each marker. Samples that did not display positivity for the markers were not included in the tables; however, all pilot study cohort samples and the relative sample rfu fold-change values were included in all of the following statistical analysis.

4.2.1 Marker expression relative to stage, gender and age

Table 12: Mean rfu fold changes for each marker relative to disease stage

Marker	**stage 0** rfu fold change (mean ± Std Dev)	**stage I** rfu fold change (mean ± Std Dev)	**Difference (p-value*)**
SCYL3	3.93 ± 2.00	2.82 ± 0.92	0.275
ENO2	3.14 ± 2.83	1.98 ± 0.97	0.569
NRBF2	2.80 ± 2.48	3.06 ± 2.22	0.827
PAGE5	2.17 ± 2.20	1.34 ± 0.84	0.693
SOX10	1.73 ± 0.76	2.10 ± 1.10	0.311
STAP1	2.66 ± 3.02	1.98 ± 0.72	0.484
SSX7	1.83 ± 0.91	1.60 ± 0.52	0.790
SPANXN1	1.57 ± 0.90	1.06 ± 0.15	0.175
*p-value obtained by Mann-Whitney U analysis; $p<0.05$ was regarded as significant			

Table 12 shows that there were no statistically significant differences between stage 0 and I patient microarray fluorescence fold change values. Interestingly, mean rfu fold change values were generally higher in stage 0 patients than in stage I (75%) patients, except for markers NRBF2 and SOX10 where stage I melanoma patients displayed higher rfu fold change values than stage 0 melanoma patients.

Pearson chi-square test and Fishers exact test were used to examine statistical significance of microarray marker expression in patients relative to melanoma TNM stage, gender and age (Table 13). The microarray patient cohort contained patients of early disease TNM stage 0 and I only. Initially, the cohort was divided into three age-groups, namely 18-34 years, 35-64 years and 65 years and above, which had been utilised to compare patient marker expression relative to age in the final ELISA experiments (section 4.4.3). However, the data from age

groups 18-34 years and 35-64 years were pooled as there was only one representative sample of the 18-34 year age group in the microarray cohort. The pooling of small sample size groups was regarded a standard method to avoid small sample size comparisons (Dawson and Trapp, 2001). Incidence percentages for each category, disease stage, gender and age, are summarised in Table 13. There was no significant difference in incidence in the pilot study patient cohort in regards to the patient‟s disease stage, gender or age. The incidence rates for the combination of the markers SOX10, STAP1 and SCYL3 and a combination of all eight markers were also included. The microarray identified fourteen (93.33%) melanoma stage 0 patients as displaying positivity for at least one markers when SOX10, STAP1 and SCYL3 were examined in combination while one (20%) of TNM stage I melanoma patient was declared positive for the combination; however this was not significant (p=0.750).

Table 13: Marker positivity in patients relative to stage, gender and age

	Incidence								
	Disease Stage			Gender			Age		
Marker	**Stage 0 n (%)**	**Stage I n (%)**	**Difference (p-value*)**	**Females n (%)**	**Males n (%)**	**Difference (p-value*)**	**18-64 years n (%)**	**65+ years n (%)**	**Difference (p-value*)**
SCYL3	13/15 (86.67%)	4/5 (80%)	0.601	3/4 (75%)	14/16 (87.5%)	0.509	11/13 (84.62%)	6/7 (85.71%)	0.730
ENO2	9/15 (60%)	2/5 (40%)	0.396	3/4 (75%)	8/16 (50%)	0.375	5/13 (38.46%)	6/7 (85.71%)	0.058
NRBF2	7/15 (46.67%)	3/5 (60%)	0.50	1/4 (25%)	9/16 (56.25%)	0.291	7/13 (53.85%)	3/7 (42.86%)	0.500
PAGE5	6/15 (40%)	2/5 (40%)	0.693	2/4 (50%)	6/16 (37.5%)	0.535	7/13 (53.85%)	1/7 (14.29%)	0.106
SOX10	4/15 (26.67%)	2/5 (40%)	0.483	1/4 (25%)	5/16 (31.25%)	0.657	3/13 (23.08%)	3/7 (42.86%)	0.336
STAP1	4/15 (26.67%)	2/5 (40%)	0.483	3/4 (75%)	3/16 (18.75%)	0.061	4/13 (30.77%)	2/7 (28.57%)	0.664
SSX7	4/15 (26.67%)	1/5 (20%)	0.634	3/4 (75%)	3/16 (18.75%)	0.061	4/13 (30.77%)	2/7 (28.57%)	0.664
SPANXN1	4/15 (26.67%)	0/5 (0%)	0.282	0/4 (0%)	4/16 (25%)	0.376	2/13 (15.38%)	2/7 (28.57%)	0.439
Combination of SOX10, STAP1 and SCYL3	14/15 (93.33%)	1/5 (20%)	0.750	4/4 (100%)	15/16 (93.75%)	0.800	12/13 (92.31%)	7/7 (100%)	0.650
Combination of all 8 markers	15/15 (100%)	5/5 (100%)	n/a	4/4 (100%)	16/16 (100%)	n/a	13/13 (100%)	7/7 (100%)	n/a
*p-value obtained by Pearson chi-square and Fishers exact analysis; $p<0.05$ was regarded as significant									

4.3 Summary of microarray data

A biomarker panel comprised of all 8 potential melanoma biomarkers would detect 20 out of 20 patient samples if a sample had to show positivity for at least one of the 8 markers; resulting in 100% sensitivity. However, the same panel would also detect 9 out 20 control samples under the same conditions, resulting in specificity of only 55% and therefore, the positive and negative predictive value of a potential blood test utilising these 8 markers combined would be 68.97% and 100% respectively (Table 14).

Table 14: Comparison of sensitivity, specificity and positive and negative predictive value of a potential diagnostic blood test utilising the optimal panel of 5 biomarkers or a panel of the 8 microarray identified potential melanoma biomarkers

	Patients (n =20)	**Controls (n = 20)**	**Sensitivity (%)**	**Specificity (%)**	**Positive and Negative predictive value (%)**
Positive for at least one of the suggested 5 biomarker panel	19	1	95	95	PPV = 95 NPV = 95
Positive for at least one of the 8 biomarkers	20	9	100	55	PPV = 68.67 NPV = 100

Table 15: Pilot study cohort reactivity against proposed 5 biomarker panel displaying 95% sensitivity and specificity (0= negative for the marker, 1= positive for the marker)

Patient sample number	PAGE5	SOX10	STAP1	SSX7	SPANXN1	Control sample number	PAGE5	SOX10	STAP1	SSX7	SPANXN1
B17	0	0	1	0	0	CC1	0	0	0	0	0
B1	1	0	0	1	0	CC13	0	0	0	0	0
B10	1	0	0	0	0	CC14	0	0	0	0	0
B11	1	1	0	0	0	CC17	0	0	0	0	0
B12	0	0	1	0	0	CC2	0	0	0	0	0
B13	0	0	0	0	1	CC20	0	0	0	0	0
B14	0	0	0	1	0	CC21	0	0	0	0	0
B15	1	0	0	0	1	CC22	0	0	0	0	0
B16	0	1	0	0	0	CC23	0	0	0	0	0
B19	0	0	0	1	1	CC24	0	0	0	0	0
B2	1	1	1	0	0	CC25	0	0	0	0	0
B22	1	0	1	0	0	CC27	0	0	0	0	0
B24	1	0	0	0	0	CC28	0	0	0	0	0
B25	1	1	0	0	0	CC29	1	0	0	0	0
B3	0	0	0	0	0	CC3	0	0	0	0	0
B5	0	1	0	1	1	CC30	0	0	0	0	0
B6	0	0	0	1	0	CC32	0	0	0	0	0
B7	0	0	1	0	0	CC33	0	0	0	0	0
B8	0	1	0	0	0	CC4	0	0	0	0	0
B9	0	1	0	0	0	CC6	0	0	0	0	0

By comparison, when a combination of a subset of 5 of these autoantibody markers were analysed, they were found to provide the highest sensitivity and specificity (95%). The panel of 5 biomarkers includes the markers PAGE5, SOX10, STAP1, SSX7 and SPANXN1. It was found that 19 out of 20 (95%) patient samples displayed positivity for at least one of the 5 markers while only one of the control samples was positive for at least 1 (5%) of the markers (Table 15). The positive and negative predictive value of a blood test utilising this biomarker panel is 95% for both PPV and NPV. The difference in incidence of expression of marker positivity between patient and control samples was established to be significant (p=0.000). As previously shown in table 10, the mean rfu fold change values of the markers STAP1 and SSX7 were significantly different in patient samples compared to controls (p=0.012 and p=0.033 respectively); however, the mean rfu fold changes of the remaining three markers, PAGE5, SOX10 and SPANXN1, contained within the subset of 5 biomarkers did not display statistically significant differences between patient and control sera (p=0.292, p=0.063 and p=0.543 respectively).

To graphically visualise and numerically confirm the diagnostic potential of the 8 identified biomarkers, ROC curve analysis was performed.

Table 16: ROC curve analysis for individual markers

Biomarker name	number of observations	ROC Area	standard error
Panel of 8 biomarkers combined	40	0.7750	0.0695
panel of 5 biomarkers combined	40	0.9500	0.0354
SCYL3	40	0.7250	0.0695
ENO2	40	0.6750	0.0732
NRBF2	40	0.6750	0.0705
PAGE5	40	0.6750	0.0615
SOX10	40	0.6500	0.0526
STAP1	40	0.6500	0.0526
SSX7	40	0.6250	0.0497
SPANXN1	40	0.6000	0.0459

Table 16, and Figures 14 and 15, summarise the area under the ROC curve for each individual marker. The standard error for each marker was also included in Table 16 to show the deviation of the sample mean from the actual mean of the population investigated. According to this ROC curve analysis, SCYL3 displays the highest potential to serve as a diagnostic blood biomarker for the early detection of melanoma (area under the curve (AUC)= 0.725) while SPANXN1 shows the lowest melanoma diagnostic biomarker potential (AUC= 0.6). The biomarkers SSX7 and SPANXN1 represented the smallest deviation of their sample mean to the mean of the actual population (standard error= 0.0497 and 0.0459 respectively). Figure 14 below illustrates the AUC for each individual marker as well as the AUC of the 5 biomarker panel, denoted in the figure as „optimal markers" while Figure 15 compares the AUC of the 5 marker panel to a combination of all 8 microarray identified potential diagnostic melanoma biomarkers.

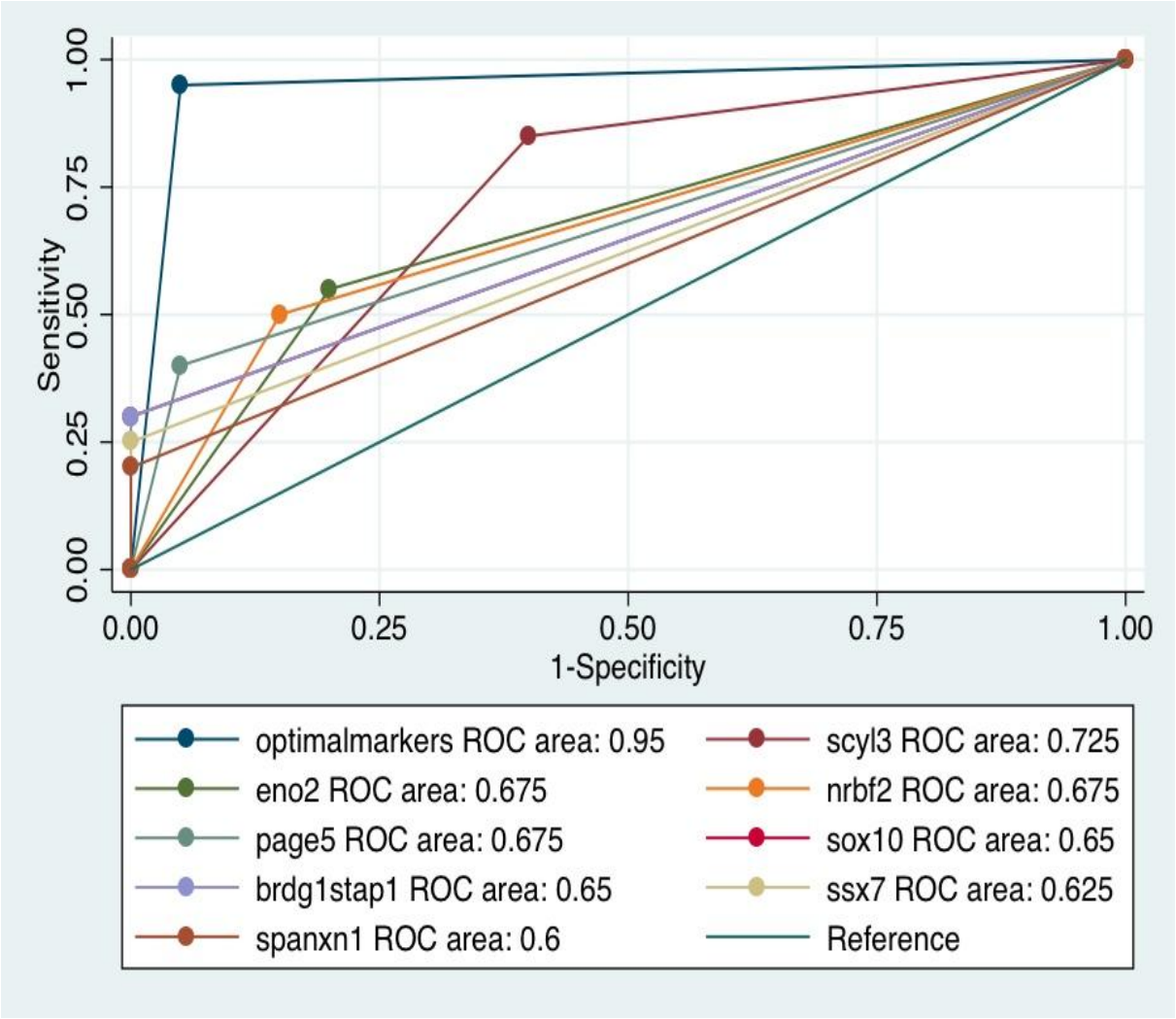


Figure 14: ROC curve displaying the area under the curve (AUC) for individual markers

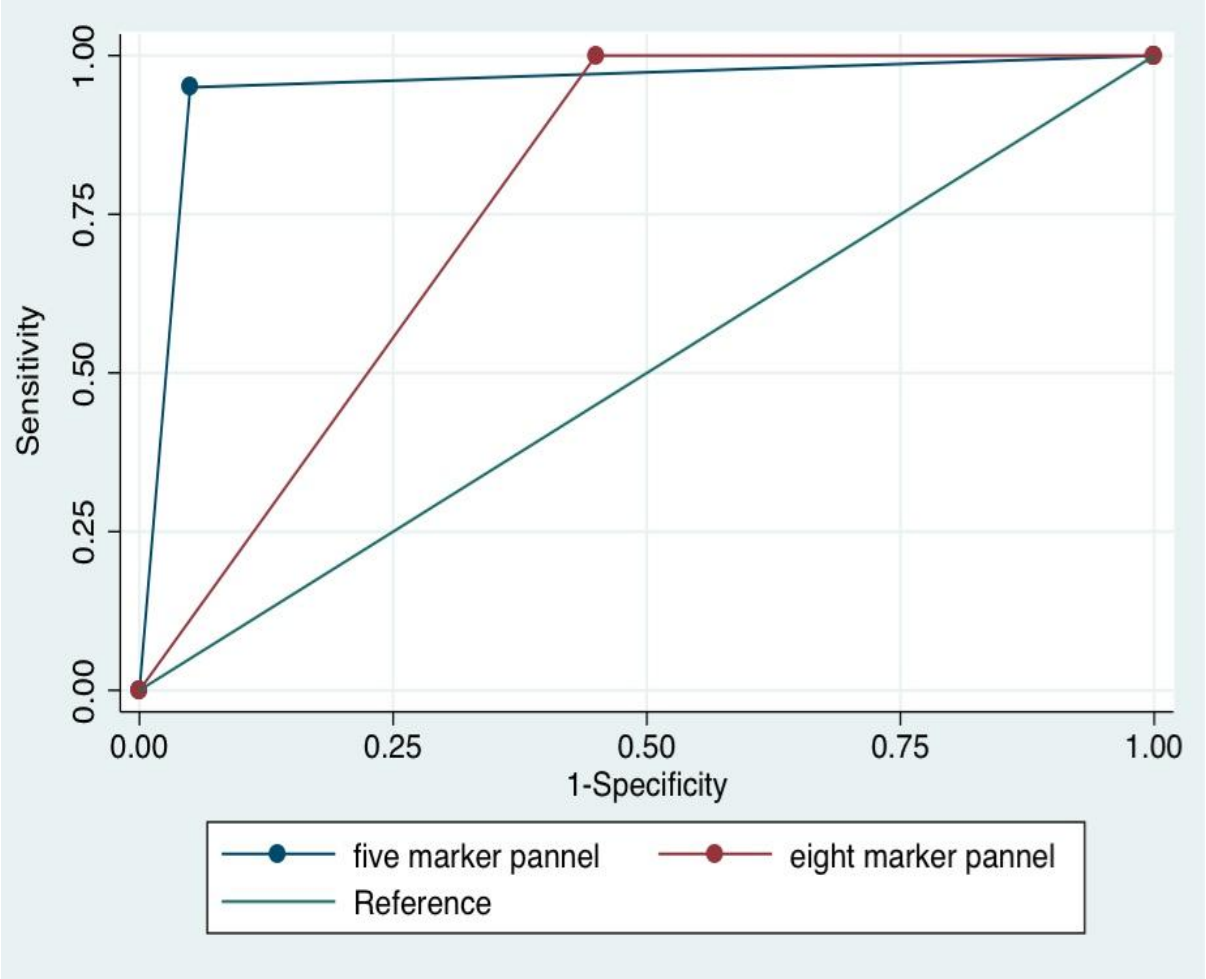


Figure 15: ROC curve comparison of a diagnostic panel of 5 biomarkers and a panel of 8 biomarkers

In summary, the microarray experiment identified 8 markers as potential diagnostic melanoma autoantibody markers with sensitivity of up to 85% and specificity of up to 100% for individual markers, while a combination of all 8 biomarkers may yield a sensitivity of 100% and a specificity of 55%. The positive and negative predictive value of a potential blood test utilising these 8 markers combined is 68.97% and 100% respectively while both the positive and native predictive value are 95% for a blood test utilising the 5 marker panel. The fold changes of rfu units showed statistically significant differences between patient and control samples for the markers SCYL3, STAP1 and SSX7 ($p=0.001$, $p=0.012$ and $p=0.033$ respectively). The markers were not identified to be stage, gender or age specific, however, these results were obtained from a small pilot study sample size ($n=40$) and larger sample sizes were required to confirm the results using an enzyme-linked immunosorbent assay.

4.4. Final SOX10, BRDG1 (STAP1) and SCYL3 ELISA

Out of the 8 identified potential melanoma biomarkers (Figure 10 and Table 11), three markers, namely SOX10, BRDG1 (STAP1) and SCYL3, were chosen for further investigation utilising an enzyme-linked immunosorbent assay (ELISA). The aim was to employ this methodology to validate the results obtained from the microarray in a larger cohort. SOX10 and BRDG1 (STAP1) were chosen, as these markers displayed the highest frequency in patients of those markers absent in the microarray controls study cohort (Table 11). The marker SOX10 was chosen over marker SSX7 as its association with melanoma development had been determined previously as described in section 5.1 of this document. There was no significant difference between patient and control samples for SOX 10 ($p=0.063$), whereas a significant difference was found for SSX7 ($p=0.033$). The lack of significance between SOX10 samples was thought to be due to small sample size of the pilot study (n=40), nevertheless, SOX10 was chosen for further analysis as it was readily commercially available. SCYL3 was chosen for further study as it displayed the highest percentage difference of frequency in patients versus controls in the microarray samples (45%) (Table 11).

4.4.1 Final ELISA study cohort

The final study cohort (n=104) for SOX10 ELISA experiment consisted of 53 patients recently diagnosed with melanoma and 51 healthy volunteers, confirmed as melanoma, cancer and autoimmune disease free. The final study cohort (n=113) for BRDG1 (STAP1) consisted of 59 patients while the final study cohort (n=114) for SCYL3 consisted of 60 patients. For both BRDG1 (STAP1) and SCYL3, the final cohort consisted of 54 controls. The patient cohort used to assess all three markers included patients at various disease stages (Table 17). None of the cohorts for any of the three markers included TNM stage IV melanoma patient samples. Of the 53 patient samples in SOX10, 42 were male patients and 11 were female patients. There were 24 male and 27 female control samples. Out of the 53 patient samples for SOX10, 26 originated from TNM stage 0 *in situ* melanoma patients, 15 samples originated from patients with TNM stage 1 melanoma, 10 samples originated from TNM stage II melanoma patients and 2 samples originated from TNM stage III melanoma patients.

Table 17: Final ELISA cohort demographical information

		Gender		Disease Stage			
Marker	Patient or Control	Male	Female	TNM 0	TNM I	TNM II	TNM III
SOX10	Patient	42	11	26	15	10	2
	Control	24	27				
BRDG1 (STAP1)	Patient	46	13	27	18	12	2
	Control	26	28				
SCYL3	Patient	46	14	27	19	12	2
	Control	26	28				

Of the 59 patient samples for BRDG1 (STAP1), 46 were male patients and 13 were female patients. There were 26 male and 28 female control samples. Out of the 59 patient samples, 27 originated from TNM stage 0 *in situ* melanoma patients, 18 samples originated from patients with TNM stage 1 melanoma and 12 samples originated from TNM stage II melanoma patients and 2 originated from TNM stage III melanoma patients. Of the 60 patient samples for SCYL3, 46 were male patients and 14 were female patients. There were 26 male and 28 female control samples. Out of the 59 patient samples, 27 originated from TNM stage 0 *in situ* melanoma patients, 19 samples originated from patients with TNM stage 1 melanoma, 12 samples originated from TNM stage II melanoma patients and 2 samples originated from TNM stage III melanoma patients. Below is a summary of the average age of the final ELISA validation study participant for each of the three markers with respect to their gender (Table 18). There was no significant difference in demographics between patients and controls.

Table 18: Average ages in years of SOX10, BRDG1 (STAP1) and SCYL3 final ELISA cohort with respect to gender

Biomarker name	Gender	Patient (years)	Control (years)	p-value
SOX10	Male	59.55	53.73	0.587
	Female	61.64	43.73	0.276
BRDG1 (STAP1)	Male	60.28	52.35	0.344
	Female	62	43.79	0.496
SCYL3	Male	60.28	52.35	0.344
	Female	62.64	43.79	0.547

4.4.2 Final SOX10, BRDG1 (STAP1) and SCYL3 ELISA results. A comparison of results where individual versus a combination of markers were used

Table 19: Mean chemiluminescence value in patients and controls for SCYL3, SOX10 and BRDG1 (STAP1)

Marker	chemiluminescence values in patients (mean ± Std Dev)	chemiluminescence values in controls (mean ± Std Dev)	Difference (p-value*)
SCYL3	0.199 ± 0.376	0.144 ± 0.109	0.102
SOX10	0.285 ± 0.224	0.213 ± 0.135	0.263
STAP1	0.099 ± 0.774	0.119 ± 0.091	0.074
*p-value obtained by Mann-Whitney U analysis; $p<0.05$ was regarded as significant			

Table 19 on the previous page summarises the final SOX10, BRDG1 (STAP1) and SCYL3 ELISA results. For each marker, the mean chemiluminescence value in samples and controls is given.

There were no statistically significant differences in chemiluminescence values between patients and controls in the final ELISA experiments for any of the three markers.

Table 20: Mean chemiluminescence values for SCYL3, SOX10 and SYAP1 relative to disease stage

Marker	stage 0 value (mean ± Std Dev)	stage I value (mean ± Std Dev)	stage II value (mean ± Std Dev)	stage III value (mean ± Std Dev)	Difference (p-value*)
SCYL3	0.155 ± 0.099	0.287 ± 0.636	0.163 ± 0.123	0.123 ± 0.018	0.997
SOX10	0.268 ± 0.222	0.347 ± 0.247	0.311 ± 0.311	0.180 ± 0.079	0.525
STAP1	0.103 ± 0.089	0.093 ± 0.063	0.103 ± 0.083	0.076 ± 0.032	0.992
*p-value obtained by Kruskal-Wallis analysis; $p<0.05$ was regarded as significant					

Table 20 shows the mean chemiluminescence values for the final ELISA patient cohort for each of the three markers tested relative to disease stage. The ELISA confirmed that when assessed by levels, the markers SOX10, BRDG1 (STAP1) and SCYL3, are not stage specific in a larger cohort as there were no statistically significant differences observed in the patients chemiluminescence values relative to disease stage.

A sample was denoted as positive for any of the three markers tested if it displayed a fold change of 1 or more above the average of all sample chemiluminescence values and above the overall background signal obtained across the plate/plates. A fold change of 2 or above, utilised in the microarray experiment, was not feasible for the determination of marker positivity on the ELISA since only 2 samples displayed a fold change above 2 and this cut-off

was therefore determined to be too high for the ELISA data and replaced by a lower cut-off value of 1.

Out of the final ELISA cohort, 20.75% of patient samples and only 1.96% of control samples displayed positivity for SOX10 alone (PPV=91.66%, NPV=54.35%), thus a statistical significance of incidence displayed between patient and control samples for SOX10 (p=0.002). For BRDG1 (STAP1), only 1.85% of all patient samples as well as 11.11% of controls exhibited BRDG1 positivity (p=0.044, PPV=14.29%, NPV=45.28%) and 6.67% of patient samples showed positivity for SCYL3 alone (PPV=80%, NPV=48.62%) while only 1.85% of control samples were denoted as positive for the marker (Table 21). Interestingly, 10 out of 53 (18.87%) patient samples and 7 out of 51 (19.61%) control samples were positive for all three markers (PPV=58.82%. NPV=50.57%). Thus 50.94% of patient samples as well as 35.29% of control samples were regarded as positive if the sample displayed positivity for at least 1 of the three markers (PPV=85.69%, NPV=55.77%). 26 out of 53 (49.06%) patients did not however show any positivity for any of the three markers indicating that other biomarkers need to be identified and included in a potential blood sample in order

Table 21: Frequency of positivity for SOX10, BRDG1 (STAP1) and SCYL3 in patients and control samples

Marker	Patients	Controls	Difference (p-value*)
SOX10 only	11/53 (20.75%)	1/51 (1.96%)	0.002
STAP1 only	1/59 (1.85%)	6/54 (11.11%)	0.044
SCYL3 only	4/60 (6.67%)	1/54 (1.85%)	0.217
combination of all three markers	10/53 (18.87%)	7/51 (19.61%)	0.329
positive for at least 1 marker	30/53 (56.60%)	22/51 (43.14%)	0.120
Positive for none of the markers	26/53 (49.06%)	33/51 (64.71%)	0.079
*p-value obtained by Pearson chi-square and Fishers exact analysis; p<0.05 was regarded as significant			

to increase the sensitivity of such a diagnostic test. 33 out of 51 (64.71%) controls did not show positivity for any of the three markers.

The following table, Table 22, summarises the sensitivity, specificity and positive and negative predictive value of the individual three markers as well as combinations of these markers.

Table 22: Summary of sensitivity, specificity and positive and negative predictive value of individual markers and/ or a combination of these markers

Marker	Sensitivity	Specificity	Positive and Negative predictive value
SOX10 only	20.75%	98.04%	PPV= 91.66% NPV= 54.35%
STAP1 only	1.85%	88.88%	PPV= 14.29% NPV= 45.28%
SCYL3 only	6.67%	98.12%	PPV= 80% NPV= 48.62%
combination of all three markers	18.87%	86.27%	PPV= 58.82% NPV= 50.57%
positive for at least 1 marker	56.60%	56.86%	PPV= 57.69% NPV= 55.77%

As illustrated in Table 22, of the markers assessed by ELISA, the marker with the highest potential for the diagnosis of melanoma patients is SOX10. According to these results, as individual serologic biomarker SOX10 exhibits 20.75% sensitivity but up to 98.04% specificity and a positive predictive value of 91.66% while it‟s negative predictive value is 54.35%. Following SOX10, the second best result was obtained when a combination of the markers was used. A sensitivity of up to 56.60% was achieved when samples exhibited positivity for at least one of the 3 markers, while it was 18.87% for samples displaying positivity for all three markers combined in patient sera. The positive and negative predictive value was 57.69% and 55.77% respectively for the panel where samples had to display positivity for at least one of the three markers while the values were 58.82% and 50.57% for the panel of a combination of all three markers.

4.4.3 ELISA-based marker expression relative to stage, gender and age

Following the comparison of the diagnostic potential of individual autoantibody melanoma biomarkers SOX10, BRDG1 (STAP1) and SCYL3 relative to combinations of these markers, the data obtained from the final ELISA experiments was analysed for statistically significant differences between patients and control marker levels and positivity with regard to TNM disease stage, gender and age.

Further analysis was performed using the Pearson chi-square test and Fishers exact test, just as for the microarray data, so as to examine statistical significance of microarray marker expression in patients relative to melanoma TNM stage, gender and age (Table 23).

There was no significant difference in incidence in the pilot study patient cohort in regards to the patient‟s disease stage, gender or age. The final ELISA experiments identified twelve (46.15%) melanoma stage 0, ten (66.67%) melanoma stage I, 5 (50%) melanoma stage II and zero (0%) of melanoma stage III patients as displaying positivity for at least one markers when SOX10, STAP1 and SCYL3 were examined in combination; however, similar to the microarray data, this was not significant (p=0.784).

Following the analysis of incidence of marker positive samples relative to disease stage, gender and age, differences in chemiluminescence values were investigated relative to these characteristics.

Table 23: Marker positivity in final ELISA cohort patients relative to stage, gender and age

	Incidence											
	Disease Stage					Gender			Age			
Marker	**Stage 0 n (%)**	**Stage I n (%)**	**Stage II n (%)**	**Stage III n (%)**	**Difference (p-value*)**	**Females n (%)**	**Males n (%)**	**Difference (p-value*)**	**18-34 years n (%)**	**34-64 years n (%)**	**65+ years n (%)**	**Difference (p-value*)**
SCYL3	9/27 (33.33%)	7/19 (36.84%)	3/12 (25%)	0/2 (0%)	0.424	7/14 (50%)	12/46 (26.09%)	0.089	2/5 (40%)	5/27 (18.52%)	12/28 (42.86%)	0.239
SOX10	9/26 (34.62%)	10/15 (66.67%)	4/10 (40%)	0/2 (0%)	0.949	7/11 (63.64%)	16/42 (38.10%)	0.119	2/5 (40%)	11/25 (44%)	10/23 (43.48%)	0.936
STAP1	8/27 (26.63%)	4/18 (22.22%)	4/12 (33.33%)	0/2 (0%)	0.735	5/13 (38.46%)	11/46 (23.91%)	0.241	2/5 (40%)	5/27 (18.52%)	9/27 (33.33%)	0.636
Combination of SOX10, STAP1 and SCYL3	12/26 (46.15%)	10/15 (66.67%)	5/10 (50%)	0/2 (0%)	0.784	7/11 (63.64%)	20/42 (47.62%)	0.273	2/5 (40%)	12/25 (48%)	13/23 (56.52%)	0.438
*p-value obtained by Pearson chi-square and Fishers exact analysis; $p<0.05$ was regarded as significant												

Patient samples were grouped according to TNM stage ranging from TNM *in situ* to TNM III. Interestingly, stage I melanoma patient chemiluminescence values for SOX10 were significantly higher than control chemiluminescence values (p=0.028, Figure 16 and Table 24) while none of the other stages in SOX10 or the other two markers displayed a statistically significant difference between chemiluminescence values. The mean and standard deviation of SOX10 chemiluminescence values for stage 0 samples were 0.26 and 0.22, for stage I the mean was 0.35 and the standard deviation 0.25, for stage II the mean was 0.31 and the standard deviation 0.31, for stage III the mean was 0.18 and the standard deviation 0.08 and the mean of control samples was 0.21 and the standard deviation 0.13.

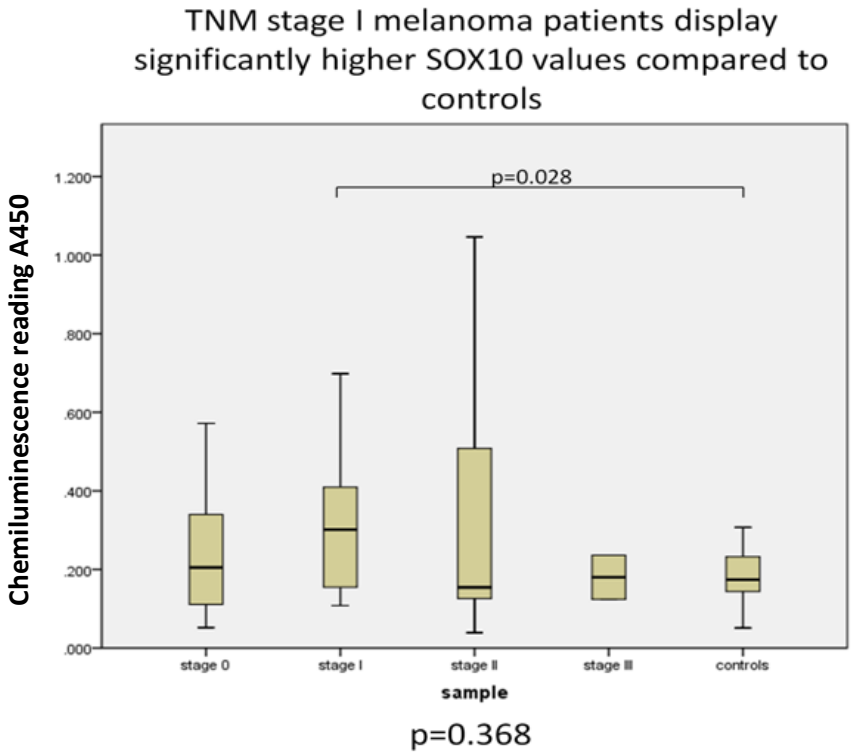


Figure 16: TNM stage I melanoma patients display significantly higher SOX10 chemiluminescence values than controls

Table 24: Statistical significance for comparison of chemiluminescence values between samples of various melanoma disease stages or between melanoma disease stages and controls

	Stage 0	Stage I	Stage II	Stage III	Controls
Stage 0					
Stage I	0.190				
Stage II	0.942	0.356			
Stage III	0.853	0.261	0.667		
Controls	0.691	0.028	0.938	0.834	

Following this, stage 0 and I samples were grouped together to represent early-stage melanoma patients with relatively low metastatic potential and stage II and III melanoma samples were grouped together to represent more advanced melanoma patients with higher metastatic potential. The chemiluminescence values of both groups were analysed for statistical differences and we found that there was no statistically significant difference between early stage and advanced stage melanoma patient samples for any of the three markers SOX10, BTDG1 (STAP1) and SCYL3 ($p=0.588$, $p=0.922$ and $p=0.958$ respectively.)

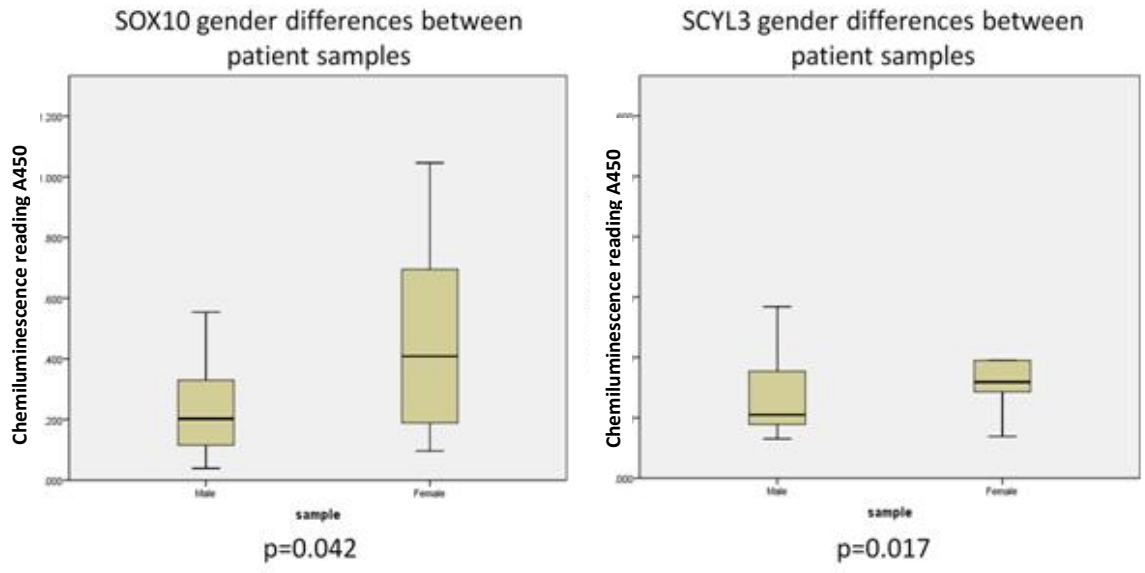


Figure 17: Female patients exhibit higher chemiluminescence values for SOX10 and SCYL3 than male patients

Furthermore, analysis showed that female patients exhibited significantly higher levels of rfu fluorescence for SOX10 than male patients (p=0.042, Figure 17) while there was no statistically significant difference between male and female control chemiluminescence values. Female patients also exhibited significantly higher levels of chemiluminescence for SCYL3 than male patients while there was also no statistically significant difference between male and female control chemiluminescence values (p=0.017, Figure 17).

To assess any age related differences between chemiluminescent values in samples, patients were stratified into three age groups 18-34 years, 35-64 years and 65 and above (65+).

There were no statistically relevant differences in patient chemiluminescence readings for SOX10, BRDG1 (STAP1) and SCYL3 with regard to age (p=0.710, p=0.412 and p=0.552 respectively).

4.5 Comparison of results of marker levels and incidence between microarray and ELISA for SOX10, BRDG1 (STAP1) and SCYL3

Similarities and differences of SOX10, BRDG1 (STAP1) and SCYL3 frequency and levels between the OGT microarray experiment and enzyme-linked immunosorbent assay were investigated. First, the rfu values from the microarray were assessed relative to the colour luminescence values for the pilot study cohort (Table 25). It is important to note when comparing results that different conditions are used in different methodologies, less sample volume on the microarray, different antibody concentrations and antigen conformations, different development times and different readouts - fluorescence on the microarray versus colour luminescence on the ELISAs may greatly affect the results obtained.

First, the mean and standard deviation of the fluorescence and luminosity for the same patient cohort against the same three human recombinant proteins were compared.

Table 25: Summary of mean, standard deviation and statistical significance of the differences of microarray rfu vales and ELISA colour luminescence values in pilot study patient and control samples (microarray data = pilot study testing; luminescence unit data = secondary study results)

Marker and Methodology	**Mean ± Standard deviation** patients	**Mean ± Standard deviation** controls	**p-value*** patients versus controls (n = 40)	**Mean ± Standard deviation** patients	**Mean ± Standard deviation** controls	**p-value*** patients versus controls (n = 104 (SOX10), 113 (STAP1), 114 (SCYL3))
SCYL3 (microarray rfu units)	6188.45 ± 3991.75	3403.68 ± 1549.59	0.006			
SCYL3 (ELISA luminescence units)	0.140 ± 0.060	0.140 ± 0.100	0.715	0.199 ± 0.376	0.144 ± 0.109	0.102
SOX10 (microarray rfu units)	3521.6 ± 4234.75	2249.16 ± 1388.61	0.133			
SOX10 (ELISA luminescence units)	0.260 ± 0.180	0.220 ± 0.120	0.653	0.285 ± 0.224	0.213 ± 0.135	0.263
STAP1 (microarray rfu units)	4852.25 ± 7845.70	2122.74 ± 1015.54	0.068			
STAP1 (ELISA luminescence units)	0.090 ± 0.050	0.110 ± 0.110	0.888	0.099 ± 0.774	0.119 ± 0.091	0.074
* $p<0.05$ was regarded as significant, statistical significance was analysed by Mann-Whitney U analysis						

As illustrated in Table 25, only SCYL3 was found to have statistically significant differences in levels between pilot study patient and control sample rfu values in the microarray experiment (p=0.006). This was not evident in the ELISA results for the pilot study cohort nor was it evident in the final study cohorts. In the ELISA experiment, none of the markers demonstrated levels of chemiluminescence in patients that were different from controls. However, the mean value of SOX10 colour luminescence was highest both in the pilot cohort

as well as in the final patient cohort, while SCYL3 followed by BRDG1 (STAP1) showed lower levels when assessed by ELISA.

Finally, both methodologies, microarray and ELISA, were compared for frequency of positivity for SOX10, BRDG1 (STAP1) and SCYL3 and for combinations of these markers (Table 26). Table 26 summarises the frequency of positivity of individual markers and combinations of these markers in the pilot study and final ELISA cohort. There was a statistically significant difference in incidence when patients were regarded positive for at least one of these three markers in the microarray ($p=0.003$). This is similarly reflected in the ELISA pilot study cohort but the statistical difference was not found to be significant ($p=0.055$). Furthermore, the ELISA identified 6 out of 20 (30%) pilot study patients as being positive for marker SOX10 alone ($p=0.010$) while in the extended cohort, 11 out of 53 (20.75%) patients were diagnosed as SOX10 positive only ($p=0.010$).

Overall, the results illustrated in Table 27 show that Microarray and ELISA results of sensitivity and specificity as well as the positive and negative predictive values for the three markers are highly similar. Both the microarray and ELISA results demonstrate that, using a combination of the three markers where patients are regarded as melanoma positive when they display positivity for at least one of the markers provides the highest sensitivity for melanoma diagnosis (90% and 55% respectively). Both methodologies also demonstrated the diagnostic potential of the individual marker SOX10 with high positive predictive values of 91.66% and 100% respectively.

Table 26: Frequency of positivity for SOX10, BRDG1 (STAP1) and SCYL3 and combinations of these markers in pilot study patient and control samples as well as final ELISA samples

Marker	Patients (microarray)	Controls (microarray)	Difference (p-value*)	Patients (Pilot study ELISA cohort)	Controls (Pilot study ELISA cohort)	Difference (p-value*)	Patients (Final ELISA cohort)	Controls (Final ELISA cohort)	Difference (p-value*)
SOX10 only n (%)	0/20 (0%)	0/20 (0%)	n/a	6/20 (30%)	0/20 (0%)	0.010	11/53 (20.75%)	1/51 (1.96%)	0.002
STAP1 only n (%)	1/20 (5%)	0/20 (0%)	0.500	0/20 (0%)	0/20 (0%)	n/a	1/59 (1.85%)	6/54 (11.11%)	0.044
SCYL3 only n (%)	8/20 (40%)	8/20 (40%)	0.626	0/20 (0%)	0/20 (0%)	n/a	4/60 (6.67%)	1/54 (1.85%)	0.217
Combination of all three markers n (%)	1/20 (5%)	0/20 (0%)	0.500	2/20 (10%)	2/20 (10%)	0.698	10/53 (18.87%)	7/51 (19.61%)	0.329
Positive for at least 1 marker n (%)	18/20 (90%)	9/20 (45%)	0.003	14/20 (70%)	8/20 (40%)	0.055	30/53 (56.60%)	22/51 (43.14%)	0.120
Positive for none of the markers n (%)	2/20 (10%)	11/20 (55%)	0.003	9/20 (45%)	14/20 (70%)	0.100	26/53 (49.06%)	33/51 (64.71%)	0.079
*p-value obtained by Pearson chi-square and Fishers exact analysis; $p<0.05$ was regarded as significant									

Table 27: Summary of sensitivity, specificity and positive and negative predictive value of individual markers and combinations of these markers in the pilot study cohort

Microarray				**ELISA**			
	sensitivity	**specificity**	**positive and negative predictive value**		**sensitivity**	**specificity**	**positive and negative predictive value**
SOX10 (n=40)	30.00%	100.00%	PPV= 91.66% NPV= 54.35%	**SOX10 (n=104)**	43.40%	84.31%	PPV= 100% NPV= 58.82%
STAP1 (n=40)	30.00%	100.00%	PPV= 14.29% NPV= 45.28%	**STAP1 (n=113)**	27.12%	68.52%	PPV= 0% NPV= 50%
SCYL3 (n=40)	85.00%	60.00%	PPV= 80% NPV= 48.62%	**SCYL3 (n=114)**	31.67%	75.93%	PPV= 0% NPV= 50%
combination of all 3 markers	5.00%	100.00%	PPV= 58.82% NPV= 50.57%	**combination of all 3 markers**	10.00%	89.47%	PPV= 50% NPV= 50%
positive for at least 1 of the markers	90.00%	55.00%	PPV= 57.69% NPV= 55.77%	**positive for at least 1 of the markers**	55.00%	73.68%	PPV= 63.64% NPV= 66.66%

5. Discussion

Melanoma is a highly aggressive skin cancer whose progression is dramatically reduced through early detection, followed by early surgical removal of the tumour (Balch et al., 2011).

The overall aim of this research was to identify highly sensitive and specific serologic autoantibody biomarkers and combinations of these markers for the early detection of melanoma. Results from this study, although preliminary, may contribute to the development of a diagnostic melanoma blood test which will aid the improvement of the disease management, further increasing the number of patients diagnosed positive for the cancer at an early stage.

We showed here that indeed melanoma early stage patients are positive for autoantibodies. We also show that microarray technology was able to identify a panel of autoantibody markers with high specificity and sensitivity for detection of early stage melanoma and that this technology is superior for detection of autoantibodies in patient sera. Our results also demonstrate that the levels of three of the identified autoantibody biomarkers may be associated with gender but are not disease stage or age specific.

5.1 Identification of 8 autoantibody biomarkers for early stage melanoma diagnosis utilising the OGT functional protein microarray

A major finding of this research project was the identification of 8 proteins exhibiting high reactivity with patient sera and low or no reactivity with control sera which were therefore considered potential diagnostic biomarkers for the early detection of melanoma patients.

The markers identified include the protein-associating with the carboxyl-terminal domain of ezrin (SCYL3), gamma-enolase (ENO2), nuclear receptor-binding factor 2 (NRBF2), G antigen family E member 1 (PAGE5), transcription factor SOX-10 or SRY sex-determining region Y-box 10 (SOX10), signal-transducing adaptor protein 1 (STAP1), synovial sarcoma, X breakpoint 7 (SSX7) and sperm protein associated with the nucleus on the X chromosome N1 (SPANXN1).

A search of the literature confirmed an association of a number of these markers with melanoma or the markers were hypothesised to be associated with melanomagenesis, or they contribute to pathways and physiological changes resulting in tumour formation or

maintenance. Some of the identified markers however, have not yet been shown to be associated with melanoma or melanoma diagnosis and may therefore represent novel diagnostic melanoma biomarkers.

Briefly, literature published on SCYL3, also known as PACE1, defines PACE1 as a novel protein that interacts with the c-terminal domain of ezrin and suggests that this biomarker may therefore play a role in cell adhesion and migration complexes in migrating cells (Sullivan et al., 2003). The cell adhesion molecule CD44 is known to interact with the ezrin family (ERM family) members and this CD44/ ezrin complex influences cell migration, cell adhesion, endothelium interactions as well as tumour progression and metastasis (Martin et al., 2003; Neisch and Fehon, 2011). Although the association of the SCYL3 protein and it‟s autoantibodies with melanoma has not been investigated previously, its association with the binding site ezrin suggests a relationship between elevated SCYL3 autoantibody levels and melanoma.

ENO2 has not yet been identified as a melanoma marker. However in a recent study, gene family member ENO1 was identified in melanoma patient sera utilising the serological proteome approach (SERPA) (section 1.12.5). The study found ENO1 levels to be elevated in patient sera compared to control sera and ENO1 mRNA was frequently expressed in various melanoma cell lines (Suzuki et al., 2010).

NRBF2, also known as CORP2, is known to be one of the regulators of keratinocyte gene expression and the literature suggests that the protein can act as a transcriptional activator (Flores et al., 2004). However, the diagnostic relevance of anti-NRBF2 levels for the diagnosis of melanoma is still unknown.

PAGE5, better known as cancer-testis antigen 16 (CT16), is frequently expressed in advanced melanoma (Scanlan et al., 2002) and recent research has established PAGE5 to act as a regulator of the expression of apoptotic and anti-apoptotic genes, thereby promoting melanoma tumour cell survival (Nylund et al., 2012). Here we have shown for the first time that PAGE5 may have potential in diagnosing early-stage melanoma patients.

SOX10 regulates many targets during neural crest development, including microphthalmia-associated transcription factor (MITF), necessary for normal generation and maintenance of melanocytes (Cronin et al., 2009; Garraway et al., 2005). High levels of SOX10 are expressed in solid melanoma tumours (Agnarsdóttir et al., 2010; Bakos et al., 2010;

Flammiger et al., 2008; Nonaka et al., 2008) and SOX10 ablation has been shown to arrest the cell cycle, inducing senescence and promoting the suppression of melanomagenesis (Cronin et al., 2013).

STAP1, also known as BRDG1, has been shown to act as a downstream target of the Tec tyrosinase kinase positive feedback loop, increasing tyrosinase stability (Ohya et al., 1999), thereby indirectly controlling melanin production (Hearing et al., 1992; Kobayashi et al., 1998) which as described in section 1.1, is crucial for prevention of melanoma tumour formation.

SSX7 was shown to be expressed in melanoma cell lines and may function as transcriptional repressor (Güre et al., 2002), capable of eliciting cellular immune responses in cancer patients. The SSX proteins are commonly regarded as highly useful targets for cancer vaccine-based immunotherapy. Here we show for the first time that SSX7 autoantibody levels may have a potential diagnostic role since patient anti-SSX7 levels were displaying statistically significantly higher than those in control sera ($p= 0.033$).

SPANXN1, also referred to as cancer-testis antigen 11.6 (CT11.6), expression has been reported in a number of tumours, including melanoma. Recent research has observed increased SPANX gene expression in benign melanoma tumours, which was higher than the expression in normal tissue but lower than that in metastatic melanoma tissue (Salemi et al., 2009), indicating the validity of the detection of increased serum levels of SPANXN1 autoantibodies in early stage melanoma patient sera.

It is reasonable to believe that following apoptosis or necrosis, tumour cells release self antigen leading to a breakdown in central tolerance resulting in the production of autoantibodies that circulate within the patient‟s circulatory system where an extensive supply of autoantibodies against various cancer-related antigens may be present (Chapman et al., 2008; Lokshin et al., 2006). Patient serum samples therefore provide a precious source of cancer antigens (Lu et al., 2008; Shoshan and Adom, 2007) which can be utilised on high-throughput platforms such as the microarray utilised in this research study to identify melanoma related diagnostic autoantibody biomarkers.

Recent studies have shown an increase in autoantibody production in several cancers (Gnjatic et al., 2009; Tan and Zhang, 2008) including cancer of the breast (Disis, 1994), lung (Diesinger et al., 2002), colon (Scanlan et al., 1998), ovary (Chatterjee, 2006) prostate (Wang

et al., 2005) and head and neck cancer (Carey, 1983), indicating a high potential of autoantibody biomarker use for the early detection of cancer.

One example of the potential of serological autoantibodies to diagnose early-stage cancer is the discovery of the extracellular protein kinase A (ECPKA) autoantibody as a potentially universal cancer biomarker. In healthy mammalian cells, cAMP-dependent protein kinase A (PKA) is an intracellular enzyme. In most cancers, this enzyme is secreted into the circulatory system. Once secreted, the protein is known as extracellular protein kinase A (ECPKA). This antibody was found to be elevated in a wide range of cancers at various stages of malignancies in different cell types including bladder, breast, cervical, colon, esophageal, gastric, liver, lung, ovarian, prostate, pancreatic, renal, small bowel, rectal, adenocystic carcinomas, melanoma, sarcoma, thyoma, liposarcoma and leiomyosarcoma compared to healthy controls. Blood ECPKA levels have been found to be decreased after surgical removal of solid tumours (Kita et al., 2004). With the assumption that this excretion results in the production of anti-ECPKA antibodies or ECPKA autoantibodies, an enzyme immunoassay measuring the IgG of this autoantibody was developed and the sensitivity and specificity of this biomarker for detecting the incidence of 20 different cancers was calculated to be 90% and 87%. Anti-ECPKA autoantibody was detected in 90% of the patient samples and in only 13% of the control samples, indicating that the presence of the ECPKA autoantibody in sera correlates with cancer incidence (Nesterova et al., 2006). These results are highly promising and outperform the results presented here of either the individual or combination of melanoma markers (see table 27). However, the proposed panel of 5 identified markers results in up to 95% sensitivity and specificity (table 14). Moreover, these markers may be melanoma specific while ECPKA is not.

5.2 The OGT functional protein microarray and in-house ELISA are appropriate methodologies to identify and validate autoantibody biomarkers

As described in section 1.13 and 3.5, the Oxford Gene Technology (OGT) microarray represented the optimal multiplex platform to enable melanoma autoantibody biomarker detection due to its versatile selection of attached human recombinant proteins including kinases, signalling proteins, transcription factors and secreted proteins which were analysed simultaneously in one assay using only minute amounts of serum sample. The results obtained from this assay are highly reliable as proteins were attached to the microarray glass slide in quadruplicate with their natural and functional three-dimensional structure still intact

and therefore conformation dependent epitopes were maintained ensuring highly-specific binding conditions. Furthermore, the maintained three dimensional protein structures were fully accessible for binding with autoantibodies present in patient sera as the addition of a biotin carboxyl carrier protein tag (BCCP) to the microarray attached human recombinant proteins or antigens resulted in the proteins being lifted up from the streptavidin-coated microarray surface, exposing all relevant binding sites for antigen-antibody binding.

A previous study has successfully utilised the Oxford gene technology functional protein microarray to identify autoantibodies. One study on prostate cancer, with a cohort of 73 participants, identified 15 potential autoantibody biomarkers for prostate cancer (McAndrew et al., 2012). A combination of these biomarkers showed both a sensitivity and specificity of greater than 90% for prostate cancer. Out of this panel, 8 of the 15 potential prostate cancer autoantibody biomarkers had previously been associated with the cancer, indicating the validity of this technology, and highlighted the ability of the array to identify novel autoantibody biomarkers. By comparison, the single biomarker PSA, currently utilised in prostate cancer screening has a sensitivity of 86% and a specificity of only 33% (Harvey et al., 2009). The findings of this prostate cancer OGT study are currently being further substantiated in a larger cohort of 1800 participants (McAndrew et al., 2012). We therefore hypothesised, that the OGT array could be utilised to similarly identify autoantibodies specific for melanoma.

When the microarray and ELISA were compared in this study for similarities in sensitivity, specificity and positive and negative predictive value of individual markers and combinations of three markers, the methodologies showed similar sensitivity and specificity outcomes for single markers (apart from marker STAP1 and SCYL3) and combinations of markers. The microarray did however display greater sensitivity.

Overall, our results indicate that the microarray is an appropriate methodology to be utilised for the identification of autoantibody biomarkers in melanoma patient sera and that the in-house ELISA represents an appropriate methodology to be utilised for the validation of the identified serological autoantibody biomarkers in a larger cohort.

5.3 Increased sensitivity and specificity through utilisation of multi-biomarker panels

While ECPKA, described in section 5.1, provides significant sensitivity and specificity, to date, no single autoantibody biomarker has been utilised as a cancer biomarker for melanoma

specifically, presumably due to the generally low sensitivity and specificity of single markers. Panels of multiple tumour-associated autoantibodies with high specificity and sensitivity are sought therefore for translation into simple biomarker panel tests for routine clinical diagnosis of early stage cancer. (Anderson and LaBaer, 2005; Caron et al., 2007; Gunawardana and Diamandis, 2007; Huang et al., 2004; Zhang et al., 2003; Zhong et al., 2004).

In this research, we confirmed that autoantibodies are suitable as diagnostic biomarkers for melanoma and 8 autoantibody biomarkers displaying high reactivity with early stage melanoma patient sera and low or no reactivity with healthy control sera were identified as potential biomarkers. Statistical analysis of the final results showed that a combination of all markers, where patients needed to exhibit positivity for at least one of the markers to be denoted as melanoma positive, had the highest diagnostic potential for early stage melanoma patients relative to healthy control samples. This was confirmed with three biomarkers by an ELISA test. Both methodologies, the functional protein microarray and the in-house indirect ELISA, showed similar results confirming that multiple markers are suitable for the identification and validation of diagnostic autoantibody biomarkers for the early diagnosis of melanoma.

The optimal autoantibody biomarker panel identified in this research study exhibits a positive and negative predictive value of 95% which is very comparable with the positive and negative predictive values of other potential autoantibody cancer biomarker panels currently in commercial use and it is therefore reasonable to be considered as a potential biomarker panel for the early diagnosis of melanoma.

The use of a combination of biomarkers for cancer diagnosis has been proven to be favourable for the detection of various cancers.

For example, in a study by Xie et al. (2011), a new multiplex assay termed the “A+PSA” assay (the autoantibody +PSA assay) was developed. This assay was aimed at providing a reliable platform which will enable the diagnosis of prostate cancer patients relative to non-malignant cases. This assay used B cell epitopes from previously defined prostate-cancer-associated antigen (PCAA), including New York oesophageal squamous cell carcinoma (NY-ESO-1), synovial sarcoma X breakpoint 2,4 (SSX-2,4), X antigen family member 1B (XAGE-1b), lens epithelium-derived growth factor (LEDGF), transferrin receptor protein 9 (p90) and alpha-methylacyl-CoA racemase (AMACR). The platform allowed the

simultaneous screening of these six autoantibodies alongside PSA and PSA screening alone in 131 pre-surgery biopsy confirmed prostate cancer patients and 121 prostatitis and/or benign prostatic hyperplasia patients. Xie et al. (2011) found that PSA alone had a sensitivity of 52% and specificity of 79% in all patients while the A+PSA platforms showed a sensitivity of 79% and a specificity of 84% in all patients. The A+PSA platform also had a decreased false positive outcome of only 16% versus 21% when PSA alone was utilised. Overall, the accuracy of the A+PSA test platform was as high as 81% while PSA alone only showed an accuracy of 65% (Xie et al., 2011).

Similarly, in a breast cancer study, the SERPA approach (section 1.12.5) was utilised by Desmetz et al. (2008) to investigate the presence of heat shock protein 60 (HSP60) autoantibodies in a cohort consisting of 49 ductal carcinoma *in situ* patients, 58 early-stage breast cancer patients, 20 patients with other types of cancer, 20 patients with various autoimmune diseases and 93 healthy controls. They showed that HSP60 autoantibodies are a potential biomarker for the diagnosis of breast cancer demonstrating a sensitivity of 31.8% while its specificity is 95.7%, resulting in an accuracy of 63.75%. A study by Chapman et al. (2007) tested seven antigens including HER2, c-myc, p53, breast cancer type 1 susceptibility protein (BRCA1), breast cancer type 2 susceptibility protein (BRCA2), Ny-ESO-1 and MUC1 with a cohort of 94 healthy controls, 97 primary breast cancer sera and 40 ductal Carcinoma in Situ (DCIS) sera. When all seven markers were used, the specificity of the assay was found to be as high as 91-98%, even when tested for individual markers only; however, the individual autoantigen assay sensitivity was only 3-23% in the DCIS sera and 8-24% in the primary breast cancer sera. By comparison, the sensitivity increased to 45% in DCIS sera and 64% in primary cancer sera with a specificity of 85% when a combined panel of 6 out of the 7 autoantigens were tested. Such a multimarker test could be used alongside other cancer detection methods such as mammography, to significantly improve in breast cancer detection.

5.4 Autoantibody biomarkers SOX10 and SCYL3 may be gender specific biomarkers but SOX10, BRDG1 (STAP1) and SCYL3 are not melanoma disease stage or age specific

When microarray identified markers, including SOX10, BRDG1 (STAP1) and SCYL3 were analysed to examine statistical significance of the microarray pilot study cohort marker expression relative to melanoma TNM stage, age and gender incidence, no statistically

significance was found which would indicate a lack of association of autoantibody levels and therefore marker expression with any of the above mentioned criteria. The lack of significant statistical difference between patient sample stages may indicate that autoantibody levels are not indicative of tumour burden. Autoantibodies may be formed early in the disease in response to abnormal proteins but are not produced at increasing levels at increasing disease stages.

When chemiluminescence values of the final ELISA cohort were compared for each marker, we found that female patient autoantibody chemiluminescence levels were significantly higher than those of male patients in the final SOX10 and SCYL3 ELISA ($p=0.042$ and $p=0.017$ respectively). This finding has not been shown previously in other cancers.

It is interesting to note that the number of female and male patient samples was independent of the primary researchers and therefore the number of female and male patient samples received mirrors the number of female and male patients being diagnosed over the course of the study. There were only 4 female patient samples and 16 male patient samples in the microarray study resulting in a 4:1 ratio of males being diagnosed with melanoma in comparison to female patients. In the final ELISA cohort, there were up to 46 male patient samples and 14 female patient samples, resulting in a 4.18:1 ratio of males being diagnosed with melanoma in comparison to females. This finding coincides with increased melanoma risk associated with males (Green et al., 2012), but fails to explain the higher association of these markers with female patients. Further research in a larger cohort is required to confirm these results.

5.5 The importance of a diagnostic blood test for melanoma

Currently, clinicians may diagnose melanoma initially through the visual examination of suspicious moles using the ABCDE rule, which confirms a lesion as melanoma positive if it exhibits an asymmetrical shape, irregular border, colour variability of the pigmentation of the skin and if the moles diameter has advanced past 6mm, indicating that the mole is evolving by displaying further symptoms such as itching, bleeding or crusting (Abbasi et al., 2004; Rigel et al., 2005). Although highly utilised, the changes observed in this rule are not highly specific to melanoma and less than 17% of all benign naevi displaying the tendency to show such changes are actually melanomas (Goulart et al., 2011). Further limitations of the ABCDE rule utilisation for the early detection of melanoma include the high likelihood of

negligence of smaller lesions through the employment of the 6mm diameter cut-off and these lesions may remain untreated (Helsing and Loeb, 2004; Rhodes, 2006). Another rule, denoted as the consumer-friendly rule to assess lesions for asymmetry and colour for melanoma suspicious lesions (AC rule) showed that 91% of the general population observed in a study, correctly diagnosed themselves as melanoma burdened while when used by dermatologists, the sensitivity of the test was 88%. The specificity of this melanoma diagnosis rule however was only 64% in the general population and 88% when utilised by dermatologists to diagnose their patients (Luttrell et al., 2011). Most skin cancers, including melanoma, are detected by skin self-examination (SSE) (Hamidi et al., 2008), with SSE sensitivity values ranging from 25% to 93% and specificity values ranging from 83% to 97% (Hamidi et al., 2010). In fact, in Australia, 44% of all melanomas (McPherson et al., 2006) and 73% of melanoma recurrences are detected by the patient or the patient‟s partner (Francken et al., 2007). Studies have also found that melanoma lesions detected by SSE are generally thinner, indicating an earlier disease stage which may greatly influence the patient‟s survival rate (Hamidi et al., 2008; Janda et al., 2011). However, lesions on hard-to-see body sites are often missed in skin self-examinations (Youl et al., 2011) which do not tend to be thorough (Weinstock et al., 2007). The success of SSE may further be compromised depending on personal factors such as the patients skin cancer knowledge, personal history and therefore higher perceived risk of skin cancer, previous conversation with a provider about sun protection, and SSE self-efficiency (Hamidi et al., 2008). On the other hand, clinical skin examination (CSE) was found to lead to earlier detection of skin cancer in general (Carli et al., 2003a) and melanoma thickness levels were found to be further decreased when compared to SSE diagnosis (Carli et al., 2003b; Hamidi et al., 2008) which ultimately led to a substantial favourable impact of CSE on melanoma mortality (Katalinic et al., 2012). There are two types of dermoscopes, utilised to aid CSE, which can detect various features of the melanoma lesion such as seborrheic keratosis (Wurm et al., 2010), vascular structures and shiny white streaks which may indicate fibrosis (Benvenuto-Andrade et al., 2007). Although dermoscopy may be regarded as the golden standard for melanoma detection (Wurm et al., 2010), it still displays a limited scope of observable structures (Hoffmann-Wellenhof et al., 2009) and requires a conceptual level of knowledge and experience to distinguish melanoma features and patterns from those of normal naevi (Rosendahl et al., 2011) as well as adding several minutes to the clinical visit (Zalaudek et al., 2008). Reflectance confocal microscopy (RCM), which uses a near infrared laser beam to penetrate the upper layers of the skin to allow definition of cellular structures and morphology of the

epidermis, dermoepidermal junction and dermis (Hoffmann-Wellenhof et al., 2009), and total body photography (TBP), which is used to document multiple lesions covering the body over time and observe changes in the lesions (Wurm et al., 2010), may also be utilised to diagnose melanoma. Both technologies are non-invasive and exhibit high sensitivity and specificity scores (Ferris and Harris, 2012; Wurm et al., 2010), but large-scale trials are still required to determine their actual usefulness in melanoma detection (Hoffmann-Wellenhof et al., 2009; Wurm et al., 2010) and both diagnostic methods furthermore elongate the time required per patient for each clinical visit.

Finally, once a biopsy has been obtained from a patient for further analysis, the collected tissue sample is analysed by cytological analysis and immunohistochemistry, using markers such as Human Melanoma Black 45 (HMB45), S100 calcium binding protein B (S100B) and Protein melan-A (MLANA) also known as melanoma antigen recognised by T-cells 1 (MART-1); to assess the presence of cutaneous melanoma and therefore diagnose the patient from which the tissue originated (Mocellin et al., 2008; Pflugfelder et al., 2013). In order to increase the accuracy of these analytical tests, it is necessary to utilise a larger number of markers with high sensitivity and specificity for cutaneous melanoma. In cases where immunohistochemistry results are indecisive, electron microscopy may be utilised (Lai et al., 1998). On occasion however, there may be difficulty in diagnosing melanoma as the phenotype and genetic makeup can resemble the cells of other cancer types (Sheffield et al., 2002).

Therefore, currently utilised techniques for the early stage diagnosis of melanoma, exhibit many advantages but also several disadvantages. Some of the disadvantages resulting from incorrect melanoma diagnosis for example through limitations in the diagnostic tests and technologies listed above, include the corresponding results of melanoma misdiagnosis such as the psychological impact on a healthy individual of a possible melanoma diagnosis, an increased number of unnecessary surgical procedures on benign lesions along with their accompanying costs and possible delays of diagnosis confirmation (Carli et al., 2004; MacKie et al., 2003). Resent focus has therefore been gathering momentum in search for biomarkers which may be used in a diagnostic melanoma blood test as an alternative diagnostic technique.

A diagnostic blood test which incorporates the autoantibody biomarkers identified in this research study, by using a functional protein microarray and validated in a larger cohort using

an indirect enzyme-linked immunosorbent assay (ELISA), may be used as a second independent test for melanoma positivity, counteracting the limitations mentioned above and providing improved and accurate melanoma diagnosis. The blood test will be less invasive than the collection of a biopsy tissue sample; it will only cause minor discomfort to the patient and can be readily performed by any trained phlebotomist employed at the appropriate clinical institution, centre or hospital. The cost of the blood test may be less extensive than unnecessary surgical skin excisions and will add less time to each clinical visit than other diagnostic methods currently in use. It is anticipated that this blood test may be used in conjunction with the currently utilised diagnostic techniques.

To date, serological biomarkers are generally not utilised to diagnose melanoma, but may prove valuable, particularly in occult melanoma cases where the primary tumour is not found and therefore none of the above mentioned techniques are applicable (Kamposioras et al., 2011). A diagnostic blood test also be useful for the diagnosis or monitoring of patients with mole counts greater than 100 across their body surface, which often pose a problem as the visual monitoring of these lesions is very time consuming and therefore clinicians may have difficulty in monitoring the patient‟s skin lesion changes. The blood test will not suffer from detection bias as may be the case for other diagnostic techniques and could eliminate the potential of negligence of suspicious lesions located on hard-to-see body sites.

Currently, following the surgical excision of their primary tumour, melanoma patients generally do not receive further treatment and PET/ CT scans are currently the only way to monitor a limited number of patients with suspected advanced melanoma (Marsden et al., 2010). Therefore, a blood test may not only be used to diagnose melanoma patients but also to monitor their disease status.

5.6 Limitations of the study

The sample size of the pilot study cohort utilised in the microarray experiment to identify the panel of 8 diagnostic early stage melanoma biomarkers was relatively small (n=40) and therefore these markers may not be optimal for melanoma. An increased sample size may produce statistical significance in areas of this research that were otherwise lacking in significance. Furthermore, although the microarray utilised in this research study consisted of 1627 human recombinant proteins derived from various tumours there may have been markers present in high levels in patient sera and absent or present in very low levels in

control sera that may have been missed in our investigation simply because they were not part of the microarray.

For the ELISA experiments, research was restricted to commercially available human recombinant proteins and these may have differed slightly from those on the microarray. It is also important to note that the ELISA methodology did not incorporate a BCCP tag as used in the microarray experiment and therefore, the three dimensional structure of the proteins used in the ELISA experiments were not lifted away from the ELISA plate surface which may have resulted in important autoantibody binding epitopes being inaccessible to autoantibody binding. Furthermore, although healthy participants of the study were asked if, to the best of their knowledge, they have ever been diagnosed with any cancer or autoimmune disease, they may have been undiagnosed for any of these conditions and may therefore not accurately represent a true control sample to which melanoma patient samples should be compared. Furthermore, the different conditions utilised in both the microarray and ELISA experiments limit the levels of comparison between the two methodologies.

5.7 Suggested future experiments

Since a combination of the markers, SOX10, BRDG1 (STAP1) and SCYL3, and levels of the single marker SOX10 showed high potential to serve as diagnostic autoantibody biomarkers for the early detection of melanoma, follow up studies with increased sample sizes should be conducted. As mentioned in the results section of this document, a panel comprising the markers PAGE5, SOX10, STAP1, SSX7 and SPANXN1 may provide up to 95% sensitivity and specificity to diagnose melanoma and therefore, this panel is suggested to be investigated further in future studies. These studies should also include a larger sample size of more advanced stages of melanoma in order to determine if the identified markers display only diagnostic or also prognostic biomarker potential. It would further be advised to test the individual markers as well as combinations of these markers against a variety of samples from patients with non-melanoma cancers to identify whether these markers are melanoma specific and since the levels of autoantibodies in patients with autoimmune diseases may greatly affect potential cancer-related diagnostic autoantibody levels, future studies should also test samples from patients with various autoimmune diseases for the markers identified as melanoma markers, to ensure that the markers are indeed truly specific. Furthermore, screening an increased sample cohort on the microarray may result in the identification of other, potentially novel diagnostic autoantibody biomarkers for the early detection of

melanoma patients. Finally, any research performed in this topic area has the potential to add to the current knowledge on melanoma formation and progression and may therefore provide future targets for therapeutic interventions.

6. Conclusion

In conclusion, melanoma is a highly aggressive skin cancer, the progression of which is dramatically reduced by early detection, followed by early treatment (Balch et al., 2011). We hypothesised that increased levels of autoantibodies in the serum of early stage melanoma patients are indicative of a positive disease status and therefore may be utilised to assist with diagnosis of melanoma at an early stage. Recent studies have shown an increase in autoantibody production in several cancers (Gnjatic et al., 2009; Tan and Zhang, 2008) including cancer of the breast (Disis, 1994), lung (Diesinger et al., 2002), colon (Scanlan et al., 1998), ovary (Chatterjee, 2006) prostate (Wang et al., 2005) and head and neck cancer (Carey, 1983), indicating a high potential of autoantibody biomarker use for the early detection of cancer.

Here we have identified a panel of 8 potential diagnostic biomarkers for the early detection of melanoma through the utilisation of the OGT functional protein microarray. We have further validated the microarray results of 3 of the identified markers in a larger cohort by performing an enzyme-linked immunosorbent assay (ELISA) and found that a panel comprised of the markers SOX10, BRDG1 (STAP1) and SCYL3, where a sample had to display positivity for at least one of the markers in order to be regarded as melanoma positive, displayed the highest diagnostic potential for early stage melanoma patients. The single marker, SOX10, also displayed high diagnostic potential. Furthermore we have confirmed that the microarray and ELISA are appropriate methodologies for the identification and validation of autoantibody biomarkers as their final sensitivity and specificity scores obtained for single markers and combinations of these markers are highly similar. Finally, we found that the markers SOX10 and SCYL3 may be gender specific as female patients showed significantly higher autoantibody levels of these markers than male patients. We did not find any patient age or disease stage associations with these markers.

The major limitation of the study was the small pilot study sample size and different experimental conditions utilised in the two methodologies compared. It is suggested that further studies on the identified melanoma biomarkers should be performed to validate the results obtained from this study in an increased sample cohort of early stage melanoma patients and patients of other cancers as well as autoimmune diseases to validate these findings. Ultimately, the detection of a panel of autoantibodies may be utilised for the

development of a routine diagnostic tool, such as a blood test, to aid in the early detection of melanoma.

7. References

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8. Appendices

8.1 Summarising figure of currently utilised methodologies to detect autoantibodies

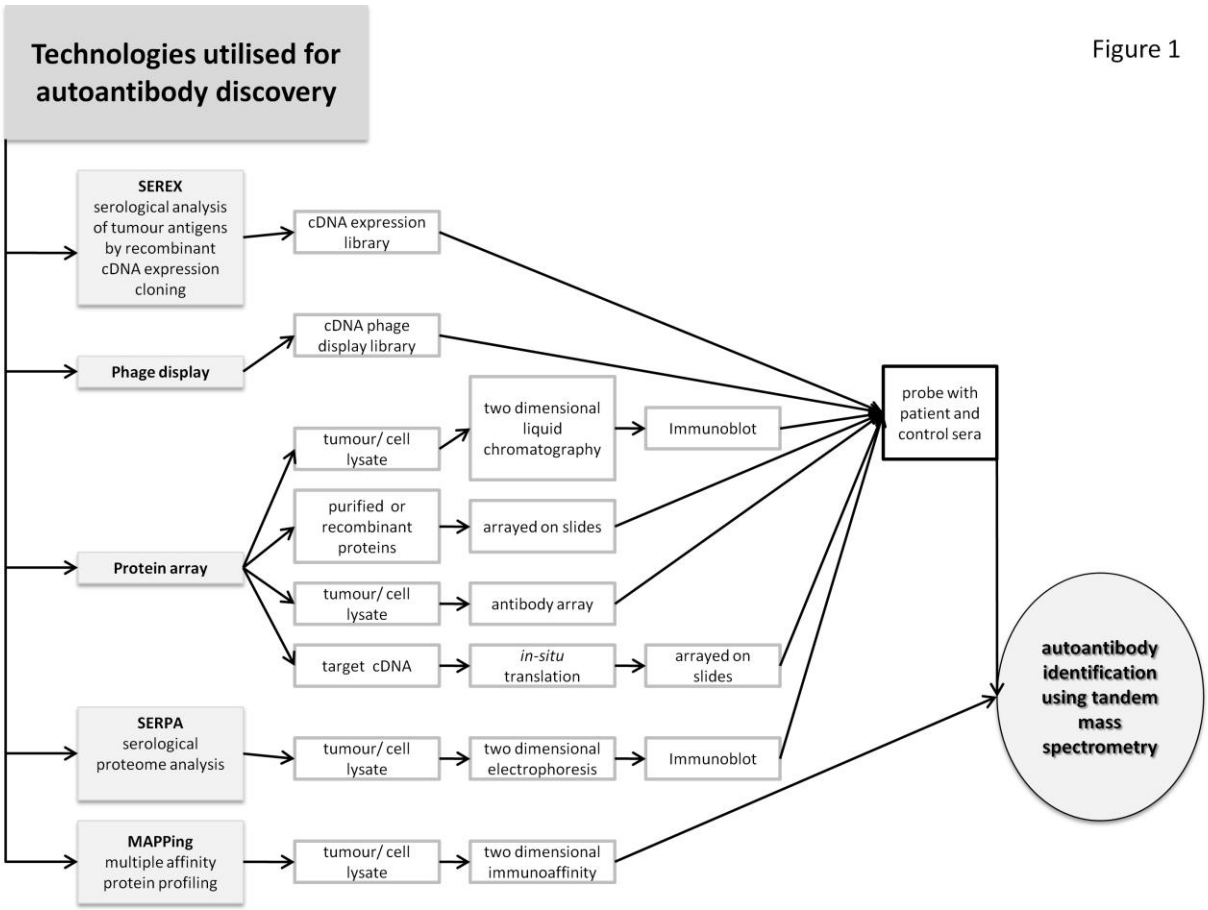
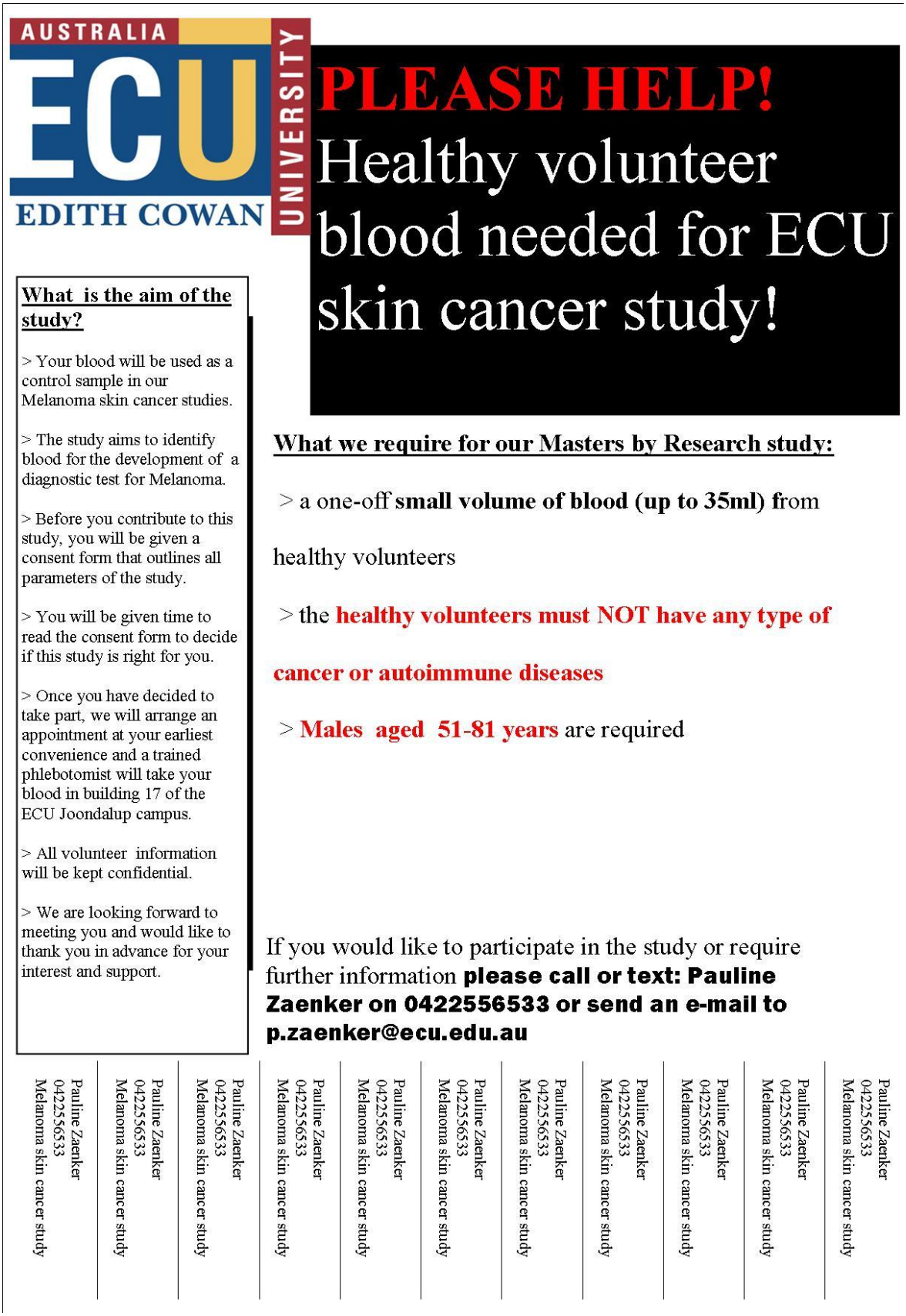


Figure 1

8.2 Ethics approved healthy volunteer flyer



8.3 Study consent form

ECU HUMAN RESEARCH ETHICS COMMITTEE

PARTICIPANT INFORMATION AND CONSENT FORMS

PARTICIPANT INFORMATION SHEET

Development of a blood test for Cutaneous Melanoma

A/Prof Mel Ziman, Mr. Mark Lee, A. Prof. Robert Pearce, Dr. Lister, Dr. Chan, Dr. Amanda Ireland, Ms. Anna Reid, Mr. James Freeman, Ms. Dr Johan Poole-Johnson, Dr. John Anson, Dr. Will Colon, Dr. Arif Anwar, Ms. Hasmawati Yahaya, Ms. Pauline Zaenker

Please take time to read the following information carefully and discuss it with your friends, family and general practitioner if you wish. Ask 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?

Edith Cowan University - in the form of a research grant, the Office of Research and Innovation strategic fund.

Contact persons:

Should you have questions about the study you may contact:

A/Prof. Mel Ziman Phone No. 6304 3640 Mobile: 041992985

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

1. 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.

2. You may wish to have a member of your family or a friend with you while you are told about the study. You may ask questions and all questions will be answered to your satisfaction.

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 serum levels of autoantibodies in the peripheral blood of patients with cutaneous melanoma relative to autoantibody serum levels in healthy individuals. It is anticipated that these autoantibodies may be used as markers to diagnose melanoma in an early-stage to improve patient survival rates.

Why is this study suitable to me?

You have been invited to participate in this study because you have been diagnosed with cutaneous melanoma, or you have been 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 as part of your routine examination by your clinician or will be provided free of charge if you are a healthy volunteer. You will then be asked to provide a blood sample.

If you agree to participate as a patient you will be asked to provide blood samples at the time of surgical removal of your tumour.

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

The study will be conducted over a two 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 cutaneous melanoma you are required to provide a small blood sample at the time of diagnosis and you may be required to give a blood sample once again over the course of two years.
- The amount of blood required for this study is small (only 9ml). Blood will be drawn into serum separation 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 or at Oxford Research Laboratories 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 or clinic 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 general 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. The results from the study may be used to commercialise a blood test for melanoma to ensure the provision of this technology for all patients 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.

The 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. When you stop participation in the study you will be reassessed as you were at the beginning of the study as a patient.

What alternatives do I have to going on this study?

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

You may wish to discuss with your doctor or the researchers how the test will benefit patient treatment in the future even though there may be 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 (9ml) 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.

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, because the trial has been completed 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 Medical Sciences at Edith Cowan University (ECU) during the study and in a locked archive for at least 5 years from the time the study is closed, and may be destroyed at any time thereafter.

Authorised representatives of the researchers, the investigating doctors, the Hospital or University Human Research Ethics Committees, Research Governance and other regulatory bodies may require access to your study records to verify study procedures and/or data. Some of your information may be sent to people in other countries for these purposes. In all cases when dealing with your information, these people are required to comply with privacy laws that protect you.

Your sample and general information about your health (no personal data) may be sent to people at other laboratories in Australia or overseas. Researchers in other laboratories will not be able to identify you as only the principle researchers at ECU will have access to your personal information and this will be kept in locked filing cabinets and on password protected computer disks.

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 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 have reviewed this study and have given their 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.

CONSENT FORM

Development of a Blood Test for Cutaneous Malignant Melanoma

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

Investigators: **A/Prof Mel Ziman, Ms. Pauline Zaenker, Mr. Mark Lee, A. Prof. Robert Pearce, Dr. Lister, Dr. Chan, Ms. Anna Reid, Ms. Dr Johan Poole-Johnson, Dr. Arif Anwar**

Participant Name:_____

Date of Birth: _____

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

2. I have been told about the possible advantages and risks of taking part in the study and I understand what I am being asked to do.

3. I have been able to have a member of my family or a friend with me while I was told about the study. I have been able to ask questions and all questions have been answered satisfactorily.

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

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

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

7. I understand that the outcome of the study may be used to commercialise a blood test for melanoma and that my sample may be sent overseas for analysis to assist with this process.

8. I understand that my sample and associated data may be used for future melanoma research but only for melanoma research conducted by researchers at Edith Cowan University.

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

Name of Participant	Signature of Participant	Date

Name of Investigator	Signature of Investigator	Date

8.4 Pilot study cohort data

Patients:

Patient sample number	**Age (yrs)**	**Gender (M/F)**	**TNM disease stage at diagnosis**	**Breslow thickness (mm)**	**Clark´s level**	**Malignancy (Yes/ No)**	**Ulceration (Yes/ No)**
B1	48	M	0	not reported	not reported	No	No
B2	76	M	0	not reported	not reported	not reported	not reported
B3	66	M	0	0.5mm	3	No	No
B5	65	M	0	not reported	not reported	No	Yes
B6	63	M	0	insitu	1	not reported	No
B7	36	M	1	0.3mm	1	not reported	Yes
B8	75	M	0	insitu	not reported	not reported	No
B9	26	M	0	insitu	not reported	not reported	not reported
B10	56	M	0	not reported	not reported	No	No
B11	57	F	I	0.35mm	2	none	none
B12	45	F	0	not reported	not reported	No	No
B13	71	M	0	not reported	1	No	No
B14	73	M	I	0.2mm	not reported	No	Yes
B15	63	M	0	insitu	TBA	No	No
B16	56	M	I	1.0mm	4	No	No
B 17	66	F	0	insitu	not reported	No	No
B19	62	M	0	1.5mm	not reported	No	No
B22	51	M	0	na	not reported	No	No
B24	46	M	0	0.28mm	not reported	No	Yes
B25	57	F	I	0.38mm	3	No	Yes

Controls:

Control sample number	**Age (yrs)**	**Sex (M/F)**
CC1 (A-J)	55	F
CC2 (A-J)	60	F
CC3 (A-J)	43	F
CC4 (A-J)	60	M
CC6 (A-J)	25	M
CC13 (A-J)	47	M
CC14 (A-J)	50	M
CC17 (A-J)	53	M
CC20 (A-J)	49	M
CC21 (A-J)	60	M
CC22 (A-J)	37	M
CC23 (A-J)	58	F
CC24 (A-J)	67	M
CC25 (A-J)	75	M
CC27 (A-J)	68	M
CC28 (A-J)	50	M
CC29 (A-J)	59	M
CC30 (A-J)	65	M
CC32 (A-J)	80	M
CC33 (A-J)	49	M

8.5 Microarray data and statistical analysis

The following tables show the samples positive for the 8 potential biomarkers identified on the OGT microarray. The sample number, participant age, gender and rfu fold change for the appropriate marker is included below as well as the mean and data range of each marker:

SCYL3:

Sample and TNM stage TNM stage 0 TNM stage I control	**Age** (years)	**Gender** (M = male, F = female)	**Fold change** values for SCYL3	**mean and data range**
B1	48	M	2.1	3.98 (2.1-7.9)
B10	56	M	5	
B11	57	F	2.3	
B12	45	F	3.7	
B13	71	M	2.4	
B14	73	M	4.2	
B15	63	M	2.7	
B16	56	M	3.3	
B19	62	M	6.2	
B2	76	M	3.7	
B22	51	M	3.9	
B25	57	F	2.3	
B3	66	M	4.9	
B5	65	M	7.5	
B6	63	M	7.9	
B8	75	M	3	
B9	26	M	2.6	
CC13	47	M	3.1	3.04 (2-4.8)
CC17	53	M	2.4	
CC2	60	F	4.8	
CC20	49	M	2	
CC22	37	M	3.4	
CC24	67	M	2.2	
CC29	59	M	4.3	
CC4	60	M	2.1	

ENO2:

Sample and TNM stage TNM stage 0 TNM stage I control	**Age** (years)	**Gender** (M = male, F = female)	**Fold change** values for ENO2	**mean and data range**
B17	66	F	2.2	4.07 (2.2-10.9)
B12	45	F	**8.4**	
B13	71	M	4.6	
B15	63	M	2.7	
B19	62	M	2.4	
B2	76	M	2.2	
B25	57	F	3.5	
B3	66	M	2.3	
B5	65	M	**10.9**	
B7	36	M	2.4	
B8	75	M	3.2	
CC13	47	M	4.4	3.03 (2.2-4.4)
CC17	53	M	2.4	
CC22	37	M	2.4	
CC24	67	M	2.9	

NRBF2:

Sample and TNM stage TNM stage 0 TNM stage I control	**Age** (years)	**Gender** (M = male, F = female)	**Fold change** values for NRBF2	**mean and data range**
B10	56	M	2.1	4.39 (2.1-10.3)
B14	73	M	4.5	
B15	63	M	3.2	
B22	51	M	2.6	
B24	46	M	4.6	
B25	57	F	2.2	
B3	66	M	**10.3**	
B5	65	M	5.8	
B6	63	M	2.4	
B7	36	M	6.2	
CC13	47	M	3.6	6.70 (3.6-10.6)
CC20	49	M	5.9	
CC3	43	F	**10.6**	

PAGE5:

Sample and TNM stage TNM stage 0 TNM stage I control	**Age** (years)	**Gender** (M = male, F = female)	**Fold change** values for PAGE5	**mean and data range**
B1	48	M	6.6	3.63 (2-7.8)
B10	56	M	2	
B11	57	F	2.4	
B15	63	M	2.6	
B2	76	M	**7.8**	
B22	51	M	2.7	
B24	46	M	2.8	
B25	57	F	2.1	
CC26	59	M	3.2	3.2

SOX10:

Sample and TNM stage TNM stage 0 TNM stage I control	**Age** (years)	**Gender** (M = male, F = female)	**Fold change** values for SOX10	**mean and data range**
B11	57	F	2.4	2.90 (2-3.9)
B16	56	M	3.9	
B2	76	M	3.5	
B5	65	M	2.2	
B8	75	M	2	
B9	26	M	3.4	

STAP1 (BRDG1):

Sample and TNM stage TNM stage 0 TNM stage I control	**Age** (years)	**Gender** (M = male, F = female)	**Fold change** values for STAP1	**mean and data range**
B17	66	F	**12**	5.00 (2-7.2)
B12	45	F	3.4	
B2	76	M	**7.2**	
B22	51	M	2.2	
B25	57	F	2	
B7	36	M	3.2	

SSX7:

Sample and TNM stage TNM stage 0 TNM stage I control	**Age** (years)	**Gender** (M = male, F = female)	**Fold change** values for STAP1	**mean and data range**
B17	66	F	**12**	5.00 (2-12)
B12	45	F	3.4	
B2	76	M	**7.2**	
B22	51	M	2.2	
B25	57	F	2	
B7	36	M	3.2	

SPANXN1:

Sample and TNM stage TNM stage 0 TNM stage I control	**Age** (years)	**Gender** (M = male, F = female)	**Fold change** values for SPANXN1	**mean and data range**
B13	71	M	2.7	2.85 (2-4)
B15	63	M	2	
B19	62	M	2.7	
B5	65	M	4	